

Molecular Microbiological Methods— Sample Handling and Laboratory Processing

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Molecular Microbiological Methods— Sample Handling and Laboratory Processing

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Foreword

A set of guidelines for collection, preservation, and molecular microbiological analyses (quantitative PCR and 16S rRNA-based taxonomic profiling) of environmental samples is described in this standard. The guidelines provided in this standard aim to provide common procedures and best practices for such analyses to be performed by different laboratories to generate comparable results.

Scope

This standard is applicable to operators, service companies, third-party laboratories, consultants, and universities that perform molecular techniques to identify microorganisms in industrial settings, including microorganisms involved in microbiologically influenced corrosion (MIC), biogenic production of hydrogen sulfide (a process also known as souring), and biofouling in industrial settings and organizations that manage assets affected by corrosion threats. This standard should be used to select appropriate procedures for sample collection, preservation, laboratory processing, and data analysis.

Rationale

As the use of Molecular Microbiological Methods (MMM) increases in numerous industries, the pursuit of consistent, comparable results by different users of these methods requires the standardization of procedures and methods.¹ This standard provides guidelines to enable different laboratories to utilize consistent methodologies to generate comparable genetic data on environmental samples.¹

Referenced Standards and Other Consensus Documents

Unless specifically dated, the latest edition, revision, or amendment of the documents listed in the table below shall apply.

AMPP/NACE/SSPC, www.amp.org:

NACE TM0212	Detection, Testing, and Evaluation of Microbiologically Influenced Corrosion on Internal Surfaces of Pipelines
NACE TM0194	Field Monitoring of Bacterial Growth in Oil and Gas Systems
NACE/ASTM G193	Standard Terminology and Acronyms Relating to Corrosion

In AMPP standards, the terms *shall* and *must* are used to state requirements and are considered mandatory. The term *should* is used to state something that is recommended, but is not considered mandatory. The term *may* is used to state something considered optional.

Section 1: General

- 1.1 Microbiological analysis of various types of samples is performed to gain an understanding of the diversity, number, and distribution of microorganisms in a given environment, often to answer a question or solve a problem. In the past decade, the use of DNA-based methods (or molecular microbiological methods [MMM]) has continually grown in sectors beyond the human microbiome and medicine where such methods have long been employed.
- 1.2 Industry is applying MMM to solve problems such as MIC, biofouling, biogenic production of the toxic and corrosive hydrogen sulfide, and other important issues; however, few end-users of molecular data in industry are well-informed as to the advantages, limitations, sample handling, processing requirements, and analytical details of MMM.
- 1.3 One major challenge is comparing MMM results that are produced by different laboratories using different methods and protocols. The lack of ability to compare results can hinder the adoption of MMM to solve important industry issues.
- 1.4 Thus, the purpose of this document is to provide an overview of the best practices to conduct MMM.

Section 2: Definitions

Abiotic: The absence of living organisms, their biological components, or the metabolic activities of living organisms.

Acid-producing Bacteria (APB): Aerobic or anaerobic bacteria that produce organic acids as a product of their metabolism. A few organisms (e.g., *Thiobacillus*), can also produce mineral acids (typically under aerobic conditions).

Aeration: (1) Exposing to the action of air. (2) Causing air to bubble through. (3) Introducing air into a solution by spraying, stirring, or similar method. (4) Supplying or infusing with air, as in sand or soil. (5) The introduction of air into the pulp in a flotation cell to form air bubbles.

Aerobic Microorganism (aerobe): A microorganism that uses oxygen as the final electron acceptor in metabolism.

Anaerobic Microorganism (anaerobe): A microorganism that does not require oxygen for metabolism.

Archaea: Unicellular microorganisms that are genetically distinct from bacteria and eukaryotes, which often inhabit extreme environmental conditions. *Archaea* include halophiles (microorganisms that may inhabit extremely salty environments), methanogens (microorganisms that produce methane), and thermophiles (microorganisms that can thrive in extremely hot environments). *Archaeoglobus* is a common *Archaea*.

Autoclave: A pressurized, steam-heated vessel used for sterilization.

Biofilm: Microbial growth at an interface in which individual cells are bound within a matrix of extracellular polymeric materials.

Biotic: Involving the presence or metabolic activities of living organisms.

Carbohydrate: Any of the group of organic compounds composed of carbon, hydrogen, and oxygen, including sugars, starches, and celluloses.

Cleaning Pig: A device inserted in a pipeline for cleaning solids or displacing liquids or hydrocarbons from within a pipeline.

Corrosion: The deterioration of a material, usually a metal, that results from a chemical or electrochemical reaction with its environment.

Corrosion Threat: Potential failure of a system vulnerability due to corrosion.

Culture Medium: A sterile solution or other substrate formulated to promote the growth of a particular type or group of microorganisms. (Also called *Growth Medium*).

Deoxyribonucleic Acid (DNA): A self-replicating material that is present in nearly all living organisms as the main constituent of chromosomes. It is the carrier of genetic information.

Dissimilatory: Metabolic reactions in which a reductant is used as an electron acceptor and material is not incorporated into the cell (e.g., dissimilatory sulfate or nitrate reduction); metabolic changes that convert complex molecules into simple ones.

DNA Sequencing: The process of determining the nucleic acid sequence – the order of nucleotides in DNA.

Eukaryotes: Cells having a true nucleus, bound by a double membrane. Prokaryotic cells have no nucleus.

Facultative: Capable of growing either with or without the presence of a specific environmental factor, e.g., oxygen.

Fungi: Nucleated, usually filamentous, spore-bearing microorganisms devoid of chlorophyll, which include molds, mildews, smuts, mushrooms, yeasts, and others. Fungi are often found to degrade fuel (e.g., fuel spoilage).

Growth: An increase in cell size, cell numbers, or both.

Growth Medium: See *Culture Medium*.

Inoculum: A small quantity of microorganisms used to start a new culture.

Inorganic Acid: A compound composed of hydrogen and a nonmetal element or radical; examples are hydrochloric acid (HCl) and sulfuric acid (H₂SO₄). A substance that yields hydrogen ions when dissolved in water and that can act as a proton donor.

Isotonic: A solution that has uniform tension; having the same osmotic pressure as the fluid phase of a cell or tissue.

Methanogens: Microorganisms that produce methane as a metabolic by-product in anoxic (i.e., oxygen-free) conditions. They are classified as *Archaea*, a group quite distinct from bacteria. Some are extremophiles and found in environments such as oil field systems, hot springs, and submarine hydrothermal vents, as well as in the “solid” rock of the Earth’s crust, kilometers below the surface. Methanogens are common *Archaea* in oil production systems; however, they are normally not measured with current culturing techniques. Methanogens are involved in MIC by consuming hydrogen at the metal surface and thereby creating a depolarization.

Microbe: See *Microorganism*.

Microbial Morphology: A branch of biology that deals with the structure and form of an organism at any stage of its life history.

Microbiologically Influenced Corrosion (MIC): Corrosion affected by the presence or activity, or both, of microorganisms. The microorganisms that are responsible for MIC are typically found in biofilms on the surface of the corroding material. Many materials, including most metals and some nonmetals, can be degraded in this manner.

Microorganism: An organism of microscopic or ultramicroscopic size. Bacteria, *Archaea*, and fungi are microorganisms. Bacteria and *Archaea* are combined and called prokaryotes. Fungi belong to eukaryotes (*Eukarya*).

Nucleotide: A compound consisting of a nucleoside linked to a phosphate group. Nucleotides form the basic structural unit of nucleic acids such as DNA.

Organic Acid: Weak acid that contains carbon (correctly classified as a carboxylic acid because it contains a carboxyl group, –COOH). Organic acids (e.g., formic, acetic, lactic) are the end product of metabolism by a variety of microorganisms.

Organism: A complex structure of interdependent and subordinate elements whose relations and properties are largely determined by their function as a whole (Also see *Microorganism*).

Phosphate Buffer: A solution made of dibasic potassium phosphate (K_2HPO_4) and sodium phosphate (Na_2HPO_4).

Polymerase Chain Reaction (PCR): A molecular technique that allows the production of large quantities of a specific DNA sequence from a DNA template using a simple enzymatic reaction without a living organism. A quantitative version of PCR is called Quantitative Polymerase Chain Reaction (qPCR).

Polysaccharide: A carbohydrate composed of many monosaccharides.

Prokaryotes: The prokaryotes are divided into two domains: the *Bacteria* and the *Archaea*. *Archaea* were originally thought to live only in inhospitable conditions such as extremes of temperature, pH, and radiation, but have been found in all types of habitats. Sulfate-reducing prokaryotes (SRP) consist of both sulfate-reducing bacteria and sulfate-reducing *Archaea*.

Quantitative Polymerase Chain Reaction (qPCR): A molecular microbiological method used to quantify the total number of microorganisms (i.e., dead and alive) or a specific genus/species of microorganisms in nearly any type of sample. qPCR can be used for both fluid and solid samples, as well as microorganisms collected via membrane filtration. This method does not underestimate microorganisms that do not grow in culture. This method uses synthetic DNA (called primers) tagged with a fluorescent molecule or synthetic DNA mixed with a DNA intercalating agent (dye) to quantify microorganisms using a modified version of polymerase chain reaction (PCR).

Ribonucleic Acid (RNA): A nucleic acid present in all living cells. Its principal role is to act as a messenger carrying instructions from DNA for controlling the synthesis of proteins, although in some viruses, RNA rather than DNA carries the genetic information.

Ringer's Solution: An aqueous solution of chlorides that is isotonic to animal tissues.

Robbins Device: A tool to provide samples of biofilms growing on submerged surfaces, typically consisting of a long chamber through which the test fluid flows, exposing a number of removable plugs or coupons to the test conditions.

Sterile: Free of any living microorganisms. Not introducing microorganisms that are foreign to the host body or subject under study.

Sulfate-reducing Archaea (SRA): A group of anaerobic *Archaea* that perform dissimilatory reduction of sulfate, resulting in sulfide formation. They are most likely to grow at reservoir conditions (140 to 203 °F [60 to 95 °C]).

Sulfate-reducing Bacteria (SRB): A group of anaerobic bacteria that perform dissimilatory reduction of sulfate, resulting in sulfide formation. They grow at a broad range of temperatures.

Sulfate-reducing Prokaryotes (SRP): A group of microorganisms that consists of both sulfate-reducing bacteria (SRB) and sulfate-reducing archaea (SRA).

16S Ribosomal RNA Gene: Gene encoding the RNA component of the 30S subunit of a prokaryotic ribosome.

Section 3: Sample Type Definition

Four different sample types are considered in this standard: Liquid, Solid, Wet Gas, and Surface. Note that for molecular analysis of microbial communities, it is not necessary to maintain the cells in a live state. Definitions and descriptions of the four different sample types are given below:

3.1 Liquid Samples

- 3.1.1** Water-based or aqueous samples are the focus of the liquid sample processing procedures described in this standard. Examples of samples containing water can include produced water (water

that is retrieved along with crude oil or gas during hydrocarbon recovery operations), injection water (water that is used to inject into oil and gas fields for facilitating hydrocarbon recovery), water from water-transporting pipelines, water from gas-transporting pipelines, and water that may be stored either in a receptacle, such as a pond or tank that is used for field injection or that has been collected following recovery operations, cooling, and heating media, firewater, and utility water. It should be noted that these types of water-based samples may not be completely free of crude oil and may contain emulsions of oil and water or particulates from recovery operations. Such samples can still be considered for the protocols described herein.

3.2 Solid Samples

3.2.1 Solid samples for the purposes of this standard are those with no discernable liquid content (e.g., samples do not flow as liquids), such that the sample must be collected and/or manipulated with a spatula or similar; it does not readily pour and could not be obtained from a pipeline (or vessel) sample point tap or drain valve. Examples of solid samples and their origin include:

3.2.1.1 Surface associated: Material collected from a pipe or vessel surface (internal or external) that has been exposed to production fluids or another environment. The solid sample may encompass sludges, corrosion products, soil, and biofilm.

3.2.1.2 Pigging residue: Material recovered during maintenance or cleaning pigging from the pig itself, the pig receiver barrel, or the fluid envelope located ahead of the pig which may be filtered to isolate any solids (see NACE TM0212, Paragraph 6.7.5).

3.2.1.3 Mudzone: Material that is located below the interface of a water body such as sea, lake, or riverbed, and is covered by mostly solid material. (See NACE/ASTM G193).

3.2.1.4 Corrosion products: Material deposited on an infrastructure surface due to a corrosion process, such as metal oxides, carbonates, and sulfides. They may be present in a wide range of sizes and morphologies and can be found in situ or transported. (See NACE TM0212, Paragraph 6.8.4).

3.3 Wet Gas Samples

3.3.1 Wet gas is natural gas that contains condensate or liquid hydrocarbons, and sometimes free water. Wet gas sampling can be divided into two types:

3.3.1.1 Representative sampling collects representative fractions of the gas and liquid streams.

3.3.1.2 Non-representative sampling extracts quantities of either the gas or liquid stream for analysis of composition.

3.3.2 Liquid can be distributed in a pipeline by either flowing along the bottom of the pipe; condensing on the top of the pipe or distributed as liquid droplets in the gas. If available, collect condensate from condensate drop out points. The sampling methods described herein are for sampling liquid droplets in the gas.

3.4 Surface Samples: Samples that are collected from surfaces (such as pipe surfaces and corrosion coupons) using sterile cotton or polyester fiberfill samplers to determine surface-attached (biofilm) microbial communities.

Section 4: Sampling and Preservation Procedures

4.1 General Requirements

- 4.1.1** This section describes the equipment requirements and sampling procedure.
- 4.1.2** As a best practice, biological samples should be maintained at low temperature to maintain the integrity of the samples and avoid population shifts due to microbial growth during transfer of the samples from the sample location to the laboratory. The samples should be maintained at temperatures of 39 °F (4 °C) or below as soon as they are retrieved from the system. In situations when maintaining the samples at low temperature is not feasible (e.g., remote locations, international shipments, etc.), addition of a chemical preservative is critical.
- 4.1.3** A minimum amount of sample should be equal to the limit of detection of quantitative PCR and 16S rRNA gene sequencing (~10 pg of ATP/unit or ~1x10³ CFU/unit determined by ATP or serial dilution, respectively). Extracting DNA from larger amounts of sample provides a better representation of the sampled population and improves the sample signal to be distinguished from the noise of unrelated microbial contaminants. However, DNA concentrations above 300 ng/mL extracted from samples shall require dilution.
- 4.1.4** In general, any materials that come into contact with a sample should be sterile, such as sampling implements used for collecting samples (e.g., swabs, spatulas), the vessels into which the samples are placed (e.g., bottles, tubes), and any other processing supplies (such as filters, tubing, valves, and filtration units). Specific sampling materials for each sample type are given in the sections below; however, common recommended sampling materials used for collecting all sample types should include the following:
- 4.1.4.1** Latex or nitrile gloves.
 - 4.1.4.2** Labels.
 - 4.1.4.3** Plastic zip-sealed bags (1 L).
 - 4.1.4.4** Permanent marker.
 - 4.1.4.5** Cooler and ice packs (if available).
- 4.1.5** Considerations about chemical preservatives: Numerous chemical preservatives are available, and they all present pros and cons; some DNA/RNA buffers may contain surfactants which may aid in homogenizing the preservative buffer with the sample.² The following types of preservatives are examples of those currently used:
- 4.1.5.1** Alcohols (such as 70% isopropanol or 95% ethanol).
 - 4.1.5.2** Commercially available DNA preservative (e.g., such as one containing guanidinium thiocyanate-based preservative). A commercially available RNA preservative may also be used (e.g., one containing quaternary ammonium sulfate).
 - 4.1.5.3** Preservative buffers (e.g., DESS [dimethyl sulfoxide, disodium EDTA, saturated NaCl], NAP [Nucleic Acid Preservation buffer]).

4.2 Collection of Liquid Samples

- 4.2.1** Different sampling materials and procedures may be required depending on whether samples are filtered and preserved immediately after collection (on-site), or whether samples are preserved without filtration before transport.