Fuel and Fuel System Microbiology:

fundamentals, diagnosis, and contamination control

Editor:
Frederick J. Passman

Fuel and Fuel System Microbiology—Fundamentals, Diagnosis, and Contamination Control

Frederick J. Passman, Editor

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Foreword

This publication, *Fuel and Fuel System Microbiology—Fundamentals, Diagnosis, and Contamination Control*, was sponsored by ASTM International Committee D02 on Petroleum Products and Lubricants. The editor was Frederick J. Passman.

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Preface

Frederick J. Passman, Ph.D[1]

The *Manual on Fuel and Fuel System Microbiology—Fundamentals, Diagnosis, and Contamination Control* augments Standard Guide D 6469[2]. It is addressed to all liquid fuel production, transportation, and consumption stakeholders. The target audience includes management, supervisory, operational, quality assurance, maintenance, inspection, and technical personnel responsible for fuel quality, fuel handling equipment integrity, or both. The material presented in this Manual is equally applicable for gasoline, diesel (including biodiesel), aviation turbine, marine, industrial gas turbine, kerosene, gasoline, and aviation gasoline fuels. Much of the information is also applicable to other fuel grades ranging from bunker to natural gas.

This manual seeks to complement the Guide D 6469 in each of four areas. Chapter 1 provides an overview of the microbiological principles underlying fuel and fuel system biodeterioration. The information contained in this chapter will enable the reader to better understand why recognizing biodeterioration is difficult yet essential.

Sampling for microbial contamination detection presents unique challenges. Both the non-homogeneous distribution of microbes and the fact that they are living beings necessitate special handling, not discussed in Standard Practice D 4057 Manual Sampling of Petroleum and Petroleum Products[3]. Consequently, Chapter 2 provides the detailed information personnel need to collect and handle samples intended for biodeterioration diagnosis.

Chapter 3 provides specific, practical recommendations for disinfecting and removing microbial contamination from fuels and fuel systems.

As noted earlier, D 6469 recommends a variety of diagnostic tests, many of which do not appear in the Annual Book of Standards, Volume 5. Since quite a few of the tests examine bottom water properties, they aren't run at fuel labs routinely. Nearly all of the methods that aren't drawn from Volume 5 come from the Annual Book of Standards, Volumes 10, 11, or 14. By incorporating the Standards from these three volumes into this Manual, it was our intention to improve test method accessibility, which would expand the diagnostic capabilities of fuel quality labs.

Our objective in developing the *Manual on Fuel and Fuel System Microbiology—Fundamentals, Diagnosis, and Contamination Control* was to provide a broad range of stakeholders with a readable, accessible insight into the nature of fuel and fuel system biodeterioration, sampling requirements, test methods, and remediation practices.

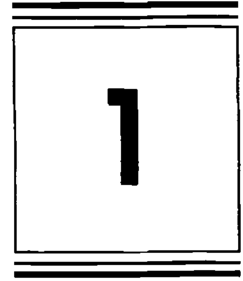
As the Editor of this Manual and Chair of the D.02.14 Task Force on Microbial Contamination, I thank those ASTM International colleagues who have been indispensably helpful in the development of both D 6469 and this document. Harry Giles and Erna Beal, Chair and Secretary of D.02.E.05 and D.02.14 have been remarkably supportive since my friend and colleague Howard Chesneau first proposed inclusion of microbial contamination in each of the product standards under the cognizance of Subcommittees D.02.A, E, and J. I offer my sincerest thanks also to Howard Chesneau, Andy Pickard, and John Bacha, who each contributed tremendously to the development of the Guide and the Manual. Sadly, John Bacha's untimely death in August, 2001 prevented him from seeing the publication of this manual. I dedicate this manual to him

[1]President, Biodeterioration Control Associates, Inc., PO Box 3659, Princeton, NJ 08543-3659.
[2]Annual Book of ASTM Standards, Vol. 05.04.
[3]*Annual Book of ASTM Standards*, Vol 05.02.

in appreciation for his contributions and many years of dedication and commitment to fuel quality science.

Finally, without the guidance and support of ASTM Staff Members Kathy Dernoga, Monica Siperko, and Holly Stupak, the Manual would never have been created. Thank you all.

Fredrick J. Passman
Princeton, New Jersey, USA



Introduction to Fuel Microbiology

Frederick J. Passman, Ph.D.[1]

INTRODUCTION

UNCONTROLLED MICROBIAL CONTAMINATION in fuels and fuel systems causes biodeterioration problems that translate into substantial economic loss. Biodeterioration's adverse economic effects constitute one *cost of quality* category.[2] Microbial contamination problems are sometimes difficult to diagnose, and require the expertise of a microbiologist experienced in biodeterioration. Often, however, well informed stakeholders can recognize microbial contamination and take effective action to control it. Consequently, if all personnel involved with fuel and fuel system stewardship have a general understanding of fuel microbiology, they will be better prepared to reduce the costs of quality caused by biodeterioration. This chapter provides an overview of microbiology fundamentals pertinent to understanding fuel and fuel system biodeterioration. It opens with an explanation of the microorganisms likely to inhabit fuel systems, and then reviews their primary activities. The next section explains how air, water-content, temperature and other key variables affect biological activity. The final section provides an overview of fuel system microbial ecology.

BIODETERIORATION

Biodeterioration refers to all processes by which organisms affect materials adversely, either directly or indirectly. Food spoilage, fouling, and microbially influenced corrosion (MIC) are well-known examples of biodeterioration. Direct, or first order biodeterioration, occurs when organisms consume a material directly, using it as a food source. Indirect biodeterioration includes all of the detrimental, incidental effects of organism activity. Indirect biodeterioration may be removed from the actual deterioration process by one or more *degrees*. The greater the number of degrees of separation (or *process steps*) that exist between biological activity and an observable deterioration process, the more difficult it becomes to demonstrate the relationship between microbial contamination and the symptoms. Organisms that participate in the biodeterioration process, either directly or indirectly, are called *biodeteriogens*.

MIC processes illustrate second and third degree biodeterioration. Microorganisms growing within biofilms on metal surfaces excrete waste products, or *metabolites*. Polymeric metabolites form the biofilm matrix. Because surfaces aren't coated uniformly, physicochemical conditions at the fluid-metal interface of biofilm-free areas will differ from those at the fluid-metal interface of biofilm covered areas. These differences provide the driving force for a variety of gradients, the most readily measured of which is the electropotential gradient, or *Galvanic cell* (measured potentiometrically in mV). Galvanic cell formation represents second-degree biodeterioration, since it's one step removed from direct bioconversion. Many metabolites are weak organic acids. Inorganic salts, such as sodium chloride salts can react with these weak acids, forming strong inorganic acids (for example, hydrochloric) that etch the metal surface with which they are in contact. Since the reaction between inorganic acids thus produced, and metal surfaces is two steps removed from the process of weak acid production, it is an example of third-degree biodeterioration.

Recognizing the possibility that organisms may be playing a subtle but pivotal role in deterioration symptomology is critical to successful root cause analysis (RCA[3]) and deterioration control. Biodeterioration includes the adverse activities of all organisms ranging from bacteria to mammals. However, microorganisms are the predominant biodeteriogens in fuels and fuel systems. This chapter provides the basic information needed to understand fuel system microbiology.

MICROBIOLOGY BASICS

Microbiology Defined

Microbiology is the branch of science devoted to the study of organisms that are too small to be seen with the naked eye.

[1]President, Biodeterioration Control Associates, Inc., PO Box 3659, Princeton, NJ 08543-3659.

[2]Costs of quality include all material, production, transportation costs attributable to quality issues. These costs include both product or system deterioration and the budgeted costs of preventing such deterioration. Under normal circumstances, the cost of problem prevention is a fraction of cost of correction. Problem correction often includes waste handling expenses, lost productivity, lost revenues and lost good will.

[3]Root cause analysis (RCA) is a formalized process for diagnosing the fundamental cause of a quality problem. A number of process management experts, most notably W. Edwards Deming [1], have detailed the details and philosophy of RCA. RCA's principal objective is to go beyond obvious, apparent cause and effect relationships by uncovering underlying causes. These causes are typically process weaknesses. By shifting the focus from individual problem events to process variables, RCA facilitates long-term quality improvement and its consequent reduction in costs of quality.

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Microbiologists study viruses, bacteria, archaea, fungi, and blue-green algae.

Viruses occupy one end of the size and complexity spectrum. Typically comprised of only some genetic material encased in a protein coat, smaller viruses may measure less than 100 Å[4] in diameter.

Fungi and blue-green algae are at the other end of the size spectrum included in the study of microbiology. Some representatives of these two groups are large enough to be visible to the naked eye. Both are *eukaryotes*.[5] Their structural complexity is comparable to that of the cells of all higher organisms. All eukaryotes contain a membrane bound nucleus and other membrane bound bodies called *organelles*. The nucleus holds most of the cell's genetic material, deoxyribonucleic acid (DNA). Neither bacteria nor archaea have internal, membrane-bound bodies. Viewed under a microscope, cells from the Bacteria or Archaea domains appear to be filled only with a grainy, gel-like substance called *protoplasm*.

Although Archaea species have been recovered from petroleum formations, and are known to grow on C_1 and C_2 hydrocarbons, there haven't been any reports of Archaea species being recovered from fuels or fuel systems, yet.[6] To date, microbiologists have recovered only bacteria, fungi and, occasionally, algae (both blue-green algae and true algae). The remaining discussion within this chapter will focus on the bacteria and fungi.

Bacteria

As noted previously, bacteria are single-cell organisms that lack the membrane-bound organelles that define all higher life forms (the eukaryotes). Historically, microbiologists characterized bacteria based on their shape and physiological characteristics. Figure 1 illustrates the most common bacterial shapes.

The primary physiological traits used to categorize bacteria include:

- Cell wall chemistry—reaction to stains
- Ability to transform into dormant endospores
- Source of energy for metabolism (sunlight, organic molecules, oxygen, or other inorganic ions)
- Requirement or tolerance for oxygen
- Requirements for specific food molecules (carbon dioxide or organic molecules)
- Ability to produce specific end-products (metabolites)
- Motility

The most commonly used stain test is the Gram stain, developed by Christian Gram in 1884. The stain neatly divided bacteria into two categories: Gram positive (G +) and Gram negative (G −). When observed under a light microscope, G + bacteria appear blue or violet colored. G − bacteria appear pink.

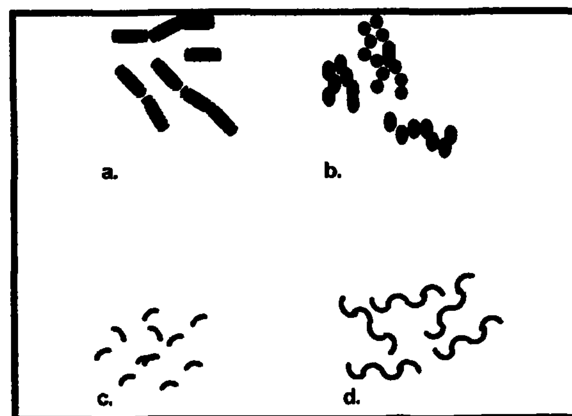

FIG. 1—Typical shapes of bacteria: (a) rods (bacilli); (b) spheres (cocci); (c) comas (vibrios); (d) spirals.

Some bacteria are able to form endospores when conditions become unfavorable. Unlike fungal spores, which are reproductive bodies, endospores are essentially dried out, dormant (inactive) cells in a protective coating. Under the right conditions, endospores regerminate into active (vegetative) cells. All of the known spore-forming bacteria are G + rods.

All cells engage in two primary types of metabolism. Energy metabolism provides the energy for all of the cell's activities. The other, *anabolism*, includes all of the processes for building new cell parts. Microbes meet their energy requirements by three means. *Photosynthetic* organisms convert light into energy directly. All other organisms get their energy from organic or inorganic molecules.[7] *Oxidative* metabolism uses inorganic molecules such as oxygen, sulfate or nitrate.[8] *Fermentative* metabolism uses organic molecules.

Microbes that depend on oxygen for oxidative metabolism are known as *aerobes*. Aerobes cannot grow in the absence of oxygen. *Obligate anaerobes* cannot tolerate oxygen. Some obligate anaerobes depend on sulfate or nitrate for their oxidative energy metabolism. Fermentative anaerobes use organic molecules. Some types of bacteria can operate as aerobes when oxygen is available, and can shift their energy metabolism to fermentation once oxygen has been depleted from their environment. These microbes are called *facultative anaerobes*. As we shall see below, facultative anaerobes play a critical role in fuel system biodeterioration.

Autotrophic bacteria can get by with carbon dioxide (CO_2) as their sole nutrient. Heterotrophic bacteria require organic molecules as food from which to manufacture new cell com-

[4]Å–Angstrom unit = 10^{-10} M or 10^{-1} μm.

[5]Current taxonomy divides all life into three domains: the bacteria, the Archaea and the eukaryotes. When initially discovered in the 1980s, the Archaea were classified among the bacteria. Subsequent research has demonstrated that the cell wall and cell membrane structures differentiate the two domains, as do other cell properties.

[6]It is quite likely that the absence of reports of Archaea in fuel systems reflects the limitations of microbiological science rather than system ecology. Methods used for Archaea sampling, isolation and cultivation are different from traditional microbiological methods. Few laboratories have the requisite expertise of facilities to recover and investigate Archaea.

[7]Strictly speaking, these microbes use inorganic molecules as terminal electron acceptors in a metabolic pathway called the electron transport system, the cell's energy generator. For a fuller discussion of these metabolic pathways, the interested reader may refer to any introductory microbiology textbook.

[8]Oxidative metabolism is driven by a series of cascading energy-exchange reactions called the *electron transport system*. The final electron exchange in this process transfers an electron from a cytochrome molecule to a *terminal electron acceptor* (O_2, NO_2^-, NO_3^-, or $SO_4^=$).

ponents. Bacteria exhibit tremendous nutritional diversity. Organic molecules ranging from methane to gigantic polymers used in composite material construction are biodegradable. As will be explained later in this chapter, not all biodegradable molecules are used as nutrients. Biodegradation includes all processes by which organisms break molecules down or otherwise transform them. Although molecules that are biodegradation products may be nutrients, they aren't necessarily so. Some may actually be more toxic that the original molecule from which they were derived. Nutrient molecules include those that provide energy, serve as building blocks for biomolecules or do both. Larger molecules generally are first broken down through a complex series of enzymatic processes before cells actually utilize derivative molecules as food. Microbial taxonomists will test bacterial isolates for their ability to grow on several hundred different nutrients in order to identify the isolate. Taxonomists will also test for characteristic metabolite production.

Some types of bacteria are mobile. Most mobile bacteria are propelled by whip-like structures called *flagella*. Flagella may be attached at the cell's ends (*polar flagella*) or attached all over (*peritrichous flagella*). Some bacterial species are propelled across surfaces by secreting a mucilaginous (or slimy) substance. This type of slime-jet driven motion is called *gliding motility*.

All of the aforementioned characteristics used to categorize microbes depend on phenotypic properties. Phenotypic properties may vary with changes in environmental conditions. Historically, this has led to considerable confusion in terms of bacterial taxonomy. More recently, as the science of molecular biology has matured, taxonomists are relying more on genotypic tests. These tests compare deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) among characterized and uncharacterized bacteria. Individual isolates are then clustered into taxonomic groupings (taxa) based on the degree (percentage) of match between their respective DNA or RNA molecules. Table 1 lists the types of bacteria most commonly recovered from fuel systems.

In summary, it's difficult to determine a bacterium's correct taxonomic designation. Although more than a million different species of bacteria have been identified, microbiologists estimate that we've only discovered 1/10,000th of the different bacterial species on earth [1]! Fortunately, for personnel responsible for controlling microbial contamination in fuel systems, non-taxonomic information is easier to obtain and generally more useful for contamination control decisions.

Fungi

Fungi comprise the second major group of microbes commonly recovered from fuel systems. The fungi include diverse types of organisms ranging from single-cell yeasts to large mushrooms [2].

TABLE 1—*Bacteria commonly recovered from contaminated fuel samples.*

Taxa	Taxa
Acinetobacter spp.	Desulfovibrio desulfuricans
Aerobacter spp.	Flavobacter spp.
Aeromonas spp.	Micrococcus spp.
Alcaligenes spp.	Pseudomonas spp.
Bacillus spp.	

TABLE 2—*Fungi commonly recovered from contaminated fuel samples.*

Taxa	Taxa
Aspergillus spp.	Paecillomyces spp.
Candida spp.	Penecillium spp.
Cephalosporium spp.	Phialophora spp.
Fusarium spp.	Rhodotorula spp.
Hormoconis resinae	Trichoderma spp.

The two types of fungi typically recovered from fuels are yeasts and molds (*fungi imperfecti*). As noted above, yeasts are single cell fungi. They reproduce by *budding*. During budding, one or more *daughter cell* develops as a bubble attached to the parent's cell wall. Once a daughter cell has matured sufficiently, it separates from the parent.

Molds form filaments (*hyphae*)—long tangled strands of cells. Filament growth occurs as cells within a filament divide. Some cells with fungal filaments transform into specialized cells that form aerial hyphae, spores and the structures that hold the spores. The pigmented spores give fungal colonies their characteristic color (for example, the green of a *Penicillium* species colony). Spores are dispersed when the spore-containing structure bursts open. Each spore can then start dividing, thereby initiating a new mold filament. In liquids, mold colonies often appear as spherical, gelatinous *fisheyes* or furry scuz-balls. At the fuel-water interface, fungi can form a dense membranous *pellicle* that may be quite strong, structurally.

More than a million different fugal species have been described, although only a few are recovered from fuels and fuel systems routinely (Table 2). Other microbes including algae and diatoms are occasionally recovered from fuel system samples. Although algae can grow on the dark, deriving their nutrition from organic molecules, there is no indication that these microbes often play a significant role in fuel or lubricant biodeterioration.

MICROBIAL ACTIVITIES

Although many microbes may not be recoverable by conventional methods, they do leave evidence of their presence and biodeteriogenic activities. This section reviews the primary activities of microorganisms. Specific tests for monitoring these activities are listed in the ASTM Guide to Microbial Contamination in Fuels and Fuel Systems (D 6469).

Nutrient Metabolism

Carbon—As discussed above, all microbes need a source of carbon, for food, and a source of energy to drive their metabolism. Like all other organisms, microbes excrete waste products. The carbonaceous waste products range from carbon dioxide to high molecular weight polymers comprised of amino acid (peptide) and sugar (glycan) chains (peptidoglycans). By tracking chemical changes in fuel systems, it's possible to track the biodeterioration process [3].

Microbes will deplete fuels of lower molecular weight, aliphatic hydrocarbons selectively, enriching the fuel for

heavier, more complex molecules. This phenomenon is most likely to be detected by comparing carbon-number distributions of two fuel samples, one from the fuel: water interface (bottom 1 cm of fuel column) and one from > 0.5 m above the interface. Biodeterioration will be reflected in boiling point distribution shifts. More sophisticated chromatographic testing can be applied to identify specific molecular changes.

Nitrogen and Sulfur—In addition to carbon, hydrogen and oxygen, all organisms require nitrogen, phosphorous, and sulfur. Microbes attack organonitrogen compounds (amines, amides, cycloamidines, amidines and nitriles) found in fuel. Some of the nitrogen is incorporated into the biomass as amino acids. The balance is excreted as ammonia (typically as the ammonium – NH_4^+ ion). Nitrifying bacteria oxidize ammonium ions to nitrite (NO_2^-) and nitrate (NO_3^-). Analyzing bottoms-water for changes in NH_4^+ (ASTM Test Method for Ammonia Nitrogen in Water, D1426), NO_2^- and NO_3^- (ASTM Test Method for Nitrite-Nitrate in Water, D3867) provides evidence of active nitrifying bacteria.

Similarly, organosulfur compounds are metabolized to provide sulfur to growing microbes. Sulfur is found in a variety of biomolecules, including, for example, the amino acids cysteine, cystine, glutathione and methionine; biotin, Coenzyme A and sulfated polysaccharides. Some microbes store granules of elemental sulfur (S^0) as energy reserves. Others oxidize organosulfur compounds, producing sulfate ($SO_4^=$) ions. Sulfur oxidizing bacteria thrive in well-aerated, strongly acidic environments (pH < 2; acidity comparable to 2.0N sulfuric acid). Sulfate reducing bacteria use $SO_4^=$ as a terminal electron acceptor (see discussion under **Microbiology Defined**), producing hydrogen sulfide (H_2S) in the process.[9]

The SRB and a few other genera of obligate anaerobes produce the enzyme *hydrogenase*. Hydrogenase plays an important role in MIC. When a galvanic cell develops on a metal surface anodic and cathodic regions form. Within the cell, electrons (e^-) flow from the anode to the cathode, giving the cathode a negative charge. Protons (actually hydrogen ions – H^+) are attracted to the cathode surface. When the concentration of H^+ and e^- ions is equal, electrons no longer flow and the surface is passivated. The hydrogenase enzyme removes H^+ ions, thereby inhibiting passivation and accelerating the electron flux. This translates into accelerated corrosion. Several excellent biocorrosion process references [4–6] provide more detail about this topic.

Carbon, hydrogen, oxygen, nitrogen, sulfur, and phosphorous are all *macronutrients*. Significant deficits in the absolute or relative concentration of any one of these elements can restrict microbe growth and proliferation. Microbes also need other elements in smaller quantities as *micronutrients*. Some of the more critical micronutrients are sodium, potassium, calcium, iron, manganese and magnesium. Many other elements may be required in trace quantities by individual species.

[9]There are actually two major classes of microbial sulfate reduction. A variety of bacteria are *assimilatory* sulfate reducers. These bacteria have enzyme systems that enable them to convert $SO_4^=$ into biomass. The group of bacteria generally identified as SRB are called *dissimulatory* sulfate reducers. They use $SO_4^=$ as a terminal electron acceptor.

Metabolites

Several types of metabolites have been mentioned in earlier sections. These include weak organic acid (C_1–C_3 dicarboxylic acids), biopolymer (slime) and inorganic molecules (NH_4^+, NO_2^-, NO_3^-, $SO_4^=$ and H_2S). Some microbes also excrete surfactants that facilitate fuel biodegradation. Biosurfactants enable hydrocarbon-degrading microbes to contact non-polar molecules, the first step in hydrocarbon metabolism. Practical consequences of biosurfactant production include bottom-water emulsification into the fuel (invert emulsion formation) and fuel emulsification into bottom-water. Invert emulsion droplets also carry polar contaminant molecules into the fuel-phase. These polar molecules can seed polymerization, reducing fuel stability and accelerating sedimentation. Weak organic acids increase a fuel's acid number and, consequently, its corrosivity.

Microbial metabolite production and dispersion is particularly important because metabolites produced in one part of a fuel system can be transported far from the place they were created. This, in turn, increases the challenge of successful root cause analysis (RCA). For example, organic acids produced by biofilm communities within a tanker or pipeline can create biodeterioration symptoms in downstream terminal or retail service tanks. Active microbes need not be present in the affected fuel system. Consider an active microbial community growing at a low point in a transportation pipeline. This community may produce a variety of low molecular weight organic acids. The acids are transported downstream and react with inorganic chlorates also present in the system. The reaction products include organic salts and hydrochloric acid. The organic salts react with fuel components, thereby decreasing the fuel's oxidative stability.

Detecting microbial metabolites can be extremely challenging. Biochemicals entering the fuel stream are diluted sufficiently to be undetectable among the diverse molecules that comprise fuel. Deteriogenic chemicals may be highly concentrated within microenvironments such as biofilms and corrosion tubercles, but go undetected because material from these niches are rarely captured in conventional fuel samples.

FACTORS AFFECTING MICROBIAL ACTIVITY

A habitat's physicochemistry determines the type of microbes able to thrive there. It also affects the metabolic activities of microbes living within that habitat. A large part of the art and science of biotechnology is devoted to creating conditions that induce specific microorganisms to produce commercially valuable chemicals or perform beneficial material transformations (for example, bioremediation processes). When fuel system stakeholders understand the physicochemical factors affecting microbial activity, they are better able to design and implement strategies to control that activity. However, this section opens with a cautionary note. Taken as a whole, the three Divisions of microbes—Archaea, Bacteria and Fungi (Eukarya)—exhibit a remarkable range of capabilities. Thriving microbial communities can be found in deep-ocean thermal vents, where temperatures exceed 120° C, and under polar ice. Some microbes require pres-

sures > 5 kPa[10]. Deep-ocean communities grow under 20 kPa pressure. Some species need only CO_2 as their sole carbon source. Invariably, when scientists investigate the biodegradability of natural and synthetic organic compounds, they discover a consortium[11] and set of conditions under which biodegradation occurs. This means that it's unlikely that we'll ever be able to create conditions throughout the petroleum distribution system that will be sufficiently inhospitable to microbes as to prevent their growth entirely.

The primary factors affecting microbial growth are:

- Air (oxygen availability)
- Water
- Temperature
- pH
- Nutrient availability
- Osmotic pressure
- Salinity

The balance of this section will review how each of these factors affects biological activity.

Air

As summarized earlier, microbes fall into three primary categories with respect to their requirement for oxygen. Aerobes require oxygen in order to be active (typically > 5 mg · L^{-1}). Anaerobes are only active in an oxygen-depleted environment (typically < 2 mg · L^{-1}). Facultative anaerobes can thrive in well-aerated and anoxic environments. Facultative anaerobes play a crucial ecological role in fuel systems. They scavenge oxygen and create anoxic conditions, favorable for obligate anaerobes, within biofilms, sludges, sediments, and other niches where fuel and water can stagnate within fuel systems.

Water

Microbes do not require free-water. They do, however, require available water as measured by *water activity* (a_w). Water activity[12] is defined as the ratio of water vapor pressure over a material (P) to that over pure water (P_o) [7]:

$$a_w = P \div P_o \tag{1}$$

Most known bacteria won't grow at $a_w < 0.95$, but some thrive where $a_w \approx 0.75$. Some fungi grow in environments where $a_w = 0.60$.

This explains how fungi (most commonly, *Hormoconis resinae*) can colonize overhead stringers in ships' fuel tanks, the undersides of floating and fixed storage tank ceilings and the top inside surfaces of underground storage tanks. Colonization and growth can occur wherever the relative humidity is sufficiently high. Volatile organic carbons (VOCs) provide the necessary food.

[10]By comparison, atmospheric pressure at sea level is 0.1 kPa.
[11]A consortium is a community comprised of two or more microbial species capable of carrying out processes that cannot otherwise be performed by any of its individual constituent species.
[12]Water activity, when expressed as %, is the relative humidity of the atmosphere in equilibrium (equilibrium relative humidity–ERH). ERH (%) = 100 x a_w measured in a closed system in which the air and fluid temperatures are equal.

Fuels containing as much as 0.1% water generally meet quality criteria such as those specified in ASTM fuel standards, for example ASTM Standard for Diesel Fuel -D78. Since fuel's ability to hold water decreases with decreasing temperature, free water availability typically increases as fuels cool. Some of the free water evaporates and coalesces on headspace surfaces. Some free water coalesces on the tank skin. The balance, that doesn't remain suspended in the fuel, drops out and accumulates as bottoms-water. A droplet of water with a diameter of 1.0 mm (~ 0.5 mm^3) can accommodate several million bacteria! The water activity within a biofilm is > 0.99. Consequently, a 2 mm slime film, coating the shell of a 50 m diameter, 10 m tall fuel storage tank provides a 6 m^3 habitat for microbes. This means that a tank with no measurable water may still have sufficient water to accommodate considerable biomass.

Temperature

In general, microbial activity follows Arrhenius kinetics.[13] Up to a certain, critical temperature, growth-rate and metabolism increase with increasing temperature. This relationship is log-linear. As temperature increases linearly, activity increased logarithmically. Above the critical (often called *optimal*) temperature, rates plummet. Although each type of microbe has a characteristic optimal growth temperature, three general patterns exist (Fig. 2).

[13]Late in the 19th century the Swedish chemist Savante Arrhenius developed a kinetic rate law. The Arrhenius rate law states that the rate of a chemical reaction increases exponentially with the absolute temperature:

$$k = A \cdot e^{-E/RT} \tag{2}$$

where k is the reaction rate, A is a constant, R is the universal gas constant ($8.134 \cdot^{-3}$ kJ $mol^{-1}K^{-1}$) and T is temperature, °K. Although not initially applied to biological systems, it was discovered to apply to microbial kinetics at sub-denaturing temperatures. Once the temperature reaches the point at which enzymes and other biomolecules begin to unfold (denature), the Arrhenius law no longer applies. Primary (amino acid sequence), secondary (protein chain folding) and tertiary (association of constituent protein chains) enzyme structural differences account, in part for the temperature preferences of psychrophiles, mesophiles, and thermophiles.

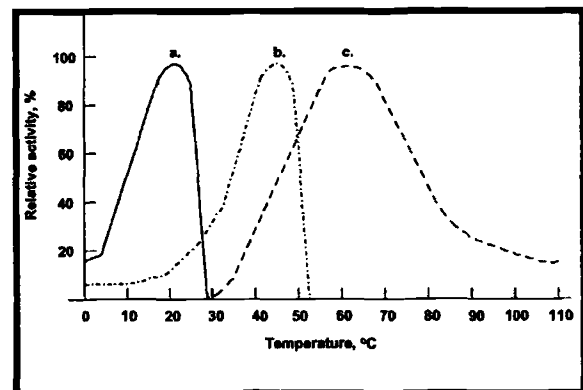

FIG. 2—Effect of temperature on microbial growth. Activity ranges for: (a) psychrophiles; (b) mesophiles; and (c) thermophiles.

Obligate psychrophiles grow optimally at temperatures below 20° C. Mesophiles prefer moderate temperatures (20–40° C). Microbes that require temperatures > 45° C are called obligate thermophiles. Representatives from each of these temperature preference groups grow in fuel systems. Consequently, fuel and fuel system biodeterioration has been reported in fuel systems ranging from the equator to the highest latitudes where tankage has been installed (Antarctica and the North Slope oilfields of Alaska). Universally, life at low temperatures takes place at a slower pace than life at high temperatures. Consequently, biodeterioration rates in tropic and temperate climates tend to be greater than those in polar climates. Over prolonged periods, biodeterioration is the same, both qualitatively and quantitatively.

pH

Bottoms-water pH typically ranges from 6.8–8.5. However, chemical and biological processes can affect pH considerably. This section reviews the concept of pH, discusses the factors that affect pH in fuel systems and summarizes the relationships between pH and microbes. It's important to recall that pH is a property of aqueous solutions. Fuels may have measurable acidity or basicity. Although these properties are related to pH, they are not the same. These fuel properties are more directly analogous to alkalinity and acidity, which are described below.

Defined as the negative log of the hydrogen ion (H^+) concentration in an aqueous solution ($-\log[H^+]$), pH is a measurement of a liquid's acid-base properties. Fluids with pH > 7.0 are considered alkaline. Those with pH < 7.0 are acidic. Neutral solution pH ≈ 7.0. The buffering capacity of a fluid is its resistance to pH change. This resistance, generally reported in mg calcium carbonate · L^{-1} (mg $CaCO_3 \cdot L^{-1}$), is called *alkalinity* or *acidity* depending on the direction of change being resisted. For example, two solutions may have the same pH. It takes more acid to decrease the more alkaline solution's pH by a given amount than it does to decrease the less alkaline solution's pH.

Alkalinophilic microbes grow only in environments in which pH > 8. Acidophiles require low pH environments (< 4.0). Some acidophiles thrive in the equivalent of 2N sulfuric acid. Most microbes prefer pHs ranging from 6–8.5. Notwithstanding the unique claim that bacteria and archaea have on life at the extremes, fungi tend to prefer a lower pH environment than bacteria. Fungal diversity is maximal in environments with pHs ranging from 4.5–6.5.

Bottoms and entrained waters in fuel systems vary considerably in both pH and alkalinity/acidity. This range reflects the chemistry of water-soluble constituents of the overlying fuel, the origins of the water, and biodeteriogenic processes that occur within the fuel system. Until recently, it was impossible to measure interstitial water[14] pH. Measurements, made possible by current microprobe technology, have demonstrated that pH and other electrochemical properties differ dramatically between interstitial and bulk water. For example, interstitial water with pH = 4.0 can be recovered from a system in which the bottoms-water pH = 7.2. The mechanisms responsible for these differences were discussed under **Microbial Activities**.

Typically, rainwater pH ranges from 6–7 and is weakly buffered. Bottoms-water pH from uncontaminated or minimally contaminated fuel systems tends to mirror rainwater. When bottom-water pH > 8 and the alkalinity is > 1,000 mg $CaCO_3 \cdot L^{-1}$, it's most likely that alkaline fuel-constituents have partitioned into the water-phase. Bottom-water pH < 6, with acidity > 150 mg $CaCO_3 \cdot L^{-1}$ indicates biodeterioration. Since it's more challenging technically, and more costly, interstitial water testing is only needed when bulk, bottoms-water pH and alkalinity data appear to conflict with other biodeterioration evidence.

Microbial growth in fuel-associated water can drive the pH up or down. Most commonly, microbially produced organic acids cause the pH to fall over time. However, alkaline metabolites such as ammonia and some polypeptides (amine chains) may also accumulate and is reflected though pH increases. Although these pH variations may affect microbial population succession (some species replacing others as the dominant members of the community), they do not limit the net biodeterioration process.

Nutrient Availability

The microbial world's nutritional diversity was discussed earlier. One of the critical factors determining whether biodeterioration will occur is nutrient availability. Recently, the author and coworkers [2] reported an average of 67% depletion of the oxygenate methyl tertiary-butyl ether (MTBE) in gasoline over microbially contaminated bottoms-waters. This extent of MTBE depletion is dramatically greater than that observed in the bioremediation literature [8]. In fluid systems, MTBE is available. In soils it's not. Recently, Salanitro et al. demonstrated > 99% depletion of MTBE in subsoil microcosms [9].

In fuel systems, microbes that are not capable of using petroleum hydrocarbons may still thrive. First, non-hydrocarbon fuel constituents may be sufficiently nutritive to support the non-hydrocarbon degraders. Second, metabolites produced by hydrocarbon degraders can sustain a variety of non-hydrocarbon degrading microbes. In fact, without a food-chain dynamic, in which one microbe's waste is another's feast, metabolite accumulation could become toxic to the microbes producing them. Consequently, nutrient molecules become available to microbes by several different means.

Fungal growth in fuel tank headspace was noted earlier. Under the right conditions, contaminant fungi utilize volatile organic carbon (VOC) vapors. Successive colonizers are then able to utilize fungal biomass and metabolites for food. Biofilm bacteria that produce biosurfactants increase the availability of non-polar fuel molecules for themselves and neighboring microbes that don't produce biosurfactants.

Microbial metabolism was reviewed under **Microbial Activities**. Two related processes may improve nutrient availability. Cometabolism occurs when an enzyme intended to attack a specific molecule is also able to carry out the same enzymatic process on non-target molecules. In cometabolism, degradation of the non-target molecule or

[14]Interstitial water is trapped within, for example, biofilm or sludge and sediment. Discrete bodies of interstitial water may be comprised on < 100 μm^3 fluid.

molecules do not benefit the microbes that produced the enzymes, but may serve as a nutrient for other microbes. In a second process, enzymes employed generally for a specific metabolic activity may attack molecules that are structurally similar to the normally targeted molecule. For example, certain *oxygenases* designed to open the rings of low molecular weight aromatics, will also cleave rings of polynuclear aromatic compounds (PNAs), thereby making the compounds more available for biodegradation by other microbes. The oxygenase producers don't use the PNAs, and therefore don't benefit.

The microenvironment of the biofilm provides countless opportunities for hydrocarbon emulsification, cometabolism and nutrient concentration. The spatial and biochemical relationships amongst biofilm population members ensure that nutrient utilization is maximal within the biofilm. Consequently, biodeterioration is less likely to occur in fuel systems from which mature biofilms are absent.

Osmotic Pressure

Osmotic pressure is the force with which water tends to move across a semi-permeable membrane. The relative concentration of solute molecules on either side of the membrane drives water flow towards the side with the higher solute concentration. Cell membranes are semi-permeable that contain various systems (typically called *pumps*) to help attenuate the effects of osmotic pressure. Without these pumps, cells would swell up and burst whenever they were in a low osmotic strength environment (for example, condensate water). In high osmotic strength environments (for example, sea water) cells would desiccate as the water flowed out though the membrane into the surrounding medium.

Common inorganic salts (for example: NaCl, KCl, HCl, NH_4Cl, $NaNO_3$; KH_2PO_4) and sugar molecules (for example: glucose, lactose, maltose, sucrose) contribute to a solution's osmolarity[15]. Just as with temperature, microbes fall into three general categories with respect to their need or tolerance for osmolarity. Some (osmophobic) bacterial species are found only in very pure (distilled) water. Others (osmophilic) can only live in salt brine. Most microbes prefer environments for which the osmolarity falls between these two extremes.

In general, microbes that thrive in higher osmolarity environments tend to be more robust. This may reflect their more complex cell envelopes (cell wall and membrane system). In practical terms, it generally takes higher antimicrobial pesticide concentrations and prolonged exposure to kill microbes that thrive in moderately high osmolarity (0.75–1.5 OsM) environments than it does to kill microbes adapted to freshwater life.

Salinity

Salinity is the concentration of total dissolved solids (TDS) in seawater as measured in g TDS $\cdot$ kg^{-1} seawater. Three salts (NaCl, $CaCl_2$, and $MgCl_2$) comprise more than 90% of the dissolved solids in seawater. Potassium phosphate and sodium sulfate are also important seawater constituents. Salinity measurements assume that all carbonates and organic molecules have been oxidized and all bromide and iodide ions have been replaced with chloride. Ocean water salinities average approximately 35 g $\cdot$ kg^{-1}. Waters with salinities ranging from 10 to 25 g $\cdot$ kg^{-1} are brackish. Fresh water salinity is near zero. Salinity can be computed from conductivity by the simple calculation:

$$\text{TDS}\ (g \cdot kg^{-1}) = 6.4\ S \cdot m^{-1} \quad (3)$$

Where $S \cdot m^{-1}$ are siemans per meter ($1\ S \cdot m^{-1} = 1$ mmho $\cdot$ cm^{-1}). Water salinity is directly related to its osmolarity, discussed in the previous section.

Dissolved solids tend to concentrate in fuel tank bottoms-water. When condensation and entrained water rejection are the primary bottoms-water sources, TDS concentrations will be comparable to fresh water. Seawater ballast will also be characteristic of its source. Tanks contaminated with rain or surface runoff water typically have brackish bottoms-water. As noted in the previous discussion about the effects of high osmotic strength, high TDS microbial communities tend to be more robust and more antimicrobial pesticide resistant than those growing in low TDS water.

Total hardness, measured as mg $CaCO_3 \cdot kg^{-1}$ water, is the concentration of calcium and magnesium in water. It should be obvious that hardness contributes to water's TDS concentration. Combined with pH and alkalinity/acidity, hardness is one of three parameters used to compute water's aggressiveness index (A.I.):

$$\text{A.I.} = \text{pH} + \log \text{alkalinity} + \log \text{hardness} \quad (4)$$

A.I. values ≤ 10 indicate that the water is highly corrosive. Values between 10 and 12 indicate mild corrosivity. Non-corrosive water will have A.I. ≥ 12.

Typically high TDS water will also be hard[16]. It follows that hard water (≥ 180 mg $CaCO_3 \cdot kg^{-1}$ water) will support a more robust microbial community than will softer water [10]. Surface runoff water and drier-bed particulates are likely to be the primary water hardness sources in fuel systems.

Alkalinity and hardness, both reported as mg $CaCO_3 \cdot kg^{-1}$, contribute to high A.I. (low corrosivity) values. In systems with hard bottoms-water, pH and alkalinity are likely to mask microbial acid production. These systems are sites where interstitial water chemistry data are critical. Localized zones of low pH and measurable acidity[17], although characteristic of MIC, are typically missed when bottoms-water samples are collected and analyzed.

Operational Factors

Several operational important factors affect microbial contamination in fuel systems. These include system configuration, fuel turnover-rate, and housekeeping practices. Although it's beyond the scope of the present chapter to discuss

[15]Osmolarity (OsM) is the sum of the molarities of solute particles in solution. The osmolarity of seawater is approximately 1 OsM. Freshwater is approximately 0.001 OsM (1mOsM).

[16]Water is generally classified into general hardness categories, based on mg $CaCO_3 \cdot kg^{-1}$concentrations: < 60 - very soft, 60 to 120 - soft, >120 to ≤ 180 - slightly hard, >180 to ≤ 230 - moderately hard, >230 to ≤ 340 - hard; >340 - very hard.

[17]When acidity data are used, A.I. = pH − log acidity + log hardness.

the merits of alternative operational practices, it's important to describe their general implications in terms of fuel system microbial ecology.

System Configuration–Vapor recovery and ventilation subsystems can be designed to minimize entry of airborne contaminants. System design determines the volume of water likely to accumulate and the ease with which it can be removed. Fuel system design dictates its fluid dynamics.

The fluid-flow dynamics in most tanks can be described as three zones. Typically there is a quiescent bottom zone. This is where water, sludge, and sediment accumulate. Except during bottoms-water draw-downs, there is little circulation within this zone. Under normal operating conditions, the fuel-water interface lies within this zone. As discussed in preceding sections, this is where most of the contaminant microbes call home. The heaviest biofilm growth accumulates on tank shell surfaces in contact with this bottom zone, or *heel*. Although the heel generally comprises less than 5% of the total tank volume, it's the primary biodeterioration zone.

In contrast, > 90% of the fluid of most tanks (the exception being long-term storage tanks) is within the high turnover zone. Although product may remain in emergency generator and strategic reserve tanks for years, turnover rates in most commercial tanks range from < 24 h to a month. Refinery and terminal tanks turnover produce 45–50 times annually. Biofilms can develop on the interior shell walls of fixed roof tanks, but are unlikely to form where floating roof gaskets scrape tanks walls clean as product is drawn from the tank. In floating roof tanks, biofilms develop below the lowest level the roof reaches during the regular ebb and flow of product. Although microbes introduced during tanker or pipeline transit, through tank vents or leaking gaskets can be recovered from bulk fuel samples, they are unlikely to be degrading the fuel as they settle to the bottom or find their way into biofilms.

The third zone is poorly characterized. It's the transition zone between the nearly zero-flow bottom region and the high turnover bulk fuel zone. At its lower limit, the transition zone is barely distinguishable from the underlying quiescent zone. Metabolites produced within the quiescent zone diffuse into the transition zone. Invert emulsion micelles and biofilm stalagmites may extend into the transition zone. Sheer forces and consequent mixing increase closer to the high turnover zone. Microbes and metabolites can be transported into the high turnover zone and, subsequently, downstream into other fuel system components. This is one mechanism by which fuel in an uncontaminated system that is downstream of a contaminated system may become unstable or corrosive. Suction line design and positioning will affect the risk of transporting contaminants downstream.

Turnover Rates—Particularly in fixed roof tanks, turnover rates can affect biofilm development. Most high turnover (retail outlet) gasoline storage tanks show little evidence of biofilm accumulation on the upper two-thirds of their interior surfaces. As gasoline is dispensed, fluid levels fall with the tank. Residual gasoline evaporates from the exposed tank surfaces rapidly, dehydrating the surface at the same time. Since tanks are customarily refilled when inventory falls to approximately one-third capacity, the bottom third of these tanks don't experience the routine flash evaporation that the upper tank surfaces do. Biofilms are able to develop below the minimum ullage line. Since evaporation rates are slower for heavier fuel grades, growth is more likely to occur above the minimum ullage line in distillate fuel tanks. Also, the desiccation effect is less pronounced in tanks with slower (> weekly) turnover rates.

High fuel throughput rates also create elevated water transport rates. Warm fuel holds more water than cooler fuel. When fuel is shipped from refineries at maximum permissible temperature, it cools enroute to downstream tankage. As the product cools, water splits out and is either transported as emulsified water or accumulates in pipeline low-points. Given sufficient time, dissociated water will coalesce and drop out of the fuel while the fuel is in terminal tankage. As throughput increases the opportunity to allow water to drop out decreases. More water moves further through the fuel distribution system. Coalescers and other water separating devices can reduce water transport, thereby reducing the amount of water available for microbes in fuel systems.

Housekeeping—The earlier discussion about the role of water suggests that attempts to keep tanks dry might be futile. Although it may not be possible to prevent all microbial activity, it's important to reduce the total volume and surface area conducive to microbial activity. In most cases, the dryer a system is kept, the lower the risk of biodeterioration problems. This links housekeeping to system design. A system capable of removing all but 10–20 ppm water (0.2–0.4 m^3 water in a 20,000 m^3 tank) is going to have fewer microbial contamination problems than one that leaves ≥ 1% water (200 m^3 in a 20,000 m^3 tank) behind.

FUEL SYSTEM MICROBIAL ECOLOGY

Throughout this chapter the importance of microbial activity as a niche process involving multiple species has been a recurring theme. This final section will provide an overview of where microbes tend to concentrate and how microbial communities cause biodeterioration.

Communities and Consortia

With rare exceptions, microbes do not exist as pure cultures in nature. This fact has profound implications on our understanding of biodeterioration, the process by which microbes cause or contribute to undesirable changes in fuels and fuel systems. In fuel systems, communities form within biofilms. Biofilms develop at system interfaces. These include the fuel: air, fuel: vessel, vessel: air, fuel: water and water: vessel interfaces. Consortia (singular–*consortium*) are communities in which the individual members, working in concert, cause things to happen that wouldn't otherwise happen. For example, sulfate-reducing bacteria (SRB) require an oxygen-free (*anoxic*) atmosphere in order to thrive and reduce sulfate to sulfide. Aerobic and facultatively anaerobic bacteria consume any oxygen that may permeate the surface region of a biofilm. Consequently, they create an environment suitable for SRB growth deep within the biofilm. Moreover, SRB prefer C_1 to C_3 dicarboxylic acids as their primary food, the same weak organic acid metabolites that were dis-

cussed above apropos of MIC. Microbes able to attack high molecular weight fuel and fuel additive molecules excrete metabolites that SRB and other microbes can digest. The microbes that use these metabolites as food prevent them from accumulating and becoming toxic to the microbes that generated the metabolites. It's not too far fetched to think of microbial consortium members as different organs in your body. The sum of their activities is dramatically greater than that of the individual members.

There are several reasons why it is so important to understand the consortium model. First, it affects the way we sample. As discussed in Graham Hill's chapter on sampling, inappropriate sampling will provide samples that yield misleading data. Second, it affects the way we process samples for root cause analysis. The vast majority of microbes found in nature are not culturable (see earlier comment under *Bacteria*). Using traditional microbiological methods, investigators often have difficulty reproducing biodeterioration phenomena in the laboratory.

For example, consider a fuel storage tank in which fuel has become corrosive. A bottom sample is taken, and a subsample is tested for viable counts (Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods - D 5465). Biodeteriogenic microbes, thriving in the system from which samples are collected, may fail to grow on the conventional nutrient media used to screen samples for microorganisms. The investigator cultivates the different microbes that were recovered successfully from the sample and then pools them to create a challenge inoculum for a test system (*microcosm*). The microcosm is designed to roughly mimic the problem system. If critical consortium members are missing, members can't effect the changes that had been mediated by the intact consortium. The investigator concludes erroneously that biodeterioration is not a potential root cause. Biodeterioration continues, uncontrolled, in the problem system. Since we don't have the tools for cultivating all microbes likely to grow in fuel systems, we must use nonconventional methods to diagnose biodeterioration. Nonconventional methods are discussed in D 6469 and Guide for Evaluating Nonconventional Microbiological Tests for Enumerating Bacteria- E 1326.

A third reason that understanding the consortium concept is its potential value for biodeterioration control. Control strategies that disrupt consortia may prove to be more cost-effective than traditional disinfection. Understanding biodeterioration dynamics enables fuel system managers to consider monitoring and housekeeping programs that prevent biodeteriogenic consortia from forming.

The relationships among microbes living within consortia are complex. Bacteria from a single taxon take on dramatically different characteristics, depending on their position within a biofilm. Again, on the analogy with higher organisms, it's much like the way somatic (germ) cells differentiate to form different organs. The genetic information within each cell is identical, but the immediate physicochemical environment causes different cells to carry out different activities and assume different gross characteristics. The University of Montana, Bozeman, Center for Biofilm Engineering website [11] provides tremendous detail, suitable for all levels of readership, on our current understanding of biofilm ecology.

Biomass and Biofilms

Historically, fuel system stakeholders concerned about biodeterioration typically focused on biomass accumulation symptoms. Premature filter plugging is the most commonly observed biomass accumulation symptom. Fuel transports flocs of biomass, sloughed-off from active biofilms, to filter media. Bacterial and fungal cells generally comprise $< 5\%$ of the total mass of a biomass floc. The balance is made up of biofilm material, inert detritus and water. To better understand how microbes plug filters, it's useful to understand the basics of biofilm development.

Initial Colonization—When a fuel system is first placed in service, it may already be contaminated with dormant microbes adsorbed onto dust particles and other construction debris. Tanks are typically hydrotested before being placed into service. Water used for hydrotesting typically harbors 10^2 to 10^6 microbes $\cdot$ mL^{-1}. Consequently, microbes often settle into fuel systems before the systems are even placed into service. Once in service, tanks breath as fuel is added and removed. During suction cycles, dust and water particles enter tanks through vents. Also, fuel may pick up microbes at each stage of the distribution channel from the refinery to the ultimate consumer.

Once microbes enter a fuel system, they tend to settle and diffuse similarly to the way entrained water does (Fig. 3). Many species of bacteria produce sticky, mucilaginous biopolymers called extracellular polymeric substances (EPS). When a bacterium contacts a surface the EPS enables the microbe to adhere. Under appropriate conditions, pioneer microbes (those first to attach) begin multiplying. Generation times for the bacteria typically recovered from fuel systems range from 0.5–6 h. Consequently, within a few hours after attachment, the pioneers form small colonies.

Biofilm Maturation—The EPS[18] matrix entrains nutrients, water and other microbes. Before long a biofilm consortium evolves[19]. As discussed above, a mature biofilm may support a single species or a diverse microbial population. A mature biofilm shares many similarities with multi-cellular organisms, feeding on the available nutrients and excreting wastes into the bulk fluid. Given the sticky, polar nature of biofilm material, inorganic particulates (for example rust) become entrapped within the glycocalyx. Thus we have microbes, their metabolites enmeshed within a slimy, watery matrix that is also laden with entrained organic, organometallic, and inorganic molecules.

Dynamic Equilibria—Mature biofilms are dynamic. Just like skin, whose outer cells die and slough off, small pieces of biofilm slough off and get transported through the suspending medium[20]. Although the apparent dimensions of biofilm communities may appear to be stable, they are being renewed constantly.

[18]The EPS matrix is also called a *glycocalyx*.

[19]In recirculating water systems, a mature biofilm may develop on a clean surface in 24–48 h. In fuel systems biofilms may require 1–3 months to mature.

[20]Biofilm floc transport depends on the relative specific gravities of the floc and suspending medium. In fuel systems, flocs may be transported by fuel or water. Heavier flocs will tend to settle into the sludge zone and lighter flocs will remain suspended in the fuel or water phase.

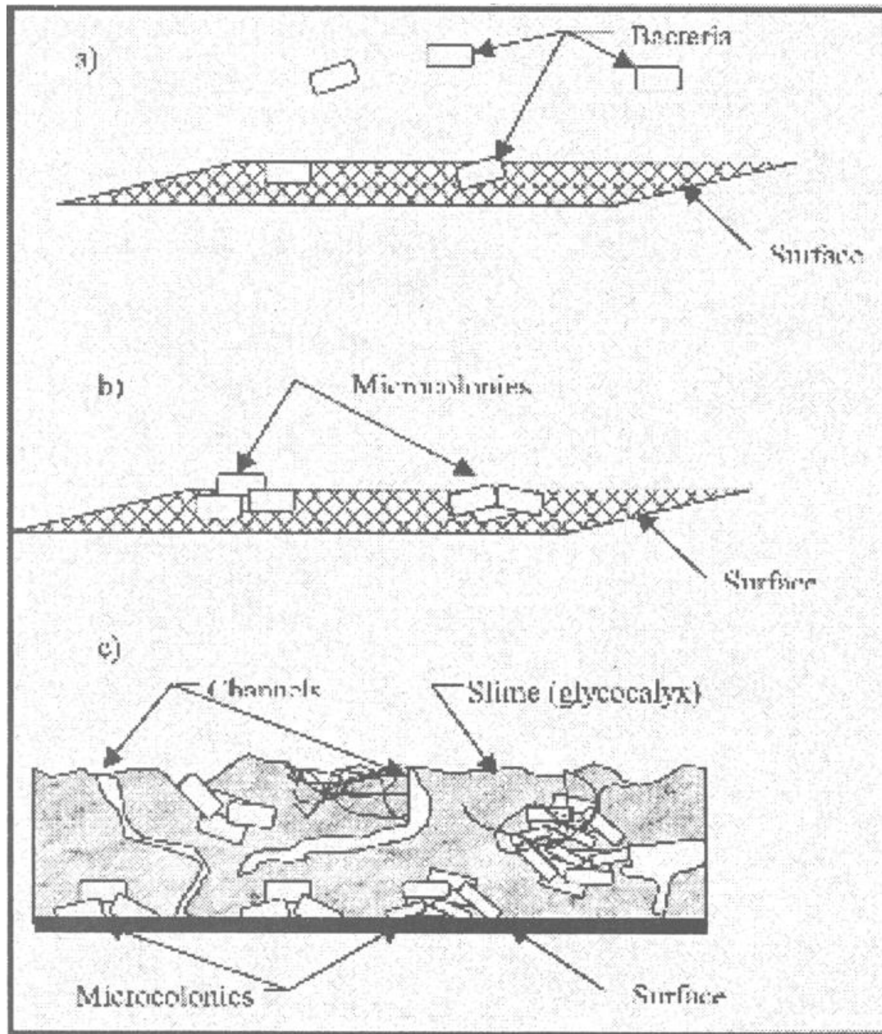

FIG. 3—Dynamics of biofilm formation: (a) Inoculation and initial settling–bacteria settle onto surface and begin producing slime; (b) Microcolonies form as initial settlers reproduce. Electrochemical gradients begin to develop between exposed and overgrown surfaces; (c) Mature biofilm. Microcolonies have developed throughout the biofilm. Channels facilitate nutrient and waste transport. Physicochemical conditions within the mature biofilm are substantially different from those in the overlying bulk fluid.

The general appearance of a biofilm reflects the conditions under which they develop. In high flow systems (for example, pipelines) biofilms will tend to be smooth and compact. Fluffy biofilms tend to form in quiescent environments (for example, the walls of storage tanks).

Biofilms forming at fuel-water interfaces mature to form thick, membranous *pellicles* or *rag layers*. A pellicle is a tough, flexible skin reminiscent of the skin that forms on the surface of paint in an open can. A rag layer is a less distinct zone that may be > 1 cm thick, in which it may be difficult to differentiate the glycocalix from invert emulsion. Virtually all (> 99%) of the biomass within fuel systems is found within biofilms (Fig. 4 [12]).

Biofilm Disruption—Any substantial system perturbance may disrupt a biofilm. Surges in flow rates and shifts between turbulent and laminar flow tend to increase floc formation (Fig. 5). Changes in fuel chemistry may also disag-

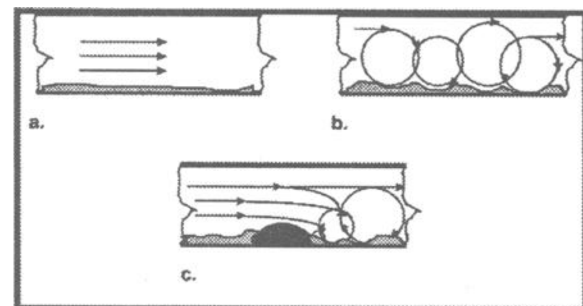

FIG. 4—Microbe and nutrient profile: fuel over water. Flow pattern: (a) laminar:uniform biofilm (gray shaded area) coverage; (b) turbulent flow:non-uniform biofilm; (c) turbulent flow caused by obstruction (weld seam) on surface.

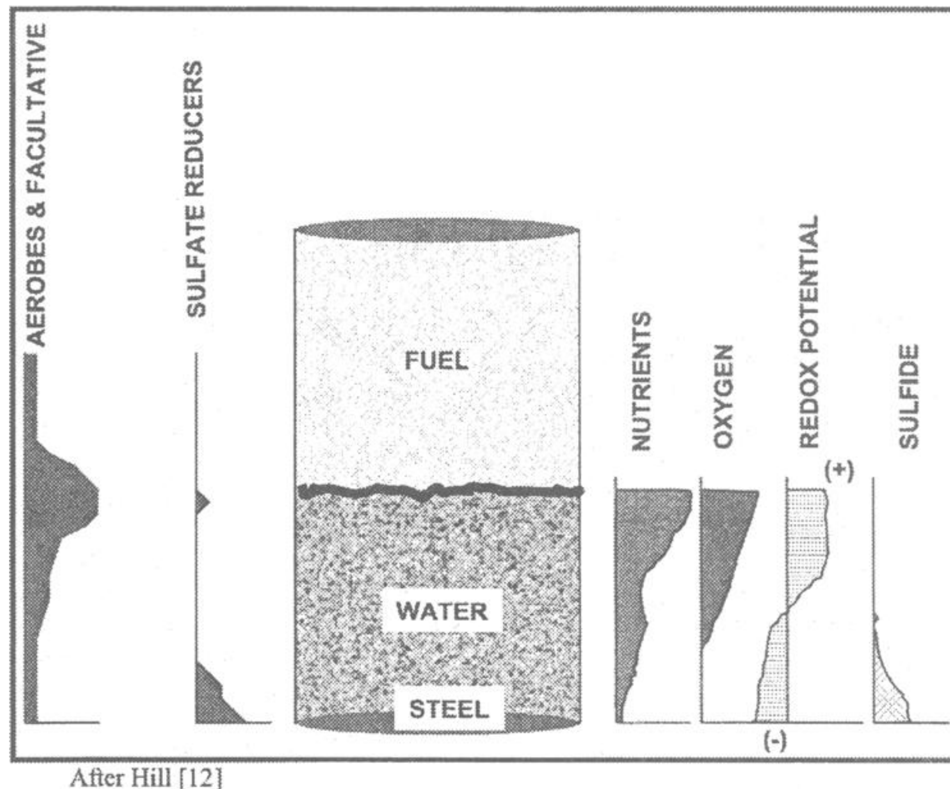

FIG. 5—Effect of flow dynamics on biofilm accumulation. Flow pattern: (a) laminar: uniform biofilm (gray shaded area) coverage; (b) turbulent flow: non-uniform biofilm; (c) turbulent flow caused by obstruction (weld seam) on surface.

gregate biofilms. Increased filter plugging often accompanies semiannual shifts between oxygenated and non-oxygenated fuels. This is a practical example of biofilm disruption. Typically biofilm disruption is a transient phenomenon after which the biofilm community adapts to the new conditions. However, some percentage of the biomass flocs released during a disruption event will be transported to and deposited onto downstream filter media. It's these flocs that plug filters. Microbes are more likely to proliferate within filter media in middle distillate and heavier grade fuel systems. This may be because gasoline systems don't trap enough water to support microbial colonization. Depth filters and coalescers are generally more susceptible than paper elements to biofouling. Even low levels of microbial contamination may disrupt coalescer function [13].

Sludge and Sediment—As noted above, those microbes that don't get incorporated into biofilms, or are embedded in biofilm flocs that don't get transported out of a tank, settle to tank bottoms. According to Hill [12] this is where most of the SRB activity occurs. Biomass comprises only a small percentage of the sludge and sediment mass[21], however, the sludge zone is an important region for the production of deteriogenic molecules.

Common Growth Zones–Figure 6 illustrates the zones of tank walls where biofilms are most likely to develop. Stable communities prefer environments that don't tend to change. The tank shell within the quiescent zone (see *Operational Factors*, above) provides a stable environment for biofilm accumulation. Since water and nutrient availability are greatest at the fuel-water interface, this is where growth tends to be heaviest. At this interface, biofilms develop within the fluid as well as on tank walls. In tanks with MIC problems, pit densities are often greatest within a few mm of where the fuel-water interface contacts the shell.

Bottom sludge and sediment provides another good environment for microbial activity. Particulates offer tremendous surface area for colonization. Because the sediment zone is typically anoxic, however, and the biological processes for breaking down fuel molecule polymers are aerobic, biological activity within the bottom sediment zone is generally less than that within the fuel-water interface biofilm. The secondary and tertiary effects of biomolecules may be significant in the sediment and sludge layer.

As discussed earlier under **Factors Affecting Microbial Activity**, microbes can colonize headspace surfaces, utilizing VOC vapors and water condensate to meet their nutrient and water requirements. Once headspace biofilms mature, microbes not able to utilize VOC vapors can proliferate, feeding off of the metabolites and cell components of the early colonizers.

[21]The atomic weights of the elements comprising biomolecules (carbon, hydrogen, oxygen, nitrogen, sulfur and phosphorous are light as compared to iron, zinc, vanadium and other heavy metal constituents routinely recovered from biofilms. Consequently, even dense, metabolically active microbial communities will comprise relatively small fractions of the total mass (dry weight) of a sludge or sediment sample. A mole of iron weighs 56 times as much as a mole of hydrogen.

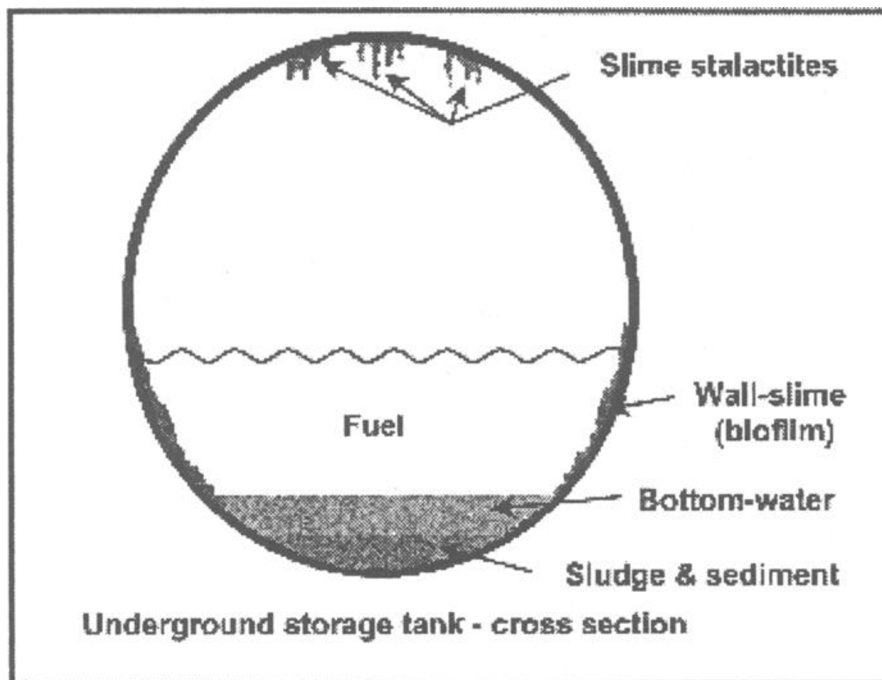

FIG. 6—Tank zones where biofilms are most likely to develop. Biofilms containing high concentrations of metal ions and asphaltenes accumulate on tank surfaces. Because biofilms are so efficient at retaining non-biological material, the biological aspect of tank wall residue is often ignored. In horizontal, underground storage tanks, most of the wall slime is concentrated in the lower third of the tank.

Community Impact

The consequences of uncontrolled microbial growth in fuel systems are diverse. Microbes use fuel molecules for food. Except for fuels in long-term storage systems, chemical changes due to direct attack are generally undetectable. The effects of the metabolic byproducts are considerably more noticeable.

Biosurfactants create emulsions and invert emulsions. Hazy fuel and rag layer formation provide easily recognized gross evidence of this biodeterioration process. Low molecular weight metabolites, particularly C_1 to C_4 carboxylic acids, react with fuel molecules, catalyzing fuel polymerization. This process causes increased sludge and sediment formation rates. As discussed earlier, weak organic acids also react with dissolved salts to form strong inorganic acids, thereby increasing fuel corrosivity. When this occurs within biofilms that are growing on system surfaces, MIC results. Rag-layer activity is more often reflected in accelerated corrosion of downstream valves and other fuel system components.

Filter plugging when it occurs, is a late symptom of uncontrolled microbial contamination. Most commonly, fuel transports flocs of biomass that have sloughed off of upstream surfaces. Under normal operating conditions, fuel filters should be able to handle at least 1,000 m^3 of fuel before plugging[22]. It's not uncommon for filters on heavily contaminated systems to plug before 100 m^3 have flowed through them. The other significant biodeterioration processes listed in this section have been operational long before this order of premature filter plugging occurs.

[22]This volume refers to typical 10 μm nominal pore-size filters used at retail dispensers. Larger filters should be able to accommodate considerably more volume.

CONCLUSIONS

Although microbes are essential to our quality of life, most non-microbiologists consider the science of microbiology to be a bit esoteric, perhaps daunting. Considering that microbes play critical roles in food production, normal bodily functions (particularly digestive tract and skin health) and waste treatment, it's paradoxical that microbiology isn't included in general science training. Uncontrolled biodeterioration costs the petroleum industry $ billions annually. Biodeterioration affects crude oil while it's still in petroleum formations. It affects petroleum handling equipment and fluid at every stage of the industry from the oilfield to the ultimate user.

In order to quantify biodeterioration's economic impact and devise cost-effective strategies for minimizing that impact, stakeholders need to understand the fundamental microbiology behind biodeterioration. ASTM D 6469 provides an overview of the nature and dynamics of fuel and fuel system biodeterioration. It also recommends a variety of tests that can be used to determine whether biodeterioration is occurring within a fuel system. This chapter was designed to provide a broader understanding of the microbiology fundamentals that drive biodeterioration.

In summary, for recognizing and controlling biodeterioration, understanding traditional microbial taxonomy is less

useful than understanding microbial activities. Although microbes utilize fuel as food, this is a relatively minor biodeterioration function. Secondary and tertiary interactions between microbial communities and fuels or fuel systems represent the greatest biodeterioration related costs of quality. Corrosion and fuel destabilization are the most common symptoms of biodeterioration, although premature filter plugging is the most frequently reported problem.

Microbiologists have barely begun to understand the diversity and complexity of the microbial world. Bodies of water as small as 7 mm^3 (~ 1 mm diameter) can be ecosystems supporting significant biodeteriogenic activity. Individual microbial species may have limited nutritional capabilities and environmental tolerances. However taken as a whole, microbes exhibit a remarkable range of capabilities. Under most conditions, multiple species co-exist within communities. The relationships amongst members of a community range from aggressive competition to absolute synergy. Many of the biodeterioration processes observed in fuel systems require intact microbial communities in order to occur.

A relatively limited number of variables define microbial growth conditions. These include temperature, pH, osmotic pressure and the availability of air, nutrients, and water. This chapter summarized the role of each of these factors.

In this chapter, several community-driven biodeterioration processes were illustrated. The role of facultatively anaerobic bacteria in creating environments suitable for obligate anaerobes was discussed. Food chain relationships were reviewed in which pioneering microbes able to utilize VOCs in one example, or non-polar fuel molecules in another example, synthesize metabolites that other microbes can utilize as food. This chapter emphasized the significance of organic acids and their effect on fuel and bottoms-water corrosivity.

Non-biological processes may also cause many of the phenomena caused by microbial activity. Consequently, biodeterioration symptoms may be incorrectly assigned to non-biological factors such as poor additive quality, refining process variances, fuel incompatibilities, and system upsets. Unaware of biological mechanisms, stakeholders may end the root cause analysis process prematurely. If readers now have greater awareness and understanding of the microbiology of fuel and fuel system biodeterioration this chapter has served its purpose.

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Sampling Methods for Detecting Microbial Contamination in Fuel Tanks and Systems

2

Graham Hill[1]

INTRODUCTION

THE OLD MAXIM, THAT ANY TEST result is only as good as the sample, is never more true than for analysis of samples drawn for the investigation of microbiological contamination in fuels and fuel systems. Probably more than any other fuel contamination type, microbial contamination will tend to have a highly heterogeneous dispersion that is likely to be in a continual state of change. There may be changes in the overall numbers of microbes present, their viability (and culturability), the relative numbers of the predominant types (genera and species) and the amounts of microbial biomass present [1]. These changes may be due to the microbial activity itself or as a consequence of tank or system operating activities. It is thus apparent that both the timing of sampling operations and selection of appropriate sampling points need careful consideration and planning. In order that those conducting the analyses can put the best possible interpretation on the results obtained, as much information as possible about the sampling needs to be conveyed to the testing laboratory.

This chapter will describe and discuss the special considerations of sampling a fuel system as part of a microbiological investigation. It will provide an overview of existing procedures and guidelines for fuel sampling and, where appropriate, comment on how these may need to be adapted for microbiological investigations. Finally, the chapter will provide general recommendations for sampling programs and appropriate sampling procedures for various types of microbiological investigation and routine microbiological monitoring.

FACTORS AFFECTING THE DISTRIBUTION OF MICROBES WITHIN FUEL TANKS AND SYSTEMS

Prior to embarking on a sampling program as part of a microbiological investigation, it is important to have an appreciation of the ways in which microbes disperse within a fuel system and the factors that may affect their distribution. Further background information can be found in Chapter 1 of this manual. Microbes will only be actively proliferating if a free water phase is present [2]. In a quiescent tank/system the water phase will be primarily located at the lowest point. In the case of a storage tank this will obviously be on the tank floor, although, depending on the condition and age of the tank, the lowest point may not always be directly beneath the normal sample access points on the tank roof. If subsidence of the tank floor has occurred, several low points may exist and water can be distributed across the tank floor in isolated pockets. Temperature changes can result in condensation of water from both fuel and air in a tank; this condensate water may collect as droplets on tank walls. In pipeline and hydrant systems, water may collect at low points and drain points, particularly where product throughputs are low compared to the pipe bore. Fuel Water Separator (FWS) units will also be prime locations for free water accumulation and microbial proliferation [3,4]. Substantial volumes may collect in FWS sumps if the units process "wet" fuel and /or where the units are not regularly drained. Water droplets may also accumulate on the outer socks of FWS coalescer elements, particularly where units are underutilized or where flow rates are low.

The water phase does not need to be large in volume to support microbial growth. It is the surface area of the fuel/water interface that is probably more important in determining the extent of microbial proliferation. Hydrocarbon nutrient levels will be particularly high in the immediate vicinity of this interface. Oxygen concentration will also be relatively high, as the fuel itself will contain a certain amount of dissolved oxygen [5]. Consequently aerobic microbial growth will be promoted. Fungal filaments may extend upwards from a cohesive mat at the interface into the fuel layer itself. These filaments may produce hydrophobic spores, which will remain suspended in the fuel phase. Bacteria and fungi (yeasts and molds) can produce surfactants that cause formation of an emulsion layer and a loss of clear distinction between fuel and water phases. Within this layer and in the fuel just above it, microbial cells may be contained within microscopic water drops. Some polymeric materials produced by microbes also show affinity for both fuel and water phases [6]. Large numbers of microorganisms may grow in slimes (biofilms) on the internal tank surfaces, particularly those surfaces in direct contact with water on the tank bottom. The anaerobic Sulphate Reducing Bacteria (SRB), an important group of microbes with regards to corrosion and sulfide generation [7], will tend to be found at the dead bottom of the tank or deep in biofilms where oxygen levels are depleted, oxidation reduction potential (Eh) is low and where they are sustained

[1]ECHA Microbiology Ltd, Unit M210 Cardiff Bay Business Centre, Cardiff CF24 5EJ, UK.

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by organic acids produced by primary aerobic fuel degraders [8]. Figure 1 shows a typical distribution of micro-organisms in a quiescent fuel storage tank.

It is apparent that although microbial growth may be restricted to areas in the immediate vicinity of free water, there may be penetration of microbes into the fuel layer. In a quiescent system this penetration will generally be no more than a few centimeters. However, if there is physical disturbance of the tank or system, particularly if the water or interface layers are disrupted, a far greater dispersion of microbes into the fuel will occur [9]. For example, tank filling operations and product movements will cause significant turbulence and disperse microbes and microbial slimes throughout the fuel where they may go on to cause fouling and filter plugging problems to fuel users [10,11].

Because the density of microbes is considerably greater than that of fuel, given time, microbes suspended in fuel phase will settle back to the tank bottom [12]. According to Stoke's Law, the velocity at which they do so is a function of the viscosity of the fuel and the density and diameter of the microbial particles. This determines that a typical individual microbial cell of say 2 μm across will settle at about 0.2 cm h^{-1} in a typical gas oil and thus could take many weeks, even months to return to the tank bottom. In practice microbes are usually aggregated into larger particles and thus settling of the majority of microbial material will be far quicker. Fuel quality control guidelines will typically stipulate that, after filling a tank, a settling time of 1 h per 1 ft (30 cm) of height of product is allowed before that tank is returned to use (e.g., Joint Inspection Group (JIG) *Guidelines for Aviation Fuel Quality & Operating Procedures for Jointly Operated Supply & Distribution Facilities*). In theory this should allow complete settling of microbial particles of greater than 25 μm across, which in practice will make up a substantial proportion of the suspended microbial material. This is why "Top" and "Upper" layer samples from tanks will generally only contain a few, if any, microorganisms. Occasionally settling of microbes to the tank bottom will be impaired; for example if microbially generated gas is trapped within the microbial aggregate or due to convection currents within the tank.

The fuel phase presents a hostile environment to most microbes. Only spores (a viable but inactive state of microbes) are capable of long-term viability in the fuel layer. Most bacteria that spoil fuel do not produce spores and hence die quickly, often within days or even hours, unless contained within water drops [5,13]. Yeasts tend to survive slightly longer [14] whereas molds, which tend to produce spores prolifically at the fuel water interface, may survive indefinitely [15]. It should not be forgotten that contamination of fuel by dead microbes can still cause operational problems such as filter plugging. The poor survival of some microbes in fuel phase also has implications for the validity of test results when there is a long delay between drawing fuel samples and conducting analyses for viable microorganisms (e.g., colony forming unit counts). Analyses for viable microbes that are conducted more than a few days after drawing fuel phase samples may significantly underestimate contamination.

In summary, it can be seen that although viable microbes and non-viable microbial material may be detected in fuel phase, the vast majority of microbes will normally be de-

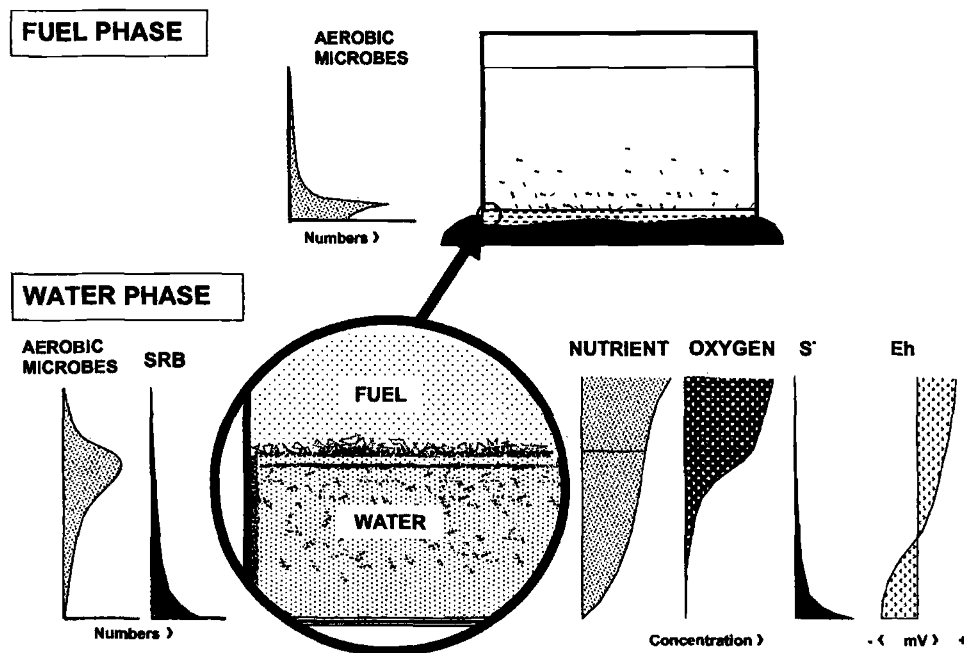

FIG. 1—Typical distribution of microbes in a quiescent contaminated fuel storage tank. The fuel phase will generally only contain *aerobic* microbes, which slowly settle to the fuel:water interface. Levels of hydrocarbon *nutrients* and oxygen will be relatively high at the interface and thus *aerobic microbial growth* is promoted in the water immediately under the fuel. Nearer the steel plate on the tank bottom, *oxygen* is depleted, oxidation reduction potential (*Eh*) becomes negative and anaerobic growth by Sulfate Reducing Bacteria (*SRB*) is promoted with consequent sulphide (*S⁼*) generation.

tected in water phase at the bottom of a fuel tank or system. (Note: adopted convention is that numbers of microbes in fuel phase tend to be quoted per liter and numbers in water phase per milliliter). Top layers of fuel will usually contain fewer microbes than lower layers. Biofilms adhered to internal tank surfaces may contain even higher numbers of microbes; as these are generally inaccessible, it is exceedingly difficult to sample microbial populations in biofilms in "in service" tanks and systems. The types and numbers of microbes detected in samples taken from a fuel tank or system at any one time, will be affected by the volume and dispersion of free water, the surface profile of tank floors, the amount of disturbance of tank contents and the subsequent length of settling time given. These factors are ideally considered prior to undertaking a sampling exercise. Sampling may then be optimized to provide the most informative data.

EXISTING GUIDANCE ON SAMPLING AS PART OF A MICROBIOLOGICAL INVESTIGATION

There are currently two recognized guideline documents that provide a background to microbiological contamination in fuels:

1. *ASTM Standard Guide to Microbial Contamination in Fuel and Fuel Systems* (ASTM D 6469)
2. Institute of Petroleum, *Guidelines for the Investigation of the Microbial Content of Fuel Boiling Below 390°C and Associated Water*

Both documents include some guidance on how to take samples for microbiological investigation. Specific guidance on sampling methodology is more extensive in the IP document, which focuses on use of microbiological testing as part of a fuel quality assessment. The ASTM document emphasises root cause analysis with a broader consideration of test methods that are useful investigative tools; microbiological tests are included in context with physical and chemical tests [16]. This chapter aims to reiterate, update, and expand on the guidance given in these documents, but does not intend to repeat all the information given therein.

Detailed guidance on drawing representative samples of fuels for chemical and physical testing is given in *ASTM Standard Practice for Manual Sampling of Petroleum and Petroleum Products* (ASTM 4057) and in ISO 3170 *Petroleum Liquids–Manual Sampling*. The procedures described are those widely employed in the oil industry and they are generally applicable to the drawing of samples for the purpose of chemical and physical tests conducted as part of a microbiological investigation. They also provide a basis for suitable procedures for the drawing of samples for microbiological testing, but require some modification for this application. Appropriate modifications are described later in this chapter.

It is not intended to cover general health and safety aspects of sampling in this chapter and the reader should refer to the above industry standards and consider relevant local legislation. The risk to health presented by the microbiological contaminants themselves should be considered. Microbial contaminants in fuel will generally be in Biohazard Category / Level 1 or 2 [17,18] and the presence of highly pathogenic organisms is very unlikely [19,20]. Because tank entry is not generally required in the drawing of samples, there will be no direct exposure of sampling personnel to large numbers of microbial contaminants, assuming routine safety precautions for the handling of petroleum products are observed. Consequently, in most cases there will probably not be a significant microbiologically related health risk to those drawing the samples. Sampling fuel systems as part of a microbiological investigation will generally not present any greater hazard to health than sampling for other purposes. It must be stressed, however, that there is an onus on those managing sampling operations to fully assess all potential hazards [21]. A significant health risk may be presented where personnel enter tanks containing residues of microbial growth. For example, inspection of an aircraft fuel tank, where internal surfaces are covered in mold growth shedding large numbers of spores, could present a hazard [22]. Expert advice should then be sought.

DEVELOPING SAMPLING PLANS FOR MICROBIOLOGICAL INVESTIGATION

The precise objectives of a microbiological investigation are ideally considered before drawing samples. The analyses to be conducted should be determined in advance, as the sampling requirements for different tests may vary. Typically, investigations are conducted in order to establish:

- Whether there is a potential for microbiologically related operational problems.
- Whether existing operational problems may have been caused by microbiological contamination.
- Whether antimicrobial control measures have been successful.

These objectives will, in particular, determine whether a water phase or fuel phase sample(s) (or both) are most appropriate for analysis [23,24]. Investigations will broadly fall into two categories [25]:

- Investigation or monitoring of contamination in a *tank* or *system*
- Investigation of *fuel* quality

Investigation of Tanks and Fuel Systems

When the onus is on assessing contamination in a tank/system, microbiological analysis of a water phase sample is most appropriate. A water phase sample will be a "worst case" sample and is not representative of contamination in the bulk of the fuel. The majority of contaminated water will remain in the tank, regardless of fuel receipts and deliveries. However, the microbes in this water have the potential to contaminate the fuel passing through the tank, with consequent adverse implications for both fuel quality and dissemination of contamination down the fuel distribution chain.

Many refineries, distribution facilities, storage facilities, and end-users now have routine sampling and microbiological testing programs that enable the extent of contamination to be monitored in their fuel tanks. "Action" and "warning" limits may be set, which, when exceeded, notify the operator that investigative or remedial measures are required. Exceeding the "warning" limit will probably instigate further in-

vestigation, whereas the "action limit" will indicate that anti-microbial measures are necessary. Further sampling should then be conducted to confirm those measures have been successful. Ideally the water phase sample will be obtained using a bottom sampler (thief), but a drain sample is sometimes used as a more easily obtainable alternative [26], particularly for small end-user tanks. Contamination in water phase is not always indicative of contaminated fuel and consequently, when water phase contamination is in excess of predetermined "warning" limits, fuel phase samples may then be analyzed for viable microbes, microbial particulate and/or by-products of microbial activity (e.g., surfactants and sulphide). The fuel phase analysis will help determine whether contamination in water phase has any bearing on fuel quality (see below). It is important to draw such fuel phase samples after consideration of any effects of product movements and settling times. Further investigation may also entail additional tests of water phase, for example, to determine the presence of microbial nutrients or physico-chemical conditions that may affect the ability of the water phase to support microbial growth. A typical sampling and testing program is shown in Fig. 2.

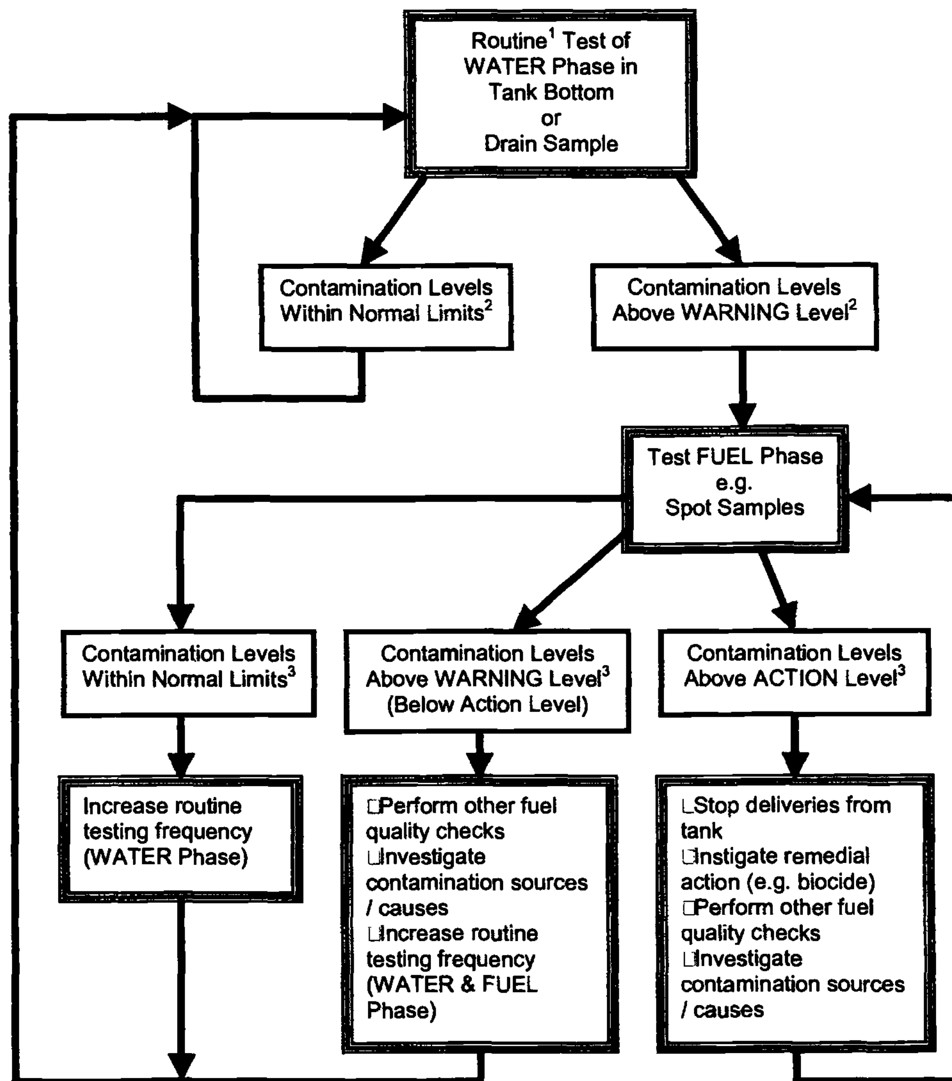

FIG. 2—Example of a microbiological sampling and testing regime for fuel storage tanks.

[1]Frequency of routine testing will be determined by numerous factors (See section above, Investigation of Tanks And Fuel Systems).

[2]Typically, counts in excess of 10^4–10^5 per mL of water phase indicate some microbial proliferation is occurring, but the water phase Warning Level should be based on "in-house" experience for the site / system / tank sampled. The types of microbes present and trends of contamination will also be important.

[3]There are no universally accepted standards or norms for levels of microbial contamination in fuels in storage tanks. "In-house" values should be set taking into account experience, expert advice, type of fuel, operating conditions, and what is practically achievable.

NB. The actions shown are for example only and may be inappropriate for some facilities and/or operations (e.g., biocides should never be added to aviation kerosene in storage).

For routine microbiological monitoring programs, the frequency in which samples are drawn for analyses will very much depend on the particular circumstances and operating conditions. ASTM D 6469 advises that sampling frequency should be such that at least three data sets are obtained during the period taken for contamination to go from negligible to significant levels. This will enable timely remedial action before operational problems are experienced. Where there are no historical data available to establish how long this period is, a sampling interval of between one and three months will in most cases be appropriate. The rate at which contamination and operational problems develop will increase with increases in water contamination, nutrient availability (e.g., water in tanks that has a high nitrate and/or phosphate content) [11] and increasing temperature (e.g., higher growth rates in summer months and in tropics) [27]. The throughput of fuel through terminals may also influence the rate at which problems develop. All these factors should be considered when initially determining the sampling interval. This can then be modified as necessary with experience.

Investigation of Fuel Quality

To assess fuel quality, analysis of fuel phase rather than water phase samples will obviously be most appropriate. Refiners and fuel distributors may need to conduct an occasional check on the microbiological quality of fuel leaving their facilities as a quality assurance measure. They may also check the quality of fuel receipts to protect themselves against introduction of contamination. End-users may occasionally check the microbiological quality of the fuel they are supplied to protect themselves against operational problems; this is particularly true of marine bunkering and aviation fuel uplifts, where the consequences of using heavily contaminated fuel may be serious. Microbiological quality checks of fuel may be incorporated as part of a routine program, but it is more often the case that fuel quality is only checked when routine monitoring of water phase in facilities (as described above) indicates that significant contamination may be present. Checks may also be made when an end-user has good reason to suspect a fuel supply is of dubious quality. The fuel samples selected must be representative of the entire fuel batch or parcel. Because of the highly heterogeneous distribution of microbial contamination, a single sample will rarely give an adequate reflection of overall contamination. Ideally several samples are needed and typically, for storage tank sampling, three spot samples (Upper, Middle, and Lower) will be taken; a single test on a proportional composite of these may be made. If this is deemed impractical a Clearance sample (or suction level) can be tested, although this will be less likely to give a full representation of fuel phase contamination. For smaller end-user tanks, one or two spot samples may be appropriate or alternatively samples drawn from the line delivering fuel to the engine, before any filtering stage. A water phase sample or tank bottom sample may also be included as part of an investigation of overall microbiological quality of fuel in a tank. If the water phase is contaminated, even if fuel layer samples are free of significant contamination at the time of sampling, with any disturbance during supply or if there is a risk of transfer of water with the fuel, then there is a potential to contaminate the fuel and the facility to which that fuel is passed.

For product deliveries and transfers through pipelines, at very least, beginning and end of transfer samples should be drawn. Alternatively, automatic sampling devices are sometimes employed but the user needs to be sure that a truly representative sample is obtained. If microbiological contamination is transferred with the product it may be contained within small pockets or slugs of water and it is difficult to ensure that this water is sampled proportionally. Pipelines may create conditions of laminar flow whereby particulate, including micro-organisms, will tend to concentrate at the centre of the line; in such cases, devices such as drip samplers, which do not sample across the entire bore of the pipeline, may fail to provide a true representation. ASTM *Standard Practice for Automatic Sampling of Petroleum and Petroleum Products* (ASTM D 4177) and ISO 3171 *Petroleum Liquids–Automatic Pipeline Sampling* provide guidance on design, installation and operation of appropriate automatic sampling equipment.

In the event of disputes where microbial contamination is alleged to have influenced a fuel's fitness for purpose, a complete range of representative samples of fuel phase, plus water phase, will ideally be tested. However, as disputes can be protracted, suitable fresh samples may no longer be obtainable when the analysis is conducted (sometimes several years after the disputed incident); in such cases exceptional caution must be exercised when interpreting results of tests for viable organisms.

In some cases, it is desirable to make occasional checks of part or all of the chain of fuel delivery, for example from Aviation Fuel storage to point of fuel uplift. It is not possible to consider all possible investigations of this type, but general principles can be applied. The critical points at which fuel quality may be affected should be identified and then representative samples taken before and after these points. For example, Filter Water Separators with coalescer units will improve fuel quality if well maintained, but will contaminate fuel if the coalescer socks become sites of microbial growth [3,4]. Samples of fuel should be taken upstream and downstream of these filters at several times during each individual fuel delivery. Ideally, several fuel batches should be investigated. Analysis of a sample from the Filter Water Separator drain will provide an indication of whether the unit is harboring microbial growth. To investigate the extent to which fuel phase microbial contamination may be passed from a tank down the distribution chain, samples should be taken after the normal settling time.

Many aircraft operators conduct routine or occasional microbiological monitoring of fuel tanks as a precautionary measure [28]. A number of unique factors need consideration particularly with regards to the wide temperature fluctuations within tanks (e.g., below −40°C in flight to above +40°C on the ground). These temperature variations will directly affect microbial growth rates and will also affect the condensation of water within tanks [29]. Ideally sampling times and the selection of tank drain points should be optimized for collection of a water phase for analysis and the experience of field maintenance staff will be important in determining this.

Fuel end-users who are investigating microbial quality problems should always try to establish the quality of fuel

supplied to them. This will be the baseline for comparison with results of samples taken from appropriate points onboard their vessel, aircraft, vehicle, or train. It will then be possible to determine whether operational problems have been encountered due to a quality problem with the fuel supply or due to growth on-board (or both) [11]. It should be remembered, however, that a negative result for a test of a single spot sample of the batch supplied will not necessarily provided assurances about its quality. Samples provided by the fuel supplier may also not always be thoroughly representative of the fuel actually delivered.

A final comment on devising sampling plans is to stress the importance of consulting field operators. They will usually have specific experience of the facility or system, which will assist in selection of appropriate sampling locations and frequencies. For example, they may be able to advise whether particular tanks are prone to slime accumulation or comment on operating conditions that promote water accumulation.

SAMPLING PROCEDURES

Techniques for taking samples for microbiological investigation can be broadly split into the following two categories according to the type of analysis that is to be performed:

- Samples for microbiological culture tests for viable microorganisms.
- Samples for analysis of chemical and/or physical properties or for microbiological analyses that do NOT involve culture tests for viable microbes.

Where samples are to be used for culture tests for viable microorganisms, precautions must be taken to ensure that microorganisms from a source other than the sampled material do not contaminate the sample; such sampling contaminants can prejudice a test result. Most microbiological tests that involve an incubation stage of several days are culture tests. Individual microbial particles in the sample are cultured in or on a nutrient medium, where they replicate many times, reaching numbers of many millions and becoming visible as a countable colony (colony forming unit or cfu) or a color change or turbidity in the growth medium [23,25]. If microbes that are not derived from the sampled material are introduced during sampling, they will also replicate during the incubation stage and thus give an artificially high result or even mask growth by genuine fuel contaminants. Examples of commonly used culture type tests that require such sampling precautions are:

- IP 385 *Determination of the viable microbial content of fuels and fuel components boiling below 390°C–Filtration and culture method* (IP 385).
- ASTM *Test Methods for Sulfate Reducing Bacteria in Water and Water Formed Deposits* (ASTM D 4412).
- NACE TMO-194 *Standard Test Method. Field Monitoring of Bacterial Growth in Oilfield Systems (Section 3 Culture Techniques)* [30].
- Standard plate counts for microbial colony forming units (various methods).
- On-site tests such as dip sticks, dip slides, and gel-based tests, which make semi-quantitative or quantitative estimates of microbial colony forming units (many brands).
- Liquid broth vials utilizing a color change or turbidity in broth to indicate microbial contamination (several brands).

It is not practical to achieve truly sterile conditions when taking samples from fuel tanks and systems. Microbes are ubiquitous and those that may contaminate samples could come from air, personnel taking the sample, the sample bottle, the sampling equipment and dirt on sampling pipes, hatches and accessories around the sampling point. Reasonable measures can, however, be taken to minimize the chances of sample contamination and consequent adverse impact on the test result. These measures are described in more detail later in this section.

For physical and chemical analyses and microbiological tests that do NOT involve a culturing stage it is not necessary to take specific precautions to eliminate ingress of microbial contaminants when taking samples. The procedures stipulated in ASTM D 4057 and ISO 3170 are appropriate. The reader is asked to reference either of these documents and also to take into consideration any specific requirements stipulated in the relevant test standard. For example, many tests for particulate in fuel and/or for filterability under standard vacuum require a large sample volume (e.g., 5 L). Many of the physical and chemical tests that may be conducted as part of a microbiological investigation are included within this manual. Examples of microbiological tests, which are NOT culture tests are: ASTM *Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water* (ASTM D 4012) and IP 472 *Determination of Fungal Fragment Content of Fuels Boiling Below 390°C* (IP 472).

It is not practical to cover within this chapter, all specific precautions for the wide variety of test methods that may be employed in a microbiological investigation. The guidelines below apply to samples to be taken for microbiological testing where a culture stage is involved. Generally the techniques described will also be good practice for samples taken for other analyses as a high degree of cleanliness is ensured during sampling. Where it is not known in advance which test methods are to be used as part of the microbiological investigation, it is recommended that the following guidelines be followed.

It should be noted that little specific reference is made in this Chapter to the collection of biofilm samples. In the course of routine fuel use and delivery operations, such samples are difficult to obtain. Tank entry is almost always required. Nevertheless, if samples of biofilm can be obtained, for example from filters and filter casings or tank surfaces, there is considerable merit in submitting these for analysis. Many of the precautions described below are applicable.

Preparations for Transport of Samples and Analyses

To minimize delays between sampling and analyses, preparations for testing and, where necessary, arrangements for transport of samples to the test laboratory should be made prior to drawing samples. Changes in the microbial population in a sample are likely to occur during transit. Precautions must be taken to ensure that, once the sample has been drawn, it continues to maintain, as far as is practicable, the same microbial population characteristics until such time as

it can be analyzed. Samples from ambient temperature systems are ideally kept cool (c. 4°C) during transit, but should never be frozen. The delay between sampling and analysis should be minimized by conducting microbiological tests as soon as possible after drawing samples; this should be within 24 h. Reliable results may not be obtained if analyses for viable microbes are conducted after 24 h. If testing within 24 h is not practicable, an expert microbiological opinion should be sought as to the reliability of test data; this will take into account factors such as sample transit and storage conditions, the time delay in conducting analysis, type of sample and whether the sample contained water. The use of on-site tests has the advantage that delays between sampling and analysis can be greatly reduced.

If an analytical laboratory is used, give them advance warning of delivery of samples so that they can be prepared for the analysis and so minimize delays. It will also assist the laboratory in their selection of test procedures and interpretation of results if they are supplied with as much information as possible about the system sampled and the reasons for and objectives of the investigation. This information might include a description of the appearance of the sample at the time of sampling, the system / tank temperature, the method used to decontaminate sampling devices / bottles (if any), the volume and depth of fuel sampled and the location and type of tank draw off points. If biocides have been used, analysis techniques may need to be adapted to neutralize these. Hence, if appropriate, a full record of recent biocide treatment (type, dose, and application dates) should be supplied with the samples.

Labeling and Chain of Custody

Always indelibly mark sample containers immediately before drawing samples. A fully detailed label should then be prepared to include the following details:

- Place at which the sample was drawn;
- Description of the material sampled;
- Tank / system / aircraft / ship reference number or name;
- Type of sample (e.g., drain / bottom / middle etc.);
- Date and time of sampling;
- Identifying mark of the operator who drew the sample.

The receiving laboratory should also record both the date and time the sample was received and date and time the sample was tested. The chain of custody from sampler to laboratory should be clearly defined and recorded.

Sample Bottles and Containers

Sample bottles should be of suitable size (500 mL is usually appropriate) and both bottle and cap should be made of material that is compatible with fuel (e.g., not polystyrene). Cork stoppers and caps that contain a cardboard liner are not recommended as they may harbor microbes. Clear glass bottles are ideal as they readily enable a preliminary visual assessment, an important part of any microbiological investigation. Polypropylene is appropriate if a more durable bottle is required for transport.

Ideally sterile sample bottles should be used, although it is not always practical to do so. Pre-sterilized polypropylene or polythene containers can be purchased from most laboratory suppliers. Sample bottles made of glass can be sterilized by heating in an oven at 160 °C for at least 2 h (ensure the cap is heat resistant or sterilize separately). Specialist laboratories may have the ability to autoclave (steam treatment at 103–138 kPa (15 to 20 psi) for 30 min) sample bottles made of glass or autoclavable plastics (e.g., polypropylene and PET (polyethylene terephthalate)). Some autoclave processes, however, leave a water condensate in bottles and thus, precautions must be taken to ensure bottles are completely dry when they have cooled down after autoclaving. Small amounts of residual water in the sample container can lead to erroneous microbiological test results for fuel phase samples [26], because any microbes in the sample will tend to concentrate in the water. Water condensate in the test bottle will also lead to erroneous visual examination.

New, clean, dust free-bottles can be used if sterile containers are not readily available when required.

Sampling Devices

Devices that are commonly used to take samples include bottle cages (for spot samples) and bottom samplers. Such devices should be thoroughly cleaned internally and externally before use (see ASTM D 4057). The rope or wire used to lower the sampling device should also be clean. Ideally the sampling device will have a screen that prevents drops from the line falling into the sample as it is raised from the tank. Because, in the case of bottom samplers, the sampled material is drawn first into the sampler itself before transfer to the sample bottle, the device should additionally be decontaminated internally by rinsing with a 70% alcohol solution; Industrial Methylated Spirit, Ethanol, or Iso-propanol are suitable. The alcohol should be thoroughly drained away and allowed to evaporate from the sample device and particular attention should then be paid to rinsing away any alcohol residues with the fuel to be sampled (see below). Alcohol residues in the sample could adversely affect the viability of fuel contaminants and thus prejudice the test result. Ideally sampling devices will be of a design that facilitates cleaning (e.g., both ends will be removable to enable internal access). Figure 3 shows a bottom-sampling device suitable for collecting samples for microbiological analyses. Sampling devices should be cleaned between each tank/system sampled. It is not absolutely necessary to clean thoroughly between sampling each layer when samples are taken in the order of top to bottom, as recommended below.

Sample Cocks and Drains

When taking samples from sample cocks, valves, and tank drain points ensure these are externally clean and then wipe with a clean lint-free cloth soaked in 70% alcohol. To reduce the chances of microbes on the outside of the outlet point entering the sample, it is recommended that while taking the sample, the outlet point does not extend into the sample container below the liquid level in the bottle (NB this is converse to recommendations in ISO 3170).

The design of the sampling point, and the location and height from which the sampling line draws within the tank should be fully understood. Lines that incorporate filters or sight glasses are generally not appropriate for taking samples

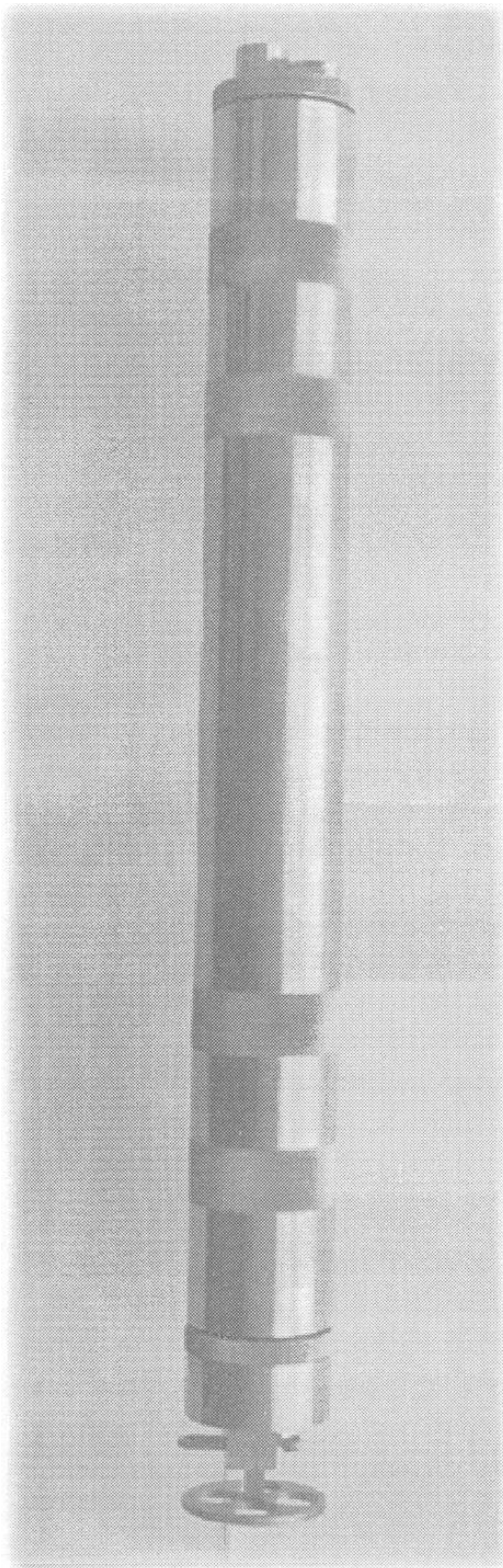

FIG. 3—Bottom sampling device suitable for the drawing of fuel tank bottom samples for microbiological analysis. The device shown is constructed of stainless steel and can be opened at both ends for easy cleaning. The device is lowered on an earthed wire or rope. A valve in the bottom of the device opens when the foot rests on the tank bottom. The foot can be adjusted so that any height from 1–30 cm above the bottom of the tank may be sampled. As the sample enters the bottom valve, air escapes from the opening on the top of the device; after lifting the device full of sample, this opening is also used to pour the sample into the sample container.

for microbiological analysis. An estimate of the volume of fluid in the sampling line (i.e., between the point it draws and the outlet) should be made and an equivalent volume flushed from the line before taking the sample.

Taking Samples

Wash hands before handling clean sampling equipment. Clean the area immediately around the sampling location as far as is practical; particular attention should be paid to cleaning away loose dirt or scale that may drop into the sample container during sampling. If necessary, decontaminate surfaces by wiping with a lint-free cloth soaked in 70% alcohol. Health and safety precautions will usually dictate that protective gloves are worn when taking samples. These gloves should be clean and of fuel resistant material.

Always avoid touching the insides of sample containers, container caps, and cleaned sampling devices. To reduce the chance of contamination from the surrounding air, only open sample containers and sampling devices for the minimum time necessary to conduct the sampling operation.

When sampling various layers in a tank, always sample from top to bottom. Before taking the true sample, always rinse the inside of the sampling devise (if used) and sample bottle with the fuel being sampled (from the top layer in large tanks). Ideally three rinses should be performed.

Note: To avoid anoxic conditions developing within the sample prior to analysis, the sample container should not be completely filled. IP Guidelines recommend the container should be no more than 80% full, whereas ASTM D 6469 recommends no more than half full. Somewhere in between these two recommendations will probably be acceptable, but much will depend on the delay between sampling and analysis; leave more headspace for a longer delay. An exception to this recommendation is when samples are to be drawn specifically for Sulfate Reducing Bacteria analysis; in this case fill samples almost to the brim to promote anoxic conditions; leave only sufficient headspace to allow for possible expansion of contents with increases in temperature during transit.

SUMMARY

It is not possible to provide detailed guidance on the drawing of samples for all possible scenarios in which microbiological testing may be undertaken. It is hoped, however, that this Chapter has highlighted all the major issues which must be considered in the planning of such investigations. Once those conducting the investigation are aware of these factors, the implementation of good practice is relatively easy and requires minimal training of sampling personnel. The principles of appropriate procedures are the same as for routine good sampling practice for petroleum products; avoiding contamination and cross-contamination, clearly identifying samples and ensuring the integrity of samples is maintained during their transfer to the testing facility. When the test laboratory is furnished with all the relevant background information and they are sure that a good sampling protocol has been followed, they will be in the best possible position to accurately and informatively interpret the test data.

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3

Remediation Techniques

Howard L. Chesneau[1]

INTRODUCTION

THE TERM FUEL SYSTEM REMEDIATION describes the various processes used to return contaminated fuel and fuel systems to an acceptable condition. This chapter provides guidance on the basic processes for decontaminating fuels and fuel systems. Contaminants included in this discussion are water, organic and inorganic particulates, sludge slime, and biomass. After completing this chapter, readers should have a general understanding of each of the remediation processes; fuel polishing, tank cleaning, disposal, and chemical treatment. Fuel polishing involves filtration processes to clean the fuel. Physical tank cleaning is the best way to ensure that a system has been cleaned thoroughly. Tank cleaning is most effective when combined with fuel polishing. Typically, preventive measures are more cost effective than corrective measures. This chapter concludes with recommendations for reducing the rate of contaminant accumulation. 'Fuel polishing' is designed to remove water and particulates from fuel in order to reduce water and sediment loads below applicable product specification criteria such as ASTM fuel specifications. Where sludge and sediment have accumulated on tank bottoms, slime has built up on tank shell surfaces, or a combination of both phenomena has occurred, fuel polishing is insufficient. Tanks thus contaminated need to be cleaned. Microbicide treatment may be needed to disrupt and kill biofilm populations (see Chapter 1).

Both biological and non-biological processes may cause fuel and fuel system deterioration that requires remediation. The selection of remediation techniques consequently depends on the deterioration symptoms and their causes. Additionally, tank size and configuration will impose logistical and economic limitations on the practicality of different remediation options. For small service tanks, tank replacement may prove less expensive than remediation options. Intermediate sized tanks (3.7–75 m^3; 1 000 to 20 000 gal US) may be cleaned using remote systems. Larger tanks may require both manual and remote cleaning processes.

Chapter 1 presented information to help operators diagnose deterioration problems. Chapter 2 discussed sampling strategies and techniques. The present chapter provides guidance on the basic processes for decontaminating fuels and fuel systems. After completing this chapter, readers should have a general understanding of each of the remediation processes, but this manual is not designed to qualify readers to perform remediation processes. This chapter offers an introduction and overview of the most commonly used remediation processes. It does not provide step-by-step standard operating procedures. Nor does it provide a complete review of the safety precautions required for each of the remediation processes. Qualified personnel should perform remediation. Typically, personnel trained to perform fuel system remediation have received considerable hazardous material handling, operations safety and first aid training as well.

The following sections review the fundamentals of fuel polishing, tank cleaning, and microbicide treatment.

FUEL POLISHING

This technique primarily involves filtration. Centrifugation is used less frequently. Although both processes can produce the same quality end-product, as defined by water and sediment content criteria, centrifugation equipment tends to be more costly and more susceptible to mechanical problems than comparably sized filtration equipment. Matching unit capacity with anticipated contaminant loads is more critical with centrifuges than it is with filtration units. Moreover, centrifuge efficiency depends on the relative differences between contaminant and fuel specific gravities. The smaller the differences, the greater centrifugal force (rotor speed) needed to separate particulates from the fuel. In contrast, filtration depends on particle size and geometry. As will be discussed below, appropriate filtration media must be selected based on the characteristics of the contaminants to be removed. Some contaminated fuels may require a combination of filtration and centrifugation in order to meet the objectives of the polishing process.

The key to successful polishing is first defining the cleanliness requirement for the particular system or use of the fuel. Although there are water and sediment criteria listed in most fuel standard specifications, certain applications may have more stringent particulate contamination criteria [1]. For example, the military acceptance criterion for sediment load in No. 2 diesel is 10 mg L^{-1} [2] by ASTM Method D5254. In contrast, most grades of diesel fuel are permitted to carry up to 0.05% ($^{v}/_{v}$) water and sediment (ASTM Specification for Diesel Fuel Oils, D 975), although most fuel suppliers and users would consider fuel with this level of contamination to be unacceptable and unfit for use. Consequently, clearly defined fuel cleanliness criteria (parameter control limits and

[1]President, Fuel Quality Services, Inc., P.O. Box 1380, Flowery Branch, GA 30542.

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the test methods by which those parameters will be measured) must be defined before the polishing process is designed.

Media Selection

Filter media selection depends on fuel grade, water content, initial particulate load, volume to be filtered, flow-rate, temperature time constraints, and cleanliness required. The same factors dictate filtration system selection. A single medium, single-stage system may be able to polish small volumes of moderately contaminated fuel adequately. More often, multi-stage systems that include some combination of coalescer and particulate filtration media, aligned in series, are needed to achieve successful polishing. As noted above, in some circumstances (for example, a contaminant with chemical and rheological properties that make it difficult to remove by filtration) a centrifugation stage may be needed to achieve the required cleanliness criteria.

Typically, filtration media are described by nominal pore size (NPS) or Beta (β) ratings. The NPS refers to the minimum size particle that the medium is designed to trap as a percentage of efficiency for that size (for example, 10 μm at 50% or 10 μm at 75%) The β-ratio [1] measures filter efficiency as the ratio of the number of standard-sized particles that are retained to those that pass through a test filter. In practice, trapped particles alter filter performance over time. Partially blocking the fluid flow-path, they help trap particles that are smaller than the filter's rated NPS. However, as particulate loads on the filter medium continue to accumulate, fluid-flow becomes increasingly restricted. This phenomenon is generally measured either as pressure differential[2] across the filtration unit as pressure increase between the pump filter. Ultimately, the medium becomes overburdened with particulates and needs to be either replaced or cleaned. Filter performance (both efficiency and capacity) is affected by the contaminants' physical and chemical properties.

Typically, fuel polishing systems are designed to provide either 0.2 μm (micron) or 0.5 μm NPS filtration. Some types of polishing might require the use of clay. Attapulgite (sometimes called Attapulgite clay or Fuller's Earth is the best medium for removing polar compounds and small amounts of suspended water. Fiberglass and resin impregnated cellulose are used for particulate removal. Attapulgite clay, diatomaceous earth, activated carbon and fibrous pads are depth filter media. This means that the flow path through the medium is torturous. Particles accumulate within the medium matrix. In contrast, membrane filters retain particles only on the upstream filter surface. Table 1 lists some of the more commonly used fuel filtration media and their applications.

The foregoing discussion illustrates the necessity of first identifying the characteristics and load of the contaminants to be removed, next defining the criteria by which the polishing effort will be monitored, then specifying the polishing system components (media, capacities, and inclusion of water separators, centrifuges, or both).

Filtration Strategies

Fuel polishing is part art, part science, and part engineering. The two primary filtration strategies are removal-replacement and recirculation [3]. Removal-replacement provides better overall results, and is generally less time consuming, but may be more challenging logistically. Recirculation can provide satisfactory results, but the filtered fuel may become recontaminated quickly if microbial debris is not removed (or killed) completely and is able to become resuspended in the bulk fuel. Recirculation filtration is easier to perform than removal-replacement filtration. Regardless of the method selected, operators should first remove as much bottom water, sludge and sediment as possible from the tank. Although well-designed filtration units can handle the bottoms materials, this gross debris may repeatedly plug filters. This adds to labor, filter costs, and hazardous waste disposal costs.

In removal-replacement filtration, fuel is filtered as it is pulled from the original tank and transferred to another tank. Once the original tank has been emptied and rendered safe for entry, personnel can physically enter the tank and clean it. The fuel may then be filtered a second time as it is transferred back into the original tank. Removal-replacement filtration requires receiver tankage and appropriate transfer piping. Special spill containment provisions may be required to accommodate temporary tankage and transfer piping. The technical and logistical requirements of tank entry and manual cleaning will be discussed below. These requirements add to the complexity of removal-replacement filtration. However, fuel that is returned to a clean tank will not be subject

[2]Pressure differential is the mathematical difference between the pressure measured at the filter housing inlet and outlet, respectively. $\Delta P = P_i - P_o$, where ΔP is the pressure differential, P_i is the pressure at the housing inlet and P_o is the pressure at the housing outlet. Pressure may be recorded in psig or kPa.

TABLE 1—*Fuel filtration media and their applications.*

Medium	Contaminant Material Removed	Filtration Stage	Nominal Particle Size Filtered (μm)
Depth media			
Activated Carbon	Hydrocarbons from water	Waste discharge	N.A.
Attapulgite clay (Fuller's Earth)[a]	Surfactants and water	Initial	5.0
Resin impregnated cellulose	Particulates	2^0 and final	0.2 to 50
Spun glass filaments	Particulates and coalescing	Pre-filter and final	0.2 to 50
Mechanical media			
Centrifugation	Water and solids	Pre-filter	>100
Slant rib	Water and solids	Pre-filter	>100
Screen	Solids	Pre-filter	>100

[a]Detergent packages may be removed. The fuel must be re-additized after use of attapulgite clay.

to rapid recontamination by slime and particulates sloughing off tank walls.

Despite the risk of early recontamination, recirculation filtration is used more often than removal-replacement filtration in practice. To perform recirculation filtration, operators place the filtration system in-line between a tank suction- line and fill- line. These lines may be integral to the fuel system, or may be installed temporarily for the duration of the filtration process. Fixed or portable pumps are used to recirculate fuel through the filtration unit. Depending on the tank size and the location of the entry points, about five to seven volume rotations (tank volume) are usually required to achieve specified fuel quality.

The reason that recirculation filtration requires multiple passes is two-fold: the moving fuel scours surfaces over which it passes, and 'cleaned' fuel re-mixes with 'dirty' fuel. Deposits on tank shell and bottom become suspended in the recirculating fuel, recontaminating fuel that has passed through the filtration media. It takes several passes for most of the readily dislodged or re-suspended debris to be removed by the filter.

Unless special provisions are made to vary the depth of the suction line inlet, recirculation filtration may not be effective for fuel stored in large tanks ($\geq$ 1 600 m^3; >70 000 gal US or > 10 000 bbl). Fuel in larger tanks will form layers or strata rather than recirculate uniformly. Consequently, fuel recirculating within a zone ranging from 1.5–2 m from the suction line inlet and return line outlet will be polished, but fuel outside this zone will not. When designing a recirculation filtration project, operators must ensure that all of the fuel in the system will pass through the filtration system. Except for this consideration, the logistical and special design considerations for recirculation filtration are generally simpler than they are for removal-replacement filtration. When done properly, recirculation filtration may substantially decrease the frequency of tank entry and cleaning. Such tank cleaning can be costly, and also disrupt normal operations at a facility.

A detailed discussion of filtration system design is beyond the scope of this chapter. What follows is a general overview of typical filtration system components.

Pumps are the heart of any filtration system. Pumps must meet both performance and safety requirements demanded by the fuel filtration process. Many fuel polishing systems utilize small volume pumps ($\leq$ 570 $L \cdot min^{-1}$; $\leq$ 150 gal US min^{-1}) because of their relatively low cost. These systems are usually adequate for small tanks (< 40 m^3; <1 000 USG) but are inadequate for larger tanks. It requires a much greater flow rate (> 1 325 $L \cdot min^{-1}$; 350 gal US $\cdot$ min^{-1}) to polish larger volumes of fuel effectively.

Filter housings comprise the second critical element of all filtration systems. Housing capacity and design depend on the anticipated throughput rates and media selections. The most rudimentary systems include a single housing designed to contain multi-functional coalescer filters. Multiple housings aligned in series enable operators to pass fuel through several media (coarse to fine). Such designs maximize the performance life of the most expensive, final polishing, filter elements. They also facilitate filtration customization to meet special, local needs.

In a series rig, the first stage is typically a coalescer, designed to remove water and other polar compounds. Subsequent stages may be used to remove increasingly fine particles. Parallel housings may be used at one or more stages to accommodate continuous filtration during filter media change-outs. All other factors being equal, filtration efficiency decreases with increasing flow-rate. Consequently, optimal filtration system size must increases with higher design flow-rates.

Suction and discharge piping and hose must accommodate both flow requirements and fitting limitations on the filtration system and the fuel system being polished.

As indicated earlier, filter medium selection depends on the initial condition of the fuel to be filtered and the desired quality of the polished fuel. Alternative medium types (activated-carbon, mineral clays, diatomaceous earth, resin-polymer fibers, etc.) and formats (depth or planar[3]) are available. Each has properties that are advantageous for certain applications and disadvantageous for others. The critical issue is to match the media with the project. Filtration specialists typically select the most appropriate media types and formats for each project. For example, precautions should be taken to avoid removing detergent additives during the polishing process.

Filtration systems require a considerable amount of peripheral equipment. Connections, control valves, gauges, and various safety devices are integral components of filtration systems. Well designed systems also accommodate waste handling.

Strict adherence to safety precautions is an essential element of all fuel polishing processes. Operators must be familiar with the filtration system as well as the fuel system being polished. Regardless of the filtration process, system grounding, spill protection, and adequate site preparation should be part of every standard operating procedure.

TANK CLEANING

Tank cleaning is the best way to ensure that a system has been decontaminated thoroughly. The overall fuel quality improvement process is enhanced substantially by filtering the fuel as it is transferred from the contaminated tank into a temporary holding tank (see previous section).

In general, although the underlying principles remain the same, the actual protocol for tank cleaning depends on tank size and configuration. Tank size and accessibility dictate the general tank cleaning approach. When possible, the process should include personnel entry. Tanks are confined spaces and present a variety of potential health and safety hazards. All personnel entering the tank must have received appropriate confined space entry training and must follow the prescribed safety procedures. Small tanks and those with inadequate provision for entry may be cleaned using remote controlled, mechanical devices. The effectiveness of a remote cleaning process depends on the tank's internal structural

[3]A planar filter may be made of cellulosic or synthetic fiber media, a membrane and / or wire screening. Planar media are differentiated from depth media in that the flow path through the medium is very short relative to the surface area of the face of the medium (upstream surface through which fuel flows). Particulates collect on the surface of planar filters.

complexity and the cleaning system design. Remote systems are typically more effective cleaning tanks with few surface irregularities or internal fittings. Remote systems are more likely to miss surface slime and debris deposits on irregular surfaces such as baffles and coamings in ships' tanks.

Pipelines and other fuel transfer systems may require remediation processes that are beyond the scope of this chapter. Because of the complexity of most pipelines and fueling systems, cleaning requires specialized skills and equipment generally possessed by professional cleaning companies. Cleaning projects should not be done without the training necessary to perform operations safely and thoroughly.

Cleaning Process–General Principles

Except for cleaning processes that use the stored fuel as the scouring agent, all tank cleaning processes share a number of common elements. These include site preparation, fuel removal, initial flushing, surface cleaning, waste removal, finishing, and restoration into service (re-commissioning).

Site Preparation—Site preparation includes all of the engineering, logistical operations, and safety processes that must be completed before tank cleaning can begin. Engineering issues focus on system design. If pre-existing, installed piping doesn't not provide a means to transfer product from the dirty tank, through the filtration system, into a holding tank and back, a temporary system should be designed and installed. Logistical issues include scheduling tank outages and sub-contractors so that the tank can be drained and cleaned with minimal disruption to normal operations. Provisions need to be made for removal of all hazardous wastes generated during the cleaning process. Safety processes address fuel transfers, spill control, hazardous chemical handling and confined space entry. All electrical, hydraulic, and manual valving systems that might affect the tank cleaning operation should be tagged appropriately. Many facilities use prepared operational checklists that must be endorsed by all affected and supervisory personnel before tank-cleaning operations can begin. Such checklists ensure that all stakeholders are fully aware of the tank cleaning process and its effect on their routine activities.

Fuel Removal—Once the site is prepared, the first step in tank cleaning is removing most of the bulk fuel. When possible, reduce the fuel inventory within the dirty tank to a minimum. As described earlier, all remaining fuel should be filtered as it is transferred to a clean receiving tank. If the fuel is contaminated, appropriate decontamination measures can be taken at this stage (removal of polar contaminants, microbicide treatment, etc.).

Tank Cleaning—Although the principles are the same, the general procedures for cleaning small tanks and large tanks will differ. Typically large tanks are cleaned by personnel working inside the vessel. Small tanks are cleaned more often using remote devices. These processes are discussed in more detail below.

Waste Removal—Wastes generated during tank cleaning may be classified as hazardous. Federal, state and local regulations define hazardous wastes, handling requirements, documentation and disposal. Regulations usually require thorough documentation of waste volume and characteristics. Manifests follow wastes from cradle to grave. In regulatory parlance, the tank owner is the waste generator and remains responsible until ultimate disposal has been documented. Licensed hazardous waste haulers can help guide waste generators through the regulatory process. Regulations prescribe the required retention of hazardous waste manifest records.

Return to Service (re-commissioning)—Once the tank has been cleaned, it should be tested to confirm that the cleaning process has not created any leaks. The tank can then be returned to service. If the original fuel is to be returned to the tank, it should be filtered once more between the temporary storage tank and the newly cleaned tank. The first batches of fuel drawn from a cleaned tank should be visually inspected to ensure cleanliness and suitability for use.

Cleaning Process—Large Tanks (Entry Required)

The general principles outlined above apply to tanks of all sizes. However, there are a number of stages in the tank cleaning process for which there are important differences depending on tank size. As noted earlier, entry is typically required for large tank cleaning.

Once bulk fuel has been removed, the tank must be prepared for entry. ASTM D 4276, Practice for Confined Space Entry, addresses the processes involved in preparing tanks for safe entry. Ventilation, preliminary cleaning or a combination of both may be needed to prepare the space for entry. High-pressure water, steam and surface-active chemicals may be used individually or in combination to achieve preliminary cleaning.

Tank entry will allow a tank operator to inspect a tank visually for the amount and type of contamination present and assess any damage that may have been caused (e.g., microbially induced corrosion). Experts should evaluate any damage that may be present and determine whether the cleaning process is likely to cause further damage. This examination may be as simple as a visual inspection by a corrosion expert, or as technical as non-destructive testing using high tech equipment.

Tank cleaning personnel will use a combination of high-pressure water or steam, scouring brushes and squeegees to remove slime and scale from tank shell surfaces (Fig. 1). They typically use shovels and squeegees to remove sludge and other debris from the tank floor. In some cases more exotic robotic cleaning equipment may be used. The method for physical cleaning should be understood by the tank owner/operator prior to its use so that the cleanliness expectations and requirements are known. Although most of the tank cleaning effort may be completed before repairs are made, final cleaning should be accomplished after repairs are completed. This will ensure that materials and debris remaining from the repair work are removed before the tank is resealed.

The cleanliness parameters should be established prior to this type of effort. In most cases, removal of all visible slime and scale is sufficient. In some cases it may be desirable to achieve a corrosion-free (shiny) surface. If water has been used to clean the tank, the tank should be dried. Water-vacuuming, squeegee-sweeping, air-drying or any combination thereof may be used. Drying with rags, even lint-free rags, may leave fibers behind. Use of isopropyl alcohol or

FIG. 1—Bulk storage tank cleaning. Confined space entry certified technician, using squeegee to clean sludge off bottom of terminal tank (high sulfur diesel). Photo courtesy of Fuel Quality Services, Inc.

similar volatile drying agent is not recommended. These agents create an unsafe atmosphere for personnel entry. Surfaces that are to be coated may require special treatments to ensure proper bonding between the coating and surface. Final tank surface treatment with a protective coating, rust preventative, or microbicide may be prescribed under certain circumstances.

Once all internal work has been completed, there should be a final safety inspection to confirm that all debris, equipment and supplies have been removed. The tank is then closed up and leak-tested. Once the tank has passed appropriate leak testing, it can be returned to service. Fuel is returned from the temporary holding tank, via the filtration system. It is advisable to treat the first fill with a fuel-soluble microbicide in order to ensure that any remaining biological contamination, or biological contamination introduced during the cleaning process, is killed. The tank cleaning process may miss biomass that has developed on the surfaces of local piping, and within cracks and crevices that are inaccessible to mechanical cleaning processes. This post-cleaning microbicide treatment is likely to cause substantial quantities of biomass to slough-off of the aforementioned surfaces, get transported through the system and plug filters. Consequently, operators should have a supply of replacement filters available to replace those that become plugged. Typically two or three filter change-outs are needed before this dislodged, dead biomass has been flushed from the system.

Although tank cleaning has been practiced for many years, much has changed. Both the methods used and the safety issues involved require significant planning and due diligence on the part of the tank owner/operator as well as the company doing the cleaning.

Cleaning Process—Small Tanks (Entry Not Required)

Another method of tank cleaning used primarily in small tanks (≤ 190 m^3; ≤ 50 000 gal US; ≤ 1 100 bbl,) employs the use of the fuel itself to do the cleaning (Fig. 2). This involves recirculating the fuel at high flow-rates (0.75–1.70 $m^3 \cdot min^{-1}$; 200 to 450 gal US min^{-1}) through filtration equipment. Some sophisticated systems employ a device that will use a hose to slide down the tank and clean the bottom, using the fuel to move debris. The dirty fuel is then filtered and the filtered fuel is returned back into the tank. It is important that the tank operator understand the limits of this type of cleaning. Many claims have been made, but verifying the results may be difficult [4].

Vehicle tanks are more difficult to clean because of tank location, the lack of entry points, and the many configurations (and internal piping or compartments) that may be involved. These tanks may be cleaned either chemically or mechanically. In some instances involving suspected corrosion, visual examination may be necessary. Optical devices that facilitate visual inspection are available commercially. Depending on tank configuration and replacement cost, it may be more cost effective to replace small tanks rather than to clean them.

ANTIMICROBIAL PESTICIDES

Antimicrobial pesticides, also referred to as microbicides or biocides, are chemicals that are used to kill microbes that contaminate fuel systems. Antimicrobials have been used to treat contaminated fuels and fuel systems since the early 1960s. Early treatments were used primarily in jet fuel systems that had experienced severe microbiological contamination and corrosion. Filter plugging and tank corrosion also justified increased use of fuel microbicides in marine systems. Increased recognition of the costs of fuel quality degradation due to uncontrolled microbial contamination has led to the increased use of fuel microbicides in many grades of fuels.

Microbicides are typically classified by their target organism. Bactericides are primarily effective against bacteria. Fungicides are primarily effective against fungi. Broad-spectrum antimicrobial pesticides, often just called microbicides, are effective against both bacteria and fungi. Although the occurrence of algae is rare in fuel systems (See Chapter 1; uncharacterized biomass is often misidentified as algae), fungicides and broad spectrum microbicides are generally effective against algae too.

Additionally, antimicrobial pesticides may be classified on the basis of their fuel and water solubility. According to this scheme, they are classified as fuel soluble, water soluble or dual soluble. The chemistries are varied as is the performance of these materials. A general list is provided in Table 2. Microbicide selection should be based on treatment objectives and appropriate approvals. In the United States, fuel soluble microbicide use is regulated under 40 CFR 150 – 189 (Pesticide Programs derived from the Federal Insecticide Fungicide and Rodenticide Act–FIFRA) and 40 CFR 79 (Fuels and Fuel Additive Regulations derived from the Clean Air Act–CAA). Microbicides used to treat fuel systems, control microbial contamination in fuel system bottoms-water, or treat off-highway fuels only, are not regulated under 40 CFR 79. In addition to governmental regulations, engine manufacturers and trade groups may specify microbicides that are approved for use in fuels used in their systems. For example, airframe and jet engine manufacturers must ap-

FIG. 2—Recirculating underground storage tank cleaning system. Suction riser from tank is at far right. Suction line (in background) leads to API separator alongside power pack set on truck (left). Fuel then flows through first-stage housing with coalescer media (center-left) and particulate final filter (center-right) before flowing through return riser (foreground-left). Photo courtesy of Fuel Quality Services, Inc.

TABLE 2—*Microbicides approved by U.S. EPA for use in fuels and fuel systems.*

PC CODE[b]	Active Ingredient(s)
001001	Dimethyl-1,3-dioxan-4-ol acetate
035602	Tetrahydro-3,5-dimethyl-2H-1,3, 5-thiadiazine-2-thione
035603	2-(thiocyanomethylthio)benzothiazolole
039002	Potassium dimethyldithiocarbamate
043901	Glutaraldehyde
046609	Alkyl*-2-imidazoline-1-ethanol *(as in fatty acids of tall oil)
064104	Sodium o-phenylphenate
068102	Methylene bis (thiocyanate)
082901	1,3,5-triethylhexahydro-s-triazine
083301	Hexahydro-1,3,5-tris (2-hydroxyethyl)-s-triazine
101801	2,2-dibromo-3-nitrilopropionamide
107104	2-methyl-3(2H)-isothiazolone
128101	4,5-dichloro-2-n-octyl-3(2H)-isothiazolone
216400	Bromo-2-nitro-1,3-propanediol
012401 + 012402	2,2-(1-methyltrimethylenedioxy)bis (4-methyl-1,3,2-dioxborinane +2,2-oxybis (4,4,6-trimethyl-1,3,2-dioxaborinane)
014503 + 034804	Disodium ethylenebis(dithiocarbamate) +sodium dimethyldithiocarbamate
035603 + 068102	2-(thiocyanomethylthio)benzothiazolole + methylene bis (thiocyanate)
043901 + 069105	Glutaraldehyde +alkyl dimethyl benzyl ammonium chloride
100801 + 100802	4,4′-(2-ethyl-2-nitrotrimethylene) dimorpholine + 4-(2-nitrobutyl) morpholine
107103 + 107104	5-chloro-2-methyl-3(2H)-isothiazolone +2-methyl-3(2H)-isothiazaolone
216400 + 107103 + 107104	Bromo-2-nitro-1,3-propanediol+ 2-methyl-3(2H)-isothiazolone+5-chloro-2-methyl-3(2H)-isothiazolone

[b]Product code as listed in California EPA Office of Pesticides Programs on-line database: http://www.cdpr.ca.gov/docs/epa/epachem.htm.

prove all additives, including microbicides, intended for use in jet fuels, and may also limit the amount of treatment. Before using a fuel treatment microbicide, ensure that it has the requisite governmental, and non-governmental approvals for your intended application.

Biocide use regulations may vary from country to country. It is important for each tank owner/operator to be familiar with national and local regulations that may apply in their area.

Typically, system antimicrobials are not used as fuel additives. Unlike most additives, microbicides are used to treat fuel systems. Since microbiological problems are generally speaking not actually part of the fuel (the exception to this is severe contamination that has physically altered some of the fuel characteristics or where the fuel has been contaminated with debris caused by the organisms), the anti-microbial treatment employed is usually not continuous.

Since microorganisms live in the water and not in the fuel (they move with and survive in fuel, but typically do not proliferate there; see Chapter 1), a treatment strategy should be employed that will best fit both the physical characteristics of the system and the intended use of the fuel. A fixed roof, long-term storage tank may be treated most effectively with a dual-soluble microbicide. The fuel solubility will ensure that the active ingredient diffuses throughout the system uniformly, reaching microbes embedded in slime accumulations high on the tank walls. Water solubility will ensure that the active ingredient reaches microbes where they grow, within high-water content micro-environments, usually on the bottom (see discussion in Chapter 1).

In contrast, water-soluble microbicides may be more advantageous in high throughput systems and in tanks that have irregular bottoms where water may be trapped and cannot be drained off. To be maximally effective, anti-microbials need 12–24 h contact time. In high throughput systems, the exposure time for a fuel soluble or dual soluble microbicide may be less than the minimal 12-h contact period. Since microbes tend to accumulate in the bottom water, as water-soluble microbicide will remain with the bottoms and thereby control microbial contamination.

Other operational and technical factors that are beyond the scope of this chapter should also be considered. Treatment regimens, doses, product handling safety, and waste disposal details are site specific. When microbicide use is being considered, detailed advice should be sought from product manufactures or industry experts.

Used alone, anti-microbial pesticides are rarely sufficient to correct a severe microbial contamination problem. Antimicrobials are most effective when used as preventive treatments or in concert with system cleaning. Shock treatment of heavily contaminated systems will cause flocs of biomass to dislodge from tank and pipeline surfaces. The dislodged biomass can cause premature filter plugging. Recall that microbicides are designed to kill microbes, but not to clean up or eliminate organic debris resulting from microbial growth. Since microbicides are unlikely to sterilize entire slime communities, biomass settling to tank bottoms provides a habitat in which surviving microbes can proliferate and accelerate the rate of system re-contamination.

CONTAMINATION CONTROL STRATEGIES

Contamination control strategies are either proactive or reactive. This section summarizes the three most common categories of contamination control.

Corrective Maintenance

Most of this chapter has addressed corrective maintenance. By definition, corrective maintenance is the set of actions taken to fix an existing problem. Operational problems have signaled the need for reaction. Either product has been degraded or system components have deteriorated. When uncontrolled microbial contamination is the primary or a major contributing cause of the a problem, corrective maintenance will include some combination of disinfection, fuel filtration and tank cleaning in addition to any other repairs necessitated by the problem.

Where the risk of failure is deemed low or the projected cost of corrective maintenance is low, relative to the projected costs of preventive or predictive maintenance, then corrective maintenance may be the most cost-effective option. Typically, however, reactive corrective maintenance is orders of magnitude more expensive than the proactive alternatives to be discussed below. Moreover, economic analysis used to justify the corrective maintenance strategic options rarely consider chronic costs of poor quality that erode profitability. The cost of poor quality is often hidden within the ill-defined category of routine maintenance costs.

Preventive Maintenance (PM)

Preventive maintenance (PM) calls for specific action items to be completed at regularly scheduled intervals. Typically, past history is used as the basis for determining maintenance action frequency. For example, if records indicate that bottom-water volumes hit maximum acceptable levels between 80 and 100 days after dewatering, then dewatering might be scheduled as a quarterly maintenance action. If bottom-water bioburdens exceed threshold levels after five to seven months, then treatment with a microbicide might be scheduled as a semi-annual maintenance action.

When the list of maintenance actions is based on well-researched needs and maintenance item scheduling is derived from historic failure analysis, preventive maintenance can be a cost effective practice for ensuring that contamination does not affect system operations adversely. The popularity of preventive maintenance peaked in the late 1980s, by which time many organizations had developed considerable data on the return on investment that PM provided. Two somewhat surprising trends were noted. First, system perturbances resulting from preventive maintenance actions caused failures that might not have occurred had systems been left alone. Second, since PM frequency was based on historic trends, failure events could and did occur between scheduled maintenance. Rigorously implemented PM did not necessarily decrease corrective maintenance costs. Consequently, over the past two decades, PM has evolved into Predictive Maintenance (PDM).

Predictive Maintenance (PDM)

Predictive maintenance is distinguished from PM by its linkage to condition monitoring. Under PDM, system condition and operational performance criteria are defined, as are the test methods by which they will be measured. Performance criteria are defined in terms of average expected values and control limits. Generally, parameter averages and control limits are derived from a combination of historical data and experimentation. Optimally the control limits should differ from the average by a value considerably greater than the standard deviation, but considerably less than the value at which operational problems occur. For example, if the maximum allowable value for suspended particulates is 100 mg $\cdot$ L^{-1} and average values are 20 $\pm$ 5.0 mg $\cdot$ L^{-1} the upper control limit (UCL) might be set at 70 mg $\cdot$ L^{-1}. The process capabilities are well within the performance criterion and it is easy to distinguish between normal variation and special cause variation (incipient problems). The UCL (note: for contaminants, such as suspended particulates, there is no lower control limit) is set low enough to permit corrective action to be completed before the fuel falls out of specification, but high enough to avoid unnecessary maintenance actions.

As long as measured parameters yield values within the control limits, no maintenance action is needed. When a parameter's value exceeds the upper control limit a sequence of predetermined response actions is initiated. In short, under PDM, PM is data driven. As illustrated above, control limits are set so that maintenance actions can be completed before operations are impacted. For example, assume that the UCL for bacterial viable count recoveries in bottom-water samples has been set at 10^6 colony forming units (CFU) $\cdot$ mL^{-1} and samples are taken and tested monthly. Under PDM both the UCL and test frequency are based upon data analysis that has indicated that bottom-water viable count recoveries > 10^6 CFU $\cdot$ mL^{-1} correlate with operational problems (for example premature filter plugging) and that after treatment, populations tend to recover to the 10^6 CFU $\cdot$ mL^{-1} level after about three months[4]. Also assume that a standard protocol has been defined for what to do in case the test result is over the UCL. The protocol may call for immediate retesting by the same method and collaborative testing by an alternative method. Following confirmation, a specific action is prescribed, for example, treat with microbicide at a specified dose and retest after 24 h.

Condition monitoring data collection may be manual or automated, continuous, semi-continuous or periodic. Depending on the system and parameter, some combination of these options may be incorporated into a condition-monitoring program. For example, consider bottoms-water measurement. Many underground storage tanks are fitted with electronic gauging devices designed to provide semi-continuous reports of both product and bottoms-water volumes. However, since these devices are subject to calibration errors, volumes are typically checked manually on a regularly scheduled basis (weekly).

Instead of recalibrating electronic ullage devices quarterly (PM), they should be recalibrated whenever the variance between direct and electronic data exceeds some criterion value (the author recommends 10%).

Designing a condition-monitoring program for microbial contamination can be problematic. First, there are no generally accepted criteria for maximum acceptable bioburdens or other indicators of biodeterioration described in Chapter 1. Second, there are few standardized fuel microbiology test methods for analysts to use. Third, under some conditions, microbial contamination can develop and grow so suddenly that it might not be detected in early stages. Finally, there are no broadly adopted practices for collecting samples to be tested for microbiological parameters. Chapter 2 addresses the sample collection issue. Notwithstanding, proper surveillance is possible and potential biological problems can be detected long before they affect product quality or system integrity. Balanced against costs of fuel and fuel system biodeterioration, well-designed and implemented microbial contamination control PDM can provide a substantial return on investment.

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[3] Anonymous, "Commercial Item Description Fuel Oil, Diesel; for Posts, Camps and Stations," U.S. Army Tank-automotive and Armaments Command, AMSTA-TR-D/210, Warren, MI, 1996.

[4] Besse, G., H. Chesneau, Christie, S., Jr. and Hayden, A., "Mobile Fuel Filtration/Additive Unit," SAE Technical Paper No. 930015," SAE, Warrendale, PA, 1993, pp. 1–6.

[4]Note: this example is for illustrative purposes only. Operators should determine the appropriate control limits and testing frequencies for their fuel systems. Both control limits and optimal test frequency may vary amongst fuel grades and fuel systems.

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Standard Test Methods for Dissolved Oxygen in Water [1]

This standard is issued under the fixed designation D 888; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

This standard has been approved for use by agencies of the Department of Defense. Consult the DoD Index of Specifications and Standards for the specific year of issue which has been adopted by the Department of Defense.

1. Scope

1.1 These test methods cover the determination of dissolved oxygen in water. Two test methods are given as follows:

	Range, mg/L	Sections
Test Method A—Titrimetric Procedure–High Level	>1.0	8 to 14
Test Method B—Instrumental Probe Procedure	0.05 to 20	15 to 23

1.2 The precision of Test Methods A and B was carried out using a saturated sample of reagent water. It is the user's responsibility to ensure the validity of the test methods for waters of untested matrices.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For a specific precautionary statement, see Note 17.

2. Referenced Documents

2.1 *ASTM Standards:*

D 1066 Practice for Sampling Steam[2]
D 1129 Terminology Relating to Water[2]
D 1193 Specification for Reagent Water[2]
D 2777 Practice for Determination of Precision and Bias of Applicable Methods of Committee D-19 on Water[2]
D 3370 Practices for Sampling Water from Closed Conduits[2]
E 200 Practice for Preparation, Standardization, and Storage of Standard and Reagent Solutions for Chemical Analysis[3]

3. Terminology

3.1 *Definitions*—For definitions of terms used in these test methods, refer to Terminology D 1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *amperometric systems,, n*—those instrumental probes that involve the generation of an electrical current from which the final measurement is derived.

3.2.2 *instrumental probes,, n*—devices used to penetrate and examine a system for the purpose of relaying information on its properties or composition. The term probe is used in these test methods to signify the entire sensor assembly, including electrodes, electrolyte, membrane, materials of fabrications, etc.

3.2.3 *potentiometric systems,, n*—those instrumental probes in which an electrical potential is generated and from which the final measurement is derived.

4. Significance and Use

4.1 Dissolved oxygen is required for the survival and growth of many aquatic organisms, including fish. The concentration of dissolved oxygen may also be associated with corrosivity and photosynthetic activity. The absence of oxygen may permit anaerobic decay of organic matter and the production of toxic and undesirable esthetic materials in the water.

5. Purity of Reagents

5.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. [4] Other grades may be used if it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

5.1.1 Reagent grade chemicals, as defined in Practice E 200, shall be used unless otherwise indicated. It is intended that all reagents conform to this standard.

5.2 Unless otherwise indicated, reference to water shall be understood to mean reagent water conforming to Type II of Specification D 1193.

6. Sampling

6.1 Collect the samples in accordance with Practices D 1066 and D 3370.

[1] These test methods are under the jurisdiction of ASTM Committee D-19 on Water and are the direct responsibility of Subcommittee D19.05 on Inorganic Constituents in Water.

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[2] *Annual Book of ASTM Standards*, Vol 11.01.

[3] *Annual Book of ASTM Standards*, Vol 15.05.

[4] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

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6.2 For higher concentration of dissolved oxygen, collect the samples in narrow mouth glass-stoppered bottles of 300-mL capacity, taking care to prevent entrainment or solution of atmospheric oxygen.

6.3 With water under pressure, connect a tube of inert material to the inlet and extend the tube outlet to the bottom of the sample bottle. Use stainless steel, Type 304 or 316, or glass tubing with short neoprene connections. Do not use copper tubing, long sections of neoprene tubing, or other types of polymeric materials. The sample line shall contain a suitable cooling coil if the water being sampled is above room temperature, in which case cool the sample 16 to 18°C. When a cooling coil is used, the valve for cooling water adjustment shall be at the inlet to the cooling coil, and the overflow shall be to a point of lower elevation. The valve for adjusting the flow of sample shall be at the outlet from the cooling coil. The sample flow shall be adjusted to a rate that will fill the sampling vessel or vessels in 40 to 60 s and flow long enough to provide a minimum of ten changes of water in the sample vessel. If the sampling line is used intermittently, flush the sample line and cooling coil adequately before using.

6.4 Where samples are collected at varying depths from the surface, a special sample bottle holder or weighted sampler with a removable air tight cover should be used. This unit may be designed to collect several 250 or 300 mL samples at the same time. Inlet tubes extending to the bottom of each bottle and the water after passing through the sample bottle or bottles displaces air from the container. When bubbles stop rising from the sampler, the unit is filled. Water temperature is measured in the excess water in the sampler.

6.5 For depths greater than 2 m, use a Kemmerer-type sampler. Bleed the sample from the bottom of the sampler through a tube extending to the bottom of a 250 to 300 mL biological oxygen demand (BOD) bottle. Fill the bottle to overflowing and prevent turbulence and the formation of bubbles while filling the bottle.

7. Preservation of Samples

7.1 Do not delay the determination of dissolved oxygen. Samples for Test Method A may be preserved 4 to 8 h by adding 0.7 mL of concentrated sulfuric acid (sp gr 1.84) and 1.0 mL of sodium azide solution (20 g/L) to the bottle containing the sample in which dissolved oxygen is to be determined. Biological activity will be inhibited and the dissolved oxygen retained by storing at the temperature of collection or by water sealing (inverting bottle in water) and maintaining at a temperature of 10 to 20°C. Complete the determination as soon as possible, using the appropriate procedure for determining the concentration of dissolved oxygen.

TEST METHOD A—TITRIMETRIC PROCEDURE —HIGH LEVEL

8. Scope

8.1 This test method is applicable to waters containing more than 1000 μg/L of dissolved oxygen such as stream and sewage samples. It is the user's responsibility to ensure the validity of the test method for waters of untested matrices.

8.2 This test method, with the appropriate agent, is usable with a wide variety of interferences. It is a combination of the Winkler Method, the Alsterberg (Azide) Procedure, the Rideal-Stewart (permanganate) modification, and the Pomeroy-Kirshman-Alsterberg modification.

8.3 The precision of the test method was carried out using a saturated sample of reagent water.

9. Interferences

9.1 Nitrite interferences are eliminated by routine use of sodium azide. Ferric iron interferes unless 1 mL of potassium fluoride solution is used, in which case 100 to 200 mg/L can be tolerated. Ferrous iron interferes, but that interference is eliminated by the use of potassium permanganate solution. High levels of organic material or dissolved oxygen can be accommodated by use of the concentrated iodide-azide solution.

10. Apparatus

10.1 *Sample Bottles*, 250 or 300 mL capacity with tapered ground-glass stoppers. Special bottles with pointed stoppers and flared mouths are available from supply houses, but regular types (tall or low form) are satisfactory.

10.2 *Pipets*, 10-mL capacity, graduated in 0.1-mL divisions for adding all reagents except sulfuric acid. These pipets should have elongated tips of approximately 10 mm for adding reagents well below the surface in the sample bottle. Only the sulfuric acid used in the final step is allowed to run down the neck of the bottle into the sample.

11. Reagents

11.1 *Alkaline Iodide Solutions*:

11.1.1 *Alkaline Iodide Solution*—Dissolve 500 g of sodium hydroxide or 700 g of potassium hydroxide and 135 g of sodium iodide or 150 g of potassium iodide (KI) in water and dilute to 1 L. Chemically equivalent potassium and sodium salts may be used interchangeably. The solution should not give a color with starch indicator when diluted and acidified. Store the solution in a dark rubber-stoppered bottle. This solution may be used if nitrite is known to be absent and must be used if adjustments are made for ferrous ion interference.

11.1.2 *Alkaline Iodide-Sodium Azide Solution I*—This solution may be used in all of these submethods except when adjustment is made for ferrous ion. Dissolve 500 g of sodium hydroxide or 700 g of potassium hydroxide and 135 g of sodium iodide or 150 g of potassium iodide in water and dilute to 950 mL. To the cooled solution add 10 g of sodium azide dissolved in 40 mL of water. Add the NaN_3 solution slowly with constant stirring. Chemically equivalent potassium and sodium salts may be used interchangeably. The solution should not give a color with starch indicator solution when diluted and acidified. Store the solution in a dark rubber-stoppered bottle.

11.1.3 *Alkaline Iodide-Sodium Azide Solution II*—This solution is useful when high concentrations of organic matter are found or when the dissolved oxygen concentration exceeds 15 mg/L. Dissolve 400 g of sodium hydroxide in 500 mL of freshly boiled and cooled water. Cool the water slightly and dissolve 900 g of sodium iodide. Dissolve 10 g of sodium azide in 40 mL of water. Slowly add, with stirring, the azide solution

to the alkaline iodide solution, bringing the total volume to 1 L.

11.2 *Manganous Sulfate Solution*—Dissolve 364 g of manganous sulfate in water, filter, and dilute to 1 L. No more than a trace of iodine should be liberated when the solution is added to an acidified potassium iodide solution.

11.3 *Potassium Biiodate Solution* (0.025 *N*)—Dissolve 0.8125 g of potassium biiodate in water and dilute to 1 L in a volumetric flask.

NOTE 1—If the bottle technique is used, dissolve 1.2188 g of biiodate in water and dilute to 1 L to make 0.0375 *N*.

11.4 *Phenylarsine Oxide Solution* (0.025 *N*)—Dissolve 2.6005 g of phenylarsine oxide in 110 mL of NaOH solution (12 g/L). Add 800 mL of water to the solution and bring to a pH of 9.0 by adding HCl (1 + 1). This should require about 2 mL of HCl. Continue acidification with HCl (1 + 1) until a pH of 6 to 7 is reached, as indicated by a glass-electrode system. Dilute to 1 L. Add 1 mL of chloroform for preservation. Standardize against potassium biiodate solution.

NOTE 2—Phenylarsine oxide is more stable than sodium thiosulfate. However, sodium thiosulfate may be used. The analyst should specify which titrant is used. For a stock solution (0.1 *N*), dissolve 24.82 g of $Na_2S_2O_3 \cdot 5H_2O$ in boiled and cooled water and dilute to 1 L. Preserve by adding 5 mL of chloroform. For a dilute standard titrating solution (0.005 *N*) transfer 25.00 mL of 0.1 *N* $Na_2S_2O_3$ to a 500-mL volumetric flask. Dilute to the mark with water and mix completely. Do not prepare more than 12 to 15 h before use.

NOTE 3—If the full bottle technique is used, 3.9007 g must be used to make 0.0375 *N*.

NOTE 4—If sodium thiosulfate is used, prepare and preserve a 0.1 *N* solution as described in Note 1. Determine the exact normality by titration against 0.025 *N* potassium biiodate solution. Dilute the appropriate volume (nominally 250 mL) of standardized 0.1 *N* $Na_2S_2O_3$ solution to 1 L. One millilitre of 0.025 *N* thiosulfate solution is equivalent to 0.2 mg of oxygen. If the full bottle technique is followed, use 37.5 mL of sodium thiosulfate solution and standardize to 0.0375 *N*.

11.5 *Starch Solution*—Make a paste of 6 g of arrowroot starch or soluble iodometric starch with cold water. Pour the paste into 1 L of boiling water. Then add 20 g of potassium hydroxide, mix thoroughly, and allow to stand for 2 h. Add 6 mL of glacial acetic acid (99.5 %). Mix thoroughly and then add sufficient HCl (sp gr 1.19) to adjust the pH value of the solution to 4.0. Store in a glass-stoppered bottle. Starch solution prepared in this manner will remain chemically stable for one year.

NOTE 5—Powdered starches such as thyodene have been found adequate. Some commercial laundry starches have also been found to be usable.

NOTE 6—If the indicator is not prepared as specified or a proprietary starch indicator preparation is used, the report of analysis shall state this deviation.

11.6 *Sulfuric Acid* (sp gr 1.84)—Concentrated sulfuric acid. One millilitre neutralizes about 3 mL of the alkaline iodide reagent.

NOTE 7—Sulfamic acid (3 g) may be substituted.

11.7 *Potassium Fluoride Solution* (400 g/L)—Dissolve 40 g of potassium fluoride in water and dilute to 100 mL. This solution is used in the procedure for eliminating ferric ion interference. Store this solution in a plastic bottle.

11.8 *Potassium Oxalate Solution* (20 g/L)—Dissolve 2 g of potassium oxalate in 100 mL of water. One millilitre of this solution will reduce 1.1 mL of the $KMnO_4$ solution. This solution is used in the procedure for eliminating ferrous ion interference.

11.9 *Potassium Permanganate Solution* (6.3 g/L)—Dissolve 6.3 g of potassium permanganate in water and dilute to 1 L. With very high ferrous iron concentrations, solution of $KMnO_4$ should be stronger so that 1 mL will satisfy the demand. This solution is used in the procedure for eliminating ferrous ion interference.

12. Procedure

12.1 *Elimination of Ferrous Ion Interference, if necessary*:

12.1.1 Add to the sample (collected as in 6.2) 0.70 mL of H_2SO_4, followed by 1.0 mL of $KMnO_4$ solution. Where high iron is present, also add 1.0 mL of KF solution. Stopper and mix by inversion. The acid should be added with a 1-mL pipet graduated in 0.1-mL divisions. Add sufficient $KMnO_4$ solution to maintain a violet tinge for 5 min. If the color does not persist for 5 min, add more $KMnO_4$ solution, but avoid excess. In those cases where more than 5 mL of $KMnO_4$ solution is required, a stronger solution of this reagent may be used to avoid dilution of the sample.

12.1.2 After 5 min, completely destroy the permanganate color by adding 0.5 to 1.0 mL of $K_2C_2O_4$ solution. Mix the sample well, and allow it to stand in the dark. Low results are caused by excess oxalate so it is essential to add only sufficient oxalate to completely decolorize the permanganate without having an excess of more than 0.5 mL. Complete decolorization should be obtained in 2 to 10 min. If the sample cannot be decolorized without a large excess of oxalate, the dissolved oxygen results will be of doubtful value.

12.2 Add 2.0 mL of $MnSO_4$ solution to the sample as collected in a sample bottle, followed by 2.0 mL of alkaline iodide-sodium azide solution well below the surface of the liquid (see Note 8 and Note 9). Be sure the solution temperature is below 30°C to prevent loss due to volatility of iodine. Carefully replace the stopper to exclude air bubbles and mix by inverting the bottle several times. Repeat the mixing a second time after the floc has settled, leaving a clear supernatant solution. Water high in chloride requires a 10-min contact period with the precipitate. When the floc has settled, leaving at least 100 mL of clear supernatant solution, remove the stopper, and add 2.0 mL of H_2SO_4, allowing the acid to run down the neck of the bottle. Restopper and mix by inversion until the iodine is uniformly distributed throughout the bottle. Titrate without delay 203 mL of original sample. A correction is necessary for the 4 mL of reagents added (2 mL of $MnSO_4$ solution and 2 mL of alkaline iodide-sodium azide solution: $200 \times [300/(300 - 4)] = 203$ mL (see Note 10)).

NOTE 8—Take care to use the correct alkaline iodide solution (11.1.1) if no nitrite is present or ferrous ion was oxidized, (11.1.2) for normal use, or (11.1.3) if there is a high organic or dissolved oxygen concentration.

NOTE 9—Two millilitres of the alkaline iodide-sodium azide solution are used to ensure better contact of the iodide-azide solution and sample with less agitation. With 250-mL bottles, 1 mL of the iodide-azide solution may be used if desired. In this procedure, as in the succeeding ones, all reagents except the H_2SO_4 are added well below the surface of the liquid.

NOTE 10—In the case where ferrous ion interference has been eliminated, a total of 6.7 mL of reagents were added (0.7 mL of acid, 1 mL of $KMnO_4$ solution, 2 mL of $MnSO_4$ solution, and 3 mL of alkaline iodide solution). The volume of sample for titration is 203 mL. A slight error occurs due to the dissolved oxygen of the $KMnO_4$ solution, but rather than complicate the correction further, this error is ignored.

12.3 Rapidly titrate the 203 mL of sample with 0.025 *N* titrating solution to a pale, straw yellow color. Add 1 to 2 mL of starch indicator. Continue the titration to the disappearance of the blue color.

NOTE 11—If the full bottle technique is used, transfer the entire contents of the bottle, 300 ± 3 mL, to a 500-mL Erlenmeyer flask and titrate with 0.0375 *N* titrating solution.

NOTE 12—At the correct end point, one drop of 0.025 *N* $KH(IO_3)_2$ solution will cause the return of the blue color. If the end point is overrun, continue adding 0.025 *N* $KH(IO_3)_2$ solution until it reappears, noting the volume required. Subtract this value, minus the last drop of $KH(IO_3)_2$ (0.04 mL) from the volume of 0.025 *N* titrating solution used. Disregard the late reappearance of the blue color, which may be due to the catalytic effect of organic material or traces of uncomplexed metal salts.

13. Calculation

13.1 Calculate the dissolved oxygen content of the sample as follows:

$$\text{Dissolved oxygen, mg/L} = \frac{T \times 0.2}{200} \times 1000 \qquad (1)$$

where:

T = 0.025 *N* titrating solution required for titration of the sample, mL.

13.2 Use Eq. 2 to convert to a standard temperature and pressure measurement.

$$\text{Dissolved oxygen, mg/L} = \frac{A}{0.698} \qquad (2)$$

where:

A = oxygen at 0°C and 760 mm Hg, mL.

NOTE 13—Each millilitre of 0.0375 *N* titrant is equivalent to 1 mg/L O_2 when the full bottle technique is used.

NOTE 14—If the percentage of saturation at 760-mm atmospheric pressure is desired, the dissolved oxygen found is compared with solubility data from standard solubility tables,[5] making corrections for barometric pressure and the aqueous vapor pressure, when necessary. See Appendix X1.

14. Precision and Bias [6]

14.1 The precision of the test method was determined by six operators in three laboratories, running three duplicates each (not six laboratories as required by Practice D 2777 – 86) using a saturated sample of reagent water. The mean concentration was 9.0 mg/L, and the pooled single operator precision in these samples was 0.052 mg/L.

[5] Carpenter, J. H., "New Measurement of Oxygen Solubility in Pure and Natural Water," Limnology and Oceanography, Vol 11, No. 2, April 1966, pp. 264–277.

[6] Supporting data for the precision statement have been filed at ASTM Headquarters. Request RR: D19 – 1070.

TEST METHOD B—INSTRUMENTAL PROBE PROCEDURE

15. Scope

15.1 This test method is applicable to waters containing dissolved oxygen in the range from 50 to 20 000 µg/L. It is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

15.2 This test method describes procedures that utilize probes for the determination of dissolved oxygen in fresh water and in brackish and marine waters that may contain dissolved or suspended solids. Samples can be analyzed in situ in bodies of water or in streams, or samples can be collected and analyzed subsequent to collection. The probe method is especially useful in the monitoring of water systems in which it is desired to obtain a continuous record of the dissolved oxygen content.

15.2.1 This test method is recommended for measuring dissolved oxygen in waters containing materials that interfere with the chemical methods, such as sulfite, thiosulfate, polythionate, mercaptans, oxidizing metal ions, hypochlorite, and organic substances readily hydrolyzable in alkaline solutions.

15.3 Dissolved oxygen probes are practical for the continuous monitoring of dissolved oxygen content in natural waters, process streams, biological processes, etc., when the probe output is conditioned by a suitably stable electronic circuit and recorded. The probe must be standardized before use on samples free of interfering materials, preferably with the azide modification of Test Method A.

16. Summary of Test Method

16.1 The most common instrumental probes for determination of oxygen dissolved in water are dependent upon electrochemical reactions. Under steady-state conditions, the current or potential can be correlated with dissolved oxygen concentrations.

NOTE 15—Steady-state conditions necessitate the probe being in thermal equilibrium with the solution, this typically taking 20 min for nonlaboratory conditions.[7]

16.1.1 Probes that employ membranes normally involve metals of different nobility immersed in an electrolyte that is retained by the membrane. The metal of highest nobility (the cathode) is positioned at the membrane. When a suitable potential exists between the two metals, reduction of oxygen to hydroxide ion occurs at the cathode surface. An electrical current is developed that is directly proportional to the rate of arrival of oxygen molecules at the cathode.

16.1.2 The thallium probe, which does not utilize a membrane, exposes a thallium electrode to the water sample. Reaction of oxygen with the thallium establishes a potential between the thallium electrode and a reference electrode. The potential is related logarithmically to dissolved oxygen concentration. The cell output decreases (theoretically 59 mV/decade at 25°C) with increased oxygen concentration.

[7] D'Aoust, B. G., Clark, M. J. R., "Analysis of Supersaturated Air in Natural Waters and Reservoirs," *Transactions of the American Fisheries Society*, Vol 109, 1980, pp. 708–724.

NOTE 16—The thallium probe has utility in waste treatment monitoring systems; it has limited application under conditions of high dissolved oxygen (>8 mg/L) and low temperature (<10°C).

16.1.3 The electronic readout meter for the output from dissolved oxygen probes is normally calibrated in convenient scales (0 to 10, 0 to 15, or 0 to 20 mg/L) with a sensitivity of approximately 0.05 mg/L. More sensitive dissolved oxygen ranges are practical through amplification in the electronic readout (including μg/L readings in boiler feed waters).

16.2 Interfacial dynamics at the probe-sample interface are a factor in probe response. Turbulence should be constant or above some minimum level as recommended by the instrument manufacturer.

16.3 Response rates of dissolved oxygen probes are relatively rapid, often as fast as 99 % in 15 s. Probe outputs may be recorded for continual monitoring or utilized for process control (see Note 15).

17. Interferences

17.1 Dissolved organic materials normally encountered in water are not known to interfere in the output from dissolved oxygen probes.

17.2 Dissolved inorganic salts are a factor in the calibration of dissolved oxygen probe.

17.2.1 Solubility of oxygen in water at a given oxygen partial pressure changes with the kind and concentration of dissolved inorganic salts. Conversion factors for seawater and brackish waters may be calculated from dissolved oxygen saturation versus salinity data if internal compensation is not included in the instrument. Conversion factors for specific inorganic salts may be developed experimentally. Broad variations in the kinds and concentrations of salts in samples can make the use of a membraned probe difficult.

17.2.2 The thallium probe measures ionic activity instead of concentration as do all ion selective electrodes. Gross changes in the concentration of dissolved salts will affect the activity coefficient of the thallous ion and thus shift the span (see 19.2.1). The thallium probe may be calibrated and operated in water of any conductivity above 100 μS, but a ten-fold change in conductivity will produce an error of approximately 20 %. Since the thallium requires a conducting path through the sample to the reference electrode, the response will become sluggish at very low conductivity. It is therefore desirable to calibrate the sensor in solutions having a conductivity greater than 100 μS.

17.3 Reactive compounds can interfere with the output or the performance of dissolved oxygen probes.

17.3.1 Membraned probes are sensitive to reactive gases that may pass through the membrane. Chlorine will depolarize the cathode and cause a high probe output. Long-term exposure to chlorine can coat the anode with the chloride of the anode metal and may eventually desensitize the probe. Hydrogen sulfide will interfere with membraned probes if the applied potential is greater than the half-wave potential of the sulfide ion. If the applied potential is less than the half-wave potential, an interfering reaction will not occur, but coating of the anode metal can occur.

17.3.2 The thallium probe is affected by interference from soluble sulfur compounds, such as hydrogen sulfide or mercaptans. Ten milligrams of hydrogen sulfide per litre of water will produce a negative error corresponding to approximately 1 mg/L of dissolved oxygen. Free halogens also will interfere with the thallium probe if present in appreciable concentrations, such as above 2 mg of chlorine per litre of water.

17.4 At dissolved oxygen concentrations below 2 mg/L, pH variation below 4 and above 10 interfere with the performance of the thallium probe (approximately ±0.05 mg/L dissolved oxygen per pH unit). The performance of membraned probes is not affected by pH changes.

17.5 Dissolved oxygen probes are temperature sensitive and temperature compensation is normally provided by the manufacturer. The thallium probe has a temperature coefficient of 1.0 mV/°C, membraned probes have a temperature coefficient of 4 to 6 %/°C dependent upon the membrane employed.

17.6 Insoluble organic or inorganic materials that can coat the surface of dissolved oxygen probes will affect the performance of either the thallium or membraned probes.

18. Apparatus

18.1 *Amperometric Probes*—Oxygen-sensitive probes of the amperometric type are normally composed of two solid metal electrodes of different nobility in contact with a supporting electrolyte that is separated from the test solution by a selective membrane. The current generated by the reduction of oxygen at the cathode is measured through an electronic circuit and displayed on a meter. Typically, the anode is constructed of metallic silver or lead and the cathode of gold or platinum. Probes are generally not affected by hydraulic pressure and can be used in the temperature range from 0 to 50°C.

18.1.1 *Semipermeable Membranes of Polyethylene or TFE-fluorocarbon* permit satisfactory oxygen diffusion and limit interference from most materials.

18.1.2 *Accessory Equipment* may involve apparatus to move the sample past the probe and to provide suitable turbulence at the membrane-sample interface.

18.2 *Potentiometric Probes*—The commonly used potentiometric probe employs a thallium-measuring electrode and a suitable reference half cell such as a saturated calomel. At 25°C and 0.1 mg/L of dissolved oxygen, the cell establishes a negative potential of approximately 817 mV. The potential decreases logarithmically in absolute value with increased dissolved oxygen concentration (theoretically, 59 mV/decade change in dissolved oxygen concentration) to approximately 688 mV at 15 mg/L of dissolved oxygen. An external millivoltage source that opposes the output of the electrometer is used to adjust the net readout of output to the desired range.

NOTE 17—Thallium and its salts are toxic. Avoid contact with the skin.

19. Apparatus Standardization

19.1 Under equilibrium conditions, the partial pressure of oxygen in air-saturated water is equal to that of the oxygen in the water-saturated air. Consequently, a probe may be calibrated in air as well as water. Consider carefully the manufacturer's recommended procedure. If it is necessary to zero the instrument, immerse the probe in water containing 1 g

of sodium sulfite and two drops of saturated cobalt chloride solution (as deoxygenation catalyst) per litre of water and adjust the instrument to read zero. If a water-saturated air calibration is necessary, follow the manufacturer's directions for its preparation.

19.2 To calibrate the probe in water, carefully obtain approximately 1 L of the type of water to be tested and saturate it with oxygen from the atmosphere by passing clean air through it. Carefully draw three replicate samples from the well mixed sample and immediately determine the dissolved oxygen concentration by Test Method A in duplicate. In the third replicate sample, immerse the probe and provide for suitable turbulence in the sample. Standardize the probe by adjusting the meter reading to the dissolved oxygen value as determined by the chemical procedure. If substances that interfere with the chemical method are present in the natural water or wastewater sample, standardize the probe using reagent water or a synthetic sample as indicated below.

19.2.1 *Fresh Water Samples* (less than 1000 mg/L of dissolved salts)—If chemical interferences are absent, use a test sample as indicated above. If interferences are present, use reagent water for membraned probes. With thallium probes, the greatest accuracy can be obtained from calibrating in a sample of the water to be tested or a synthetic sample similar to the test sample.

19.2.2 *Salt Water Samples and Membraned Probes* (greater than 1000 mg/L of dissolved salts)—Use a sample of clean water having the same salt content as the test material. If a sample free from substances that interfere with the azide method is not available, prepare a synthetic standardization sample by adding the same salts contained in the sample until the two solutions have the same electrical conductance within 5 %. High concentrations of dissolved salts are not a problem with the thallium probe.

19.3 *Temperature Coefficient*—Systems are available with automatic temperature compensation that permit direct measurements in milligrams per litre of dissolved oxygen. The temperature compensation of membraned probes corrects for changes in membrane characteristics including boundary-layer effects at the membrane-water interface and the changes in solubility of oxygen in water. The temperature compensation of thallium probes corrects for the changes characteristic of oxidation/reduction systems (see Note 15). It is necessary that the probe is in thermal equilibrium with the solution to be measured for satisfactory temperature correction.

19.3.1 For those instrumental systems using membraned probes that are not temperature-compensated, the following procedure is recommended to obtain the temperature coefficient. Measure the oxygen content in water samples for five temperatures over a ±10°C range greater and less than the expected sample temperature. By a least-squares procedure, or graphically in a semilog plot of Y versus T, calculate the slope and intercept constant as follows:

$$\operatorname{Log} y = B/T + A \tag{3}$$

where:

y = scale factor, milligrams of dissolved oxygen per litre per microampere of electrode current,
B = slope constant,
T = temperature, °C, and
A = intercept constant.

This relationship is linear on a semilog plot only over a range of ±10°C. Over larger ranges an equation of higher degree is necessary to reflect the curvature of the relationship.

19.3.2 If the thallium probe is utilized in a circuit without temperature compensation, the observed output in millivolts must be corrected for the temperature sensitivity of the measuring cell that has a temperature coefficient of 1.0 mV/°C. The measuring cell's output will increase (apparent dissolved oxygen concentration decrease) with an increase in temperature,

$$MV_R = MV_0 - 1.0\,(T_o - T_R) \tag{4}$$

where:

MV_R = millivolts of output at reference temperature,
MV_O = millivolts of output observed,
T_R = reference temperature, °C, and
T_o = temperature at the observed output, °C.

19.4 *Correction for Content of Dissolved Salts*—If the concentration of salts is above 1000 mg/L, it will be necessary to correct for the effect of the salts in the relationship between oxygen partial pressure and concentration and also for the activity of thallium ion. For any given salt, a series of experimental data should be obtained in which solutions are prepared by dissolving varying weights of the salt in reagent water in the range of interest. The solutions plus a reagent water control are aerated at constant temperature until oxygen saturation is achieved. Determine the oxygen concentration of each solution by the chemical method and, at the same time, obtain probe readings. Determine the ratio A for each solution as follows:

$$A = O/R \tag{5}$$

where:

O = actual dissolved oxygen concentration, mg/L, as determined by Test Method A, and
R = reading of the probe meter.

For the reagent water control to which the probe is calibrated, the value of A is 1.0. Prepare a plot with salt concentration as abscissa and the ratio A as ordinate. Use the developed curve for calculation of the dissolved oxygen content of salt waters.

20. Sampling

20.1 *Bottle Samples*—Collect a bottle sample by the procedure described in Practice D 1066 or Practices D 3370. Collect the samples in 300-mL BOD bottles or other suitable glass-stoppered bottles, preventing entrainment or solution of atmospheric oxygen. If analysis is delayed beyond 15 min, cool the sample below 5°C and hold at this temperature until analyzed. Make the dissolved oxygen determination without further temperature adjustment using the appropriate temperature coefficient. It will be necessary to have the probe

at the temperature of the sample or otherwise compensate for instability due to heat flow from probe to sample.

20.2 *In Situ Samples*—An effective use of the instrumental probes is for the direct, in situ determination of dissolved oxygen. By this means, sample handling problems are avoided, and data may be obtained quickly at various locations in a body of water without concern for the change in oxygen during storage or handling.

21. Procedure

21.1 Consider carefully the manufacturer's recommendations on the use of equipment to obtain satisfactory operation.

21.2 Provide for suitable turbulent flow past the membrane of membraned probes or past the thallium probe. This may, under some circumstances, be achieved adequately in flowing streams. However, in large bodies of water, it may be necessary to employ mechanical stirring or pumping of water past the probe. For accurate results, it is important that comparable degrees of turbulence be employed both for calibration and utilization.

21.3 If the probe is not automatically compensated for temperature changes, record the temperature of the water at the sample probe at the time of dissolved oxygen measurement. To avoid heat-flow effects, it is important that temperature equilibrium be established between sample and probe.

21.4 Recalibrate the probe whenever the comparison with reference samples (19.2) indicates an absolute error of more than ±0.2 mg/L of dissolved oxygen or other value that is compatible with the desired accuracy.

21.4.1 Careful handling is required with membraned probes to avoid rupturing the thin membrane.

21.4.2 Recalibrate the probe after replacing the membrane or cleaning the probe in accordance with the manufacturer's directions. For a period of a few hours after a membrane replacement, the probe output may drift, and frequent recalibration may be required.

21.5 Probes can become fouled by oil, grease, biological growths, etc., and cleaning may be required. Some of the techniques currently in use include air-blasting, brush cleaning, and ultrasonic cleaning systems.

21.6 The probe may be utilized in situ or the sample may be transferred to a sampling station that houses the probe and associated equipment.

21.6.1 In situ placement of the probe is preferable from the consideration that sample handling is not involved. However, in situ installations may be impractical because of problems with vandalism, severe climate conditions (freezing, etc.), and difficulty in probe recovery for maintenance.

21.6.2 The use of sample transfer systems is practical when proper consideration is given to design features such as line size, rates of transfer, kind of pump and location, practicality for cleaning the transfer system, and other maintenance.

21.6.3 Examine unattended probes at least once per week and recalibrate when required depending upon condition and service. Recalibration may be accomplished by using a portable probe that has been placed into position next to the unattended probe and that has been properly calibrated as outlined in 19.2.

22. Calculation

22.1 For uncompensated probes, correct the observed meter reading for the difference of the observed temperature from the standardization temperature by the factors developed in 19.3.

22.2 For wastewaters with varying salt contents, make corrections utilizing the data developed in 19.4.

23. Precision and Bias

23.1 The precision of this test method was determined by six operators in three laboratories running three duplicates each (not six laboratories as required by Practice D 2777 – 86) using a saturated sample of reagent water. The mean concentration was 9.0 mg/L, and the pooled single-operator precision in these samples was 0.029 mg/L.

24. Keywords

24.1 analysis; dissolved; oxygen; probe; titrimetric; water

APPENDIX

(Nonmandatory Information)

X1. OXYGEN SATURATION VALUES

X1.1 *Oxygen Saturation Values in Water and Elevations*—The solubility of oxygen in water at various temperatures and elevations under an atmospheric pressure of 760 mm is shown in Table X1.1.

X1.2 *Oxygen Saturation Values in Water and Salt Waters*—The solubility of oxygen in water exposed to water saturated air under an atmospheric pressure of 760 mm is shown in Table X1.2 at several temperatures and concentrations of sea water to illustrate the effects of salt concentration and temperature. The solubility versus dissolved salt concentration can vary considerably with the nature of the salts in solution.

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TABLE X1.1 Solubility of Oxygen (mg/L) at Various Temperatures and Elevations (Based on Sea Level Barometric Pressure of 760 mm Hg) [12]

Temperature, °C	Elevation, Feet above Sea Level						
	0	1000	2000	3000	4000	5000	6000
0	14.6	14.1	13.6	13.2	12.7	12.3	11.8
2	13.8	13.3	12.9	12.4	12.0	11.6	11.2
4	13.1	12.7	12.2	11.9	11.4	11.0	10.6
6	12.4	12.0	11.6	11.2	10.8	10.4	10.1
8	11.8	11.4	11.0	10.6	10.3	9.9	9.6
10	11.3	10.9	10.5	10.2	9.8	9.5	9.2
12	10.8	10.4	10.1	9.7	9.4	9.1	8.8
14	10.3	9.9	9.6	9.3	9.0	8.7	8.3
16	9.9	9.7	9.2	8.9	8.6	8.3	8.0
18	9.5	9.2	8.7	8.6	8.3	8.0	7.7
20	9.1	8.8	8.5	8.2	7.9	7.7	7.4
22	8.7	8.4	8.1	7.8	7.7	7.3	7.1
24	8.4	8.1	7.8	7.6	7.3	7.1	6.8
26	8.1	7.8	7.6	7.3	7.0	6.8	6.6
28	7.8	7.5	7.3	7.0	6.8	6.6	6.3
30	7.5	7.2	7.0	6.8	6.5	6.3	6.1
32	7.3	7.1	6.8	6.6	6.4	6.1	5.9
34	7.1	6.9	6.6	6.4	6.2	6.0	5.8
36	6.8	6.6	6.3	6.1	5.9	5.7	5.5
38	6.6	6.4	6.2	5.9	5.7	5.6	5.4
40	6.4	6.2	6.0	5.8	5.6	5.4	5.2

TABLE X1.2 Solubility of Oxygen (mg/L) at Various Temperatures and Chlorinity (Based on Sea Level Barometric Pressure of 760 mm Hg) [12]

Temperature,° C	Chlorinity, %					
	0	4.0	8.0	12.0	16.0	20.0
0	14.6	13.9	13.2	12.5	11.9	11.3
2	13.8	13.2	12.5	11.9	11.4	10.8
4	13.1	12.5	11.9	11.3	10.8	10.3
6	12.4	11.8	11.3	10.8	10.3	9.8
8	11.8	11.3	10.8	10.3	9.8	9.4
10	11.3	10.8	10.3	9.8	9.4	9.0
12	10.8	10.3	9.8	9.4	9.0	8.6
14	10.3	9.9	9.4	9.0	8.6	8.3
16	9.9	9.4	9.0	8.6	8.3	8.0
18	9.5	9.1	8.7	8.3	8.0	7.6
20	9.1	8.7	8.3	8.0	7.7	7.4
22	8.7	8.4	8.0	7.7	7.4	7.1
24	8.4	8.1	7.7	7.4	7.1	6.9
26	8.1	7.8	7.5	7.2	6.9	6.6
28	7.8	7.5	7.2	6.9	6.6	6.4
30	7.5	7.2	7.0	6.7	6.4	6.2
32	7.3	7.0	6.7	6.5	6.2	6.0
34	7.1	6.8	6.5	6.3	6.0	5.8
36	6.8	6.6	6.3	6.1	5.8	5.6
38	6.6	6.4	6.1	5.9	5.6	5.4
40	6.4	6.2	5.9	5.7	5.4	5.2

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Designation: D 1067 – 02

Standard Test Methods for Acidity or Alkalinity of Water[1]

This standard is issued under the fixed designation D 1067; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

This standard has been approved for use by agencies of the Department of Defense.

1. Scope *

1.1 These test methods[2] cover the determination of acidity or alkalinity of all types of water. Three test methods are given as follows:

	Sections
Test Method A (Electrometric Titration)	7 to 15
Test Method B (Electrometric or Color-Change Titration)	16 to 24
Test Method C (Color-Change Titration After Hydrogen Peroxide Oxidation and Boiling)	25 to 33

1.2 In all of these test methods the hydrogen or hydroxyl ions present in water by virtue of the dissociation or hydrolysis of its solutes, or both, are neutralized by titration with standard alkali (acidity) or acid (alkalinity). Of the three procedures, Test Method A is the most precise and accurate. It is used to develop an electrometric titration curve (sometimes referred to as a pH curve), which defines the acidity or alkalinity of the sample and indicates inflection points and buffering capacity, if any. In addition, the acidity or alkalinity can be determined with respect to any pH of particular interest. The other two methods are used to determine acidity or alkalinity relative to a predesignated end point based on the change in color of an internal indicator or the equivalent end point measured by a pH meter. They are suitable for routine control purposes.

1.3 When titrating to a specific end point, the choice of end point will require a careful analysis of the titration curve, the effects of any anticipated changes in composition on the titration curve, knowledge of the intended uses or disposition of the water, and a knowledge of the characteristics of the process controls involved. While inflection points (rapid changes in pH) are usually preferred for accurate analysis of sample composition and obtaining the best precision, the use of an inflection point for process control may result in significant errors in chemical treatment or process control in some applications. When titrating to a selected end point dictated by practical considerations, (*1*) only a part of the actual neutralizing capacity of the water may be measured, or (*2*) this capacity may actually be exceeded in arriving at optimum acidity or alkalinity conditions.

1.4 A scope section is provided in each test method as a guide. It is the responsibility of the analyst to determine the acceptability of these test methods for each matrix.

1.5 Former Test Methods C (Color-Comparison Titration) and D (Color-Change Titration After Boiling) were discontinued. Refer to Appendix X4 for historical information.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

D 596 Practice for Reporting Results of Analysis of Water[3]
D 1129 Terminology Relating to Water[3]
D 1192 Specification for Equipment for Sampling Water and Steam in Closed Conduits[3]
D 1193 Specification for Reagent Water[3]
D 1293 Test Methods for pH of Water[3]
D 2777 Practice for Determination of Precision and Bias of Applicable Methods of Committee D-19 on Water[3]
D 3370 Practices for Sampling Water from Closed Conduits[3]
D 5847 Practice for Writing Quality Control Specifications for Standard Test Methods for Water Analysis[4]
E 200 Practice for Preparation, Standardization, and Storage of Standard and Reagent Solutions for Chemical Analysis[5]

3. Terminology

3.1 *Definitions*—The terms in these test methods are defined in accordance with Terminology D 1129.

3.1.1 Certain uses of terminology exist in the water treatment industry which may differ from these definitions. A discussion of terms is presented in Appendix X1.

[1] These test methods are under the jurisdiction of ASTM Committee D19 on Water and are the responsibility of Subcommittee D19.05 on Inorganic Constituents in Water.

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[2] The basic procedures used in these test methods have appeared widespread in the technical literature for many years. Only the particular adaptation of the electrometric titration appearing as the Referee Method is believed to be largely the work of Committee D-19.

[3] *Annual Book of ASTM Standards*, Vol 11.01.
[4] *Annual Book of ASTM Standards*, Vol 11.02.
[5] *Annual Book of ASTM Standards*, Vol 15.05.

*A Summary of Changes section appears at the end of this standard.

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4. Significance and Use

4.1 Acidity and alkalinity measurements are used to assist in establishing levels of chemical treatment to control scale, corrosion, and other adverse chemical equilibria.

4.2 Levels of acidity or alkalinity are critical in establishing solubilities of some metals, toxicity of some metals, and the buffering capacity of some waters.

5. Purity of Reagents

5.1 Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.[6] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

5.2 Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type I. In addition, reagent water for this test shall be free of carbon dioxide (CO_2) and shall have a pH between 6.2 and 7.2 at 25°C. Other reagent water types may be used provided it is first ascertained that the water is of sufficiently high purity to permit its use without adversely affecting the precision and bias of the test method. Type III water was specified at the time of round robin testing of this test method. A procedure for the preparation of carbon dioxide-free water is given in Practice E 200.

6. Sampling

6.1 Collect the sample in accordance with Specification D 1192 and Practices D 3370 as applicable.

6.2 The time interval between sampling and analysis shall be as short as practically possible in all cases. It is mandatory that analyses by Test Method A be carried out the same day the samples are taken; essentially immediate analysis is desirable for those waste waters containing hydrolyzable salts that contain cations in several oxidation states.

TEST METHOD A—ELECTROMETRIC TITRATION

7. Scope

7.1 This test method is applicable to the determination of acidity or alkalinity of all waters that are free of constituents that interfere with electrometric pH measurements. It is used for the development of a titration curve that will define inflection points and indicate buffering capacity, if any. The acidity or alkalinity of the water or that relative to a particular pH is determined from the curve.

8. Summary of Test Method

8.1 To develop a titration curve that will properly identify the inflection points, standard acid or alkali is added to the sample in small increments and a pH reading is taken after each addition. The cumulative volume of solution added is plotted against the observed pH values. All pH measurements are made electrometrically.

9. Interferences

9.1 Although oily matter, soaps, suspended solids, and other waste materials may interfere with the pH measurement, these materials may not be removed to increase precision, because some are an important component of the acid- or alkali-consuming property of the sample. Similarly, the development of a precipitate during titration may make the glass electrode sluggish and cause high results.

10. Apparatus

10.1 *Electrometric pH Measurement Apparatus*, conforming to the requirements given in Test Methods D 1293.

11. Reagents [6]

11.1 *Hydrochloric Acid, Standard* (0.02 *N*) (see Note 1)—Prepare and standardize as directed in Practice E 200, except that the titration shall be made electrometrically. The inflection point corresponding to the complete titration of carbonic acid salts will be very close to pH 3.9.

NOTE 1—Sulfuric acid of similar normality may be used instead of hydrochloric acid. Prepare and standardize in like manner.

11.2 *Sodium Hydroxide, Standard* (0.02 *N*)—Prepare and standardize as directed in Practice E 200, except that the titration shall be made electrometrically. The inflection point corresponding to the complete titration of the phthalic acid salt will be very close to pH 8.6.

12. Procedure

12.1 Mount the glass and reference electrodes in two of the holes of a clean, threehole rubber stopper chosen to fit a 300-mL, tall-form Berzelius beaker without spout, or equivalent apparatus. Place the electrodes in the beaker and standardize the pH meter, using a reference buffer having a pH approximating that expected for the sample (see Test Methods D 1293). Rinse the electrodes, first with reagent water, then with a portion of the sample. Following the final rinse, drain the beaker and electrodes completely.

12.2 Pipette 100 mL of the sample, adjusted, if necessary, to room temperature, into the beaker through the third hole in the stopper. Hold the tip of the pipette near the bottom of the beaker while discharging the sample.

12.3 Measure the pH of the sample in accordance with Test Methods D 1293.

12.4 Add either 0.02 *N* acid or alkali solution, as indicated, in increments of 0.5 mL or less (see Note 2). After each addition, mix the solution thoroughly. Determine the pH when the mixture has reached equilibrium as indicated by a constant reading (see Note 3). Mechanical stirring, preferably of the magnetic type, is required for this operation; mixing by means of a gas stream is not permitted. Continue the titration until the necessary data for the titration curve have been obtained.

NOTE 2—If the sample requires appreciably more than 25 mL of standard solution for its titration, use a 0.1 *N* solution, prepared and standardized in the same manner (see Practice E 200).

[6] *Reagent Chemicals, American Chemical Society Specifications.* American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopoeia and National Formulary*. U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

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NOTE 3—An electrometric titration curve is smooth. with the pH changing progressively in a single direction, if equilibrium is achieved after each incremental addition of titrant, and may contain one or more inflection points. Ragged or irregular curves may indicate that equilibrium was not attained before adding succeeding increments. The time required will vary with different waters as the reaction rate constants of different chemical equilibria vary. In some instances the reaction time may be an interval of a few seconds while other slower, more complex reactions may require much longer intervals. It is important, therefore, that the period be sufficient to allow for any significant pH changes, yet consistent with good laboratory practices.

12.5 To develop a titration curve, plot the cumulative milliliters of standard solution added to the sample aliquot against the observed pH values. The acidity or alkalinity relative to a particular pH may be determined from the curve.

13. Calculation

13.1 Calculate the acidity or alkalinity, in milliequivalents per liter, using Eq 1:

$$\text{Acidity (or alkalinity), meq/L (epm)} = AN \times 10 \quad (1)$$

where:
A = standard acid or alkali required for the titration, mL, and
N = normality of the standard solution.

14. Report

14.1 Report the results of titrations to specific end points as follows: "The acidity (or alkalinity) to pH at °C = meq/L (epm)."

14.2 Appropriate factors for converting milliequivalents per liter (epm) to other units are given in Practice D 596.

15. Precision and Bias [7]

15.1 The precision and bias data presented in Table 1 for this test method meet the requirements of Practice D 2777.

15.2 The collaborative test of this test method was performed in reagent waters by six laboratories using one operator each, using three levels of concentration for both the acidity and alkalinity.

15.3 Precision and bias for this test method conforms to Practice D 2777–77, which was in place at the time of collaborative testing. Under the allowances made in 1.4 of D 2777–98, these precision and bias data do meet existing requirements for interlaboratory studies of Committee D19 test methods.

[7] Supporting data are available from ASTM Headquarters. Request RR:D19-1149.

TABLE 1 Determination of Precision and Bias for Acidity and Alkalinity by Electrometric Titration (Test Method A)

Amount Added, meq/L	Amount Found, meq/L	S_t	S_o	Bias, %
		Acidity		
48.30	48.76	1.25	0.44	+0.94
23.00	22.61	0.68	0.27	−1.67
17.10	16.51	0.71	0.26	−3.47
		Alkalinity		
4.90	5.00	0.39	0.12	+2.12
2.46	2.45	0.14	0.06	−0.00
0.51	0.56	0.15	0.05	+10.59

TEST METHOD B—ELECTROMETRIC OR COLOR-CHANGE TITRATION

16. Scope

16.1 This test method covers the rapid, routine control measurement of acidity or alkalinity to predesignated end points of waters that contain no materials that buffer at the end point or other materials that interfere with the titration by reasons that may include color or precipitation.

17. Summary of Test Method

17.1 The sample is titrated with standard acid or alkali to a designated pH, the end point being determined electrometrically or by the color change of an internal indicator.

18. Interferences

18.1 Natural color or the formation of a precipitate while titrating the sample may mask the color change of an internal indicator. Suspended solids may interfere in electrometric titrations by making the glass electrode sluggish. Waste materials present in some waters may interfere chemically with color titrations by destroying the indicator. Variable results may be experienced with waters containing oxidizing or reducing substances, depending on the equilibrium conditions and the manner in which the sample is handled.

19. Apparatus

19.1 *Electrometric pH Measurement Apparatus*—See 10.1.

20. Reagents

20.1 *Bromcresol Green Indicator Solution* (1 g/L)—Dissolve 0.1 g of bromcresol green in 2.9 mL of 0.02 *N* sodium hydroxide (NaOH) solution. Dilute to 100 mL with water.

20.2 *Hydrochloric Acid, Standard* (0.02 *N*) (Note 1)—See 11.1, except that the acid may be standardized by colorimetric titration as directed in Practice E 200 when an indicator is used for sample titration.

20.3 *Methyl Orange Indicator Solution* (0.5 g/L)—Dissolve 0.05 g of methyl orange in water and dilute to 100 mL.

20.4 *Methyl Purple Indicator Solution* (1 g/L)—Dissolve 0.45 g of dimethyl-aminoazobenzene-O-carboxylic acid, sodium salt, in approximately 300 mL of water. To this solution add 0.55 g of a water-soluble blue dye-stuff, Color Index No. 714, [8] and dissolve. Dilute to 1 L with water. This indicator is available commercially in prepared form.

20.5 *Methyl Red Indicator Solution* (1 g/L)—Dissolve 0.1 g of water-soluble methyl red in water and dilute to 100 mL.

20.6 *Phenolphthalein Indicator Solution* (5 g/L)—Dissolve 0.5 g of phenolphthalein in 50 mL of ethyl alcohol (95 %) and dilute to 100 mL with water.

[8] Refers to compounds, bearing such number, as described in "Color Index," Society of Dyers and Colourists, Yorkshire. England (1924). American Cyanamid Company's "Calcocid Blue AX Double" has been found satisfactory for this purpose.

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NOTE 4—Specially denatured ethyl alcohol conforming to Formula No. 3A or 30 of the U. S. Bureau of Internal Revenue may be substituted for ethyl alcohol (95 %).

20.7 *Sodium Hydroxide, Standard* (0.02 *N*)—See 11.2, except that the alkali may be standardized by colorimetric titration as directed in Practice E 200 when an indicator is used for sample titration.

21. Procedure

21.1 Depending on the method of titration to be used, pipette 100 mL of the sample, adjusted, if necessary, to room temperature, into a 300-mL, tall-form beaker or a 250-mL, narrow-mouth Erlenmeyer flask. Hold the tip of the pipette near the bottom of the container while discharging the sample.

21.2 Titrate the aliquot electrometrically to the pH corresponding to the desired end point (see Note 5). When using an indicator, add 0.2 mL (see Note 6) and titrate with 0.02 *N* acid (for alkalinity) or 0.02 *N* NaOH solution (for acidity) until a persistent color change is noted (see Note 7). Add the standard solution in small increments, swirling the flask vigorously after each addition. As the end point is approached, a momentary change in color will be noted in that portion of the sample with which the reagent first mixes. From that point on, make dropwise additions.

NOTE 5—The choice of end point will have been made to provide optimum data for the intended use or disposition of the water. When an indicator is used, those listed in 20.1 and 20.3 through 20.6 are used most frequently; others may be employed if it is to the user's advantage. Color change and endpoint data for indicators listed herein are presented in Appendix X2 and Table X2.1.

NOTE 6—After some practice, slightly more or less indicator may be preferred. The analyst must use the same quantity of phenolphthalein at all times, however, because at a given pH, the intensity of one-color indicators depends on the quantity.

NOTE 7—If the sample requires appreciably more than 25 mL of 0.02 *N* solution for its titration, use a smaller aliquot, or a 0.1 *N* reagent prepared and standardized in the same manner (see Practice E 200).

22. Calculation

22.1 Calculate the acidity or alkalinity, in milliequivalents per liter, using Eq 2:

$$\text{Acidity (or alkalinity), meq/L (epm)} = (AN/B) \times 1000 \quad (2)$$

where:

A = standard acid or alkali required for the titration, mL,
N = normality of the standard solution, and
B = sample titrated, mL.

23. Report

23.1 Report the results of titration as follows: "The acidity (or alkalinity) to at °C = meq/L (epm)," indicating the pH and the temperature at which it was determined, or the name of the indicator used, for example, "The acidity to methyl orange at °C = meq/L (epm)."

24. Precision and Bias [7]

24.1 The precision and bias data presented in Table 2 for this test method meet the requirements of Practice D 2777.

24.2 The collaborative test of this test method was performed in reagent waters by six laboratories using one operator each, using three levels of concentration for both the acidity and alkalinity.

TABLE 2 Determination of Precision and Bias for Acidity and Alkalinity by Electrometric or Color-Change Titration (Test Method B)

Amount Added, meq/L	Amount Found, meq/L	S_t	S_o	Bias, %
		Acidity		
48.30	49.06	0.802	0.589	+ 1.57
23.00	22.83	0.610	0.455	−0.74
17.10	16.84	0.334	0.146	−1.52
		Alkalinity		
4.90	4.88	0.156	0.034	−0.41
1.92	1.80	0.080	0.014	−6.25
0.51	0.50	0.044	0.024	−1.96

24.3 Precision and bias for this test method conforms to Practice D 2777–77, which was in place at the time of collaborative testing. Under the allowances made in 1.4 of D 2777–98, these precision and bias data do meet existing requirements for interlaboratory studies of Committtee D19 test methods.

TEST METHOD C—COLOR-CHANGE TITRATION AFTER HYDROGEN PEROXIDE OXIDATION AND BOILING

25. Scope

25.1 This test method is intended specifically for mine drainage, surface streams receiving mine drainage, industrial waste waters containing waste acids and their salts, and similar waters bearing substantial amounts of ferrous iron or other polyvalent cations in a reduced state.

25.2 Because the oxidation and hydrolysis of ferrous iron generate acidity, a reliable measure of acidity or alkalinity is obtained only when complete oxidation is achieved and hydrolysis of ferric salts is completed (see Appendix X3). In many instances, the concentration of ferrous iron is such that a 2-min boiling period is not sufficient to assure complete oxidation. In this test method, hydrogen peroxide is added prior to boiling to accelerate the chemical reactions needed for equilibrium.

25.3 This test method may be used to determine approximate alkali requirements for neutralization and to assure comparability of results when both alkaline and acid flows are under consideration in mine drainage treatment.

26. Summary of Test Method

26.1 The pH of the sample is determined. Standard acid is added as needed to lower the pH to 4.0 or less. Hydrogen peroxide (H_2O_2) is added, the solution boiled, and finally either titrated while hot to the phenolphthalein end point, or cooled and titrated electrometrically with standard alkali to pH = 8.2, the desired end point.

27. Interferences

27.1 Natural color or the formation of a colored precipitate during boiling may mask the color change of the phenolphthalein end point, requiring a pH meter for the titration. Suspended solids may cause sluggishness in electrometric titrations; however, compensation is made by a 15-s pause between alkali

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additions or by dropwise addition of titrant when the designated pH is approached.

27.2 The standard acid added prior to boiling neutralizes volatile components, for example, bicarbonates which contribute to the alkalinity and, hence, minimizes this source of error.

28. Apparatus

28.1 *Electrometric pH Measurement Apparatus*—See 10.1.

29. Reagents

29.1 *Hydrogen Peroxide* (H_2O_2, 30 % Solution).

29.2 *Phenolphthalein Indicator Solution* (5 g/L)—See 20.6.

29.3 *Sodium Hydroxide, Standard* (0.02 *N*)—Prepare and standardize as directed in Practice E 200.

29.4 *Sulfuric Acid, Standard* (0.02 *N*)—Prepare and standardize as directed in Practice E 200.

NOTE 8—Hydrochloric acid of similar normality may be used instead of sulfuric acid. Prepare and standardize in like manner.

30. Procedure

30.1 Pipette 50 mL of the sample into a 250-mL beaker.

30.2 Measure the pH of the sample (see Test Methods D 1293). If the pH is above 4.0, add 5-mL increments of standard H_2SO_4 to lower the pH to 4.0 or less (see Note 8).

30.3 Add only 5 drops of H_2O_2.

30.4 Heat the sample to boiling and continue to boil for 2 to 4 min.

30.5 If the sample is discolored, cool to room temperature and titrate electrometrically with standard NaOH solution to pH = 8.2, corresponding to the desired end point. If the sample is colorless, titrate to the phenolphthalein color change while hot.

31. Calculation

31.1 Calculate the acidity in milliequivalents per liter using Eq 3 or Eq 4:

31.1.1 Where no sulfuric acid is added:

$$\text{Acidity (boiled and oxidized), meq/L (epm)} = (BN_b/S) \times 1000 \quad (3)$$

31.1.2 Where sulfuric acid is added:

$$\text{Acidity (boiled and oxidized), meq/L (epm)} = [(BN_b - AN_a)/S] \times 1000 \text{ (see Note 9)} \quad (4)$$

where:

A = H_2SO_4 added to sample, mL,
B = NaOH solution required for titration of sample, mL,
N_a = normality of the H_2SO_4,
N_b = normality of the NaOH solution, and
S = sample used, mL.

NOTE 9—Minus acidity represents excess alkalinity contributed by constituents such as bicarbonates.

32. Report

32.1 Report the results of titrations as follows: "The acidity (boiled and oxidized) to pH (or phenolphthalein) = meq/L (epm)."

33. Precision and Bias [7]

33.1 The precision and bias data presented in Table 3 for this test method meet the requirements of Practice D 2777.

TABLE 3 Determination of Precision and Bias for Acidity by Color-Change Titration After Hydrogen Peroxide Oxidation and Boiling (Test Method C)

Amount Added, meq/L	Amount Found, meq/L	S_t	S_o	Bias, %
		Acidity		
48.30	49.06	1.28	0.43	+1.57
23.00	23.00	0.46	0.37	0.00
0.07	0.15	0.12	0.69	+106.0

33.2 The collaborative test of this test method was performed in reagent waters by six laboratories using one operator each, using three levels of concentration for both the acidity and alkalinity.

33.3 Precision and bias for this test method conforms to Practice D 2777–77, which was in place at the time of collaborative testing. Under the allowances made in 1.4 of D 2777–98, these precision and bias data do meet existing requirements for interlaboratory studies of D19 test methods.

34. Quality Control

34.1 In order to be certain that analytical values obtained using these test methods are valid and accurate within the confidence limits of the test, the following QC procedures must be followed when analyzing acidity or alkalinity.

34.1.1 *Calibration and Calibration Verification*:

34.1.1.1 Calibrate according to Test Method D 1293.

34.1.1.2 Verify instrument calibration after standardization by analyzing a pH solution.

34.1.1.3 If calibration cannot be verified, recalibrate the instrument.

34.1.2 *Initial Demonstration of Laboratory Capability*:

34.1.2.1 If a laboratory has not performed the test before, or if there has been a major change in the measurement system, for example, new analyst, new instrument, etc., a precision and bias study must be performed to demonstrate laboratory capability.

34.1.2.2 Analyze seven replicates of a standard solution prepared from an Independent Reference Material containing a mid-range concentration acidity or alkalinity. The matrix and chemistry of the solution should be equivalent to the solution used in the collaborative study. Each replicate must be taken through the complete analytical test method including any sample pretreatment steps. The replicates may be interspersed with samples.

34.1.2.3 Calculate the mean and standard deviation of the seven values and compare to the acceptable reanges of bias in sections 15,24, or 33 (depending on the method used). This study should be repeated until the recoveries are within the limits given in 15,24, or 33. If a concentration other than the recommended concentration is used, refer to Test Method D 5847 for information on applying the *F* test and *t* test in evaluating the acceptability of the mean and standard deviation.

34.1.3 *Laboratory Control Sample (LCS)*:

34.1.3.1 To ensure that the test method is in control, analyze a LCS containing a mid-range concentration of acidity or

alkalinity with each batch or 10 samples. If large numbers of samples are analyzed in the bath, analyze the LCS after every 10 samples. The LCS must be taken through all of the steps of the analytical method, including sample pretreatment. The result obtained for the LCS shall fall within ± 15 % of the known concentration.

34.1.3.2 If the result is not within the precision limit, analysis of samples is halted until the problem is corrected, and either all the samples in the batch must be reanalyzed, or the results must be qualified with an indication that they do not fall within the performance criteria of the test method.

34.1.4 *Duplicate*:

34.1.4.1 To check the precision of samaple analyses, analyze a sample in duplicate with each batch.

34.1.4.2 Calculate the standard deviation of the duplicate values and compare to the precision in the collaborative study using an *F* test. Refer to 6.4.4 of Test Method D 5847 for information on applying the *F* test.

34.1.4.3 If the result exceeds the precision limit, the batch must be reanalyzed or the results must be qualified with an indication that they do not fall within the performance criteria of the test method.

34.1.5 *Independent Reference Material (IRM)*:

34.1.5.1 In order to verify the quantitative value produced by the test method, analyze an Independent Reference Material (IRM) submitted as a regular sample (if practical) to the laboratory at least once per quarter. The concentration of the IRM should be in the concentration mid-range for the method chose. The value obtained must fall within the control limits established by the laboratory.

35. Keywords

35.1 acidity; alkalinity; titrations; water

APPENDIXES

(Nonmandatory Information)

X1. DISCUSSION OF TERMS

X1.1 The terms, acidity and alkalinity, as used in water analysis may not be in accord with generally accepted terminology with a neutral point at pH 7. In water analysis, a pH of about 4.5 is frequently the end point for titration of alkalinity and a pH of about 8.2 for acidity.

X1.2 In addition to free hydroxide, alkalinity may be produced by anions that tend to hydrolyze; these include carbonate, bicarbonate, silicate, phosphate, borate, arsenate, aluminate, possibly fluoride, and certain organic anions in waste waters. All the effects due to these anions are lumped together in an alkalinity analysis.

X1.3 The factors causing acidity in water are also complex. Acidic materials encountered in water analysis include, in addition to free organic and mineral acids, uncombined dissolved gases, and acids formed on hydrolysis of salts of weak bases and strong acids. Hydrolyzable salts of aluminum and ferric and ferrous iron in mine drainage and certain industrial waste waters, are common causes of acidity. Acidity determinations on waters containing ferrous iron are further complicated by air oxidation of ferrous to the ferric state and subsequent hydrolysis to produce additional acidity.

X1.4 Since some water samples change on storage, analyses must be made without delay or results may be of little value. Interpretation of acidity and alkalinity data should be made cautiously. For a more thorough understanding of the subject, it is recommended that the analyst review the literature[9,10,11]. Then, the analyst may be able to develop an interpretation of his data better suited to his particular needs.

[9] Hem, J. D., "Study and Interpretation of The Chemical Characteristics of Natural Water," *Geological Survey Water-Supply Paper 1473*, 1959, pp. 92–100.

[10] Rainwater, F. H., and Thatcher, L. L., "Methods for Collection and Analysis of Water Samples," *Geological Survey Water-Supply Paper 1454*, 1960, pp. 87–95.

[11] Sawyer, C. N., *Chemistry for Sanitary Engineers*, McGraw-Hill Book Co., Inc., New York, NY, 1960, pp. 211–227.

X2. INTERNAL ACID-BASE INDICATORS

TABLE X2.1 pH End Points Equivalent to Color Change of Indicators

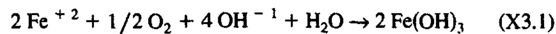
Indicator	Range		End Point	
	pH	Color	pH	Color
Phenolphthalein	8.0 to 10	colorless-red	8.2	pink
Methyl orange	3.2 to 4.4	pink-yellow	4.2	pink-orange
Methyl purple [A]	4.8 to 5.5	purple-green	4.9	gray-purple
Methyl red	4.2 to 6.2	pink-yellow	5.5	orange
Bromcresol green	4.0 to 5.4	yellow-blue	4.5	green
Bromphenol blue	3.0 to 4.6	yellow-blue	3.7	green

[A] Available as a prepared reagent from most chemical supply houses.

X2.1 Table X2.1 is provided as a guide in the selection of a titration indicator for determinations of acidity and alkalinity.

X3. USES OF THE HYDROGEN PEROXIDE TEST METHOD

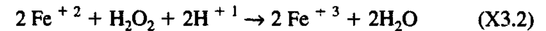
X3.1 The hydrogen peroxide test method is particularly suitable for assessing the acidity of mine drainage waters that are discharged into public streams. Under such conditions, all ferrous iron is rapidly oxidized to the ferric state, resulting in the precipitation of $Fe(OH)_3$:



$$2\,Fe^{+2} + 1/2\,O_2 + 4\,OH^{-1} + H_2O \rightarrow 2\,Fe(OH)_3 \quad (X3.1)$$

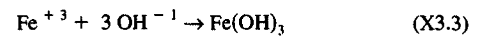
X3.2 The presence of ferrous ion in waters creates a twofold problem. First, the solubility of ferrous ion at a pH of 8.2 (phenolphthalein end point) is appreciable and the full acidity potential of the water cannot be assessed by direct titration to this end point. Second, at a pH of 8.2, soluble ferrous iron is rapidly oxidized by atmospheric oxygen. Subsequent hydrolysis of the resultant ferric ion immediately decreases the pH, resulting in a fading end point.

X3.3 Both problems can be avoided by oxidizing the ferrous ion with hydrogen peroxide prior to titration:



$$2\,Fe^{+2} + H_2O_2 + 2H^{+1} \rightarrow 2\,Fe^{+3} + 2H_2O \quad (X3.2)$$

During the subsequent titration, the ferric ion is precipitated as ferric hydroxide:



$$Fe^{+3} + 3\,OH^{-1} \rightarrow Fe(OH)_3 \quad (X3.3)$$

Note that in Eq X3.2 and Eq X3.3, the net effect is that two hydroxyl ions are consumed for each ferrous ion originally present, although the end product in each case is ferric hydroxide.

X4. RATIONALE FOR DISCONTINUATION OF TEST METHODS

X4.1 *Color-Comparison Titration:*

X4.1.1 This test method was discontinued in 1988. The test method may be found in its entirety in the 1988 *Annual Book of ASTM Standards*, Vol 11.01.

X4.1.2 This test method is applicable to routine control used in determining the acidity or alkalinity to a particular end point of waters containing no materials that buffer at the end point or interfere with the titration due to color or precipitation, or other reasons.

X4.1.3 The sample is titrated with standard acid or alkali to a predesignated pH, the end point being determined by comparison of the color developed by an added indicator with the color of a standard buffer solution containing the same added indicator.

X4.1.4 This test method was discontinued because there were insufficient laboratories interested in participating in another collaborative study to obtain the necessary precision and bias as required by Practice D 2777.

X4.2 *Color-Change Titration After Boiling:*

X4.2.1 This test method was discontinued in 1988. The test method may be found in its entirety in the 1988 *Annual Book of ASTM Standards*, Vol 11.01.

X4.2.2 This test method is applicable to routine control measurement of acidity or alkalinity of waters containing concentrations of slowly hydrolyzable materials sufficient to significantly delay attainment of equilibrium conditions at a titration end point. It is particularly applicable to mine drainage, industrial waste waters carrying waste acids, and similar waters. Volatile components contributing to the acidity or alkalinity of the water may be lost during sample pretreatment.

X4.2.3 The sample aliquot (acidified if alkaline) is boiled to accelerate chemical reactions for attaining equilibrium conditions, cooled, and titrated with standard acid or alkali to a predesignated end point. Titration is carried out by means of an internal indicator using the color-change procedure described in Test Method B (Electrometric or Color-Change Titration).

X4.2.4 This test method was discontinued because there were insufficient laboratories interested in participating in another collaborative study to obtain the necessary precision and bias as required by Practice D 2777.

SUMMARY OF CHANGES

This section identifies the location of selected changes to these test methods that have been incorporated since the last issue. For the convenience of the user, Committee D-19 has highlighted those changes that may impact the use of these test methods. This section may also include descriptions of the changes or reasons for the changes, or both.

(1) Sections 15, 24, and 33 and Tables 1, 2, and 3 were added.

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This standard is subject to revision at any time by the responsible technical committee and must be reviewed every five years and if not revised, either reapproved or withdrawn. Your comments are invited either for revision of this standard or for additional standards and should be addressed to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend. If you feel that your comments have not received a fair hearing you should make your views known to the ASTM Committee on Standards, at the address shown below.

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Designation: D 1126 – 02

Standard Test Method for Hardness in Water[1]

This standard is issued under the fixed designation D 1126; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

This standard has been approved for use by agencies of the Department of Defense.

1. Scope

1.1 This test method covers the determination of hardness in water by titration. This test method is applicable to waters that are clear in appearance and free of chemicals that will complex calcium or magnesium. The lower detection limit of this test method is approximately 2 to 5 mg/L as $CaCO_3$; the upper limit can be extended to all concentrations by sample dilution. It is possible to differentiate between hardness due to calcium ions and that due to magnesium ions by this test method.

1.2 This test method was tested on reagent water only. It is the user's responsibility to ensure the validity of the test method for waters of untested matrices.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

D 1066 Practice for Sampling Steam[2]

D 1129 Terminology Relating to Water[2]

D 1193 Specification for Reagent Water[2]

D 3370 Practices for Sampling Water from Closed Conduits[2]

D 5847 Practice for Writing Quality Control Specifications for Standard Test Methods for Water Analysis[3]

3. Terminology

3.1 *Definitions:*

3.1.1 *equivalent per million (epm), n*—a unit chemical equivalent weight of solute per million unit weights of solution.

3.1.2 *laboratory control sample, n*—a solution with certified hardness.

3.1.3 For definitions of other terms used in this test method, refer to Terminology D 1129.

4. Summary of Test Method

4.1 Calcium and magnesium ions in water are sequestered by the addition of disodium ethylenediamine tetraacetate. The end point of the reaction is detected by means of Chrome Black T[4], which has a red color in the presence of calcium and magnesium and a blue color when they are sequestered.

5. Significance and Use

5.1 Hardness salts in water, notably calcium and magnesium, are the primary cause of tube and pipe scaling, which frequently causes failures and loss of process efficiency due to clogging or loss of heat transfer, or both.

5.2 Hardness is caused by any polyvalent cations, but those other than Ca and Mg are seldom present in more than trace amounts. The term hardness was originally applied to water in which it was hard to wash; it referred to the soap-wasting properties of water. With most normal alkaline water, these soap-wasting properties are directly related to the calcium and magnesium content.

6. Interferences

6.1 The substances shown in Table 1 represent the highest concentrations that have been found not to interfere with this determination.

6.2 The test method is not suitable for highly colored waters, which obscure the color change of the indicator.

7. Reagents

7.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society.[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use

[1] This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.05 on Inorganic Constituents in Water.

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[2] *Annual Book of ASTM Standards*, Vol 11.01.

[3] *Annual Book of ASTM Standards*, Vol 11.02.

[4] 3–Hydroxy–4-(1–hydroxy-2–napththyl) azo-7–nitro–1 naphthalenesulfonic acid, sodium salt, Color Index 14645.

[5] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

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TABLE 1 Freedom of Reaction from Interferences

Substance	Maximum Concentration Without Interference in the Total Hardness Test, mg/L	Maximum Concentration Without Interference in the Calcium Hardness Test, mg/L
Aluminum, Al^{+++}	20	5
Ammonium, NH_4^+	[A]	2 000
Bicarbonate, HCO_3^-	...	500
Bromine, Br	...	2
Cadmium, Cd^{++}	20	...
Carbonate, CO_3^{--}	1 000	50
Chloride, Cl^-	10 000	...
Chlorine, Cl	...	2
Chromate, CrO_4^{--}	500	500
Cobalt, Co^{++}	0.3	...
Copper, Cu^{++}	20	2
Iron, ferric, Fe^{+++}	10[B]	20
Iron, ferrous, Fe^{++}	10[B]	20
Lead, Pb^{++}	20	5
Manganese, Mn^{++}	1[C]	10[C]
Nickel, Ni^{++}	0.5[D]	...
Nitrate, NO_3^-	500	500
Nitrite, NO_2^-	500	500
Phosphate, PO_4^{---}	100	...
Silicate, SiO_3^{--}	200	100
Strontium, Sr^{++}	[E]	[E]
Sulfate, SO_4^{--}	10 000	10 000
Sulfite, SO_3^{--}	500	500
Tannin, Quebracho	200	50
Tin, stannic, Sn^{++++}	10	5
Tin, stannous, Sn^{++}	10	5
Zinc, Zn^{++}	20	5

[A] No data are available.

[B] Iron will not interfere in concentrations up to 200 mg/L. However, the red color of the end point may return in about 30 s.

[C] Manganese will not interfere in concentrations up to 10 mg/L if a few crystals of $K_4Fe(CN)_6 \cdot 3H_2O$ are added to the buffer immediately before use.

[D] Accurate results can be obtained in the presence of 1 mg/L nickel, but the end point is slow under these conditions.

[E] If strontium is present, it will be titrated with calcium and magnesium.

without lessening the accuracy of the determination.

7.2 *Purity of Water*—Unless otherwise indicated, reference to water shall be understood to mean reagent water conforming to Specification D 1193, Type I. Other reagent water types may be used provided it is first ascertained that the water is of sufficiently high purity to permit its use without adversely affecting the precision and bias of the test method. Type II water was specified at the time of round robin testing of this test method.

7.3 *Ammonium Hydroxide Solution* (1 + 4)—Mix 1 volume of NH_4OH (sp gr 0.90) with 4 volumes of water.

7.4 *Buffer Solution*—Prepare the buffer solution in three steps as follows:

7.4.1 Dissolve 40 g of sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) in 800 mL of water.

7.4.2 Dissolve 10 g of sodium hydroxide (NaOH), 10 g of sodium sulfide ($Na_2S \cdot 9H_2O$), and 10 g of potassium sodium tartrate ($KNaC_4O_6 \cdot 4H_2O$) in 100 mL of water.

7.4.3 When cool mix the two solutions and add 1 g of magnesium disodium ethylenediamine tetraacetate, having a magnesium-to-EDTA mole ratio of 1 to 1. Make up to 1 L with water. Keep the solution bottle stoppered when not in use. The reagent will be effective for at least 1 month.

7.5 *Calcium Solution, Standard* (1 mL = 0.20 mg $CaCO_3$)—Dissolve 0.2000 g of $CaCO_3$ in 3 to 5 mL of HCl (1 + 4). Dilute to 1 L with water.

7.6 *Calcium Indicator*—Use powdered hydroxynaphthol blue,[6] or grind solid hydroxynaphthol blue to 40 to 50 mesh size.

7.7 *Hardness Indicator*—The hardness indicator can be prepared, stored, and used in liquid or powder form.

7.7.1 *Hardness Indicator Solution*—Dissolve 0.5 g of Chrome Black T[3] in 50 mL of diethanolamine or triethanolamine. Store the solution in a dark-colored bottle. This solution has a storage life of several months.

7.7.2 *Hardness Indicator Powder*—Grind 0.5 g of Chrome Black T[3] with 100 g of powdered sodium chloride. Use a dark-colored bottle for storage. The powder has a storage life of at least 1 year.

7.8 *Hydrochloric Acid* (1 + 4)—Mix 1 volume of concentrated hydrochloric acid (sp gr 1.19) with 4 volumes of water.

7.9 *Disodium Ethylenediamine Tetraacetate (Na_2H_2 EDTA) Solution, Standard* (1 mL = 1.0 mg $CaCO_3$)—Dissolve 3.8 g of disodium ethylenediamine tetraacetate dihydrate in approximately 800 mL of water. Adjust the pH of the solution to 10.5 with NaOH solution (50 g/L). Determine the concentration of this solution using the standard calcium solution, and that procedure in Section 9 that will be used for the sample analysis (9.1, 9.2, or 9.3). Adjust the concentration of the EDTA so that 1 mL will be equivalent to 1.0 mg of $CaCO_3$. Store the standard EDTA in polyethylene, plastic, or hard rubber bottles and restandardize monthly.

7.10 *Sodium Hydroxide Solution* (50 g/L)—Dissolve 50 g of sodium hydroxide in water and dilute to 1 L.

8. Sampling

8.1 Collect the sample in accordance with Practice D 1066 or Practices D 3370 as applicable.

9. Procedure

9.1 *Hardness*—Measure 50 mL of clear sample into an opaque white container or a clear colorless container utilizing a white background. Adjust the pH of the sample to 7 to 10 by adding NH_4OH solution or HCl solution. Add 0.5 mL of buffer solution, and approximately 0.2 g of hardness indicator powder or 2 drops of liquid and stir. Add standard Na_2H_2EDTA solution slowly from a burette with continuous stirring until the color changes from red to blue. Complete the titration within 5 min after the buffer addition. If the titration requires more than 20 mL of the titrating solution, dilute the sample and repeat the test.

9.2 *Low Hardness*—Determine low-hardness values (0.5 to 5.0 ppm as $CaCO_3$) in accordance with 9.1, but use a 100 mL sample and titrate by means of micro-burette. When employing a 100-mL sample, add twice the quantity of the reagents as indicated in 9.1.

9.3 *Calcium Hardness*—Measure 50 mL of the sample into an opaque white container, or a clear colorless container utilizing a white background. Add 2 mL of NaOH solution and

[6] 3–Hydroxy-4 (2–hydroxy–4 sulfo–1 naphthyl) azo–2, 7–naphthalenedisulfonic acid, trisodium salt.

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stir. Add approximately 0.2 g of calcium indicator and stir. Add standard Na_2H_2EDTA solution slowly from a burette with continuous stirring until the color changes from red to royal blue. Complete the titration within 5 min after the NaOH addition. If the titration requires more than 15 mL of the titrating solution, dilute the sample and repeat the test.

10. Calculations

10.1 Calculate the hardness, epm, of the sample as follows:

$$\text{Hardness, epm} = 20\, C/S \quad (1)$$

where:
epm = equivalent parts per million; milliequivalents per liter,
C = standard Na_2H_2EDTA solution added in titrating hardness, mL, and
S = sample taken, mL.

10.1.1 Calculate the calcium hardness, epm, of the sample as follows:

$$\text{Calcium hardness, epm} = 20\, D/S \quad (2)$$

where:
epm = equivalent parts per million; milliequivalents per liter,
D = standard Na_2H_2EDTA solution added in titrating calcium hardness, mL, and
S = sample taken for test, mL.

10.1.2 Calculate the magnesium hardness, epm, of the sample as follows:

$$\text{Magnesium hardness, epm} = E - F \quad (3)$$

where:
epm = equivalent parts per million; milliequivalents per liter,
E = hardness, epm, and
F = calcium hardness, epm.

10.2 Calculate the hardness as calcium carbonate of the sample as follows:

$$\text{Hardness, mg/L as } CaCO_3 = 1000\, C_1/S_1 \quad (4)$$

where:
C_1 = standard Na_2H_2EDTA solution added in titrating hardness, mL, and
S_1 = sample taken, mL.

10.2.1 Calculate the calcium hardness as calcium carbonate of the sample as follows:

$$\text{Calcium hardness, mg/L as } CaCO_3 = 1000\, D_1/S_1 \quad (5)$$

where:
D_1 = standard Na_2H_2EDTA solution added in titrating calcium hardness, mL, and
S_1 = sample taken, mL.

10.2.2 Calculate the magnesium hardness as calcium carbonate of the sample as follows:

$$\text{Magnesium hardness, mg/L as } CaCO_3 = G - H \quad (6)$$

where:
G = hardness, mg/L as $CaCO_3$, and
H = calcium hardness, mg/L as $CaCO_3$.

11. Precision and Bias [7]

11.1 The single operation and overall precision of the total hardness test method within its designated range for 6 laboratories, which include a total of 6 operators analyzing each sample on 3 different days may be expressed as follows:

$$S_o = 0.0047\, X + 0.40$$
$$S_T = 0.0078\, X + 1.80$$

where:
S_o = pooled single-operator precision, mg/L,
S_t = overall precision, mg/L, and
X = hardness concentration, mg/L.

11.2 The single operator and overall precision of the calcium hardness test method within its designated range for 6 laboratories, which include a total of 6 operators analyzing each sample on 3 different days may be expressed as follows:

$$S_o = 0.0052\, X + 0.37$$
$$S_t = 0.025\, X + 0.61$$

where:
S_o = pooled single-operator precision, mg/L
S_t = overall precision, mg/L, and
X = calcium hardness concentration, mg/L.

11.3 Recoveries of known amounts of hardness and calcium hardness in a series of prepared standards for the same laboratories and operators are as shown in Table 2.

11.4 These data apply to reagent water only. It is the analyst's responsibility to ensure the validity of this test method for waters of untested matrices.

11.5 Precision and bias for this test method conforms to Practice D 2777 - 77, which was in place at the time of collaborative testing. Under the allowances made in 1.4 of D 2777 - 98, these precision and bias data do meet existing requirements for interlaboratory studies of Committee D19 test methods.

TABLE 2 Statistical Information, Total Hardness

Amount Added, mg/L	Amount Found, mg/L	Bias	% Bias	Statistically Significant
11.0	11.4	+ 0.4	+ 3.6	No
45.0	46.3	+ 1.3	+ 2.9	No
206.	206.	0.0	0.0	No
450.	453.	+ 3.	+ 0.7	No
Calcium Hardness				
6.2	6.1	−0.1	−1.6	No
25.0	24.9	−0.1	−0.4	No
125.	126.	+ 1.0	+ 0.8	No
250.	250.	0.0	0.0	No

12. Quality Control (QC)

12.1 The following quality control information is recommended for the determination of hardness in water.

12.2 A check standard shall be analyzed at a minimum frequency of 10 % throughout the batch analysis. The value of the check standard shall fall between 80 % and 120 % of the true value.

[7] Supporting data are available from ASTM International Headquarters. Request RR: D19-1125.

12.3 A Laboratory Control Sample shall be analyzed with each batch of samples at a minimum frequency of 10 %.

12.4 If the QC for the sample batch is not within the established control limits, reanalyze the samples or qualify the results with the appropriate flags, or both (Practice D 5847).

12.5 Blind control samples should be submitted by an outside agency in order to determine the laboratory performance capabilities.

13. Keywords

13.1 analysis; calcium carbonate hardness; hardness; titration; water

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Designation: D 1293 – 99

Standard Test Methods for pH of Water[1]

This standard is issued under the fixed designation D 1293; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

This standard has been approved for use by agencies of the Department of Defense.

1. Scope

1.1 These test methods cover the determination of pH by electrometric measurement using the glass electrode as the sensor. Two test methods are given as follows:

	Sections
Test Method A—Precise Laboratory Measurement	8 to 15
Test Method B—Routine or Continuous Measurement	16 to 24

1.2 Test Method A covers the precise measurement of pH in water utilizing at least two of seven standard reference buffer solutions for instrument standardization.

1.3 Test Method B covers the routine measurement of pH in water and is especially useful for continuous monitoring. Two buffers are used to standardize the instrument under controlled parameters, but the conditions are somewhat less restrictive than those in Test Method A.

1.4 Both test methods are based on the pH scale established by NIST (formerly NBS) Standard Reference Materials.[2]

1.5 Neither test method is considered to be adequate for measurement of pH in water whose conductivity is less than about 5 μS/cm. Refer to Test Methods D 5128 and D 5464.

1.6 Precision and bias data were obtained using buffer solutions only. It is the user's responsibility to assure the validity of these test methods for untested types of water.

1.7 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

D 1066 Practice for Sampling Steam[3]

D 1067 Test Methods for Acidity or Alkalinity of Water[3]

D 1129 Terminology Relating to Water[3]

D 1192 Specification for Equipment for Sampling Water and Steam in Closed Conduits[3]

D 1193 Specification for Reagent Water[3]

D 2777 Practice for Determination of Precision and Bias of Applicable Methods of Committee D-19 on Water[3]

D 3370 Practices for Sampling Water from Closed Conduits[3]

D 5128 Test Method for On-Line pH Measurement of Water of Low Conductivity[3]

D 5464 Test Methods for pH Measurement of Water of Low Conductivity[3]

E 70 Test Method for pH of Aqueous Solutions with the Glass Electrode[4]

3. Terminology

3.1 *Definitions*—For definitions of terms used in these test methods, refer to Terminology D 1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *pH, n*—the pH of an aqueous solution is derived from E, the electromotive force (emf) of the cell

glass electrode | solution ‖ reference electrode

(where the double vertical line represents a liquid junction) when the electrodes are immersed in the solution in the diagrammed position, and E_s is the electromotive force obtained when the electrodes are immersed in a reference buffer solution.

With the assigned pH of the reference buffer designated as pH_s, and E and E_s expressed in volts is the following:[5]

$$pH = pH_s + \frac{(E - E_s)F}{2.3026\,RT}$$

where:

F = Faraday,

R = gas constant, and

T = absolute temperature, t (°C) + 273.15.

The reciprocal of $F/2.3026\,RT$ is known as the slope of the electrode, and is the expected difference in observed voltage for two measurements one pH unit apart. Values of the slope at various temperatures are given in Table 1.

4. Summary of Test Method

4.1 The pH meter and associated electrodes are standardized

[1] These test methods are under the jurisdiction of ASTM Committee D19 on Water and are the direct responsibility of Subcommittee D19.03 on Sampling of Water and Water-Formed Deposits, Surveillance of Water, and Flow Measurement of Water.

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[2] "Standard Reference Materials: Standardization of pH Measurements" Wu and Koch, NBS Special Publications No. 260-53, 1988.

[3] *Annual Book of ASTM Standards*, Vol 11.01.

[4] *Annual Book of ASTM Standards*, Vol 15.05.

[5] Bates, R. G., *Determination of pH: Theory and Practice*, 2nd Ed., J. Wiley and Sons, New York, 1973, p. 29.

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TABLE 1 Slope Factor at Various Temperatures

Temperature,° C	Slope, millivolts
0	54.20
5	55.19
10	56.18
15	57.17
20	58.17
25	59.16
30	60.15
35	61.14
40	62.13
45	63.13
50	64.12
55	65.11
60	66.10
65	67.09
70	68.09
75	69.08
80	70.07
85	71.06
90	72.05
95	73.05

against two reference buffer solutions that closely bracket the anticipated sample pH. The sample measurement is made under strictly controlled conditions and prescribed techniques.

5. Significance and Use

5.1 The pH of water is a critical parameter affecting the solubility of trace minerals, the ability of the water to form scale or to cause metallic corrosion, and the suitability of the water to sustain living organisms. It is a defined scale, based on a system of buffer solutions[2] with assigned values. In pure water at 25°C, pH 7.0 is the neutral point, but this varies with temperature and the ionic strength of the sample.[6] Pure water in equilibrium with air has a pH of about 5.5, and most natural uncontaminated waters range between pH 6 and pH 9.

6. Purity of Reagents

6.1 Reagent grade chemicals shall be used in all tests, except as specifically noted for preparation of reference buffer solutions. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.[7] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

6.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type I.

7. Sampling

7.1 Collect samples in accordance with Practice D 1066, Specification D 1192, or Practices D 3370, whichever is applicable.

TEST METHOD A—PRECISE LABORATORY MEASUREMENT OF pH

8. Scope

8.1 This test method covers the precise measurement of pH in water under strictly controlled laboratory conditions.

9. Interferences

9.1 The glass electrode reliably measures pH in nearly all aqueous solutions and in general is not subject to solution interference from color, turbidity, colloidal matter, oxidants, or reductants.

9.2 The reference electrode may be subject to interferences and should be chosen to conform to all requirements of Sections 10 and 12. Refer also to Appendix X1.3.

9.3 The true pH of an aqueous solution or extract is affected by the temperature. The electromotive force between the glass and the reference electrode is a function of temperature as well as pH. The temperature effect can be compensated automatically in many instruments or can be manually compensated in most other instruments. The temperature compensation corrects for the effect of changes in electrode slope with temperature but does not correct for temperature effects on the chemical system being monitored. It does not adjust the measured pH to a common temperature; therefore, the temperature should be reported for each pH measurement. Temperature effects are discussed further in Appendix X1.2.

9.4 The pH response of the glass electrode/reference electrode pair is imperfect at both ends of the pH scale. The indicated pH value of highly alkaline solutions may be too low, by as much as 1 pH, depending on electrode composition and sample conditions. See X1.5.1. The indicated pH value of strong aqueous solutions of salts and strong acids having a pH less than 1, will often be higher than the true pH value. Interferences can be minimized by the selection of the proper glass and reference electrodes for measurements in highly alkaline or acidic solutions.

9.5 A few substances sometimes dispersed in water appear to poison the glass electrode. A discussion of this subject is given in Appendix X1.4.

10. Apparatus

10.1 *Laboratory pH Meter*—Almost all commercially available meters are of the digital type and will have either manual or automatic calibration, and either manual or automatic temperature (slope) correction. All four types are permissible. However, readability to 0.01 pH is essential (Section 14), and the ability to read in millivolts is useful in troubleshooting.

10.2 *Glass Electrode*—The pH response of the glass electrode shall conform to the requirements set forth in 12.1 through 12.5. The glass electrode lead wire shall be shielded. New glass electrodes and those that have been stored dry shall be conditioned and maintained as recommended by the manufacturer.

[6] The relative acidity or alkalinity measured by pH should not be confused with total alkalinity or total acidity (for example, Test Methods D 1067). Thus, 0.1 *M* HCl and 0.1 *M* acetic acid have the same total acidity, but the HCl solution will be more acidic (approximately pH 1 versus pH 3.).

[7] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

10.3 *Reference Electrode*—This may be used as separate "half cell," or it may be purchased integral with the glass pH electrode body, as a combination electrode. The internal reference element may be calomel (mercury/mercurous chloride), silver/silver chloride, or an iodide-iodine redox couple. For best performance, the reference element should be the same type in both the reference electrode and inside the pH electrode. For all three types, the junction between the reference filling solution and the sample may be either a flowing or nonflowing junction. The flowing liquid junction-type unit ensures that a fresh liquid junction is formed for each measurement and shall be used for Test Method A determinations. If a saturated calomel electrode is used, some potassium chloride crystals shall be contained in the saturated potassium chloride solution. If the reference electrode is of the flowing junction type, the design of the electrode shall permit a fresh liquid junction to be formed between the reference electrode solution and the buffer standard or tested water for each measurement and shall allow traces of solution to be washed from the outer surfaces of the electrodes. To ensure the desired slow outward flow of reference electrode solution, the solution pressure inside the liquid junction should be kept somewhat in excess of that outside the junction. In nonpressurized applications, this requirement can be met by maintaining the inside solution level higher than the outside water level. If the reference electrode is of the nonflowing junction type, these outward flow and pressurization considerations do not apply. The reference electrode and junction shall perform satisfactorily as required in the standardizing procedure described in 12.1 through 12.5. A discussion of reference electrodes is given in Appendix X1.3.

10.4 *Temperature Compensator*—The thermocompensator is a temperature-sensitive resistance element immersed in the water sample with the electrodes. The thermocompensator automatically corrects for the change in slope of the glass electrode (with change of temperature) but does not correct for actual changes in sample pH with temperature. The automatic thermocompensator is not required if the water temperature is essentially constant and the analyst chooses to use the manual temperature compensation feature of the pH meter.

11. Reagents

11.1 *Reference Buffer Solutions*—The pH values of the reference buffer solutions measured at several temperatures are listed in Table 2. Table 3 identifies each buffer salt by its National Institute of Standards and Technology (NIST) number and provides a recommended drying procedure prior to use. The current renewal of each NIST standard reference material should be used. Keep the five reference buffer solutions with pH less than 9.5 in bottles of chemically resistant glass. Keep the calcium hydroxide solutions in a plastic bottle that is nonporous to air (that is, polypropylene or high density polyethylene). Keep all the reference buffer solutions well-stoppered and replace if a visible change is observed.

11.1.1 *Borax Reference Buffer Solution* (pH_s= 9.18 at 25°C)—Dissolve 3.80 g of sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$) in water and dilute to 1 L.

11.1.2 *Calcium Hydroxide Reference Buffer Solution* (pH_s= 12.45 at 25°C)—Prepare pure calcium hydroxide ($Ca(OH)_2$) from well-washed calcium carbonate ($CaCO_3$) of low-alkali grade by slowly heating the carbonate in a platinum dish at 1000°C and calcining for at least 45 min at that temperature. After cooling in a dessicator, add the calcined product slowly to water with stirring, heat the resultant suspension to boiling, cool, and filter through a funnel having a fritted-glass disk of medium porosity. Collect the solid from the filter, dry it in an oven at 110°C, and crush it to a uniform and fine granular state. Prepare a saturated calcium hydroxide solution by vigorously shaking a considerable excess (about 3 g/L) of the fine granular product in water at 25°C in a stoppered plastic bottle (that is, polypropylene or high density polyethylene) that is essentially nonporous to gases. Allow the gross excess of solid to settle

TABLE 2 pH_s of Reference Buffer Solutions[A]

Temperature, °C	Tetroxalate Solution	Tartrate Solution	Phthalate Solution	Phosphate Solution	Borax Solution	Sodium Bicarbonate Sodium Carbonate	Calcium Hydroxide Solution
0	1.67	...	4.00	6.98	9.46	10.32	13.42
5	1.67	...	4.00	6.95	9.39	10.25	13.21
10	1.67	...	4.00	6.92	9.33	10.18	13.00
15	1.67	...	4.00	6.90	9.28	10.12	12.81
20	1.68	...	4.00	6.88	9.23	10.06	12.63
25	1.68	3.56	4.00	6.86	9.18	10.01	12.45
30	1.68	3.55	4.01	6.85	9.14	9.97	12.29
35	1.69	3.55	4.02	6.84	9.11	9.93	12.13
40	1.69	3.55	4.03	6.84	9.07	9.89	11.98
45	1.70	3.55	4.04	6.83	9.04	9.86	11.84
50	1.71	3.55	4.06	6.83	9.02	9.83	11.71
55	1.72	3.55	4.07	6.83	8.99	...	11.57
60	1.72	3.56	4.09	6.84	8.96	...	11.45
70	1.74	3.58	4.12	6.85	8.92	...	...
80	1.77	3.61	4.16	6.86	8.89	...	...
90	1.79	3.65	4.19	6.88	8.85	...	...
95	1.81	3.67	4.21	6.89	8.83	...	...

[A]For a discussion of the manner in which these pH values were assigned, see Bates, R. G., "Revised Standard Values for pH Measurements from 0 to 95°C," *Journal of Research*, NBS, Vol 66A, 1962, p. 179. The reference values were obtained without a liquid junction, which has an uncertainty of ±0.005. Liquid junction electrode values may have an uncertainty of ±0.012, with uncertainty± 0.03 for the tetroxalate and the $Ca(OH)_2$. More recent values have been published in *pH Measurement* by Helmuth Galster, VCH Publishers, Inc., New York, 1991.

TABLE 3 National Institute of Standards and Technology (NIST) Materials for Reference Buffer Solutions

NIST Standard Reference Material Designation	Buffer Salt[A]	Drying Procedure
187	Borax (sodium tetraborate decahydrate)	Drying not necessary (this salt should not be oven-dried)
186	disodium hydrogen phosphate	2 h in oven at 130°
186	potassium dihydrogen phosphate	2 h in oven at 130°C
185	potassium hydrogen phthalate	2 h in oven at 110°C
188	potassium hydrogen tartrate	drying not necessary
189	potassium tetroxalate dihydrate	should not be dried
191	sodium bicarbonate	should not be dried
192	sodium carbonate	2 h in oven at 275°C
2193	calcium carbonate	see NIST material certificate

[A]The buffer salts listed can be purchased from the Standard Reference Materials Program, National Institute of Standards and Technology, Gaithersburg, MD 20899.

and filter the solution with suction through a fritted-glass funnel of medium porosity. The filtrate is the reference buffer solution. Contamination of the solution with atmospheric carbon dioxide renders it turbid and indicates need for replacement.

11.1.3 *Phosphate Reference Buffer Solution* (pH_s= 6.86 at 25°C)—Dissolve 3.39 g of potassium dihydrogen phosphate (KH_2PO_4) and 3.53 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4) in water and dilute to 1 L.

11.1.4 *Phthalate Reference Buffer Solution* (pH_s= 4.00 at 25°C)—Dissolve 10.12 g of potassium hydrogen phthalate ($KHC_8H_4O_4$) in water and dilute to 1 L.

11.1.5 *Tartrate Reference Buffer Solution* (pH_s= 3.56 at 25°C)—Shake vigorously an excess (about 75 g/L) of potassium hydrogen tartrate ($KHC_4H_4O_6$) with 100 to 300 mL of water at 25°C in a glass-stoppered bottle. Filter, if necessary, to remove suspended salt. Add a crystal of thymol (about 0.1 g) as a preservative.

11.1.6 *Tetroxalate Reference Buffer Solution* (pH_s= 1.68 at 25°C)—Dissolve 12.61 g of potassium tetroxalate dihydrate ($KHC_2O_4 \cdot H_2C_2O_4 \cdot 2H_2O$) in water and dilute to 1 L.

11.1.7 *Sodium Bicarbonate—Sodium Carbonate Reference Buffer Solution* (pH_s= 10.01 at 25°C)—Dissolve 2.092 g of sodium bicarbonate ($NaHCO_3$) and 2.640 g of sodium carbonate (Na_2CO_3) in water and dilute to 1 L.

11.2 *Other Buffer Solutions*—A buffer solution other than that specified may be used as a working standard in the method providing that in each case such a solution is first checked against the corresponding reference buffer solution, using the procedures of the method, and is found to differ by not more than ± 0.02 pH unit.

11.3 *Commercial Buffer Solutions*—Commercially available prepared buffer solutions are not acceptable for the standardization in Test Method A.

12. Standardization of Assembly

12.1 Turn on the instrument, allow it to warm up thoroughly, and bring it to electrical balance in accordance with the manufacturer's instructions. Wash the glass and reference electrodes and the sample container with three changes of water or by means of flowing stream from a wash bottle. Form a fresh liquid junction if a sleeve-type reference junction is used. Note the temperature of the water to be tested. If temperature compensation is to be manual, adjust the temperature setting of the meter to correspond to the temperature of the water to be tested and allow time for all buffers, solutions, and electrodes to equilibrate thermally.

12.2 Select at least two reference buffer solutions, the pH_s values of which closely bracket the anticipated pH (refer to Table 2). Warm or cool the reference solutions as necessary to match within 2°C the temperature of the solution to be tested. Fill the sample container with the first reference buffer solution and immerse the electrodes. Stir the solution as described in 13.3.

12.3 Set the pH_s value of the reference buffer solution at the temperature of the buffer, as read from Table 2 or interpolated from the data therein, according to the manufacturer's instructions.

12.4 Empty the sample container and repeat, using successive portions of the reference buffer solution, until two successive readings are obtained without adjustment of the system. These readings should differ from the pH_s value of the buffer solution by not more than ± 0.02 pH unit.

NOTE 1—If the temperature of the electrode differs appreciably from that of the solution to be tested, use several portions of solution and immerse the electrodes deeply to assure that both the electrodes and the solution are at the desired temperature. To reduce the effects of thermal lag, keep the temperature of electrodes, reference buffer solutions, and the wash as close to that of the water sample as possible.

12.5 Wash the electrodes and the sample container three times with water. Place the second reference buffer solution in the sample container, and measure the pH. Set the temperature corrected value of the second reference buffer solution according to the meter manufacturer's instructions. Use additional portions of the second reference buffer solution, as before, until two successive readings differ by not more than ± 0.02 pH unit. The assembly shall be judged to be operating satisfactorily if the reading obtained for the second reference buffer solution agrees with its assigned pH_s value within 0.05 (or less) pH units.

12.6 If only an occasional pH determination is made, standardize the assembly each time it is used. In a long series of measurements, supplemental interim checks at regular intervals are recommended. Inasmuch as commercially available pH assemblies exhibit different degrees of measurement stability, conduct these checks at intervals of 30 min, unless it is ascertained that less frequent checking is satisfactory to ensure the performance described in 12.2 to 12.5.

13. Procedure

13.1 Standardize the assembly with two reference buffer solutions as described in 12.2 to 12.5 and then wash the electrodes with three changes of water or by means of a flowing stream from a wash bottle.

13.2 Place the water sample in a clean glass beaker provided with a stirring bar and either a thermometer (for meters with manual temperature compensation) or an ATC probe (for meters with automatic temperature compensation).

13.3 Stir during the period of pH measurement at a rate that will prevent splashing and that will avoid loss or gain of acidic or basic gases by interchange with the atmosphere. When necessary, stir briskly enough to intermix the phases of a nonhomogeneous water sample. Stop the stirrer during periods of measurement if fluctuations in readings are observed. (See Appendix X1.3.4 and X1.4.3).

13.4 Insert the electrodes and determine a preliminary pH value (since this value may drift somewhat, it should be considered an estimated value). Measure successive portions of the water sample until readings on two successive portions differ by no more than 0.03 pH unit, and show drifts of less than 0.02 pH unit in 1 min. Two or three portions will usually be sufficient if the water is well buffered.

13.5 Record the pH and temperature of the sample.

13.6 Measure the pH of slightly buffered waters (that are in equilibrium with air) essentially as described in 13.1 to 13.5, but measure the pH of successive portions until the readings for two successive portions differ by no more than 0.1 pH unit. Six or more portions may be necessary.

NOTE 2—Take special precautions if the sample is not in equilibrium with the carbon dioxide of the atmosphere protecting the sample from exposure to the air during measurement. *Measurement of unbuffered or slightly buffered samples is more reliably made in flow-type cells as described in Note 4.* Test Methods D 5464 describe additional precautions that should be taken if the electrical conductivity of the sample is less than about 5 μS/cm.

14. Report

14.1 Report the temperature of the measurement of the nearest 1°C.

14.2 Report the pH of the test solution to the nearest 0.01 pH unit when the pH measurement lies between 1.0 and 12.0.

14.3 Report the pH of the test solution to the nearest 0.1 pH unit when the pH measurement is less than 1.0 or greater than 12.0.

15. Precision and Bias [8]

15.1 The information summarized in this section was derived from an interlaboratory study performed in 1973 on four buffer solutions having pH values of approximately 3.7, 6.5, 8.2, and 8.4. Eleven laboratories (fourteen operators, with one laboratory providing four operators) analyzed each solution in duplicate and replicated the analysis on another day for a total of 224 determinations. A variety of commercial meters was used in this study. It is assumed that all measurements were made at room temperature.

15.2 Statistical treatment of the data conforms to the recommendations of Practice D 2777. Further information, based on a different statistical interpretation, can be found in Test Method E 70.

15.3 *Precision*—The overall and single-operator precision of this test method varies with pH as shown in Fig. 1.

15.4 *Bias*—The pH values of the buffer solutions, as determined using a gaseous hydrogen electrode, are compared with values obtained using this test method in Table 4.

15.5 Precision and bias data were obtained using buffer solutions only. It is the user's responsibility to assure the validity of the standards for untested types of water.

TEST METHOD B—ROUTINE OR CONTINUOUS MEASUREMENT OF pH

16. Scope

16.1 This test method is used for the routine measurement of pH in the laboratory and the measurement of pH under various process conditions.

17. Summary of Test Method

17.1 A direct standardization technique is employed in this test method for routine batch samples. Two buffers are used to standardize the instrument under controlled parameters, but the conditions are somewhat less restrictive than those in Test Method A. An indirect standardization procedure is used on flowing systems in which grab samples are removed periodically in order to compare a monitored pH value (of the system) with the reading of a laboratory pH meter.

18. Interferences

18.1 For information on interferences, see Section 9 and Appendix X1.4.

19. Apparatus

19.1 *Laboratory pH Meter*—See 10.1.

19.2 *Glass Electrode*—See 10.2.

19.3 *Reference Electrode*—See 10.3.

19.4 *Temperature Compensator*—See 10.4.

19.5 *Process pH Measurement Instrumentation*—Instruments that are used for process pH measurements are generally much more rugged than those which are used for very accurate measurements in the laboratory.

19.5.1 *Electrode Chamber*—For process pH measurements; the electrodes and thermocompensator are mounted in an electrode chamber or cell.

19.5.1.1 *Flow-Through Chamber* completely encloses the electrodes and the sample is piped to and from the chamber in a flow-through configuration. Commercially available chambers generally can tolerate temperatures as high as 100°C over a pH range from 0 to 14, and pressures up to 1034 kPa (approximately 150 psi).

19.5.1.2 *Immersion Type Chamber*, suitable for measurement in open streams or tanks, shields but does not completely enclose the electrodes. Immersion-style chambers are available for use at depths to 30 m (100 ft).

19.5.2 *Signal Transmission*—The glass electrode is usually a high-impedance device from which only an extremely small current can be drawn. Shielded cable must be used to connect the electrode to the pH analyzer. The signal can frequently be transmitted up to 300 m (approximately 1000 ft) with no loss in accuracy if the manufacturer's recommendations are followed carefully. However, long runs are vulnerable to electrical

[8] Supporting data for these test methods have been filed at ASTM Headquarters. Request Research Report RR: D19-1111.

D 1293

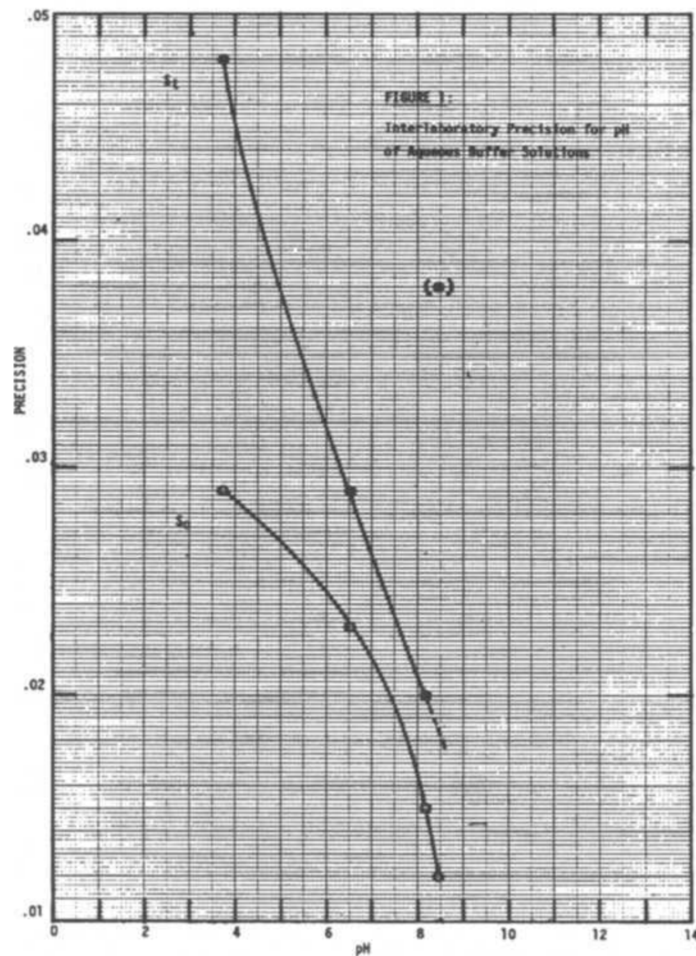

FIG. 1 Interlaboratory Precision for pH of Aqueous Buffer Solutions

TABLE 4 Determination of Bias

pH Expected	pH Found	Bias, ± %[A]	Statistically Significant (95 % Confidence Level)
3.714	3.73	+0.48	No
6.517	6.53	+0.20	Yes
8.147	8.19	+0.53	Yes
8.470	8.45	−0.24	Yes

[A]Since pH is a logarithmic function, this value may be misleading. It may be more useful to calculate bias as the difference between the values for pH Expected and pH Found.

noise pickup and high impedance signal leakage. The signal is usually amplified for distances greater than 5 m (approximately 16 feet).

19.5.3 *pH Signal Retransmission*—The electrical output signal of on-line pH instrumentation shall be electrically isolated from the electrode measuring circuit to prevent ground loop problems when measuring pH in a grounded sample and connecting the output signal to a computer, control system, data acquisition system, or other grounded equipment.

20. Reagents

20.1 *Commercial Buffer Solutions*—Commercially available prepared buffer should be adequate for the standardization in Test Method B. These commercial buffer solutions usually have pH values near 4, 7, and 10, the exact pH and use temperature being provided by the purveyor of the specific buffer. The pH 10 buffer is especially susceptible to contamination from atmospheric carbon dioxide, and frequently used or partially filled bottles are particularly vulnerable to this error.

20.2 For more information on reagents, see Section 11.

21. Standardization of Assembly

21.1 Turn on the analyzer, allow it to warm up thoroughly in accordance with the manufacturer's instructions. Wash the electrodes, the thermocompensator, and the sample container with three changes of water or by means of flowing stream from a wash bottle. Form a fresh liquid junction if a sleeve reference electrode junction is used. If manual temperature compensation is to be used, note the temperature of the water sample and adjust the temperature dial of the meter to correspond.

21.2 *Direct Standardization*:

21.2.1 Select two reference buffer solutions that have pH_s values that bracket the anticipated pH of the water sample. Warm or cool the reference solution to within 2°C of the

temperature of the water sample.

21.2.2 Fill the sample container with the first reference buffer solution and immerse the electrodes. Set the known pH_s of the reference buffer solution according to the instrument manufacturer's instructions. Repeat with successive portions of the reference buffer solution until two successive instrument readings are obtained which differ from the pH_s value of the buffer solution by no more than 0.02 pH unit.

21.2.3 Wash the electrodes and sample container three times with water. Place the second reference buffer solution in the sample container, and measure the pH. Adjust the slope control only until the reading corresponds to the temperature corrected value of the second reference buffer solution. Use additional portions of the second reference buffer solution, as before, until two successive readings differ by not more than 0.02 pH unit.

21.2.4 If only an occasional pH determination is made, standardize the assembly each time it is used. In a long series of measurements, supplement initial and final standardizations by interim checks at regular intervals. As commercially available pH assemblies exhibit different degrees of measurement stability, conduct these checks at intervals of 30 min, unless it is ascertained that less frequent checking is satisfactory to ensure performance. For continuous on-line measurements, the frequency of calibration shall be determined by experience since it is highly application dependent.

21.3 *Indirect Standardization*:

21.3.1 This procedure is to be employed when it is not convenient or practical to remove the electrodes from the flowing stream or container on which the pH is being determined. Use of a laboratory pH meter or an additional analyzer is required.

21.3.2 Standardize the laboratory pH meter or additional process analyzer as outlined in 21.2.

21.3.3 Collect a grab sample of the water from the immediate vicinity of the electrodes or from the discharge of a flow-through chamber. Measure the pH of this grab sample immediately, using the standardized laboratory pH meter.

21.3.4 Adjust the standardization control on the process analyzer until the reading corresponds to the pH of the grab sample. Repeat the grab sampling, analyzing, and adjusting procedure until two successive readings are obtained that differ by no more than 0.05 pH unit or within an acceptable accuracy.

NOTE 3—Indirect standardization as described above cannot be employed when the pH of the water being tested fluctuates by more than 0.05 pH unit. The standardization shall be accomplished in the shortest possible time if the pH is fluctuating. It is absolutely essential that the grab sample be representative of the water in contact with the electrodes of the analyzer being standardized. The integrity of the grab sample shall be maintained until its pH has been measured by the standardized meter, and its temperature shall remain constant. An alternate procedure giving greater flexibility is available using commercially-available process analyzers which provide a *hold* function. This function is manually activated at the time a grab sample is taken. It holds the pH value on the display, and allows time for the grab sample to be measured and its value to be used for calibration. After this standardization, the *hold* feature is deactivated.

21.3.5 Indirect standardization is a one-point calibration and does not establish the proper response of the electrodes over a pH range.

22. Procedure, Batch Samples

22.1 Standardize the assembly as described in 21.2 and wash the electrodes with three changes of water or by means of a flowing stream from a wash bottle.

22.2 Place the water sample in a clean glass beaker provided with a thermometer and a stirring bar. Stir during the period of pH measurement at a rate that will prevent splashing and that will avoid loss or gain of acidic or basic gases by interchange with the atmosphere. When necessary, stir briskly enough to intermix the phases of a nonhomogeneous water sample.

22.3 Insert the electrodes and determine a preliminary pH value (the reading may drift). Measure successive portions of the water sample until readings on two successive portions differ by no more than 0.05 pH unit. Two portions will usually be sufficient if the water is well-buffered.

22.4 Record the pH and temperature of the sample.

NOTE 4—*Continuous Determination of pH*—Make the selection of the electrodes and the electrode chamber to suit the physical and chemical characteristics of the process water. Locate a submersion style electrode chamber so that fresh representative sampling is provided continuously across the electrodes. Agitation may be required to improve homogeneity. Process pH measurements generally employ automatic temperature compensation. The pH value is usually displayed continuously and can be noted at any specific time. Also, record the successive pH values frequently to provide a permanent record. If the temperature of the sample fluctuates significantly with time, the temperature should also be recorded to interpret the pH values correctly.

23. Report

23.1 Report the temperature of measurement to the nearest 1°C.

23.2 Report the pH to the nearest 0.1 pH unit.

24. Precision and Bias [8]

24.1 Because of the wide variability in measurement conditions and the changeable character of the pH of many process waters, the precision of this test method is probably less than that of Test Method A; however, a precision of 0.1 pH unit should be attainable under controlled conditions.

24.2 Precision and bias data were obtained using buffer solutions only. It is the user's responsibility to assure the validity of this test method for untested types of water.

25. Keywords

25.1 hydrogen ion concentration; pH; pH buffer solution

APPENDIX

(Nonmandatory Information)

X1. MISCELLANEOUS NOTES ON GLASS ELECTRODE MEASUREMENTS AND EQUIPMENT

X1.1 Meaning of the Term pH

X1.1.1 The term pH historically has referred to the hydrogen ion activity of a solution and has been expressed as the logarithm to the base 10 of the reciprocal (negative logarithm) of the activity of hydrogen ions at a given temperature, as follows:

$$pH = \log 1/(H^+) = -\log(H^+)$$

where:

(H^+) = activity of hydrogen ions.

X1.1.2 Although this expression is helpful in giving theoretical meaning to the term pH and can be used as an approximate definition, it may not be rigorously related to empirical pH measurements. The definition given in 3.2.1 has gained wide acceptance.

X1.2 Temperature Effects

X1.2.1 The effects of temperature on electrometric pH measurements arise chiefly from two sources: (*1*) temperature effects that are common to all electrometric measurements and (*2*) variations of sample pH with temperature. The first category includes the effect of temperature on the factor $F/2.3026\ RT$ that occurs in the definition of pH (see 3.2). Values of this factor for various temperatures are given in Table 1. When electrodes are moved from a solution at one temperature to a solution at another, time is required for internal reference elements to reach the new temperature and, if saturated solutions are involved (for example, calomel or silver chloride electrodes), for the elements to come to a new equilibrium. During this period, some drifting may be observed. The extent of the problem will depend on the nature of the reference elements and their location within the electrodes.

X1.2.2 Secondly, because of changes in activity coefficients and equilibrium constants with temperature, the pH of a sample will change with temperature. These changes are independent of the method of measurement. In general, the rate of change of pH with temperature is not constant, and it may be positive or negative. The data in Table 2, showing changes in pH_s of buffer solutions with temperature, are typical examples. Process samples with known temperature coefficients may take advantage of solution temperature compensation available on some process analyzers to provide readout of pH referenced to 25°C.

X1.3 Reference Electrodes

X1.3.1 In making pH measurements with the glass electrode, the reference electrode used to complete the cell assembly functions simply as a source of reproducible potential. The absolute value of the reference electrode potential is of no consequence owing to the way the measurements are made. Saturated calomel, silver/silver chloride, and iodide-iodine redox references are all widely used and have proven themselves to be satisfactory reference electrodes at normal room temperatures. The calomel is the least satisfactory at elevated temperatures, and the iodide-iodine is the least affected by changing temperatures. Depending on the environmental conditions, other electrodes may serve satisfactorily as reference electrodes.

X1.3.2 If a saturated calomel electrode is used under significantly changeable temperature conditions, care must be taken to see that sufficient solid potassium chloride is present at all the temperatures to ensure solution saturation throughout, both in the free solution in the electrode tube and in the solution permeating the electrode element. The electrode must be given 5 or 10 min to accommodate itself to a new temperature condition before a pH measurement is made. If the temperature falls appreciably, crystallization of potassium chloride may cause plugging of the liquid junction; one result may be high resistance and false or erratic potential at the junction. Any such accumulation of potassium chloride should therefore be removed by aqueous washing.

X1.3.3 Reference electrodes of the unsaturated type have been used preferentially in continuous mechanized pH monitoring where the temperature is likely to fluctuate. The selected potassium chloride concentration is frequently saturation at the lowest temperature of use (for example, approximately 3.3 *N* for 0°C). Such a reference electrode has the advantage of being free from the annoying effects caused by variable solubility, but take considerable care to prepare the required concentration and to maintain the prescribed value under plant operating conditions. Follow the instrument manufacturer's recommendations on choosing and maintaining reference electrodes.

X1.3.4 Reference electrodes are available with any number of means to establish the liquid junction. These include, but are not limited to, dependence on the porosity of wood, fibrous materials, glass-encased noble metal, ground-glass sleeves, ceramic frits, and nonflowing polymeric bodies. Most offsets and fluctuations in readings as a result of stirring are due to effects at the liquid junction of the reference electrode.[9] For laboratory use, cleanable junctions (usually of a sleeve type of construction or having renewable elements) will give more consistent performance in "dirty" samples.

X1.4 Faulty Glass Electrode Response and Restorative Techniques

X1.4.1 *Detecting Faulty Electrodes*—The pH measuring assembly is standardized with two reference buffer solutions (see 12.2) to verify the response of the electrode combination at different pH values. Standardization also detects a faulty glass or reference electrode or an incorrect temperature compensator. The faulty electrode is indicated by a failure to obtain a reasonably correct value for the pH of the second reference

[9] Brezinski. D. P., "Kinetic, Static and Stirring Errors of Liquid Junction Reference Electrodes," *The Analyst*, 1983, 108. 425.

buffer solution after the meter has been standardized with the first. A cracked glass electrode will often yield pH readings that are essentially the same for both standards and should be discarded. Even though a normal glass electrode responds remarkably well to moderate pH changes, it is not necessarily fully responsive, and may miss the rigid requirements of 12.5, if, for example, the pH span is made as great as 5 pH units (phthalate to borax).

X1.4.2 *Imperfect pH Response*—The pH response of the glass electrode may be impaired by a few coating substances (certain oily materials or even some particulates). When the faulty condition is disclosed by the check with the two reference buffer solutions, the electrode can frequently be restored to normal by an appropriate cleaning procedure.

X1.4.3 *Stirring Errors*—If readings drift or are noisy only when the solution is stirred, there are two likely causes: (*1*) the sample is poorly buffered, and the pH is affected by air or CO_2 or (*2*) the reference junction is clogged or malfunctioning.

X1.4.4 *Glass Electrode Cleaning Techniques*—Where emulsions of free oil and water are to be measured for pH, it is absolutely necessary that the electrodes be cleaned thoroughly after each measurement. This may be done by washing with soap or detergent and water, followed by several rinses with water, after which the lower third of the electrodes should be immersed in HCl (1 + 9) to remove any film that may have been formed. Rinse the electrode thoroughly by washing it in several changes of water before returning it to service. Process pH analyzers used for continuous measurement may be provided with an ultrasonic cleaner to lessen or even eliminate the need for manual cleaning of electrodes.

X1.4.5 Thorough cleaning with a suitable solvent may be necessary after each measurement if the sample contains stickly soaps or suspended particles. If this fails, a chemical treatment designed to dissolve the particular deposited coating may prove successful. After the final rinsing of the electrode in the cleaning solvent, immerse the lower third of the electrodes in HCl (1 + 9) to remove a possible residual film. Wash the electrode thoroughly in several changes of water before subjecting it to the standardization procedure.

X1.4.6 Protein coatings may be removed by a 1 to 2 min soak of the bulb in a 30 % solution of a commercial hypochlorite bleach (approximately 1.5 % NaOCl). This should be followed by a rinse in 1 + 9 HCl:water and thorough washing with water.

X1.4.7 If an electrode has failed to respond to the treatment suggested in X1.4.3, try a more drastic measure as a last resort. This drastic treatment, which will limit the life of the electrode and should be used only as an alternative to discarding it, is immersing it in chromic acid cleaning solution for a period of several minutes (or longer if necessary). Chromic acid is particularly effective in cleaning foreign substances from the surface of the glass, but it also has a dehydrating effect on the glass. Consequently allow an electrode so treated, after thoroughly rinsing, to stand in water overnight before using it for measurements. Finally, if the electrode fails to respond to the chromic acid solution, it may be subjected to mild etching in ammonium bifluoride solution. Immerse the electrode for about 1 min in a 20 % solution of ammonium bifluoride (NH_4HF_2) in water, in a polyethylene cup. The bifluoride actually removes a portion of the bulb glass, and should be used only as a last resort (and then only infrequently). Follow the fluoride etch by thorough rinsing and conditioning as is recommended for a new electrode. The electrode manufacturer may have additional suggestions, specific to his own product.

X1.4.8 Techniques for cleaning flow cell electrodes include the use of ultrasonics, brushes, and high-velocity submerged jets.

X1.5 Special Measurements Techniques

X1.5.1 *Measurements on Alkaline Waters*—Although most modern pH glass formulations give good results in alkaline solutions, there can be an error if the solution is quite alkaline and contains high levels of sodium. This effect is greater at elevated temperatures. If in doubt, check with the electrode manufacturer.

X1.5.2 Carbon dioxide from the air tends to react with an alkaline water and to change its pH. Make all measurements with alkaline waters or buffer solutions as quickly as possible, with the water exposed to the air no longer than is absolutely necessary.

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Designation: D 1426 – 98

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Standard Test Methods for Ammonia Nitrogen In Water [1]

This standard is issued under the fixed designation D 1426; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

This standard has been approved for use by agencies of the Department of Defense.

1. Scope

1.1 These test methods cover the determination of ammonia nitrogen, exclusive of organic nitrogen, in water. Two test methods are included as follows:

	Sections
Test Method A—Direct Nesslerization	7 to 15
Test Method B—Ion Selective Electrode	16 to 24

1.2 Test Method A is used for the routine determination of ammonia in steam condensates and demineralizer effluents.

1.3 Test Method B is applicable to the determination of ammonia nitrogen in the range from 0.5 to 1000 mg NH_3N/L directly in reagent and effluent waters. Higher concentrations can be determined following dilution. The reported lower range is based on multiple-operator precision. Lower limits have been obtained by two of the twelve laboratories participating in the round robin.

1.4 Both test methods A and B are applicable to surface and industrial waters and wastewaters following distillation. The test method for distillation given in Appendix X1 has been used in the past to meet requirements for predistillation of samples being analyzed for ammonia.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

1.6 The distillation method now appears as Appendix X1 and is provided as nonmandatory information only. The automated colorimetric phenate method has been discontinued.

2. Referenced Documents

2.1 *ASTM Standards:*

D1066 Practice for Sampling Steam[2]

D1129 Terminology Relating to Water[2]

D1192 Specification for Equipment for Sampling Water and Steam in Closed Conduits[2]

D1193 Specification for Reagent Water[2]

D2777 Practice for Determination of Precision and Bias of Applicable Methods of Committee D-19 on Water[2]

D3370 Practices for Sampling Water[2]

E60 Practice for Photometric and Spectrophotometric Methods for Chemical Analysis of Metals[3]

E275 Practice for Describing and Measuring Performance of Ultraviolet, Visible, and Near Infrared Spectrophotometers[4]

2.2 *APHA Standard*:

Standard Methods for the Examination of Water and Waste Water [5]

3. Terminology

3.1 *Definitions*—For definitions of terms used in these test methods, refer to Terminology D 1129.

4. Significance and Use

4.1 Nitrogen is a nutrient in the environment and is necessary to sustain growth of most organisms. It exists in several forms such as nitrate, nitrite, organic nitrogen such as proteins or amino acids, and ammonia.

4.2 Ammonia is a colorless, gaseous compound with a sharp distinctive odor. It is highly soluble in water where it exists in a molecular form associated with water and in an ionized form as NH_4^+. The extent of association or ionization is dependent on the temperature and pH. It may also be toxic to aquatic life. The extent of toxicity is dependent upon species and extent of dissociation.[6] Ammonia may occur in water as a product of anaerobic decomposition of nitrogen containing compounds or from waste streams containing ammonia.

5. Purity of Reagents

5.1 Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such

[1] These test methods are under the jurisdiction of ASTM Committee D-19 on Water and are the direct responsibility of Subcommittee D19.05 on Inorganic Constituents in Water.

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[2] *Annual Book of ASTM Standards*, Vol 11.01.

[3] *Annual Book of ASTM Standards*, Vol 03.05.

[4] *Annual Book of ASTM Standards*, Vol 03.06.

[5] Available from American Public Health Association, 1015 15th St. N.W., Washington, DC 20005.

[6] *Quality Criteria for Water*, USEPA-440/9-76-023, July 26, 1976, pp. 16–24.

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specifications are available.[7] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

5.2 Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type I. In addition, this water shall be free of ammonia nitrogen. Such water is best prepared by the passage of distilled water through an ion-exchange resin. These resins should also be selected so that organic compounds which might subsequently interfere with the ammonia determination will be removed. Regeneration of the ion-exchange materials should be carried out in accordance with the instructions of the manufacturer.

6. Sampling

6.1 Collect the sample in accordance with Practice D 1066, Specification D 1192, and Practices D 3370, as applicable.

6.2 Preserve the samples by the addition of 1 mL of concentrated sulfuric acid per litre and store at 4°C. The pH should be 2.0 or less. Analyze the samples within 24 h of sampling. Do not use mercuric chloride as a preservative.

TEST METHOD A—DIRECT NESSLERIZATION

7. Scope

7.1 This test method is suitable for the rapid routine determination of ammonia nitrogen in steam condensates and demineralized water. See Appendix X1 for the distillation test method.

8. Summary of Test Method

8.1 A sample aliquot is Nesslerized directly and the ammonia content determined colorimetrically.

9. Interferences

9.1 Glycine, urea, glutamic acid, cyanates, and acetamide hydrolyze very slowly in solution on standing, but, of these, only urea and cyanates will hydrolyze on distillation at a pH of 9.5. Glycine, hydrazine, and some amines will react with Nessler's reagent to give the characteristic yellow color in the time required for the test. Similarly, volatile alkaline compounds such as hydrazine and the amines will influence titrimetric results. Some organic compounds such as ketones, aldehydes, alcohols, and some amines may cause an off color on Nesslerization. Some of these, such as formaldehyde may be eliminated by boiling off at a low pH prior to Nesslerization. Residual chlorine must be removed prior to the ammonia determination by pretreatment of the sample.

9.2 Turbid samples may be clarified with $ZnSO_4$ and NaOH solution; the precipitated $Zn(OH)_2$ is filtered off, discarding the first 25 mL of filtrate, and the ammonia is determined on an aliquot of the remaining clear filtrate by direct Nesslerization. Ammonia can be lost in basic conditions. Check procedure with a standard solution.

10. Apparatus

10.1 *Nessler Tubes*— Matched Nessler tubes[5] about 300 mm long, 17-mm inside diameter, and marked for 50 mL at 225± 1.5 mm from inside the bottom.

10.2 *Photometer*—Filter photometer or spectrophotometer suitable for absorbance measurements at 425 nm. Filter photometers and photometric practices used in this test method shall conform to Practice E 60. Spectrophotometers shall conform to Practice E 275.

10.3 *Stoppers*—Rubber, size No. 2, to fit Nessler tubes. These stoppers shall be boiled in H_2SO_4(1 + 99), rinsed, boiled in NaOH solution (1 g/L), rinsed, allowed to stand in dilute Nessler reagent for 30 min, and then rinsed again.

11. Reagents

11.1 *Ammonia Nitrogen Solution, Standard* (1 mL = 0.01 mg N)—Dry reagent grade ammonium sulfate ($(NH_4)_2SO_4$) for 1 h at 100°C. Accurately weigh 4.718 g and dissolve in water. Dilute to 1 L in a volumetric flask. Pipet 10 mL of this stock solution to a 1-L volumetric flask and dilute to volume with water.

11.2 *Disodium Dihydrogen Ethylenediamine Tetraacetate Solution* (500 g/L)—Dissolve 500 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate in water containing 100 g of NaOH. Gently heat to complete dissolution. Cool and dilute to 1 L.

11.3 *Nessler Reagent*— Dissolve 100 g of anhydrous mercuric iodide (HgI_2) and 70 g of anhydrous potassium iodide (KI) in a small volume of water. Add this mixture slowly, with stirring, to a cooled solution of 160 g of sodium hydroxide (NaOH) in 500 mL of water. Dilute the mixture to 1 L. Store the solution in the dark for five days and filter twice, either through a fritted glass crucible or glass fiber filter before using. If this reagent is stored in a chemically resistant bottle out of direct sunlight, it will remain stable up to a period of 1 year.

NOTE 1—This reagent should give the characteristic color with ammonia within 10 min after addition, and should not produce a precipitate with small amounts of ammonia (0.04 mg in a 50-mL volume). The solution may be used without 5-day storage if it is filtered through a 0.45 μm membrane (previously rinsed with reagent water Type I (see Specification D 1193)) shortly before use.

NOTE 2—Mercury and its salts are hazardous materials. They should be stored, handled and dispensed accordingly. Disposal of solutions must be made by legally acceptable means.

11.4 *Sodium Hydroxide Solution* (240 g/L)—Dissolve 240 g of NaOH in water and dilute to 1 L.

11.5 *Sodium Potassium Tartrate Solution* (300 g/L)—Dissolve 300 g of sodium-potassium tartrate tetrahydrate in 1 L of water. Boil until ammonia-free and dilute to 1 L.

11.6 *Zinc Sulfate Solution* (100 g/L)—Dissolve 100 g of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) in water and dilute to 1 L.

12. Calibration

12.1 Prepare a series of standards containing the following volumes of standard ammonia nitrogen solution diluted to 50

[7] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

D 1426

mL with water: 0.0, 1.0, 3.0, 5.0, 8.0, and 10.0 mL. Mix, add 1 mL of Nessler reagent, and remix. After 20 to 30 min, using a photometer suitable for absorbance measurement at 425 nm and a compensatory blank (Nesslerized ammonia-free water), prepare a calibration curve based on a series of these standards.

12.2 If a visual comparison method is used, prepare a series of 14 Nessler tubes containing the following volumes of standard ammonia nitrogen solution diluted to 50 mL with water: 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.7, 2.0, 2.5, 3.0, 3.5, and 4.0 mL. Mix, add 1 mL of Nessler reagent, and remix.

13. Procedure

13.1 If the sample contains turbidity, add 1 mL of $ZnSO_4$ solution to a 100-mL aliquot and mix. Add NaOH solution with gentle mixing until the pH is about 10.5. Allow to settle and filter using a water-washed, moderately-retentive filter paper, discarding the first 25 mL of the filtrate. Dilute a portion of the filtrate or clear sample, containing not more than 0.1 mg of ammonia nitrogen, to 50 mL in a Nessler tube. Add 2 drops of sodium potassium tartrate solution (or disodium dihydrogen ethylenediamine tetraacetate) to prevent cloudy tubes, and mix. Add 1 mL of Nessler solution and measure photometrically at a wavelength of 425 nm.

13.2 If a visual comparison method is used, select a volume containing not more than 0.04 mg of ammonia nitrogen and dilute to 50 mL. Mix, add 1 mL of Nessler reagent, and remix. Compare the color developed after 10 min with the previously prepared standards. If the ammonia nitrogen concentration is below 0.008 mg (in the 50-mL tube) compare after 30 min.

14. Calculation

14.1 Calculate the ammonia concentration in mg/L of nitrogen in the original sample, using Eq 1:

$$\text{Ammonia nitrogen, mg/L} = [(A \times 1000)/S] \quad (1)$$

where:
A = ammonia nitrogen observed, mg, and
S = sample, mL.

14.2 Calculate the ammonia concentration in mg/L of ammonia in the original sample, using Eq 2:

$$\text{Ammonia, mg/L} = E \times 1.22 \quad (2)$$

where:
E = ammonia nitrogen, mg/L.

15. Precision and Bias [8]

15.1 The precision of this test method was measured without the use of any distillation procedure by nine laboratories in reagent water only at four levels in the range from 30 to 100 mg NH_3-N/L, and each concentration was done in triplicate. The test method was tested in reagent water because steam condensates and demineralized effluents are similar to reagent water.

15.2 Analysts using Test Method A in any matrix other than a steam condensate or demineralized effluent must show the applicability of this test method to that matrix.

15.3 The precision of Test Method A in reagent water was 0.04 mg/L at 1.0 mg NH_3-N/L. Other precision data are shown in Table 1.

15.4 *Bias*—Recoveries of known amounts of ammonia from reagent water are shown in Table 1. No distillation procedure was used in this test.

TEST METHOD B—ION SELECTIVE ELECTRODE

16. Scope

16.1 This test method is applicable to the measurement of ammonia in reagent and effluent water.

17. Summary of Test Method

17.1 The sample is made alkaline with sodium hydroxide to convert ammonium ion to ammonia. The ammonia thus formed diffuses through a gas-permeable membrane of an ion selective electrode (ISE) and alters the pH of its internal solution which, in turn, is sensed by a pH electrode. The potential is measured by means of a pH meter or an ISE meter. If the pH meter is used, the ammonia content is determined from a calibration curve; if the ISE meter is used, the ammonia content is read directly from the meter.

18. Interferences

18.1 Volatile amines are positive interferences.

18.2 Mercury, if present, forms ammonia complexes, thus causing negative interference.

18.3 Organic compounds that form ammonia readily (within 5 min) under alkaline conditions are a positive interference. In general, this should not be a problem because the interfering concentrations may have to be greater than 100 mg/L. Among the inorganic compounds, hydrazine sulfate has yielded a reading of 0.2 mg/L of NH_3 as N when its concentration was 100 mg/L as N.

19. Apparatus

19.1 *Electrode*, gas-sensing, ammonia, incorporating an internal reference electrode and a diffusion-type membrane.

19.2 *Meter*, one of the following:

19.2.1 *pH Meter*, digital or expanded millivolt scale, accurate to ±0.1 mV.

19.2.2 *ISE Meter*, with direct-reading concentration scale.

19.3 *Electrode Holder*, for mounting the electrode at 20° to the vertical.

19.4 *Stirrer*, magnetic, with TFE-fluorocarbon-coated stirring bars.

19.5 *Heat Barrier*, 6-mm thick cork board placed underneath the beaker to insulate the sample solution from heat generated by the magnetic stirrer.

TABLE 1 Determination of Precision and Bias for Test Method A—Direct Nesslerization Method (Photometric at 425 nm)

Amount Added, mg/L	Matrix Water	Mean Recovery, %	Precision, mg/L		Bias, %
			S_t	S_o	
0.120	Reagent	89	0.011	0.003	−10.8
0.200	Reagent	98	0.013	0.002	−2.5
0.350	Reagent	98	0.021	0.002	−1.7
1.000	Reagent	101	0.042	0.014	+1.4

[8] Supporting data are available from ASTM Headquarters. Request RR:D19-1015.

20. Reagents

20.1 *Ammonia, Solution, Stock* (1000 mg NH_3 as N/L)—Dry reagent-grade ammonium sulfate ($(NH_4)_2SO_4$) for 1 h at 100°C. Accurately weigh 4.718 g and dissolve in water in a 1-L volumetric flask. Dilute to volume with water. This solution is stable for at least three months.

20.2 *Ammonia, Solution, Intermediate* (100 mg NH_3 as N/L)—Pipet 100 mL of the 1000-mg/L standard solution to a 1-L volumetric flask and dilute to volume with water. This solution is stable for one month.

20.3 *Ammonia, Solution, Working* (10, 1, and 0.1 mg NH_3 as N/L)— Quantitatively transfer 100, 10, and 1 mL of the 100-mg/L standard solution into separate 1-L volumetric flasks. Dilute each to volume with water. Prepare these solutions daily before use.

20.4 *Ammonium Chloride Solution* (5.4 g/L)—Dissolve 5.4 g of ammonium chloride (NH_4Cl) in water and dilute to 1 L. This solution is used only for soaking the electrode.

20.5 *Sodium Hydroxide Solution* (400 g/L)—Dissolve 400 g of sodium hydroxide (NaOH) in water. Cool and dilute to 1 L.

21. Calibration

21.1 *pH Meter*—Refer to the manufacturer's instruction manual for proper operation of the pH meter. Prepare calibration curves using a minimum of three standard solutions (see 20.3), bracketing the expected concentrations of the samples.

21.1.1 Treat the standards as directed in 22.1 and measure the potential of each standard and record in millivolts. The standards and the sample must be at the same temperature, preferably about 25°C.

21.1.2 Using semilogarithmic graph paper, plot the concentration of ammonia nitrogen in milligrams per litre on the log axis against the corresponding electrode potential, in millivolts, on the linear axis.

21.1.3 Check the calibration curve every 3 h when analyzing a series of samples.

21.2 *ISE Meter*—Refer to the manufacturer's instruction manual for proper operation of the meter. Prepare calibration curves with three standard solutions (see 20.3), bracketing the expected concentrations of the samples.

21.2.1 Check the calibration curve every 3 h when analyzing a series of samples; otherwise, calibrate daily.

22. Procedure

22.1 *Sample Treatment*:

22.1.1 Transfer 100 mL of the sample (or an aliquot diluted to 100 mL) to a 150-mL beaker. The sample temperature must be the same as that of the standards used in calibration (see 21.1 and 21.2).

22.1.2 Add the stirring bar and mix on the magnetic stirrer. Do not mix so rapidly that air bubbles are drawn into the solution.

22.1.3 Immerse the electrode into the sample, positioning it at an angle 20° to the vertical, making sure that no air bubbles are trapped on the membrane of the electrode. All precautions recommended by the manufacturer should be observed to ensure accurate measurements.

22.1.4 Add 1.0 mL of NaOH solution (see 20.5) to the sample. The NaOH solution should be added just prior to measurement because ammonia may be lost to the atmosphere from a stirred alkaline solution.

22.1.5 Check the pH of the sample with pH paper. The pH must be greater than 11.0. If less than 11.0, add additional NaOH solution (see 20.5) in 0.1-mL increments until the pH of the solution exceeds 11.0.

22.1.6 When the electrode comes to equilibrium, measure the electrode potential of the ammonia nitrogen concentration as directed in 22.2 (see Note 3).

NOTE 3—The time required for the electrode to come to equilibrium is dependent on the ammonia content of the sample. For concentrations above 0.5 mg/L, the response time is about 30 s.

22.2 *Sample Measurement*—Determine the ammonia nitrogen concentration by means of a pH meter or a specific-ion meter.

22.2.1 *pH Meter*—Record the observed potential in millivolts and convert to milligrams per litre of ammonia nitrogen by means of the calibration curve (see 21.1.2).

22.2.2 *ISE Meter*—Record the concentration reading directly from the logarithmic scale as milligrams of ammonia nitrogen per litre.

23. Calculation

23.1 Report the ammonia nitrogen content in milligrams per litre. If necessary calculate for dilution of original sample.

24. Precision and Bias [9]

24.1 The precision of this test method was tested without the use of any distillation procedure by twelve laboratories in reagent water and effluent waters at six levels in the range from 0.04 to 750 mg NH_3-N/L, and each concentration was done in triplicate.

24.2 Analysts using Test Method B in any matrix other than reagent water or effluent waters must show the applicability of this test method to that matrix.

24.3 The precision of Test Method B in reagent water was 0.11 mg/L at 0.8 mg NH_3-N/L and 0.3 mg/L at 0.8 mg NH_3-N/L in effluent waters. Other precision data are shown in Table 2.

[9] Supporting data are available from ASTM Headquarters. Request RR:D19-1052.

TABLE 2 Precision and Bias of Test Method B—Ion Selective Electrode

Amount Added, mg/L	Matrix Water	Mean Recovery,%	Precision, mg/L		Bias, %
			S_t	S_o	
0.04	Reagent	200	0.05	0.01	+ 100
	Effluent	100	0.03	0.00	0
0.10	Reagent	180	0.05	0.01	+ 80
	Effluent	470	0.61	0.01	+ 370
0.80	Reagent	105	0.11	0.04	+ 5
	Effluent	105	0.30	0.06	+ 5
20	Reagent	95	2	1	–5
	Effluent	95	3	2	–5
100	Reagent	98	5	2	–2
	Effluent	97	...	...	...
750	Reagent	97	78	12	–3
	Effluent	99	106	10	–1

24.4 *Bias*—Recoveries of known amounts of ammonia, without the use of any distillation procedure, from both reagent water and effluent water were 95 % at 0.8 mg NH_3-N/L. Other recovery data are shown in Table 2.

25. Keywords

25.1 ammonia; analysis; calorimetric; electrode; water

APPENDIX

(Nonmandatory Information)

X1. DISTILLATION TEST METHOD

X1.1 Distillation Apparatus

X1.1.1 An all-glass still consisting of a 1- or 2-L flask, preferably double-necked to facilitate sample addition. The center neck is connected in series with a spray trap (Kjeldahl), a water-cooled condenser, and a long narrow delivery tube which extends nearly to the bottom of a suitable receiver marked at 300 or 350 mL. The outer neck carries a glass-stoppered funnel to facilitate sample addition. The outlet of this funnel shall extend below the liquid level in the flask. In the distillation of ammonia it is also permissible to use the regular Kjeldahl distillation apparatus. When using such apparatus, the 800-mL Kjeldahl flask shall be used.

X1.2 Reagents

X1.2.1 *Borate Buffer Solution*—Add 88 mL of a 4 g/L (see X1.2.5) NaOH solution to 500 mL of a 5.04-g/L sodium tetraborate ($Na_2B_4O_7$) solution and dilute to 1 L.

X1.2.2 *Boric Acid Solution* (20 g/L)—Dissolve 20 g of boric acid (H_3BO_3) in water and dilute to 1 L.

X1.2.3 *Dechlorinating Agent*—Dissolve 1.0 g of sodium arsenite ($NaAsO_2$) in ammonia-free water and dilute to 1 L. One millilitre of this solution will remove 1 mg/L of residual chlorine from the 500-mL sample.

X1.2.4 *Sodium Hydroxide Solution* (240 g/L)—Dissolve 240 g of NaOH in 1 L of water.

X1.2.5 *Sodium Hydroxide Solution* (4 g/L)—Dissolve 4 g of NaOH in 1 L of water.

X1.3 Procedure

X1.3.1 *Distillation*— Remove residual chlorine by adding the appropriate quantity of dechlorinating agent (see X1.2.3). To 500 mL of water add 25 mL of borate buffer and adjust the pH to 9.5 with 6 *N* NaOH solution (see X1.2.4) using a pH meter. Distill until two 50-mL portions of the distillate are shown to be ammonia-free. After the still has cooled, add sample containing not more than 0.4 mg of ammonia nitrogen and water to attain a final volume of about 550 mL. Distill 300 mL at a rate of 6 to 10 mL/min into 50 mL of H_3BO_3 solution (see X1.2.2). Remove the receiver and mix. Collect an additional 50 mL to check for complete ammonia removal.

X1.4 Ammonia Determination

X1.4.1 The distillation procedure (see X1.3) should be followed by the use of either Test Method A or B.

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Designation: D 3867 – 99

Standard Test Methods for Nitrite-Nitrate in Water[1]

This standard is issued under the fixed designation D 3867; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope *

1.1 These test methods cover the determination of nitrite nitrogen, nitrate nitrogen, and combined nitrite-nitrate nitrogen in water and wastewater in the range from 0.05 to 1.0 mg/L nitrogen. Two test methods[2] are given as follows:

	Sections
Test Method A—Automated Cadmium Reduction	9 to 16
Test Method B—Manual Cadmium Reduction	17 to 24

1.2 These test methods are applicable to surface, saline, waste, and ground waters. It is the user's responsibility to ensure the validity of these test methods for waters of untested matrices.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For specific hazard statements, see Note 1 and Note 2.

2. Referenced Documents

2.1 *ASTM Standards:*

D 992 Test Method for Nitrate Ion in Water[3]
D 1129 Terminology Relating to Water[4]
D 1141 Specification for Substitute Ocean Water[5]
D 1192 Specification for Equipment for Sampling Water and Steam in Closed Conduits[4]
D 1193 Specification for Reagent Water[4]
D 1254 Test Method for Nitrite Ion in Water[6]
D 2777 Practice for Determination of Precision and Bias of Applicable Methods of Committee D-19 on Water[4]
D 3370 Practices for Sampling Water from Closed Conduits[4]
E 60 Practices for Photometric and Spectrometric Methods for Chemical Analysis of Metals[7]
E 275 Practice for Describing and Measuring Performance of Ultraviolet, Visible, and Near Infrared Spectrophotometers[8]

3. Terminology

3.1 *Definitions:* For definitions of terms used in these test methods, refer to Terminology D 1129.

4. Summary of Test Methods

4.1 A filtered sample is passed through a column containing copper-coated cadmium granules to reduce nitrate ion to nitrite ion. The combined nitrite-nitrate nitrogen is determined by diazotizing the total nitrite ion with sulfanilamide and coupling with *N*-(1-naphthyl)ethylenediamine dihydrochloride to form a highly colored azo dye that is measured spectrophotometrically.

4.2 The nitrite ion originally present in the sample can be determined separately by carrying out the procedure and omitting the cadmium reduction step.

4.3 The nitrate ion can be calculated as the difference between the combined nitrite-nitrate nitrogen and the nitrite nitrogen.

5. Significance and Use

5.1 Both test methods use identical reagents and sample processing. The only difference between the two methods is that one test method is automated and the other is manual. The ranges and interferences are identical.

5.2 The automated test method is preferred when large numbers of samples are to be analyzed. The manual test method is used for fewer samples or when automated instrumentation is not available.

5.3 These test methods replace Test Methods D 1254 (Nitrite) and D 992 (Nitrate). The nitrite test method (Test Method D 1254) used a reagent which is considered to be a potential carcinogen. The nitrate test method (Test Method D 992) has been shown to have relatively large errors when used in wastewaters and also has greater manipulative difficulties than the test method described herein.

[1] These test methods are under the jurisdiction of ASTM Committee D-19 on Water and are the responsibility of Subcommittee D19.05 on Inorganic Constituents in Water.

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[2] Methods similar to these appear in *Methods of Chemical Analysis of Water and Wastes*, 2nd edition, U.S. Environmental Protection Agency.

[3] Discontinued; see 1983 *Annual Book of ASTM Standards*, Vol 11.01.

[4] *Annual Book of ASTM Standards*, Vol 11.01.

[5] *Annual Book of ASTM Standards*, Vol 11.02.

[6] Discontinued; see 1980 *Annual Book of ASTM Standards*, Part 31.

[7] *Annual Book of ASTM Standards*, Vol 03.05.

[8] *Annual Book of ASTM Standards*, Vol 03.06.

***A Summary of Changes section appears at the end of this standard.**

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6. Interferences

6.1 Turbid samples must be filtered prior to analysis to eliminate particulate interference. Furthermore, sample turbidity results in a buildup on the reduction column that restricts sample flow.

6.2 Sample color that absorbs at wavelengths between 520 and 540 nm interferes with the absorbance measurements. When color is suspect, analyze a sample blank, omitting the *N*-(1-naphthyl)ethylenediamine dihydrochloride from the color reagent.

6.3 Oil and grease in the sample coat the surface of the cadmium and prevent complete reduction of nitrate to nitrite. This interference is usually removed by filtration prior to analysis. If filtration is not adequate, the interference can be removed by preextracting the sample with an *n*-hexane or a solid phase extraction (SPE) filter.

6.4 Certain metal ions, in concentrations above 35 mg/L, may cause an interference. For example, Hg (II) and Cu (II) may form colored complex ions having absorption bands in the region of color measurement. Iron and manganese are other reported examples of interference.

6.5 Excessive amounts of chlorine will deactivate the reducing column. Chlorine might be present in some Type II water. The use of chlorine-containing Type II water will lead to a negative interference because nitrite and chlorine do not normally coexist. This is of particular importance when preparing standards or spiked samples.

6.6 In acid samples (pH less than 4.5) nitrate is not reduced in the cadmium column. To overcome this interference, the sample must be neutralized to a pH of between 6 and 8 prior to analysis.

7. Purity of Reagents

7.1 Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, when such specifications are available.[9] Other grades may be used, provided it is first ascertained that the reagent is of sufficient high purity to permit its use without lessening the accuracy of the determination.

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type I. Other reagent water types may be used, provided it is first ascertained that the water is of sufficiently high purity to permit its use without adversely affecting the bias and precision of these test methods. Type II water was specified at the time of round-robin testing of these test methods.

8. Sampling and Sample Preservation

8.1 Collect the sample in accordance with Specification D 1192 and Practices D 3370, as applicable.

8.2 When nitrite ion is to be determined separately, analyze as soon as possible after sampling. Even when sterile bottles are used, bacteria naturally present in the water may cause conversion of all or part of nitrite ion to other forms such as nitrate or ammonia. Ammonia and natural amines, which are frequently present in natural waters, may react with nitrites to form nitrogen. If samples are to be stored for 24 h or less, preserve the sample by refrigeration at 4°C. If the sample must be stored for more than 24 h, preserve it by the addition of 2 mL of chloroform per litre (11.8 and 11.9) in addition to refrigeration at 4°C.

NOTE 1—**WARNING:** Chloroform is toxic and is a suspected human carcinogen. Use with adequate ventilation or in a fume hood. Wear prescribed protective equipment. Use of chloroform is discouraged, since its use renders the solution a hazardous waste.

NOTE 2—**CAUTION:** The common prescribed use of sulfuric acid or mercury compounds as preservatives is discouraged. Sulfuric acid does not necessarily inhibit oxidation and mercury compounds should be avoided to prevent environmental pollution. Mercuric chloride is known to deactivate the column.

TEST METHOD A—AUTOMATED CADMIUM REDUCTION

9. Scope

9.1 The applicable range of this test method is from 0.05 to 1 mg/L of nitrite or nitrate nitrogen. The range may be extended upward by dilution of an appropriate aliquot. Many workers have found that this test method is reliable for nitrite and combined nitrite-nitrate levels to 0.01 mg N/L. However, the precision and bias data presented in this test method are insufficient to justify application of this test method in the 0.01 to 0.05 mg/L-N range.

9.2 This test method is applicable to surface, saline, waste, and ground waters. It is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

10. Apparatus

10.1 *Automated Analysis System* [10] consisting of:

10.1.1 *Sampler.*

10.1.2 *Manifold or Analytical Cartridge.*

10.1.3 *Colorimeter* equipped with a 15- or 50-mm tubular flow cell and 540 ± 10-nm filters.

10.1.4 *Recorder or Electronic Data Acquisition Device.*

10.1.5 *Digital Printer (Optional).*

10.1.6 *Continuous Filter (Optional).*

10.2 *Reduction Columns*—Choose the appropriate reduction column for the manifold system. A schematic drawing of the manifold system is shown in Fig. 1 and the cartridge system is shown in Fig. 2.

10.2.1 *Reduction Column,* a glass tube 8 by 50 mm with the ends reduced in diameter to permit insertion into the system (see Fig. 1).

10.2.2 *Reduction Column,* a U-shaped glass tubing, 350-mm length and 2-mm inside diameter.

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[9] "Reagent Chemicals, American Chemical Society Specifications," American Chemical Society, Washington, D.C. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K. and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

[10] The apparatus described is commercially available. ASTM does not undertake to ensure anyone utilizing an automated analysis system against liability of infringement of patent or assume such liability.

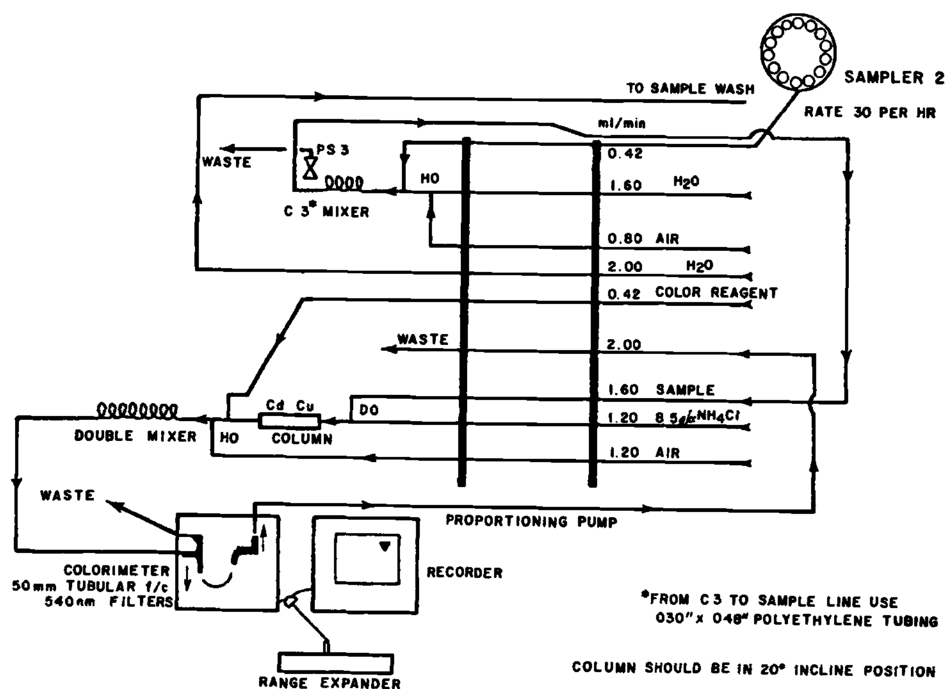

FIG. 1 Nitrite-Nitrate Manifold

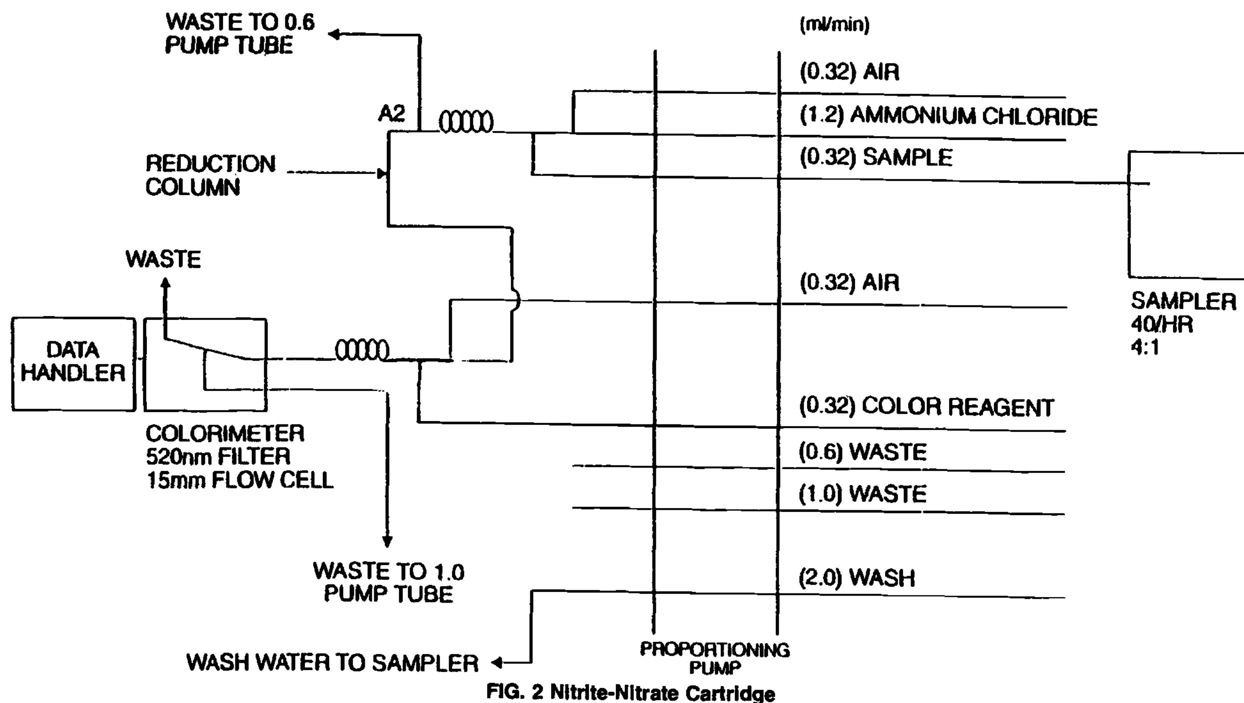

FIG. 2 Nitrite-Nitrate Cartridge

NOTE 3—A pump tube with 0.081-in. (2.1-mm) inside diameter can be used in place of the 2-mm glass tube.

11. Reagents

11.1 *Ammonium Chloride Solution* (85 g/L)—Dissolve 85 g of ammonium chloride (NH_4Cl) in water and dilute to 1 L. Add 0.5 mL wetting agent.[11]

11.2 *Cadmium*, 40 to 60 mesh, granulated.[12]

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[11] A 30 % aqueous solution of Brij® 35, a polyoxyethylene compound with dodecyl alcohol (sp gr 1.18 to 1.22) has been found satisfactory for this purpose.

[12] Different sizes of granulated cadmium may be used. The analyst should ensure that adequate reduction occurs with the size chosen.

11.3 *Color Reagent*— Add the following to 800 mL of water, while stirring constantly: 100 mL of concentrated phosphoric acid (H_3PO_4), 10 g of sulfanilamide, and 0.5 g of *N*-1-(naphthyl)ethylenediamine dihydrochloride. Stir until dissolved. Add 1 mL of wetting agent,[11] and dilute to 1 L with water. This solution is stable for about a month when stored in a brown bottle in a dark cool place.

11.4 *Copper Sulfate Solution* (20 g/L)—Dissolve 20 g of copper sulfate pentahydrate ($CuSO_4{\cdot}5\ H_2O$) in 500 mL of water. Dilute to 1 L.

11.5 *n-Hexane.*

11.6 *Hydrochloric Acid* (1 + 1)—Slowly add 50 mL of concentrated hydrochloric acid (HCl) to 40 to 45 mL of water and dilute to 100 mL.

11.7 *Nitrate Solution, Stock* (1.0 mL = 1.0 mg NO_3-N)—Dry potassium nitrate (KNO_3) in an oven at 105°C for 24 h. Dissolve 7.218 g in water in a 1-L volumetric flask. Dilute to the mark with water. This solution is stable for up to 1 month with refrigeration. If longer stability is required or refrigeration is not available, add 2 mL of chloroform as a preservative and store in a dark bottle. This solution is stable for 6 months. (See Note 1.)

11.8 *Nitrate Solution, Standard* (1.0 mL = 0.01 mg NO_3-N)—Dilute 10 mL of stock nitrate solution (11.7) to 1 L with water and store in a dark bottle. Prepare fresh as needed.

11.9 *Nitrite Solution, Stock* (1.0 mL = 1.0 mg NO_2-N)—Place about 7 g of potassium nitrite (KNO_2) in a tared 125-mL beaker and dry for about 24 h to a constant weight in a desiccator containing a suitable dessicant. Adjust the weight of the dry potassium nitrite to 6.072 g. Add 50 mL of water to the beaker, stir until dissolved, and transfer quantitatively to a 1000-mL volumetric flask. Dilute to the mark with water store in a sterilized bottle under refrigeration. Prepare fresh as needed.

NOTE 4—Potassium nitrite is easily oxidized, so use only fresh bottles of this reagent.

11.10 *Nitrite Solution, Standard* (1.0 mL = 0.01 mg NO_2-N)—Dilute 10 mL of stock nitrite solution (11.9) to 1 L with water. This solution is unstable; prepare fresh as needed.

12. Preparation of Reduction Column

12.1 *Cadmium Granules Treatment*—Clean and copperize new or used cadmium granules in the following manner:

12.1.1 Clean about 10 g of cadmium granules by washing with dilute HCl (11.6) and rinsing with water.

12.1.2 Swirl the clean cadmium in 100-mL portions of copper sulfate solution (11.4) in a beaker for 5 min or until the blue color partially fades. Decant and repeat with fresh copper sulfate until the first visible brown colloidal precipitate appears.

12.1.3 Wash the granules with water at least 10 times to remove all of the precipitated copper.

12.2 *Filling the Reduction Column*:

12.2.1 Insert a small plug of glass wool in one end of the column (10.2).

12.2.2 Fill the column with water to prevent the entrapment of air bubbles during the filling operation.

12.2.3 Fill the column with copper-cadmium granules, tap to pack the granules, and plug the open end with glass wool.

12.3 *Installation of Reduction Column*—Install the copper-cadmium reduction column in the automatic analyzer system. Purge the system with ammonium chloride solution (11.1) using water in the sample line. Observe the following precautions while installing the reduction column:

12.3.1 Place the column in the manifold system in an upflow 20° incline to minimize channeling (see Fig. 1).

12.3.2 Fill all pump tubes with reagents before inserting the column in the cartridge system to prevent the entrapment of air bubbles.

12.4 *Reduction Column Storage*—When it is not in use, put the sample line in water and purge the column with ammonium chloride solution and water.

NOTE 5—Do not allow air to enter the column and do not let the cadmium granules become dry. If this occurs, refill the column with freshly treated cadmium granules.

13. Calibration

13.1 Using the standard nitrate solution (11.8) prepare calibration standards by pipetting specified volumes of the standard solution into 100-mL volumetric flasks and diluting to the mark with water. Table 1 specifies the millilitres of standard solution required.

13.2 Prepare at least one calibration standard from the standard nitrite solution at the same concentration as one of the nitrate standards to verify the efficiency of the reduction column. Repeat this when a suspected loss in NO_3-N reduction is observed.

NOTE 6—When the sample to be analyzed is a saline water, use substitute ocean water (SOW) to prepare the standards (Specification D 1141). Run a reagent water blank in addition to a SOW blank because the reagents used to prepare SOW frequently contain nitrite or nitrate, or both. Adjust this curve for the contaminant level in SOW.

13.3 Develop the color and determine the absorbance of each standard as directed in the procedure (14.5).

13.4 Prepare a standard curve by plotting the peak heights of each processed calibration standard against its known concentrations.

14. Procedure

14.1 *Removal of Interferences*—Remove interferences (Section 6) by the following procedures:

14.1.1 For turbidity removal, when suspended solids are present, filter the sample through a glass-fiber filter or a 0.45-µm filter. Alternatively, use a continuous filter (10.1.6) as an integral part of the system to remove particulate matter. Centrifugation can be used as an option.

TABLE 1 Concentration of Calibration Standards, Automated Cadmium Reduction

NO_3-N or NO_2-N, mg/L	mL Standard Solution/100 mL
0.01	0.1
0.02	0.2
0.04	0.4
0.1	1.0
0.2	2.0
0.4	4.0
0.7	7.0
1.0	10.0

14.1.2 For oil and grease removal, if necessary after filtration, adjust the pH of the sample to 2 with concentrated HCl. Extract with two 25-mL portions of *n*-hexane (11.5) in a separatory funnel. Discard the *n*-hexane layer after each extraction. Alternatively, solid-phase extraction filters may be used.

14.1.3 For pH adjustment, determine the pH of the sample with a pH meter. Adjust the pH to within the range from 6 to 8 with concentrated HCl or concentrated NH_4OH, if needed.

14.1.4 For correction for color interferences, if there is a possibility that the color of the sample might absorb in the photometric range from 530 ± 10 nm, determine the background absorbance. Replace the color reagent with a similar reagent where just the *N*-1-(naphthyl) ethylenediamine dihydrochloride is omitted and analyze the sample for background color absorbance as directed in the following procedure. Repeat the analysis using the complete color reagent.

14.2 Depending on the model of analysis system available, set up the manifold and complete the system as shown in Fig. 1 or Fig. 2.

NOTE 7—When determining nitrite alone, omit the reduction column from the manifold system.

14.3 Turn on the colorimeter and the recorder and allow them both to warm up for 30 min.

14.4 Obtain a stable baseline with all reagents, feeding water through the sample line.

14.5 Place the appropriate nitrate and nitrite calibration standards in the sampler in order of decreasing concentration of nitrogen. Fill the remainder of the sample tray with unknown samples.

14.6 For the manifold system, sample at a rate of 30/h, 1 + 1 cam. For the cartridge system, use a 40/h, 4 + 1 cam and a common wash.

14.7 Switch the sample line from water to sampler and begin the analysis, continuing until all unknowns have been analyzed.

15. Calculation

15.1 Determine the concentration of nitrate or nitrite nitrogen in the samples in milligrams per litre by comparing the peak heights of the samples with the standard curves (13.4) manually or by a computer-based data handler.

NOTE 8—If the background color absorbance has been measured (14.1.4), calculate the net absorbance by subtracting the background absorbance from the measured absorbance of the color developed sample. Use the net absorbance to determine the concentration of nitrogen in the sample.

15.2 Where separate values are required for nitrite-nitrogen and nitrate-nitrogen, calculate the nitrate-nitrogen by subtracting the nitrite-nitrogen from the total nitrate-nitrite nitrogen content.

16. Report

16.1 Report the following information:

16.1.1 Report the nitrogen content in milligrams per litre as:

16.1.1.1 Nitrite-Nitrogen (NO_2-N), mg/L,

16.1.1.2 Nitrate-Nitrogen (NO_3-N), mg/L, and

16.1.1.3 Combined Nitrate-Nitrite Nitrogen (NO_3, NO_2-N), mg/L.

17. Precision and Bias [13]

17.1 *Precision Statement:*

17.1.1 *Nitrite*—Based on the results of six operators in five laboratories, the overall and single-operator precision of this test method for nitrite within its designated range for reagent water and selected water matrices (including surface, saline, waste, and ground waters) varies with the quantity being tested in accordance with Table 2. No data were rejected as outliers for this statistical evaluation.

17.1.2 *Nitrate*—The precision of this test method for nitrate within its designated range for reagent water and selected water matrices may be expressed as follows (concentrations are given in mg/L):

Reagent Water	$S_T = 0.0400$
	$S_O = 0.0296$
Water Matrix	$S_T = 0.0437$
	$S_O = 0.0300$

17.2 This section on precision and bias conforms to Practice D 2777 – 77, which was in place at the time of collaborative testing. Under the allowances made in 1.5 of Practice D 2777 – 86, these precision and bias data do meet existing requirements for interlaboratory studies of Committee D-19 test methods.

17.3 *Bias Statement*— Recoveries of known amounts of nitrites-nitrates from reagent water and selected water matrices are shown in Table 3.

17.4 It is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

TEST METHOD B—MANUAL CADMIUM REDUCTION

18. Scope

18.1 The applicable range of this test method is from 0.05 to 1 mg/L of nitrite or nitrate nitrogen. The range may be extended upward by dilution of an appropriate aliquot. Many workers have found that this test method is reliable for nitrite and combined nitrite-nitrate levels to 0.01 mg N/L. However, the precision and accuracy data presented in this test method are insufficient to justify application of this test method in the 0.01 to 0.05 mg/L-N range.

18.2 This test method is applicable to surface, saline, waste, and ground waters. It is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

19. Apparatus

19.1 *Reduction Column* as shown in Fig. 3. The column

[13] Precision and bias data are available from ASTM Headquarters. Request Research Report RR:D19-1058.

TABLE 2 Interlaboratory Precision for Nitrite Found in Selected Matrices

Water Matrix:				
Concentration (x), mg/L	0.05	0.09	0.42	0.80
S_T	0.024	0.006	0.033	0.049
S_O	0.012	0.005	0.029	0.043
Reagent Water:				
Concentration (x), mg/L	0.05	0.09	0.42	0.80
S_T	0.021	0.005	0.019	0.032
S_O	0.009	0.002	0.011	0.006

TABLE 3 Reagent Water, Automated Cadmium Reduction

Radical	Amount Added, mg/L	Amount Found, mg/L	Bias, %	Statistically Significant 95 % Level
Nitrite-nitrogen	0.050	0.042	−16	no
	0.090	0.096	+ 6	yes
	0.420	0.416	−1	no
	0.800	0.798	0	no
Nitrate-nitrogen	0.050	0.044	−11	no
	0.090	0.092	+ 2	no
	0.420	0.404	−4	yes
	0.850	0.828	−3	no
		Water Matrix		
Radical	Amount Added, mg/L	Amount Found, mg/L	Bias, %	Statistically Significant 95 % Level
Nitrite-nitrogen	0.050	0.060	+ 20	no
	0.090	0.097	+ 8	yes
	0.420	0.427	+ 2	no
	0.800	0.790	−1	no
Nitrate-nitrogen	0.050	0.053	+ 6	no
	0.090	0.081	−10	no
	0.420	0.396	−6	yes
	0.850	0.828	−3	no

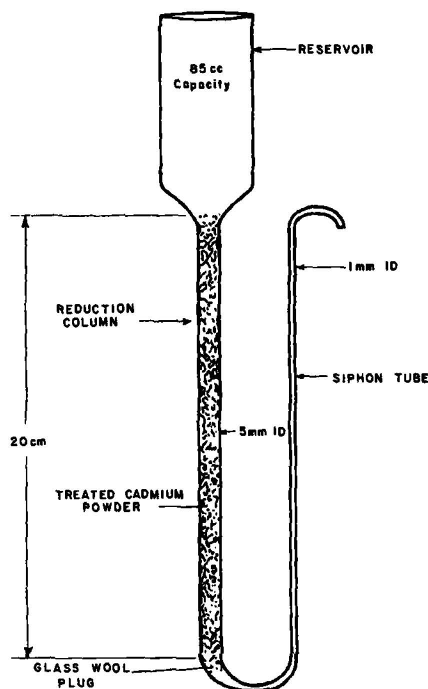

FIG. 3 Cadmium Reduction Column

shall be laboratory fabricated from the following:

19.1.1 *Pipet*, volumetric, 100-mL capacity, with 200-mm delivery stem. The top of the bulb shall be cut off before the constriction for the entry stem and the delivery tip curved to form a shallow hook.

19.1.2 *Tubing*, glass or vinyl, 1-mm inside diameter (1⁄16in.) and about 350-mm (14 in.) in length.

NOTE 9—If glass tubing is used, it must be bent at the lower end to connect with the curved tip of the pipet and parallel to the pipet. Its upper end is then bent over to form an inverted U-siphon. This last bend should be level with the top of the lower stem of the pipet. With this arrangement, liquid placed in the reservoir flows out of the system and stops when the level of the liquid just covers the cadmium powder packing.

19.2 *Cylinders*, graduated, 50-mL capacity.

19.3 *Test Tubes*, 15-mL capacity.

19.4 *Photometer*—A spectrophotometer or filter photometer suitable for use at 543 nm and equipped with absorption cells providing a light-path length of 1 cm. Spectrophotometers shall conform to Practice E 275. Filter photometers and photometric practices prescribed in this method shall conform to Practice E 60.

19.5 *Pipets*, serological, 1 and 10-mL capacity, calibrated to 0.1 mL, to deliver with ejection.

20. Reagents

20.1 *Ammonium Chloride Solution* (85 g/L)—Dissolve 85 g of ammonium chloride (NH_4Cl) in water and dilute to 1 L.

20.2 *Cadmium*, 40 to 60 mesh granulated.[12]

20.3 *Color Reagent*— Add the following to 800 mL of water, while stirring constantly: 100 mL of concentrated phosphoric acid (H_3PO_4), 10 g of sulfanilamide, and 0.5 g of *N*-(1-naphthyl)ethylenediamine dihydrochloride. Stir until dissolved. Dilute to 1 L with water. This solution is stable for about a month when stored in a brown bottle in a dark cool place.

20.4 *Copper Sulfate Solution* (20 g/L)—See 11.4.

20.5 *n-Hexane*..

20.6 *Hydrochloric Acid* (1 + 1)—See 11.6.

20.7 *Nitrate Solution, Stock* (1.0 mL = 1.0 mg NO_3-N)—See 11.7.

20.8 *Nitrate Solution, Standard* (1.0 mL = 0.01 mg NO_3-N)—See 11.8.

20.9 *Nitrite Solution, Stock* (1.0 mL = 1.0 mg NO_2-N)—See 11.9.

NOTE 10—Potassium nitrite is easily oxidized, and only fresh bottles of this reagent are to be used.

20.10 *Nitrite Solution, Standard* (1.0 mL = 0.01 mg NO_2-N)—See 11.10.

21. Preparation of Reduction Column

21.1 *Cadmium Granules Treatment*—Clean and copperize new or used cadmium granules in accordance with 12.1.

21.2 *Filling the Reduction Column*:

21.2.1 Insert a small plug of glass wool in the tip of the pipet.

21.2.2 Fill the column with water to prevent the entrapment of air bubbles during the filling operations.

21.2.3 Pour sufficient cadmium powder into the apparatus to produce a column 300 mm in length; tap to pack the powder.

21.2.4 Wash the column thoroughly with ammonium chloride solution (20.1).

NOTE 11—Use a flow rate no greater than 8 mL/min. If the rate is too fast, slow it down by constricting the end of the siphon outlet or by raising the height of the siphon tube. Flow rates of less than 5 mL/min unnecessarily increase the time for analysis and may cause low results.

21.3 *Storing the Column*—When not in use, cover the cadmium in the column with ammonium chloride solution. Do

not allow air to enter the packing, nor the packing to dry out. If this occurs, prepare another column.

22. Calibration

22.1 Using the nitrate standard solution (20.8) prepare calibration standards by pipetting specified volumes of the standard solution into 100-mL volumetric flasks and diluting to the mark with water. Table 4 specifies the millilitres of standard solution required.

22.2 Prepare at least one calibration standard from the nitrite standard solution (20.10) at the same concentration as one of the nitrate standards to verify the efficiency of the reduction column.

NOTE 12—When the sample to be analyzed is a saline water, use substitute ocean water (SOW) to prepare the standards (Specification D 1141). A reagent water blank should be run in addition to a SOW blank because the reagents used to prepare SOW frequently contain nitrite or nitrate, or both. Adjust this curve for the contaminant level in SOW.

22.3 Treat the standards as directed in 23.2.

22.4 Develop the color and measure the absorbance of each standard as directed in 23.3 for nitrite standards or in 23.4 for nitrate standards.

22.5 Prepare a standard curve by plotting the absorbance of each processed calibration standard against its known concentration.

NOTE 13—The nitrite standard will fall on the nitrate standard curve if the reduction of nitrate was complete. If the nitrite standard is 5 % higher, prepare a fresh reduction column and repeat the analysis of the nitrate standards. Analyze a nitrate standard every 4 hours of continuous testing. The reduction column will usually last for several weeks of continuous analysis.

23. Procedure

23.1 *Removal of Interferences*—Remove interferences (Section 6) by the following procedures:

23.1.1 For turbidity removal, when suspended solids are present, filter the sample through a glass fiber filter or 0.45-µm filter.

23.1.2 For oil and grease removal, adjust the pH of the sample to 2 with concentrated HCl. Extract with two 25-mL portions of trichloro-trifluoroethane (20.5) in a separatory funnel. Discard the lower trichlorotrifluoroethane layer after each extraction.

23.1.3 For pH adjustment, determine the pH of the sample with a pH meter. If the pH is less than 6 or greater than 8, adjust the pH to within the range from 6 to 8 with concentrated HCl or concentrated NH_4OH.

23.1.4 For correction for color interferences, if there is a possibility that the sample might absorb at 543 nm, determine the background absorbance. Dilute the sample as directed in 23.2. Replace the color reagent with a similar reagent where just the *N*-(1-naphthyl)-ethylenediamine dihydrochloride is omitted and follow the procedure as directed in 23.3. Using the complete color reagent, repeat the analysis as directed in 23.3 for nitrite determination or in 23.4 for combined nitrite-nitrate determination.

TABLE 4 Concentration of Calibration Standards, Manual Cadmium Reduction

NO_3-N or NO_2-N, mg/L	mL Standard Solution/100 mL
0.04	0.4
0.1	1
0.2	2
0.4	4
0.7	7
1.0	10

23.2 *Sample Treatment*— To a 20-mL sample add 80 mL of ammonium chloride solution (20.1). Mix well.

23.3 *Nitrite Determination*:

23.3.1 Pipet a 10-mL portion of the treated sample into a 15-mL test tube.

23.3.2 Pipet 3 mL of color reagent (20.3) into the test tube, mix, and let stand for 15 min.

23.3.3 Using water in the reference cell, determine the absorbance of the solution at 543 nm and record.

23.4 *Combined Nitrite-Nitrate Determination*:

23.4.1 Pour about 60 mL treated sample (23.2) into the reservoir of the reduction column on top of any liquid in the column.

NOTE 14—The cadmium in the reduction column will be covered by the ammonium chloride storage solution or by a previous sample because it should *never* be allowed to dry out.

23.4.2 Place a clean 50-mL graduate under the siphon outlet and allow approximately 30 mL of effluent to collect. Discard this initial 30 mL.

23.4.3 Place a clean 50-mL graduate under the siphon outlet and collect an additional 25 mL of effluent. Do not remove all the liquid from the column. Allow enough solution to remain in the column to completely cover the copper-cadmium granules.

23.4.4 Pipet a 10-mL portion of the collected effluent into a 15-mL test tube.

23.4.5 Pipet 3.0 mL of color reagent (20.3) into the test tube, mix, and let stand for 15 min.

23.4.6 Using water in the reference cell, determine the absorbance of the solution at 543 nm and record.

24. Calculation

24.1 From the standard curve (22.5) obtain the nitrogen content in mg/L which corresponds to the absorbance.

NOTE 15—If the background color absorbance has been measured (23.1.4), calculate the net absorbance by subtracting the background absorbance from the measured absorbance of the color developed sample. Use the net absorbance to determine the concentration of nitrogen in the sample.

24.2 Where separate values are required for nitrite-nitrogen and nitrate-nitrogen, calculate the nitrate-nitrogen by subtracting the nitrite-nitrogen (23.3) from the combined nitrite-nitrate nitrogen content (23.4).

25. Report

25.1 Report the following information:

25.1.1 Report the nitrogen content in milligrams per litre as:

25.1.1.1 Nitrite-Nitrogen (NO_2-N), mg/L,

25.1.1.2 Nitrate-Nitrogen (NO_3-N), mg/L, and

25.1.1.3 Combined Nitrite-Nitrate Nitrogen (NO_2, NO_3-N), mg/L.

D 3867

26. Precision and Bias [13]

26.1 Based on the results from eight operators from seven laboratories, the precision of this test method within its designated range for reagent water and water matrices selected (see 17.1.1) may be expressed as follows (concentrations are given in mg/L):

Reagent Water

Nitrite-Nitrogen	$S_T = 0.039\ X + 0.0108$
	$S_O = 0.010$
Nitrate-Nitrogen	$S_T = 0.033$
	$S_O = 0.014$

Water Matrix

Nitrite-Nitrogen	$S_T = 0.0901\ X + 0.035$
	$S_O = 0.0841\ X + 0.019$
Nitrate-Nitrogen	$S_T = 0.057$
	$S_O = 0.038$

26.2 *Bias Statement*—Recoveries of known amounts of nitrite and nitrate from reagent water and water matrices selected are shown in Table 5.

26.3 One set of nitrate results were rejected as outliers. The nitrite results from this laboratory were acceptable.

26.4 It is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

TABLE 5 Reagent Water, Manual Cadmium Reduction

Radical	Amount Added, mg/L	Amount Found, mg/L	Bias, %	Statistically Significant 95 % Level
Nitrite	0.05	0.05	0	no
	0.09	0.09	0	no
	0.42	0.42	0	no
	0.80	0.82	+ 2	yes
Nitrate	0.05	0.03	−40	yes
	0.09	0.08	−11	no
	0.42	0.43	+ 2	no
	0.85	0.81	−5	yes
		Water Matrix		
Radical	Amount Added, mg/L	Amount Found, mg/L	Bias, %	Statistically Significant 95 % Level
Nitrite	0.05	0.04	−20	no
	0.09	0.06	−33	yes
	0.42	0.36	−10	no
	0.80	0.75	−6	yes
Nitrate	0.05	0.04	−20	no
	0.09	0.10	−11	no
	0.42	0.41	−2	no
	0.85	0.79	−7	yes

27. Keywords

27.1 cadmium reduction method; groundwater; nitrate; nitrite; saline water; waste water; water

SUMMARY OF CHANGES

This section identifies the location of selected changes to these test methods that have been incorporated since the last issue. For the convenience of the user, Committee D-19 has highlighted those changes that may impact the use of these test methods. This section may also include descriptions of the changes or reasons for the changes, or both.

(1) Techniques for the removal of interferences due to oil and grease were revised.

(2) Techniques for preservation of samples were revised.

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Designation: D 4012 – 81(Reapproved 2002)

Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water[1]

This standard is issued under the fixed designation D 4012; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers the measurement of adenosine triphosphate (ATP) in microorganisms in concentrations normally found in laboratory cultures, waters, wastewaters, and in plankton and periphyton samples from waters.

1.2 Knowledge of the concentration of ATP can be related to *viable biomass* or metabolic activity, or by utilizing an average concentration (or amount) of ATP per cell, an estimated count of microorganisms can be obtained in the case of unispecies cultures.

1.3 This test method offers a high degree of sensitivity, rapidity, accuracy, and reproducibility. However, extreme care must be taken at each step in the analysis to ensure meaningful and reliable results.

1.4 The analyst should be aware that the precision statement pertains only to determinations in reagent water and not necessarily in the matrix being tested.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water[2]

D 1193 Specification for Reagent Water[2]

3. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminology D 1129.

4. Summary of Test Method

4.1 The biomass in the sample can be determined by direct ATP extraction when cell counts are greater than 20 000 microorganisms per millilitre. When the cell counts are less than 20 000 microorganisms per millilitre, the sample may be concentrated using either centrifugation or filtration prior to ATP extraction.

4.2 The ATP is extracted from the sample with boiling 0.02 *M* tris buffer.

4.3 A carefully measured aliquot of the ATP extract is mixed with a standard quantity of buffered luciferin-luciferase reaction mixture and the light produced in the resulting reaction is measured with an appropriate photometric analyzer.

4.4 The data obtained from the test can be expressed in terms of ATP content or biomass.

5. Significance and Use

5.1 A rapid and routine procedure for determining biomass of the living microorganisms in cultures, waters, wastewaters, and in plankton and periphyton samples taken from surface waters is frequently of vital importance. However, classical techniques such as direct microscope counts, turbidity, organic chemical analyses, cell tagging, and plate counts are expensive, time-consuming, or tend to underestimate total numbers. In addition, some of these methods do not distinguish between living and nonliving cells.

5.2 The ATP firefly (luciferin-luciferase) method is a rapid, sensitive determination of viable microbial biomass. ATP is the primary energy donor for life processes, does not exist in association with nonliving detrital material, and the amount of ATP per unit of biomass (expressed in weight) is relatively constant. (ATP per cell varies with species and physiological state of the organism.)

5.3 This test method can be used to:

5.3.1 Estimate viable microbial biomass in cultures, waters, and wastewaters.

5.3.2 Estimate the amount of total viable biomass in plankton and periphyton samples.

5.3.3 Estimate the number of viable cells in a unispecies culture if the ATP content (or if the average amount of ATP) per cell is known.

5.3.4 Estimate and differentiate between zooplanktonic, phytoplanktonic, bacterial, and fungal ATP through size fractionation of water, and wastewater samples.

5.3.5 Measure the mortality rate of microorganisms in toxicity tests in entrainment studies, and in other situations

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[1] This test method is under the jurisdiction of Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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[2] *Annual Book of ASTM Standards*, Vol 11.01.

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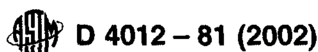


where populations or assemblages of microorganisms are placed under stress.

6. Interferences

6.1 Reagents must be of high purity so that background light emission is held to a minimum for the measurement of ATP.

6.2 ATP-free glassware, prepared by the procedure in 7.5, is required for the determination of ATP.

6.3 Luciferase is a protein and as such can be inhibited or denatured by the presence of heavy metals, high salt (NaCl) concentrations, and organic solvents, in the sample. The ATP luciferase reaction is also affected by certain phosphate buffers, inorganic salts, and by high magnesium concentrations.

6.4 Other energy-mediating compounds, such as adenosine diphosphate, cytidine-5-triphosphate, and inosine-5-triphosphate also react with luciferase to produce light, but as compared to ATP they are usually present only in small amounts and do not constitute a significant source of error.

6.5 High-viscosity samples may not mix adequately with the reagents upon injection. If this occurs, reaction rate may be reduced (reaction will go to completion, but the reaction rate will be decreased with improper mixing) or the results may not be reproducible.

7. Apparatus

7.1 *ATP Photometers or Liquid Scintillation Spectrometers*—may be used. The stability of the instrument should be checked before each use with a standard light source available from the manufacturer. It is advisable to maintain a record of the instrument response to permit detection of any unstability or changes in response levels.

7.2 *Vacuum Filtration System* (0.45-μm membrane filters).

7.3 *Precision Syringe*, 50-μL. A constant-rate injection attachment is recommended.

7.4 *Automatic Pipets and Disposable Tips*.

7.5 *ATP-Free Glassware*—Rinse chemically clean glassware three times with 0.2 *N* HCl, rinse three times with tris buffer (8.8), and rinse three times with low-response water (8.6).

7.6 *Reaction Vial*, 6 by 49-mm.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.[3] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall conform to Specification D 1193, Type II.

8.3 *ATP Standard Solution*—Weigh 119.3 mg of crystalline adenosine 5′-triphosphate-disodium salt using ATP-free glassware. Dissolve the ATP in 100 mL of fresh 0.02 *M* tris buffer containing 29.2 mg of EDTA (Na_2H_2 EDTA·$2H_2O$) and 120 mg of $MgSO_4$ (the resulting concentration is 1 mg of ATP/mL). The material may be dispensed in 1.0-mL aliquots and stored at – 20°C until required.

8.4 *Extraction Reagent*—ATP can be extracted from samples by various reagents and procedures. The most commonly used extracting reagent is boiling tris buffer (see 8.8).

8.5 *Hydrochloric Acid* (17 mL/L)—Add 17.0 mL of HCl (sp gr 1.19) to a 1-L volumetric flask and bring to volume with water.

8.6 *(LR) Water, Low-Response*—(Sterile ATP-free water may be prepared by treatment in a suitable system involving carbon treatment with deionization, filtration glass distillation, or sterilization by autoclaving and stored under refrigeration in stoppered flasks.

8.7 *Luciferase/Luciferin Reaction Mixture*—This material is commercially available and should be prepared in accordance with the supplier's instructions. Note the following when preparing this material:

8.7.1 Clean glassware must be used.

8.7.2 The luciferase/luciferin reaction mixture must be mixed gently without shaking.

8.8 *Tris Buffer* (0.02 *M*) *(Tris(Hydroxymethyl) Aminomethane)*—Dissolve 2.5 g of the buffer crystals in 1 L of deionized water. Bring to pH 7.75 using HCl (pH meter). Sterilize by autoclaving for 30 min at 121°C, 15 psi (103 kPa) pressure, and store refrigerated in stoppered flasks.

NOTE 1—Bacteria may live and multiply in the LR water and tris buffer; this can introduce an ATP interference. The quality of the LR water and tris buffer should be periodically tested.

9. Precaution

9.1 This standard may involve the use of hazardous materials, operations, and equipment. It is the responsibility of whoever uses this standard to establish appropriate safety practices and to determine the applicability of regulatory limitations prior to use.

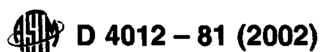
10. Collection

10.1 The sample sites should correspond as closely as possible to those selected for chemical, biological, and microbiological sampling, so that there is maximum correlation of results. The sample collection method will be determined by study objectives. To collect a sample, use a nonmetallic water sampling bottle. Extraction procedures should be performed immediately after collection. The sample may be stored 2 to 3 h if necessary if the temperature and lighting conditions are maintained; for example, do not place a warm sample from a well-lighted area into a cool, dark ice chest.

11. ATP Extraction Procedures

11.1 Accurate determinations of ATP require quantitative extraction of ATP from the sample. Separate the cells from any possible free (extracellular) ATP and other interfering materials by filtration, centrifugation, sample washing, etc. Omit the separation step if the sample is known to be free of soluble ATP or interfering material. After separation, lyse the cell wall to free ATP for subsequent analysis. Perform three replicate

[3] "Reagent Chemicals, American Chemical Society Specifications," American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."



(triplicate) analyses on each sample to ensure the efficiency, reliability, and reproducibility of the method employed.

11.2 *Procedure A—Boiling 0.02 M Tris Filtration Method*:

11.2.1 Filter 100 mL of sample through a 0.45-μm membrane filter.

11.2.2 Remove the filter as soon as the filtration is complete. Do not allow the filter to dry. Break the vacuum just as the last of the water passes through the filter and quickly transfer the filter and place in 5.0 mL of boiling 0.02 *M* tris buffer.

11.2.3 Heat for 5 to 10 min at 100°C in a water bath.

11.2.4 If the analysis is not to be performed immediately, the extracted sample may be stored at – 20°C for a period up to 6 months.

11.3 *Procedure B—Boiling 0.02 M Tris Buffer Without Filtration Method*:

11.3.1 Add 1.0 mL of sample to approximately 35 mL of 0.02 *M* tris buffer pH 7.75 in a 50-mL Erlenmeyer flask that has reached a temperature of at least 98°C in a boiling water bath.

11.3.2 Maintain boiling temperature for 2 to 4 min.

11.3.3 Cool to room temperature.

11.3.4 Analytically transfer to a 50-mL volumetric flask and bring up to volume with 0.02 *M* tris buffer. If the analysis is not to be performed immediately, the extracted sample may be stored at – 20°C for a period up to 6 months.

11.3.5 If marine water samples are extracted directly without filtrations, it is especially important to dilute the sample with 0.02 *M* tris buffer to avoid inhibition of the luminescence reaction by NaCl.

12. Standardization Curve

12.1 Pipet 1.0 mL of the standard ATP solution containing 1 mg of ATP/mL into a 1-L volumetric flask and bring up to volume with 0.02 *M* tris buffer. Call this Solution A. Solution A will contain 1.00 μg ATP/mL. Then make the following serial dilutions:

12.1.1 1.0 mL of Solution A + 9 mL of 0.02 *M* tris = Solution B, Solution B = 1.00×10^{-1} μg ATP/mL.

12.1.2 1.0 mL of Solution A + 99 mL 0.02 *M* tris = Solution C, Solution C = 1.00×10^{-2} μg ATP/mL.

12.1.3 1.0 mL of Solution C + 9 mL 0.02 *M* tris = Solution D, Solution D = 1.00×10^{-3} μg ATP/mL.

12.2 The above concentrations should be used when preparing a curve for normal laboratory samples. For oligotrophic waters additional dilutions are required. Standards can be prepared and frozen, then thawed as needed.

12.3 A minimum of three replicate determinations of each of the standard solutions (Solutions A, B, C, and D) should be used to prepare a calibration curve. These solutions should be chosen so that they contain concentrations of ATP at the lower end, upper end, and midway in the range of ATP concentrations that the analyst suspects (or knows) to be present in the samples to be analyzed.

12.4 Determine (triplicate measurements) the instrument response to the reagent blank, consisting of sterile extractant. Subtract the instrument response to the blank from the response to dilutions of the standard and plot the results versus ATP concentration on log-log paper. If the microorganisms are not concentrated by filtration before the ATP is extracted, test the water carrying the microorganisms for the presence of agents that might interfere with the ATP-luciferase reaction. This is done by spiking a suitable volume of filtered water, that does not contain soluble extracellular ATP (see 10.1) from the samples and the unfiltered sample with a known amount of ATP to determine the percent recovery.

13. ATP Measurement

13.1 Rinse the microlitre syringe three times with 0.2 *N* HCl (8.5) by drawing acid into the entire 50 μL; rinse three times with 0.02 *M* tris buffer solution to neutralize any remaining acid.

13.2 Add sufficient volume of extract from 11.2 or 11.3 to the luciferin-luciferase mixture and measure the response with a suitable ATP analyzer.

NOTE 2—Some systems required the luciferin-luciferase mixture to be injected into the sample.

13.3 Repeat rinse (13.1) between each sample.

13.4 Convert the instrument reading to ATP units per millilitre using the standard curve. Account for the total sample volume filtered or the volume actually analyzed, or both, as appropriate.

14. Precision and Bias

14.1 The precision data were obtained by using standard ATP solutions as it was not possible to prepare a standard reference water sample for ATP content, which would represent a true environmental sample. Furthermore, because of the unstable nature of ATP, it was not possible to prepare and ship "unknown" standard solutions to all participating laboratories. For these reasons, the following procedure was used:

14.2 Each participating laboratory prepared and calibrated a luminescence meter covering the concentration range from 0.5 to 100μ g/L ATP. Participating laboratories were instructed to prepare" Pseudo Unknown" ATP samples with concentration levels of 0.4, 4.0, 16.0, 64.0, and 96 μg/L in 0.02 *M* tris buffer. These solutions were prepared by an independent person in the laboratory and the "Pseudo Unknown" ATP samples were then analyzed for 3 days. Each sample was run in triplicate and ATP concentration was determined using the calibration curve prepared during the same day. The round robin included seven people and six laboratories. The results are tabulated in Table 1.

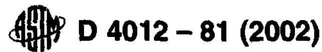
 D 4012 – 81 (2002)

TABLE 1 Precision and Bias

Known Concentration, µg/L	Single-Operator Precision, S_o, µg/L	Overall Precision, S_T	± % Bias, µg/L
0.4	0.166	0.162	6.9
4.0	0.540	0.521	0.3
16.0	1.171	1.479	0.7
64.0	3.180	4.251	0.8
96.0	5.516	5.343	0.9

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(9) St. John, J. B., "Determination of ATP in Chlorella with the Luciferin-Luciferase Enzyme System," *Analytical Biochemistry*, Vol 37, 1970, pp. 409–416.

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Designation: D 4412 – 84 (Reapproved 2002)

Standard Test Methods for Sulfate-Reducing Bacteria in Water and Water-Formed Deposits[1]

This standard is issued under the fixed designation D 4412; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 These test methods cover the procedure for the detection and enumeration by the most probable number (MPN) technique of sulfate-reducing bacteria in water or water-formed deposits.

1.2 Two media preparations are provided. Medium A which is prepared with reagent grade water, and Medium B which is prepared using the water to be sampled as the water source. Medium B is offered for those special conditions where sulfate-reducing bacterial strains have adapted to atypical non-fresh water environment.

1.3 For the isolation and enumeration of thermophilic sulfate-reducing bacteria encountered in waters associated with oil and gas production, all broths, dilution blanks, and incubations must be maintained at temperatures of at least 45°C and preferably within 5°C at the sample temperature.

1.4 The sensitivity of these test methods can be increased by purging the dilution blanks and tubes of media with nitrogen immediately prior to use.

1.5 The analyst should be aware that adequate collaborative data for precision and bias statements as required by Practice D 2777 are not provided. See Section 11 for details.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water[2]

D 1193 Specification for Reagent Water[2]

D 2777 Practice for Determination of Precision and Bias of Applicable Methods of Committee D19 on Water[2]

D 3370 Practices for Sampling Water from Closed Conduits[2]

2.2 *APHA Standard:*

Standard Methods for the Examination of Water and Wastewater, Fifteenth Edition[3]

3. Terminology

3.1 *Definitions*—For definitions of terms used in these test methods, refer to Terminology D 1129.

3.2 *Definitions of Terms Specific to This Standard:* —For a description of the term MPN used in these test methods, refer to literature.[4]

4. Summary of Test Methods

4.1 Water and water deposit samples and dilutions of these samples are dispensed into tubes of Starkey's medium (A or B) following five tube MPN procedures. The tubes are sealed with liquid paraffin, and incubated at 20°C for 21 days.[4] Positive reactions are indicated by the deposit of a black precipitate.

5. Significance and Use

5.1 Sulfate-reducing bacteria are widely distributed in marine and fresh water muds which, in consequence, frequently are laden with the hydrogen sulfide produced by these organisms during dissimilatory sulfate reduction.

5.2 It has been reported that Desulfovibrio can form as much as 10 g of sulfide per litre during active multiplication. Sulfate-reducing bacteria can cause the external or internal corrosion of water or wastewater pipelines and pipelines for petroleum and natural gas. The formation of galvanic cells by massive growth of sulfate-reducing bacteria under suitable conditions makes the corrosion much worse than just the effect of the hydrogen sulfide on the metal or concrete.

6. Apparatus and Materials

6.1 *Anaerobic Incubator*, 20°C, if available, or conventional 20°C incubator.[5]

6.2 *Pipets*, sterile, 1 mL and 10 mL," calibrated" to deliver.

6.3 *Test Tubes*, with close fitting or airtight caps; 16 by 150 mm and 20 by 150 mm.

6.4 *Test Tube Racks*, of sufficient size to contain 16 and 20-mm tubes.

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[1] These test methods are under the jurisdiction of ASTM Committee D19 on Water and are the direct responsibility of Subcommittee D19.24 on Water Microbiology.

Current edition approved Oct. 26, 1984. Published February 1985.

[2] *Annual Book of ASTM Standards*, Vol 11.01.

[3] Available from American Public Health Association, 1015 18th St. N.W., Washington, DC 20036.

[4] Bonde, G. J., "Bacterial Indicators of Water Pollution," *A Study of Quantitative Estimation*, Teknisk Forlag, Copenhagen, 1963.

[5] For thermophilic organisms use a 45°C incubator.

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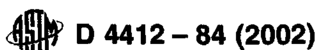

7. Reagents

7.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society,[6] when such specifications are available.

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean Reagent Water Type II conforming to Specification D 1193. In addition, reagent water used for these test methods must be sterile.

7.3 *Starkey's Medium A*—[7] (modified):

Sodium lactate ($C_3H_5NaO_3$)	3.5 g
Ammonium chloride (NH_4Cl)	1.0 g
Dipotassium, hydrogen orthophosphate (K_2HPO_4)	0.5 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	2.0 g
Sodium sulfate (Na_2SO_4)	0.5 g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.1 g
Thioglycollic acid	0.1 g
Ammonium ferrous sulfate or ferrous ammonium sulfate ($(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$)	0.001g
Water (H_2O)	1 L

7.3.1 Double strength medium (2×) is prepared as above except 500 mL of water are used instead of 1 L.

7.3.2 Heat to dissolve and dispense 9 mL of medium per single strength tube, and 10 mL per double strength tube.

7.3.3 Tubes should be of sufficient capacity to contain 1 mL of inoculum plus 9 mL of single strength medium or 10 mL of inoculum plus 10 mL of 2× medium.

7.3.4 pH of medium should be 7.2 after autoclave sterilization, at 121°C for 15 min.

7.4 *Starkey's Medium B*—The medium is similar to that described in 7.3, 7.3.1, and 7.3.2 with the following modification:

7.4.1 Water collected from the sample collection site is used to prepare the medium outlined in 7.3. The water sample is filtered to remove particulates (1.2 µm membrane filter) and the pH is recorded.

7.4.1.1 After preparing the Medium B following 7.3.1, 7.3.2, and 7.3.3, and prior to dispensing, check and adjust pH, if necessary to that of the original water used, then filter sterilize the medium by passage through 0.2-µm filter and asceptically dispense into presterilized tubes.

7.5 *Hydrogen Sulfide Test Reagent*:

7.5.1 *Ferric Chloride Stock Solution* ($FeCl_3 \cdot 6H_2O$)—Dissolve 13.5 g of ferric chloride in a mixture of 250 mL of water and 250 mL of HCl (sp gr 1.19). Store in an airtight amber container. Prepare fresh monthly.

7.5.2 *p-Aminodimethylaniline Dihydrochloride Stock Solution*

p-Aminodimethylaniline dihydrochloride ($C_8H_{12}N_2 \cdot 2HCl$)	1.0 g
HCl (6 N)	500 mL

Dissolve 1 g of p-aminodimethylaniline dihydrochloride in 500 mL of 6 N HCl. Store for up to 1 month in an amber airtight container.

7.6 *Liquid Paraffin*—Heavy, sterile, or sterile mineral oil.

7.7 *Buffered Dilution Water*—Stock Solution

7.7.1 Dissolve 34.0 g of KH_2PO_4 in 500 mL of water, adjust pH to 7.2 with 1 N NaOH and dilute to 1 L with distilled water. This is called the stock phosphate solution.

7.7.2 Dissolve 38 g of $MgCL_2$ in 1 L of distilled water.

7.8 *Buffered Dilution Water, Working Solution*—Add 1.25 mL of stock buffered dilution water and 5 mL of $MgCl_2$ solution to 500 mL of water. Bring to 1 L with water. Mix well and dispense as 90 mL dilution blanks in screw-capped bottles. Sterilize by autoclaving at 121°C for 15 min.

8. Procedure

8.1 Clean and disinfect the area with a cleaning solution that leaves no residue.

8.2 Set out and label five replicate tubes of 10-mL double-strength Starkey's medium, A or B, in the test tube rack.

8.3 Set out and label five replicate tubes of 10-mL single-strength Starkey's medium, A or B, for each mL of sample or mL of sample dilution to be tested. Use two sets of five replicate 10-mL tubes, each to contain 1 mL of sample or 1 mL of 1/10 dilution of sample.

8.4 Prior to sample inoculation, heat tubes of media and dilution blanks in a water bath to 60°C then cool rapidly to 20°C to ensure minimal oxygen levels.

8.5 Shake sample thoroughly,[8] at least 25 times; make dilutions starting with 10 mL of sample into one 90-mL dilution blank.

8.6 Pipet 10 mL of sample into each double-strength broth and 1 mL of sample or diluted sample into each set of five single-strength broths.

8.7 Maintain anaerobic conditions by layering 2 to 3 ml of sterile liquid paraffin in each tube.

8.8 Recap tubes and incubate at 20°C for 21 days.

8.9 Include sterile water samples with each test as negative controls.

8.10 Positive reaction is indicated by the deposit of a black precipitate (sulfide).

8.11 Confirm dubious results by the addition of 0.5 mL of ferric chloride reagent followed by 0.5 mL of p-aminodimethylaniline reagent to the MPN tube. Add reagent to the bottom of the tube using syringe or long Pasteur pipet. A positive reaction, blue color, occurs within 10 min if H_2S is present.

9. Calculation

9.1 Compute the number of positive findings resulting from multiple-portion decimal dilution planting as the combination of positives and recorded in terms of the Most Probable Number[3] (MPN).

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[6] "Reagent Chemicals, American Chemical Society Specifications," American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."

[7] Starkey, R. L., "Characteristics and Cultivation of Sulfate-Reducing Bacteria," *Journal of the American Water Works Association*, Vol 40, 1948, pp. 1291–1298.

[8] Organisms do not appear to be hypersensitive to small amounts of oxygen.

9.2 When more than three series of tubes are employed in a decimal series of dilutions, use the results from only three of these used in computing the MPN, for example:

10 mL	1 mL	0.1 mL	0.01 mL				
5/5	5/5	2/5	0/5	=	5-2-0 × 10	=	490/100 mL
5/5	4/5	2/5	0/5	=	5-4-2	=	220/100 mL
5/5	3/5	1/5	1/5	=	5-3-2	=	140/100 mL
5/5	0/5	0/5	0/5	=	5-0-0	=	23/100 mL

10. Report

10.1 Report the results as number of sulfate-reducing bacteria per 100 mL of sample.

11. Precision and Bias [9]

11.1 Due to the instability of the organisms, round robin testing can not be carried out. Statements can only be made on the precision of the MPN procedure.

11.2 Unless a large number of portions of sample are examined, the precision of the MPN is rather low. For example, even when the sample contains one organism per millilitre, about 37 % of tubes inoculated with 1 mL of sample may be expected to yield negative results because of irregular distribution of the bacteria in the sample and the multiple attachment of bacteria to particles. When five tubes, each with 1 mL of sample, are employed under these conditions, a completely negative result may be expected less than 1 % of the time. Thus, even when five tubes are employed for each dilution, the precision of the results obtained is not of a high order.

11.3 See the Research Report for results of a single laboratory, two operator study.

[9] Supporting data for these test methods have been filed at ASTM Headquarters. Request RR: D-19-1116.

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An American National Standard

Standard Guide for Microbial Contamination in Fuels and Fuel Systems[1]

This standard is issued under the fixed designation D 6469; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide provides personnel who have a limited microbiological background with an understanding of the symptoms, occurrence, and consequences of chronic microbial contamination. The guide also suggests means for detection and control of microbial contamination in fuels and fuel systems. This guide applies primarily to gasoline, aviation, boiler, industrial gas turbine, diesel, marine, and furnace fuels (see Specifications D 396, D 910, D 975, D 1655, D 2069, D 2880, D 3699, D 4814, and D 6227) and fuel systems. However, the principals discussed herein also apply generally to crude oil and all liquid petroleum fuels.

1.2 This guide is not a compilation of all of the concepts and terminology used by microbiologists, but it does provide a general understanding of microbial fuel contamination.

1.3 The values in SI units are to be regarded as the standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

D 130 Test Method for Detection of Copper Corrosion from Petroleum Products by the Copper Strip Tarnish Test[2]
D 396 Specification for Fuel Oils[2]
D 445 Test Method for Kinematic Viscosity of Transparent of Opaque Liquids (the Calculation of Dynamic Viscosity)[2]
D 515 Test Methods for Phosphorus in Water[3]
D 664 Test Method for Acid Number of Petroleum Products by Potentiometric Titration[2]
D 888 Test Methods for Dissolved Oxygen in Water[3]
D 910 Specification for Aviation Gasolines[2]
D 974 Test Method for Acid and Base Number by Color-Indicator Titration[2]
D 975 Specification for Diesel Fuel Oils[2]
D 1067 Test Methods for Acidity or Alkalinity of Water[3]
D 1126 Test Method for Hardness in Water[3]
D 1293 Test Methods of pH of Water[3]
D 1298 Test Method for Density, Relative Density (Specific Gravity), or API Gravity of Crude Petroleum and Liquid Petroleum Products by Hydrometer Method[2]
D 1331 Test Methods for Surface and Interfacial Tension of Solutions of Surface-Active Agents[4]
D 1426 Test Methods for Ammonia Nitrogen in Water[3]
D 1655 Specification for Aviation Turbine Fuels[2]
D 1744 Test Method for Water in Liquid Petroleum Products by Karl Fischer Reagent[2]
D 1976 Test Method for Elements in Water by Inductively-Coupled Argon Plasma Atomic Emission Spectroscopy[3]
D 2068 Test Method for Filter Blocking Tendency of Distillate Fuel Oils[2]
D 2069 Specification for Marine Fuels[2]
D 2274 Test Method for Oxidation Stability of Distillate Fuel Oil (Accelerated Method)[2]
D 2276 Test Method for Particulate Contaminant in Aviation Fuel by Line Sampling[2]
D 2880 Specification for Gas Turbine Fuel Oils[2]
D 3240 Test Method for Undissolved Water in Aviation Turbine Fuels[5]
D 3241 Test Method for Thermal Oxidation Stability of Aviation Turbine Fuels (JFTOT Procedure)[5]
D 3242 Test Method for Acidity in Aviation Turbine Fuel[5]
D 3325 Practice for Preservation of Waterborne Oil Samples[6]
D 3326 Practice for Preparation of Samples for Identification of Waterborne Oils[6]
D 3328 Test Methods for Comparison of Waterborne Petroleum Oils by Gas Chromatography[6]
D 3414 Test Method for Comparison of Waterborne Petroleum Oils by Infrared Spectroscopy[6]
D 3699 Specification for Kerosine[5]
D 3867 Test Methods for Nitrite-Nitrate in Water[3]
D 3870 Practice for Establishing Performance Characteristics for Colony Counting Methods in Microbiology[6]
D 4012 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water[6]

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[1] This test method is under the jurisdiction of ASTM Committee D02 on Petroleum Products and Lubricantsand is the direct responsibility of Subcommittee D02.14on Stability and Cleanliness of Liquid Fuels.

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[2] *Annual Book of ASTM Standards*, Vol 05.01.

[3] *Annual Book of ASTM Standards*, Vol 11.01.

[4] *Annual Book of ASTM Standards*, Vol15.04.

[5] *Annual Book of ASTM Standards*, Vol 05.02.

[6] *Annual Book of ASTM Standards*, Vol 11.02.

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D 4057 Practice for Manual Sampling of Petroleum and Petroleum Products[5]
D 4176 Test Method for Free Water and Particulate Contamination in Distillate Fuels (Visual Inspection Procedures)[5]
D 4412 Test Methods for Sulfate-Reducing Bacteria in Water and Water-Formed Deposits[6]
D 4418 Practice for Receipt, Storage, and Handling of Fuels for Gas Turbines[5]
D 4454 Test Method for Simultaneous Enumeration of Total Respiring Bacteria in Aquatic Systems by Microscopy[6]
D 4478 Test Methods for Oxygen Uptake[7]
D 4814 Specification for Automotive Spark-Ignition Engine Fuel[5]
D 4840 Guide for Sampling Chain of Custody Procedures[3]
D 4860 Test Method for Free Water and Particulate Contamination in Mid-Distillate Fuels (Clear and Bright Numerical Rating)[3]
D 4870 Test Method for Determination of Total Sediment in Residual Fuels[5]
D 4952 Test Method for Qualitative Analysis for Active Sulfur Species in Fuels and Solvents (Doctor Test)[5]
D 5304 Test Method for Assessing Distillate Fuel Storage Stability by Oxygen Overpressure[8]
D 5452 Test Method for Particulate Contamination in Aviation Fuels by Laboratory Filtration[8]
D 6217 Test Method for Particulate Contamination in Middle Distillate Fuels by Laboratory Filtration[8]
D 6227 Specification for Grade 82 Unleaded Aviation Gasoline[8]
D 6426 Test Method for Determining Filterability of Distillate Fuel Oils[9]
E 177 Practice for the Use of the Terms Precision and Bias in ASTM Test Methods[10]
E 1259 Test Method for Evaluation of Antimicrobials in Distillate Fuels (Based on Preliminary Screening and Compatibility)[11]
E 1326 Guide for Evaluating Nonconventional Microbiological Tests Used for Enumerating Bacteria[11]

2.2 *Institute of Petroleum Standards:*[12]
IP 385 Determination of the Viable Microbial Content of Fuels and Fuel Components Boiling Below 390°C—Filtration and Culture Method
IP Guidelines for the Investigation of the Microbial Content of Fuel Boiling Below 390°C and Associated Water
IP Proposed Method BY Determination of Fungal Fragment Content of Fuels Boiling Below 390°C

2.3 *Government Standards:*[13]
40 CFR 79 Fuels and Fuel Additives Registration Regulations
40 CFR 152 Pesticide Registration and Classification Procedures

2.4 *Other Standards:*[14]
Test Method 2540 D. Total Suspended Solids Dried at 103–105°C

3. Terminology

3.1 *Definitions:*

3.1.1 *aerobe, n*—an organism that requires oxygen to remain metabolically active.

3.1.1.1 *Discussion*—Aerobes use oxygen as their terminal electron acceptor in their primary energy-generating metabolic pathways. Aerobes require oxygen for survival, using *aerobic* metabolic processes to generate energy for growth and survival.

3.1.2 *aggressiveness index (A.I.), n*—the value computed from the sum of the pH + log alkalinity + log hardness of water sample where both alkalinity and hardness are reported as milligram $CaCO_3L$.

3.1.2.1 *Discussion*—As A.I. decreases, water becomes more corrosive. At A.I. $\geq$ 12, water is noncorrosive. At 10 $\leq$ A.I. < 12, water is moderately corrosive. At A.I. < 10, water is strongly corrosive.

3.1.3 *anaerobe, n*—an organism that cannot grow or proliferate in the presence of oxygen.

3.1.3.1 *Discussion*—Anaerobes use molecules other than oxygen in their primary energy-generating metabolic pathways, such as sulfate, nitrate, ketones, and other high-energy organic molecules. Although anaerobes may survive in the presence of oxygen, anaerobic growth typically occurs only in an oxygen depleted environment.

3.1.4 *anoxic, adj*—oxygen free.

3.1.5 *antimicrobial, n*—see biocide.

3.1.6 *bacterium (pl. bacteria), n*—a single cell microorganism characterized by the absence of defined intracellular membranes that define all higher life forms.

3.1.6.1 *Discussion*—All bacteria are members of the biological diverse kingdoms *Prokaryota* and *Archaebacteriota*. Individual taxa within these kingdoms are able to thrive in environments ranging from sub-zero temperatures, such as in frozen foods and polar ice, to superheated waters in deep-sea thermal vents, and over the pH range < 2.0 to > 13.0. Potential food sources range from single carbon molecules (carbon dioxide and methane) to complex polymers, including plastics. Oxygen requirements range from obligate anaerobes, which die on contact with oxygen, to obligate aerobes, which die if oxygen pressure falls below a species specific threshold.

3.1.7 *bioburden, n*—the level of microbial contamination (*biomass*) in a system.

3.1.7.1 *Discussion*—Typically, bioburden is defined in terms of either biomass or numbers of cells per unit volume or mass or surface area material tested (g biomass / mL; g biomass / g; cells / mL sample, and so forth). The specific parameter used to define bioburden depends on critical properties of the system evaluated and the investigator's preferences.

[7] Discontinued: see 1994 *Annual Book of ASTM Standards*, Vol 11.02.
[8] *Annual Book of ASTM Standards*, Vol 05.03.
[9] *Annual Book of ASTM Standards*, Vol 05.04.
[10] *Annual Book of ASTM Standards*, Vol 14.02.
[11] *Annual Book of ASTM Standards*, Vol 11.05.
[12] Available from Institute of Petroleum, 61 New Cavendish St., London, W.I., England.
[13] Available from Superintendent of Documents, U.S. Government Printing Office, Washington. D.C. 20402.
[14] Available from American Public Health Association, Washington, D.C.

3.1.8 *biocide, n*—a poisonous substance that can kill living organisms.

3.1.8.1 *Discussion*—Biocides are further classified as bactericides (kill bacteria), fungicides (kill fungi), and microbiocides (kill both bacterial and fungi). They are also referred to as *antimicrobials*.

3.1.9 *biodeterioration, n*—the loss of commercial value or performance characteristics, or both, of a product (fuel) or material (fuel system) through biological processes.

3.1.10 *biofilm, n*—a film or layer of microorganisms, biopolymers, water, and entrained organic and inorganic debris that forms as a result of microbial growth and proliferation at phase interfaces (liquid-liquid, liquid-solid, liquid-gas, and so forth) (synonym: *skinnogen layer*).

3.1.11 *biomass, n*—density of biological material per unit sample volume, area, or mass (g biomass / g (or / mL or / cm^2) sample).

3.1.12 *biosurfactant, n*—a biologically produced molecule that acts as a soap or detergent.

3.1.13 *consortium (pl. consortia), n*—microbial community comprised of more than one, species that exhibits properties not shown by individual community members.

3.1.13.1 *Discussion*—Consortia often mediate biodeterioration processes that individual taxa cannot.

3.1.14 *depacifying, adj*—the process of removing hydrogen ions (protons) from the cathodic surface of an electrolytic cell, thereby promoting continued electrolytic corrosion.

3.1.15 *deplasticize, v*—the process of breaking down polymers in plastics and similar materials, resulting in loss of the material's structural integrity.

3.1.16 *facultative anaerobe, n*—a microorganism capable of growing in both oxic and anoxic environments.

3.1.16.1 *Discussion*—Facultative anaerobes use oxygen when it is present, and use either organic or inorganic energy sources (nitrate, sulfate, and so forth) when oxygen is depleted or absent.

3.1.17 *fungus (pl. fungi), n*—single cell (yeasts) or filamentous (molds) microorganisms that share the property of having the true intracellular membranes (organelles) that characterize all higher life forms (*Eukaryotes*).

3.1.18 *metabolite, n*—a chemical substance produced by any of the many complex chemical and physical processes involved in the maintenance of life.

3.1.19 *microbial activity test, n*—any analytical procedure designed to measure the rate or results of one or more microorganism processes.

3.1.19.1 *Discussion*—Examples of microbial activity tests include loss or appearance of specific molecules or measuring the rate of change of parameters, such as acid number, molecular weight distribution (carbon number distribution), and specific gravity.

3.1.20 *microbially induced corrosion (MIC), n*—corrosion that is enhanced by the action of microorganisms in the local environment.

3.1.21 *mold, n*—form of fungal growth, characterized by long strands of filaments (hyphae) and, under appropriate growth conditions, aerial, spore-bearing structures.

3.1.21.1 *Discussion*—In fluids, mold colonies typically appear as soft spheres; termed *fisheyes*.

3.1.22 *obligate aerobe, n*—microorganism with an absolute requirement for atmospheric oxygen in order to function.

3.1.22.1 *Discussion*—Obligate aerobes may survive periods in anoxic environments but will remain dormant until sufficient oxygen is present to support their activity.

3.1.23 *obligate anaerobe, n*—microrganism that cannot function when atmospheric oxygen is present.

3.1.23.1 *Discussion*—Obligate anaerobes may survive periods in oxic environments but remain dormant until conditions become anoxic.

3.1.24 *oxic, adj*—an environment with a sufficient partial pressure of oxygen to support aerobic growth.

3.1.25 *shock treatment, n*—the addition of an antimicrobial agent sufficient to cause rapid and substantial (several orders of magnitude) reductions in number of living microbes in a fluid or system receiving that concentration.

3.1.26 *skinnogen, n*—synonymous with *biofilm*.

3.1.26.1 *Discussion*—Generally applied to a biofilm formed at the fuel-water interface.

3.1.27 *sour, v*—to increase the concentration of hydrogen sulfide.

3.1.28 *sulfate reducing bacterial (SRB), pl., n*—any bacteria with the capability of reducing sulfate to sulfide.

3.1.28.1 *Discussion*—The term SRB applies to representatives from a variety of bacterial taxa that share the common feature of sulfate reduction ($SO_4^=$ to $S^=$). SRB are major contributors to MIC.

3.1.29 *taxa, pl., n*—the units of classification of organisms, based on their relative similarities.

3.1.29.1 *Discussion*—Each *taxonomic unit* (group of organisms with greatest number of similarities) is assigned, beginning with the most inclusive to kingdom, division, class, order, family, genus, and species. Bacteria and fungi are often further classified by strain and biovariation.

3.1.30 *viable titer, n*—the number of living microbes present per unit volume, mass, or area.

3.1.30.1 *Discussion*—Viable titer is reported in terms of either colony forming units (CFU) or most probable number (MPN) per millilitre, milligram, or centimetre squared.

4. Summary

4.1 Microbes may be introduced into fuels as products cool in refinery tanks. Bacteria and fungi are carried along with dust particles and water droplets through tank vents. In seawater ballasted tanks, microbes are transported with the ballast. Vessel compartments ballasted with fresh, brackish, or seawater, all of which may contain substantial numbers of microbes, may easily become contaminated with the microbes transported with the ballast water. See Section 6 for more a detailed discussion.

4.2 After arriving in fuel tanks, microbes may either stick to overhead surfaces or settle through the product. Some microbes will adhere to tank walls, whereas others will settle to the fuel/water interface. Most growth and activity takes place where fuel and water meet. The tank bottom fuel/water interface is the most obvious fuel/water boundary. However, there is also a considerable area of fuel/water interface on the interior surface of tank-shells. Typically, fuel and system

deterioration is caused by the net activity of complex microbial communities living within slimy layers called *biofilms*. Biofilms may be found on tank roofs, shells, at the fuel/water interface, and within bottom sludge/sediment. Section 7 provides greater detail.

4.3 Obtaining representative samples may be challenging. For best results, samples should be collected from the interface zones, especially the fuel/water interface, described in 4.2. Refer to Section 8 for more details.

4.4 Sample analysis includes gross observations as well as a battery of physical, chemical, and microbiological tests. Because biodeterioration shares symptoms with other fuel and fuel-system degradation processes, it is critical to subject samples to a sufficient range of appropriate tests to permit accurate root-cause diagnosis. Section 9 provides more information on examining and testing samples.

4.5 Microbial contamination control requires a well designed strategy that considers system design, sampling and analysis, and preventive and remedial treatment. See Section 11 for details.

4.5.1 Good system design minimizes contaminant entry and provides for adequate sampling, water removal, and periodic cleaning and inspection.

4.5.2 Effective monitoring programs cost-effectively balance biodeterioration risks with sampling and analytical costs.

4.5.3 Remedial efforts may include fuel filtration, reconditioning, disposal, biocide treatment, or tank/system cleaning, or combination thereof. Health, safety, and environmental considerations are critical to proper tank remediation.

5. Significance and Use

5.1 This guide provides information addressing the conditions that lead to fuel microbial contamination and biodegradation and the general characteristics of and strategies for controlling microbial contamination. It compliments and amplifies information provided in Practice D 4418 on handling gas-turbine fuels. More detailed information may be found in the IP Guidelines.

5.2 This guide focuses on microbial contamination in refined petroleum products and product handling systems. Uncontrolled microbial contamination in fuels and fuel systems remains a largely unrecognized but costly problem at all stages of the petroleum industry from crude oil production through fleet operations and consumer use. This guide introduces the fundamental concepts of fuel microbiology and biodeterioration control.

5.3 This guide provides personnel who are responsible for fuel and fuel system stewardship with the background necessary to make informed decisions regarding the possible economic or safety, or both, impact of microbial contamination in their products or systems.

6. Origins of Microbial Contamination

6.1 The high temperature characteristic of distillation and other refinery processes sterilize refinery stocks used in fuel blending. However, conditions in refinery tankage, transport systems, terminal tankage, and users' system tankage may lead to microbial contamination and possible biodeterioration.

6.2 In refinery tankage, water can condense and coalesce as product cools. Tank vents draw moisture from the outside atmosphere and may allow precipitation to enter the tank. Moreover, product withdrawal creates a partial vacuum that pulls pollen, dust, and other microbe-carrying particulates through tank vents. Consequently, refinery products tanks are the first stage of petroleum handling where significant microbial contamination can occur.

6.3 In transport by means of tanker or pipeline, additional water may be introduced by condensation. In contrast to pipelines, condensate is not the major source of additional water. Rather, inadequate cargo compartment stripping, use of water as false bottoms to facilitate complete cargo discharge, and other incidental, intentional water use provide substantial water to fuel tanks. Biofilms can form on tanker or pipeline surfaces where they entrain water, inorganic particles, and nutrients to support growth. Such growth can slough off and be carried to terminal and end user tankage (see 6.4). In terminal tanks, turnover rates may be a week or longer, allowing particulates (including biofilm flocs) to settle into the sludge and sediment zone before product is drawn from the tank. As turnover rates increase, the likelihood of drawing biomass with fuel also increases, due to reduced settling times. Population densities of less than two millions cells/mL will have no effect on fuel clarity. Consequently, contaminated fuel is rarely detected visually at the terminal rack.

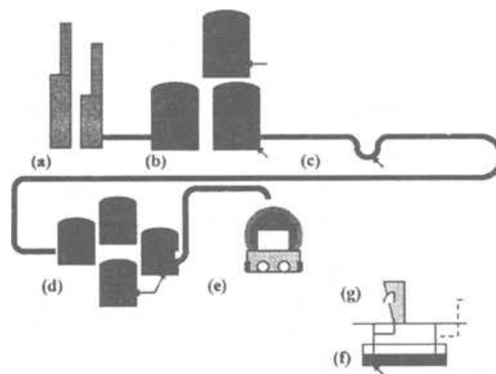
6.4 End-user tank materials and configurations are varied, reflecting use applications that range from small reservoirs (< 3 L) on power appliances (chain-saws, mowers, and so forth) to large (> 4000 L) day tanks feeding major power generation and propulsion engines. Location (above or below ground) and proximity to the point of combustion will also vary. End-use tanks accumulate water and bioburden that can lead to engine failure through fuel starvation resulting from filter or feed line plugging, or both. Moreover, MIC may compromise fuel tank integrity, leading to leakage. Substantial water volumes may be introduced into fuel tanks intentionally. In some ships, water is used as ballast and may occupy greater than 80 % of the total tank volume. At some tank farms, a layer of water is used to reduce the risk of ground-water, contamination due to fuel leakage.

7. Occurrence and Impact

7.1 Microbes require water as well as nutrients. Consequently, they concentrate at sites within fuel systems where water accumulates (see Fig. 1).

7.1.1 Water is essential for microbial growth and proliferation. Even negligible traces of water are sufficient to support microbial populations.

7.1.2 Nutrients are divided into macro-nutrients and micronutrients. Carbon, hydrogen, oxygen, nitrogen, sulfur, and phosphorus (CHONSP) comprise the macro-nutrients, and most of these are readily available in fuels. Only phosphorous is likely to be growth limiting in most fuel systems. A variety of elements, including calcium, sodium, potassium, iron, magnesium, manganese, copper, cobalt, nickel, and other metals, are required in trace quantities. None of these elements is limiting in fuel systems. Fuel systems that provide both the requisite water and nutrients will support microbial growth and proliferation.



NOTE 1—Legend—(*a*) Refinery distillation towers; (b) refinery product tanks; (c) fuel transportation pipeline (low points in pipeline trap water); (d) distribution terminal tanks; (e) commercial dispensing rack and tank truck; (f) retail/fleet underground storage tank; (g) retail/fleet dispensing system; arrows indicate sites where water and biologicals tend to accumulate.

FIG. 1 Fuel Distribution System

7.1.3 The rate of microbial growth increases with increasing temperature within the *physiological range* (temperature range within which growth occurs) of a given microorganism. Microbes are generally classified into three groups, based on their temperature preferences/requirements. Some microbes require low temperatures (<20°C). Others thrive in superheated environments (>100°C). However, the physiological range of the microbes most commonly recovered from fuel tanks is 0°C to 35°C, with growth optimal between 25°C and 35°C.

NOTE 1—The risk of uncontrolled microbial contamination is generally greatest in tropical regions. However, in the absence of adequate housekeeping practices, microbial contamination problems can also occur in fuel systems located in cold climates.

7.1.4 Water pH is generally not a controlling factor in fuel systems. Most contaminant microbes can tolerate pH's ranging from 5.5 to 8.0. As with temperature, there are microbes that prefer acidic environments (some grow in the equivalent of 2N sulfuric acid) and others that grow in alkaline systems with pH > 11. Fuel tank bottom-water pH is usually between 6 and 9.

7.2 As water activity tends to be greatest at interface zones, this is where microbes are most likely to establish communities, or biofilms. Numbers of microbes within biofilms are typically orders or magnitude greater than elsewhere in fuel systems. Biofilms can form on tank overheads, at the bulk-fuel, bottom-water interface, and on all system surfaces.

7.2.1 Using fuel hydrocarbon vapors as their carbon source, microbes can colonize tank overheads, where condensation provides the necessary water activity. Biofilms on overheads generally look like slimy stalactites.

7.2.2 The biofilm that develops at the fuel-water interface (sometimes called the skinnogen layer because of its tough membranous characteristics) represents a unique micro-environment relative to either the overlying fuel or underlying water. Nutrients from both the overlying fuel and underlying water are concentrated in this third-phase.

7.2.3 Whereas a 1-mm thick biofilm on a tank wall may seem negligible, it is 100 times the thickness of most fungi, and 500 to 1000 times the longest dimension of most bacteria. This seemingly thin film provides a large reservoir for microbial activity. Within the biofilm micro-environment, conditions can be dramatically different from those in the bulk product.

7.2.4 The microbial ecology of biofilms is complex. Microbial consortia (communities) give the biofilm community characteristics that cannot be predicted from analysis of its individual members.

7.2.4.1 Biofilms are formed when early colonizers, or pioneers, secrete mucous-like biopolymers that protect cells from otherwise harsh environmental conditions.

7.2.4.2 These biopolymers trap nonpolymer producing microbes, that then become part of the biofilm community, and cations that act as ligands that strengthen biofilm structural integrity.

7.2.4.3 Aerobes and facultative anaerobes (bacteria that grow aerobically under oxic conditions and anaerobically under anoxic conditions) scavenge oxygen, creating conditions necessary for obligate anaerobes to grow and proliferate.

7.2.4.4 Some bacterial and fungal species produce biosurfactants that create invert emulsions, which in-turn make nonpolar fuel components available for use as food.

7.2.4.5 Microbes able to attack hydrocarbons directly excrete waste products that other consortium members use as food. The net effect is a change in pH, oxidation-reduction (or redox) potential, water activity, and nutrient composition that has little resemblance to the environment outside the biofilm.

7.2.4.6 The biofilm consortium acts like a complex bioreactor, causing several types of significant changes to the fuel and fuel system.

7.2.4.7 Biofilm communities are directly involved in MIC that can result in pinhole leaks in tanks and pipelines. The problem of MIC is a consequence of several microbial processes.

7.2.4.8 First, the heterogeneity of biofilm accumulation creates electropotential gradients between zones of covered and uncovered surfaces.

7.2.4.9 SRB and other anaerobes use the hydrogen ions, thereby depacifying the electrolytic cell and accelerating the corrosion reactions. The hydrogen sulfide generated by biological sulfate reduction sours the fuel, causing copper corrosion test (see Test Method D 130) failure. Moreover, toxic hydrogen sulfide trapped within bottom sludge can be a safety hazard to personnel entering gas-freed tanks.

7.2.4.10 Microbes growing anaerobically produce low molecular weight organic acids (formate, acetate, lactate, pyruvate, and others). These acids accelerate the corrosion process by chemically etching the metal surface. There are data demonstrating that biofilm communities can deplasticize the polymers used in fiberglass synthesis. Such activity can result in catastrophic tank failure and is most likely to occur along the longitudinal centerline (the same place of the greatest frequency of MIC pinholes).

7.3 Biodeterioration shares many symptoms with nonbiological fuel deterioration processes. Without an adequate battery of tests, the root cause of a given fuel degradation problem may be misdiagnosed. The following paragraphs discuss symptoms caused by microorganisms. However, many of these symptoms may also be caused by nonbiological factors.

7.3.1 Biosurfactants facilitate water transport into the fuel phase and some fuel additive partitioning into the water phase. Other metabolites may accelerate fuel polymerization. Produced at concentrations that are difficult to detect against the complex chemistry of fuel components, these metabolites can have a significant deleterious effect on fuel stability. Although most of the change occurs within a few centimeters of the biofilm-fuel interface, product mixing can distribute metabolites throughout the fuel system.

7.3.2 The most commonly recognized symptom of microbial contamination is filter plugging. Two distinct mechanisms can cause this problem. When flocs of biomass are transported through the fuel system and are trapped in the filter medium, they can restrict flow. Direct observation of filters plugged by this mechanism reveals masses of slime on the filter element's external surfaces. Alternatively, microbial contaminants may colonize filter media. The biopolymers they produce within the filter medium's matrix eventually plug the filter.

8. Sampling

8.1 Bottom samples, as described in Practice D 4057, provide the best material for evaluating microbial contamination.

8.2 Because sample analyses may be performed by more than one laboratory, good sample chain of custody procedures should be followed (see Guide D 4840).

8.3 Both biological and nonbiological deterioration processes continue in a sample during the period between collection and analysis. Ideally, all testing should be accomplished at the sampling site, within a few minutes after a sample is drawn. As this is rarely possible, good practices for preserving and preparing samples for analysis should be following (see Practices D 3325 and D 3326).

8.4 Samples for pH, alkalinity/acidity, and dissolved oxygen determinations should be tested within 1 h after sampling.

8.5 Samples for microbiological testing should be kept on ice for transport to the laboratory. Tests should be performed within 1 h and no later than 36 h after sampling. Samples stored at higher temperatures, or for longer times, may show the presence of microbial contamination that does not represent actual fuel system conditions.

8.5.1 Samples for microbiological testing should be collected in new, unused containers.

8.5.2 If microbiological tests are not going to be completed within an hour after sample collection, the container should not be more than half-full. This provides adequate headspace to minimize the risk of conditions within the sample container becoming anoxic. Samples to be examined for anaerobic bacteria should be filled completely to maintain oxygen depleted or anoxic conditions.

8.6 Sampling intervals should be set so that there are at least three sets of data obtained during the period between system changes. For example, if microbial loads take six months to exceed criteria levels after biocide treatment, then tests should be performed every 1.5 to 2 months. This provides a compromise between controlling monitoring costs and detecting potential problems before they affect operations.

9. Examination and Testing

9.1 Some analytical methods can be performed in the field under less than optimal conditions, but many others will require the services of a laboratory with specialized equipment.

9.2 *Gross Observations*:

9.2.1 Gross observations, such as color, odor, clarity, and appearance of the fuel/water interface, are made during routine housekeeping and change over practices. When careful records are kept, they can identify changes in operating practices and environmental conditions that result in increased levels of microbial contamination. Gross observations should be made whenever a sample is drawn from the tank.

9.2.2 Check any accessible tank surfaces for the presence of microbial mats or slime. Their presence is evidence of microbial infestation.

9.2.3 Observe an interface sample that contains both water and fuel (see Fig. 2).

9.2.3.1 Uncontaminated samples contain either no water or only two clear and bright phases. Turbidity in the fuel phase (see Test Methods D 4176 and D 4860) indicates a significant problem, which might be due to microbial activity, high water content, surfactant contamination, or chemical instability.

9.2.3.2 Emulsified brown to red to black material in either phase indicates the presence of microbes. These colors generally reflect the presence of iron oxide or iron hydroxide, or both. Formation of these precipitates may involve microbial activity or may be the result of nonbiological processes.

9.2.3.3 The presence of a third phase (the *rag* phase or *cuff* layer) between the fuel and water suggests a microbial problem, although the rag layer may also be formed nonbiologically due to fuel component polymerization or inorganic precipitate formation, or both.

9.2.3.4 Presence of significant amounts of precipitated material in jars containing tank or pipeline samples further suggests the presence of microbes.

9.2.3.5 Hydrogen sulfide and other atypical (rancid) odors may indicate heavy microbial contamination.

9.2.3.6 Compare the gross properties (see Test Method D 4176) or near-bottom fuel (from 5 to 10 cm above fuel-water interface or tank bottom) with those of the bottom sample fuel-phase. Differences in clarity or color indicate that there are differences in fuel chemistry. Microbial activity may be the cause.

9.2.4 Pipe or filter blockage also indicates that severe infestation may be present in a fuel system. Test Method

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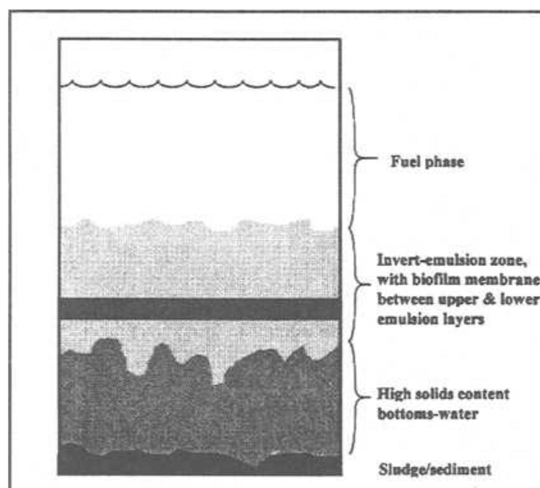

FIG. 2 Schematic of Fuel Tank Bottom Sample with Significant Microbial Contamination and Biodeterioration

D 5452 can be used to predict a fuel's filter plugging tendencies.

9.2.5 The major advantage to gross observations is their speed and simplicity. Their principal disadvantage is that gross changes typically occur late in the biodeterioration process, after significant damage has occurred.

9.3 *Physical Testing*:

9.3.1 Physical testing requirements for fuels are listed in each product grade specification (see Specifications D 396, D 910, D 975, D 1655, D 2069, D 2880, D 3699, D 4814, and D 6227).

9.3.2 Microbial contamination is most likely to affect filterability (see Test Methods D 2068, D 2276, D 4870, D 5452, D 6217, and D 6426) and oxidation stability (see Test Methods D 2274, D 3241, and D 5304). Severe contamination can eventually affect viscosity (see Test Method D 445) and density (see Test Method D 1298). Consequently, when these parameters are outside the specification range, microbial contamination should be considered as one of the possible causes.

9.3.3 Physical tests for bottom-water samples are not included in fuel standards. One particularly useful test is determination of suspended solids by filtration (see Test Method 2540 D). This test determines the mass of material that is present in the contaminated water sample.

9.3.4 The measurement of interfacial tension (see Test Methods D 1331) is sometimes used as a surrogate for direct surface active by-product (fatty acids and proteins) analysis. The presence of these biosurfactants is indicative of microbial activity. This test method is most useful if baseline data (fresh fuel over sterile water) are available. Both near-bottom fuel and bottom-water should be tested periodically to determine changes in their respective contact angles (interfacial tension).

9.4 *Chemical Testing*:

9.4.1 Chemical testing should be performed on the fuel phase, water-phase, and filter material, if available.

9.4.2 ASTM fuel product specifications include chemical tests. Microbial contamination may contribute to changes in any chemical property of a fuel. Consequently, a microbial cause should be considered when investigating a fuel's failure to meet chemical specifications. Several additional standard fuel chemistry tests facilitate diagnosis of fuel diodeterioration. In addition, there are a variety of commercially available test kits for many of the parameters discussed below. Typically the test kits provide a simplified means for obtaining data. In many circumstances, data from test kits are sufficient for contamination monitoring and diagnostic purposes. Operators or laboratory personnel intending to substitute a simplified test should confirm that the simplified test meets their specific needs and expectations. Guide E 1326 offers suggestions for evaluating the suitability of nonconventional or nonstandard test methods.

9.4.3 Acid/base (see Test Methods D 664, D 974, and D 3242) number indicates the extent to which the fuel composition has changed through production of fatty acid by-products, carbon dioxide, or other by-products of metabolism or non-biological processes.

9.4.4 Water content (see Test Method D 1744 or D 3240) provides information related to the amount of water present in the fuel phase. Water is critical to the successful establishment of a microbial population in the fuel phase.

9.4.5 Copper corrosion (see Test Method D 130) testing may indicate the presence of anaerobic microbes, sulfate-reducers, and *clostridia* and other protein fermenters that also produce hydrogen sulfide (H_2S). The copper corrosion strip test is rapid and economical and is included in some fuel specifications.

9.4.6 Proteins, lipids, and fatty acids in the fuel and bottom-water are directly proportional to biomass.[15]

9.4.7 Gas chromatography/mass spectroscopy (GC/MS) fingerprinting may reveal compositional changes in the fuel components that can be evaluated using simulated distillation methods and elemental analysis. Certain types of changes are expected in a product contaminated with microbes. These include a diminution in alkanes (normal, iso-, and cyclo-) relative to uncontaminated fuels.

[15] Gerhardt, Phillip, ed., *Manual of Methods for General Bacteriology*, American Society for Microbiology, 1981.

9.4.8 Water phase tests are not included in fuel specifications but provide critical information about fuel system biodeterioration.

9.4.8.1 Bottom-water samples should be measured for pH (see Test Methods D 1293). Bottom-water pH is normally alkaline (> pH 7.0). Samples with pH <7.0 are suspect for microbial activity.

9.4.8.2 Acidity or alkalinity (see Test Methods D 1067) data are used to compute the aggressiveness index of bottom-water. The alkalinity of uncontaminated bottom-water under middle distillate fuel typically ranges from 75 to 150 mg $CaCO_3$/ L. Under gasoline that has been augmented with amine-based anticorrosion additives, alkalinities > 2500 mg $CaCO_3$ are not unusual. To be most useful, baseline bottom-water alkalinity should be determined using fresh fuel over local tap or freshly collected rainwater, or any readily available fresh water with a known low (< 50 mg $CaCO_3$/L) alkalinity. If total alkalinity falls below half of the baseline value, microbial activity is the most likely cause. Microbial activity is also likely in bottom-water samples with any measurable acidity, as the by-products or microbial metabolism tend to be acidic and readily partition into the water phase. These include the by-products of hydrocarbon metabolism: acetate, butyrate, formate, and others, as well as carbon dioxide.

9.4.8.3 Although not affected directly by microbial growth, hardness (see Test Method D 1126) is one of three variables used to compute aggressiveness index. Moreover, hard water (> 200 mg $CaCO_3$/ L) tends to support more robust microbial communities than does soft water.

9.4.8.4 Compute A.I. from pH, alkalinity/acidity, and hardness data.

(*a*) Water with A.I. > 12 is considered noncorrosive. If A.I. is between 10 and 12, the water is moderately corrosive, and if A.I. <10, it is strongly corrosive. Low bottom-water A.I. is a strong indication of biological activity.

9.4.8.5 Dissolved oxygen (see Test Methods D 888) in bottom-water from uncontaminated tanks is typically at 50 to 75 % saturation. Moreover, the oxygen concentration is an uncontaminated sample that is aerated to saturation will remain greater than 50 % saturation for at least 4 h after aeration is discontinued. Consequently, a bottom-water sample with an oxygen concentration less than 50 % of the saturation concentration, or a sample in which the oxygen concentration decreases by more than 50 % in a 4 h period is likely to have a significant bioburden.

9.4.8.6 Metal ion analysis by inductively-coupled argon plasma atomic emission spectroscopy (see Test Method D 1976) provides a measure of the extent to which metals from tanks, pipes, and other equipment surfaces have been solubilized. Microbes involved in MIC can directly or indirectly cause solubilization of metals from system components. Consequently, the presence of increased soluble iron over time strongly suggests bacterial activity. Changes in aluminum and manganese (along with iron) are also indicative of corrosion.

9.4.8.7 Nitrogen analysis (see Test Methods D 1426 and D 3867) is useful because nitrate-reducing bacteria convert nitrate ions to nitrite and ammonia.

9.4.8.8 Sulfate reducers produce hydrogen sulfide from sulfate ions. Increased sulfide concentrations in bottom-waters (see Test Methods D 4952) are characteristic of biodeterioration in fuel systems.

9.4.8.9 Fuel components that have partitioned into the water-phase can be analyzed by Test Methods D 3328 or Test Method D 3414. Biosurfactants increase petroleum emulsification into bottom-water.

9.4.8.10 Phosphorus is often a growth-limiting nutrient in bottom-water. Determine phosphorus in bottom-water samples using Test Methods D 515.

9.5 *Microbiological Testing*:

9.5.1 In contrast to many of the gross observations, physical and chemical tests, there are no generally accepted criteria for acceptable microbial contamination levels. In many applications, any detectable microbes in the fuel-phase trigger corrective action. However, populations of greater than 10^5 cells/mL may be tolerable in bottom-water. As discussed in Section 10, it is critical to monitor contamination trends and to correlate microbiological data with operational, chemical, and physical data in order to define the role of microbial contamination in specific fuel or system problems

9.5.2 Direct microscopic examination enables the analyst to directly examine all sample phases (fuel, water, filter medium, and so forth). A skilled analyst can assess the relative distribution of different groups of microbes and enumerate total cell numbers (see Test Method D 4454). IP Proposed Method BY provides a technique for recovering and observing fungal fragments from fuel samples.

9.5.3 Electron microscopy (Scanning Electron Microscopy (SEM) or Time of Flight SEM) is useful in special circumstances.[16] These have a practical resolution of about 25 nm, compared with about 300 nm for conventional light microscopy, and a depth-of-field at least 300 times greater. However, both methods currently require water to be removed, which may alter surface structures creating artifacts.

9.5.4 Enumeration methods estimate microbial loads from the growth of microorganisms either in liquid or on solid nutrient growth media (see Practice D 3870).

9.5.4.1 On solid media, viable titer estimates are computed from the number of colonies that form on the inoculated growth medium. The three most common solid media viable titer methods are the pour-plate, spread-plate, and membrane filtration methods.

9.5.4.2 In liquid media, viable titer estimates are based on turbidity development or color change, or both. Liquid media are better suited for estimating viable titers in fuel-phase samples, because fuel hydrophobicity (tendency to reject water) interferes with sample dispersion across a solid medium's surface.

9.5.4.3 Viable titer methods are most meaningful for detecting subpopulations with specific physiological attributes. Examples include hydrocarbon or additive mineralization and sulfate reduction (see Test Methods D 4412).

9.5.4.4 The choice of medium has a large effect upon what microbes are recovered and constitutes a bias introduced into

[16] Lawrence, J. R., Korber, D. R., Wolfaardt, G. M., and Caldwell, D. E.," Analytical Imaging and Microscopy Techniques," Chaper 5 in *Manual of Environmental Microbiology*, ed. C. J. Hurst, American Society for Microbiology, 1997.

this type of testing. Frequently, microbes that are important components of the ecosystem being studied do not grow in the media used for viable titers.

9.5.4.5 Typically sample volumes from 0.01 to 1.0 mL are used to inoculate solid media, and 1.0 to 5.0 mL are used to inoculate liquid media. Given the nonuniform (heterogeneous) distribution of biomass in fuel systems, it is difficult to get a truly representative sample. Any given sample of water or hydrocarbon may not come from where the bulk of the microbes are growing at the site. Dislodging bacteria attached to equipment surfaces is not easy. Population counts based on planktonic (suspended) microbes generally severely underestimate the total population present at the site.

9.5.4.6 Several suppliers offer solid media on dip-slides and liquid media in pre-measured vials. These devices provide data comparable (precision and accuracy) to viable titers determined by standard laboratory test procedures while offering convenience for field use.

9.5.4.7 Since viable recoveries from fuel are typically less than 1 CFU/mL, samples can be filtered through 0.22 and 0.45-μm filters to concentrate bacteria and fungi respectively. It is useful to filter up to 1000 mL of fuel in order to decrease detection limits from 1 CFU/mL (from the plate count method described in 9.5.4.5) to 1 CFU/L (see IP 385).

9.5.5 Microbial activity tests may be used as an alternative or supplement to viable titer tests. These tests typically require some skill and laboratory equipment to complete but provide a more direct indication of actual or potential biodeterioration.

9.5.5.1 A variety of chemical and potentiometric test methods can be used to measure the rate of molecule production or disappearance. Examples include periodic measurements of dissolved oxygen (see Test Methods D 888) to determine the rate of oxygen consumption (see Test Methods D 4478) and periodic spectroscopic analyses to measure changes in relative concentrations of fuel components, primarily molecular species.

9.5.6 Cell component measurements provide a fourth approach to quantifying bioburdens. Over the past two decades, numerous test methods have been developed and automated for analyzing clinical samples for specific cell constituents. A growing number of these test methods have been adapted for environmental and industrial testing. Adenosine triphosphate (ATP) (see Test Method D 4012), catalase activity, nucleic acid, and protein concentrations are four examples of molecules routinely monitored in industrial systems. Guide E 1326 provides more guidance on test method selection.

10. Data Interpretation

10.1 The critical element for all data interpretation is change measurement. This, in turn, depends on three factors.

10.2 Tests should be selected based on their ability to guide system management decisions. This means that test results should indicate conditions of the system, which if not properly controlled could result in biodeterioration.

NOTE 2—The mere presence of recoverable microbes in bulk fuel indicates heavy contamination levels. In contrast, values from 1×10^3 to 1×10^5 CFU/mL in bottom-water may be acceptable if all other fuel and system conditions are normal.

10.3 The precision and accuracy of each test method needs to be known to determine whether observed changes are real or are within the limits of experimental error (see Practice E 177). Once test precision and variation are defined, criteria levels can be assigned. For fuel samples, the criteria listed in the respective ASTM standards can be used. For water and system samples, managers should define criteria that meet their specific needs.

10.4 Different microbiological growth media will yield different results. Therefore, consistency in sampling and test procedure is important for establishing baseline and special-cause variance conditions.

11. Strategies for Controlling Microbial Growth

11.1 *System Design*—Strategies for controlling microbiological growth should begin with system design.

11.1.1 All tanks should be designed to facilitate water and bottom-solids removal, minimize contaminant entry, and facilitate sample collection.

11.1.2 Bulk tank bottom geometry should be cone-down (concave) with sufficient slope to permit sludge and sediment to migrate toward a central sump.

11.1.3 Floating of roof tanks should be fitted with a non-floating supraroof (false roof) to minimize precipitation and debris accumulation on the floating roof surface and to protect the above-floating-roof shell-surface from the environment. If this is not possible or economically feasible, tank bottoms should be checked daily for free water. Any free water found should be drained from the tank.

11.1.4 Horizontal, cylindrical, underground storage tanks should be fitted with a second access port at the opposite end from the fill-pipe to allow dipping the tank for water at both ends and pumping out bottom-water from the low-end.

11.1.5 During system design, biodeterioration control considerations are typically subordinate to other issues. Moreover, existing systems are not likely to be redesigned to accommodate biodeterioration prevention programs. Consequently, the other components of biodeterioration control strategies become more important.

11.2 *Sampling and Analysis*:

11.2.1 An adequate sampling and analysis program can be the most critical element of an effective biodeterioration control strategy. Although the details of the sampling program will depend on system operations and configuration, minimal sampling requirements will include bulk fuel and near-bottom fuel/bottom-water samples. Suitable sampling locations and techniques are paramount in determining both the presence and the extent of biological contamination. Microbiological contamination generally resides at a low point in a storage vessel or pipeline. Another potential location is filters. Biological contamination requires water to metabolize fuel, and filter media provide a perfect collection area for both water and the ability to hold matter.

11.2.2 In dealing with living organisms, it is important to sample with equipment that is free of contamination. Both samplers and containers should be sterile if possible. Sample containers should be new and kept closed prior to use.

11.2.3 Once contamination has been detected, further investigation and analysis can determine the extent of the problem.

11.3 *Remediation*:

11.3.1 Remediation really begins with prevention. Because microbes require water, an intensive water removal program is important. Many storage systems have been poorly designed in relationship to water removal, so extra effort may be necessary to determine the best method of removal. The best advice that can be given is that for each location, a sampling procedure may have to be written to accommodate the differences in tank and piping design.

11.3.2 After removing water from a system, a representative sample from that water may be tested for presence of biological activity. If the test is positive, it is likely that contamination is present in the facility. Additional steps, such as chemical treatment or tank cleaning, or both, may be required.

11.3.3 For systems that have high microbial loads, but no other gross evidence of contamination, water removal and biocide treatment usually suffice.

11.3.4 Water removal is never 100 % effective. Most tank configurations make it impossible to remove all water. Most bulk tanks with installed water removal systems still retain water after draining. Tank bottom configuration has a major impact on water removal capabilities. Flat and convex bottomed tanks retain the most water. Concave (optional) tank bottoms, with sumps drawing from the lowest point, retain the least amount of water.

11.3.5 Not all free-water accumulates in tank bottoms. The biofilm layer that accumulates on tank walls is typically greater than 90 % water. This creates a substantial, but difficult to sample, habitat for microorganisms. Data from bottom-water samples are used to estimate the likelihood of significant tank surface contamination. Generally, evidence of significant bottom-water contamination is predictive of significant tank surface contamination. In the absence of performance problems, acceptable bottom-water contamination levels also indicate acceptable tank-surface contamination levels. However, if performance problems indicate that there is significant microbial contamination, but bottom-water data are either negative or equivocal, the bottom-water data may not be a satisfactory indicator of overall microbial contamination within the tank.

11.4 *Biocide Use*:

11.4.1 By definition, biocides are toxic materials. They can, however, be used and handled safely. Users are advised to review and comply with the safety, handling, and disposal requirement information provided in each product's material safety data sheet (MSDS) and technical information literature.

11.4.2 In the United States two groups within the Environmental Protection Agency regulate fuel biocide use. The pesticides group issues restrictions for all industrial biocides (see 40 CFR 152), while the air quality group issues restrictions for all fuel additives (see 40 CFR 79). Only biocides meeting both pesticide and fuel additive restrictions should be used to treat fuel systems. In addition, end-user groups (for example, the aviation industry and the U.S. Department of Defense) may place further restrictions on biocide selection options. Finally, biocides are required by law to be be registered in the state(s) in which they are used. Outside the United States different countries have unique biocide regulatory requirements. Always check with manufacturers or the appropriate local authorities.

11.4.3 There are three major groups of fuel biocides: fuel soluble, water soluble, and universally soluble.

11.4.3.1 Fuel soluble biocides are unstable or insoluble in water. Their principal advantage is that they reside in the fuel phase and can be transported throughout the fuel system. Their primary disadvantage is that they are typically inactivated by water, where the microbes tend to grow.

11.4.3.2 Water-soluble biocides are insoluble in fuel. They tend to be inexpensive and are best used to shock-treat bottom-water contamination in tanks that are not drained routinely. The microbes found in bottom-water can contribute to the wastewater treatment process. Consequently, there is little value in using a biocide to kill microbes in water that is destined for waste treatment. Water-soluble biocides do not persist in the fuel phase enough to diffuse into system surface biofilms. Consequently, they tend to be effective only against bottom-water populations.

11.4.3.3 Universally soluble biocides are stable in both fuel and water. Typically, these products are primarily fuel-soluble, with sufficient water solubility to perform in both phases. Like fuel soluble biocides, universally soluble products can be transported throughout the fuel system. Their water solubility makes them equally effective against biofilm and bottom-water microbes. Their principal disadvantage is their high cost relative to the other two fuel biocide groups.

11.4.3.4 Biocide treatment frequency and dose levels are both system and biocide product specific. Test Method E 1259 addresses fuel biocide performance testing. Fuel system operators should consult with manufacturers or others with biocide-use expertise before using fuel treatment biocides.

11.5 *Tank Cleaning*:

11.5.1 The option of tank cleaning is expensive, potentially disruptive, and risky. A plan of action needs to be carefully considered before this step is utilized. Care should be exercised in the selection of a contractor because of the liability of a system's owner is exposed to should a spill occur or waste be disposed of improperly.

11.5.2 Heavily contaminated systems generally require tank and pipe cleaning in conjunction with biocide treatment. The most effective programs include a three-step process.

11.5.2.1 Systems are first shock-treated with biocide. Biocide-treated surface biofilms will slough-off system walls and accumulate in tank bottoms.

11.5.2.2 Next, systems are cleaned. There are a variety of tank-cleaning strategies offered by service companies.

NOTE 3—Certain commonly used materials, such as strong detergents, are not suitable for cleaning aviation fuel systems. Any cleaning material used should first be checked for compatibility with both the fuel and fuel system.

11.5.2.3 Fuel system operators should evaluate alternative recommendations to ensure that the proposed methods meet their needs.

11.5.2.4 After cleaning, the freshly charged fuel system should be retreated with a second biocide dose. This treatment decontaminates surfaces that may not have been reached either by the initial dose (biocide is consumed as it kills microbes) or subsequent cleaning (microbes protected by surface irregularities, called *aspersions*, may escape mechanical cleaning). If

stored fuel had been temporarily removed to accommodate tank cleaning, filtering it as it is pumped back into the cleaned tank may reduce the risk of recontaminating the tank with microbes that may be present in the original fuel.

12. Keywords

12.1 biocides; biodegradation; biodeterioration; biological contamination; contamination; fuel quality; microbial contamination; microbially induced corrosion; microbiological testing; sampling

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Designation: E 1259 – 01

Standard Test Method for Evaluation of Antimicrobials in Liquid Fuels Boiling Below 390°C[1]

This standard is issued under the fixed designation E 1259; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method is designed to evaluate antimicrobial agents for the prevention of microbially influenced deterioration of liquid fuels (as defined by Specification D 396, D 910, D 975, D 1655, D 2069, D 2880, D 3699, D 4818 and D 6227), system deterioration, or both.

1.2 Knowledge of microbiological techniques is required for these procedures.

1.3 It is the responsibility of the investigator to determine whether Good Laboratory Practice (GLP) is required and to follow them where appropriate (40 CFR, 160), or as revised.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

D 396 Specification for Fuel Oils[2]

D 910 Specification for Aviation Gasolines[2]

D 975 Specification for Diesel Fuel Oils[2]

D 1655 Specification for Aviation Turbine Fuels[2]

D 2069 Specification for Marine Fuels[2]

D 2880 Specification for Gas Turbine Fuels[3]

D 3699 Specification for Kerosine[3]

D 4814 Specification for Automotive Spark-Ignition Engine Fuel[4]

D 6227 Specification for Grade 82 Unleaded Aviation Gasoline[4]

D 6469 Guide to Microbial Contamination in Fuels and Fuel Systems[4]

2.2 *Federal Standards:*

40 CFR, Part 79, Fuels and Fuel Additives Registration Regulations[5]

40 CFR, Part 152, Pesticide Registration and Classification Procedures[5]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *antimicrobials, n*—chemical agents that are cidal or static to microorganisms.

3.1.2 *microbial-induced deterioration, n*—decomposition/degradation of material (fuel) or making unsuitable for use, as a result of metabolic activity or the presence of microbes

3.1.3 *microbicide, n*—an agent that kills microbes: bacterial vegetative cells, fungal vegetative cells and spores, algae and protozoa. This term is applied to chemical agents that kill microbes.

4. Summary of Test Method

4.1 This test method is conducted on a reference fuel, and determinines the antimicrobial efficacy under well-defined conditions that include specific inocula *Pseudomonas aeruginosa*, American Type Culture Collection, (ATCC) No. 33988, *Hormoconis resinae*, ATCC No. 20495, and *Yarrowia tropicalis* (formerly *Candida tropicalis*, ATCC No. 18138; as well as water/fuel ratios, and time of containment. It is designed for destructive sampling at regular intervals during bottom water buildup. This test method allows for impact of fuel/water partitioning and time, on the antimicrobial agent, as well as the effect of continual rechallenge. Every 2 weeks, water phase is increased by 0.25 % while concomitantly, a paired system is destructively tested. Thus, at 4 weeks, there is an increase in the water phase to 0.5 %, at 6 weeks 0.75 %, and at 8 weeks 1.0 %. At each sampling time interval, treated and untreated aliquots are checked for the three types of organisms in the initial inoculum. These counts are coupled with gross observations of each system for biofilm formation and interfacial growth.

5. Significance and Use

5.1 Guide D 6469 details the types of problems associated with uncontrolled microbial growth in fuels and fuel systems. Treatment with effective antimicrobial agents is one element of contamination control strategy.

5.2 The procedure should be used to evaluate the relative efficacy of microbicides in distillate fuels. The effect of environmental conditions, such as a variety of fuel additives,

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[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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[2] *Annual Book of ASTM Standards*, Vol 05.01.

[3] *Annual Book of ASTM Standards*, Vol 05.02.

[4] *Annual Book of ASTM Standards*, Vol 05.03.

[5] Available from the Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

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metal surfaces, and climatology, are variables that can be included in specific tests using this protocol.

5.3 This method addresses product performance issues only. Regulatory Agencies restrict and control the use of both pesticides (in the U.S.:40 CFR 152) and fuel additives (40 CFR 79). Regardless of performance in this method, antimicrobials must only be used in compliance with applicable regulations. Specific industries, for example, the aviation industry, may place further restrictions on chemicals used for fuel treatment.

6. Apparatus

6.1 *Colony Counter*—Any of several types, for example, a Quebec Colony Counter may be used.

6.2 *Incubator*—Any incubator capable of maintaining temperature of 30 to 35°C may be used.

6.3 *Sterilizer*—Any suitable steam sterilizer capable of producing the conditions of sterility is acceptable.

6.4 *Separatory Funnels*—Sixteen 1-L funnels.

6.5 *Ring Stand*, suitable for supporting separatory funnel.

6.6 *Vortex*—Mixer.

7. Reagents and Materials

7.1 *Petri Dishes*—100 by 15 mm required for performing standard plate count.

7.2 *Bacteriological Pipets*—10.0 mL and 1.1, or 2.2 mL capacity.

7.3 *Water Dilution Bottles*—Any sterilizable glass container having a 150–200 mL capacity and tight closure may be used.

7.4 *Fuel.*[6]

7.5 *Synthetic Bottom Water.*[7]

7.6 *Soy Peptone Casein Digest Agar.*

7.7 *Sabouraud Dextrose Agar.*

7.8 *Agar, Bacteriological Grade.*

7.9 *Potassium Tellurite Solution*—sterile 1 %.

7.10 *Gentamicin Sulfate*—50 µg/mL.

7.11 *Plate Count Agar.*

7.12 *Potato Dextrose Agar.*

8. Inoculum

8.1 *Inoculum Preparation and Maintenance*:

8.1.1 *Inoculum Revitalization*—Cultures are *Pseudomonas aeruginosa*, ATCC No. 33988, *Hormoconis resinae*, ATCC No. 20495, and *Yarrowia tropicalis* (formerly *Candida tropicalis*), ATCC No. 18138. Obtain lyophilized preparations from ATCC. Before initiating fuel antimicrobial tests, revitalize each of the three cultures in accordance with the instructions contained with each culture.

8.1.2 *Maintenance and Preparation of Inocula*—All three cultures are transferred from slants of a specified agar, (*a*) *Pseudomonas aeruginosa* (Plate Count Agar), (*b*) *Hormoconis resinae* Potato Dextrose Agar), and (*c*) *Yarrowia tropicali* (Potato Dextrose Agar) to synthetic bottom water medium in a suitable size screw-cap glass bottle (French square), and then overlaid with 10 times the volume of fuel. This two-phase system is kept at room temperature (18-24°C) for seven days, and the interface with half the bottom water is transferred weekly to a similar system weekly until used. The bacterial levels expected are about 10^7 CFU/mL, the yeast levels 10^6 CFU/mL, and mold levels 10^4 spores/mL. For the test inoculum, the bacteria are diluted 1:100 while yeast and molds are diluted 1:10. The counting of the inoculum is done directly from the prepared synthetic bottom water mixture at time zero, just prior to adding inoculum to each setup, and at each subsequent time point.

NOTE 1—**Caution:** In the distillate fuel industry, additives, including biocides, are calculated on a weight per weight basis so that the specific gravity of both the fuel and the biocide (if a liquid formulation) must be taken into account.

9. Procedure

9.1 *The Setup*—For each biocide, four setups are needed plus four for the control. A typical evaluation could include three levels of the biocide in addition to the control system with no biocide.

$$4 \times 3l + 4c = 16ts \quad (1)$$

where:
l = level,
c = control, and
ts = total setups.

9.1.1 To each l-L funnel, add 800 mL of test fuel and the appropriate level of stock biocide so that the desired concentration of biocide is achieved.

9.1.2 Stock biocide is made by adding it to a solvent that will dissolve the biocide.

9.1.3 Next, add 2 mL of synthetic bottom water medium (0.25 % of fuel volume) containing the appropriate levels of each organism. Close the funnel. Shake vigorously for 10 s. Place in ring stand. Open stopper slightly to allow volatile gas to escape. Close and leave for two weeks.

9.2 *Sampling*—At 2, 4, 6, and 8 weeks, the following protocol is observed. The bottom water fraction including the fuel/water interface and a minimal amount of fuel is bled from the separatory funnel and mixed vigorously in a vortex for 10 s. Before settling, aliquots are removed for bacteria, yeast and mold plate counts. Dilutions, 1:100, 1:1000, and 1:10000, are prepared in sterile synthetic bottom water. These will be used for pour platings for bacteria and yeast, respectively. In addition, three 0.1 mL portions will be used for bacteria and yeast pour plates, and for spread plates for mold counts. For estimating *Pseudomonas aeruginosa*, use soy casein digest agar; for *Yarrowia tropicalis*, use Sabouraud's Dextrose Agar with gentamicin 0.5 µg/mL; and for *Hormoconis resinae*, use 0.01 % potassium tellurite in 1.5 % bacteriological agar.

9.3 *Reinoculation*—Immediately after the sacrificing (destructive sampling) of one-system biocide level, 2 mL of synthetic bottom water solution containing additional inoculum is added to each of the three remaining setups/biocide level. Again, the procedure described in 8.1.2 and 9.1.3 is repeated. After two more weeks, 9.2 is again repeated and the process is repeated at weeks 6 and 8.

[6] Representative fuel samples from each product grade are available from all petroleum refiners.

[7] Items 7.5-7.12 are available from a variety of media manufacturers and chemical supply companies.

10. Results

10.1 *Comparison of Test and Control*—At each interval, the microbiological counts for treated systems will be compared with those of the untreated systems. In addition, gross observation of the condition of each system will be made with the intent of using these data as part of the evaluation.

NOTE 2—*Yarrowia* readily outgrows *Hormoconis* in Sabouraud making distinction of both groups difficult, if not impossible. *Hormoconis resinae* is able to grow in a simple, unsupplemented agar, albeit slowly (about 5 days incubation with a tellurite reduction as an indicator of growth). Under these minimal nutritional conditions, the potassium tellurite may also be inhibitory to the yeast.

11. Precision and Bias

11.1 It is not practical to specify the precision of the procedure in Test Method E 1259 because detection and enumeration of microorganisms is subjective and not absolute. Since there is no accepted reference material suitable for the procedure in Test Method E 1259, bias has not been determined.

12. Keywords

12.1 antimicrobials; aviation fuels; biodeterioration; diesel; distillate fuels; gasoline; gas-turbine fuels; marine fuels; microbially-induced deterioration

APPENDIX

(Nonmandatory Information)

X1. ALTERNATIVE PROCEDURES

X1.1 Setups using 1-L French squares or other suitable container, can substitute for separatory funnels. Although the interface is not so readily discerned, the bottom water can be removed by careful pipetting.

X1.2 *Fuel*—Potentially, the inclusion of additives may not only increase the growth rate of the inoculum but may also affect the efficacy of the biocide. In any specific testing for fuel suppliers, it would be unrealistic to exclude these additives from the study (see also Practice D 4054).

X1.3 *Bottom Water Level and Time of Storage*—The test described here does not consider either long term storage with minimal bottom water (for example, 8 weeks at 0.25 % water) or shock dosing a heavily contaminated system with a water soluble biocide, or bleed off of bottom water with loss of biocide.

X1.4 *Corrosion*—There is provision for use of metal coupons for both evaluation of biocide corrosivity and microbial-induced corrosion.

X1.5 *Inoculum*—For specific testing, it may be advisable to use contaminated fuel as an inoculum after determination of the identity of the contaminant.

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Designation: E 1326 – 98

Standard Guide for Evaluating Nonconventional Microbiological Tests Used for Enumerating Bacteria[1]

This standard is issued under the fixed designation E 1326; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 The purpose of this guide is to assist users and producers of nonconventional tests in determining the applicability of the test for processing different types of samples and evaluating the accuracy of the results. Conventional procedures such as the Heterotrophic (Standard) Plate Count, the Most Probable Number (MPN) method and the Spread Plate are widely cited and accepted for the enumeration of microorganisms. However, these methods have their limitations, such as performance time and degree of accuracy. It is these limitations that have recently led to the marketing of a variety of non-conventional procedures, test kits and instruments.

1.2 A conventional test is one that is widely accepted and published as a standard microbiological method or related procedure. A new, nonconventional test method will attempt to provide the same information through the measurement of a different parameter. This guide is designed for comparing levels of bacteria recovered from samples by the Heterotrophic Plate Count Procedure to the equivalent units determined with a nonconventional test.

1.3 It is recognized that the Heterotrophic Plate Count does not recover all microorganisms present in a product or a system[2] (**1**). When this problem occurs during the characterization of a microbiological population, alternate standard enumeration procedures may be necessary, as in the case of sulfate-reducing bacteria. At other times, chemical methods that measure the rates of appearance of metabolic derivatives or the utilization of contaminated product components might be indicated. In evaluating nonconventional tests, the use of these alternate standard procedures may be the only means available for establishing correlation. In such cases, this guide can serve as a reference for those considerations.

1.4 Since there are so many types of tests that could be considered nonconventional, it is impossible to recommend a specific test protocol with statistical analyses for evaluating the tests. Instead, this guide should assist in determining what types of tests should be considered to verify the utility and identify the limitations of the nonconventional test.

2. Referenced Documents

2.1 *ASTM Standards:*

D 3870 Practice for Establishing Performance Characteristics for Colony Counting Methods in Bacteriology[3]

D 5245 – 92 Practice for Cleaning Laboratory Glassware, Plasticware and Equipment used in Microbiological Analysis[3]

D 5465 – 93 Practice for Determining Microbial Counts From Waters Analyzed by Plating Methods[3]

E 691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method[4]

3. Summary of Guide

3.1 ASTM standard practices are referenced for use by producers and users to determine the potential utility of the nonconventional test. Users of tests who are unequipped for performing standard microbiological tests are given recommendations for seeking out microbiological laboratories that could perform collaborative studies to evaluate and verify the information generated with the nonconventional tests.

4. Significance and Use

4.1 This guide should be used by producers and potential producers of nonconventional tests to determine the accuracy, selectivity, specificity, and reproducibility of the tests, as defined in Practices E 691 and D 3870. Results of such studies should identify the limitations and indicate the utility or applicability of the nonconventional test, or both, for use on different types of samples.

4.2 Nonconventional test users and potential users should employ this guide to evaluate results of the nonconventional test as compared to their present methods. Practices D 5245 and D 5465 should be reviewed in regards to the conventional microbiological methods employed. If conventional methods have not been used for monitoring the systems, then guidelines are included for obtaining microbiological expertise.

4.3 Utilization of a nonconventional test may reduce the time required to determine the microbiological status of the system and enable an improvement in the overall operating efficiency. In many cases, the findings of a significantly high level of bacteria indicates the need for an addition of an

[1] This guide is under the jurisdiction of ASTM Committee E-35 on Pesticides and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this guide.

[3] *Annual Book of ASTM Standards*, Vol 11.02.

[4] *Annual Book of ASTM Standards*, Vol 14.02.

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antimicrobial agent. By accurately determining this in a shorter time period than by conventional methods, treatment with antimicrobial agents may circumvent more serious problems than if the treatment were postponed until conventional results were available. If the antimicrobial treatment program relies on an inaccurate nonconventional test, then unnecessary loss of product and problems associated with inappropriate selection or improper dosing with antimicrobial agents would exist.

4.4 Since many methods based on entirely different chemical and microbiological principles are considered, it is not possible to establish a unique design and recommend a specific method of statistical analyses for the comparisons to be made. It is only possible to present guides that should be followed while performing the experiments. It is also recommended that a statistician be involved in the study.

5. Procedures

5.1 In order to determine the utility of the nonconventional test, evaluate and compare the results to those obtained with a previously accepted standard method. Often, the Heterotrophic Plate Count is entirely satisfactory for this purpose (**2**); however, understand its limitations before it is used as the basis for evaluating methods that measure other parameters indicative of microbial life (metabolic activity, concentration of cell constituents, or whole cell numbers). The variety of methods used for the Heterotrophic Plate Count are listed in Table 1. When this method is not a suitable standard, use alternative standard enumeration methods or methods for measuring the rate of the appearance of derivatives or the rate of disappearance of components of the product in which the microbial contamination is being measured—where such phenomena are known to be correlated to microbial contamination levels. No single method is universally applicable; consequently, it is imperative to determine the rationale for employing any given measurement procedure and to select a standard that will permit the determination of whether or not the nonconventional method achieves the objectives defined in the scope of the procedure.

5.2 A knowledge of standard microbiological technique is required for this procedure. If that expertise is not currently available in-house, then consult an outside testing laboratory. Many industrial microbiology laboratories are certified for the analysis of drinking water by the EPA or the state government (a listing of these laboratories can be obtained from the regional EPA office or the state government). There are also other microbiology laboratories that specialize in processing samples from different industries; these are often listed as "Laboratories—Testing" in the telephone book. It is important that this document be referenced when undertaking an evaluation with an outside laboratory.

5.3 For each method, first make an enumeration of all major sources of variability. For example, if a nonconventional test method is involved and if more than a single analysis can be conducted with a single test, consider the variability within and between tests. For plates, it is important to consider the variability between plates obtained from aliquots of the same sample. It is also important to prepare samples covering the entire range of values (for example, counts per milliliter) of interest. Each such value is referred to as a level. Thus, the levels must cover the range of interest.

5.4 At each level, analyze replicate samples, both by the method under study, and by the standard method. The number of replicates depends on the number of sources of variability. Thus, in the previous-mentioned example of nonconventional test, it would be advisable to analyze at each level at least two replicates of each (preferably more) in at least two nonconventional tests (preferably more). At the same time, analyze replicates by the Heterotrophic Plate Count, resulting in several replicate plates. The scheme shown in Table 2 illustrates such a procedure; in this case, three replicates are analyzed at any given level using three nonconventional tests, while five replicate plates are counted by the Heterotrophic Plate Count. (These numbers will vary according to the method.)

5.5 Using the example of Table 2, the data of the new method would be analyzed and compared with the Heterotrophic Plate Count method for determining precision, as well as (*1*) within-test variability; (*2*) between-test variability; and (*3*) between-plate variability.

5.6 Again, using the example of Table 2, the nine values by the new method and the five values by the Heterotrophic Plate Count are averaged for all levels and then plotted. A curve, using appropriate statistical procedures, must then be fitted to these points. This curve is the calibration line of the new method versus the Heterotrophic Plate Count, and it can be

TABLE 1 Comparison of Selected Heterotrophic Plate Count Procedures for Samples from Various Sources

	Water 5	Dairy 6	Environment 7	Food 8	Cosmetic 8	Paper 9	Pharmaceutical 10
Media	TGE, SM, R2A or m-HPC	SM	SM or TGE	SM	ML	TGE	SCD
Dilution, H_2O	KH_2PO_4+ $MgCl_2$	KH_2PO_4	KH_2PO_4	KH_2PO_4	MLB	H_2O	KH_2PO_4
Incubation, °C	35 ± 0.5 20 or 28 (R2A)	32 ± 1	35 ± 0.5	35	30 ± 2	36 ± 0.5	30–35
Incubation, h	48 ± 3 72 ± 4 (bottled water) 72–168 (R2A medium)	48 ± 3	48	48 ± 2	48	48	48–72
Amount of Agar, mL	10–12 (Pour Plate) 15 (Spread Plates) 5 (Membrane Filter)	10–12	10+	12–15	Spread Plates	15–20	15–20

TGE = Tryptone Glucose Extract Agar
SM = Standard Methods Agar (Tryptone Glucose Yeast Agar)
ML = Modified Letheen Agar
MLB = Modified Letheen Broth
SCD = Soybean Casein Digest Agar
R2A = Low-Nutrient Media (which may not be available in dehydrated form)
m-HPC = Formerly called m-SPC Agar (used for membrane filtration)

TABLE 2 Scheme for Analysis at a Given Level

Test	New Method		Heterotrophic Plate Count	
	Replicate Within Test	Determination	Plate	Determination
1	1	1	1	1
	2	2	2	2
	3	3	3	3
			4	4
			5	5
2	1	4		
	2	5		
	3	6		
3	1	7		
	2	8		
	3	9		

used to convert values obtained by the new method into equivalent units of the Heterotrophic Plate Count.

6. Report

6.1 The standard deviations obtained by the new method can be converted, by appropriate statistical procedures, into equivalent units of the standard method by using the calibration line for conversion. A comparison with the standard method can then be made to determine the precision of the new method.

6.2 In view of the complexity of the problem and variety of situations that can arise, it is not possible to recommend further procedures and statistical methods, or both. A more detailed discussion of statistical methods may be found in the *Statistical Manual of the Association of Official Analytical Chemists* (**3**) and in Chapter 14, "The Comparison of Method of Measurements," of *The Statistical Analysis of Experimental Data* (**4**).

7. Precision and Bias

7.1 A precision and bias statement cannot be made for this guide.

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(**3**) Youden, W. J., and Steiner, E. H., *Statistical Manual of the Association of Official Analytical Chemists*, Second Printing, Association of Official Analytical Chemists, Arlington, VA 22209, 1979.

(**4**) Mandel, J., *The Statistical Analysis of Experimental Data*, Dover, 1984.

(**5**) "Standard Methods for the Examination of Water and Wastewater," American Public Health Association, New York, NY, 19th ed., 1995 or most current.

(**6**) "Standard Methods for the Examination of Dairy Products," American Public Health Association, New York, NY, 16th ed., 1993 or most current.

(**7**) "Microbiological Methods for Monitoring the Environment," Environmental Monitoring and Support Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio, *EPA 600/8-78-017*, December 1978.

(**8**) FDA Bacteriological Analytical Manual, Food and Drug Administration Staff, 1995, AOAC International, Arlington, VA, 8th ed., or most current.

(**9**) "Microbiological Examination of Process Water and Slush Pulp," (proposed review of Official Method T631 om-79), Technical Association of the Pulp and Paper Industry, Technology Park, Atlanta, GA, April 5, 1984, or most current.

(**10**) "Microbial Limits-Total Aerobic Microbial Count," *U.S. Pharmacopoeia XXIII-National Formulary*, U.S. Pharmacopoeia Convention, Inc., Rockville, MD, 1995 or most current.

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IP IP 385/99

Determination of the viable aerobic microbial content of fuels and fuel components boiling below 390 °C — Filtration and culture method

This standard does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

Foreword

Knowledge of microbiological techniques is required for the procedures described in this standard.

1 Scope

This standard describes two procedures for the determination of the viable microbial content of fuels and fuel components boiling below 390 °C. Procedure A is suitable for enumeration of viable microbial units up to 25 000 per litre. Procedure B is suitable for enumeration of viable microbial units above 25 000 per litre.

The procedures employ two selective microbiological growth media which nominally allow separate enumeration of bacteria and fungi. But as the media are not exclusively selective, it is possible that the determination of viable microbial units from the medium which favours bacterial growth will also include some fungi and vice versa. The microbiological procedures for distinguishing bacterial growth and for distinguishing between yeasts and moulds within the fungal content are not within the scope of this standard.

NOTE 1 If the approximate contamination level is unknown it is recommended both procedures are carried out.

NOTE 2 Some of the principles described in procedure B can also be used to determine the viable microbial content of water associated with the fuel. However procedures to determine the microbial content of water are not given in this standard.

2 Normative references

The following document contain provisions which, through reference to this text, constitute provisions of this standard. At the time of publication, the edition indicated was valid. All standards are subject to revision, and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below.

Institute of Petroleum's *Guidelines for the investigation of microbial content of fuel boiling below 390 °C and associated water,* 1996.

3 Principle

After separation of any water phase, known volumes of fuel are filtered aseptically through membrane filters. Viable microorganisms collected on the filters are assayed by either placing the filters directly onto agar growth media or eluting the micro-organisms, diluting the eluent if necessary, and placing aliquots of the eluent and / or dilutions of the eluent onto agar growth media. After incubation the number of colonies are counted and from these the number of viable bacteria and fungi present in a given volume of the original fuel sample are calculated.

4 Materials and reagents

Unless otherwise specified, use only reagents of recognised analytical grade and only distilled or deionized water.

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4.1 ¼ Strength Ringer's Solution

Sodium chloride	2,25 g
Potassium chloride	0,105 g
Calcium chloride $6H_2O$	0,12 g
Sodium bicarbonate	0,05 g
Water	1 l

Dissolve salts in 1 l of distilled or deionized water and sterilise by placing in an autoclave (5.19) at 121 °C ± 2 °C for 15 min. Dispense 10 ml aliquots aseptically into sterile bottles (5.17).

NOTE 3 ¼ Strength Ringer's salts are available in tablet form from various manufacturers.

4.2 Detergent solution

Polyoxyethylene(20)sorbitan monooleate (Tween 80 ®[1]) - 0,1% (*V/V*) aqueous solution sterilised by passing through a 0,2 µm membrane filter (5.21) into a sterile vessel, or placing in an autoclave (5.19) at 121 °C ± 2 °C for 15 min.

4.3 Alcohol

Ethanol, Propan-2-ol or Industrial Methylated Spirit, if necessary sterilise by passing through a 0,2 µm membrane filter (5.21) into a sterile vessel.

4.4 Malt Extract Agar (MEA)

The pH of this medium favours the growth of fungi (moulds and yeasts) over that of bacteria.

Malt Extract	30 g/l
Mycological Peptone	5 g/l
Agar	15 g/l

Suspend 50 g of the MEA in 1 l of distilled water and boil to dissolve. Adjust the pH to 5,4 ± 0,2 using either 1 mol/l hydrochloric acid (4.6) or sodium hydroxide 10% (*m/V*) (4.7). Place in an autoclave at 115 °C ± 2 °C for 10 min. Cool to approximately 50°C and pour approximately 20 ml into a number of 90 ml Petri dishes (5.7) and allow to cool.

Take one Petri dish containing the MEA at 20°C - 30°C and using a flat surface electrode and pH meter check that the pH is in the range 5,4 ± 0,2. If the reading is outside this range reject the batch and make a fresh mixture.

NOTE 4 The addition of an antibiotic, chlortetracycline, will inhibit bacterial growth. Alternatively further lowering the pH of the medium to 3,5 - 4,0 with lactic acid (4.9) will improve inhibition of bacterial growth.

If the medium is required at pH 3,5 cool to 47 °C and acidify with 10 % lactic acid. Once acidified do not re-heat the medium.

If the pH 5,4 medium is required to be inhibited with an antibiotic, add 1 ml of a 0.1 % aqueous solution of chlortetracycline (filter sterilised) per 100 ml of MEA, mix by shaking and immediately pour into the Petri dishes.

NOTE 5 MEA is available in dehydrated form from various manufacturers. If such material is used follow the manufacturers instructions regarding sterilization. Prepoured plates, with or without added antibiotic, can be purchased.

NOTE 6 An alternative medium to MEA can be used, providing its ability to promote comparable growth of yeasts and moulds which are likely to be encountered in tested samples can be demonstrated.

NOTE 7 Alternative antibiotics may be used providing their ability to inhibit growth of bacteria but not yeasts and moulds has been validated.

4.5 Tryptone Soya Agar, TSA (Soya bean Casein Digest Agar)

This medium favours the growth of bacteria. Yeasts and moulds may also develop but usually not so luxuriantly as on the selective MEA medium.

Tryptone	15,0 g/l
Soya peptone	5,0 g/l
Sodium chloride	5,0 g/l
Agar	15,0 g/l

Suspend 40 g of the TSA in 1 l distilled water. Bring to the boil and dissolve completely. Sterilize by placing in an autoclave (5.19) at 121 °C ± 2 °C for 15 min. Pour approximately 20 ml into 90 ml Petri dishes (5.7) and allow to cool. Take one Petri dish containing the TSA at approximately 20°C - 30°C and using a flat surface electrode and pH meter check the pH. If this is found to be

1) Tween is the registered trademark of Atlas Chemical Industries Inc.

outside the required range of pH (7,3 ± 0,3) reject the batch and make a fresh mixture.

NOTE 8 TSA is available in dehydrated form from various manufacturers. If such material is used follow the manufacturers instructions regarding sterilization. Prepoured plates can be purchased.

NOTE 9 Alternative media to TSA can be used, providing their ability to promote comparable growth of bacteria which are likely to be encountered in tested samples can be demonstrated.

4.6 Hydrochloric acid, 1 mol/l.

4.7 Sodium hydroxide, 10 % (*m/V*) aqueous

4.8 Chlortetracycline (optional), 0,1 % (*m/V*) aqueous sterilized by passing through a 0,2 µm filter (5.21).

4.9 Lactic Acid (optional), 10 % (*m/V*) aqueous sterilized by passing through a 0,2 µm filter (5.21).

5 Apparatus

5.1 Measuring cylinders, glass, nominal capacity 100 ml and 1 l.

5.2 Pipettes, glass or sterile disposable plastic, nominal capacity 1 ml with 0.1 ml graduations, and nominal capacity 10 ml, or adjustable volume pipettor and sterile disposable plastic tips.

5.3. Mixed esters of cellulose membrane filters, presterilized, preferably gridded, 47 mm diameter, nominal pore size 0,45 µm.

NOTE 10 Whilst the recommended filter material is mixed esters of cellulose the selection of membrane material will depend on individual preference and fuel type.

5.4 Filter holder assembly, single or manifold.

5.5 Filter flask, of sufficient capacity to receive all the sample being filtered and the washings.

5.6 Vacuum source, not more than 66 kPa vacuum.

5.7 Petri dishes, disposable plastic or glass, sterile, nominal diameter 90 mm.

5.8 Forceps, blunt tipped.

5.9 Incubator, capable of maintaining a temperature of 25 °C ± 2 °C or any other temperature, as appropriate, ± 2 °C.

5.10 pH meter and flat pH electrode

5.11 Scalpel or scissors

5.12 Glass beaker and cover, nominal capacity 500 ml.

5.13 Gas burner

5.14 Spreading rod, glass.

5.15 Conical flask, glass 2 l capacity.

5.16 Vortex mixer

5.17 Universal bottles, glass, screw capped, 30 ml nominal capacity.

5.18 Aluminium foil

5.19 Autoclave, capable of maintaining a temperature 115 °C ± 2°C and 121 °C ± 2°C.

5.20 Oven, capable of maintaining a temperature of 170°C ± 5°C.

5.21 Membrane filter (optional), for sterilizing liquids, nominal pore size 0,2 µm.

6 Apparatus sterilization

6.1 Glass apparatus (5.1, 5.2, and 5.14)

Cover orifices with aluminium foil or place in a sterilizing can as appropriate and place in an oven (5.20) and sterilise at 170 °C ± 2 °C for 1 h or place in an autoclave (5.19) at 121 °C ± 2 °C for 15 min. If autoclaved ensure that the glassware is dry before use. Plug mouthpieces of pipettes with non-absorbent cotton wool.

6.2 Glass bottles (5.17)

Loosen caps and place in an autoclave (5.19) at 121 °C ± 2 °C for 15 min.

6.3 Filter assembly

The flask which receives the filtered fuel and wash solutions need not be sterilized.

Do not sterilise complete assembly with membrane filter in place as this can lead to distortion or cracking of the membrane.

Either:

a) Cover orifices with aluminium foil and sterilise in an oven (5.20) at 170°C ± 2°C for 1 h or,

b) Place the apparatus in an autoclave (5.19) at 121°C ± 2°C for 15 min and dry before use.

6.4 Forceps, scalpel, scissors and glass spreading rod

Place in a covered glass beaker (5.12) containing sufficient alcohol (4.3) to cover the working ends of these instruments. Immediately prior to use remove the instrument from the alcohol and pass the working end through a burner flame. After use return the instrument to the alcohol.

CAUTION Alcohol is highly flammable. Care shall be taken to prevent the ignition of the alcohol contained in the beaker.

6.5 Plastic disposable pipette tips

Place in a suitable rack or holder, cover and place in an autoclave (5.19) at 121 °C ± 2 °C for 15 min.

7 Sampling

Guidance on how to draw and store samples for microbial testing is given in the Institute of Petroleum's *Guidelines for the investigation of microbial content of fuel boiling below 390 °C and associated water.*

8 Sample preparation

8.1 Allow sample to stand for 1 h and then examine visually.

8.2 If the sample contains free water allow it to settle and then separate the water phase and associated particulate matter by pipetting from the bottom of the sample bottle.

NOTE 11 Further analysis by microscopy and conventional microbiological culture techniques can be conducted on the water phase and associated particulate matter if required.

8.3 Shake the fuel phase of sample.

8.4 Sub-sample test portions of the fuel phase using a sterile pipette (5.2) for quantities up to 10 ml or sterile measuring cylinders (5.1) for larger quantities.

9 Procedure

9.1 Sample filtration

Place a sterile 0,45 µm pore filter (5.3) on the filter support using sterile forceps (5.8). Assemble the filter holder (5.4). Apply suction and filter the test portion through the membrane filter.

For procedure A either filter two test portions (see notes 12 and 13) through two filters or, after filtration and rinsing of a single test portion through one filter, divide the filter into two.

For procedure B filter a single test portion through one filter (see notes 12 and 13).

Record the volume of fuel filtered.

NOTE 12 It is recommended that aliquots of 50 ml are filtered; however the choice of volumes will be dictated by volume of the sample and the level of contamination expected and the filterability of the fuel. Filtration of larger sample volumes will increase test sensitivity and hence is recommended for fuels which require a high standard of microbial cleanliness such as aviation kerosene.

NOTE 13 When an adequate quantity of fuel is available, the test should be carried out at least in triplicate and if possible a greater number of replicates made.

9.1.1 Filter detergent wash

Maintaining suction, wash the membrane filter free of fuel with a 10 ml aliquot of sterile detergent solution (4.2).

9.1.2 Filter rinse

Whilst maintaining suction, wash the membrane filter free of detergent solution with three successive 10 ml portions of sterile 3 Strength Ringer's solution (4.1).

9.1.3 Remove the suction, dismantle the filtration apparatus carefully and using sterile forceps, remove the filter. Either divide the filter into two or use the whole filter and proceed in accordance with 9.2 or 9.3.

9.2 Procedure A — Placing filters directly on agar growth media

If prepoured plates are to be used examine for the presence of microbial colonies before use. Reject any which show evidence of microbial growth. Also examine plates for free moisture. If free moisture is present dry the plates before use by either leaving them unstacked on the laboratory bench for 1 h or place the plates unstacked in an incubator at 37 °C ± 2 °C until dry.

Transfer either the two membrane filters, or the two halves of the single filter, exposed surface up, onto the surface of the MEA (4.4) and TSA media (4.5) in the Petri dishes. Ensure good contact between membrane filter and medium.

9.3 Procedure B — Elution of micro-organisms

9.3.1 Elution of microorganisms from membrane filter

If prepoured plates are to be used examine for the presence of microbial colonies before use. Reject any which show evidence of microbial growth. Also examine plates for free moisture. If free moisture is present dry the plates before use by either leaving them unstacked on the laboratory bench for 1 h or place the plates unstacked in an incubator at 37 °C ± 2 °C until dry.

Using sterile forceps transfer the membrane filter to a sterile Petri dish. Cut the membrane filter into strips using a sterile scalpel or scissors and use the sterile forceps to transfer the strips to 10 ml of sterile 3 Strength Ringer's solution (4.1) in a sterile Universal bottle (5.17).

Mix the filter strips in the eluent using a vortex mixer (5.16) for 30 s to elute microorganisms from their surface.

9.3.2 Transfer of eluted microorganisms to culture media

If prepoured plates are to be used examine for the presence of microbial colonies before use. Reject any which show evidence of microbial growth. Also examine plates for free moisture. If free moisture is present dry the plates before use by either leaving them unstacked on the laboratory bench for 1 h or place the plates unstacked in an incubator (5.9) at 37 °C ± 2 °C until dry.

If required make ten-fold serial dilutions in sterile 3 Strength Ringer's solution of the eluent.

Using a pipette (5.2) place 0,1 ml of the mixed eluent, and serial dilutions if prepared, onto Petri dishes containing the MEA (4.4) medium and the TSA (4.5) medium and using a freshly flamed glass spreading rod (5.14) spread the eluent and each serial dilution onto the MEA and TSA media.

NOTE 14 Replicating the procedure will improve the precision.

9.4 Incubation of Agar Media

9.4.1 Place the dishes in an incubator (5.9) controlled at 25 °C ± 2 °C for 5 days. Invert Petri dishes containing TSA.

NOTE 15 It is recommended that the dishes are examined for growth after 3 days and again after 5 days. This should ensure that the slow developing colonies are not missed and that small colonies are not missed through overgrowth.

NOTE 16 The incubation temperature should reflect the temperature at which microbial proliferation may occur in the sampled fuel system. 25 °C is suitable for most ambient systems but an appropriately higher incubation temperature can be used when the temperature of the system sampled exceeds 30 °C.

9.4.2 After the allotted incubation period examine the dishes and record the number of colony forming units on the TSA and MEA media. Do not agitate the plates or remove the lids whilst examining. If procedure A has been followed and the colonies can not be differentiated either repeat the test using procedure B or take a smaller sample.

NOTE 16 If practicable, colonies on each medium should be identified by colour, morphology and microscopic examination; colony counts of bacteria,

yeasts and moulds can then be recorded separately. Because some yeasts grow well on the TSA medium (or alternative bacterial media) as well as on the MEA medium it is advisable to identify colony types on TSA by microscopy to determine whether they are yeasts or bacteria. If yeasts do grow on TSA, yeast colonies should be counted on MEA and TSA and the highest colony counts used to calculate numbers per litre as described in 10.1 or 10.2 below.

Recommendations for optimum colony numbers and colony count confidence limits are given in annex A.

10 Calculation

10.1 Procedure A

Calculate the number of colony forming units per litre, *N*, in the sample from the colony counts on the TSA plates, and the fungi per litre in the sample from the colony counts on the MEA plate (see note 16) using the following equation:

$$N = \frac{CC \times 1000}{V}$$

where:

CC is the colony count (see 9.4.2);

V is the volume of the fuel filtered, in millilitres.

If for each medium duplicate aliquots were filtered, average the results of the duplicate estimations. If only halves of membrane filters were used for Procedure A multiply the colony count by two.

10.2 Procedure B

Calculate the number of colony forming units per litre, *N*, in the sample from the colony count on the TSA plate, and the fungi from the colony count on the MEA plate (see note 16) using the following equation:

$$N = \frac{CC \times 10^5 \times DF}{V}$$

where:

CC is the colony count, average of replicate plates (see 9.4.2);

DF is the dilution factor of the eluent (if no dilution of the eluent is made then DF = 1);

V is the volume of the fuel filtered, in millilitres.

If for each medium duplicate aliquots were filtered, average the results of the duplicate estimations.

11 Expression of results

Report the number of colony forming units as counts per litre.

12 Test report

The test report shall contain at least the following information:

a) a reference to this standard;

b) the result of the test (see clause 11);

c) sufficient detail to identify the fuel tested;

d) any deviation, by agreement or otherwise, from the procedure specified;

e) any unusual observations before, during or after testing;

f) the date of the test.

Annex A
(informative)

Optimum Colony Counts and Colony Count Confidence Limits

A.1 General

The accuracy of culture methods for the enumeration of microbes can be poor and is affected by both determinable and indeterminable factors. A principle indeterminable factor is the heterogeneity of microbial distribution in the material being sampled. The principle determinable inaccuracy is dependent on the number of colonies on a plate. This inaccuracy decreases as the number of colonies on the plate increases, up to a limit when overcrowding effects inhibit growth and/or the user can no longer discern separate colonies. Another factor is the inability of some organisms to grow on the enumeration media. Whilst techniques are employed to keep the determinable inaccuracies to a minimum, the precision that can be expected for the analysis of dissolved chemical species is not possible for this test.

A.2 Optimum colony counts

It is recommended that plates or filters containing less than 20 colonies or more than 300 colonies should not be counted. However this upper limit for colony counts is dependent on the ability of the user to discern individual colonies. Provided that a sufficiently large volume is filtered and that both Procedures A and B are used, it will usually be the case that at least one assay plate will have a colony count within the recommended range.

Where microbial contamination is low the use of plates containing less than 20 colonies may be unavoidable. In such cases it should be appreciated that accuracy and precision will be low.

A.3 Colony count confidence limits

The precision of the test is dependent on the number colonies that form on the agar plate and may be indicated by quoting 95% confidence limits. These limits define the range within which, with a 95% probability, the true colony count lies. The confidence limits for counts of colonies obtained when a single sample is placed on an agar plate or, passed through a membrane filter are given in Table A.1 below. This assumes that the distribution of organisms within the fuel sample or the aqueous extract is random and conforms to a Poisson series.

Table A.1 — 95% confidence limits

Number of colonies counted	95% confidence limit
200	172 - 228
100	80 - 120
80	62 - 98
50	36 - 64
30	19 - 41
20	11 - 29
16	8 - 24
10	4 - 16
6	1 - 11

Increased precision may be achieved by preparing three or more replicate plates from the fuel and calculating the mean colony count (see notes 13 and 14). The 95 % confidence limits for the mean of the replicates may be determined by standard statistical techniques.

Determination of fungal fragment content of fuels boiling below 390 °C

This standard does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations.

FOREWORD A knowledge of microbiological techniques is required for the procedures described in this standard.

1 Scope

This standard describes a method for the collection of fungal fragments contained in a sample and an estimation of their number.

NOTE 1 The method may also be used to assist in the diagnosis of the fragments, see annex A.

2 Principle

A known volume of fuel is filtered through either a 0,45 μm or a 0,8 μm membrane filter marked with a grid of 3 mm squares. The number of fungal fragments are counted and from the number present on the counted grids and the volume of fuel filtered the fungal fragment content per litre is calculated.

3 Apparatus

3.1 Filter holder assembly.

3.2 Filter flask, of sufficient capacity to receive all the sample being filtered.

3.3 Membrane filters, cellulose ester, 47 mm diameter, white, 0,45 μm or 0,8 μm pore size, marked with a square grid.

3.4 Stoppered glass measuring cylinder, 100 ml nominal capacity.

3.5 Microscope.

3.6 Microscope slides and covers.

3.7 Separating funnel glass, 500 ml capacity.

3.8 Glass beaker, 250 ml capacity.

3.9 Screw capped bottle, 500 ml capacity.

4 Membrane filter calibration

Measure the diameter of the filter holder and calculate the filtration area. Calculate the membrane factor, C_{f}, using the following equation.

$$C_{\mathrm{f}} = A_{\mathrm{t}}/A_{\mathrm{s}}$$

where

A_{t} is the total filtration area, in square millimetres;

A_{s} is the single grid area, in square millimetres.

5 Sample preparation

5.1 Examine and record the appearance of the sample as received.

5.2 If no water is observed proceed in accordance with 5.3. If free water is present either:

a) pour the sample into a sterile separating funnel (3.7) and run off the water into a sterile screw capped bottle (3.9) for further examination, if required, then return the sample to its original container;

or,

b) decant the fuel direct into the stoppered measuring cylinder (3.4).

5.3 Shake the sample and disperse any sediment which may have settled to the bottom.

6 Procedure

6.1 Assemble the filter holder (3.1) and connect it to the filter flask (3.2).

6.2 Aseptically place the membrane filter (3.3) in the filter holder.

6.3 Shake the sample and pour 100 ml into the stoppered measuring cylinder.

6.4 Apply suction to the filter.

6.5 Pour the contents of the measuring cylinder through the membrane filter, shaking the contents of the measuring cylinder before each addition.

6.6 Shake the sample and refill the measuring cylinder and repeat the filtering operation until either 500 ml of sample has been filtered or the filtration rate becomes very slow. Record the amount of fuel filtered.

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NOTE 2 Some fuels have a high fungal fragment count and this can result in less than 100 ml of sample being filtered.

6.7 Continue to apply suction until the filter membrane is substantially fuel free.

6.8 Disconnect the suction and remove the filter membrane.

6.9 Place the filter membrane onto the microscope slide (3.6). Wet with one to two drops of filtered fuel, cover with a cover slip (3.6) and place on the microscope stage (3.5).

6.10 Proceed in accordance with one of the following, as applicable.

a) Scan a 3 × 3 grid of nine squares for fungal fragments at a magnification of 250 to 400 and record the number and nature of the fungal fragments present in the nine grid squares. Include those fragments which lie across the top and right-hand boundary but exclude those which lie across the bottom and left-hand boundary.

b) When a high number of fungal fragments are present, scan adjacent entire grid squares until a minimum of 100 fungal fragments are counted. Record the number and nature of the fungal fragments present and the number of grid squares counted.

NOTE 3 Filter membranes may be cut to aid microscope slide mounting.

7 Calculation

Calculate the fungal fragments content per litre, F, using the following equation:

$$F = \frac{F_c C_f}{N_g V}$$

where

C_f is the membrane filter conversion factor;

F_c is the fragment count for N_g grid squares;

N_g is the number of grid squares counted;

V is the volume of fuel filtered (see 6.7), in litres.

8 Expression of result

Report the fungal fragments per litre to the nearest 10 fragments. If applicable give an indication of their viability (see annex A).

9 Precision

The precision of this method has not been determined.

10 Test report

The test report shall contain at least the following information:

a) sufficient detail to identify the fuel tested;

b) a reference to this standard;

c) the result of the test (see clause 8);

d) the date of the test;

e) the appearance of the sample as received (see 5.1);

f) the appearance of the fragments (see annex A);

g) any deviation, by agreement or otherwise, from the procedure specified.

Annex A (informative)

Interpretation of result

The appearance of fungal fragments can provide additional information.

Small broken fragments can be considered to be derived from old, dying and disintegrating mycelia, which may have been in the fuel for some time.

The presence of larger, branching filaments are often viable and indicate recent detachment from an active mycelium growing in the water bottom or on the wall of the tank from which the sample was taken.

Fragments other than those of fungal hyphae may be found in some fuel samples. Examples of these are paper, glass fibre, rock-wool, cotton, polypropylene, rayon and wool. The operator should be able to distinguish fungal material from these other possible contaminants.

Glossary

Frederick J. Passman, Ph.D.[1]

RECOGNIZING THAT MANY OF this Manual's readers may be unfamiliar with microbiological and filtration terms used in Chapters 1, 2, and 3, this glossary has been compiled as a quick reference. Additional definitions may be found in the *Terminology* section of each of the ASTM standards compiled in this Manual. In particular, Guide D 6469 *Microbial Contamination in Liquid Fuels and Fuel Systems* provides definitions for 30 terms relevant to the discussion of microbial contamination.

Where possible, the definitions provided in this glossary were drawn from the ASTM Dictionary of Engineering, Science, and Technology. The source standard and responsible committee are listed after each definition. Several definitions are drawn from other sources. When this was done, the source was identified after the definition. Finally, the author conjured or embellished definitions for a few terms, or added as *discussion*. These definitions are identified with the author's initials: FJP.

alga (pl. *algae*), *n.*–any of a group of chiefly aquatic mono cellular plants with chlorophyll often masked by a brown or red pigment. **D 6161**, D19

antimicrobial pesticide, *n.*–chemical additive registered under 40CFR152, for use to inhibit growth, proliferation or both of microorganisms. (Synonyms: biocide, microbicide) **E 2169**, E35

anoxic, *adj.*–oxygen free. **D 6469**, D02

bacterium (pl. *bacteria*), *n.*–a simple, single cell microorganism characterized by the absence of defined intracellular membranes that define all higher life forms.

Discussion—All bacteria are members of the biologically diverse kingdoms: *Prokaryota* and *Archaebacteriota* (recently assigned kingdom status as the Archaea). Individual taxa (phyla, families, genera, species and strains) within these kingdoms are able to thrive in environments ranging from sub-zero temperatures such as in frozen foods and polar ice, to superheated waters in deep-sea thermal vents, and over the pH range < 2.0 to > 13.0. Potential food sources range from single carbon molecules (carbon dioxide and methane) to large hydrocarbons and complex polymers, including plastics. Oxygen requirements range from obligate anaerobes, which die on contact with oxygen, to obligate aerobes, which die if oxygen pressure falls below a minimum threshold which is species specific. **D 6469**, D02

Beta-ratio (β_x where x = particle size), *n.*–the ratio of number of particles of known size ($> x\ \mu m$) entering a filter to the number of those particles passing through that filter.

Discussion–for example if 500 particles $\geq 10\ \mu m$ diameter are filtered and 50 pass through the filter, $\beta_{10\mu m} = 10$. Filter performance, in terms of particle retention, increases as the beta-ratio increases.

FJP adapted from *Filtration Technology*, Parker Filtration, Cleveland OH, 1997, pp. 1–284.

biocide, *n.*–a poisonous substance that can kill living organisms. **D 6469**, D02

biodeteriogen, *n.*–an organism capable of causing biodeterioration.

http://www.rocmaquina.es/ingles/Publications/technic/biodeterioration.htm

biodeterioration, *n.*–the loss of commercial value and/or performance characteristics of a product (fuel) or material (fuel system) through biological processes. **D 6469**, D02

biofilm, *n.*–a film or layer of microorganisms, biopolymers, water, and entrained organic and inorganic debris that forms as a result of microbial growth and proliferation and proliferation at phase interfaces (liquid-liquid, liquid-solid, liquid-gas, etc.). (synonym: *skinnogen layer*) **D 6469**, D02

biomass, *n.*–density of biological material per unit sample volume, area or mass (g biomass / g (or / mL or / cm^2) sample). **D 6469**, D02

biosurfactant, *n.*–a biologically produced molecule that acts as a soap or detergent. **D 6469**, D02

Discussion—These materials may produce and stabilize emulsions of water in fuel. FJP

coalescer, *n.*–a filter element designed to cause water droplets to coalescence.

coalescence, *n.*–the merging of two or more liquid particles to form a single (larger) liquid particle. **E 1620**, E29

consortium (pl. **consortia**), *n.*–microbial community comprised of two or more than one species that exhibits properties not shown by individual community members.

Discussion—Consortia often mediate cause or create biodeterioration processes that individual taxa cannot. **D 6469**, D02

depth filter, *n.*–filtration medium comprised of either fibers (for example: spun glass) or particles (for example: activated carbon or clay) designed to entrap contaminants both within the matrix and on the surface of the medium.

[1] President, Biodeterioration Control Associates, Inc., PO Box 3659, Princeton, NJ 08543-3659.

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Discussion—As particles are trapped within a depth filter's matrix, they improve its performance until such time as the particle load impedes fluid flow and the benefits of filtration efficiency are offset by the disadvantages of flow restriction.

FJP adapted from: http://www.pall.com/catalogs/oem_health/concepts.asp

Filter water separator *(FWS)*, *n.*–a device used in fuel distribution systems for removal of solids (usually down to 1 μm) and water (usually down to < 15 ppm) from fuel.

Discussion—Typically the device will consist of several coalescer elements and a separator element. API / IP 1581

fuel polishing, *n.*–a process to clean fuel in which filtration, centrifugation or both are used to clarify fuel by removing water, particulates, or both. **FJP**

fungus (pl. **fungi**), *n.*–single cell (yeasts) or filamentous (molds) microorganisms that share the property of having the true intracellular membranes (organelles) that characterize all higher life forms (*Eukaryotes*). **D 6469**, D02

metabolite, *n.*–a chemical substance produced by any of the many complex chemical and physical processes involved in the maintenance of life. **D 6469**, D02

microbially influenced corrosion (MIC), *n.*–corrosion that is initiated or enhanced by the action of microorganisms in the local environment. **D 6469**, D02

Discussion—MIC can cause pitting corrosion in steel tanks and pipes.

microbicide, *n.*–see antimicrobial pesticide.

nominal pore size (NPS), *n.*–the minimum size particle that the medium is designed to trap as a percentage of efficiency for that size (for example, the NPS for filter that retains a minimum of 95% of all particles ≥ 5.0 μm is 95% at 5.0 μm).

Discussion—In common usage, only the pore dimension is stated. In the example given above, the filter would be described simply as a 5 micron NPS filter.

FJP adapted from *Filtration Technology*, Parker Filtration, Cleveland OH, 284 pp., 1997

porosity, *n.*–the ratio of the volume of air or void contained within the boundaries of a material to the total volume (solid matter plus air or void) expressed as a percentage. **D 123**, D13

Discussion—Porosity (n) = $V_{void} \div V_{total}$ where V_{void} is the volume occupied by fluids (fuel, air and water) and V_{total} is the total volume of the filter.

rag layer, *n.*–in inhomogeneous invert emulsion of fuel in water that may develop as a layer between two phases, such as water and fuel.

Discussion—the rag layer may be comprised of bubbles ranging from < 5 μm to > 1 mm which may be visible, along with entrained sediments. The blend of entrained oil and sediment particles typically gives the rag layer a dirty appearance. (Synonyms: invert emulsion layer; Lacy emulsion layer). GCH & FJP

sediment load, *n.*–a general term that refers to material in suspension or in transport, or both; it is not synonymous with either discharge or concentration. **D 4410**, D19

suspended solids (SS), *n.*–solid organic and inorganic particles that are held in suspension in a liquid. **D 6161**, D19

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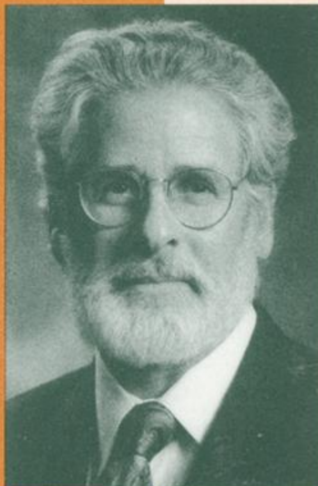

Frederick J. Passman

Dr. Passman has over 25 years' experience in environmental-industrial microbiology. After receiving his A.B. in Microbiology from Indiana University, Dr. Passman entered the U.S. Navy, where he served as an Engineering Officer aboard a destroyer. He left active duty and entered the Reserves in 1973 in order to pursue his Ph.D. in marine microbiology at the University of New Hampshire.

Since 1973, Dr. Passman has conducted research and consulted to government and private industry on topics as diverse as composting municipal sewage sludge, U.S. EPA criteria for various groups of toxic substances in fresh-water systems, microbially enhanced oil recovery, and microbial contamination control in industrial process-fluids. Before founding BCA, Inc., in spring 1992, Dr. Passman was the Business Manager of ANGUS Chemical Company's Biocide Division. Dr. Passman is a member of the American Society for Microbiology, ASTM International, Biodeterioration Society, International Society for Stability and Handling of Liquid Fuels, Society for Industrial Microbiology, and Society for Tribology and Lubrication Engineering (STLE). He is an Associate Editor for *Lubrication Engineering*, Chair, STLE Annual Meeting Education Course Committee and member of the editorial board for the *International Journal of Biodegradation and Biodeterioration*. Dr. Passman has received STLE's Wilber Deutsch Memorial Award for writing excellence. He has more than 30 publications to his name.

Dr. Passman's civilian career paralled his Naval Reserve career, during which he served as the Selected Reserve Executive Officer of a minesweeper, the Selected Reserve Executive Officer of a destroyer and as Commanding Officer of five Naval Reserve Commands, including three training units, a naval control of shipping office and a military sealift command unit. During his Naval Reserve career, Dr. Passman was awarded four Naval Commendation Medals. He retired at the rank of Captain in 1998.

Dr. Passman has chaired a Task Group on Fuel Microbiology within ASTM Subcommittee D02.14 Stability and Cleanliness of Liquid Fuels since its inception in 1989. He has also received an ASTM Award of Appreciation for his efforts within D02.14. Dr. Passman is also an active member of ASTM Subcommittees E34.50 Health and Safety of Metalworking Fluids and E35.15 Antimicrobial Pesticides and has drafted ASTM Standards for each of these committees.

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