PART I

STANDARD TECHNIQUES IN GEOMICROBIOLOGY

1 General Geochemistry and Microbiology Techniques

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Abstract

Geomicrobiological investigations benefit from knowledge of geochemical and biological systems at different scales, including information about both the abiotic and the biotic components. Gathering this information requires analysis and characterization of both abiotic and biotic components of the target system. The techniques presented in this chapter were selected to cover a variety of needs in geomicrobiological studies, including general sample collection and storage, organic and inorganic compound quantification, and best practices for cultivation, observation, and analysis of microorganisms and microbial communities. In this chapter, introductions and discussions for common techniques provide the reader with a basic understanding of the technique itself, which samples can be analyzed using the technique, and how to prepare samples for analysis. Detailed methods are provided for select techniques, and citations to standard methods are provided for techniques whenever available. For techniques that are rapidly evolving, recent developments and applications are discussed.

1.1 Field Sampling and Sample Collection, Preservation, and Storage

Field samples are important resources to answer many geomicrobiological research questions. They can provide information on target systems under real conditions that laboratory-synthesized samples cannot. Before samples can be collected from the field, a sampling design must be established. For design planning, care must be taken to prevent introduction of bias, misrepresentation, and insufficient data in the final dataset. If, for example, a research question is aimed at understanding the relationship between rhizosphere microbial communities and tree roots in a forest ecosystem, collecting samples exclusively from the rhizosphere of oak trees might bias the results. In another example, when trying to understand the soil carbon (C) content of a 100 acre agricultural field, it would be a poor sampling design to collect all replicate samples from the area within a square half acre, because data from one specific area does not accurately and/or sufficiently represent the entire field; likewise, collecting one replicate sample from each edge of the field would also be a poor sampling design, because edge effects (e.g., transition from agricultural field to nonagricultural field) may be captured in the samples, and the results may not accurately represent the conditions in the agricultural field. A thorough discussion of sampling strategies for field sample collection is presented by Thompson (2012).

The following sections discuss common strategies and methods for sample collection, preservation, and storage for geochemical and microbiological analyses. Special considerations for each analysis type are examined.

1.1.1 Samples for Geochemical Analyses

For geochemical analyses, once a sampling design is established, the next step is to decide *how* the samples will be collected. To decide how to collect samples, many variables must be considered: (1) Is the sample solid or liquid? (2) What analyte(s) will be measured? (3) Is the analyte sensitive to light? (4) Is the analyte sensitive to atmospheric gases (e.g., O_2, CO_2)? and (5) When is the best time to sample? Liquid samples are commonly collected in high-density polyethylene (HDPE) or glass bottles that have been cleaned (e.g., acid-washed to remove contaminant metals, combusted to remove residual carbon) prior to sample collection. If the target analyte is known to react with or sorb to either of these materials, alternative materials (e.g., Teflon) should be used for sample collection. Solid samples (e.g., soils, rocks, and minerals) can be collected in plastic storage bags, paper bags, or geological sample bags, including plastic-lined bags; if C is a target analyte, paper bags and unlined geological sample bags should not be used. If an analyte reacts with light, the sample can be collected in a UV-blocking or opaque container (e.g., amber glass, brown HDPE, plastic bag placed inside larger opaque container, or aluminum foil-wrapped container). For analytes that react with atmospheric gases, care must be taken to prevent interaction with and exchange of atmospheric gases into the sample during and after collection. Glass sample containers fitted with airtight lids provide the best protection from potential interactions and exchanges. Further, whenever possible, samples should be sealed with no ambient headspace in the container to avoid interactions with atmospheric gases (e.g., CO_2, H_2, or S-bearing gases) that might contaminate the sample; small bottles of inert gas (e.g., Ar or N_2) can be brought to the field to purge headspace in containers for sensitive samples. Another consideration for sample collection is when to sample; some systems have natural cycles (e.g., diurnal or seasonal), which can contribute to sampling errors if not considered carefully.

Ideally, samples are collected and analyzed immediately to most accurately capture the information present at the time of sample collection, though this is rarely possible. The next best option is to analyze the samples as soon as possible. When this is not possible either, preservation methods may be used to stabilize analytes by preventing physical, chemical, and/or biological reactions that would otherwise alter those analytes between the time of sample collection and analysis. Common preservation methods for aquatic samples include sterilization, reduction in temperature, filtration, and acidification. Sterilization inactivates biological processes and prevents alterations to analytes, especially analytes that are nutrients for organisms (e.g., C-, N-, and S-bearing compounds). It can be achieved with radiation, though this process is expensive and has the potential to alter some analytes. Alternatively, a reduction in biological activity can be achieved with a reduction in temperature (≤4 °C). Caution should be used when reducing the temperature of samples where the analyte may form mineral precipitates under lower-temperature conditions. Filtration removes suspended particles from the sample, which can either sorb or desorb

analytes and affect their concentrations, and allows the determination of the "dissolved" fraction of analytes; typically, 0.45 μm filters are used, but 0.2 μm filters may be used to remove smaller particles. Filters and filtration methods should be carefully chosen to avoid significant analyte loss during filtration (Batley, 1989; Horowitz et al., 1992). Acidification reduces interferences from atmospheric CO_2 and stabilizes dissolved metals, preventing their sorption to the container material and/or precipitation from solution. Concentrated acid (e.g., HCl) is added to bring the pH of the sample down to 1.5 ± 0.5. Table 1 in Uzoukwu (2000) provides a detailed list of parameters and suitable container materials and preservation methods for common geochemical analytes in aquatic samples. For solid samples, the most common preservation method is drying. Moist solid samples are susceptible to continued physical, chemical, and biological reactions, which are minimized upon drying. Most analytes are not significantly affected by drying, with the notable exception of those that are easily oxidized under ambient atmospheric conditions (e.g., Fe(II)) (Bates, 1993; Tan, 1996). Drying should be achieved at room temperature using forced air flow (e.g., a fan or exhaust hood) to hasten the process; oven drying should be avoided if possible, and an oven above 35 °C should never be used. Depending on the analyte and the analytical technique (and potential interferences), preservation of samples may not be required. However, in all cases, methods that alter, destroy, or contaminate analytes should be avoided entirely.

Storage time between sample collection and analysis should be minimized as much as possible. For some analytes (e.g., pH, Fe(II), Mn, nitrate, organics, and nutrients), time is of the essence, and despite best efforts to preserve the sample, analyte alterations will occur over time. Other analytes (e.g., total metal content), however, may be stable for long periods of time. As a rule of thumb, solid samples can be stored longer than water samples, though exceptions exist in both cases. The method of storage (e.g., room temperature, chilled, or frozen) will depend on the analyte(s) of interest; if unsure, default to chilled (~4 °C) but reduce fluctuations in temperature over time (i.e., either keep chilled or keep unchilled). Methods for long-term storage of samples should prevent contamination to and alteration of the sample, and eliminate temperature and moisture fluctuations as much as possible.

1.1.2 Samples for Microbiological Analyses

In addition to the considerations discussed for geochemical analyses (see Section 1.1.1), special considerations must be made when collecting samples for microbiological analyses. The first consideration is how best to prevent contamination of the sample with microbial cells and biomolecules from the sample collector(s), collection gear and tools, and surrounding environment (e.g., surfaces and air). Conditions for collecting and handling samples in the field are not as ideal as working in a controlled laboratory environment, and achieving and maintaining an aseptic working environment in the field is a constant challenge. Working carefully and frequently (re)sterilizing surfaces are common approaches to minimizing potential contamination risks. To minimize contamination risks from sample collectors, collectors should wear sterile gloves during sample collection and frequently sterilize their gloves with either 70% ethanol or isopropyl alcohol in water. If multiple samples are being collected, gloves should be

thoroughly rinsed and sterilized, or preferably changed, between samples to prevent cross-contamination of samples. Collectors should also be careful to avoid breathing directly on the sample and not to touch the sample with any part of their body, including hair and clothing that has not been sterilized. It should be noted that ribonuclease (RNase) activity is abundant in bodily fluids, such as perspiration, skin oils, and saliva. Therefore, ungloved hands can introduce RNase contamination that compromises sample accuracy. To minimize contamination risks from collection gear and tools, all surfaces that will contact the sample should be thoroughly and frequently sterilized. Field gear can be sterilized with 70% ethanol or isopropyl alcohol, or by heat sterilization using a small, portable torch. Tools and gear that cannot withstand these treatments, or those with scratches or porous surfaces, should not be used to collect samples for microbiological analyses, as they are difficult to sterilize and increase risks of contamination. Alternatively, manufacturer-sterilized disposable tools can be used. When working with sensitive, valuable paleoecological samples (e.g., ancient glacial ice and permafrost sediments), every precaution should be employed to prevent contamination of the sample (e.g., wearing sterile caps and facemasks in addition to sterile gloves, and spiking drilling equipment with recognizable microorganisms to identify contamination). Microbes and/or their biomolecules are ubiquitous in all environments, resulting in an extremely high risk of contamination of paleoecological samples by contemporary microbial signatures. Even trace amounts of contaminant contemporary microbes or their biomolecules can cause inaccurate microbiological analysis results, especially when nonspecific cultivation or molecular amplification is used. A detailed discussion of precautions, controls, and criteria for reducing the risk of contamination of ice and permafrost samples, both in the field and in the laboratory, has been given by Willerslev et al. (2004).

The second consideration that must be made when collecting samples for microbiological analyses is selecting the appropriate container to store and transport the sample once it is collected. Commonly used containers include manufacturer-sterilized disposable sampling bags (e.g., Whirlpak brand bags), as well as sterilized vials, tubes, and bottles, which are rigid and thus provide structural protection for samples that are sensitive to compaction during transport. These container options are inexpensive and available in multiple sizes or materials to accommodate different sample volumes and analysis requirements. Special attention should be paid when the sample will be analyzed for biomolecules such as nucleic acids and proteins. If nucleic acids are to be analyzed, field samples should be collected in sterile, nuclease-free, nonpyrogenic containers for best performance. If peptides and proteins are to be analyzed, adsorption of the target biomolecules to the container surface should be considered, as these amphiphilic biomolecules can easily sorb to most surfaces. Choosing the optimal container helps avoid inaccurate measurements and false conclusions due to unpredictable peptide/protein loss (Goebel-Stengel et al., 2011). Other containers can also be used, but above all else, the selected container must be sterile prior to use to prevent contamination of the sample by the container. In addition to sterilization, labware used to collect samples for nucleic acid analyses should be pretreated to eliminate nucleic acids and nucleases for best performance. Labware can either be heated (e.g., 250+ °C for 2+ hours) or treated with commercially available surface decontaminants that can eliminate nucleic acids and nucleases.

The third consideration when collecting field samples for microbiological analyses is to maintain the sample under appropriate conditions both in the field and during transport. Due to the sensitivity of microbes to environmental changes and the subsequent shifts in cellular activity and community composition and structure, field samples should ideally be processed and analyzed immediately upon collection. If immediate processing is not feasible, samples should be preserved immediately upon collection. If field samples are to be analyzed using culture-dependent methods (e.g., cultivation and isolation), it is imperative to maintain the microbial community in the original sample as close to native as possible. This is difficult to achieve, as living organisms respond to small changes in the surrounding environment (e.g., temperature, humidity, and O_2 level) at the molecular, cellular, and population levels. Chilling samples can slow microbial metabolism and changes to the community, but will not prevent changes to the community. Usually, samples are kept on ice (~4 °C) in a cooler or at room temperature during collection and transport, and are processed for culture-dependent analyses as soon as feasibly possible to minimize potential changes and biasing of analysis results. If field samples are to be analyzed using culture-independent analyses (e.g., nucleic acid and protein analyses), samples are commonly frozen in dry ice or liquid nitrogen as quickly as possible and kept in the dark in the field to avoid degradation of biomolecules by enzymes such as nucleases and proteases, as well as by hydrolysis, oxidation, UV exposure, and other physicochemical processes. This is especially critical for samples that will be analyzed for ribonucleic acid (RNA); RNA is more susceptible than deoxyribonucleic acid (DNA) to degradation, because RNases are ubiquitous in the environment and the ribose sugar in RNA contains a 2′-hydroxyl group, which acts as a nucleophile in reactions with nucleases. Additionally, samples can be transferred to specific RNA storage and stabilization solutions (e.g., commercially available RNAlater solutions) before freezing to minimize degradation. When dry ice or liquid nitrogen is unavailable, samples should be kept on ice in the dark and transported to the laboratory as quickly as possible, although some degradation is expected to occur under these conditions. While there are commercially available reagents designed to simultaneously stabilize nucleic acids and proteins in tissue samples at room temperature, those reagents may or may not be suitable for environmental samples, given the complicated physical and biochemical characteristics of many environmental samples. The field-frozen samples should be transferred to a −20 °C freezer, or preferably a −80 °C freezer, upon arrival at the laboratory and should remain frozen until sample processing begins. Freeze-thaw cycles must be avoided as much as possible, as biomolecules will rapidly degrade during repeated freeze-thaw cycles.

1.1.3 Samples for Both Geochemical and Microbiological Analyses

Collecting field samples that will be used for both geochemical and microbiological analyses requires some preplanning. For example, considerations need to be made regarding whether strategies and methods for sample collection, preservation, and storage for one type of analyses will also accommodate the other type of analyses. Ideally, the same field sample would be used for both types of analyses, but extracting multiple aliquots from the same sample for multiple analyses has the potential to introduce contamination into the sample, especially if the tools used to extract the aliquots are not sterile or do not meet the

needs of specific analyses (e.g., using tools containing nucleases to prepare aliquots for nucleic acid analyses). If all requirements for both geochemical and microbiological analyses have been met, once in the laboratory, a small aliquot of the sample can be archived appropriately and used strictly for microbiological analyses to minimize contamination, and the remaining sample can be subsampled for geochemical analyses. However, in cases where the preservation and/or storage methods required for geochemical and microbiological analyses differ (e.g., room temperature vs. frozen, acidified vs. non-acidified, wet vs. dried), this approach is not feasible. Instead, replicate field samples should be collected and each replicate sample preserved and stored according to the requirements for the analyses to be performed on that replicate sample.

1.2 Geochemical Techniques

Investigating the large variety of materials and analytes in geochemical studies requires an equally large variety of techniques, from simple benchtop wet chemistry to extremely sophisticated approaches, and most often a combination of multiple approaches. In this section, basic approaches to geochemical investigations are presented. Considerations for collecting, preserving, and storing samples are discussed, followed by techniques applied for quantification of inorganic and organic compounds.

1.2.1 Inorganic Compounds

Geochemical studies rely heavily on the analysis of inorganic compounds to elucidate information and trends from samples under investigation. In this section, common analytical techniques used to quantify and characterize inorganic components are presented, including spectrophotometric and atomic spectroscopic techniques. Technique overviews, analysis considerations and limitations, sample preparation requirements, and analysis-specific data processing procedures are covered. For common analytes, step-by-step methods are also provided.

1.2.1.1 Spectrophotometric Techniques

Spectrophotometric techniques allow users to quantify the concentration of an analyte by measuring the absorbance of energy (i.e., light) by the analyte or the analyte after reaction with a photosensitive compound. The principles of spectrophotometric techniques are based on adaptations to the Beer–Lambert Law, which states that the absorbance is linearly proportional to the concentration of the absorbing species and the pathlength of the sample (Kaur, 2007; Tan, 1996). Because it is difficult to measure absorbance directly, most techniques directly measure transmittance and calculate the corresponding absorbance. Today, capabilities exist to perform spectrophotometric analyses in the ultraviolet, visible, and infrared regions of the electromagnetic spectrum. Infrared spectroscopy is discussed in more detail in Chapter 12.

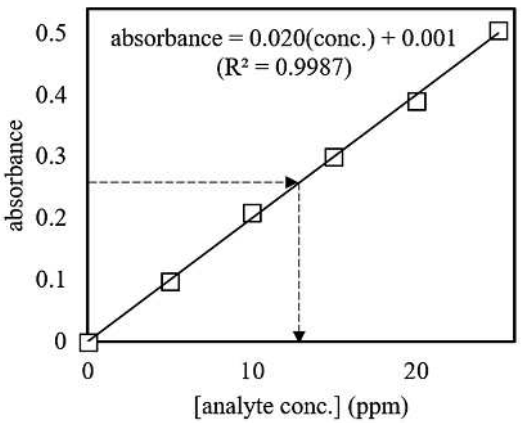

Figure 1.1 Example calibration curve for an analyte ranging in concentration from 0 to 25 ppm. The resulting calibration equation is included for illustration. The arrows indicate the calculation performed to determine the analyte concentration using the absorbance value from the sample and the calibration curve. For some techniques, absorbance will be replaced by the concentration-dependent metric (e.g., intensity, peak height, peak area) specific to the technique.

This section introduces colorimetric assay and ion chromatography (IC) methods, which are common spectrophotometric techniques used in analyzing geochemical samples. Colorimetric assays quantify concentrations of a single analyte using a reagent to produce a reagent–analyte complex, which absorbs light at a characteristic wavelength. Generally speaking, these assays are selective for the target analyte and are not sensitive to complex sample matrices. However, potential interfering compounds for each assay are discussed later. IC quantifies multiple analytes from a single sample by passing the mixture through sorbent-packed columns that separate the analytes based on chemical properties of both the sorbent and the analytes. For both colorimetric assay and IC analyses, analyte concentrations are calibrated by treating a set of standards using the same method used to treat samples. The standards are analyzed, and the resulting calibration curve is used to calculate the concentration of the analyte in a sample (Figure 1.1). The r^2 values for all analyses included in this section should be ≥0.99; if the resulting r^2 value is lower, create a fresh set of standards and start over.

1.2.1.1.1 Colorimetric Assay – Iron

Due to its prevalence in geomedia and biological tissues and its importance in redox cycles, iron (Fe) is an element of common interest in geochemical investigations. In some cases, researchers are interested in understanding the redox cycling of Fe and want to measure both the oxidized ferric (Fe(III)) and reduced ferrous (Fe(II)) valences in their sample. In other cases, researchers are interested in quantifying Fe extracted from Fe minerals. Common Fe mineral extraction methods include the dithionite-citrate-bicarbonate (DCB) method for extracting long- and short-range order Fe minerals (Jackson, 2005; Mehra and

Jackson, 1960; Pansu and Gautheyrou, 2006), the HCl method for extracting short-range order Fe minerals (Lovley and Phillips, 1986), and the Tamm's reagent method for extracting colloidal Fe (Blakemore, 1968). Whatever the case, the ability to quantify Fe is a useful geochemical technique.

Aqueous and extracted Fe concentrations can be quantified using simple colorimetric assays (Verschoor and Molot, 2013), including the ferrozine method (Stookey, 1970; Viollier et al., 2000). The ferrozine method is widely used and a preferred Fe assay for several reasons. First, the method is rapid, requires few chemicals, and is relatively inexpensive to perform. Second, the ferrozine method uses a non-reducing reagent to generate the color compound and results in more accurate quantification of ferrous ions, unlike other methods (e.g., TPTZ) that use strong reducing agents and overestimate ferrous content. Third, the method is easily adapted. A common method adaptation is selecting a reagent buffer that is appropriate for the experimental samples. The most common reagent buffer is ammonium acetate, but sodium acetate, 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES) and 1,4-piperazinediethanesulfonic acid (PIPES) buffers are also commonly used. The method can also be easily adapted to accurately quantify Fe within the ppb-to-ppm range.

The ferrozine reagent forms a color compound with ferrous iron only. Thus, ferrous Fe can be directly quantified using the ferrozine method. Ferric Fe, however, must first be reduced to ferrous iron before quantification using this method. To reduce ferric iron, ascorbic acid or hydroxylamine hydrochloride reductants are routinely used. Ascorbic acid is often preferred because it is an efficient reducing agent at room temperature. However, it loses its reducing ability shortly after being mixed with the sample (~2 h), and reduced Fe can reoxidize; if samples are prepared and immediately analyzed, this is not a concern. Hydroxylamine does not lose its reducing ability over time and has a long shelf-life once the reagent is prepared, but it must be purified to remove contaminant Fe before it can be used in the assay. If more than ~2 h will elapse between sample preparation and analysis, hydroxylamine is the preferred reductant.

There are several considerations to be made when performing the ferrozine method. First, the ferrozine method is best performed in an anoxic environment, such as in a glovebox or glovebag with an N_2 atmosphere, to eliminate the risk of oxidation of ferrous Fe. When working with low concentrations of Fe, it is safest to also sparge the ferrozine reagent with N_2 to remove dissolved oxygen. Second, adaptations made to the method may alter the ferrozine and/or reductant reaction. For each adaptation, new method controls should be performed to verify and optimize that method. For example, changing a buffer system may yield a longer color development period, necessitating a kinetics control to determine the reaction time required to reach color optimization. Third, color development for the ferrous–ferrozine compound is achieved between pH 3 and 8, and is most stable between pH 4 and 6 (Schilt and Hoyle, 1967). If an alternative buffer system is selected, it should be adjusted to and stable within this pH range and should not absorb energy at 562 nm. Lastly, Si in the sample can interfere with the ferrozine method. The orthophenanthroline (phen) method (Fortune and Mellon, 1938; O'Connor et al., 1965) is the appropriate Fe assay to use if high concentrations of Si are expected in a sample (e.g., in samples extracted from Si-rich materials). Other interfering