

9240 IRON AND SULFUR BACTERIA*

9240 A. Introduction

The group of nuisance organisms collectively designated “iron and sulfur bacteria” is morphologically and physiologically heterogeneous, having in common the ability to transform or deposit significant amounts of iron or sulfur, usually in the form of objectionable slimes. However, iron and sulfur bacteria are not the sole producers of bacterial slimes and in some cases may be associated with slimes of other bacteria.

The iron and sulfur bacteria may be filamentous or single-celled, autotrophic or heterotrophic, aerobic or anaerobic. The taxonomic position of these bacteria is very diverse. They are studied as iron and sulfur bacteria, because these elements and their transformations may be important in water treatment and distribution systems and may be especially bothersome in waters for industrial use, such as cooling and boiler waters. Iron bacteria may cause, or be associated with, fouling and plugging of wells.

* Approved by Standard Methods Committee, 2000.

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Also, the growth of these bacteria may result in consumer complaints of red water in distribution systems for potable water and sulfate-reducing bacteria may cause rusty water and tuberculation of pipes. These organisms also may cause odor, taste, frothing, color, and increases in turbidity in waters.

The nutrient supply for iron and sulfur bacteria may be wholly or partly inorganic. Many of these bacteria can grow under oligotrophic conditions when attached to a substrate in flowing water. This seems quite important in the case of certain sulfur bacteria utilizing small amounts of hydrogen sulfide or in the case of organisms such as *Gallionella*, which obtain their energy from the oxidation of ferrous iron. *Thiobacillus ferrooxidans* contributes to the problem of acid mine drainage and can be identified by tests for transformation of ferrous to ferric iron or oxidation of reduced sulfur compounds under conditions of low pH. Temperature, light, pH, and oxygen supply are critical to the growth of iron and sulfur bacteria. Under different environmental conditions some bacteria may appear either as iron or as sulfur bacteria.

9240 B. Iron Bacteria

1. General Characteristics

“Iron bacteria” are considered to be capable of metabolizing reduced iron present in their aqueous habitat and of depositing it in the form of hydrated ferric oxide on or in their mucilaginous secretions. A somewhat similar mechanism is used by bacteria that utilize manganese. The large amount of brown slime so produced will impart a reddish tinge and an unpleasant odor to drinking water and may render the supply unsuitable for domestic or industrial purposes. These bacteria obtain energy by the oxidation of iron from the ferrous to the ferric state; the ferric form is precipitated as ferric hydroxide $[\text{Fe}(\text{OH})_3]$. Iron may be obtained from the pipe itself if it is made of iron or from the water within it. Because of the energetics involved, the amount of ferric hydroxide deposited is very large in comparison with the enclosed cells.

Some bacteria that do not oxidize ferrous iron may cause it to be dissolved or deposited indirectly. In their growth, either they liberate iron by utilizing organic radicals to which the iron is attached or they alter environmental conditions to permit the solution or deposition of iron. Under these conditions, less ferric hydroxide may be produced, but taste, odor, and fouling may be engendered.

2. Collection of Samples and Identification

a. Sampling and examination procedures: Examples of iron bacteria are shown in Figures 9240:1 through 9240:6. Identifi-

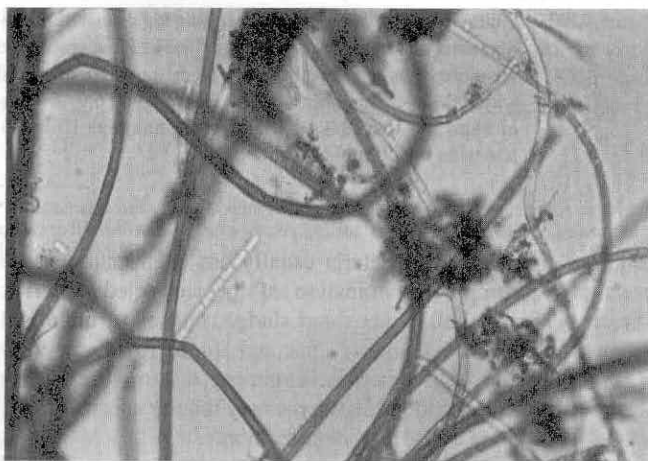


Figure 9240:1. Filaments of *Crenothrix polyspora* showing variation of size and shape of cells within the sheath. Note especially the multiple small round cells, or “conidia,” found in one of the filaments. This distinctive feature is the reason for the name *polyspora*. Young growing colonies usually are not encrusted with iron or manganese. Older colonies often exhibit empty sheaths that are heavily encrusted. Cells may vary considerably in size: Rod-shaped cells average 1.2 to 2.0 μm in width by 2.4 to 4.5 μm in length; coccoid cells of “conidia” average 0.6 μm in diameter.

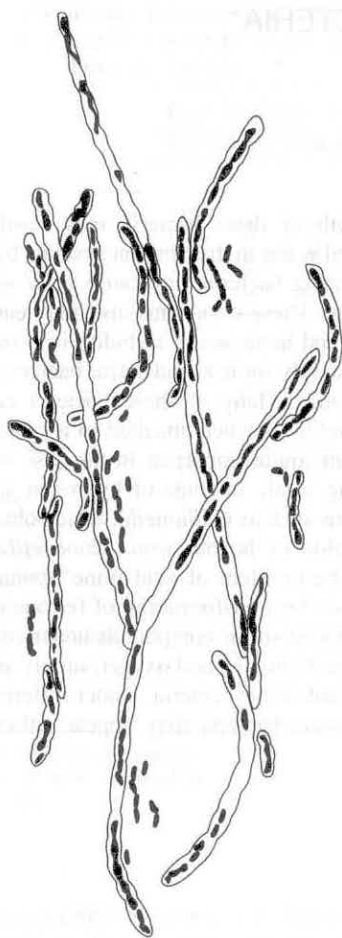


Figure 9240:2. Filaments of *Sphaerotilus natans*, showing cells within the filaments and some free “swarmer” cells. Filaments show false branching and areas devoid of cells. Individual cells within the sheath may vary in size, averaging 0.6 to 2.4 μm in width by 1.0 to 12.0 μm length; most strains are 1.1 to 1.6 μm wide by 2.0 to 4.0 μm long.

cation of nuisance iron bacteria usually has been made on the basis of microscopic examination of the suspected material. Directly examine bulked activated sludge, masses of microbial growth in lakes, rivers, and streams, and slime growths in cooling-tower waters. Suspected development of iron bacteria in water wells or in distribution systems may require special efforts to secure samples useful for identification.

Settle or centrifuge samples drawn directly from wells and examine sediment microscopically. Place a portion of sediment on a microscope slide, cover with a cover slip, and examine under a low-power microscope for filaments and iron-encrusted filaments. The material trapped by filters placed in front of back-surge valves often has yielded excellent specimens of iron bacteria. Water pumped from wells may be passed through a 0.45- μm membrane filter and the filter examined microscopically after drying and clearing with immersion oil applied directly to the membrane. Phase-contrast microscopes have made possible the examination of unstained culture material. Use india ink or lactophenol blue for staining when conventional light

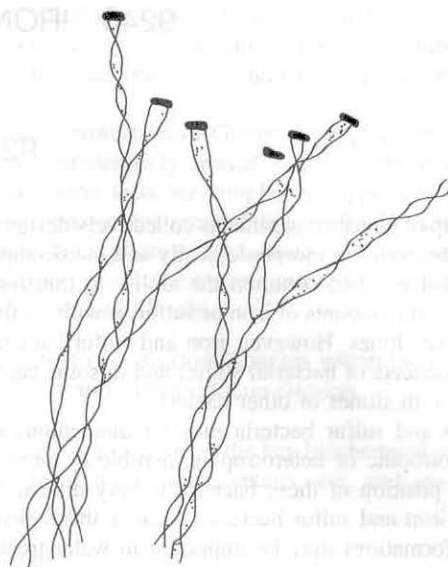


Figure 9240:3. Laboratory culture of *Gallionella ferruginea*, showing cells, stalks excreted by cells, and branching of stalks where cells have divided. A precipitate of inorganic iron on and around the stalks often blurs the outlines. Cells at tip of stalk average 0.4 to 0.6 μm in width by 0.7 to 1.1 μm in length.

microscopy is used. Also, iron bacteria have been observed when the epifluorescence microscopic method (Section 9216B) was used.

Continued heavy deposition of iron caused by the oxidation of ferrous iron by air or by other environmental changes often hides the sheaths or stalks of iron bacteria. The cells within the filaments often die and disintegrate and the filaments tend to be fragmented or crushed by the mass of the iron precipitate.

To dissolve iron deposits place several drops of 1N HCl at one edge of cover slip and draw it under the cover slip by applying filter or blotting paper to the opposite edge. Reducing compounds such as sodium ascorbate also may be used to dissolve deposits and permit observation of cellular structure. To verify that the material is iron, add a solution of potassium ferrocyanide to a sample on a slide, cover, and draw 1N HCl under the cover slip. A blue precipitate of Prussian blue will form as iron around cells or filaments is dissolved.

b. Flowcell procedure for collection of iron-related biofilm sample: The flowcell sampling procedure provides a means of collecting biofilm material from pipeline flow (e.g., discharge at a wellhead or flow within a pipeline) for examination by microscopy. This sampling method permits collection of biofilm samples from systems that do not reliably yield samples useful for microscopy via grab sampling.

Numerous procedures for collecting biofouling microflora, including iron bacteria, have been described.¹⁻⁵

In the following procedure water is trickled through an enclosed chamber containing collection surfaces⁶ using a modified flowcell device.⁵ Because systems to be sampled are not sterile, quality control procedures must be performed to ensure that the flowcell is not the source of the bacteria obtained in the sample.

1) Apparatus—The flowcell collection assembly (Figure 9240:7) consists of:

a) Sealable flowcell chamber made of polycarbonate plastic with removable end plates, ring gasket (fits in end-plate groove),

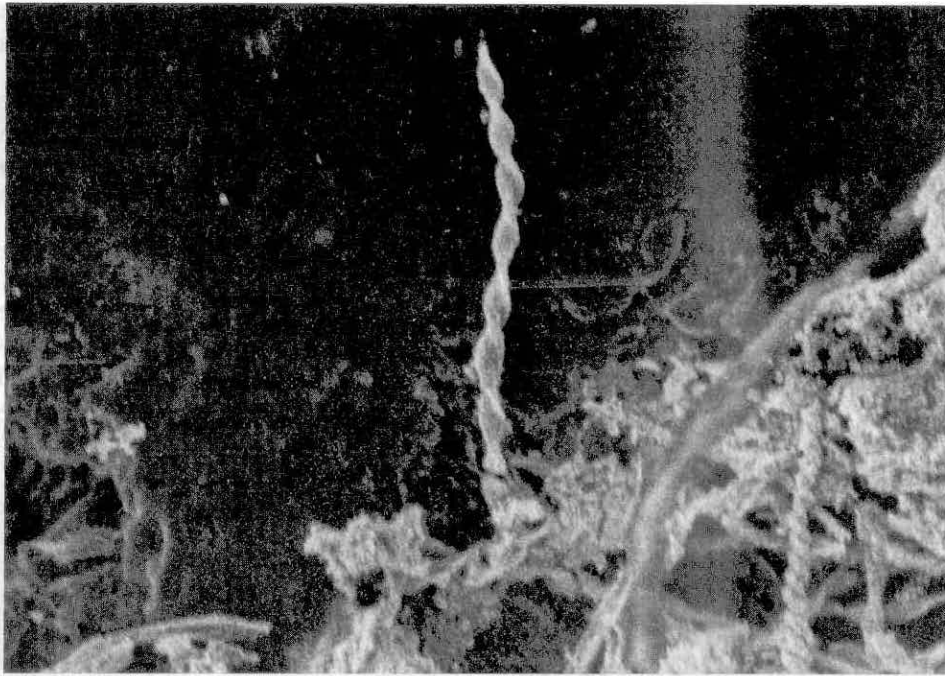


Figure 9240:4. Stalk of *Gallionella ferruginea*. Fragmented stalks appear golden yellow to orange when examined under the microscope. Magnification is 2000 X.

one or more wing-nut bolts, and inlet and outlet taps. Hoses from inlet are adapted to configuration of sampling point (e.g., a hose connection). Flowcell assembly is fitted with an inlet stopcock valve and backflow prevention check valve.

b) Insert element within flowcell chamber, with microscope slides or other coupons of interest. Sterilize insert assembly with its collection surfaces by autoclaving or by gas (depending on heat tolerance of selected coupons). Do not open or handle until ready to use.

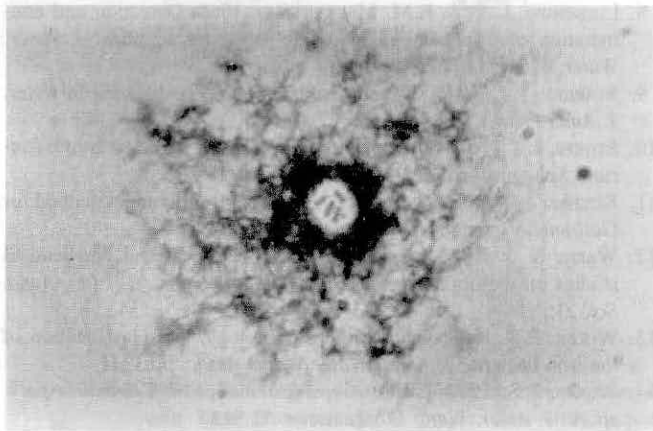


Figure 9240:5. Single-celled iron bacterium *Siderocapsa treubii*. Magnification is 1000X. Cells are surrounded by a deposit of ferric hydrate. Individual cells average 0.4 to 1.5 μm width by 0.8 to 2.5 μm in length.

2) Sampling procedure—Sample iron bacteria and other biofouling (e.g., sulfur-oxidizing slime) as follows:*

To install apparatus, assemble flowcell unit, attachment hoses, valves, and fittings from clean parts without encrustation or discoloration. Disinfect flowcell chamber assembly by immersing in a chlorine solution of approximately 100 mg/L for 10 min. Rinse with treated water or the water to be sampled. Keep end plate and hose ends from touching nonchlorinated surfaces.

* A minimum amount of sediment may collect in the flowcell, but it does not appear to have any effect on biofilm collection, nor does it appear to have an effect due to gradient formation.

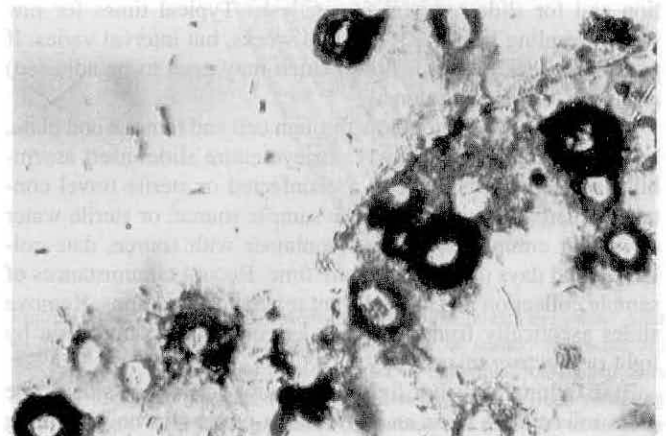


Figure 9240:6. Multiple colonies of *Siderocapsa* spp.

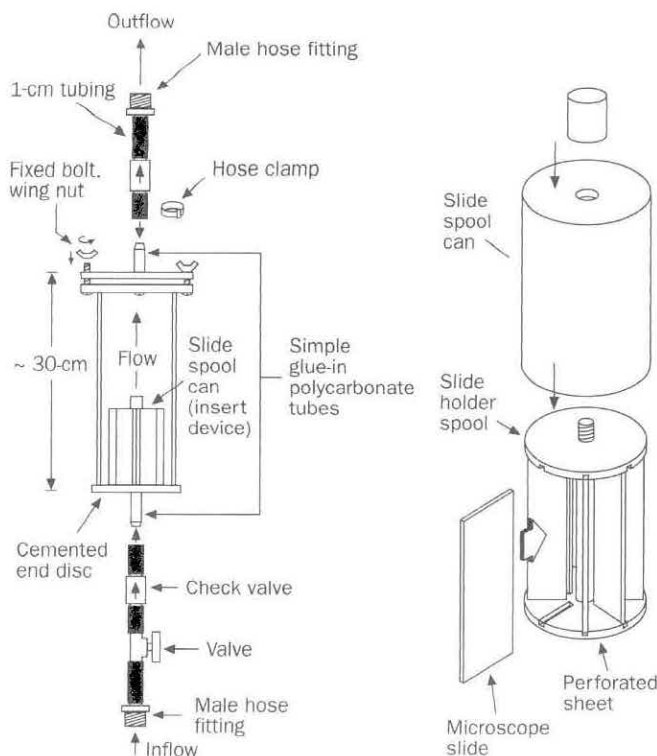


Figure 9240-7. Schematic of flowcell: (left) assembly; (right) slide holder insert.

Alternatively, if materials permit, sterilize the assembly whole. Attach inlet hose to wellhead tap. Mount assembly so that flow enters at bottom and exits at top. Open spigot and inlet valves so that flowcell fills slowly to rim of opening. Overflow to remove any additional disinfectant. Unwrap sterilized slide-holder insert, handling only by the nut that secures the covering sleeve (preferably with sterile latex gloves). Place insert into filled water column and let it settle to the bottom. Install end plate and secure. Turn on water flow and establish a trickle flow of approximately 0.4 to 1 L/min. Test seals, connections, and joints for leaks, and purge air from flowcell chamber. Establish a time period for inspecting slides for signs of iron or other discoloration and for slide removal for analysis. Typical times for raw water sampling from wells is 1 to 4 weeks, but interval varies. If possible, check daily for flow (which may need to be adjusted) and signs of discoloration.

To remove slides, stop flow through cell and remove end plate, keeping chamber full. Slowly retrieve entire slide insert assembly. Immediately transfer to a disinfected or sterile travel container filled with water from the sample source, or sterile water of similar composition. Label container with source, date collected, and days of slide exposure time. Record circumstances of sample collection and any relevant related observations. Remove slides aseptically from the insert assembly for examination by light or electron microscopy.

3) Examination—For light microscopy, clean one side of the glass microscope slide and fit a clean cover slip on remaining biofilm-coated side. Alternatively, subsample a biofilm-coated coupon for culturing of microflora present, electron microscopy,

or other elemental or crystallinity analyses relevant to the biofouling or its associated effects on the exposed coupon.

4) Alternative procedures—The method of immersing slides can be adapted for wells by suspending the slides in the well water column and retrieving them after a period of time, as for the flowcell method. This procedure also is suitable for collecting biofilms consisting predominantly of sulfur-oxidizing bacteria. Modifications are necessary for such factors as: security (tamper-free operation), high temperature or pressure, or situations in which the outflow from the flowcell has to be contained (e.g., contaminated ground or process water).

c. Detection and identification: A cultural method called the iron bacteria presence test has been developed for the detection of iron bacteria.⁷

Identify organisms by comparing with available drawings or photographs of iron bacteria.^{4,7-22}

3. References

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9240 C. Sulfur Bacteria

1. General Characteristics

The bacteria that oxidize or reduce significant amounts of inorganic sulfur compounds exhibit a wide diversity of morphological and biochemical characteristics. One group, the sulfate-reducing bacteria, consists mainly of single-celled forms that grow anaerobically and reduce sulfate, SO_4^{2-} , to hydrogen sulfide, H_2S . One member of this group, *Desulfonema*, is multicellular and exhibits gliding motility. A second group, the photosynthetic green and purple sulfur bacteria, grows anaerobically in the light and uses H_2S as a hydrogen donor for photosynthesis. Members of a third colorless filamentous group are mixotrophic and utilize organic sources of carbon but may get their energy from the oxidation of reduced sulfur compounds. The sulfide is oxidized to sulfur or sulfate. A fourth group, the aerobic sulfur-oxidizers, oxidizes reduced sulfur compounds aerobically to obtain energy for chemoautotrophic growth.

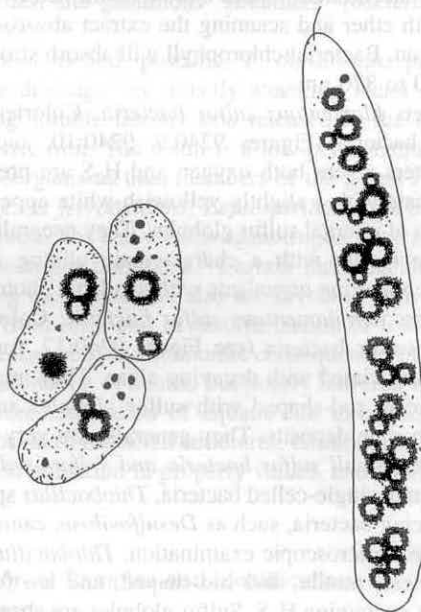


Figure 9240:8. Photosynthetic purple sulfur bacteria. Large masses of cells have brown-orange to purple color—may appear chalky if there is a large amount of sulfur within the cells. (Left) cells of *Chromatium okenii* (5.0 to 6.5 μm wide by 8 to 15 μm long) containing sulfur globules; (right) *Thiospirillum jenense* (3.5 to 4.5 μm wide by 30 to 40 μm long)—cell contains sulfur globules and polar flagellum is visible.

The sulfur bacteria of most importance in the water and wastewater field are the sulfate-reducing bacteria, which include *Desulfovibrio*, and the single-celled aerobic sulfur-oxidizers of the genus *Thiobacillus*. The sulfate-reducing bacteria contribute greatly to tuberculations and galvanic corrosion of water mains and to taste and odor problems in water. *Thiobacillus*, by its production of sulfuric acid, has contributed to the destruction of concrete sewers and the acid corrosion of metals.

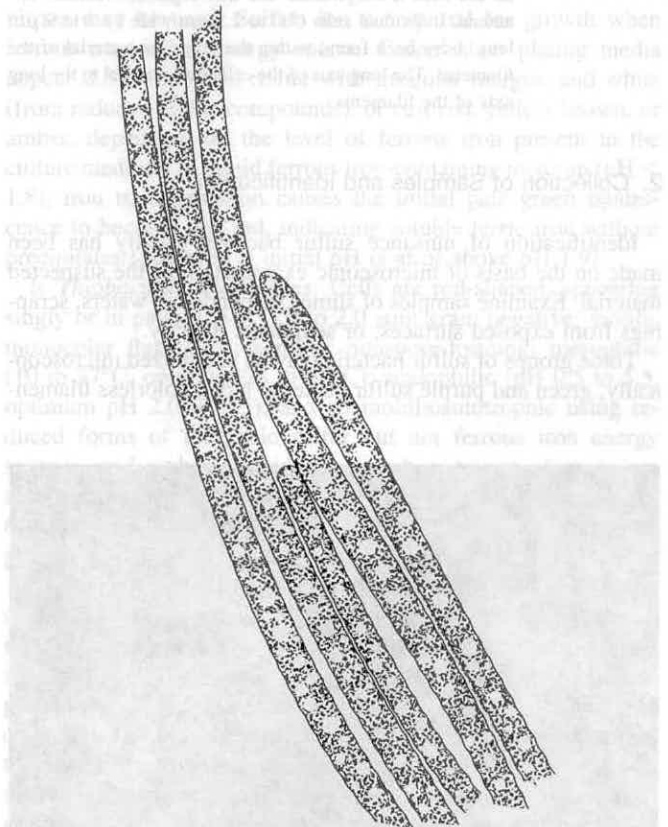


Figure 9240:9. Colorless filamentous sulfur bacteria: *Beggiatoa alba* trichomes, containing globules of sulfur. Filaments are composed of a linear series of individual rod-shaped cells that may be visible when not obscured by light reflecting from sulfur granules. Trichomes are 2 to 15 μm in diameter and may be up to 1500 μm long; individual cells, if visible, are 4.0 to 16.0 μm long.

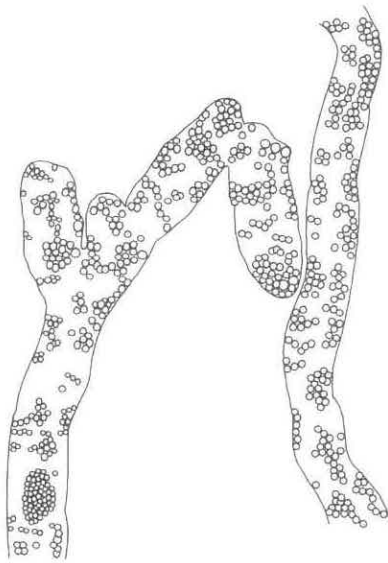


Figure 9240:10. Colorless filamentous sulfur bacteria: portion of a colony, showing branching of the mucoid filament, identified as *Thiodendron mucosum*.¹ Because the name *Thiodendron* previously had been used in bacterial taxonomy, its use here is illegitimate and this organism remains unnamed. Individual cells (1.0 to 2.5 μm wide by 3 to 9 μm long) have been found within the jelly-like material of the filaments. The long axis of the cells runs parallel to the long axis of the filaments.

2. Collection of Samples and Identification

Identification of nuisance sulfur bacteria usually has been made on the basis of microscopic examination of the suspected material. Examine samples of slimes suspended in waters, scrapings from exposed surfaces, or sediments directly.

Three groups of sulfur bacteria may be recognized microscopically; green and purple sulfur bacteria; large, colorless filamentous

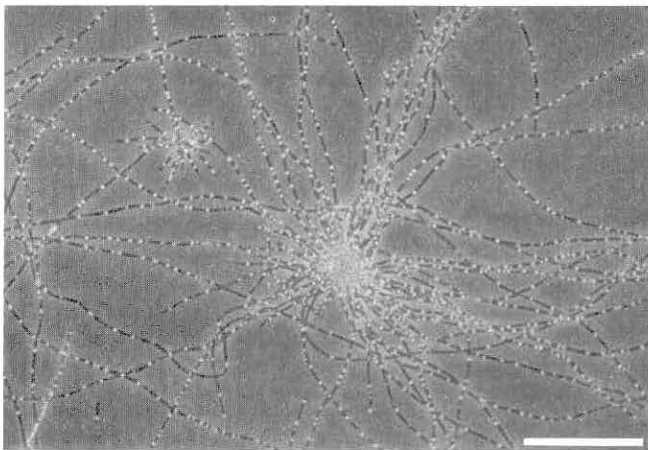


Figure 9240:11. *Thiothrix unzii* after 24 h in lactate-thiosulfate medium. Bright inclusions in filaments are sulfur granules. Bar = 40 μm . Reproduced with permission of the American Society for Microbiology.

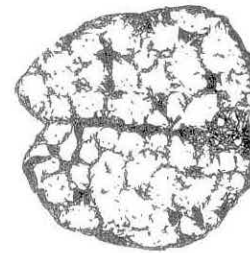


Figure 9240:12. Colorless nonfilamentous sulfur bacteria: dividing cell of *Thiovolum majus*, containing sulfur globules. Cells may measure 9 to 17 μm in width by 11 to 18 μm in length and are generally found in nature in a marine littoral zone rich in organic matter and hydrogen sulfide.

tous sulfur bacteria; and large, colorless, nonfilamentous sulfur bacteria. The fourth group, consisting of sulfate-reducing bacteria and sulfur-oxidizing bacteria of the genus *Thiobacillus*, cannot be identified by appearance alone.

a. Green and purple sulfur bacteria:

1) Green sulfur bacteria most frequently occur in waters containing H_2S . They are small, ovoid to rod-shaped, nonmotile organisms, generally less than 1 μm in diameter, and with a yellowish-green color in masses. Sulfur globules are seldom if ever deposited within the cells.

2) Purple sulfur bacteria (Figure 9240:8) occur in waters containing H_2S . They are large, generally stuffed with sulfur globules, and often so intensely pigmented as to make individual cells appear red. Large, dense, highly colored masses are detected easily by the naked eye. The presence of photosynthetic bacteria in concentrated masses can be confirmed by extracting the mass with ether and scanning the extract absorbance in the infrared region. Bacterial chlorophyll will absorb strongly in the range of 660 to 870 nm.

b. *Colorless filamentous sulfur bacteria:* Colorless filamentous sulfur bacteria (Figures 9240:9, 9240:10, and 9240:11) occur in waters where both oxygen and H_2S are present. They may form mats with a slightly yellowish-white appearance due to deposition of internal sulfur globules. They generally are large and may be motile with a characteristic gliding movement. Identify by comparing organisms with available photographs.¹⁻⁴

c. *Colorless nonfilamentous sulfur bacteria:* Colorless, nonfilamentous sulfur bacteria (see Figure 9240:12, for example) usually are associated with decaying algae. They are extremely motile, ovoid to rod-shaped with sulfur globules and possible calcium carbonate deposits. They generally are very large.

d. *Colorless small sulfur bacteria and sulfate-reducing bacteria:* The small single-celled bacteria, *Thiobacillus* spp., and the sulfate-reducing bacteria, such as *Desulfovibrio*, cannot be identified by direct microscopic examination. *Thiobacillus* types are small, colorless, motile, and rod-shaped, and are found in an environment containing H_2S . Sulfur globules are absent. Identify *Thiobacillus* types, *Desulfovibrio*, or other sulfate-reducing bacteria physiologically.

3. References

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9240 D. Bacteria Living in Acidic Environments

1. General Discussion

Exposure of sulfide-containing ores and pyrite-laden coal to water and oxygen may produce mineral acid and soluble-metal-bearing waters.¹ The low-pH (typically pH 2.5 to 3.0) waters that discharge from mines are called acidic mine drainages.² Central to these transformations are the acidophilic iron and sulfur bacteria, which include, but may not be limited to, *Thiobacillus ferrooxidans*, *T. thiooxidans*, and *Leptospirillum ferrooxidans*. Considerable strain variation exists for the acidophilic thiobacilli, and these genetic differences may be reflected in the natural behavior of the organisms on mineral substrates.

The iron- and sulfur-oxidizing bacteria are chemolithoautotrophic; hence, they sustain synthesis and energy requirements through fixation of carbon dioxide and oxidation of reduced inorganic forms of iron or sulfur. Other acidophilic and acid-tolerant bacteria, which derive nutrition from the metabolism of organic compounds, have been recovered from acidic mine waters.³ The ecology of these organisms is not well understood; however, they are ubiquitous in the mine water environment and may be isolated directly from water, decaying vegetation, ferric oxyhydroxides, and gelatinous "streamers" (bacteria embedded in glycocalyx).

T. ferrooxidans and, possibly, *T. thiooxidans* participate in acidic mine drainage⁴ by directly attacking metal sulfides and transforming soluble ferrous iron released in the oxidation of pyrite to ferric iron. The result is a low-pH, mineralized water. Other microorganisms than members of the genus *Thiobacillus*, for example, *L. ferrooxidans*, *Leptospirillum*-like bacteria, acidophilic mixotrophs and chemoorganotrophs, may assist in producing mine-water pollutants.⁵ Certain thermoacidophilic, sulfur-oxidizing archaeobacteria also are involved with leaching of metal ions from ores, and in desulfurization of fossil fuels.

The environmental and economic consequences of acidic mine water contamination of neutral but poorly buffered watercourses include severe retardation of aquatic life throughout the food chain, corrosion of impacted structures, elimination of a drinking water source, reduction in property values, and overall aesthetic degradation.

2. Collection of Samples and Identification

Iron- and sulfur-oxidizing thiobacilli in natural samples cannot be identified by direct microscopic examination because the bacteria have no peculiar morphology, the presence of colloidal iron may interfere with the detection of the bacteria, and large numbers are required to be seen. In most cases, no special precautions are needed to collect specimens for laboratory use; however, collect samples in pre-sterilized containers and trans-

port to the laboratory at reduced temperatures. The essential characteristics of bacterial species reported in connection with acidic mine water environments and bioleaching are presented below.⁶⁻⁹

a. Thiobacillus ferrooxidans: Cells are rod-shaped, appearing singly or in pairs; 0.3 to 0.5×1.0 to $1.7 \mu\text{m}$, gram negative; motile; monopolar flagellated; aerobic; nonspore-forming; mesophilic (10 to 37°C , optimum 30 to 35°C); acidophilic (pH 1.3 to 4.5 , optimum pH 2.5 to 2.8); and chemolithoautotrophic using reduced forms of inorganic sulfur (elemental sulfur, thiosulfate, and tetrathionate), ferrous iron, and sulfidic minerals as energy sources, and carbon dioxide as a carbon source. Chemolithotrophic growth on reduced copper, uranium, and selenium may occur. Ammonium nitrogen is the typical nitrogen source, but nitrate may be used. Sulfate ion is required for growth when ferrous iron is the energy source. Colonies on plating media appear 0.5 to 1.0 mm, round with irregular margin, and white (from reduced sulfur compounds), or rust red, yellow brown, or amber, depending on the level of ferrous iron present in the culture medium. In liquid ferrous iron-containing medium (pH < 1.8), iron transformation causes the initial pale green opalescence to become rust red, indicating soluble ferric iron without precipitates (observed if initial pH is at or above pH 1.9).

b. Thiobacillus thiooxidans: Cells are rod-shaped, appearing singly or in pairs; 0.5×1.0 to $2.0 \mu\text{m}$; gram negative; motile; monopolar flagellated; aerobic; nonspore-forming; mesophilic (10 to 37°C , optimum 28 to 30°C); acidophilic (pH 0.5 to 5.5 , optimum pH 2.0 to 3.5); and chemolithoautotrophic using reduced forms of inorganic sulfur but not ferrous iron energy sources, and carbon dioxide as a carbon source. Ammonium nitrogen serves as nitrogen source. On solid culture media containing thiosulfate, colonies are circular, 0.5 to 1.0 mm, transparent or whitish yellow from sulfur deposition.

c. Thiobacillus acidophilus (*T. organoparus*): Cells are rod-shaped; 0.5 to 0.8×1.0 to $1.5 \mu\text{m}$; gram negative; some strains motile; mono- and disubterminal flagella; aerobic; nonspore-forming; mesophilic (25 to 37°C , optimum 27 to 30°C); acidophilic (pH 1.5 to 5.5 , optimum pH 2.5 to 3.0); and facultatively chemolithotrophic using reduced forms of inorganic sulfur (autotrophic), glucose or some organic acids (heterotrophic), or a mixture of glucose and reduced sulfur (mixotrophic). Ammonium and urea nitrogen serve as nitrogen sources. On solid culture media containing glucose, mature colonies are 1.0 to 2.0 mm, round, convex, and slightly translucent to cream to rose colored, depending on age.

d. Leptospirillum ferrooxidans: Cells are curved (vibroid); 0.2 to 0.4×0.9 to $1.1 \mu\text{m}$; as spirilla, $8.0 \mu\text{m}$; may form chains of 3 to 12 cells giving false *Spirillum*-like morphology; gram negative; motile; nonspore-forming; monopolar flagellated; aerobic;

optimally mesophilic (30°C) with facultatively thermophilic strains (40 to 45°C); optimally acidophilic (pH 2.5 to 3.0); chemolithotrophic using ferrous iron but not reduced sulfur forms of energy. Ammonium nitrogen serves as nitrogen source. Colony appearance typical of iron-oxidizing thiobacilli in size and coloration.

e. Sulfolobus thermosulfidooxidans: Cells rod-shaped, 0.6 to 0.8 × 1.0 to 3.0 μm; may appear in aggregates; gram positive; may be encapsulated; endospore-forming; nonmotile; aerobic; optimally thermophilic (50 to 55°C), optimally acidophilic (pH 1.7 to 2.4); and facultatively chemolithotrophic using sulfidic materials, elemental sulfur, and ferrous iron (autotrophic) or yeast extract, glucose, sucrose, and glutamic acid (heterotrophic). Ammonium nitrogen serves as nitrogen source.

f. Sulfolobus acidocaldarius: Cells irregularly spheroidal with lobes; 0.8 to 1.0 μm; gram negative (archaeobacterial cell wall); nonspore-forming; nonmotile; aerobic; thermophilic (55 to 85°C, optimum 70°C); acidophilic (pH 1.0 to 6.0, optimum 2.0 to 3.0); and mixotrophic on elemental sulfur and heterotrophic on several organic compounds. Colonies vary in size from small to large.

g. Acidianus brierleyi: Cells spheroidal with lobes; 1.0 to 1.5 μm; gram negative (archaeobacterial cell wall); nonspore-forming; nonmotile; facultatively aerobic; thermophilic (45 to 75°C, optimum 70°C); acidophilic (pH 1.0 to 6.0, optimum 1.5 to 2.0); and facultatively chemolithotrophic on sulfidic minerals, elemental sulfur (anaerobic with hydrogen as electron donor), and ferrous iron (autotrophic), yeast extract (heterotrophic), or elemental sulfur and organic compounds (mixotrophic).

h. Acidiphilium cryptum: Cells rod-shaped, 0.3 to 0.5 × 0.6 to 1.5 μm; gram negative; motile; monopolar or dilateral flagella; aerobic; weakly catalase-positive, mesophilic (31 to 41°C), acidophilic (pH 2.5 to 5.9); chemoorganotrophic using complex, soluble organic matter (e.g., trypticase and yeast extract with glucose); acetate inhibits growth. Colonies on organic media are 0.5 to 2.0 mm, circular with irregular margins, and white; colonies on ferrous iron media without exogenous carbon source are small (maximum 0.3 mm) and lobate.

3. Enumeration, Enrichment, and Isolation

The acidophilic bacteria previously described can all be grown on appropriate culture media, although sometimes with difficulty. There are no good means of enumerating acidophilic iron and sulfur bacteria. Procedures frequently have been calibrated with axenic strains and may not accurately reflect the growth characteristics of naturally occurring organisms.

Several culture media have been formulated for the isolation and mass culture of *Thiobacillus ferrooxidans* but strain differences preclude the availability of a universal culture medium. Problems associated with the enumeration of this species on membrane filters and solidified culture media long have been recognized.¹⁰ The task of obtaining axenic cultures is further complicated by the invariable association of acidophilic, heterotrophic bacteria with *T. ferrooxidans*.

The chemolithotrophic and mixotrophic iron- and sulfur-oxidizing thiobacilli are not fastidious and may be grown on several mineral salt formulations with appropriate energy sources. In-

clusion of a surfactant* in the sample to be plated¹¹ may enhance the colony size of *T. ferrooxidans*. In isolation media for the iron-oxidizing bacteria, keep level of ferrous iron relatively low (20 mM) with a final pH of 2.2 to 2.5 to minimize precipitation of ferric iron salts.

Species of *Sulfolobus* are more difficult to isolate and manipulate. The media described below are only suggestive. More complete discussions of the isolation and cultivation of the organisms are available in literature.^{9,11}

The mineral salts formulations provided below may be appropriate basal salts for acidophilic heterotrophic bacteria when used in conjunction with suitable organic carbon and energy sources. Solidifying agents given in connection with specific media may be used with any basal salts solution as desired.

a. Ferrous-tryptone soya broth (FeTSB),¹² for *Thiobacillus ferrooxidans* and acidophilic heterotrophs:

Ferrous sulfate, FeSO ₄ ·7H ₂ O	40.0 g
Ammonium sulfate, (NH ₄) ₂ SO ₄	1.8 g
Magnesium sulfate, MgSO ₄ ·7H ₂ O	0.7 g
Tryptone soya broth (dehydrated)	0.35 g
Agarose†	14.0 g

Dissolve ferrous sulfate in reagent-grade water and bring volume to 200 mL. Acidify with H₂SO₄ to pH 2.0. Filter-sterilize using a sterile, acid-tolerant 0.22-μm membrane filter. Dissolve remaining ingredients (excluding agarose) in reagent-grade water; adjust pH to 2.5 with H₂SO₄ and bring volume to 1 L; sterilize by autoclaving (121°C, 15 min). Prepare liquid medium by combining both solutions and reagent-grade water at a ratio of 1:14:5, respectively. To prepare solid medium, suspend agarose in 500 mL reagent-grade water, autoclave, and cool to 55°C. Separately warm and mix ferrous sulfate and mixed ingredient solutions at 55°C and aseptically add agarose in same ratio, substituting agarose suspension for reagent-grade water.

A modification of FeTSB medium, FeTSBo medium,¹³ is reported to improve enumeration of acidophilic and moderately thermophilic bacteria. FeTSBo medium contains 0.5% agarose, 0.75% ferrous iron, and 1 mM (final concentration) zinc sulfate. Prepare initially in the same way as solid FeTSB, then divide into two volumes at a ratio of 2:1 and hold at 50°C. Add active culture of acidophilic, heterotrophic bacteria (e.g., *Acidiphilium*-like strain SJH)¹³ to the larger volume at 2.5% w/v, mix thoroughly, and pour into presterilized petri dishes (20 mL). Upon solidification, overlay 10 mL of uninoculated medium. Dry plates for 10 min before use.

*b. Medium 9K*¹⁴ maintenance medium for *Thiobacillus ferrooxidans*; acidophilic bacteria:

Ferrous sulfate, FeSO ₄ ·7H ₂ O	44.22 g
Ammonium sulfate, (NH ₄) ₂ SO ₄	3.0 g
Potassium chloride, KCl	0.1 g
Dipotassium hydrogen phosphate, K ₂ HPO ₄	0.5 g
Magnesium sulfate, MgSO ₄ ·7H ₂ O	0.5 g
Calcium nitrate, Ca(NO ₃) ₂ ·4H ₂ O	0.01 g
Reagent-grade water	1 L

* For example, 0.5% Tween 80, or equivalent.

† Type I; Sigma or equivalent.

Dissolve ferrous sulfate in 300 mL reagent-grade water, acidify to pH 2 to 3 with H_2SO_4 and filter-sterilize. Dissolve remaining salts in 700 mL reagent-grade water, acidify to pH 2.5 with H_2SO_4 , and sterilize by autoclaving (121°C , 15 min). Prepare the liquid medium by combining both solutions. To prepare a solid medium, suspend 5 g agarose in 250 mL reagent-grade water, autoclave, and combine with 450 mL ferrous sulfate solution and 300 mL mixed salts solution at 55°C .

c. *TSM 1 medium*¹⁵ for enumeration of *Thiobacillus ferrooxidans* on plates or as nutrient base for membrane filters:

Ferrous sulfate, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	22.0 g
Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$	3.0 g
Potassium chloride, KCl	0.1 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.5 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
Calcium nitrate, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	0.01 g
Agarose†	5.0 g
Reagent-grade water	1 L

Dissolve ferrous sulfate in 150 mL reagent-grade water, acidify to pH 2.5 with H_2SO_4 , and filter-sterilize. Dissolve remaining salts in 600 mL reagent-grade water, acidify to pH 2.5 with H_2SO_4 , and sterilize by autoclaving (121°C , 15 min). To prepare medium, suspend agarose in 250 mL reagent-grade water, autoclave (121°C , 15 min), and cool to 60°C . Mix sterile ferrous sulfate and mixed salts solutions at 60°C ; aseptically add agarose suspension; pour into prewarmed, sterile petri plates.

d. *ISP medium*¹⁶ for isolation of *Thiobacillus ferrooxidans* and acidophilic heterotrophic bacteria on plates:

Ferrous sulfate, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	33.4 g
Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$	6.0 g
Potassium chloride, KCl	0.2 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1.0 g
Calcium nitrate, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	0.02 g
Purified agar	7.0 g
Reagent-grade water	1 L

Dissolve ferrous sulfate in 300 mL reagent-grade water, acidify to pH 2.5 with H_2SO_4 , and filter-sterilize. Dissolve remaining salts in 550 mL reagent-grade water, acidify to pH 3.0 with H_2SO_4 , and sterilize by autoclaving (121°C , 15 min). To prepare medium for iron-oxidizing bacteria, suspend purified agar in 150 mL reagent-grade water, autoclave (121°C , 15 min), and cool to 60°C . Mix sterile ferrous sulfate and mixed salts solutions at 60°C ; aseptically add agar suspension.

e. *Enrichment medium* for *Thiobacillus acidophilus*:¹⁷

Ferrous sulfate, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	44.22 g
Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$	3.0 g
Potassium chloride, KCl	0.1 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.5 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
Calcium nitrate, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	0.01 g
Glucose	0.1 g
Reagent-grade water	1 L

Use medium 9K, supplemented to contain 0.01% glucose, to enrich *Thiobacillus acidophilus*; adjust pH to 3.5. Inoculate

enrichment medium with acidic mine water and incubate for several days. Transfer inoculum from the enrichment to fresh medium 9K, modified by reducing ferrous iron concentrations from 9 g/L to 10 mg/L and increasing glucose level from 100 mg/L to 10 g/L in four successive steps. To prepare an isolation medium 9K for *Thiobacillus acidophilus*, modify preparation of 9K solid medium (§ b above) by reducing ferrous iron concentration to 10 mg/L, adding 10 g/L glucose, and adjusting final pH to 4.5.

f. *Medium of Norris and Kelly*,¹⁸ for growth and maintenance of *Leptospirillum ferrooxidans*: *L. ferrooxidans* is difficult to grow on solid media; strain differences may require different formulations to produce satisfactory results.

Ferrous sulfate, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	13.9 g
Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$	0.2 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.2 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g
Reagent-grade water	1 L

To produce liquid medium, dissolve ferrous sulfate in 300 mL reagent-grade water, acidify to pH 1.5 with H_2SO_4 , and filter-sterilize. Dissolve remaining salts in 700 mL reagent-grade water, acidify to pH 1.5 with H_2SO_4 , and sterilize by autoclaving (121°C , 15 min). Make solid medium for isolating *L. ferrooxidans* by adding silica gel as a hardening agent.

g. *Medium of Starkey*,¹⁹ for isolation and maintenance of *Thiobacillus thiooxidans*:

Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$	0.2 g
Calcium chloride, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.25 g
Dipotassium hydrogen phosphate, K_2HPO_4	3.0 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
Ferrous sulfate, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01 g
Sulfur energy source: Either	
Sodium thiosulfate stock solution	5 mL
or	
Sulfur, elemental	10.0 g

If sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) is used as the energy source, prepare stock solution by dissolving 10.0 g in 100 mL reagent-grade water; filter-sterilize. Dissolve remaining salts in 950 mL reagent-grade water, adjust to pH 4.0 with H_2SO_4 , and sterilize by autoclaving (121°C , 15 min). Add 5 mL stock thiosulfate to mixed salts solution, mix, and bring volume to 1 L.

If elemental sulfur is used, pasteurize in an empty 1-L flask under flowing steam for 1 h on each of 3 consecutive days before preparation of medium. Dissolve mineral salts and sulfur in 1 L reagent-grade water, adjust pH to 4.0, and sterilize by autoclaving (121°C , 15 min). Prepare solid isolation/culture medium from maintenance medium by using a suitable gelling agent, and adjusting volumes and concentrations appropriately.

h. *Modified Allen medium*,²⁰ for enrichment, isolation, and maintenance of *Sulfolobus* and *Acidianus*:

Basal medium:	
Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$	1.3 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.28 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.25 g
Calcium chloride, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.25 g
Reagent-grade water	1.0 L

† Biorad High MR-1620001 or equivalent.

Dissolve ingredients in 1 L reagent-grade water, adjust pH to 2.0 to 3.0 with H_2SO_4 , and sterilize by autoclaving (121°C, 15 min).

Trace mineral salts:

Ferric chloride, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	20.0 g
Manganese chloride, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1.8 g
Sodium borate, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$	4.5 g
Zinc sulfate, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.22 g
Cupric chloride, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.05 g
Sodium molybdate, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	0.03 g
Vanadyl sulfate, $\text{VO}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$	0.04 g
Cobalt sulfate, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$	0.01 g
Reagent-grade water	1.0 L

Dissolve mineral salts in 950 mL reagent-grade water; adjust pH to <1.5 with H_2SO_4 , bring volume to 1 L, and filter-sterilize.

To prepare liquid medium, aseptically add 1 mL mineral salts solution to basal medium. When promoting lithotrophic growth on elemental sulfur (5 to 10 g/L), adjust pH to higher initial value to compensate for production of sulfuric acid from sulfur oxidation (pasteurize elemental sulfur as in preparation of Starkey medium, ¶ g above). To enhance lithotrophic growth, add yeast extract with or without glucose in small amounts (0.05 to 0.2 g/L) by aseptically transferring from sterile stock to medium. Heterotrophic growth is promoted by increasing final concentration of yeast extract to 1 g/L and glucose to 2 g/L in sterile medium.

Sulfolobus spp. may be enriched selectively, aerobically at 85°C in modified Allen medium containing yeast extract only at 1 g/L. Primary enrichment of *Acidianus* spp. can be accomplished by anaerobic incubation (80:20 H_2 : CO_2 , 300 kPa) in the same mineral salts solution supplemented with 0.2 g/L yeast extract and 5.0 g/L elemental sulfur. *A. brierleyi* is enhanced selectively by incubating at 65°C, and maintained by transferring to a variation of the medium containing 1 g/L yeast extract without sulfur, followed by aerobic incubation. *A. infernus* is favored by anaerobic incubation of the primary enrichment medium at 85°C followed by aerobic incubation subculturing to same medium with a reduced yeast extract concentration of 0.1 g/L.

To prepare solid isolation medium, make a double-strength salts solution and combine with equal volumes of a gelling agent in conjunction with an overlay technique.

For polysilicate:

Sodium silicate solution, 1.38 kg $\pm$ 0.01 kg/L 6.0 mL

Dissolve 6 mL silicate solution in 94 mL reagent-grade water and mix with 5 g sodium bentonite. Rapidly adjust pH to 6.0 with H_2SO_4 ²¹ while vigorously stirring mixture. Repeat using nutrient solution in place of water. Make trial preparations to determine proper volume of acid (firmness of silica gel increases with decreasing pH) and sodium silicate solution for best results. Poured plates harden in 2 to 5 min; incubate at temperatures up to 80°C in a humidified atmosphere. Before use, equilibrate hardened plates with equal volumes of liquid media by carefully superimposing sterile liquid medium on the hardened plates and withdrawing the liquid in 5 sequential treatments over 24 h.

For other gelling agents:

Gelling agent§	24 g
Reagent-grade water	2 L

Prepare two concentrations, 16 g/L and 8 g/L, of gelling agent solution in reagent-grade water, sterilize by autoclaving, and cool to 70°C. Combine the 16 g/L solution with an equal volume of prewarmed, double-strength modified Allen medium (or medium 9K) to which the following have been added:

Tetrathionate	20.0 mM
Sodium chloride, NaCl	2.0 mM
Casamino acids or yeast extract	2.0 g/L
Magnesium chloride, MgCl_2	16.0 mM

Pour medium into petri dishes and let solidify. Combine the 8-g/L gelling agent solution with an equal volume of double-strength medium previously inoculated with a suitable level of cells from an enrichment culture; pour over solid base layer and let harden. Incubate at 65°C in sealed plastic bags.

*i. Acidiphilium medium*³ for isolation and maintenance of *Acidiphilium* spp.:

Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$	2.0 g
Potassium chloride, KCl	0.1 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.5 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
Glucose stock solution	100 mL
Reagent-grade water	1.0 L

Prepare glucose stock solution by dissolving 50.0 g glucose in 500 mL reagent-grade water. Sterilize by autoclaving (121°C, 15 min). Dissolve salts in 900 mL reagent-grade water (pH adjusted to 3.0 with H_2SO_4) and autoclave (121°C, 15 min). Aseptically add 100 mL sterile glucose stock solution (alternatively use yeast extract or trypticase at 0.01% final concentration). To prepare solid medium suspend 12 g agar in 150 mL reagent-grade water, dissolve mineral salts in 750 mL reagent-grade water, and autoclave both (121°C, 15 min); cool to 60°C and aseptically combine 100 mL glucose stock, agar, and mineral salts solution.

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§ Consisting of Gelrite, Kelco, Monsanto, or equivalent.

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9240 E. Enumeration, Enrichment, and Isolation of Iron and Sulfur Bacteria

There are no good means of enumerating iron and sulfur bacteria other than the sulfate-reducing bacteria and the thiobacilli. Laboratory cultivation and isolation of pure cultures is difficult and successful isolation is uncertain. This is especially true of attempts to isolate filamentous bacteria from activated sludge or other sources where many different bacterial types are present.

1. Media

a. Casitone-glycerol-yeast autolysate broth (CGY), for the Sphaerotilus group: This medium may not be available in dehydrated form and may require preparation from the basic ingredients. It may be solidified by adding 1.5% agar.

Casitone	5.0 g
Glycerol	10.0 mL
Yeast autolysate	1.0 g
Reagent-grade water	1 L

b. Isolation medium (iron bacteria): This medium may not be available in dehydrated form and may require preparation from the basic ingredients:

Glucose	0.15 g
Ammonium sulfate, (NH ₄) ₂ SO ₄	0.5 g
Calcium nitrate, Ca(NO ₃) ₂	0.01 g
Dipotassium hydrogen phosphate, K ₂ HPO ₄	0.05 g
Magnesium sulfate, MgSO ₄ ·7H ₂ O	0.05 g

Potassium chloride, KCl	0.05 g
Calcium carbonate, CaCO ₃	0.1 g
Agar	10.0 g
Cyanocobalamin	0.01 mg
Thiamine	0.4 g
Reagent-grade water	1 L

c. Maintenance (SCY) medium (iron bacteria): This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Sucrose	1.0 g
Casitone	0.75 g
Yeast extract	0.25 g
Trypticase soy broth without dextrose	0.25 g
Agar	10.0 g
Cyanocobalamin	0.01 mg
Thiamine	0.4 mg
Reagent-grade water	1 L

d. Mn agar No. 1: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Manganous carbonate, MnCO ₃	2.0 g
Beef extract	1.0 g
Ferrous ammonium sulfate, Fe(NH ₄) ₂ (SO ₄) ₂	150 mg
Sodium citrate	150 mg
Yeast extract	75 mg
Cyanocobalamin	0.005 mg
Agar	10.0 g

Reagent-grade water 1 L

Prepare and sterilize the medium without cyanocobalamin. Separately sterilize cobalamin by filtration and add aseptically just before medium solidifies.

*e. Mn agar No. 2:*¹ Prepare fresh each time from basic ingredients:

Manganous sulfate, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$	10 mg
Agar	15.0 g
Natural water	1 L

f. Iron oxidizing medium (*Thiobacillus ferrooxidans*): This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Basal salts:

Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$	3.0 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.05 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.05 g
Potassium chloride, KCl	0.05 g
Calcium carbonate, CaCO_3	0.1 g

Energy source:

Ferrous sulfate, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 14.74% solution (w/v) ... 300 mL

Separately sterilize basal salts and energy source and combine when cool. Store in the refrigerator and discard after 2 weeks. A precipitate will form and the medium will be opalescent and green. The pH should be 3.0 to 3.6.

g. Ferrous sulfide agar (*Gallionella ferruginea*): This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Agar layer:

Ferrous sulfide, FeS (washed precipitate and liquid)	500.00 mL
Sodium sulfide, Na_2S	15.6 g
Ferrous ammonium sulfate, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$	78.4 g
Boiling reagent-grade water	1 L
Agar (liquid) (30 g/L)	500.00 mL

Liquid overlay:

Ammonium chloride, NH_4Cl	1.0 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.5 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g
Calcium chloride, CaCl_2	0.1 g
Reagent-grade water	1 L

Prepare FeS by reacting equal molar quantities of Na_2S and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ in boiling reagent-grade water. Let precipitate settle from the hot solution in a completely filled and stoppered bottle. Wash precipitate four times by decanting supernatant and replacing with boiling water. Store FeS in a glass stoppered bottle completely filled with additional boiling water.

Add equal volumes of FeS and 3% agar at 45°C . Prepare slants in screw-capped tubes. Prepare liquid overlay, bubble CO_2 through it for 10 to 15 s, and add several milliliters to agar slant. Modifications to this medium and procedure are available.²⁻⁴

A variation of the basic medium requires adding 0.5 mL formalin (40% formaldehyde solution) to a screw-capped dilution bottle containing 10 mL FeS agar and 100 mL liquid overlay. This variation may not always work.^{3,4} Add 0.001%

bromthymol blue and 0.004% bromocresol purple to liquid overlay.

h. Sulfate-reducing medium: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Sodium lactate	3.5 g
Beef extract	1.0 g
Peptone	2.0 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	2.0 g
Sodium sulfate, Na_2SO_4	1.5 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.5 g
Ferrous ammonium sulfate, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$	0.392 g
Calcium chloride, CaCl_2	0.10 g
Sodium ascorbate	0.10 g
Reagent-grade water	1 L

pH should be 7.5 ± 0.3 after sterilization. Prepare medium excluding ferrous ammonium sulfate and sodium ascorbate, dispense in screw-capped test tubes, and sterilize by autoclaving (121°C , 15 min). Use completely filled tubes; separately sterilize extra medium to be added to tubes for filling. On day of use, prepare separate solutions of ferrous ammonium sulfate (3.92 g/100 mL) and sodium ascorbate (1.00 g/100 mL), sterilize by filtration through a $0.45\text{-}\mu\text{m}$ membrane filter, and aseptically add 0.1 mL each solution/10 mL basal medium.

i. Thiosulfate oxidizing medium (*Thiobacillus thioparus*): This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Sodium thiosulfate, $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	10.0 g
Dipotassium hydrogen phosphate, K_2HPO_4	2.0 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 g
Calcium chloride, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.1 g
Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$	0.1 g
Ferric chloride, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.02 g
Reagent-grade water	1 L

pH should be 7.8 after sterilization by autoclaving (121°C , 15 min). Separately sterilize $\text{Na}_2\text{S}_2\text{O}_3$ and $(\text{NH}_4)_2\text{SO}_4$ and add before use. If this medium is used to isolate *Thiobacillus thioparus*, check isolates to ensure they are autotrophs.

j. Sulfur medium (*Thiobacillus thiooxidans*): This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Sulfur, elemental	10.0 g
Potassium dihydrogen phosphate, KH_2PO_4	3.0 g
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$	0.3 g
Calcium chloride, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.25 g
Ferric chloride, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.02 g
Reagent-grade water	1 L

pH should be 4.8 after sterilization by autoclaving (121°C , 15 min). Weigh sulfur into 250-mL flasks using 1 g/flask. Add 100 mL medium to each flask and sterilize with intermittent steam (30 min for each of 3 consecutive d).

k. Media for Beggiatoa and mixotrophic strains of Thiobacillus:

1) *Extracted hay:* Extract hay or grass at least five times by boiling in water for 30 min, with two rinses in cold water between extractions. Dry the extracted hay, place 10 to 30 blades in a large test tube, and sterilize by autoclaving (121°C , 15 min).

2) *Basal medium*

Ammonium chloride, 4% solution	5 mL
Dipotassium hydrogen phosphate, K_2HPO_4 , 1% solution	1 mL
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$, 1% solution	1 mL
Calcium sulfate, $CaSO_4 \cdot 2H_2O$, saturated solution	20 mL
Trace elements [see ¶3 below]	5 mL
Reagent-grade water	968 mL

3) *Trace elements*

Reagent-grade water	920 mL
Zinc sulfate, $ZnSO_4 \cdot 7H_2O$, 0.1% solution	10 mL
Manganous sulfate, $MnSO_4 \cdot 4H_2O$, 0.02% solution	10 mL
Copper sulfate, $CuSO_4 \cdot 5H_2O$, 0.00005% solution	10 mL
Boric acid, H_3BO_3 , 0.1% solution	10 mL
Cobalt nitrate, $Co(NO_3)_2$ or $CoCl_2 \cdot 6H_2O$, 0.01% solution	10 mL
Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$, 0.01% solution	10 mL
EDTA solution, 2% EDTA with 7% ferrous sulfate, $FeSO_4 \cdot 7H_2O$, with 1 mL conc HCl/100 mL	20 mL

4) *MP agar*: This medium may not be available in dehydrated form and should be made fresh before use.

Basal medium	1 L
Sodium acetate	0.1 g
Sodium sulfide solution [see ¶ 5 below]	3 mL
Agar	15 g

Adjust pH to between 7.0 and 7.5 and heat to dissolve agar. Sterilize by autoclaving for no more than 15 min and cool in a 45 to 50°C water bath. Add sodium sulfide solution immediately before pouring plates. If the medium is to be used for screw-capped tubes the sulfide may be added before autoclaving.

5) *Sodium sulfide solution*: Make up and separately autoclave a 10% solution of $Na_2S \cdot 9H_2O$.

6) *MY agar*: This medium may not be available in dehydrated form and should be made fresh before use.

Basal medium	1 L
Sodium acetate	0.1 g
Nutrient broth powder*	0.1 g
Yeast extract*	0.1 g
Sodium sulfide solution [see ¶ 5 above]	3 mL
Agar	15 g

Adjust pH to between 7.0 and 7.5 and heat to dissolve agar. Sterilize by autoclaving for no more than 15 min and cool in a 45 to 50°C water bath. Add sodium sulfide solution immediately before pouring plates. If the medium is to be used for screw-capped tubes the sulfide may be added before autoclaving.

1. *Media for heterotrophic strains of Thiobacillus*: Make the media 3) through 9) with solutions 1) and 2), plus indicated additives. After adding all ingredients, adjust pH to 7.2 to 7.5. For solid media add 12 g agar/L.

1) *MSV*

Ammonium sulfate, $(NH_4)_2SO_4$	0.5 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.11 g
Potassium dihydrogen phosphate, KH_2PO_4	0.085 g

Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	0.1 g
Calcium chloride, $CaCl_2 \cdot 2H_2O$	0.05 g
Ferric chloride, $FeCl_3 \cdot H_2O$	0.002 g
EDTA	0.003 g
Vitamin mix	1 mL
Reagent-grade water	1 L

2) *Vitamin mix*

Calcium pantothenate	0.01 g
Niacin	0.01 g
Biotin	0.0005 g
Cyanocobalamin	0.0005 g
Folic acid	0.0005 g
Pyridoxine	0.01 g
p-aminobenzoic acid	0.01 g
Coccarboxylase	0.01 g
Inositol	0.01 g
Thiamine	0.01 g
Riboflavin	0.01 g
Reagent-grade water	100 mL

3) *AcS*

Sodium acetate	0.15 g
Sodium sulfide, $Na_2S \cdot 9H_2O$	0.187 g
MSV	1 L

4) *SS*

Sucrose	0.15 g
Sodium sulfide, $Na_2S \cdot 9H_2O$	0.187 g
MSV	1 L

5) *GS*

Glucose	0.15 g
Sodium sulfide, $Na_2S \cdot 9H_2O$	0.187 g
MSV	1 L

6) *SUC*

Sodium succinate	0.15 g
MSV	1 L

7) *I*

Glucose	0.15 g
MSV	1 L

8) *S*

Sodium sulfide, $Na_2S \cdot 9H_2O$	0.187 g
MSV	1 L

9) *LT*

Sodium lactate	0.5 g
Sodium thiosulfate, $Na_2S_2O_3$	0.5 g
MSV	1 L

* Difco or equivalent.

2. Iron Bacteria

a. *Sphaerotilus-Leptothrix*:

1) Iron bacteria, especially those belonging to the *Sphaerotilus-Leptothrix* group, thrive in media too dilute to support the proliferation of more rapidly growing organisms. One medium⁵ is partially selective for *Sphaerotilus* (BOD dilution water, Section 5210B, supplemented with 100 mg/L sodium lactate). Dispense 50 mL of this medium into French square bottles and autoclave at 69 kPa for 15 min. To inoculate sample add 25-mL portions of stream water or 1-, 5-, and 10-mL portions of settled wastewater or process liquor to duplicate bottles of medium. Incubate at 22 to 25°C for 5 d and observe for filamentous growth. Isolate pure cultures by picking a filament from the BOD-lactate broth and streaking on 0.05% meat extract agar. After incubating for 24 h at 25°C, pick typical curling filaments with the aid of a dissecting microscope and transfer to caseitone-glycerol-yeast autolysate (CGY) broth. If a pellicle with no underlying turbidity develops in 2 to 3 d, transfer a filament to a CGY agar slant, incubate at 25°C until growth is visible, and store in a refrigerator. In addition, extracted alfalfa straw or pea straw may be used for enrichments.⁴

A detailed key for identifying filamentous microorganisms in complex mixtures such as wastewater and activated sludge is available.⁶

2) Isolation and maintenance media have proven quite successful for identifying various groups of filamentous organisms, including iron bacteria.⁷ Prepare agar slants of these media and aseptically pipet 3 mL sterile, dechlorinated tap water onto surface of slants. Inoculate tubes and incubate at room temperature until turbid growth has developed in liquid layer. The cells will remain viable for 3 months in the refrigerator.

3) Another good maintenance medium for cultivating the *Sphaerotilus* group is CGY.⁸

4) *Leptothrix* (*Sphaerotilus discophorous*) can be distinguished from *Sphaerotilus natans* by its ability to oxidize manganous ion. Use Mn agar No. 1 as the differential medium.⁹ Alternatively, *Leptothrix* may be grown by direct plating on Mn agar No. 2.¹

b. *Thiobacillus ferrooxidans*: Although this organism also is a sulfur-oxidizing bacterium,^{10,11} its main importance has been in acid mine drainage. A medium suitable for enumeration of the MPN is available.¹² Some oxidation of iron occurs during sterilization but the loss of ferrous iron is not appreciable. The medium has a precipitate (probably ferrous and ferric phosphates), is opalescent and green, has a pH of 3.0 to 3.6, and contains 9000 mg/L ferrous iron. Growth of the organism is manifested by a decrease in pH and an increase in concentration of oxidized iron. With practice and use of uninoculated controls, an increase of deep orange-brown color can be seen in positive enrichment tubes or flasks as compared to negative ones. Shake test-tube dilutions daily because these organisms are highly aerobic.

c. *Gallionella ferruginea*: For cultivation of this organism use ferrous sulfide agar.^{13,14} Inoculate tubes with a drop of suspension of a suspected *Gallionella* deposit. Growth at room temperature usually occurs in 18 to 36 h and appears as a white deposit on sides of test tube. The ring of colonies occurring at a certain level reflects a balance between upward diffusion of ferrous ions and downward diffusion of oxygen molecules. Supplementation

of ferrous sulfide agar with formalin to isolate pure cultures may be successful.^{1,4,15}

d. *Other iron bacteria*: An acid-tolerant (pH 3.5 to 5.0) filamentous iron-oxidizing *Metallorgenium* has been isolated with a medium¹⁶ containing (NH₄)₂SO₄, 0.1%; CaCO₃, 0.01%; MgSO₄, 0.02%; K₂HPO₄, 0.001%; potassium acid phthalate, 0.4%; and 250 mg/L ferrous iron from an acidified FeSO₄·7H₂O solution. Add 0.4% formalin to 100 mL of the isolating medium in a 250-mL erlenmeyer flask.

For heterotrophic iron-precipitating bacteria¹⁷ use a ferric ammonium citrate medium consisting of: (NH₄)₂SO₄, 0.5 g/L; NaNO₃, 0.5 g/L; K₂HPO₄, 0.5 g/L; MgSO₄·7H₂O, 0.5 g/L; and ferric ammonium citrate, 10.0 g/L. Adjust pH to 6.6 to 6.8 and sterilize by autoclaving (121°C, 15 min). To make the medium solid add 15 g agar/L.

Alternatively grow iron bacteria by combining 20 mL liquid broth medium, 10 mL raw water or inoculum, and 3 g iron oxide. Incubate 48 to 72 h at 25°C on a wrist-action shaker to produce good, visible growth.

3. Sulfur Bacteria

a. *Sulfate-reducing bacteria*:

1) To enumerate sulfate-reducing bacteria such as *Desulfovibrio*, use a sulfate-reducing medium.¹⁸ Inoculate tubes and fill completely with sterile medium to create anaerobic conditions. For comparative purposes, incubate one or two uninoculated controls with each set of inoculated tubes. To sample volumes greater than 10 mL, pass sample through a 0.45-μm membrane filter and transfer filter to screw-cap test tube with medium. If sulfate-reducing bacteria are present, tubes will show blackening within 4 to 21 d of incubation at 20 to 30°C.

2) An agar medium suitable for growth and enumeration of sulfate-reducing bacteria also is available.¹⁹ The medium consists of trypticase soy agar, 4.0%, fortified with additional agar, 0.5%, to which is added 60% sodium lactate (0.4% v/v), hydrated magnesium sulfate, 0.2%, and ferrous ammonium sulfate, 0.2%. Adjust pH to 7.2 to 7.4 and sterilize by autoclaving (121°C, 15 min). Medium should be clear and free from precipitate. Inoculate all plates within 1 or at most 4 h after agar hardens to prevent saturation with oxygen. To prevent moisture condensation on petri dish covers, replace covers with sterile absorbent tops until 10 to 15 min after agar hardens. Place uninverted plates in desiccator or Brewer jars and replace atmosphere with tank hydrogen or nitrogen by successive evacuation and gas replacement. Alternatively, use a disposable anaerobic generating system.[†] Incubate at room temperature (21 to 24°C) or at 28 to 30°C, the optimum temperature for these organisms. Growth and blackening around the colonies is typical of sulfate-reducing bacteria and may occur at any time between 2 and 21 d, although the usual time is 2 to 7 d.

3) Media suitable for enumeration or isolation of various species of sulfate-reducing bacteria are available.²⁰⁻²²

b. *Photosynthetic purple and green sulfur bacteria*: Because these organisms are so specialized and rarely cause problems in water and wastewater treatment processes, methods for their isolation and enumeration are not included here. In certain in-

† GasPak, BBL, or equivalent.

stances they can be beneficial because of their ability to oxidize hydrogen sulfide and thus reduce odor. An excellent review is available.²³ Also, media formulations and methods for cultivating specific members of this group of bacteria are available.²³

c. *Thiobacillus* spp. The growth and physiology of different species of the single-celled sulfur-oxidizing bacteria of the genus *Thiobacillus* have been evaluated carefully.^{24,25} Media²⁶ suitable for enumeration of *Thiobacillus thio-parus* and *Thiobacillus thio-oxidans* by an MPN technique are listed in 9240E.1. Inoculate medium and incubate for 4 to 5 d at 25 to 30°C. Growth of thiobacilli produces elemental sulfur, which sinks to the bottom with a coincident decrease in pH and turbidity of the medium. Chemical tests for formation of sulfate are necessary to confirm presence of *Thiobacillus*.

d. Filamentous sulfur-oxidizing bacteria:

1) *Beggiatoa*—*Beggiatoa* exist in most aquatic habitats where sulfide and oxygen are both present,²⁷ including fresh and marine water, sediments, and wastewater systems. Because *Beggiatoa* is a multicellular bacterium there is no accurate method of determining the number of viable cells in a sample. To determine the population of *Beggiatoa* make a direct microscopic count of the number of filaments.

Marine *Beggiatoa*s may be quite large, up to 100 µm or more in diameter.²⁸ They have not been grown in the laboratory. However, small marine species (up to 5 µm diam) have been isolated.²⁹ The media for isolating freshwater and marine *Beggiatoa*s differ slightly.

a) Enrichment—Inoculate a tube of extracted hay, ¶ 1k1) above, with enough water and a little mud from the sample site to fill the tube to a depth of at least 8 cm. Incubate for at least 1 week and examine for the presence of "tufts" or "puff-balls" consisting of tangled filaments of *Beggiatoa*. Examine by phase-contrast microscopy for the presence of individual filaments.³⁰ If no "puff-balls" are found, continue incubation for another week and repeat the examination. Continue examining the enrichment for up to 4 weeks before discarding it.

b) Isolation—With sterilized fine-tipped forceps and using a dissecting microscope, transfer tufts of *Beggiatoa* from the enrichments to a small petri dish containing sterile basal medium, ¶ 1k2) above. Shake tufts with forceps to remove adherent bacteria and transfer tufts to a new petri dish with sterile basal medium. Continue until tufts have been washed at least five times.

Transfer tufts to a "drying plate" containing basal medium and 1.6% agar for about 1 min to remove excess fluid, then transfer tufts to the center of separate plates of either MY or MP medium,^{30,31} ¶s 1k3) and 4) above. Incubate plates at room temperature or below and examine with a dissecting microscope every 5 to 10 h for the presence of gliding filaments of *Beggiatoa*. Select filaments that have glided well away from the other filaments and appear to be uncontaminated and transfer them to separate plates of the same medium with a sterile, flattened wire, inoculating needle, or toothpick. Take a little agar with the filament to avoid drying the filament during transfer. Examine the first transfer plates every 5 to 10 h as before and transfer pure filaments to fresh media.

2) *Thiothrix*—Obligately mixotrophic *Thiothrix* have been isolated and characterized from sulfur springs and other bodies of flowing water that contain sulfide.^{31,32} Heterotrophic strains have been isolated and characterized from activated sludge

wastewater treatment plants.³³⁻³⁵ While the techniques for the isolation of the mixotrophic and heterotrophic strains are similar, isolation media differ.

a) Isolation of mixotrophic *Thiothrix*—Collect tufts of the bacterium from rocks, water pipes, or other substrates. Using a dissecting microscope, pick up filaments with fine-tipped forceps, shake to remove contaminating bacteria, and transfer to sterile basal medium, ¶ 1k2) above. Repeat at least five times to try to obtain filaments with little or no contamination.

With a sterile Pasteur pipet transfer separate drops to the edge of either MP or MY agar [¶s 1k3) and 4), above] petri dishes and tip the dishes so that the drops run from one side of the dish to the other. Draw off excess moisture with the pipet and incubate plates at room temperature or below for about 48 h. Examine plates under a dissecting microscope for the appearance of typical filamentous colonies. With sterile toothpicks, pick colonies that are widely separated and transfer individually to fresh plates of the same medium. Streak transferred material with a wire loop. After about 48 h incubation, examine plates and restreak colonies that appear to be pure.

b) Isolation of heterotrophic *Thiothrix*—There are two different procedures to use, depending on the concentration and size of the *Thiothrix* filaments in the sample.³⁴

In one procedure, wash individual filaments or rosettes of large strains of *Thiothrix* several times in MSV broth, ¶ 1l 1) above, by transfer with a Pasteur pipet while observing with a dissecting microscope. After several washings transfer filaments to a small amount of MSV (1 to 3 mL) and plate on one or more of the solid media, ¶s 1l 3)–9) above.

If the filaments are small or scarce concentrate the sample by centrifuging. Then dilute 1:5 in MSV, sonicate at 30 W for 10 s, and wash three times by centrifugation at 1900 × g for 2 to 5 min. The supernatant contains free filaments that are used to inoculate one or more of the solid media, ¶s 1l 3)–9) above.

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9245 NITRIFYING BACTERIA*

9245 A. Introduction

1. General Discussion

Nitrifying bacteria convert ammonia to nitrate via nitrite. This process is referred to as nitrification. These bacteria are found in a variety of shapes, including rods, curved rods, spheres, spirals, and lobular forms, and range in size from approximately 0.3 to 11.7 μm . Nitrifying bacteria are aerobic, gram-negative, and either heterotrophic, chemolithotrophic (i.e., obtain energy from simple inorganic chemical compounds such as ammonia or nitrite and utilize carbon dioxide to make sugars for cellular material)^{1,2} or facultative chemolithotrophic. This section discusses only the chemolithotrophic nitrifiers because of their importance in drinking water systems^{3,4} and wastewater.⁵

Chemolithotrophic nitrifiers, once classified in the family *Nitrobacteraceae*, have been reclassified in separate subdivisions (alpha, beta, and gamma) in the division *Proteobacteria*. Intracellular membranes are characteristic of nitrifying bacteria. Two groups of nitrifiers (ammonia oxidizers and nitrite oxidizers) are necessary for successful completion of the nitrification process. Ammonia-oxidizing bacteria (AOB) convert ammonia to nitrite, and nitrite-oxidizing bacteria (NOB) convert nitrite to nitrate. The nitrification process used by these bacteria to obtain their energy source has a low energy yield; therefore, the growth rate of nitrifiers is much slower than that of most heterotrophic bacteria.⁶

The most commonly isolated AOB are classified in the *Nitrosospira* and *Nitrosomonas* genera. *Nitrosomonas europaea* has been the most widely studied species of AOB. *Nitrobacter* has been the NOB genus most commonly identified from freshwater. Identification of nitrifying bacteria can be tedious, in part because of the slow growth rate of nitrifiers and their poor

* Approved by Standard Methods Committee, 2001.
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isolation and colony development on agar culture plates. Nitrifying bacteria can be identified by observing growth on selective inorganic media, by detecting nitrite or nitrate production, by using electron microscopy to observe the physical structure of the internal membranes, or by using molecular methods to target DNA sequences that are specific for the microbial group of interest.

Nitrifying bacteria can occur in a variety of habitats, including fresh water, marine, soil, brackish water, wastewater, and treated drinking water. Nitrifiers are generally sensitive to visible and ultraviolet light; therefore, they are often found in light-free environments. In wastewater treatment, nitrification is a very important process in removing ammonia-nitrogen, which can be toxic to some forms of aquatic wildlife. In addition, nitrification is often an indicator of the overall efficiency of the purification process. Factors affecting the efficiency of nitrification are retention time, temperature, pH, dissolved oxygen, nitrogen, organic matter, heterotrophic and nitrifier populations, and toxic substances. Commonly found genera in activated sludge are *Nitrosomonas* and *Nitrobacter*.⁵

Depending on the treatment application and the disinfectant, nitrification can be either a benefit or a nuisance to the water industry. Nitrification is beneficial whenever the elimination of natural sources of ammonia is desirable. In such a situation, nitrifying bacteria are allowed to increase to high levels in the filter beds or in the clarifiers, where they convert the ammonia to nitrate.⁴ Because ammonia exerts an appreciable chlorine demand whereas nitrate does not, nitrification results in lower costs for chlorination. In the filter beds, elimination of free ammonia may lead to increased biological stability of the water in the distribution system. However, nitrification in drinking water distribution systems that use chloramines can significantly affect water quality.

The oxidation of nitrite to nitrate by NOB either does not occur as a biotic process in chloramine-treated water or occurs slowly in the presence of the disinfectant.³ This leads to an accumulation of nitrite in the water (incomplete nitrification). Nitrite reduces oxidizing agents such as chlorine and chloramine,⁴ which can lead to substantial increases in levels of heterotrophic plate count (HPC) bacteria and possibly coliforms and opportunistic pathogens. These bacteria also may increase in number because they utilize the organic compounds secreted by AOB. Consequently, nitrification can impair a utility's ability to meet the provisions of the U.S. Environmental Protection Agency's Surface Water Treatment Rule (which requires utilities to maintain a disinfectant residual or to have an HPC level below 500 CFU/mL) and possibly the Total Coliform Rule.

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9245 B. Multiple-Tube Method

1. Principle

The multiple-tube (or titerwell) method is based on selective growth of ammonia-oxidizing and nitrite-oxidizing bacteria in specialized media, followed by spot tests and statistical computation of most probable numbers (MPN) of nitrifying bacteria.

2. Apparatus

Sterile test tubes or titerwell plates: If plates are used, use sizes with 6 and 24 wells.

3. Reagents and Media

a. Nitrite spot test reagents:

1) *Sulfanilic acid:* Use 8 g sulfanilic acid in 1 L 5N acetic acid (1 part glacial acetic to 2.5 parts water).

2) *N,N-dimethyl-1-naphthylamine:* Use 5 g (or 5 mL) in 1 L 5N acetic acid. CAUTION: This solution may be carcinogenic; avoid all contact with skin.

b. *Nitrite/nitrate spot test reagent — diphenylamine:*^{1,2} Dissolve 0.2 g diphenylamine in 100 mL conc H₂SO₄. Let solution equilibrate for 12 h.

c. *Reagent-grade water*, low in total organic carbon. Use for preparing culture media.

d. *Ammonia-oxidizing bacteria (AOB) medium:*^{3,4}

MgSO ₄ ·7H ₂ O	0.04	g
(NH ₄) ₂ SO ₄	0.5	g
KH ₂ PO ₄	0.2	g
CaCl ₂ ·2H ₂ O	0.04	g
Chelated iron*	0.0005	g
Phenol red†	0.0005	g
Reagent-grade water	1	L

Dispense ingredients into reagent-grade water and adjust pH to the range 7.8 to 8.1 with diluted NaOH, allowing for pH

* Sequestrene 138, Fe EDTA, or equivalent.

† Not required, but useful for indicating bacterial growth in batch cultures.

changes during autoclaving. Autoclave medium at 121°C for 15 min. If pH after autoclaving exceeds desired range, readjust pH with sterile NaOH or HCl. Dispense medium into tubes so that ratio of sample to medium is approximately 1:10.

e. Nitrite-oxidizing bacteria (NOB) medium:^{5,6}

KNO ₂	0.30	g
MgSO ₄ ·7H ₂ O	0.1875	g
KHCO ₃	1.5	g
K ₂ HPO ₄	0.5	g
KH ₂ PO ₄	0.5	g
NaCl	0.1875	g
CaCl ₂ ·2H ₂ O	0.0125	g
FeSO ₄ ·7H ₂ O	0.01	g
Reagent-grade water	1	L

Dispense other ingredients into reagent-grade water and adjust pH to 7.8 to 8.1 with diluted NaOH, allowing for pH changes during autoclaving. If pH after autoclaving exceeds desired range, readjust pH with sterile NaOH or HCl. Dispense medium into tubes so that ratio of sample to medium is approximately 1:10.

4. Procedure

a. Sample collection: Collect water samples in sterile bottles, using aseptic technique. Dark bottles are recommended. If the sample contains chloramines, dechlorinate with sodium thiosulfate. Store the bottles on ice in a dark container immediately after sample collection. See Section 9060 for more detailed information on sample collection, preservation, and storage.

b. Multi-tube procedure: The use of screw-cap tubes may be desirable to prevent desiccation. Pre-sterilized titerwell plates, which are compact and convenient when incubator space is limited, may be used instead of tubes with modifications to volumes. For example, dispense 1.5 mL sterile medium in each well of the 24-titerwell plate (maximum capacity of each well is 3 mL) and 8 mL medium for the 6-welled plates.

Use spot-test procedures to test water samples qualitatively for nitrite and nitrate before selecting dilutions. If a sample yields positive results for nitrite or nitrate, dilute it sufficiently with medium to ensure that nitrite or nitrate is not detectable, before initiating the assay. Before dispensing the sample, gently agitate the sample bottle. Use a minimum of three 10-fold serial dilutions with 5, 10, or 15 tubes or wells at each dilution. The number of tubes or wells and the sample volumes selected depend on the quality and character of the water to be analyzed. Minimize the samples' exposure to light by keeping them covered or shielded. Inoculate the media at approximately a 1:10 ratio of sample to medium. For the 24-well plates, inoculate 0.1 mL sample or dilution into the wells. For 1.0-mL sample portions, use 6-welled tier plates (maximum capacity of well, 12 mL). Cross-contamination can easily occur between wells; therefore, use caution when inoculating sample. To avoid splashing, place tip of pipet on side of well when dispensing sample, and keep plates level to prevent spillage from adjacent wells. Include blanks and positive control with each batch of samples. *Nitrosomonas europaea* (ATCC 19718) is recommended as a positive control. Use one well per row of six wells as a blank. To prevent desiccation of the medium during incubation, wrap plates in sealable plastic bags.

Incubate tubes or titerwells at 25 to 30°C for 23 to 28 d for ammonia oxidizers and at 23 to 28°C for 23 or more d for nitrite-oxidizers.^{4,6,7} To recover the slower-growing nitrifiers, incubation of the samples may be extended for several additional weeks until there are no additional positive reactions.

After the prescribed incubation period, identify positive tubes or wells as follows:

1) AOB medium—Look for a pH change in the media (indicated by a color change from red to yellow) as a preliminary indicator of growth. For confirmation, take a 1-drop portion from each tube or well and place on a clean spot plate. Add 1 drop each of sulfanilic acid and *N,N*-dimethyl-1-naphthylamine onto each portion for the determination of nitrite, indicating the presence of active AOB. Positive tubes or wells are indicated by a deep red color within 1 min. For additional verification, add 1 drop of diphenylamine to 1 drop of sample on a clean spot plate to determine nitrite/nitrate production. Positive tubes or wells are identified by development of a blue color and are scored positive; the absence of color is scored negative.^{1,2} Tubes or wells that are negative by *N,N*-dimethyl-1-naphthylamine but positive by diphenylamine indicate that the process of nitrification was completed to nitrate.

2) NOB medium — Take a 1-drop portion from each tube or well and place on a clean spot plate. Add 1 drop each of sulfanilic acid and *N,N*-dimethyl-1-naphthylamine onto each portion to determine nitrite consumption by nitrite-oxidizers. The absence of red color development within 1 min indicates the presence of nitrite-oxidizers; the presence of red color indicates the absence of nitrite-oxidizers. For additional verification, add 1 drop of diphenylamine to 1 drop of sample on a clean spot plate to determine the presence of nitrite and/or nitrate production. Positive tubes or wells are identified by development of a blue color. The absence of a blue color is scored negative.^{1,2}

5. Calculations

Calculate most probable number (MPN)/mL by using a standard MPN table (Section 9221C), or MPN program. Although MPN values have been shown to underestimate the density of nitrifying bacteria,⁸ this method is useful for quantifying nitrifiers in water and wastewater.⁹

6. References

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9250 DETECTION OF ACTINOMYCETES*

9250 A. Introduction

1. General Discussion

Earthy-musty odors affect the quality and public acceptance of municipal water supplies in many parts of the world. They are among the naturally occurring odors that plant operators find most difficult to remove by conventional treatment. As early as 1929, it was assumed that these odors could be attributed to volatile metabolites formed during normal actinomycete development.¹ Two such compounds, geosmin and 2-methylisoborneol, have been isolated²⁻⁸ and identified as the agents responsible for earthy-musty odor problems in surface water.⁸⁻¹⁰ Both, however, are produced also by some filamentous blue-green algae.¹¹⁻¹⁵ Geosmin and 2-methylisoborneol have threshold odor concentrations well below the microgram-per-liter level. Thus, traces of these products are sufficient to impart a disagreeable odor to water or a muddy flavor to fish. In areas periodically plagued by this problem, it is prudent to enumerate actinomycetes. Identification of their relative abundance in a drinking water source can provide yet another means to assess water quality. The methods described are well-established techniques that have been used with success in the isolation and enumeration of actinomycetes related to public water supplies.^{16,17} Actinomycetes also have been recognized as a cause of disruptions in wastewater treatment. Massive growths are capable of producing thick foam in the activated sludge process.^{18,19}

Of the general properties of actinomycetes, the most striking is their fungal-type morphology. Although actinomycetes were looked upon initially as fungi, later research revealed that they were filamentous, branching bacteria.²⁰ The actinomycetes are represented most commonly by saprophytic forms that have an extensive impact on the environment by decomposing and transforming a wide variety of complex organic residues. Widely distributed in nature, actinomycetes constitute a considerable proportion of the population of soil and lake and river muds. Most actinomycetes from which geosmin and 2-methylisoborneol have been identified are members of the genus *Streptomyces*, which is considered the most likely to be significant in water supply problems.

2. Samples

a. *Collection:* Collect samples as directed in Section 9060A.

b. *Storage:* Analyze samples as promptly after collection as possible. Store water samples below 10°C if they cannot be processed promptly.

3. References

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* Approved by Standard Methods Committee, 2000.

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9250 B. Actinomycete Plate Count

1. General Discussion

A plating method using a double-layer agar technique has been adapted for determining actinomycete density. Because only the thin top layer of the medium is inoculated with sample, surface colonies predominate and identification and counting of colonies is facilitated.

2. Preparation and Dilution

Prepare and dilute samples as directed in Section 9215 or 9610. Dilutions up to 1:1000 (10^{-3}) usually are suitable for raw water, while treated waters may be examined directly. For soil samples, use dilutions from 1:1000 (10^{-3}) to 1:1 000 000 (10^{-6}).

3. Medium

Starch-casein agar:

Soluble starch	10.0 g
Casein	0.3 g

Potassium nitrate, KNO_3	2.0 g
Sodium chloride, NaCl	2.0 g
Dipotassium hydrogen phosphate, K_2HPO_4	2.0 g
Magnesium sulfate, hydrate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.05 g
Calcium carbonate, CaCO_3	0.02 g
Ferrous sulfate, hydrate, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01 g
Agar	15.0 g
Reagent-grade water	1 L

No pH adjustment is required. Medium is used to prepare double-layer plates. Store medium for bottom layer in bulk or in tubes in 15-mL amounts. Store medium for surface layer in tubes in 17.0-mL amounts.

4. Procedure

a. *Plating:* Prepare three plates for each dilution to be examined. Aseptically transfer 15 mL of sterile starch-casein agar to a petri dish and let agar solidify, thus forming the bottom layer. To a test tube containing 17.0 mL liquefied starch-casein agar at

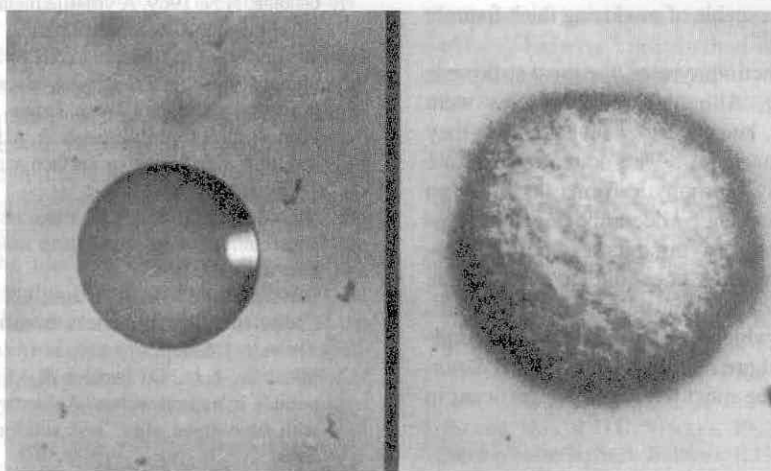


Figure 9250:1. Bacterial colonies—typical colony type vs. actinomycete colony type, 50 $\times$. Left: A typical bacterial colony characterized by a smooth mucoid appearance and a relatively distinct smooth border. Right: An actinomycete colony characterized by the mass of branching filaments that result in the fuzzy appearance of its border and by the dull powdery appearance of the spore-laden, aerial hyphae.

TABLE 9250:I. GENERAL MACROSCOPIC PROPERTIES OF BACTERIAL COLONIES ON SOLID MEDIUM

Characteristic	Typical Colony Type	Actinomycete Colony Type*
Appearance	Shiny or opalescent	When young it is composed of hyphae, but in some species these may later fragment. Substrate and surface hyphae have no distinctive color. As the colony matures, fluffy aerial hyphae that carry spores form and give to colonies of different species various colors and sometimes a chalky appearance. Soluble pigments, either melanin or brightly colored type, that diffuse into the medium, also are common.
Texture	Soft	Strong and leathery
Degree of adherence to solid medium	Weak	Strong
Edge of colony	Regular, continuous, and not different from colony as a whole	Irregular, intermittent, slightly less dense than colony as a whole, and of hyphal appearance

* Actinomycetes are authentic bacteria by all modern criteria, except for their hyphal character and mode of spore formation.

45 to 48°C, add 2 mL of appropriately diluted sample and 1 mL of the antifungal antibiotic, cycloheximide,* prepared in reagent-grade water (1 mg/mL) and sterilized by autoclaving for 15 min at 121°C. Pipet 5 mL of inoculated agar over the hardened bottom layer with gentle swirling to obtain even distribution of the surface layer.

b. *Incubation:* Invert and incubate at 28°C until no new colonies appear. Usually this requires 6 to 7 d.

c. *Counting:* Plates suitable for counting contain 30 to 300 colonies. Identify actinomycetes by gross colony appearance. If necessary, verify by microscopic examination at a magnification of 50 to 100×, as shown in Figure 9250:1. Actinomycete colonies, because of filamentous growth, typically have a fuzzy colonial border. Table 9250:I lists the distinguishing character-

istics commonly used to differentiate actinomycete from other bacterial colonies. Cycloheximide generally suppresses fungal growth; however, fungal colonies, if present, can be recognized by their wooly appearance. Microscopically, fungi reveal a considerably larger cell diameter than actinomycetes.

5. Calculation

Report actinomycetes per milliliter of water or gram (dry weight) of soil. If three plates are used per sample, the average number of colonies on all plates (total number of colonies/3), times 2, times the reciprocal of the dilution (10/1, 100/1, 1000/1, etc.) equals the actinomycete colony count per milliliter of original sample. For solid or semisolid samples, correct for water content and report actinomycete colonies per gram, dry weight, of sample.

* Actidione®, Upjohn and Company, Kalamazoo, MI, or equivalent.

9260 DETECTION OF PATHOGENIC BACTERIA*

9260 A. Introduction

1. General Discussion

One purpose of drinking water and wastewater treatment is to reduce the numbers of viable organisms to acceptable levels, and to remove or inactivate all pathogens capable of causing human disease. Despite the remarkable success of water treatment and sanitation programs in improving public health, sporadic cases and point-source outbreaks of waterborne diseases continue to

occur. Water and wastewater may contain a wide variety of bacteria that are opportunistic or overt pathogens of animals and humans. Waterborne pathogens enter human hosts through intact or compromised skin, inhalation, ingestion, aspiration, and direct contact with the mucous membranes of the eye, ear, nose, mouth, and genitals. This section provides an introduction to the etiologic agents responsible for diseases transmitted by drinking and recreational waters in the U.S.

Over 80 genera of bacteria that are nonpathogenic for humans have their natural habitat in water. In addition, some opportunistically pathogenic bacteria (*Pseudomonas*, *Serratia*, *Acinetobacter*, *Chromobacterium*, *Achromobacter*, *Aeromonas*, etc.) occur naturally in water. Other opportunists (*Bacillus*, *Enter-*

* Approved by Standard Methods Committee, 1997.

Joint Task Group: 20th Edition—Nelson P. Moyer (chair), Terry C. Covert, Peter Feng, Laura B. Kornstein, James D. Oliver, Carol J. Palmer, Christine Paszko, M. Shahamat, William A. Yanko.

obacter, *Klebsiella*, *Actinomyces*, *Streptomyces*, etc.) are sometimes washed into water from their natural habitat in soil or on vegetative matter. Opportunistic pathogens also may be seeded from regrowth and biofilms in water treatment plants and distribution systems.

Water contamination and disease transmission may result from conditions generated at overloaded and/or malfunctioning sanitary waste disposal and potable water treatment systems. In addition, common outdoor recreational activities such as swimming (including pools and hot tubs), boating, camping, and hiking, all place humans at risk of waterborne diseases from ingestion or direct contact with contaminated water.¹ Outbreaks of gastroenteritis, pharyngoconjunctivitis, folliculitis, otitis, and pneumonia are associated with these recreational activities. Overcrowded parks and recreational areas contribute to the contamination of surface and groundwater.

National statistics on outbreaks of waterborne diseases have been compiled in the U.S. since 1920.^{2,3} Since 1971, the Centers for Disease Control and Prevention, the U.S. Environmental Protection Agency, and the Council of State and Territorial Epidemiologists have maintained a collaborative surveillance program on waterborne disease outbreaks of drinking water and recreational water origin.⁴ A summary of waterborne diseases in the U.S. has been published.⁵ Summary data from outbreaks reported through the national waterborne disease surveillance system for drinking water and recreation from 1985 to 1994 are shown in Table 9260:I.

Laboratory diagnosis of infectious disease depends on isolation of the etiologic agent or demonstration of antibody response in the patient. Environmental microbiological examinations are conducted for compliance monitoring of the environment, to trouble-shoot problems in treatment plants and distribution systems, and in support of epidemiological investigations of disease outbreaks. Ideally, the public health microbiologist can contribute expertise in both clinical and environmental microbiology, thereby facilitating epidemiological investigations.

When testing for pathogens in environmental samples, it usually is advisable to include analyses for indicator organisms. Besides coliform indicators (total coliform, fecal coliform, and *E. coli*), fecal streptococci, enterococci, *Clostridium perfringens*, and *Aeromonas* have been proposed as indicators of water quality. No single indicator provides assurance that water is pathogen-free. The choice of monitoring indicator(s) presupposes an understanding of the parameters to be measured and the relationship of the indicator(s) to the pathogen(s). Some bacterial pathogens, such as *Pseudomonas*, *Aeromonas*, *Plesiomonas*, *Yersinia*, *Vibrio*, *Legionella*, and *Mycobacterium*, may not correlate with coliform indicators. Traditional bacterial indicators also may not correlate with viruses or parasites in pristine waters or groundwaters, and they may be of limited utility in estuarine and marine waters. Nevertheless, tests for total and fecal bacteria and *E. coli* are useful, because it is rare to isolate bacterial enteric pathogens in the absence of fecal contamination.

Other more general indicators also may be of value for assessing the potential for pathogen contamination and interpreting culture results. Heterotrophic plate count provides information about the total numbers of aerobic organotrophic bacteria and an indication of the total organic composition of the aquatic environment. Physicochemical factors, such as turbidity, pH, salinity, temperature, assimilable organic carbon, dissolved oxygen, bio-

TABLE 9260:I. SUMMARY DATA FROM WATERBORNE BACTERIAL DISEASE OUTBREAKS, 1985-94

Type of Water	Variable	Number
Drinking water	Total outbreaks	21
	Agent:	
	<i>Shigella</i>	12
	<i>Campylobacter</i>	6
	<i>Salmonella</i>	2
	<i>E. coli</i> O157:H7	1
	System:	
	Noncommunity	10
	Community	8
	Individual	3
	Source:	
	Well	17
	Lake	2
	Spring	1
	Cistern	1
	Cause:	
	Untreated groundwater	9
	Distribution system deficiency	7
	Treatment deficiency	4
	Unknown	1
Recreational water	Total outbreaks	71
	Agent:	
	<i>Pseudomonas</i>	44
	<i>Shigella</i>	17
	<i>Legionella</i>	6
	<i>Leptospira</i>	2
	<i>E. coli</i> O157:H7	2
	Location:	
	Hotel/motel	23
	Outdoor recreation area (surface water)	21
	Home	14
	Spa or public swimming pool	5
	Resort	4
	Apartment complex/condominium	4
	Source:	
	Whirlpool/hot tub	47
	Lake/pond	20
	Swimming pool	3
	Stream	1

chemical oxygen demand, and ammonia may provide useful information about contamination or the potential of water to support bacterial growth. For treated waters, chlorine residual should be measured at the sample collection point.

This section contains methods for *Salmonella*, *Shigella*, pathogenic *E. coli*, *Campylobacter*, *Vibrio cholerae*, *Leptospira*, *Legionella*, *Yersinia enterocolitica*, *Aeromonas*, and *Mycobacterium*. Methods for isolation and enumeration of *P. aeruginosa* are found in Section 9213E and F. Methods for other pathogens are found elsewhere.⁶

The methods outlined below may be used to analyze samples associated with disease outbreaks, or in other studies on the occurrence of pathogens in water and wastewater. Methods for recovery of bacterial pathogens from water and wastewater have not changed significantly in the past 30 years. The methods presented below are not standardized, and the procedures may need modification to fit a particular set of circumstances. No

single procedure is available for reliable detection of any pathogen or group of pathogens. Because the presence of pathogens is intermittent and the survival times in the environment are variable, routine examination of water and wastewater for pathogenic bacteria is not recommended. Even in outbreak situations, the recovery of pathogens from water and wastewater may be limited by lack of facilities, untrained personnel, inadequate methods, and high costs.

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9260 B. General Qualitative Isolation and Identification Procedures for *Salmonella*

Rather than a specific protocol for *Salmonella* detection in water, a brief summary of methods suitable for recovery of these organisms is given. Methods currently available have been used in numerous field investigations to demonstrate *Salmonella* in both fresh and marine water environments. The occurrence of *Salmonella* in water is highly variable; there are limitations and variations in both the sensitivity and selectivity of accepted *Salmonella* isolation procedures for the detection of the more than 2300 *Salmonella* serotypes currently recognized. Thus, a negative result by any of these methods does not imply the absence of salmonellae, nor does it imply the absence of other pathogens.

1. Concentration Techniques

Salmonella are ubiquitous in the environment and can be detected at low concentrations in most surface waters. These organisms are usually present in small numbers compared to coliforms; therefore, it is necessary to examine a relatively large sample to isolate the organisms.¹

a. Swab technique: Prepare swabs from cheesecloth 23 cm wide, folded five times at 36-cm lengths, and cut lengthwise to within 10 cm from the head into strips approximately 4.5 cm wide. Securely wrap the uncut or folded end of each swab with 16-gauge wire for use in suspending the swab in water. Place the swabs in kraft-type bags and sterilize at 121°C for 15 min. Place swab just below the surface of the sampling location for 1 to 3 d.^{2,3} (Longer swab exposure will not increase entrapment of pathogens.) Gauze pads of similar thickness may be substituted. During sampling, particulate matter and microorganisms are concentrated from the water passing through or over the swab. After exposure, retrieve the swab, place it in a sterile plastic bag, ice, and send to the laboratory. Maximum storage-transit time allowable is 6 h. Do not transport swabs in enrichment media; ambient transport temperature may cause sufficient proliferation of competitive organisms to mask salmonellae. In the laboratory, place pad or portions of it in enrichment media. When flasks of enrichment medium containing iced swabs are to be incubated at 40 to 41°C, place flasks in a 44.5°C water bath for 5 min before incubation in an air incubator.

b. Diatomaceous earth technique: Place an absorbent pad (not a membrane filter) on a membrane filter funnel receptacle, assemble funnel, and add 2.5 g sterile diatomaceous earth* to pack the funnel neck loosely. Apply vacuum and filter 2 L of sample. After filtration, disassemble funnel, divide resulting "plug" of diatomaceous earth and absorbent pad in half aseptically with a knife-edged, sterile spatula, and add to suitable enrichment media. Alternatively, place entire plug in enrichment medium.

c. Large-volume sampler: Use a filter composed of borosilicate glass microfibers bonded with epoxy resin to examine several liters or more of sample, provided that sample turbidity does not limit filtration.⁴ The filter apparatus consists of a 2.5- × 6.4-cm cartridge filter and a filter holder.† Sterilize by autoclaving at 121°C for 15 min. Place sterile filter apparatus (connected in series with tubing to a 20-L water bottle reservoir and vacuum pump) in the 20-L sample container appropriately calibrated to measure volume of sample filtered. Apply vacuum and filter an appropriate volume. When filtration is complete, remove filter and place in a selective enrichment medium.

d. Membrane filter technique: To examine low-turbidity water, filter several liters through a sterile 142-mm-diam membrane of 0.45-μm pore size.⁵ For turbid waters, precoat the filter: make 1 L of sterile diatomaceous earth suspension (5 g/L reagent-grade water) and filter about 500 mL. Without interrupting filtration, quickly add sample (1 L or more) to remaining suspension and filter. After filtration, place membrane in a sterile blender jar containing 100 mL sterile 0.1% (w/v) peptone water and homogenize at high speed for 1 min. Add entire homogenate to 100 mL double-strength selective enrichment medium. Alternatively, use multiple 47-mm-diam membrane filters to filter the sample. Immerse each membrane aseptically in 50 mL single-strength selective enrichment medium and incubate.

Qualitative detection of *Salmonella* in suspect potable water also may be achieved successfully by further analysis of selected M-Endo MF cultures (from 100 mL sample volume) that contain significant background growth and total coliforms.⁶ After completing routine coliform count, place entire filter with mixed

* Celite, World Minerals, Inc., Lompoc, CA or equivalent.

† Balston Type AA filter with Type 90 holder, or equivalent.

growth into 10 mL tetrathionate broth (containing 1:50 000 brilliant green dye) for *Salmonella* enrichment before differential colony isolation on brilliant green agar. This unique approach requires no special large sample collections and can be an extension of the routine total coliform analysis.

2. Enrichment

Selectively enrich the concentrated sample in a growth medium that suppresses growth of coliform bacteria. Sample enrichment is essential, because the pathogens usually are present in low numbers and solid selective media for colony isolation are somewhat toxic, even to pathogens. No single enrichment medium can be recommended that allows optimum growth of all *Salmonella* serotypes. Use two or more selective enrichment media in parallel for optimum detection. Elevated incubation temperatures including 40, 41.5, and 43°C and the addition of brilliant green dye to media help suppress background growth and may improve detection of *Salmonella*, but these modifications also suppress growth of some serotypes, including *Salmonella typhi*.

a. *Selenite cystine broth* inhibits gram-positive and nonpathogenic enterobacteria while allowing for recovery of most species of *Salmonella*, including *Salmonella typhi*. Optimum incubation time for maximum recovery of *Salmonella* is 48 h at 35 to 37°C. Repeat streaking from tubes with turbidity several times during first day, and daily up to 5 d to increase potential recovery of all serotypes that may be present. Transfer 1 mL selenite broth culture to a fresh tube of same medium for continued incubation to enrich further *Salmonella* growth and enhance recovery of streak plates.

b. *Selenite-F broth* allows for optimum recovery of most *Salmonella* species, including *Salmonella typhi*, after 24 h at 35 to 37°C. This increased recovery of *Salmonella* is accompanied by a slight decrease in selectivity when compared to selenite cystine. Most significantly, *E. coli* growth is not inhibited. Repeat streaking from tubes with turbidity several times during first day, and daily up to 5 d to increase potential recovery of all serotypes that may be present. Transfer 1 mL selenite broth culture to a fresh tube of same medium for continued incubation to enrich further *Salmonella* growth and enhance recovery of streak plates.

c. *Tetrathionate broth*, incubated at 35°C, inhibits coliforms and Gram-positive bacteria, permitting selective enrichment of most *Salmonella* species, including *S. typhi*. It has been reported that tetrathionate broth is more selective for *Salmonella* than selenite-based media when incubated for 48 h at 43°C. While this formulation is highly selective, it is unable to inhibit *Proteus mirabilis*, which shows optimum growth. Growth of *Proteus* and *Citrobacter* can be inhibited with addition of brilliant green (see Section 9260B.3a). Incubation at 43°C and addition of brilliant green also will inhibit some species of *Salmonella*, including *S. typhi*.

3. Selective Growth

Further separation of pathogens from the remaining nonpathogenic bacterial population is facilitated by proper choice of incubation temperature for primary enrichment followed by secondary differentiation on selective solid media.⁷ These factors,

incubation temperature, enrichment medium, and isolation medium, are interrelated. No one combination is optimum for recovery of all *Salmonella* serotypes. Method comparisons are encouraged to determine the best combination for a given circumstance.

Solid media commonly used for enteric pathogen detection may be classed into three groups: (a) differential media with little or no inhibition toward nonpathogenic bacteria, such as EMB (containing sucrose); (b) selective media containing bile salts or sodium desoxycholate as inhibitors,⁸ such as MacConkey's agar, desoxycholate agar, or xylose lysine desoxycholate (XLD) agar; and (c) selective media containing brilliant green dye, such as brilliant green agar or bismuth sulfite agar. Any medium selected must provide optimum suppression of coliforms while permitting good recovery of the pathogenic group. Great skill at screening for these pathogens is necessary because of the competing growth of various nonpathogens. Streaking duplicate plates, one heavily and one lightly, often aids in recognition of enteric pathogens in the presence of large numbers of interfering organisms.

a. *Brilliant green agar*: Typical well-isolated *Salmonella* colonies grown on this medium are pinkish white with a red background. *S. typhi* and a few other species of *Salmonella* grow poorly because of the brilliant green dye content. Lactose-fermenters not subject to growth suppression will form greenish colonies or may produce other colorations. Occasionally, slow lactose-fermenters (*Proteus*, *Citrobacter*, and *Pseudomonas*) will produce colonies resembling those of a pathogen. Suppress spreading effect of pseudomonads by increasing agar concentration to 2%. In some instances, *Proteus* has been observed to "swarm"; reduce this tendency by using agar plates dried to remove surface moisture. If suspect *Salmonella* colonies are not observed after 24 h incubation, reincubate for an additional 24 h to permit slow-growing or partially inhibited organisms to develop visible colonies. If typical colonies are not observed or if the streak plate is crowded, isolate in pure culture a few colonies for biochemical characterization. Non-lactose-fermenting colonies in close proximity to lactose-fermenting colonies may be masked.

b. *Bismuth sulfite agar* (Wilson and Blair medium⁹): Luxuriant growth of many *Salmonella* species (including *S. typhi*) can be expected on this medium. Examine bismuth sulfite plates after 24 h incubation for suspect colonies; reincubate for 24 h to detect slow-growing strains. Typical colonies usually develop a black color, with or without a metallic sheen, and frequently this blackening extends beyond the colony to give a "halo" effect. A few species of *Salmonella* develop a green coloration; therefore, isolate some of these colony types when typical colonies are absent. As with brilliant green agar, typical colony coloration may be masked by numerous bordering colonies after 48 h incubation. A black color also is developed by other H₂S-producing colonies, for example, *Proteus* and certain coliforms.

c. *Xylose lysine desoxycholate agar*: Compared to brilliant green dye, sodium desoxycholate is only slightly toxic to fastidious *Salmonella*. *Salmonella* and *Arizona* organisms produce black-centered red colonies. Coliform bacteria, *Proteus*, and many *Enterobacter* produce yellow colonies. Optimum incubation time is 24 h. If plates are incubated longer, an alkaline reversion and subsequent blackening occur with H₂S-positive nonpathogens (*Citrobacter*, *P. vulgaris*, and *P. mirabilis*).

d. Xylose lysine brilliant green agar: This medium is especially good for *Salmonella* from marine samples. The brilliant green inhibits many *Proteus*, *Enterobacter*, and *Citrobacter* species.

4. Biochemical Reactions

Many enteric organisms of little or no pathogenicity share certain major biochemical characteristics with *Salmonella*. The identification of pathogens by colony characteristics on selective solid media has limitations inherent in the biological variations of certain organisms and cannot be relied on for even tentative identification. Suspected colonies grown on selective solid media must be purified and further characterized by biochemical reactions; final verification is based on serological identification. Usually a large number of cultures will be obtained from the screening procedure.

Commercially available differential media kits (see Section 9225) may be used as an alternative to Phases 1, 2, and 3 described below, before serological confirmation. These kits give 95 to 98% agreement with conventional tests, although more significant tests will be necessary to achieve further differentiation among strains of *Enterobacteriaceae*.

When such kits are not used, follow a sequential pattern of biochemical testing that will result in a greater saving of media and time for laboratory personnel.¹⁰

Phase 1—Preliminary screening, phenylalanine deaminase activity: Discard phenylalanine deaminase-positive cultures immediately as indicative of the *Proteus* group. In this test, spot isolates on phenylalanine agar and incubate for 24 h at either 35 or 37°C. Phenylalanine deaminase activity is indicated by a green zone that develops around the colony after flooding of the plate with a 0.5M FeCl₃ solution. Subject phenylalanine deaminase-negative cultures to the biochemical tests of Phase 2.

Phase 2—Biochemical tests: The tests used are:

Medium	Purpose of Test
TSI	Fermentation pattern, H ₂ S production
LIA	Lysine decarboxylase activity, H ₂ S production
Urea broth	Urease production

Conformance to the typical biochemical patterns of the *Salmonella* determines whether to process cultures further (Phase 3). Aberrant cultures may be encountered that do not conform to all the classical reactions attributed to each pathogenic group. In all cases, therefore, review reactions as a whole and do not discard cultures on the basis of a small number of apparent anomalies.

Phase 3—Fermentation reactions: Test fermentation reactions in dextrose, mannitol, maltose, dulcitol, xylose, rhamnose, and inositol broths to characterize further the biochemical capabilities of the isolates. This additional sorting reduces the possible number of positive cultures to be processed for serological confirmation. If the testing laboratory is equipped for serological confirmation (see 9260B.5), this series of biochemical tests may be eliminated.

5. Genus Identification by Serological Techniques

Upon completion of the recommended biochemical tests, inoculate the suspected *Salmonella* pure culture onto a brain-heart infusion agar slant and incubate for 18 to 24 h at 35 to 37°C. With wax pencil (china marker), divide an alcohol-cleaned glass slide into four sections. Prepare a dense suspension of test organism by suspending growth from an 18- to 24-h agar slant in 0.5 mL 0.85% NaCl solution. Place a drop of *Salmonella* "O" polyvalent antiserum in the first section and antiserum plus 0.85% NaCl in the second section. Using a clean inoculating loop, transfer a loopful of bacterial suspension to the third section containing 0.85% NaCl solution and to the fourth section containing 0.85% NaCl solution plus antiserum. Gently rock slide back and forth. If agglutination is not apparent in the fourth section at the end of 1 min, the test is negative. All other sections should remain clear.

When biochemical reactions are characteristic of *S. typhi* and the culture reacts with "O" polyvalent antiserum, check other colonies from the same plate for Vi antigen reaction. If there is no agglutination with *Salmonella* Vi antiserum, the culture is not *S. typhi*. Identification of *Salmonella* serotypes requires determination of H antigens and phase of the organism as described by Edwards and Ewing.¹⁰ Isolates yielding biochemical reactions consistent for *Salmonella* and positive with polyvalent "O" antiserum may be identified as "*Salmonella* sp., serotype or bio-serotype undetermined." If species identification is necessary, send isolates confirmed as *Salmonella* by biochemical tests and polyvalent "O" antisera to reference laboratories for further analysis.

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9260 C. Immunofluorescence Identification Procedure for *Salmonella*

The direct fluorescent antibody (FA) technique is a rapid and effective means of detecting salmonellae in freshwater and seawater samples. It may be used as a screening technique to provide rapid results for large numbers of samples, such as those from recreational or shellfish-harvesting waters. Positive FA tests are presumptive evidence for the presence of *Salmonella*. Because of potential cross-reactivity of antibodies, positive FA results should be confirmed by other methods. Sample volumes used depend on the degree of contamination. Where gross pollution is present, use smaller samples. When background information is absent, analyze a 2-L sample, using the diatomaceous earth concentration technique.

1. Apparatus for Fluorescence Microscopy

Standard fluorescent antibody microscopy equipment may be obtained separately or in a package containing the essential instrumentation (a-f):

- a. *Light microscope* with microscope stand.
- b. *Light source*, providing energy in the short-wavelength region of the spectrum. A high-pressure mercury 200-W arc enclosed in a quartz envelope, a 75- to 150-W xenon high-pressure lamp, or a low-voltage 100-W quartz halogen lamp may satisfy this requirement. A significant portion of the energy should be emitted in the ultraviolet and blue region of the spectrum.
- c. *Power pack* to provide constant voltage and wattage output for the selected lamp.
- d. *Basic filters* including heat-absorbing filter (KG-1 or KG-2, or equivalent); red-absorbing filter (BG-38 or equivalent); exciter filter (BG-12 or equivalent, BG-12 being also a blue filter); and barrier filter (OG-1 or blue-absorbing filter). New interference excitation filters (KP500 or equivalent) having very high transmission in the blue portion of the spectrum (490 nm) are available. Barrier or suppression filters used with these have a sharp cutoff at 500 to 510 nm.
- e. *Optics*: The fluorescence microscope must have high-quality optics. A 100 $\times$ objective with an iris diaphragm to reduce the numerical aperture (N.A.) for dark-field work is essential.

Because the N.A. is similar for all 100 $\times$ objectives (1.25 to 1.30), base selection on desire for a flat-field (plano) lens.

f. *Cardioid dark-field condenser* for illuminating specimen: A 95 $\times$ oil immersion objective with built-in iris diaphragm is desirable. True dark-field illumination can be achieved only if the objective N.A. is smaller than the condenser N.A., i.e., of the illuminating cone of light. (Difference in N.A. between objective and condenser should be at least 0.05.) Reduce N.A. of an oil immersion objective by using the built-in diaphragm or by putting a funnel stop onto the objective.

g. *FA pre-cleaned micro slides*, 7.6- $\times$ 2.5-cm, 0.8- to 1.0-mm thickness.

h. *Cover glass for FA slides*, No. 1 1/2, 0.16- to 0.19-mm thickness.

i. *Staining assembly* consisting of dish, cover, and slide rack with handle. Five dishes are required; for Kirkpatrick's fixative, 95% ethanol, first PBS rinse, second PBS rinse, and reagent water.

j. *Moist chamber* used to incubate slides containing smears with added conjugate. A simple chamber consists of water-saturated toweling with a culture dish bottom (150 by 20 mm) placed over the wet toweling.

2. Reagents

a. *Nondrying immersion oil*, Type A (low fluorescence, PCB-free).*

b. *FA Kirkpatrick fixative*, consisting of 60 mL absolute ethanol, 30 mL chloroform, and 10 mL formaldehyde.†

c. *Phosphate-buffered saline (PBS)*: Add 10 g buffer‡ to 1000 mL freshly prepared distilled water. Stir until the powder dissolves completely. Adjust with 0.2N NaOH to pH 8.0.

d. *FA mounting fluid*: Use standardized reagent-grade glycerine adjusted to pH 9.0 with 0.2N NaOH and intended for mounting slides to be viewed with the FA microscope.

* R.P. Cargille Laboratories, Inc., Cedar Grove, NJ, or equivalent.

† Difco No. 3188 or equivalent.

‡ Difco Bacto-FA Buffer, dried, or equivalent.

e. *Reagent (laboratory pure) water*: Use double-distilled water from an all-glass still or other high-quality analytical-grade laboratory water.

f. *FA Salmonella panvalent conjugate* is a fluorescein-conjugated anti-*Salmonella* globulin. To rehydrate, add 5 mL reagent water to a vial or conjugate. Determine working dilution (see ¶ 5e). Store unused rehydrated conjugate in a freezer, preferably at -60°C . Avoid repeated freezing and thawing.

g. *Zn-CdS:Ag phosphor particle*.||

3. Concentration Technique

Place an absorbent pad on a membrane filter funnel and add sufficient sterile diatomaceous earth# to pack funnel neck loosely. Filter 2 L of sample. Rinse funnel with 50 to 100 mL sterile phosphate-buffered dilution water or 0.1% peptone water. Disassemble funnel and remove resulting "plug" of diatomaceous earth and the absorbent pad. Repeat with a second 2-L sample.

4. Enrichment

Immerse one plug and absorbent pad in a flask containing 300 mL selenite cystine broth. Immerse second plug and absorbent pad in a flask containing 300 mL tetrathionate broth supplemented with 3 mL 1:1000 aqueous solution of brilliant green dye and 3 mg L-cystine. Incubate at either 35 or 37°C for 24 h.

5. Fluorescent Antibody Reaction and Analysis

a. Prepare spot plates of brilliant green agar (BGA) and xylose lysine brilliant green (XLBG) agar by placing 1 drop (about 0.01 mL, delivered with a wire or sterile plastic loop) of the enrichment medium (selenite cystine or tetrathionate broth) at each of four separate points on the agar surface.¹ Space drops on agar plate so that FA microscope slide will cover two inoculation points. This is essential because glass slide impression smears of the inoculated points will be made after incubation of plates.

b. Incubate BGA and XLBG plates at $37 \pm 0.5^{\circ}\text{C}$ for 2.5 to 3 h. After incubation, micro CFUs will develop. Make impression smears by taking a clean FA microscope glass slide and placing it over two inoculated points on the medium. Press down lightly, being careful not to move glass slide horizontally. Do not apply too much pressure, because it will cause movement of the slide and collection of additional agar. Repeat this process for the other two inoculation points and for inoculation points on second agar medium. Prepare a total of four FA slides in this manner.

c. Air-dry smears and fix for 2 min in Kirkpatrick's fixative. Rinse slides briefly in 95% ethanol and let air dry. Do not blot.

d. Cover fixed smears with 1 drop of *Salmonella* panvalent conjugate. Before use, dilute commercial conjugate and determine appropriate working dilution. Most batches are effective at a 1:4 dilution but this will vary with the type of fluorescence equipment used, light source, alignment, magnification, cultures, etc. Determine working dilution (titer) of each lot of conjugate.

e. To determine conjugate titer use a known 18- to 24-h *Salmonella* culture grown in veal infusion broth and make smears on FA glass slide. Dilute conjugate and treat as outlined in c and d above. For example, if the following results are obtained:

Dilution of Conjugate	Fluorescence
1:2	4+
1:4	4+
1:6	4+
1:8	2+
1:10	1+

use the second highest dilution giving 4+ fluorescence. In the above example use a 1:4 dilution of conjugate. Diluting conjugate insures minimum cross-reactivity. Prepare fresh diluted conjugate daily.

f. After covering each smear with 1 drop of appropriate dilution of conjugate, place slides in a moist chamber to prevent evaporation of staining reagent. After 30 min wash away excess reagent by dipping slides into phosphate-buffered saline (pH 8.0). Place slides in second bath of buffered saline for 10 min. Remove, rinse in distilled water, and drain dry. Do not blot.

g. Place a small drop of mounting fluid (pH 9.0) on the smear and cover with a No. 1½ cover slip. Seal edges of cover slip with clear fingernail polish. Examine sealed slides within a few hours while fluorescence is of optimum intensity. Examine under a fluorescence microscope unit fitted with appropriate filters.

h. Include a positive control slide with each set of samples. This checks conjugate reactivity and FA equipment generally.

6. Recording and Interpreting Results

The intensity of organisms fluorescing in any given field is important in assessing positive *Salmonella* smears. If the majority of cells present fluoresce (4+ or 3+) the smear is positive. Carefully scrutinize smears showing only a few scattered fluorescing cells. Critical examination of cellular morphology may distinguish between these cells and salmonellae. The degree of fluorescence is the criterion on which positivity is based. Consider weakly fluorescing cells (2+ and 1+) negative. Confirm all positive FA results by cultural techniques (see Section 9260B).

Reaction	Description	Fluorescence Intensity
Positive	Brilliant yellow-green fluorescence, cells sharply outlined	4+
Positive	Bright yellow-green fluorescence, cells sharply outlined with dark center	3+
Negative	Dull yellow-green fluorescence, cells not sharply outlined	2+
Negative	Faint green fluorescence discernible in dense areas, cells not outlined	1+
Negative	No fluorescence	0

7. Quantitative Immunofluorescence Microspectrofluorometric Microscopy

To make such analyses use a system consisting of analyzing and illumination sections. The analyzing section includes an

§ Difco or equivalent.

|| General Electric or equivalent.

Celite, World Minerals Inc., Lompoc, CA, or equivalent.

eyepiece monochromator assembly and a photomultiplier-photometer. The eyepiece uses a beam splitter that reflects to the monochromator and the observer's eye, allowing for simultaneous visual observation and quantitative analysis of the yellow-green fluorescence intensity. The photometer package provides meter readout in milliamperes so that visual observation of fluorescence can be correlated with objective reading. Microspectrofluorometry can be done with a conventional fluorescence microscope.

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9260 D. Quantitative *Salmonella* Procedures

This procedure describes one approach for estimating *Salmonella* density in water samples. Other methods have been described in the literature and a comparative study is recommended to select the best quantitative method for any given application. The following procedure must be modified for use with solid or semisolid samples.

Because of the high ratio of coliform bacteria to pathogens, large samples (1 L or more) are required. Any concentration method in Section 9260B.1 may be used but preferably concentrate the sample by the membrane filter technique (Section 9260B.1d). After blending the membrane with 100 mL sterile 0.1% (w/v) peptone water, use a quantitative MPN procedure by proportioning homogenate into a five-tube, three-dilution multi-

ple-tube procedure using either selenite cystine, selenite-F, or tetrathionate broth as the selective enrichment medium (See Section 9260B.3). Incubate for 24 h as specified or required for the enrichment medium used and streak from each tube to plates of brilliant green and xylose lysine desoxycholate agars. Incubate for 24 h at 35°C. Select from each plate at least one, and preferably two to three, colonies suspected of being *Salmonella*, inoculate a slant each of triple sugar iron (TSI) and lysine iron (LIA) agars, and incubate for 24 h at 35°C. Test cultures giving a positive reaction for *Salmonella* by serological techniques (see Section 9260B.5). From the combination of *Salmonella* negative and positive tubes, calculate the MPN/1.0 L of original sample (see Section 9221C).

9260 E. *Shigella*

Shigellosis is an acute gastrointestinal disease of humans, caused by four species or serogroups of the genus *Shigella*, *S. dysenteriae* (Group A), *S. flexneri* (Group B), *S. boydii* (Group C), and *S. sonnei* (Group D). Shigellae invade the intestinal mucosa, producing dysentery characterized by abdominal pain, fever, and diarrhea. The infectious dose for *Shigella* spp. is low, and most cases result from person-to-person transmission. When outbreaks occur, they are usually associated with fecal contamination of foods and, less frequently, water. The shigellosis case rate has gradually risen in the U.S. over the past 30 years from 6 cases/100 000 population in 1965 to 12 cases/100 000 population in 1995.¹ In the U.S., *S. sonnei* (66.5%) is the most common cause of shigellosis, followed by *S. flexneri* (16.4%), *S. boydii* (1.1%), and *S. dysenteriae* (0.5%). The serogroup is not reported for 15.5% of cases.

Shigellosis is most common among children. Outbreaks from direct transmission have been reported in schools, day-care centers, and institutions providing custodial care. Waterborne outbreaks are associated with fecal contamination together with inadequate chlorination of private or noncommunity water supplies, as the result of

cross-connections between wastewater and potable water lines, and from exposure to fecally contaminated recreational waters.

Shigellae are sensitive to chlorination at normal levels, and they do not compete favorably with other organisms in the environment. Their survival time is measured in hours and days, and is a function of the extent of pollution, as well as physical conditions such as temperature and pH. Shigellae survive up to 4 d in river water. However, the time required to establish a laboratory diagnosis by culture of patient specimens (1 to 2 d) makes it improbable that shigellae can be recovered from an environmental source unless there is a continuous source of contamination such as wastewater seepage. Shigellae can survive in a viable but nonculturable state after 21 d.² The public health significance of nonculturable shigellae in the environment is unknown.

Methods for the reliable quantitative recovery of shigellae from the environment are not yet available. Culture of shigellae is usually either not attempted or unsuccessful. Methods that have resulted in isolation of *Shigella* include membrane filtration^{3,4} and centrifugation^{5,6} with or without subsequent broth enrichment. Recently, the polymerase chain reaction (PCR) has

shown promise for detection of shigellae in environmental samples.⁷⁻⁹

1. Sampling and Storage

Collect a water sample in a sterile 1-L container. Collect soil, sediment, sludge, or other samples in plastic bags* or glass or plastic bottles. Hold samples at 2 to 8°C until they are processed. Process samples as soon as possible after collection.

2. Enrichment

Choose a selective enrichment medium to minimize accumulation of volatile acid by-products derived from growth of potentially antagonistic bacteria. Selenite F broth has been used successfully to recover shigellae from water and sand.^{5,6} While GN broth facilitates better recovery of shigellae from stools than Selenite F broth, the only reported attempt to use GN broth as an enrichment for membrane filters for isolation of shigellae failed to recover the organism.¹⁰

Alternatively, use reduced-strength nutrient medium adjusted to pH 8.0 (0.15 g tryptic soy broth, added directly to the sample). During outbreak investigations, the enrichment medium may be made selective by incorporation of antibiotics to which the clinical isolates have shown resistance, such as tetracycline and streptomycin at concentrations of 150 µg/mL.¹¹

3. Membrane Filter Procedure

This procedure is suitable for low-turbidity potable and surface waters with low concentrations of coliform bacteria. Filter 100-mL to 1-L samples through 0.45-µm pore size membranes and place filters face up on the surface of XLD or MacConkey agar plates; incubate plates at 35°C overnight. Where growth is confluent, sweep growth from plate and inoculate GN or Selenite F broth enrichments; incubate for 6 h and streak onto MacConkey and XLD plates for colony isolation. Pick colorless colonies (lactose nonfermenters) from membrane or plates to TSI and LIA slants; incubate overnight at 35°C. For biochemical reactions and serological grouping, see ¶ 5 below.

4. Centrifugation Procedure

This procedure is suitable for surface waters, wastewater, and sediments. Centrifuge 200- to 250-mL water samples at 1520 × g for 15 min and pour off all but last 2 mL of supernatant. Resuspend pellet and add 8 mL Selenite F or GN broth. Incubate suspension for 24 h at 35°C. Mix suspension and inoculate one loopful to each of several MacConkey and XLD plates. Streak plates for isolation and incubate overnight at 35°C. Examine plates for colorless colonies, and pick suspect colonies to TSI and LIA slants; incubate at 35°C overnight. For biochemical reactions and serological grouping, see ¶ 5 below.

For solid samples (sediments, soil, sludge, etc.) suspend 10 g sample in 100 mL Selenite F or GN broth and mix thoroughly. Incubate suspension overnight at 35°C. Resuspend sediment and streak one loopful onto each of several MacConkey and XLD

TABLE 9260:II. REACTIONS OF COMMON BACTERIA ON TSI AND LIA MEDIA

Organism	TSI*	LIA*
<i>Shigella</i>	K/A—	K/A—
<i>Salmonella</i>	K/Ag+	K/A+
<i>Escherichia</i>	A/Ag—	K/K—
<i>Proteus</i>	A/Ag+ or K/Ag+	R/A+
<i>Citrobacter</i>	A/Ag+	K/A+
<i>Enterobacter</i>	A/Ag—	K/A—
<i>Aeromonas</i>	A/A—	K/A—
<i>Yersinia</i>	A/A— or K/A—	K/A—
<i>Plesiomonas</i>	K/A—	K/A—

* Fermentation reactions = slant/butt; H₂S production = + or —; K = alkaline, A = acid, R = red (deaminase reaction); g = gas produced.

agar plates; incubate overnight at 35°C. Pick colorless colonies to TSI and LIA slants, and proceed as above. For biochemical reactions and serological grouping, see ¶ 5 below.

5. Biochemical Identification and Serological Grouping

Examine the TSI and LIA slants for the reactions shown in Table 9260:II. Cultures that are presumptively identified as *Shigella* spp. are serogrouped by a slide agglutination test using polyvalent and group specific antisera. Refer cultures to a public health reference laboratory if molecular typing is desirable for outbreak-related strains.

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* WhirlPak®, Ziploc®, or equivalent.

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9260 F. Pathogenic *Escherichia coli*

Escherichia coli is a normal inhabitant of the human digestive tract; however, some *E. coli* cause diarrheal diseases in humans.¹ These pathogenic *E. coli* are classed into five groups: enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), and the newly recognized group called enteroadherent-aggregative *E. coli* (EA-AggEC) for its aggregative or "stacked-brick"-like adherence to cultured mammalian cells.² Pathogenic *E. coli* can be grouped on the basis of serology but, because they are classed on the basis of distinct pathogenic factors, definitive identification requires the determination of the characteristic virulence properties associated with each group. These include: plasmid-mediated cell invasion, plasmid-mediated colonization and enteroadherence factors, production of several potent cytotoxins, hemolysins, as well as heat-labile and stable enterotoxins.³

Although pathogenic *E. coli* have most often been implicated in foodborne illness, several major waterborne outbreaks have been reported.⁴ Outbreaks have involved both water supplies⁵⁻⁷ and recreational waters.^{8,9} Some *E. coli* pathogens have a low infectious dose.

1. Examination Procedures

The pathogenic *E. coli* groups are phenotypically diverse; hence, no standard microbiological methods have been developed for these pathogens. Unlike typical *E. coli*, some pathogenic groups like EIEC do not ferment lactose³; hence, coliform methods based on lactose fermentation are not suitable for detection of EIEC. Also, many fecal coliform confirmation or enrichment procedures use elevated incubation temperature, which is inhibitory to the growth of EHEC.¹⁰ Elevated temperatures and sodium lauryl sulfate used in lauryl tryptose broth (LTB) for MPN analysis also have been found to cause the loss of plasmid, which encodes many of the virulence-associated factors.¹¹

Pathogenic *E. coli* that ferment lactose and are not affected by elevated temperatures still can be presumptively distinguished from non-*E. coli* by the MPN fecal coliform procedure (9221E) or the fecal coliform membrane filter method (9222D) followed by serotyping and virulence analysis. These methods, as well as methods from other sources,¹² also have been modified to detect specific pathogenic groups. Regardless of the method, however, when testing for pathogenic *E. coli*, first identify isolates as *E. coli* either by conventional biochemical testing or by using commercially available biochemical identification kits (see Section 9260B.4) before serotyping and assaying for the virulence factors associated with the respective pathogenic groups.

a. EHEC O157:H7: The following procedure is a modification of the standard total coliform fermentation technique (9221B) for detecting *E. coli* O157:H7 in water.¹³ Inoculate a

100-mL sample into 50 mL 3× lauryl tryptose broth (LTB) and incubate at 35°C for 24 h. Serially dilute the sample, spread plate (0.1 mL) onto sorbitol MacConkey agar (SMAC)* and incubate at 35°C for 18 to 24 h. EHEC O157:H7 form colorless colonies because they do not ferment, or are slow fermenters of, sorbitol. Pick ten sorbitol-negative colonies, transfer individually into LTB-MUG (4-methylumbelliferone glucuronide; 0.1 g/L)¹⁴ and incubate at 35°C for 18 to 24 h. EHEC O157:H7 ferment lactose, but do not have β-glucuronidase activity to hydrolyze MUG, so cultures will appear gas-positive and nonfluorescent. Assay these for positive glutamate decarboxylase activity,¹³ then identify biochemically as *E. coli*.

Larger volumes of sample also may be examined by the following procedure modified from a procedure for detecting O157:H7 in food.¹⁵ This procedure has not been tested for use in water analysis; however, it has been used extensively to detect O157:H7 bacteria in apple juice. Centrifuge 200 mL sample at 10 000 × g for 10 min. Resuspend pellet in 225 mL EHEC enrichment broth (EEB) and incubate at 35°C for 6 h. Spread plate 0.1 mL from EEB and a 1:10 dilution of EEB onto tellurite cefixime SMAC (TC SMAC). Both EEB and TC SMAC contain antibiotics to reduce growth of normal flora bacteria; therefore, they are best suited for highly contaminated samples. Incubate EEB sample and TC SMAC at 35°C for 18 to 24 h. Observe TC SMAC plates for isolated, colorless colonies. If none are evident, serially dilute the overnight EEB sample and replate onto TC SMAC. Test colorless colonies for positive indole reaction and identify biochemically as *E. coli* before serotyping and virulence analysis for the Shiga toxin genes.

b. EPEC, ETEC, EIEC: With the exception of EIEC, use either the MPN fecal coliform procedure (9221E) or the fecal coliform membrane filter method (9222D) for presumptive isolation of these pathogenic *E. coli* groups from water. Alternatively plate presumptive positive samples onto selective media, such as LES Endo and MacConkey (MAC) agars (see Section 9221B.3, Completed Phase). In food analysis, L-EMB agar also has been used. For EIEC, which ferment lactose slowly or not at all, the MPN method is not useful; however, the membrane filter method (9222D) can be used. In food testing for EIEC, Hektoen agar (HE), *Salmonella-Shigella* (SS) agar and MAC are used for selective plating, but HE and MAC appear less inhibitory and are best suited for the isolation of EIEC.¹⁰ In the analysis of each pathogenic *E. coli* group, preferably pick 10 typical (lactose-positive) and 10 atypical (lactose-negative) colonies for biochemical identification. Identify all isolates as *E. coli* before serological typing and analysis for the group-specific virulence factors.

* Oxoid USA, Columbia, MD; Difco, Detroit, MI.

2. Serotyping

For definitive identification, serotype for the O:H antigens any isolates presumptively identified as pathogenic *E. coli* by microbiological methods. Polyvalent antisera are available commercially, but only for the common serotypes. Several anti-O157 and anti-H7 latex agglutination kits are available for typing O157:H7 isolates. Serotype information also is essential for epidemiological investigations.

3. Virulence Analysis

The pathogenic potential of an *E. coli* isolate can be determined only by testing for its distinctive virulence properties. A simple antibody-bound latex agglutination kit and several enzyme linked immunosorbent assay kits are available for testing Shiga cytotoxins of EHEC†. An agglutination kit also is available for testing labile and stable enterotoxins of ETEC‡, but analysis of other virulence factors may require bioassays using animal models, tissue cultures, or other antibody and nucleic-acid-based molecular methods. A partial listing of commercially available assays and media for pathogenic *E. coli* is available.¹² Most of the assays are specific for EHEC O157:H7 and introduced only recently for food analysis; hence, few have been evaluated by collaborative studies.

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† VEROTEST, MicroCarb; Premier EHEC, Meridian; Verotox-F, Denka Seiken.
‡ VET-RPLA, Unipath.

9260 G. *Campylobacter jejuni*

Campylobacters are commonly found in the normal gastrointestinal and genitourinary flora of wild animals, birds, and domestic animals including sheep, cattle, swine, goats, and chickens.¹ *Campylobacter* infections often are acquired by the fecal oral route, often as zoonoses through exposure to infected animals. Large outbreaks have resulted from contaminated milk, uncooked meat or fowl, and contaminated water systems.² *Campylobacter* has been reported to be the most common cause of bacterial enteritis worldwide.³

Waterborne transmission of *Campylobacter* has resulted from drinking untreated surface water, contamination of

groundwater with surface water, faulty disinfection, and contamination by wild bird feces.⁴ In remote mountain areas, the infection has been associated with drinking surface water from cold mountain streams.⁵ Occurrence of *campylobacters* in surface water is variable and appears to be seasonally dependent, with lowest levels occurring in summer. Survival in surface water is affected by both temperature and sunlight.⁶ Between 1978 and 1986, 57 outbreaks of campylobacteriosis were reported, including 11 waterborne outbreaks, 7 of which occurred in community water supplies.

1. Water Collection and Filtration Method

Collect large-volume water samples in sterile 10-L plastic containers. Process samples immediately after collection or store at 4°C and process as soon as possible. Filter one to several liters of the water through a 0.45- or 0.22- μ m-pore-size, 47-mm-diam, cellulose nitrate membrane filter. Remove filter and place face down on selective medium (see isolation section). Incubate microaerophilically at 42°C for 24 h. Remove filter from the plate and place it face down on another selective plate. Incubate both plates at 42°C for up to 5 d.⁷

For turbid water pre-filtration is necessary. Use a stainless steel filtration device with a 1.5-L reservoir.* Assemble with the following filter sequence: Place a 142-mm, 3.0- μ m filter on the screen inside reservoir with a 124-mm prefilter on top. In the bottom tubing adapter place a 47-mm, 1.2- μ m filter. Then place Swinnex filter holders in parallel with a 47-mm, 0.65- μ m filter in the upstream filter holder and a 47-mm, 0.45- μ m filter in the downstream holder. Add 1 L sample to the reservoir, seal, and apply pressure of about 350 kPa. After filtration, remove the 0.45- μ m-pore-size filter and culture on selective plate medium as described above.

2. Isolation

Campylobacter isolation requires use of selective media containing antimicrobial agents, microaerophilic atmosphere (5% O₂, 10% CO₂, and 85% N₂), and 42°C incubation temperature, to suppress the growth of most common bacteria.⁸ The thermophilic campylobacters (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) grow well at 42°C. However, other campylobacters (*C. jejuni* subsp. *doylei* and *C. fetus*) do not grow well at 42°C; incubate plates at both 37°C and 42°C for optimal isolation of these bacteria.⁹ Microaerophilic conditions can be provided by using commercially available systems and equipment.[†]

Several selective media for plating campylobacters are commercially available. Skirrow's medium contains blood agar base with lysed horse blood, trimethoprim, vancomycin, and polymyxin B. Campy-BAP contains *Brucella* agar base with sheep blood, trimethoprim, vancomycin, polymyxin B, amphotericin B, and cephalothin (to which some campylobacters are sensitive). Butzler's medium contains thioglycollate agar with sheep blood, bacitracin, novobiocin, cycloheximide, and cefazolin. Preston's medium contains *Campylobacter* agar base with horse blood, cycloheximide, rifampicin, trimethoprim, and polymyxin B. Other media, such as *Campylobacter* blood-free selective medium and *Campylobacter* charcoal differential agar, can be used to isolate campylobacters.¹⁰ Use of enrichment broth will improve recovery of campylobacters.

Several enrichment media, such as *Campylobacter* broth, Campy-thio broth, Gifu anaerobe-modified semisolid medium, and Preston medium, are used to enhance recovery of campylobacters.⁹ Add 10 mL water sample to 10 mL *Campylobacter* enrichment broth tubes in duplicate, and incubate cultures at 37°C and 42°C for 8 h or overnight. Pre-enrichment of water

sample in a selective enrichment broth for 4 h at 37°C may be important for recovery of stressed cells of *C. jejuni* that show less tolerance to elevated growth temperatures. For pre-enrichment of water sample, add 10 mL water to 10 mL enrichment medium and incubate culture for 4 h at 37°C, then transfer the cultures to another incubator at 42°C for overnight incubation.^{11,12}

C. jejuni may be induced to a nonculturable state in water, and it is not clear whether pre-enrichment or enrichment will facilitate isolation of these bacteria.¹³ Use of a decreased substrate concentration enhances metabolic activity in nonculturable campylobacters from water.¹⁴

3. Identification

a. Culture examination: Examine *Campylobacter* plates at 24 and 48 h for characteristic colonies, which can range from flat, spreading colonies that cover the entire surface of the plate, to very small, convex, translucent colonies. Colony colors range from gray to yellowish or pinkish.

b. Microscopy identification: *Campylobacter* spp. do not stain well by the conventional Gram stain. If safranin is used as a counterstain, apply it for 2 to 3 min; carbol fuchsin is a better alternative. Even 24-h cultures of campylobacters appear pleomorphic in stained smears, and cells range from small Gram-negative rods and coccoid forms to longer rods that may show an "S" or seagull shape, and long spirals, particularly from older cultures.¹⁵

c. Motility test: *Campylobacter* normally are motile by a single polar flagellum at one or both ends. Suspend cells in Mueller-Hinton or nutrient broth, and observe motility using phase microscopy or brightfield microscopy with reduced illumination. Do not use saline or distilled water because they may inhibit motility.⁸ Young cells are 0.2 to 0.8 μ m wide by 1.5 to 5 μ m long, curved or spiral, and motile with darting or corkscrew-like motion.¹⁶

d. Biochemical tests: Despite numerous studies, campylobacters remain relatively difficult to rapidly identify, classify, and type biochemically.¹⁷ Campylobacters do not ferment or oxidize carbohydrates, and they are inert in most biochemical media used to characterize bacterial isolates.¹⁸ Although no standard methods for the characterization of campylobacters have been published, oxidase, catalase, nitrite and nitrate reduction, H₂S production, hippurate hydrolysis, resistance to various agents, temperature tolerances, and growth requirements are among the common phenotypic tests used to characterize campylobacters.³

4. Serological Identification Tests

Commercially available kits[‡] for serotyping campylobacters are available. These kits use latex particles coated with polyvalent immunoglobulins for several *Campylobacter* species. They are designed for rapid presumptive identification of the thermophilic, enteropathogenic *Campylobacter* species (*C. jejuni*, *C. coli*, and *C. lari*); use in accordance with manufacturer's instructions.¹⁹

* Millipore No. 316 or equivalent.

† Campy Pak II, BioBag Environmental Chamber or BioBag Type Cfj, Becton Dickinson; Gas Generating Kit System BR56 or Campy Gen. Oxoid; Poly Bag System, Fisher Scientific; or equivalents.

‡ Such as Campyslide, BBL Microbiology Systems; Meritec-Campy, Meridian Diagnostics; and Microscreen, Mercia Diagnostics.

Other techniques that are not widely available in all laboratories include lectin agglutination, cellular fatty acid profiles, nucleic acid probes, polymerase chain reaction, and other genomic methods that can be used in reference and research laboratories for detection and identification of campylobacters.³

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9260 H. *Vibrio cholerae*

Vibrio cholerae is the causative agent of cholera, a waterborne illness with symptoms ranging from mild to severe and potentially fatal diarrheal disease.^{1,2} This is a well-defined species on the basis of biochemical tests and DNA studies, but the serotypes within the species can be quite diverse in their ability to produce infection. The O1 serogroup is associated with epidemic and pandemic cholera, especially in developing countries. The current (seventh) pandemic has affected over 100 countries, including the United States, with over one million reported cases and 10 000 deaths.³ The newly identified O139 Bengal serogroup⁴ also is capable of producing epidemic cholera. In contrast, the great majority of non-O1/non-O139 strains, which are more common in the environment, do not produce cholera toxin, and are not associated with epidemic cholera. However, these strains occasionally are associated with potentially fatal extra-intestinal infections. *V. cholerae* occurs as part of the normal microflora in estuarine areas, with non-O1/non-O139 strains being much more common than are O1 strains.

1. Concentration Techniques

Levels of *V. cholerae* in natural waters and sewage usually are quite low. Thus, methods of concentration or enrichment usually are employed. One method for isolating *V. cholerae* O1 from contaminated waters is placement of Moore swabs in flowing wastewater for periods up to 1 week, followed by placement into enrichment media at a 1:1 (weight/volume) ratio.⁵

2. Enrichment Procedures

Samples are enriched in alkaline peptone broth (1% peptone, 1% NaCl, pH 8.4), using appropriate concentration of broth relative to sample volume. Incubate enrichment cultures for 6 to 8 h at 35°C, then streak a loopful of the enrichment broth onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar and incubate these plates at 35°C for 18 to 24 h.⁶ Other enrichment and plating media have been reviewed.^{7,8}

3. Selective Growth

Suspected *V. cholerae* colonies appear yellow, a result of sucrose fermentation. A variety of other sucrose-fermenting vibrios also appear on TCBS, however, including *V. fluvialis*, *V. furnissii*, *V. alginolyticus*, *V. metschnikovii*, *V. cincinnatiensis*, and *V. carchariae*.²

4. Presumptive Tests to Differentiate *V. cholerae*

The following key tests are used to identify *V. cholerae*:

Test	Reaction
Gram-negative rod	+
Cytochrome oxidase	+
Glucose fermented (no gas)	+
Growth in nutrient broth:	
No NaCl added	+
8% NaCl added	—
Arginine dihydrolase	—
Ornithine decarboxylase	+
ONPG hydrolysis	+

After isolation on TCBS, streak presumptive *V. cholerae* isolates to a nonselective medium, such as trypticase soy agar, containing a minimum of 0.5% NaCl.

5. Classification of Isolates as *V. cholerae*

The tests listed below may be used for a more extensive phenotypic characterization of *V. cholerae*.⁷ To determine the serogroup, use agglutination assays.

Test	Reaction
ONPG	+
Nitrate reduction	+
Indole	+
O/129 sensitivity:	
10 mg	+
150 mg	+
Swarming	—
Luminescence	v*
Thornley's arginine dihydrolase	—
Lysine decarboxylase	+
Ornithine decarboxylase	+
Growth at 42°C	+
Growth at % NaCl:	
0%	+
3%	+
6%	v*
8%	—
10%	—
Voges-Proskauer reaction	v*
Gas from glucose fermentation	—
Fermentation to acid:	
L-Arabinose	—
m-Inositol	—
D-Mannose	v*
Sucrose	+

Test	Reaction
Enzyme production:	
Alginase	—
Amylase	+
Chitinase	+
Gelatinase	+
Lipase	+
Utilization as sole source of carbon:	
γ-Aminobutyrate	—
Cellobiose	—
L-Citrulline	—
Ethanol	—
D-Gluconate	+
D-Glucuronate	—
L-Leucine	—
Putrescine	—
Sucrose	+
D-Xylose	—

* v = variable, differs for strains within the species.

6. Serological Identification

Slide agglutination with polyvalent antisera can be used to identify the serogroups of *V. cholerae*. Polyvalent antiserum for *V. cholerae* O1 is available commercially.* The O1 serogroup can be further divided into two primary serotypes, Ogawa and Inaba.

7. Biotypes of Serogroup O1 *V. cholerae*

V. cholerae can be divided into two biotypes or biovars, classical and El Tor, which differ in several characteristics. The El Tor biotype currently is the most important biotype.

Test ⁷	Biovar	
	Classical	El Tor
Hemolysis of sheep erythrocytes	—	v*
Voges-Proskauer reaction	—	+
Chicken erythrocyte agglutination	—	+
Antibiotic sensitivity:		
Polymyxin B (50 IU)	+	—
Bacteriophage susceptibility:		
Mukerjee classical phage IV	Lysis	No lysis
Mukerjee El Tor phage 5	No lysis	Lysis

* v = different reaction within the serovar.

8. Other Procedures

Environmental samples also may be examined by fluorescent-antibody techniques, but the number of *V. cholerae* cells in aquatic samples is generally quite low.⁷ Nucleic acid probes are not routinely used for the identification of *V. cholerae*, although DNA probes are extremely useful in determining which strains of this species contain the cholera toxin gene.² This distinction is especially important in examining environmental isolates of *V. cholerae* because the great majority of these strains lack the cholera toxin gene.

* Difco or equivalent.

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9260 I. *Leptospira*

Leptospira spp. are motile, aerobic spirochetes that require fatty acids for growth.¹ Serum or polysorbate enrichments must be incorporated into artificial media, and some pathogenic strains may require CO₂ upon initial isolation. Leptospire are divided into two groups, based on their pathogenicity and growth characteristics. The saprophytic leptospire are assigned to the Bi-flexa Complex, and the pathogenic leptospire make up the Interogens Complex. Pathogenic strains have an optimal growth temperature of 28 to 30°C, and they grow over a pH range from 5.2 to 7.7. Saprophytic strains prefer a growth temperature between 5 and 10°C below pathogenic strains. Leptospire prefer alkaline conditions, and they persist longest in warm, moist environments protected from sunlight. Under favorable temperature and pH conditions, leptospire survive for 3 to 5 d in damp soil and up to 10 d in natural waters. They survive for 12 to 14 h in undiluted wastewater, up to 3 d in aerated wastewater, and up to 4 weeks in sterile tapwater at pH 7. Nonpathogenic leptospire are ubiquitous, and they have been isolated from municipal water supplies.² Generally, pathogenic leptospire require an animal host and do not survive and propagate in the environment.

Leptospirosis is a worldwide zoonotic disease of wild animals.³ Reservoirs of leptospire in wildlife include deer, foxes, raccoons, skunks, opossums, muskrats, and rodents. Domestic animals harboring leptospire include horses, cattle, goats, pigs, and sheep. Dogs may become infected but cats are spared. Humans are incidental hosts.

Humans acquire leptospirosis (Weil's disease) directly from animals, and from occupational or recreational exposure to urine-contaminated water^{4–6} or environmental surfaces. Swimming and other water sports,⁷ travel to tropical areas with occupational or recreational exposure to surface waters,⁸ and natural disasters that affect sewer systems and runoff^{9,10} increase risk of the disease. Outbreaks of leptospirosis associated with drinking water are extremely unusual, and are invariably caused by contamination of domestic water reservoirs with urine of infected rodents.¹¹

Leptospirosis ranges from mild nonspecific febrile illness to severe or fatal renal, hepatic, or meningeal disease.^{12,13} Leptospire enter through imperfections in the skin, through mucous

membranes, or by ingestion of contaminated water. Urine of infected animals and humans may contain 10⁶ to 10⁸ organisms/mL. Leptospire may be shed into the environment up to 3 months after clinical recovery from disease.

Diagnosis of disease in animals and humans usually is based upon serology, darkfield examination of urine sediments, examination of histopathological stains, or culture of the organism from urine or tissues. Recently polymerase chain reaction (PCR) methods have been introduced for diagnosis and typing of leptospire.

While leptospirosis remains relatively common in tropical regions of the world, only 40 to 120 cases/year have been reported in the U.S. over the past 30 years. Leptospirosis was dropped from the list of notifiable diseases in 1994.

Leptospire are recovered from environmental sources with great difficulty.^{14–17} Because both saprophytic and pathogenic strains of leptospire may be recovered from environmental samples, their presence has no public health significance apart from an epidemiological context.

1. Sample Collection

Collect water samples of 100 mL to 1 L in sterile containers for transport to the laboratory at ambient temperature within 72 h of collection. Multiple samples from each sample site usually are required for successful isolation because finding leptospire in 10 to 20% of samples of surface waters receiving farm runoff is considered a high yield. Leptospire find their ecological niche at the interface between sediment and shallow water. Gently agitate the water to bring some of the sediment to the surface of shallow bodies of water to improve the probability of recovering organisms.¹⁸ For soil samples, collect 10 to 20 g of soil in sterile bottles or plastic bags. Use a small, tightly sealed container to protect sample from drying. A small amount of sterile deionized water may be added to soil samples to prevent drying.

2. Sample Processing

Centrifuge a portion of a water sample at 5000 × g for 10 min and examine sediment by darkfield microscopy for leptospire.

Their presence indicates that conditions are favorable for leptospire survival, but does not differentiate saprophytic from pathogenic forms. In the laboratory, thoroughly mix soil samples with three volumes of sterile deionized water and let coarse particulate material settle by gravity. Process remaining suspension as a water sample. *Leptospira* can pass through 0.22- μ m membrane filters (§ a below); this ability has been exploited to separate them from other bacteria in environmental samples and in mixed cultures. Similarly, guinea pig inoculation (§ b below) has been used as a biological filter for isolation of leptospires from contaminated samples.

a. *Filtration method*: Filter surface water samples through filter paper* to remove coarse debris before membrane filtration. Occasionally, samples may have to be passed through a series of prefilters of decreasing pore sizes (8- μ m, 4- μ m, 1- μ m, 0.65- μ m, and 0.45- μ m) to prevent clogging of the final 0.22- μ m filter.

b. *Animal inoculation method*: Filter water through a 0.45- μ m membrane filter and inoculate 1 to 3 mL intraperitoneally into weanling guinea pigs. After 3 to 6 d, inject a small amount of sterile saline and withdraw fluid for darkfield examination. If leptospires are seen, perform a cardiac puncture to obtain blood for inoculation of culture media. If no leptospira are seen by darkfield examination, record rectal temperatures daily until a fever spike indicates infection, then repeat the darkfield examination of peritoneal fluid for leptospires. Exsanguinate guinea pigs at 4 weeks and save serum for serological tests. Culture blood, kidney, and brain of guinea pigs with serological evidence of infection. Details of the method are described elsewhere.¹⁹

3. Culture

Cultures of environmental samples usually will be contaminated with other bacteria unless the samples are filtered through a 0.22- μ m membrane filter before inoculation. Filtration also may be used to isolate leptospires from mixed cultures, by direct filtration or another method.²⁰ Unless sample filtration is used in conjunction with selective media or animal inoculation, a culture contamination rate of 60 to 80% is not uncommon. The amount of sample cultured will depend on the amount of particulate material in the sample. Generally, culture sample volumes from a few drops to 3.5 mL.

a. *Culture media*: Pathogenic leptospires have been cultured in liquid, semisolid, and solid media, but not all pathogenic strains will grow on solid media. Optimal pH of culture media is 7.2 to 7.4 and optimal incubation temperature is 30°C. Leptospires are sensitive to detergents, so keep glassware free of detergent residues. When using serum enrichments in culture media, use serum free of antibody to leptospires. Bovine serum albumin shows manufacturer and lot variations; test new batches for their ability to support growth of leptospires.

Modifications of the Ellinghausen-McMullough formulation (EMJH) that incorporate bovine serum albumin fraction V and polysorbates are used as serum replacements.²¹⁻²⁴ EMJH base is available commercially. Neomycin is used in culture media at concentrations between 5 and 25 μ g/mL to inhibit competing microflora, but it may be toxic to some strains.²⁵ 5-fluorouracil is

used at 100 or 200 μ g/mL in culture media, but it too is toxic for some strains, particularly at concentrations above 100 μ g/mL.²⁶

b. Culture methods:

1) Direct culture method—To recover leptospires from surface waters, place a few drops of water in EMJH liquid medium and incubate overnight at 30°C. Filter inoculated medium through a 0.22- μ m membrane filter into a sterile tube and reincubate at 30°C for up to 6 weeks.

2) Dilution method—When samples may contain reasonable numbers of organisms in the presence of inhibitors or competing microflora, prepare 10-fold dilutions in duplicate, and inoculate 0.1 mL undiluted sample and each dilution into EMJH medium. One tube of each pair may be made selective by addition of a single 30- μ g neomycin antimicrobial susceptibility disk to the media before incubation. Incubate cultures at 20 to 30°C for up to 4 months.

3) Animal inoculation method—Add 1 to 2 drops of heart blood from infected guinea pigs to each of three to five tubes of EMJH medium. Incubate cultures at 20°C for up to 4 months.

c. *Culture examination*: Leptospires usually are detected in cultures of environmental samples within 7 to 14 d; however, incubate and examine cultures weekly for 6 weeks before discarding them as negative. Observe tubes for a lightly turbid ring of growth just below the surface of the medium. This band of maximum turbidity at the zone of optimal oxygen tension is referred to as Dinger's ring. Remove a drop of the culture weekly for darkfield examination and prepare subcultures if motile leptospires are observed. Generally, saprophytic leptospires grow at lower temperatures, and form rings closer to the surface of culture media than pathogenic serovars. Cultures remain viable in semisolid media for at least 8 weeks at room temperature.

4. Identification

Experience and skill are required to differentiate artifacts from leptospires by darkfield microscopy. The biochemical tests previously thought to differentiate between pathogenic and saprophytic serovars do not reliably predict pathogenicity of leptospires, and they are not recommended. *Leptospira* are identified to serogroup by the microscopic agglutination test using reference antisera. Identification to serovar requires use of adsorbed antisera that are available only in reference laboratories. Over 200 serotypes of *Leptospira* are known.

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9260 J. *Legionella*

The Legionellaceae have been implicated in outbreaks of disease occurring since 1947.¹ Two forms of disease are recog-nized: a pneumonic form called Legionnaires' Disease and a nonpneumonic form called Pontiac fever. The first species was isolated following the historic outbreak associated with the Le-gionnaires' Convention in Philadelphia, Pa., in 1976. Epidemi-ological findings and animal studies have shown that the organ-ism is transmitted via the airborne route² and is ubiquitous in moist environments. The reservoirs for most outbreaks have been either contaminated air conditioning cooling tower water or contaminated potable water distribution systems.^{3,4} *Legionella* species also have been isolated in non-disease-related circum-stances from a wide variety of aquatic environments such as lakes, streams, reservoirs, and sewage.^{5,6} The organisms are able to survive for prolonged periods in laboratory distilled and tap water.⁷

The Legionellaceae are composed of a single genus, *Legio-nella*, and more than 35 different species.⁸ The organisms are Gram-negative, aerobic, non-spore-forming bacteria. They are 0.5 to 0.7 μm wide and 2 to 20 μm long. They possess polar, subpolar, and/or lateral flagella. With the exception of *L. oakrid-gensis*, all require cysteine and iron salts for growth.

Although *Legionella* originally were isolated in guinea pigs and embryonated hen's eggs, it has been shown that plating directly on artificial media is more sensitive than animal inocu-lation for *L. pneumophila*.⁹ The most widely used medium is an

ACES (N-2-acetamideo-2-aminoethanesulfonic acid) buffered (pH 6.9) charcoal yeast extract (BCYE) agar supplemented with cysteine, ferric pyrophosphate, and optimally, alpha-ketoglu-tarate (BCYE-alpha).¹⁰

No one medium will be optimal for the recovery of *Legionella* from every environmental site; thus different selective media with various antibiotic combinations in a BCYE base may be necessary.¹⁰⁻¹² Also, pretreating samples with hydrochloric acid-potassium chloride, pH 2.2, is useful for eliminating non-*Legio-nella* organisms.¹³ The two most commonly used selective me-dia are GPVA medium (BCYE-alpha supplemented with glycine anisomycin, vancomycin, and polymyxin B) and CCVC medium (BCYE-alpha supplemented with polymyxin B, cephalothin, vancomycin, and cycloheximide). The GPVA medium is less inhibitory to some *Legionella* species. Use CCVC medium in combination with a less selective medium.

Recovery of legionellae from environmental water samples sometimes is difficult. Legionellae may take up to a week to grow on plate media, and even with acid pretreatment and the addition of antibiotics to the medium, faster-growing organisms may overgrow legionellae. In addition, other organisms, includ-ing *Pseudomonas* spp., secrete into surrounding media bacterial products that can inhibit *Legionella* growth.¹⁴

Rapid methods for detecting *Legionella* utilizing direct fluores-cent antibody staining (DFA) or polymerase chain reaction technol-ogy (PCR) also are available and may be more sensitive than

culture-based assays.¹⁵⁻¹⁷ DFA can be quantitative, but may be subject to interference due to cross-reactivity with other organisms.¹⁸ The PCR method is semiquantitative. Both DFA and PCR may not distinguish between viable and nonviable bacteria.

1. Sample Collection

Collect water samples from the littoral zone or from cooling towers, condenser coils, storage tanks, showers, water taps, etc. In most instances, a 1-L water sample is sufficient. Larger volumes of water (1 to 10 L)⁶ may be needed in water having low bacterial counts. In addition to collecting water samples, it may be useful to swab various fixtures (e.g., shower heads) and plate directly on selective media. Transport samples to the laboratory in insulated containers. Refrigerate samples that cannot be processed immediately. Treat chlorinated water with sodium thiosulfate (see Section 9060A.2).

2. Immunofluorescence Procedure

Centrifuge 100 mL at $3500 \times g$ for 30 min at room temperature and reconstitute the sedimented material in 6 to 10 mL filter-sterilized (0.2- μ m filter) water from sample. Prepare smears for DFA by filling two 1.5-cm circles on a microscope slide with the concentrate. Air-dry sample smears, gently heat-fix, treat with 10% formalin for 10 min, rinse with phosphate-buffered saline (pH 7.6), and react with specific fluorescent antibodies.^{6,19} The DFA procedure lacks specificity²⁰ and cannot determine viability. Some environmental bacteria (i.e., *Pseudomonas* spp. and *Xanthomonas-Flavobacterium* group) cross-react with the *Legionella* DFA reagents.

To determine whether organisms are viable, use secondary staining with a tetrazolium dye.¹⁸ Confirm *Legionella* using direct isolation procedures.

3. Media and Reagents

a. Buffered charcoal yeast extract alpha base:¹⁹

Norit SG charcoal.....	2.0 g
Yeast extract.....	10.0 g
ACES buffer.....	10.0 g
Ferric pyrophosphate, soluble	0.25 g
L-cysteine HCl-H ₂ O	0.4 g
Agar.....	17.0 g
Potassium alpha-ketoglutarate	1.0 g
Reagent-grade water	1.0 L

Dissolve yeast extract, agar, charcoal, glycine, and alphaketoglutarate in approximately 850 mL water; boil. Dissolve 10 g ACES buffer in 100 mL warm water, adjust pH to 6.9 with 1N KOH and add. Autoclave 15 min at 121°C. Cool to 50°C. Dissolve 0.4 g cysteine and 0.25 g ferric pyrophosphate in 10 mL of water each and filter sterilize separately (0.22 μ m). After base has cooled, add cysteine, ferric pyrophosphate, and dyes in that order. Adjust pH to 6.9 with sterile 1N KOH and dispense.

b. GPVA medium:^{11,12*}

Glycine.....	0.3 %
Polymyxin B	100 units/mL
Vancomycin	5 μ g/mL
Anisomycin.....	80 μ g/mL

To cooled BCYE-alpha base with glycine, add filter-sterilized antibiotics and mix. Adjust pH to 6.9 with sterile 1N KOH and dispense.

c. CCVC medium^{11†}

Cephalothin.....	4 μ g/ml
Colistin.....	16 μ g/mL
Vancomycin	0.5 μ g/mL
Cycloheximide	80 μ g/mL

To cooled BCYE-alpha base add filter-sterilized antibiotics and mix. Adjust to pH 6.9 with sterile 1N KOH and dispense.

d. Acid treatment reagent,¹¹ pH 2.0 (0.2M KCl/HCl):

Solution A—0.2M KCl (14.9 g/L in distilled water).

Solution B—0.2M HCl (16.7 mL/L 10N HCl in distilled water).

Mix 18 parts of Solution A with 1 part of Solution B. Check pH against a pH 2.0 standard buffer. Dispense into screw-cap tubes in 1.0-mL volumes and sterilize by autoclaving.

e. Alkaline neutralizer reagent¹¹ (0.1N KOH):

Stock solution—0.1N KOH (6.46 g/L in deionized water). Dilute 10.7 mL of stock solution with deionized water to 100 mL. Dispense into screw-cap tubes in convenient volumes and sterilize by autoclaving. The pH of *d* and *e* combined in equal volumes should be 6.9.

4. Sample Preparation

a. *Low-bacterial-count water*: Concentrate water that has a low total bacterial count either by filtration¹¹ or continuous-flow centrifugation.²¹ Filter samples through sterile 47-mm filter funnel assemblies containing a 0.2- μ m porosity polycarbonate filter.‡ After filtration, immediately remove the filter aseptically and place it in a 50-mL centrifuge tube or similar-size vessel containing 10 mL sterile tap water or phosphate buffer. If more than one filter is required to concentrate a sample, combine them.

b. *High-bacterial-count water*: Process water that has a high total bacterial count directly. Place 10 mL sample in a 50-mL centrifuge tube or similar-size vessel containing 10 mL of sterile tap water or phosphate buffer.

c. *Sample dispersion*: Disperse organisms from filter or aggregates by mixing with a vortex mixer (3×30 s).

d. *Plating*: Plate acid-treated and non-acid-treated samples on two types of BCYE: plain and selective with antibiotics.

1) No acid treatment—Inoculate three plates each of BCYE-alpha and selective BCYE-alpha (GPVA or CCVC) with 0.1 mL of suspension. Spread with a sterile smooth glass rod. Save remainder of specimen for acid treatment and store at 4°C.

2) Acid treatment—Place 1.0 mL of suspension in a sterile 13 $\times$ 100-mm screw-capped tube containing 1.0 mL acid treatment reagent and mix. Final pH of mixture should be approximately 2.2. Let stand for 15 min at room temperature, neutralize by

† This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

‡ Nuclepore Corp., 7035 Commerce Circle, Pleasanton, CA, or equivalent.

* Available commercially.

adding 1.0 mL alkaline neutralizer reagent, and mix. Inoculate 0.1 mL onto three plates each of BCYE-alpha and selective BCYE-alpha (GPVA or CCVC) and spread with a sterile smooth glass rod.

3) Incubation—Incubate all plates at 35°C in a humidified atmosphere (>50%) for up to 10 d. A candle jar or humidified CO₂ incubator (2 to 5% CO₂) is acceptable.

e. Total bacterial count examination: Determine the adequacy of processing for each high-bacterial-count water. Some samples may require dilution, concentration, or animal inoculation. If the total count of the acid-treated sample exceeds 300 colonies on BCYE selective medium, make a further 10-fold dilution of the sample stored at 4°C. Repeat acid-treatment and plating.

If the total count of the non-acid-treated sample is less than 30 colonies on BCYE agar, concentrate and treat the collected water as previously described for low-bacterial-count water.

5. Examination of Cultures of Legionellae

With the aid of a dissecting microscope, examine all cultures daily after 48 h incubation for the presence of opaque bacterial colonies that have a "ground-glass" appearance. Place plates with *Legionella*-like colonies in a biological safety cabinet equipped with a burner, a bacteriological needle, and a loop. Aseptically pick each suspect colony onto BCYE-alpha agar and a BCYE agar plate prepared without L-cysteine. Streak the inoculated portion of each plate with a sterile loop to provide areas of heavy growth and incubate for 24 h.

Reincubate plates without growth an additional 24 h. Plates demonstrating growth on only BCYE-alpha agar are presumptive for *Legionella*. Confirm *Legionella* by slide agglutination or direct immunofluorescence. If these confirmatory techniques are not available, send subcultures of the presumptive legionellae to a reference laboratory for further identification. Because there are many serotypes in some species, especially *L. pneumophila*, investigation of environmental sites as possible reservoirs of epidemic-causing strains may be useful.²² Effective investigatory techniques include monoclonal antibody subtyping, electrophoretic isoenzyme analysis, restriction endonuclease tests, and plasmid analysis.

6. Polymerase Chain Reaction Procedure

A test kit utilizing the polymerase chain reaction (PCR) is available commercially[§] and has been used successfully in an epidemiological investigation of an outbreak of Pontiac fever.²³ Perform tests according to manufacturer's instructions. The kit provides sample processing reagents, PCR primers, detection strips, and positive and negative controls. Specific probes allow for the detection of twenty-five *Legionella* species as well as specific detection of *Legionella pneumophila*. The test is semi-quantitative, based on a colorimetric comparison to control strips equivalent to 10³ cells/mL.

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§ Enviroamp PCR, Perkin-Elmer Roche, Alameda, CA.

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9260 K. *Yersinia enterocolitica*

Yersinia enterocolitica is a gram-negative bacterium that can cause acute gastroenteritis and can be found in water in cold or temperate areas of the United States. Many wild, domestic, and farm animals are reservoirs of this organism, including wild animals associated with water habitats (beavers, minks, muskrats, nutrias, otters, and raccoons).^{1,2} The organism can grow at temperatures as low as 4°C with a generation time of 3.5 to 4.5 h if at least trace amounts of organic nitrogen are present.³ Most environmental strains of *Y. enterocolitica* and the closely related species, *Y. kristensenii*, *Y. frederiksenii*, and *Y. intermedia*, generally are considered nonpathogenic, but disease outbreaks have been associated with environmental sources. Some strains lacking classic virulence markers also may be associated with disease.⁴ *Y. enterocolitica* has become recognized worldwide as an important human pathogen and in several countries it is nearly as common as *Salmonella* and *Campylobacter* as a leading cause of acute or chronic enteritis.⁵ *Y. enterocolitica* usually is associated with sporadic cases of gastroenteritis in the U.S.; however, epidemiologic investigations suggest that the predominant pathogenic serotype isolated in the U.S. has been changing.⁴ *Y. enterocolitica* serogroup O:3 has replaced O:8 as the most common species recovered from patients, reflecting the same pattern seen in other parts of the world.^{4,5} Two reported incidents of waterborne gastroenteritis possibly caused by *Yersinia* occurred during the period 1971 to 1978.^{3,6,7}

Yersinia has been isolated from untreated surface and ground waters in the Pacific Northwest, New York, and other regions of North America, with highest isolations during the colder months.⁸⁻¹⁰ Concentrations have ranged from 3 to 7900 CFU/100 mL. Laboratory tests used to isolate and enumerate yersiniae do not discriminate between pathogenic and nonpathogenic strains. *Yersinia* isolations do not correlate with levels of total and fecal coliforms or total plate count bacteria.⁹ There is little information on *Yersinia* survival in natural waters and water treatment processes.

In studies of *Yersinia* in chlorinated-dechlorinated secondary effluent and receiving (river) water, the organism was isolated in

27% of the effluent samples, 9% of the upstream samples, and 36% of the downstream samples.¹¹ Mean total and fecal coliform reductions in effluent chlorination were 99.93 and 99.95%, respectively. In a survey of untreated and treated (chlorination or filtration plus chlorination) drinking water supplies, *Yersinia* was found in 14.0 and 5.7% of the samples, respectively.⁹ Of water samples with less than 2.2 coliforms/100 mL, 15.9% were *Yersinia*-positive. *Yersinia* isolation did not correlate with presence of total or fecal coliforms in this study. Another study confirmed that *E. coli* also is not a good indicator for *Yersinia* in water and that *Y. enterocolitica* O:3 strains harboring a virulence plasmid have enhanced resistance to chlorine compared to non-virulent strains.¹²

Because of the existence of animal reservoirs, widespread occurrence and persistence of *Yersinia* in natural and treated water in at least some geographic areas, the evidence for possible waterborne outbreaks, and the lack of definitive information on its reduction by treatment processes, this pathogen is of potential importance in drinking water.

1. Concentration and Cultivation

A membrane filter method for enumerating and isolating *Yersinia enterocolitica* is available.¹³ The method may be used for examining large volumes of low-turbidity water and for presumptively identifying the organism without transferring colonies to multiple confirmatory media.

Filter sample through a membrane filter (see Section 9260B.1d). Place membrane filter on a cellulose pad saturated with m-YE recovery broth. Incubate for 48 h at 25°C. Aseptically transfer the membrane to a lysine-arginine agar substrate and incubate anaerobically at 35°C. After 1 h, puncture a hole in the membrane next to each yellow to yellow-orange colony with a needle, transfer the membrane to a urease-saturated absorbent pad, and incubate at 25°C for 5 to 10 min. Immediately count all distinctly green or deep bluish-purple colonies. The green or bluish colonies are sorbitol-positive, lysine- and arginine-nega-

tive, and urease-positive. They may be presumptively identified as *Y. enterocolitica* or a closely related *Yersinia* species. Additional biochemical testing is necessary to determine species. Reasonably simple tests have been described to screen isolates for pathogenicity.¹⁴ Comprehensive biochemical and serological characterization or the use of molecular methods is necessary to confirm virulence, but these methods are not generally available.

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9260 L. Aeromonas

1. Introduction

Aeromonas spp. are natural inhabitants of aquatic environments worldwide. These Gram-negative, facultatively anaerobic, glucose-fermenting organisms have been isolated from groundwater, treated drinking water, surface waters, wastewater, sludge, and sediment. Their populations are seasonal in all natural waters, with the highest numbers present in warmer months. Aeromonads cause serious diseases of aquatic animals and represent an economic threat to the aquaculture industry. The motile aeromonads have emerged as a serious microbial threat to human populations, especially the immunocompromised.¹

As a result of recent taxonomic studies, *Aeromonas* bacteria have been removed from the family *Vibrionaceae* and established as the sole genus of the new family *Aeromonadaceae*. The genus *Aeromonas* comprises 14 recognized and 2 proposed DNA hybridization groups with 13 named phenospecies and 4 unnamed genospecies. The extreme difficulty of phenotypically differentiating aeromonads and the unavailability of DNA hybridization techniques in most laboratories have lead clinical

microbiologists to report aeromonads as *A. hydrophila*, *A. sobria*, or *A. caviae*, according to a published classification scheme.² Environmental microbiologists usually combine all motile, mesophilic aeromonads into the *Aeromonas hydrophila* complex, or simply report isolates as *A. hydrophila*. These practices obscure understanding of the medical and public health significance of aeromonads isolated from clinical specimens, environmental samples, and public water supplies; identification of *Aeromonas* isolates according to established taxonomic principles is preferable.³

While no waterborne outbreaks of gastroenteritis attributed to aeromonads have implicated public drinking water supplies in the U.S., this does not mean that none have occurred. The epidemiologic association between ingestion of untreated well water and subsequent *Aeromonas* gastrointestinal illness has been widely documented. Numerous cases and outbreak investigations of water- and food-transmitted illnesses caused by aeromonads have been reported.⁴ Outbreaks of gastroenteritis caused by aeromonads have occurred in custodial care institutions, nursing homes, and day-care centers. *Aeromonas* contam-

ination of drinking water has been documented as a cause of travelers' diarrhea.⁵

For many years, *Aeromonas* have been considered nuisance organisms by environmental microbiologists because they were reported to interfere with coliform multiple tube fermentation (MTF) methods. While aeromonads comprise 12% of bacteria isolated from drinking water by presence-absence methods, no data have demonstrated inhibition of coliform organisms by aeromonads in drinking water. Slight turbidity of LTB tubes, with or without a small bubble of gas in the inverted tube, is suggestive of aeromonads. When the MTF method is used for drinking water samples, cultures producing turbidity at 35°C that remain clear at 44.5°C are suggestive of aeromonads. The presence of aeromonads can be verified by subculturing a loopful of turbid broth to a MacConkey plate and screening colorless colonies for gelatinase and oxidase production. No data are available to support invalidation of coliform MTF tests based on turbidity of tubes in the absence of gas production.

The ecology of mesophilic aeromonads in aquatic environments, including water treatment plants and distribution systems, has been reviewed.⁶ The Netherlands and the Province of Quebec have established drinking water standards for *Aeromonas* at 20 CFU/100 mL for water leaving the treatment plant, and 200 CFU/100 mL for distribution system water. Canada has established an *Aeromonas* MCL of 0 (zero) for bottled water. A resuscitation method for recovery of aeromonads in bottled water has been published.⁷

The ability to isolate, enumerate, and identify aeromonads from water and wastewater sources is important because of their role in causing human and animal disease, their ability to colonize treatment plants and distribution systems, and their presence and distribution as alternative indicators of the trophic state of waters. The diversity of aeromonads in drinking water plants and distribution systems was shown by several investigators.⁸⁻¹⁰

Many media and methods have been proposed for the isolation and enumeration of aeromonads.^{11,12} The methods presented below represent a compromise, because no single enrichment method, isolation medium, or enumeration method is capable of recovering all aeromonads present in a water sample. The methods were chosen on the basis of reproducibility of results, objectivity of interpretation, availability of materials, and specificity of the method for detection of aeromonads in the presence of other heterotrophic bacteria. Consult the literature for additional methods for use in special circumstances.¹³

2. Sample Collection

Collect water samples in sterile screw-capped glass or plastic bottles or plastic bags.* Sample volumes of 200 mL to 1 L are sufficient for most analyses. For chlorinated waters, add sodium thiosulfate (see Section 9060A.2). The potentially toxic effect of heavy metals is neutralized by adding EDTA (see Section 9060A.2).

Transport samples to the laboratory at 2 to 8°C within 8 h. Samples for presence-absence analyses may be transported at ambient temperatures within 24 h. Grab samples are most common. Moore swabs (see 9260B.1a) have been used for waste-

water sampling, and Spira bottles have been used for tapwater sampling.¹³ Both of these methods are used in conjunction with enrichment in 1% alkaline peptone water (APW), pH 8.6.¹³ Place sediment and sludge samples in bottles or bags and submit in same way as water samples.

3. Enrichment Methods

Do not use enrichment methods for ecological studies because the predominant strain(s) will overgrow other organisms. Reserve enrichments for presence-absence tests for aeromonads in drinking water, foods, stools, or for monitoring aeromonad populations in wastewater or marine environments, where organisms may be present in low numbers or require resuscitation due to injury from exposure to inimical agents or hostile physical environments. For isolation of aeromonads from clear water samples, filter through 0.45- μ m membrane filters, place filters in a bottle with 10 mL APW, incubate overnight at 35°C, and inoculate to plating media for isolation. Optimally, to sample clear water intended for drinking, filter a volume of water through a mini-capsule filter†, decant residual water from inlet, plug ends with sterile rubber stoppers, and fill filter with APW, pH 8.6, through syringe port. Incubate filter at 35°C for 6 h or overnight and streak loopfuls of broth onto selective and differential plating media.¹⁴

4. Enumeration Methods

a. Spread plates: Enumerate samples expected to contain predominantly aeromonads in high numbers (sludge, sediments, wastewater effluents, polluted surface waters, etc.) directly by spreading 0.1-mL portions of decimal dilutions on ampicillin dextrin agar (ADA)¹⁵⁻¹⁷ plates. Incubate plates at 35°C overnight and count bright yellow colonies 1 to 1.5 mm in diameter. Presumptively identify colonies using the screening methods below.

b. Membrane filtration (MF): Enumerate aeromonads in drinking water samples or other low-turbidity waters by using MF procedures with ADA medium and incubating aerobically overnight at 35°C. Filter sample volumes equivalent to 1 mL, 10

† Gelman 12123 or equivalent.

TABLE 9260:III. REACTIONS OF ENTERIC BACTERIA ON TSI AND LIA MEDIA

Organism	TSI Reactions*	LIA Reactions*
<i>Shigella</i>	K/A—	K/A—
<i>Salmonella</i>	K/Ag+	K/K+
<i>Escherichia</i>	A/Ag—	K/A—
<i>Proteus</i>	A/Ag+ or K/Ag+	R/A+
<i>Citrobacter</i>	A/Ag+	K/A+
<i>Enterobacter</i>	A/Ag—	K/A—
<i>Aeromonas</i>	A/A—	K/A—
<i>Yersinia</i>	A/A— or K/A—	K/A—
<i>Klebsiella</i>	A/Ag—	K/A—

* Fermentation reactions = slant/butt, H₂S production = + or —, K = alkaline, A = acid, R = red (deaminase reaction), g = gas produced.

* WhirlPak[®], ZipLoc[®], or equivalent.

TABLE 9260:IV. REACTIONS OF AEROMONAS AND ENTERIC BACTERIA ON KAPER'S MEDIUM

Organism	Fermentation Pattern*	Motility	H ₂ S	Indole
<i>Aeromonas hydrophila</i>	K/A	+	—	+
<i>Klebsiella pneumoniae</i>	A/A	—	—	—
<i>Klebsiella oxytoca</i>	A/A	—	—	+
<i>Escherichia coli</i>	K/K or K/A	+	—	+
<i>Salmonella</i> spp.	K/K, K/A, A/K or A/A	+	+	—
<i>Enterobacter</i> spp.	K/K, K/N or N/N	+	—	—
<i>Proteus</i> spp.	R/K or R/A	+	+	+
<i>Yersinia enterocolitica</i>	K/K, K/N or N/N	—	—	+
<i>Citrobacter</i> spp.	K/K or K/A	+	+	—
<i>Serratia</i> spp.	K/K, K/N, or N/N	+	—	—

* K = alkaline; A = acid; N = neutral; R = red (deamination reaction).

mL, and 100 mL. To achieve a countable plate (1 to 30 colonies), prepare decimal dilutions when aeromonads are present in high numbers. Count bright yellow colonies, 1 to 1.5 mm in diameter, and pick to screening media.

c. *Multiple-tube fermentation tests (MTF)*: Multiple-tube fermentation tests using APW, pH 8.6, or trypticase soy broth (TSB) containing ampicillin at 30 µg/mL (TSB30) have been applied to foods; however, they have not been used for enumeration of aeromonads in water samples. Some aeromonads are sensitive to ampicillin and will not grow in TSB30 medium. ADA without agar has been used to enumerate aeromonads in drinking water.⁶ Use MTF methods only for clean samples such as groundwater or treated drinking water samples, because the effect of competing microflora present in surface waters on recovery of aeromonads in broth media has not been studied adequately. Similarly, the correlation between MTF population estimates and other enumeration methods has not been examined adequately for matrices other than foods.

5. Screening Tests

Pick 3 to 10 colonies resembling aeromonads on differential and selective plating media or membrane filters and stab-inoculate into deeps of Kaper's multi-test medium¹⁹ or one tube each of triple sugar iron (TSI) agar and lysine iron agar (LIA). Incubate cultures at 30°C for 24 h. Perform a spot oxidase test on growth taken from the LIA slant. Do not test for oxidase on growth from TSI slants, MacConkey agar, or other selective or differential media, because acid production interferes with the oxidase reaction. Reactions of enteric bacteria on TSI and LIA media are shown in Table 9260:III. When Kaper's medium is used instead of TSI/LIA slants, colonies may be picked and inoculated onto sheep blood agar plates; incubate at 35°C overnight to provide growth for the oxidase test and to record hemolysin production. Cultures are identified presumptively using Kaper's medium according to the characteristics shown in Tables 9260:IV. If species identification is desirable, submit presumptively identified *Aeromonas* cultures to a reference laboratory. Cultures with potential public health or regulatory significance may be subtyped using various molecular methods to determine clonality for outbreak investigations and troubleshooting of treatment plant or distribution system problems.¹

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9260 M. *Mycobacterium*

The genus *Mycobacterium* comprises over 70 characterized species that are non-motile, spore-forming, aerobic, acid-fast bacilli measuring 0.2 to 0.6×1 to $10 \mu\text{m}$. Most organisms in this genus are saprophytes, but some species are capable of causing disease in humans. The primary pathogens in this group include *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy, respectively. Recently there has been an increase in the incidence of disease caused by nontuberculosis mycobacteria, probably related to the increasing numbers of immunocompromised patients.¹⁻³ In the genus *Mycobacterium*, the most important opportunistic pathogens include *M. avium-intracellulare*, *M. kansasii*, *M. marinum*, and *M. simiae*, which are capable of causing disease when the immune system is compromised. Some of the common hosts and environmental reservoirs of *Mycobacteria* are shown in Table 9260:V.

Because of the complex nature of the cell wall, which is rich in lipids and therefore has a hydrophobic surface, this genus is resistant to many common disinfectants. As a result, several members of this genus are becoming important waterborne pathogens in the immunocompromised population. *Mycobacteria* also are acid-fast and extremely slow-growing. Some species such as *M. avium-intracellulare* require from 3 to 8 weeks to form colonies on culture media.

Mycobacterium avium and *Mycobacterium intracellulare* exhibit overlapping properties, making speciation extremely difficult. As a result, these two pathogens are grouped together and called *M. avium-intracellulare* or referred to as the MAC complex. Organisms from this group are ubiquitous in the environment and have been isolated from potable water systems, including those in hospitals⁴⁻⁶ as well as from soil and dairy products. This pathogen causes a chronic pulmonary disease in immunocompetent hosts that is clinically and pathologically indistinguishable from tuberculosis; it also causes disseminated disease in immunocompromised hosts. The primary route of transmission is believed to be through ingestion, but increasing numbers of cases originate in the respiratory tract, indicating an aerosol route of transmission.

1. Sample Collection and Concentration

Mycobacteria typically constitute a minority of the microflora, especially in finished waters, and require sample concentration. Collect water samples in sterile 1-L polypropylene containers. For finished, disinfected waters, add 1 mL 10% sodium thiosul-

fate solution/L water collected. Transport samples to laboratory immediately after collection. If samples cannot be analyzed immediately, store at 4°C and begin analysis within 24 h of sampling.

2. Screening Water Samples by Direct Fluorescent Assay

Before committing the sample to a lengthy culture incubation, survey for acid-fast bacteria by using a combination solution of Auramine-Rhodamine (A-R) fluorescent dye.^{7*} Auramine and Rhodamine nonspecifically bind to mycolic acids and resist decolorization by acid alcohol.⁸

Filter a minimum of 500 mL finished water or 100 mL source water (depending on turbidity), through a sterile 0.45- μm -porosity, 47-mm-diam black filter. Aseptically transfer filter to a sterile polypropylene 50-mL tube and add 5 mL of buffered dilution water. Resuspend organisms from filter by vortexing for 2 min. Aspirate suspension and aseptically transfer to a sterile 15-mL polypropylene centrifuge tube. Centrifuge suspension at 5000 g for 10 min and discard all but about 0.5 mL of supernatant. Resuspend pellet by vortexing. Transfer 100 mL of the concentrate to a clean glass slide and air-dry and heat-fix at 60 to 70°C for 2 h or overnight. Primary stain the smear with A-R (15 min), decolorize with acid-alcohol† for 2 to 3 min, rinse with deionized water, apply secondary potassium permanganate coun-

* Catalog #40-090, Remel, Lenexa, KS, or equivalent.

† Truant-Moore or equivalent.

TABLE 9260:V. MYCOBACTERIA OF WATERBORNE OR UNKNOWN ORIGIN

<i>Mycobacterium</i> Species	Environmental Contaminant	Reservoir
<i>M. kansasii</i>	Rarely	Water, swine, cattle
<i>M. marinum</i>	Rarely	Fish, water
<i>M. simiae</i>	No	Primates, possibly water
<i>M. scrofulaceum</i>	Possibly	Soil, water, foodstuffs
<i>M. szulgai</i>	No	Unknown
<i>M. avium-intracellulare</i>	Possibly	Soil, water, swine, cattle, birds
<i>M. xenopi</i>	Possibly	Water
<i>M. ulcerans</i>	No	Unknown
<i>M. fortuitum</i>	Yes	Soil, water, animals, marine life
<i>M. chelonae</i>	Yes	Soil, water, animals, marine life

TABLE 9260:VI. PHENOTYPIC CHARACTERISTICS OF CLINICALLY SIGNIFICANT ENVIRONMENTAL MYCOBACTERIA*

<i>Mycobacterium</i> Species	Growth Rate	Pigmentation	Urease	Nitrate Reduction	Hydrolysis of Polyoxyethylene Sorbitan Monooleate†
<i>M. kansasii</i>	S	P	±	+	+
<i>M. marinum</i>	S	P	+	—	+
<i>M. simiae</i>	S	P	±	—	—
<i>M. scrofulaceum</i>	S	S	±	—	—
<i>M. szulgai</i>	S	S/P	+	+	±
<i>M. xenopi</i>	S	S	—	—	—
<i>M. avium-intracellulare</i>	S	N	—	—	—
<i>M. ulcerans</i>	S	N	±	—	—
<i>M. fortuitum</i>	R	N	+	+	±
<i>M. chelonae</i>	R	N	+	—	±

* S = slow (3 to 8 weeks), R = rapid (7 d or longer), P = photochromogenic, S = scotochromogenic, N = nonphotochromogenic, S/P = scotochromogenic at 37°C and photochromogenic at 24°C.

† Tween 80®.

terstain (no longer than 2 to 4 min), rinse, and let air-dry. Examine smear at 100 × and 400 × with a microscope fitted with a BG-12 or 5113 primary filter with a OG-1 barrier filter. Acid-fast organisms will stain yellow-orange on a black background. To confirm for acid-fastness, apply a traditional acid-fast stain (Ziehl-Nielsen with Kenyon modification) directly to the prepared smear following the A-R stain.

For wastewater or highly turbid source waters, collect a 10-mL subsample and transfer to a sterile polypropylene 15-mL tube. Centrifuge at 5000 g for 10 min and discard all but about 0.5 mL of supernatant. Follow slide preparation procedure and staining as above.

3. Decontamination and Culture Methods

Mycobacteria grow very slowly on laboratory media. Therefore, eliminate from the sample naturally occurring organisms that can out-compete and overgrow the mycobacteria. Various isolation and identification methods have been described for the recovery of mycobacteria, especially in the hospital environment.⁴⁻⁶ Decontamination of the sample concentrate is required for the selection for mycobacteria before culture. In addition, the matrix may affect the success of the recovery of mycobacteria. Several methods (*a* through *c* below) are detailed for recovering mycobacteria from water samples; determine which method performs best with the matrix to be examined.

a. Filter 500-mL water sample through a sterile 0.45-μm-porosity, 47-mm-diam filter. Aseptically transfer filter to a sterile polypropylene 50-mL tube. Add 5 mL sterile distilled water and resuspend organisms off the filter by shaking with two 5-mm glass beads for 1 h on a mechanical shaker.⁹ Add a 3% sodium lauryl sulfate, 1% NaOH solution.¹⁰ Spread portions of this suspension onto a selective agar medium as described in ¶ 4 below.

b. Filter 500-mL water sample through sterile 0.45-μm-porosity, 47-mm-diam filter. Aseptically transfer filter to a sterile polypropylene 50-mL tube. Add 5 mL sterile distilled water and resuspend organisms off the filter by shaking with glass beads for 5 min on a mechanical shaker. Add 10 mL 1M NaOH for 20 min followed by centrifugation at 8600 g at 4°C for 15 min. Discard supernatant and add 5 mL 5% oxalic acid for 20 min. Re-centrifuge, discard supernatant, and add 30 mL sterile distilled water to neutralize. Centrifuge again, and resuspend in 0.7

mL distilled water.¹¹ Use portions of this material for selective growth (¶ 4 below).

c. Add 20 mL 0.04% (w/v) cetylpyridinium chloride (CPC) to 500-mL water sample and leave at room temperature for approximately 24 h. Filter sample and wash filter with 500 mL sterile water.¹² A study of decontamination methods for the isolation of mycobacteria from drinking water samples found a CPC concentration of 0.005% (w/v) to yield the highest isolation rate and lowest contamination rate for the water examined.¹³

4. Selective Growth

Culture all samples in duplicate. After sample decontamination, either spread portions of the concentrates or use sterile forceps to place filters on selective media. One common egg-based medium that successfully isolates mycobacteria from environmental concentrates is Lowenstein-Jensen agar. An agar-based medium containing cycloheximide (7H10) is a general growth medium for mycobacteria as well. Place plates in humid chambers or gas-permeable bags to prevent dehydration, and incubate at 37°C. Additional plates also can be incubated at 30°C in a humidified chamber to detect mycobacteria that grow optimally at lower temperatures. Examine plates periodically during a 3- to 8-week incubation period. Count suspect colonies (acid-fast coccobacilli) and subculture to a tube of 7H9 broth. After 5 d, remove subsamples and stain with Ziehl-Nielsen stain with Kenyon modification. Subculture coccobacillary acid-fast organisms further onto 7H10 plates. Conduct phenotypic tests (Table 9260:VI) as a first step toward identification. If biochemical tests do not allow speciation, use other methods, such as fatty acid profile by HPLC or GLC, serological typing, and/or molecular tests such as DNA probes, and RFLP, which have been used for rapid detection of a limited number of species.¹⁴

Although phenotypic tests have been the standard for species identification, there are several inherent problems in this approach. First, because initial identification of mycobacteria can take 3 to 8 weeks, observing biochemical changes entails additional time for the isolates (especially those of nontuberculosis mycobacteria) to metabolize specific substrates or to exhibit certain characteristics. Second, phenotypic traits are not stable; thus some species of mycobacteria are untypable by conventional methods.

One approach that can successfully speciate *Mycobacterium* is sequencing amplified rDNA.¹⁵ The method produces objective results in 2 d, gives reproducible results (due to the stability of the 16S rRNA), and can identify new species. These techniques have been used in clinical diagnostic laboratories, and now are available in some full-service environmental testing laboratories.

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9510 DETECTION OF ENTERIC VIRUSES*

9510 A. Introduction

This section is currently undergoing substantial revision; consensus on proposed changes could not be reached in time for this publication. The methods below are reprinted from the 20th Edition of *Standard Methods*.

1. Occurrence

Viruses excreted with feces or urine from any species of animal may pollute water. Especially numerous, and of particu-

lar importance to health, are the viruses that infect the gastrointestinal tract of man and are excreted with the feces of infected individuals. These viruses are transmitted most frequently from person to person by the fecal-oral route. However, they also are present in domestic sewage which, after various degrees of treatment, is discharged to either surface waters or the land. Consequently, enteric viruses may be present in sewage-contaminated surface and ground waters that are used as sources of drinking water. The viruses known to be excreted in relatively large numbers with feces include polioviruses, coxsackieviruses, echoviruses, and other enteroviruses, adenoviruses, reoviruses,

* Approved by Standard Methods Committee, 1996.

rotaviruses, the hepatitis A (infectious hepatitis) virus(es), and the Norwalk-type agents that can cause acute infectious nonbacterial gastroenteritis. With the possible exception of hepatitis A, each group or subgroup consists of a number of different serological types; thus more than 100 different human enteric viruses are recognized.¹⁻⁴

In temperate climates enteroviruses occur at peak levels in sewage during the late summer and early fall. However, hepatitis A virus (HAV), Norwalk-type viruses, and rotaviruses may be important exceptions because the incidence of the diseases due to these viruses increases in the colder months. Quantitative information on seasonal patterns of occurrence in water and wastewater of these latter viruses is lacking because they cannot be assayed readily with conventional cell culture techniques. The Norwalk-type viruses have not been cultivated in any cell cultures, although immunochemical assay methods have been developed to detect them as antigens.^{5,6} Human rotaviruses and HAV have been cultivated recently in cell cultures, but the techniques are difficult and require concomitant use of immunoassays such as immunofluorescence to detect virus growth or gene probes.⁷⁻¹¹

Viruses are not normal flora in the intestinal tract; they are excreted only by infected individuals, mostly infants and young children. Infection rates vary considerably from area to area, depending on sanitary and socioeconomic conditions. Viruses usually are excreted in numbers several orders of magnitude lower than those of coliform bacteria. Because enteric viruses multiply only within living, susceptible cells, their numbers cannot increase in sewage. Sewage treatment, dilution, natural inactivation, and water treatment further reduce viral numbers. Thus, although large outbreaks of waterborne viral disease may occur when massive sewage contamination of a water supply takes place,¹² waterborne transmission of viral infection and disease in technologically advanced nations depends on whether minimal quantities of viruses are capable of producing infections. It has been demonstrated that infection can be produced experimentally by a very few virus units,¹³ although the risk of infection increases with increasing ingested doses.¹⁴ The risk of infection incurred by an individual in a community with a water supply containing a very few virus units has not been determined. Risk analysis has suggested that significant risk of infection could result from low numbers of enteric viruses present in a drinking water supply.¹⁵ The percentage of individuals who develop clinical illness may be as low as 1% for poliovirus and as great as 97% for hepatitis A.¹⁵

Most recognized waterborne virus disease outbreaks in the U.S. have been caused by obvious sewage contamination of untreated or inadequately treated private and semipublic water supplies. Virus disease outbreaks in community water supply systems usually are caused by contamination through the distribution system.¹⁶

2. Testing for Viruses

The routine examination of water and wastewater for enteric viruses is not recommended now. However, in special circumstances such as wastewater reclamation, disease outbreaks, or special research studies, it may be prudent or essential to conduct

virus testing. Such testing should be done only by competent and specially trained water virologists having adequate facilities.^{10,17}

Laboratories planning to concentrate viruses from water and wastewater should do so with the clear understanding that the available methodology has important limitations.¹⁷ Even the most current methods for concentrating viruses from water still are being researched and continue to be modified and improved. The efficiency of a virus concentration method may vary widely depending on water quality. Furthermore, none of the available virus detection methods have been tested adequately with representatives from all of the virus groups of public health importance. Most virus concentration methods have achieved adequate virus recoveries with water or wastewater samples that have been contaminated experimentally with known quantities of a few specific enteric viruses. Although method effectiveness in field trials is difficult to evaluate, some virus concentration methods have been used successfully to recover naturally occurring enteric viruses. Some of these methods require large equipment for sample processing and virus assay and identification procedures usually require cell culture and related virology laboratory facilities.

Detecting viruses in water through recovery of infectious virus requires three general steps: (a) collecting a representative sample, (b) concentrating the viruses in the sample, and (c) identifying and estimating quantities of the concentrated viruses. Particular problems associated with the detection of viruses of public health interest in the aquatic environment are: (a) the small size of virus particles (about 20 to 100 nm in diameter), (b) the low virus concentrations in water and the variability in amounts and types that may be present, (c) the inherent instability of viruses as biological entities, (d) the various dissolved and suspended materials in water and wastewater that interfere with virus detection procedures, and (e) the present limitations of virus estimation and identification methods.

3. Selection of Concentration Method

The densities of enteric viruses in water and wastewater usually are so low that virus concentration is necessary, except possibly for raw sewage in certain areas or seasons.¹⁸ Numerous methods for concentrating waterborne enteric viruses have been proposed, tested under laboratory conditions with experimentally contaminated samples, and in some cases used to detect viruses under field conditions.^{19,20}

Virus concentration methods often are capable of processing only limited volumes of water of a given quality. In selecting a virus concentration method consider the probable virus density, the volume limitations of the concentration method for that type of water, and the presence of interfering constituents. A sample volume less than 1 L and possibly as small as a few milliliters may suffice for recovery of viruses from raw or primary treated sewage. For drinking water and other relatively nonpolluted waters, the virus levels are likely to be so low that hundreds or perhaps thousands of liters must be sampled to increase the probability of virus detection.

Three different techniques used to concentrate viruses from water are described herein: adsorption to and elution from microporous filters (Methods B and C); aluminum hydroxide adsorption-precipitation (Method D); and polyethylene glycol (PEG) hydroextraction-dialysis (Method E).^{19,20} A separate tech-

nique (Method F) for recovering viruses from solids in small volumes of water also is described. Virus concentration by adsorption to and elution from microporous filters can be used for both small volumes of wastewater and large volumes of natural and finished waters. The aluminum hydroxide adsorption-precipitation and PEG hydroextraction-dialysis methods are impractical for processing large fluid volumes. However, they are suitable for concentrating viruses from wastewater or other waters having relatively high virus densities and for second-step concentration (reconcentration) of viruses in primary eluates obtained by processing large sample volumes through microporous filters.

4. Recovery Efficiencies

In examining a particular water include a preliminary evaluation of virus recovery efficiency. To do this add a known quantity of one or more test virus types to the required volume of sample, process the sample by the concentration method, and assay the concentrate for test viruses to determine virus recovery efficiency. Ideally, such seeded samples should be used whenever field samples are processed. If seeded samples are used concurrently with field samples, take appropriate steps, including disinfection and sterilization and the use of aseptic technique, to prevent accidental contamination of samples.

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9510 B. Virus Concentration from Small Sample Volumes by Adsorption to and Elution from Microporous Filters

1. General Discussion

Viruses can be concentrated from aqueous samples by reversibly adsorbing them to microporous filters and then eluting them from the filters in a small liquid volume.¹ The virus-containing sample is pressure-filtered through microporous filters having large surface areas to which viruses adsorb, presumably by both electrostatic and hydrophobic interactions.² Two general types of adsorbent filters are available: electronegative (negative surface charge) and elec-

tropositive (positive surface charge). The former filters are composed of either cellulose esters or fiberglass with organic resin binders. They adsorb viruses most efficiently in the presence of multivalent cations such as Al^{3+} and Mg^{2+} and/or at low pH, usually pH 3.5. The latter filters are composed of either fiberglass or cellulose and a positively charged organic, polymeric resin. They adsorb viruses efficiently over a wide pH range without added polyvalent salts. If the sample is neutral or acidic, it can be processed with these filters without chemical conditioning.

Electropositive filters have given virus recoveries comparable to those with electronegative filters.³⁻⁵ They have been used in field studies,^{6,7} and were evaluated with a variety of virus types⁸⁻¹³ and waters.

Adsorbed viruses usually are eluted from the surfaces of microporous filters by pressure-filtering a small volume of eluent fluid through the filters in situ. The eluent is either a slightly alkaline proteinaceous fluid such as beef extract or a more alkaline buffer such as glycine-NaOH, pH 10.5 to 11.5. If glycine-NaOH is used as eluent, preferably use pH 10.5 because of the greater likelihood of virus inactivation at the higher pH.^{14,15}

Microporous filter methods suffer from three main limitations. Sample suspended matter tends to clog the adsorbent filter, thereby limiting the volume that can be processed and possibly interfering with the elution process.¹⁶ Dissolved and colloidal organic matter in some waters can interfere with virus adsorption to filters, presumably by competing with viruses for adsorption sites,¹⁷⁻¹⁹ and they also can interfere with virus elution. Finally, viruses adsorbed to suspended matter may be removed in any clarification procedure applied before virus adsorption. These solids-associated viruses are lost from the sample unless special efforts are made to recover the solids and process them for viruses.¹⁶ A method for recovering solids-associated viruses from small volumes of water and wastewater is given in Section 9510F. Despite these limitations, virus concentration by adsorption to and elution from microporous filters is a most promising technique for detecting viruses.

2. Equipment and Apparatus

a. *Adsorbent filter holder*, 47-, 90-, or 142-mm diam, equipped with pressure relief valve.

b. *Pressure vessel*, 12- or 20-L capacity.

c. *Positive pressure source* up to about 400 kPa with regulator: laboratory air line, air pump, or cylinder of compressed air or nitrogen gas.

d. *Autoclavable vinyl plastic tubing* with plastic or metal connectors (quick-disconnect type), for connecting positive pressure source, pressure vessel, and filter holder in series.

e. *pH meter*.

f. *Beakers*, 50- to 500-mL.

g. *Laboratory balance*.

h. *Graduated cylinders*, 25- to 100-mL.

i. *Pipets*, 1-, 5-, and 10-mL.

3. Materials

a. *Electronegative virus adsorbent filter*: Use either:

1) *Cellulose nitrate filter*, 0.45- μ m porosity.*

2) *Fiberglass-acrylic resin filter*, 0.45- μ m porosity.† Filter media available commercially only as flat sheets can be cut to the desired disk diameter with scissors.

b. *Electropositive virus adsorbent filter*: Use either:

1) *Surface modified cellulose and filter aid disk depth-filter*.‡

2) *Surface modified cellulose and filter aid thin-sheet medium*, 0.20- μ m porosity.§

c. *Prefilter*: Use one or more cellulose nitrate or fiberglass-acrylic resin filters or equivalent, with porosities greater than 0.45 μ m to prevent clogging of the virus adsorbent filter by suspended matter. Place prefilters on top of the 0.45- μ m-porosity virus adsorbent filter in the same filter holder.

4. Reagents

a. *Hydrochloric acid*, HCl, 0.1, 1.0, and 10N.

b. *Sodium hydroxide*, NaOH, 0.1, 1.0, and 10N.

c. *Aluminum chloride*, $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, 0.15N, or magnesium chloride, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5N (necessary only for electronegative filters).

d. *Sodium thiosulfate*, $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 0.5% (w/v).

e. *Sodium chloride*, 0.14N, pH 3.5: Dissolve 8.18 g in 1 L reagent-grade water and adjust to pH 3.5 with HCl (necessary only for electronegative filters).

f. *Virus eluent*: Use either:

1) *Glycine-NaOH*, pH 10.5 or 11.5: Prepare 0.05M glycine solution, autoclave, and adjust to pH 10.5 or 11.5 with 1 to 10N NaOH. Add phenol red, 0.0005%, as a pH indicator.

2) *Beef extract*, 3%, pH 9.0: Dissolve 30 g beef extract paste or 24 g beef extract powder in 1000 mL reagent-grade water, adjust to pH 9.0 with 1 to 10N NaOH, and sterilize by autoclaving.

g. *Glycine-HCl*, pH 1.5: Prepare 0.05M glycine solution, autoclave, and adjust to pH 1.5 with 1 to 10N HCl. Add phenol red, 0.0005%, as a pH indicator.

h. *Nutrient broth*, 10X, pH 7.5: Dissolve 8.0 g nutrient broth in 90 mL reagent-grade water, adjust to pH 7.5, dilute to 100 mL with reagent-grade water, and sterilize by autoclaving.

i. *Antibiotics*: Use either:

1) *Penicillin-streptomycin*, 10X: Contains 5000 IU penicillin/mL and 5000 μ g streptomycin/mL. Use commercially available form or prepare by dissolving powdered sodium or potassium penicillin-G and streptomycin sulfate in reagent-grade water and sterilizing by filtration. Store frozen.

2) *Gentamycin-kanamycin*, 100X: Contains 5000 μ g/mL each of gentamycin (base) and kanamycin (base). Prepare by combining aseptically equal volumes of commercially available sterile gentamycin and kanamycin solutions, 10 000 μ g/mL, respectively, or by dissolving powdered gentamycin sulfate and kanamycin sulfate in reagent-grade water and sterilizing by filtration. Store refrigerated or frozen.

j. *Hanks balanced salt solution*, 10X: Use commercially available form or prepare following a standard protocol.²⁰

k. *Sodium hypochlorite*, 5.25% available chlorine (household bleach).

5. Procedure

a. *Sterilization of apparatus, materials, and reagents*: Most reagents, virus adsorbent filters, filter holders, tubing, and labware can be sterilized by autoclaving or made virus-free by streaming steam. To sterilize filters load into their holders; if

* Type HA, Millipore Corp., Bedford, MA, or equivalent.

† No. 8025-035, Filterite Corp., Timonium, MD, or equivalent.

‡ Zeta-plus 50S or 60S, CUNO, Meriden, CT, or equivalent.

§ 1-MDS Virozorb, CUNO, Meriden, CT, or equivalent.

several filters are to be placed in one holder, place filter with smallest porosity on the bottom with progressively larger filters on top. Do not use an automatic drying cycle when autoclaving virus adsorbent filters. Sterilize apparatus and material that cannot be autoclaved or treated with streaming steam by treating with 10-mg/L free chlorine solution, pH 7.0, for 30 min and rinse or flush with 50-mg/L sterile $\text{Na}_2\text{S}_2\text{O}_3$ solution. Do not treat adsorbent filters with chlorine. Use aseptic technique during all virus concentration operations to prevent extraneous microbial contamination.

b. Sample size and choice of filter size: Sample size and, hence, filter diameter depend partly on water quality and the probable virus concentration. Single-stage microporous filter adsorption-elution methods have been used to recover viruses from 100 mL raw sewage on 47-mm-diam filters²¹ and from 3.8 to 4.6 L secondary and tertiary sewage effluent on 90- or 142-mm-diam filters.^{18,21,22} Based on the diameter and solids-holding characteristics of the filters, the scale and volume capacity of the apparatus and materials, and the quality of the samples, the practical limits for sample size are 20, 8, and 2 L for 142-, 90-, and 47-mm-diam filters, respectively.

c. Choice of filter type: Virus adsorption to electropositive filters decreases above pH 8 and pH adjustment below this value may be necessary for optimal virus adsorption.⁴ Virus recovery from raw sewage may be less than with electronegative filters.¹⁰

d. Sample collection and storage: Collect samples aseptically in sterile containers. If they contain residual chlorine, immediately add $\text{Na}_2\text{S}_2\text{O}_3$ solution to give a final concentration of 50 mg/L. Process samples as soon as possible after collection; do not hold samples for more than 2 h at up to 25°C or 48 h at 2 to 10°C. Do not freeze samples unless they cannot be processed within 48 h; then freeze and store at -70°C or less.

e. Sample processing of electronegative filters: Adjust sample to pH 3.5 and 0.0015N AlCl_3 or to between pH 6.0 and 3.5 and 0.1N MgCl_2 . Make sample adjustments either in a pressure vessel or in another appropriate container. Mix sample vigorously during addition of 1.0 or 0.1N HCl and AlCl_3 solution (1 part solution to 100 parts sample) or MgCl_2 solution (1 part solution to 50 parts sample). Because AlCl_3 is an acid salt, it may decrease sample pH slightly. Do not let sample pH fall below 3.0.

Place sample in a pressure vessel connected to a source of positive pressure and connect pressure vessel outlet to inlet of virus adsorbent filter holder. With pressure relief valve on filter holder opened, apply a slight positive pressure to purge air from filter holder. When sample just begins to flow from pressure relief valve, quickly close valve and continue filtration at a rate not exceeding 28 mL/min/cm² of filter area (about 130, 250, and 4000 mL/min for 47-, 90-, and 142-mm-diam filters, respectively). After filtering entire sample let positive pressure source purge excess fluid from filter holder.

Wash filters with 0.14N NaCl to remove excess Al^{3+} or Mg^{2+} from virus adsorbent filter. Use about 1.5 mL NaCl solution/cm² filter area (25, 100, and 240 mL for 47-, 90-, and 142-mm-diam filters, respectively). Place wash solution in a pressure vessel connected to filter holder inlet, use positive pressure to filter solution through virus adsorbent filter, discard filtrate, and let positive pressure purge virus adsorbent filter of excess wash solution.

Elute viruses from filters with a recommended eluent. Use about 0.45 mL eluent/cm² filter surface area (about 7.5, 28, and 71 mL for 47-, 90-, and 142-mm-diam filters, respectively). With pressure relief valve on filter holder open, add eluent to filter holder so that it completely covers filter surface. When eluent begins to discharge from pressure relief valve, quickly close valve. If pH 11.5 glycine-NaOH is the eluent, place a sterile beaker under filter outlet and apply positive pressure so that filtrate flows slowly from filter holder outlet. Collect filtrate in sterile beaker and, when filtrate no longer flows, slowly increase pressure to force retained fluid from filters. Quickly check eluate (filtrate) pH. If it is less than 11.0, elute with additional pH 11.5 glycine-NaOH until an eluate with a pH $\geq$ 11.0 is obtained. Immediately after checking pH, adjust eluate to a pH between 9.5 and 7.5 with pH 1.5 glycine-HCl or 0.1N HCl while mixing vigorously. Complete elution and eluate pH adjustment to 7.5 to 9.5 in 5 min or less to avoid the possibility of appreciable virus inactivation.

If pH 10.5 glycine-NaOH is the eluent, proceed as with pH 11.5 glycine-NaOH, but pass the eluate through the filters a total of five times. For each elution, collect the filtrate, readjust to pH 10.5 with 1.0 or 0.1N NaOH, and then pass through the filter. After the fifth elution, adjust filtrate to pH 7.4 with glycine-HCl, pH 1.5, or 0.1N HCl.

If 3% beef extract, pH 9.0, is the eluent, place a sterile beaker under filter outlet, apply a slight positive pressure to eluent-containing filter holder so that filtrate flows slowly from the outlet, and collect filtrate. Slowly increase pressure to force additional retained fluid from filters.

Measure eluate volume and add 1/10 of the measured volume each of penicillin-streptomycin or gentamycin-kanamycin, Hanks balanced salt solution, and 10X nutrient broth (add last item to glycine eluates only). Adjust sample to pH 7.4 with glycine-HCl or 0.1N HCl while mixing vigorously. Store at either 4 or -70°C, depending on the time until virus assay. Maximum storage at 4°C is 48 h.

f. Processing of electropositive filters: Processing for electropositive filters is identical to that for electronegative filters except that addition of Al^{3+} and Mg^{2+} and sample pH adjustments are unnecessary; because Al^{3+} and Mg^{2+} are not added, it is not necessary to wash filters with 0.14N NaOH before elution. If sample pH is greater than 8.0, adjust to less than pH 8 by adding 1.0 or 0.1N HCl.

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9510 C. Virus Concentration from Large Sample Volumes by Adsorption to and Elution from Microporous Filters

1. General Discussion

This section describes a two-stage process for concentrating viruses from large sample volumes. Viruses in eluate volumes too large to be conveniently and economically assayed directly in cell cultures, such as those obtained from processing large volumes of water through cartridge or large disk filters, can be concentrated further (reconcentrated) by several alternative methods. Viruses in proteinaceous eluates can be reconcentrated by either "organic flocculation,"^{1,2} aluminum hydroxide adsorption-precipitation (Section 9510D), or polyethylene glycol hydroextraction-dialysis (Section 9510E). These reconcentration techniques can be used for both proteinaceous and organic buffer eluates from all types of water. Organic flocculation, now used widely, involves precipitating viruses by acidifying eluates to pH 3.5, recovering the precipitate by centrifugation, and then resuspending it in a small volume of alkaline buffer.¹

Additionally, viruses in nonproteinaceous eluates such as glycine-NaOH can be reconcentrated by adsorption to and elution from small microporous filters. The eluate is adjusted to pH and ionic conditions for optimum virus adsorption, filtered through a secondary adsorbent, and adsorbed viruses are eluted with a small volume of eluent. This procedure can be used only for reconcentrating primary eluates obtained from processing drinking water and other highly finished waters because of potential

interfering substances likely to be present in primary eluates from natural and less finished waters.

Figure 9510:1 shows the alternative microporous filter adsorption-elution and reconcentration methods.

For general information on microporous filter techniques, see Section 9510B.1.

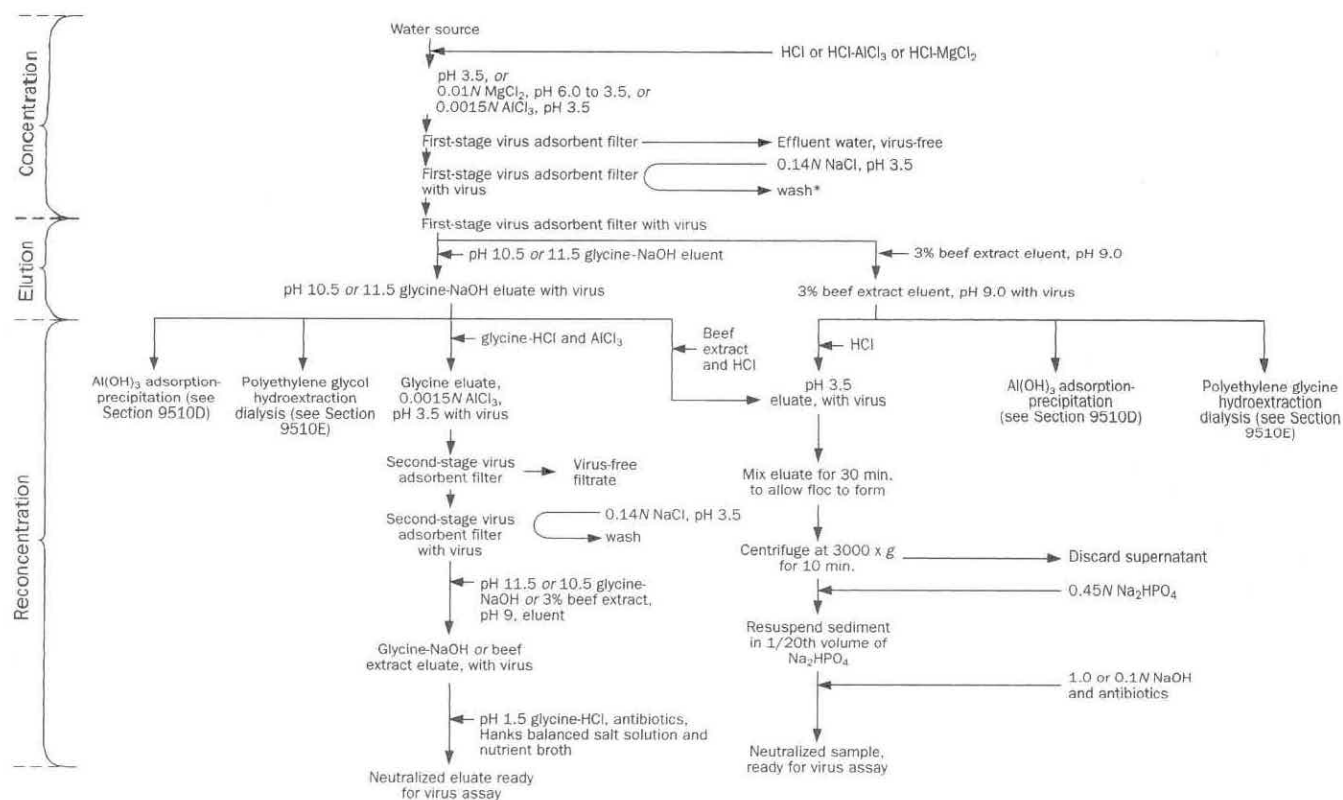
2. Equipment and Apparatus

a. Apparatus for first-stage concentration (Figure 9510:2):

- 1) *First-stage virus adsorbent filter holder.*
- 2) *Chemical additive system.* Use either:
 - a) *Fluid proportioner* with four feed pumps (quadraplex) and a mixing chamber.*
 - b) *Venturi-type proportioning injector*† with plastic or metal connectors (quick-disconnect type) and a length of vinyl tubing for the chemical feed line.³ To feed two separate additives, attach a "Y" or "T" connector and two lengths of vinyl tubing to the chemical feed port, or alternatively, use two separate proportioning injectors. It may be necessary to use a bypass system with the injector to prevent loss of chemical feed due to back pressure

* Johanson and Son Machine Corp., Clifton, NJ, or equivalent.

† Models 202-P, 203-P or 204-P, Dema Engineering Co., St. Louis, MO, or equivalent.



*When using MgCl₂ or AlCl₃ to enhance virus adsorption.

Figure 9510:1. Two-stage microporous filter adsorption-elution method for concentrating viruses from large volumes of water with electronegative filters.

from the water line.⁴ This bypass system consists of "T" pipe fittings on the injector inlet and outlet ports connected by a length of flexible hose with an in-line shut-off/control valve (see Figure 9510:2).

Proportioning injectors available commercially will process water at flow rates of 3 to 33 L/min with water-to-chemical feed ratios between 10 to 1 and 1110 to 1. Select equipment and operating conditions providing a water-to-chemical feed ratio of 100 to 1.

- 3) *Water flow meter.*
- 4) *Pressure gauge, 0 to 400 kPa.*
- 5) *Vinyl plastic tubing, autoclavable, with plastic or metal connectors (quick-disconnect type).*
- 6) *Pressure relief valve (optional).*
- 7) *Carboys, 20- to 50-L, or similar containers.*
- 8) *Positive pressure source up to 400 kPa with regulator: laboratory air line, positive pressure pump, or cylinder of compressed air or nitrogen gas.*
- 9) *Pump (if source water is not under pressure).*

b. *pH meter.*

c. *Laboratory balance.*

d. *Beakers, 2- or 4-L.*

e. *Pressure vessel, 4-L.*

f. *Graduated cylinders, 1- and 2-L.*

g. *Pipets, 1-, 5-, and 10-mL.*

h. *Centrifuge with rotor and buckets for 250- to 500-mL-capacity bottles.‡*

i. *Centrifuge bottles, 250- to 500-mL.*

3. Materials

a. *First-stage electronegative virus adsorbent filters:* Use one of the following:

- 1) 293-mm-diam, 8.0- and 1.2- μ m-porosity cellulose nitrate filter series.§
- 2) 17.8-cm-long, 8.0- μ m-porosity fiberglass-epoxy filter tube.||
- 3) 25.4-cm-long, 0.25- or 0.45- μ m-porosity fiberglass-acrylic resin pleated filter cartridge.#

b. *Second-stage electronegative virus adsorbent filters:* 47-mm-diam, 3.0-, 0.45-, and 0.25- μ m-porosity fiberglass-acrylic resin filter series. Use to reconcentrate highly finished water samples only.#

c. *First-stage electropositive adsorbent filters:* Use one of the following:

‡ Required for alternative reconcentration procedure using 3% beef extract.

§ Millipore Corp., Bedford, MA, or equivalent.

|| Balston, Inc., Lexington, MA, or equivalent.

Filterite Corp., Timonium, MD, or equivalent.

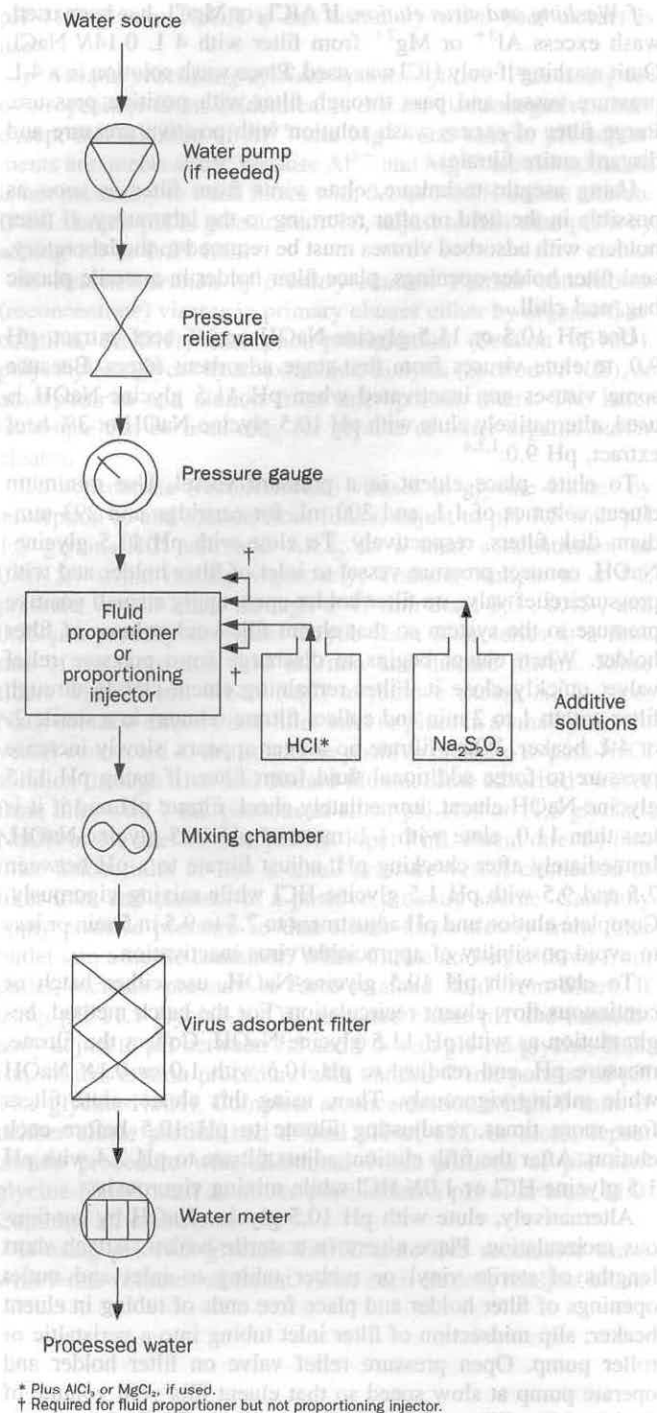


Figure 9510:2. Schematic of apparatus for first-stage concentration with negatively charged filters.

1) 293-mm-diam surface modified cellulose and filter aid filters.**

** 50S, 60S, or 1-MDS Virozorb, CUNO, Meriden, CT, or equivalent.

2) 25-cm-long, 0.20- μ m-porosity surface modified thin-sheet media pleated filter cartridge.††

4. Reagents

- Hydrochloric acid, HCl, 0.06, 1,†† and 6N.
- Sodium hydroxide, NaOH, 10N.
- Aluminum chloride, $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, 0.15 and 6N.‡‡
- Magnesium chloride, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10N.‡‡
- Sodium thiosulfate, $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 0.5% (w/v).
- Sodium hypochlorite, 5.25% available chlorine (household bleach).
- Eluent: Use either:
 - Glycine-NaOH, pH 10.5 or 11.5: See Section 9510B.4f1). Use within 2 h of pH adjustment.
 - Beef extract, 3%, pH 9.0.§§ See Section 9510B.4f2).
- Elate neutralizing solution: Use either:
 - Glycine-HCl, pH 1.5: Prepare 0.05M glycine solution and adjust to pH 1.5 with 6N HCl. Add phenol red, 0.0005%, as a pH indicator. Use within 2 h of pH adjustment.
 - HCl, 1.0N.
- Nutrient broth, 10X, pH 7.5: Dissolve 8.0 g nutrient broth in 90 mL distilled water, adjust to pH 7.5 with 10N NaOH, dilute to 100 mL with distilled water, and sterilize by autoclaving.
- Disodium phosphate, 0.45N: Dissolve 40.2 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ in 1 L distilled water and sterilize by autoclaving.
- Antibiotics: See Section 9510B.4i.
- Sodium chloride, 0.14N: Dissolve 8.18 g NaCl in 1 L distilled water (necessary only with electronegative filters).
- Hanks balanced salt solution, 10X: See Section 9510B.4j.

5. Procedure

When using electronegative filters, follow ¶s a–f below for production of primary eluate. When using electropositive filters, first see ¶ g for procedural modifications.

a. *Sterilization of apparatus, materials, and reagents:* See Section 9510B.5a.

b. *Sample size:* For drinking water use a minimum sample of 400 L, although 2000 L or more may have to be processed to detect viruses at a concentration of 1 to 2 infectious units/400 L.

c. *Preparation of feed solutions for electronegative filters:* Use an HCl additive solution to adjust sample pH to 3.5 for virus adsorption to filters. If acidification to pH 3.5 is inadequate for obtaining maximum virus adsorption, add either AlCl_3 or MgCl_2 solution.

When only HCl is used, prepare additive solution as follows: Determine concentration of HCl additive solution by titrating a 1-L sample of dechlorinated water to pH 3.5 with 0.06N HCl and noting volume required. The volume, in milliliters, of titrant required is equal to the volume of 6N HCl needed/L distilled water for making the additive solution. Make at least 5 L additive solution for 400 L of sample.

†† 1-MDS Virozorb, CUNO, Meriden, CT, or equivalent.

‡‡ Recommended for first-stage virus adsorption with electronegative filters only.

§§ For alternative reconcentration procedure: organic flocculation.

When AlCl_3 is used to enhance virus adsorption use pH 3.5 and a final concentration of added AlCl_3 of 0.0015*N*. Because AlCl_3 is an acid salt, titrate a 1-L sample to about pH 4.0 with 0.06*N* HCl, add AlCl_3 to a concentration of 0.0015*N* and continue titration to pH 3.5, noting volume of titrant used. Prepare additive solution by adding titrant volume (mL) of 6.0*N* HCl/L of 0.15*N* AlCl_3 .

When MgCl_2 is used to enhance virus adsorption, use a pH between 3.5 and 6.0 and a final concentration of added MgCl_2 of 0.1*N*. To prepare additive solution titrate a 1-L sample to desired pH with 0.06*N* HCl as previously described and note volume of titrant used. Add the titrant volume of 6.0*N* HCl/L 10*N* MgCl_2 to make the additive solution.

d. Preparation of chemical additive system:

1) When using a fluid proportioner, operate at a pressure of 100 to 700 kPa and a water flow rate of 4 to 40 L/min. Adjust each of the four chemical additive pumps of the proportioner for a ratio of 1 to 200 (1 part chemical additive to 200 parts water). Use two pumps, operating reciprocally, for each additive so that the overall dilution for each additive is 1 to 100. One additive is either HCl, HCl- AlCl_3 or HCl- MgCl_2 ; the other additive, 0.5% $\text{Na}_2\text{S}_2\text{O}_3$, is needed only when processing samples containing chlorine. Place lines from the two pumps of each additive solution into the additive containers and manually operate the pump metering rods to fill feed lines and purge them of air. Connect fluid proportioner to source water and operate briefly without a virus adsorbent in place. Sample conditioned water from proportioner outlet and check pH. The pH should be 3.5 ± 0.3 .

2) When using a Venturi-type proportioning injector, connect injector assembly to water source and to adsorbent filter inlet, and place additive feed line(s) into additive container(s). Position valve on injector outlet to drain line position (away from adsorbent filter). Begin flow of sample. Adjust screw-operated control valve on chemical feed of proportioning injector until water collected from drain line is at the desired pH as measured with a pH meter. If $\text{Na}_2\text{S}_2\text{O}_3$ is used to neutralize chlorine, check to insure that chlorine is absent. Connect virus concentrator assembly to source water by attaching concentrator inlet hose to valved outlet of a pressurized water source or to outlet of a water pump, the inlet of which has been placed in the source water. Operate for several minutes without a virus adsorbent in place to purge the unit of chlorine solution. Collect a sample from outlet of meter to insure absence of chlorine.

e. First-stage concentration: After preparing concentration apparatus and additive solutions and checking conditioned water for proper pH and absence of chlorine, attach a virus adsorbent filter to outlet of chemical additive system. Attach water meter and effluent hose to virus adsorbent outlet. Record initial meter reading and add to this value the desired volume to be processed plus an additional 1 or 2% (to account for volume of either 1 or 2 additive solutions, respectively). This gives meter reading at which sampling is to be stopped. Turn on water and start a timer (or record starting time). Shortly after filtration begins collect a sample from filter outlet and check for absence of chlorine and for appropriate pH value. Also check flow rate. Do not use a flow rate above 40 L/min. Recheck pH and chlorine residual several times during sample processing, or monitor continuously. When desired volume has been processed, turn water off. Purge filter holder of excess water with positive pressure from an air or nitrogen gas source.

f. Washing and virus elution: If AlCl_3 or MgCl_2 has been used, wash excess Al^{3+} or Mg^{2+} from filter with 4 L 0.14*N* NaCl. Omit washing if only HCl was used. Place wash solution in a 4-L pressure vessel and pass through filter with positive pressure. Purge filter of excess wash solution with positive pressure and discard entire filtrate.

Using aseptic technique, elute virus from filter as soon as possible in the field or after returning to the laboratory. If filter holders with adsorbed viruses must be returned to the laboratory, seal filter holder openings, place filter holder in a sterile plastic bag, and chill.

Use pH 10.5 or 11.5 glycine-NaOH or 3% beef extract, pH 9.0, to elute viruses from first-stage adsorbent filters. Because some viruses are inactivated when pH 11.5 glycine-NaOH is used, alternatively elute with pH 10.5 glycine-NaOH or 3% beef extract, pH 9.0.^{1,3,4}

To elute, place eluent in a pressure vessel. Use minimum eluent volumes of 1 L and 300 mL for cartridge and 293-mm-diam disk filters, respectively. To elute with pH 11.5 glycine-NaOH, connect pressure vessel to inlet of filter holder and with pressure relief valve on filter holder open, apply a small positive pressure to the system so that eluent fills void volume of filter holder. When eluent begins to discharge from pressure relief valve, quickly close it. Filter remaining eluent slowly through filter within 1 to 2 min and collect filtrate (eluate) in a sterile 2- or 4-L beaker. When filtrate no longer appears, slowly increase pressure to force additional fluid from filter. If using pH 11.5 glycine-NaOH eluent, immediately check filtrate pH and if it is less than 11.0, elute with 1 L more of pH 11.5 glycine-NaOH. Immediately after checking pH, adjust filtrate to a pH between 7.5 and 9.5 with pH 1.5 glycine-HCl while mixing vigorously. Complete elution and pH adjustment to 7.5 to 9.5 in 5 min or less to avoid possibility of appreciable virus inactivation.

To elute with pH 10.5 glycine-NaOH, use either batch or continuous-flow eluent recirculation. For the batch method, begin elution as with pH 11.5 glycine-NaOH. Collect the filtrate, measure pH, and readjust to pH 10.5 with 1.0 or 0.1*N* NaOH while mixing vigorously. Then, using this eluate, elute filters four more times, readjusting filtrate to pH 10.5 before each elution. After the fifth elution, adjust filtrate to pH 7.4 with pH 1.5 glycine-HCl or 1.0*N* HCl while mixing vigorously.

Alternatively, elute with pH 10.5 glycine-NaOH by continuous recirculation. Place eluent in a sterile beaker. Attach short lengths of sterile vinyl or rubber tubing to inlet and outlet openings of filter holder and place free ends of tubing in eluent beaker; slip midsection of filter inlet tubing into a peristaltic or roller pump. Open pressure relief valve on filter holder and operate pump at slow speed so that eluent fills void volume of filter holder. When eluent begins to discharge from pressure relief valve, quickly close it. Increase pump speed so that eluent recirculates through filter assembly and beaker at a minimum flow rate of 100 mL/min. After 5 min recirculation, remove filter inlet tube from beaker and pump remaining fluid from filter assembly. Connect filter inlet to positive pressure source to force additional eluent from filter. Adjust eluate to pH 7.4 with pH 1.5 glycine-HCl or 1.0*N* HCl while mixing vigorously.

To elute with 3% beef extract, pH 9.0, follow the procedure described above for pH 11.5 glycine-NaOH. Adjust collected filtrate to pH 7.4 with pH 1.5 glycine-HCl or 1*N* HCl while mixing vigorously. The 5 min time limit to complete elution with

pH 11.5 glycine-NaOH is not necessary when beef extract is used.

g. Sample processing of electropositive filters: Processing for electropositive filters is identical to that for electronegative filters except that addition of Al^{3+} and Mg^{2+} and sample pH adjustments are unnecessary; because Al^{3+} and Mg^{2+} are not added, it is not necessary to wash filters with 0.14N NaOH before elution. If the sample pH is greater than 8.0, adjust to less than pH 8 by adding 1.0 or 0.1N HCl.

h. Reconcentration of primary eluates: Further concentrate (reconcentrate) viruses in primary eluates either by organic flocculation, $\text{Al}(\text{OH})_3$ adsorption-precipitation (Section 9510D), polyethylene glycol hydroextraction-dialysis (Section 9510E), or adsorption to and elution from microporous filters. The latter technique can be used only for glycine or other organic buffer eluates.

To concentrate (reconcentrate) viruses in glycine eluates by adsorption to and elution from filters, adjust to pH 3.5 with pH 1.5 glycine-HCl and add AlCl_3 to a final concentration of 0.0015N while mixing vigorously. Transfer sample to a 4-L pressure vessel. Filter through a 47-mm-diam 3.0-, 0.45-, and 0.25- μm -porosity fiberglass-acrylic resin filter series at a flow rate of no more than 130 mL/min and discard filtrate. Rinse filters with 25 mL 0.14N NaCl to remove excess Al^{3+} . Pipet NaCl solution directly into filter inlet or place in a small pressure vessel connected to the inlet. Use positive pressure to pass NaCl solution through filter and discard filtrate. Elute adsorbed viruses from filter with 7-mL portions of either pH 10.5 or 11.5 glycine-NaOH or 3% beef extract, pH 9.0. Pipet 7 mL eluent directly into filter holder inlet or into a small pressure vessel connected to filter inlet and connect to a positive pressure source. Carefully apply positive pressure so that eluate flows slowly from filter outlet into a sterile container. When filtrate no longer flows from outlet, increase pressure to force retained fluid from filters. If using pH 11.5 glycine-NaOH, measure eluate pH and immediately adjust to pH between 7.5 and 9.5 with pH 1.5 glycine-HCl. Repeat this elution procedure with another 7-mL portion of pH 11.5 glycine-NaOH. Complete reconcentration within 5 min. If neither eluate portion had a final pH of 11.0 or more, repeat elution procedure with additional 7-mL portions of pH 11.5 glycine-NaOH until an eluate portion has a pH of at least 11.0. Combine all eluates.

If using pH 10.5 glycine-NaOH, elute five successive times with 7-mL volumes of eluent. After each elution, readjust eluate

to pH 10.5 with 0.1N NaOH while mixing vigorously. After the fifth elution, adjust eluate to pH 7.4 with pH 1.5 glycine-HCl or 0.1N HCl while mixing vigorously.

If using 3% beef extract, pH 9.0, elute with two 7-mL volumes, combine filtrates, and adjust to pH 7.4 if necessary.

Measure total eluate volume. For glycine eluates, add 1/10th the measured sample volume of 10X Hanks balanced salt solution and 10X nutrient broth. To all eluates add appropriate volumes of antibiotics (1/10th volume penicillin-streptomycin or 1/100th volume gentamycin-kanamycin, or both). Store at 4 or -70°C , depending on time until virus assay.

Further concentrate viruses in beef extract eluates by precipitation at pH 3.5 (organic flocculation). Viruses in glycine eluates also can be reconcentrated by this technique by first supplementing them with beef extract to a final concentration of 1 to 3%. Use sterile beef extract paste (about 80% beef extract) or sterile 20% beef extract solution made from powder to bring glycine eluates to the desired beef extract concentration. While mixing vigorously, adjust eluate to pH 3.5 by adding 1N HCl dropwise. Continue to mix at slow speed for 30 min and centrifuge at $3000 \times g$ for 10 min. Decant and discard supernatant. With vigorous mixing, resuspend sediment in 1/20 the initial sample volume of 0.45N Na_2HPO_4 . Add antibiotics (1/10 final sample volume penicillin-streptomycin, 1/100 final sample volume gentamycin-kanamycin, or both) and while mixing vigorously adjust to pH 7.4 with 1.0 or 0.1N NaOH. Check electrical conductivity of sample. If conductivity is $> 13\,000\ \mu\text{mhos}$, dialyze sample against Hanks balanced salt solution before assay. Store at 4 or -70°C , depending on time until virus assay.

6. References

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9510 D. Virus Concentration by Aluminum Hydroxide Adsorption-Precipitation

1. General Discussion

Viruses can be concentrated from small volumes of water, wastewater, and adsorbent filter eluates by precipitation with aluminum hydroxide.¹⁻⁴ This process probably involves both electrostatic interactions between the negatively charged virus surface and the positively charged aluminum hydroxide $[\text{Al}(\text{OH})_3]$ surfaces and coordination of the virus surface by hydroxo-aluminum complexes.⁵ Viruses are adsorbed to an

$\text{Al}(\text{OH})_3$ precipitate that is either added to the sample or formed in the sample from a soluble aluminum salt and a base such as sodium carbonate (Na_2CO_3) or sodium hydroxide (NaOH). Viruses are allowed to adsorb to the $\text{Al}(\text{OH})_3$ precipitate and the virus-containing precipitate is collected by filtration or centrifugation. The recovered precipitate may be inoculated directly into laboratory hosts for virus assay or the viruses are eluted from the precipitate with an alkaline buffer or a proteinaceous solution before virus assay.

The major limitations of this method are that sample size is limited to perhaps a few liters, soluble organic matter can interfere with virus adsorption, and virus recovery from the precipitate may be incomplete. Virus adsorption may be improved by forming the $\text{Al}(\text{OH})_3$ precipitate in the sample instead of adding it preformed. Although virus adsorption can be maximized by using large amounts of $\text{Al}(\text{OH})_3$, the adsorbed viruses become more difficult to elute. Therefore, some intermediate amount of $\text{Al}(\text{OH})_3$ is used to achieve maximum virus recovery. Also, $\text{Al}(\text{OH})_3$ is a relatively nonspecific adsorbent so that other substances may be concentrated with viruses. The presence of such impurities may cause the concentrated sample to be toxic for the cell cultures normally used for virus assay.

Several modifications of the $\text{Al}(\text{OH})_3$ adsorption-precipitation procedure have been used to concentrate viruses from water, wastewater, and eluates from adsorbent filters. Initially, preformed $\text{Al}(\text{OH})_3$ precipitates were made by adding Na_2CO_3 to AlCl_3 solutions and the $\text{Al}(\text{OH})_3$ precipitate was resuspended in 0.15N NaCl. This was added to the wastewater and the mixture was stirred gently for 1 h or more to allow viruses to adsorb to the precipitate. The precipitate was recovered by filtration, resuspended in cell culture media, and inoculated into cell cultures.^{3,4} More recent procedural modifications include: (a) $\text{Al}(\text{OH})_3$ precipitate formation within the sample,^{1,2,6-8} (b) recovery of the $\text{Al}(\text{OH})_3$ precipitate by centrifugation followed by elution of viruses from the precipitate with alkaline eluents,^{1,2,6,7} and (c) a large-volume method in which the precipitate is formed in the sample and collected on a cartridge filter, and viruses are eluted from the precipitate on the filter with alkaline eluent.⁹ The method described here is for relatively small sample volumes and uses $\text{Al}(\text{OH})_3$ that is either preformed or generated within the sample. The latter modification is preferable because some viruses are not adsorbed efficiently by preformed precipitates.¹⁰

2. Equipment and Apparatus

- a. *Centrifuge*, with rotor and buckets, capable of operating at about $1900 \times g$.
- b. *Centrifuge bottles and tubes*.
- c. *Beaker*, 100-mL or larger.
- d. *pH meter*.
- e. *Magnetic stirrer and stirring bars* or alternative mixing device.
- f. *Graduated cylinders*, 100-mL or larger.
- g. *Pipets*, 1-, 5-, and 10-mL.
- h. *Laboratory balance*.
- i. *Vacuum-type filter holder or Buchner filter funnel*,* 47-mm diam or larger.
- j. *Filter flask*,*
- k. *Spatula*,* flat blade, metal or autoclavable plastic.
- l. *Vacuum source*,* vacuum pump or laboratory vacuum line.

3. Materials

Filter:* Fiberglass-acrylic resin filter† or microporous filter, 0.45- μm porosity,‡ 47-mm diam or larger. To prevent virus adsorption, filter 0.1% polyoxyethylene sorbitan monooleate solution (§ 4h) through the filters, using about 1 mL solution/ cm^2 of filter surface area. Rinse filter with distilled water, using about 10 mL/ cm^2 of filter surface area. Sterilize treated filters by autoclaving.

4. Reagents

- a. *Hydrochloric acid*, HCl, 0.1 and 1.0N.
- b. *Sodium hydroxide*, NaOH, 0.1 and 1.0N.
- c. *Sodium carbonate*, Na_2CO_3 , 4N§.
- d. *Aluminum chloride*, AlCl_3 , 0.075N§ or 0.9N.
- e. *Sodium chloride*, NaCl, 0.14N.
- f. *Beef extract*, 3%, pH 7.4: Dissolve 3 g beef extract paste or 2.4 g beef extract powder in 90 mL distilled water, adjust to pH 7.4 with 1.0 or 0.1N NaOH, dilute to 100 mL with distilled water, and sterilize by autoclaving.
- g. *Antibiotics*: Use either:
 - 1) *Penicillin-streptomycin*, 10X, containing 5000 IU penicillin/mL and 5000 μg streptomycin/mL. Available commercially or prepare by dissolving powdered sodium or potassium penicillin-G and streptomycin sulfate in distilled water and sterilizing by filtration.
 - 2) *Gentamycin-kanamycin*, 100X, containing 5000 μg /mL each of gentamycin base and kanamycin base (Section 9510B.4i).
- h. *Polyoxyethylene sorbitan monooleate*,|| 0.1% (v/v) in distilled water.

5. Procedure

a. *Sterilization of apparatus, materials, and reagents*: See Section 9510B.5a.

b. *Preparation of preformed $\text{Al}(\text{OH})_3$ precipitate*: While mixing 100 mL 0.075N AlCl_3 at room temperature, slowly add 4N Na_2CO_3 solution to form precipitate and adjust to pH 7.2. Continue mixing for 15 min and, if necessary, add more Na_2CO_3 to maintain pH 7.2. Centrifuge at $1100 \times g$ for 15 min and discard supernatant. Resuspend sediment in 0.14N NaCl and recentrifuge. Discard supernatant, resuspend sediment in 0.14N NaCl, and sterilize by autoclaving. Cool, centrifuge again, decant supernatant, and resuspend $\text{Al}(\text{OH})_3$ sediment in 50 mL sterile 0.14N NaCl. Store at 4°C.

c. *Sample size, collection, and storage*: Process samples of no more than several liters because the method is too cumbersome and time-consuming for larger volumes. See Section 9510B.5d for sample collection and storage procedures.

d. *Sample processing*: Do not prefilter sample^{11,12} because substantial virus losses can occur. Adjust sample to pH 6.0 with

† Millipore AP20 or equivalent.

‡ Millipore HA or equivalent.

§ For alternative procedure using preformed $\text{Al}(\text{OH})_3$ precipitate.

|| Tween 80®, ICI United States, Inc., Wilmington, DE, or equivalent. Required for optional method for collecting $\text{Al}(\text{OH})_3$ precipitate from sample.

* Required for optional method for collecting $\text{Al}(\text{OH})_3$ precipitate from sample.

1.0 or 0.1N HCl while mixing vigorously. Form $\text{Al}(\text{OH})_3$ precipitate in sample by adding 1 part 0.9N AlCl_3 solution to 100 parts sample to give a final 0.009N Al^{3+} concentration. Check sample pH and re-adjust to 6.0 with 1.0 or 0.1N NaOH or HCl, if necessary. Mix slowly for 15 min at room temperature.

Alternatively, use preformed $\text{Al}(\text{OH})_3$ precipitate by adding 1 part stock $\text{Al}(\text{OH})_3$ suspension/100 parts sample and mix slowly for 2 h at 4 to 10°C to allow for virus adsorption.

Collect virus-containing $\text{Al}(\text{OH})_3$ precipitate by centrifugation or filtration. To collect precipitate by centrifugation, centrifuge at $1700 \times g$ for 15 to 20 min, discard supernatant, and resuspend sediment in 1/1000 to 1/20 original sample volume of 3% beef extract, pH 7.4.

To collect precipitate by filtration, vacuum filter sample through a treated filter (§ 3 above) held in a vacuum-type filter holder or Buchner funnel, using additional filters if filter clogs before entire sample is filtered. Carefully scrape precipitate from filter(s) with a sterile spatula and resuspend in 1/1000 to 1/20 original sample volume of 3% beef extract, pH 7.4.

Regardless of collection method, vigorously mix the $\text{Al}(\text{OH})_3$ beef extract suspension and, if necessary, adjust to pH 7.4 with 0.1N HCl or NaOH. Continue mixing for a total of 10 min. Centrifuge at $1900 \times g$ for 30 min. Decant supernatant, add 1/10 the volume of the concentrate of penicillin-streptomycin solution or 1/100 volume of gentamycin-kanamycin and store at 4 or -70°C.

6. References

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9510 E. Hydroextraction-Dialysis with Polyethylene Glycol

1. General Discussion

Polyethylene glycol (PEG) hydroextraction is an ultrafiltration process in which the sample is placed in a cellulose dialysis bag and exposed to PEG, a hygroscopic material. Water and microsolutes leave the sample by passing across the semipermeable dialysis membrane into the hygroscopic PEG.¹ Viruses and other macrolutes, including PEG, cannot cross the dialysis membrane. The sample volume in the dialysis bag is reduced by water loss to the PEG, thereby concentrating viruses and other macrolutes. The viruses retained in the dialysis bag are recovered by opening the bag, collecting the remaining sample, and eluting any viruses possibly adsorbed to the inner walls of the bag with a small volume of slightly alkaline proteinaceous solution such as 3% beef extract, pH 9.0. The collected concentrate and eluate are combined and assayed for viruses.

The main limitations of this method are that only small samples (less than 1 L) can be processed conveniently, virus elution from the walls of the dialysis bag may be incomplete unless the elution is done painstakingly, and other macrolutes in the sample that are concentrated with viruses may interfere with virus assays by being cytotoxic.

Initial investigations of this method reported low and highly variable virus recoveries from wastewater.^{2,3} The type of dialysis tubing and eluent solution as well as the thoroughness of the elution step have been found to influence virus recovery efficiency. More recently, with modified procedures, efficient and consistent virus recoveries have been obtained from wastewater and from adsorbent filter eluates.^{4,5}

2. Equipment and Apparatus

- a. Beakers, 100-mL or larger.
- b. Graduated cylinders, 100-mL or larger.
- c. Dialysis tubing clamps.*
- d. Pan, approximately $30 \times 30 \times 12$ cm, autoclavable.
- e. Magnetic stirrer and stirring bars or alternative mixing device.
- f. Centrifuge, with rotor and buckets, capable of operating at about $1900 \times g$.
- g. pH meter.

* Fisher Scientific No. 8-670-11A or equivalent.

- h. *Pipets*, 1-, 5-, and 10-mL.
- i. *Tape roller*[†] or similar device to aid in washing the inside walls of dialysis bags with eluting fluid.
- j. *Ultrasonic disruptor-emulsifier*,[‡] probe type, capable of generating 100 W of acoustical output.

3. Materials

- a. *Dialysis tubing*, seamless, regenerated cellulose, 4.8-nm average pore diameter.§
- b. *Polyethylene glycol (PEG)*,|| dry flakes.

4. Reagents

See Section 9510D.4.

5. Procedure

- a. *Sterilization of apparatus, materials, and reagents*: See Section 9510B.5a. Do not sterilize PEG.
- b. *Sample size, collection, and storage*: Process samples of no more than a few hundred milliliters. See Section 9510B.5d for sample collection and storage procedures.
- c. *Preparation of dialysis tubing*: Cut a length of dialysis tubing long enough to accommodate entire sample. Close one end with a clamp. Do not tie knots to close dialysis tubing. Fill tubing bag with distilled water, sterilize by autoclaving, and let cool.
- d. *Sample processing*: Aseptically remove dialysis bag from distilled water and drain. Fill bag with sample and close open end with a second clamp. Place bag in a pan containing a 5-cm layer of PEG, making sure that bag does not touch pan walls. Cover tubing with an additional 5 cm PEG and store at 4°C (for about 18 h) until sample volume has been reduced to no more than a few milliliters. (If PEG 6000 is used the process time is

reduced to 4 to 6 h.) Although sample may be allowed to dewater completely, do not let it remain in this state.

Remove dialysis bag from PEG and quickly wash PEG from outside of bag with sterile distilled water. Remove clamp from one end of bag and carefully collect sample concentrate. Add about 1/200 to 1/20 the original sample volume of 3% beef extract, pH 9.0, and clamp closed. Thoroughly wash inside walls of bag with beef extract by rubbing fluid from one end to the other several times using either fingers or a roller device. Remove clamp from one end of bag and collect fluid, kneading or squeezing to recover the last traces. Add recovered fluid to previously collected sample concentrate.

Adjust to pH 7.5 with 1.0 or 0.1N HCl while mixing vigorously. To disperse solids-associated viruses in sample, stir overnight (about 18 h) in the cold (about 4°C) or treat with ultrasonics at 100 W for 1 to 2 min. Prevent sample temperature from rising above 37°C during ultrasonic treatment by chilling in an ice bath. Centrifuge at $1900 \times g$ for 30 min. Decant supernatant, add 1/10 the volume of the concentrate of penicillin-streptomycin solution or 1/100 volume of gentamycin-kanamycin, and store at 4 or -70°C.

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[†] Optional, Fisher Scientific No. 14-245-21 or equivalent.

[‡] Optional.

[§] Made by Union Carbide Corp. and available from many scientific supply companies.

^{||} Carbowax 20 000 or 6000 or equivalent.

9510 F. Recovery of Viruses from Suspended Solids in Water and Wastewater

1. General Discussion

Viruses in the aquatic environment often are associated with solids or particulate matter, either adsorbed to particulate surfaces or embedded within the solid.¹⁻³ Both freely suspended and solids-associated viruses are concentrated from water by the methods described above. There is evidence that solids-associated viruses are not eluted efficiently from adsorbent filters or from $\text{Al}(\text{OH})_3$ precipitates and organic flocs. Recovery of solids-associated viruses by microporous filter methods employing in-situ elution is inconsistent.² Solids-associated viruses on adsorbent filters are eluted more efficiently by disrupting filters in elution fluid than by in-situ elution,² but this is cumbersome and

time-consuming, especially for large-diameter disk filters and cartridge filters.

For small volumes of water and wastewater, solids-associated viruses can be recovered expediently by separating the solids by centrifuging, decanting the supernatant, and eluting viruses from the solids by resuspending in a small volume of eluent.⁴ Viruses in the supernatant can be concentrated by one of the procedures described in Sections 9510B, C, D, or E. Viruses eluted from the resuspended solids are separated from the solids by centrifuging and are assayed directly or concentrated further by organic flocculation.^{5,6} Major limitations of these methods are incomplete virus elution and poor virus recoveries due to interferences from sample constituents.

2. Equipment and Apparatus

- Centrifuge*, with rotor and buckets for 250- to 1000-mL-capacity bottles, capable of operating at about $1250 \times g$.
- Centrifuge bottles*, 250- to 1000-mL.
- pH meter*.
- Laboratory balance*.
- Graduated cylinder*, 250-mL or larger.
- Beaker*, 250-mL or larger.
- Sample bottles*, 250-mL or larger.
- Magnetic stirrer and stirring bars*, or alternative mixing device.
- Pipets*, 1-, 5-, and 10-mL.

3. Reagents

- Hydrochloric acid*, HCl, 0.1 and 1.0N.
- Sodium hydroxide*, NaOH, 0.1 and 1.0N.
- Eluent*: Dissolve 10 g beef extract, 1.34 g disodium phosphate heptahydrate, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 0.12 g citric acid in 90 mL distilled water, adjust to pH 7.0 with 1N HCl or NaOH, dilute to 100 mL with distilled water, and sterilize by autoclaving.
- Antibiotics*: See Section 9510B.4i.

4. Procedure

a. *Sterilization of apparatus, materials, and reagents*: See Section 9510B.5a.

b. *Sample size, collection, and storage*: Collect and process samples of no more than 10 L, depending on capacity of centrifuge. See Section 9510B.5d for sample collection and storage procedures.

c. *Sample processing*: Aseptically transfer 250- to 1000-mL sample volumes to centrifuge bottles and centrifuge at $1250 \times g$ for 20 min. Decant and pool supernatants for subsequent processing for viruses by one of the methods for water or wastewater described previously.

Elute viruses from the sedimented solids by resuspending in eluent. Use 40 mL eluent per quantity of sediment from 250 mL

of original sample. Pool resuspended sediments from multiple centrifuge bottles in a sterile beaker. Alternatively, keep resuspended sediments from small numbers of centrifuge bottles in the bottles and process them individually. While vigorously mixing with a magnetic stirrer, adjust to pH 7.0 by slowly adding 1N NaOH or HCl, if necessary. Reduce mixing speed and continue mixing for 30 min. During this period, check sample pH and readjust to pH 7.0 as necessary. As an alternative to mixing for 30 min, sonicate samples at 100 W for 15 min in a rosette cooling cell maintained at 4°C. Return sample to centrifuge bottles. Centrifuge at $1250 \times g$ and 4°C for 15 min, collect supernatant for subsequent assay or further concentration, and discard the sediment.

If desired, further concentrate viruses from this supernatant by organic flocculation (see Section 9510E). For supernatants that will be assayed directly for viruses with no further concentration, adjust to pH 7.4, add 1/10 the volume of sample of penicillin-streptomycin or 1/100 volume of gentamycin-kanamycin and store at 4 or -70°C .

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9510 G. Assay and Identification of Viruses in Sample Concentrates

1. Storage of Sample Concentrates

Because it often is impossible to assay sample concentrates immediately, store them at room temperature (about 25°C) for up to 2 h or at refrigerator temperatures (4 to 10°C) for up to 48 h to minimize virus losses. Freeze samples requiring storage longer than 48 h at -70°C or less. Do not freeze samples at -10 to -20°C because extensive inactivation of some enteric viruses may occur. Store sample concentrates from finished waters in separate freezers or physically separated from other virus-containing material in common freezers.

2. Decontamination of Sample Concentrates

Sample concentrates, especially those from wastewater, are likely to be contaminated with bacteria and fungi that can overgrow cell cultures and interfere with virus detection and assay. Do not decontaminate by centrifugation or filtration because virus losses are likely to occur. For many samples, especially those from finished waters, contamination is controlled adequately by antibiotics such as penicillin-streptomycin or gentamycin-kanamycin that are added immediately after the sample is obtained. To provide additional protection against fungal contamination, add amphotericin B or nystatin at concentrations of 2.5 and 50 $\mu\text{g/mL}$, respectively.¹ If penicillin-streptomycin or

gentamycin-kanamycin are inadequate, use one or more additional antibiotics such as aureomycin, neomycin, or polymyxin B. To maximize the antibiotic effects, incubate samples for 1 to 3 h at 25 to 37°C after adding the antibiotics. Bacterial destruction is further enhanced by freezing at -70°C after incubation with antibiotics. Keep samples frozen until assayed for viruses. To determine if antibiotic treatment has been effective, plate a small subsample on a general-purpose medium such as plate count agar by the spread plate technique and incubate at 37°C for 24 to 48 h.

If extensive bacterial contamination persists after antibiotic treatment, treat with chloroform. Add 1/10 volume of sample of chloroform (CHCl_3) and mix vigorously for 30 min at room temperature or homogenize 1 to 2 min at 4 to 10°C. For phase separation, centrifuge at $\geq 1000 \times g$ or store overnight in a refrigerator. Separate sample (upper layer) from CHCl_3 (bottom layer) by aspirating with a pipet and bubble with filter-sterilized air for about 15 min to remove dissolved CHCl_3 . It may be necessary to place sample in a sterile, shallow container and expose it to the atmosphere in a sterile air environment (laminar air flow clean bench or biological safety cabinet) for up to several hours to remove remaining traces of CHCl_3 . Do not use ether to decontaminate samples because of the hazard of explosion or fire.

3. Laboratory Facilities and Host Systems for Virus Assay

Because viruses are obligate, intracellular parasites, they grow (multiply) only in living host cells. This ability to multiply in, and thereby destroy, their host cells is the basis for virus detection and assay. The two major host cell systems for human enteric viruses are whole animals (usually mice) and mammalian cell cultures of primate origin.

A complete description of facilities, equipment, materials, and methods for conducting virus assays is beyond the scope of this book; see standard handbooks on virology and cell culture.¹⁻⁴ Virus assay is beyond the capability of most water and wastewater microbiology laboratories. It should be done only by a trained virologist working in specially equipped virology laboratory facilities. Take particular care to prevent samples or inoculated hosts from becoming contaminated with viruses from other sources and to prevent virus cross-contamination arising from sample concentrates or inoculated hosts. Process and handle samples in a Class II Type I biological safety cabinet⁵ or in a "sterile" room or cubicle. The use of such cabinets or facilities is mandatory for testing drinking water or other finished water samples.

There is no single, universal host system for all enteric viruses. Some enteric viruses, notably hepatitis A virus, human rotaviruses, and Norwalk-type gastroenteritis viruses, cannot be assayed routinely in any convenient laboratory host systems. However, most of the known enteric viruses can be detected by using two or more cell culture systems and perhaps suckling mice. The latter previously were considered essential for the detection of group A coxsackie-viruses, but recent studies indicate that the RD cell line may be nearly as sensitive as suckling mice for the isolation of these viruses as well as other enteroviruses.^{6,7} In general, the more different host systems used, the greater the enteric virus recovery rate. However, the number of different

host systems used is limited by practical and economic considerations.

There have been numerous comparative studies on relative sensitivities of various cell culture systems for enteric virus detection,⁶⁻²⁰ but no systematic, comprehensive study has been reported for enteric virus recoveries from water and wastewater. Primary or secondary human embryonic kidney (HEK) cell cultures appear to be the single most sensitive host system for enteric virus isolations, but they are becoming increasingly more difficult to obtain regularly and, when available from commercial sources, they are expensive. Primary or secondary African green, cynomolgus, or rhesus monkey or baboon kidney cells are sensitive hosts for many enteroviruses and reoviruses, but are not particularly suitable for recovering adenoviruses or group A coxsackieviruses. BGM, a continuous line derived from African green monkey kidney cells, may be comparable in sensitivity to primary monkey kidney cells for enteric virus recovery.^{7,18,21,25,26} A number of other continuous cell lines as well as human fetal diploid cell strains have been evaluated for enteric virus recoveries. Some human fetal diploid cell strains give virus isolation rates comparable to primary monkey kidney cells, but plentiful supplies of specific human fetal diploid cell strains are not readily available and many are difficult to maintain. Furthermore, each different cell strain must be characterized for virus susceptibility. Most continuous cell lines generally are less effective than primary cells, but comparable isolation rates for some enteric virus groups have been obtained with Hep-2¹¹ and HeLa^{17,25} cells.

Assay the entire sample concentrate for enteric viruses, using at least two different host systems and dividing entire sample equally among the hosts. Preferably use primary (or secondary) HEK cells with either primary (or secondary) monkey kidney or BGM cells for the recovery of most enteroviruses, adenoviruses, and reoviruses. Additional use of either suckling mice or RD cells provides for enhanced recovery of group A coxsackieviruses. Different host systems may be substituted for these if it is demonstrated that they have equivalent sensitivity.

4. Virus Quantitation Procedures for Sample Concentrates

a. Advantages and disadvantages of different quantitation procedures: Virus assays in suckling mice or other animals are quantal assays and in cell cultures they can be done either by quantal (most probable number or 50% endpoint) or enumerative (plaque) methods. Selection between cell culture assay methods depends on the sample and the choice between achieving either maximum virus sensitivity or maximum precision and accuracy in estimating virus concentration. The plaque technique generally is more precise and accurate than the quantal assay because relatively large numbers of individual infectious units can be counted directly as discrete, localized areas of infection (plaques). Quantal assays are more sensitive than monolayer plaque assays, but are less sensitive than an agar cell suspension plaque assay.²⁶

Because virus plaques are discrete areas of infection arising from a single infectious virus unit, it is relatively easy to recover viruses from individual plaques and then to inoculate them into additional cell cultures to obtain a pure virus culture for identification. However, large proportions of so-called "false-positive" plaques that do not confirm as virus-positive when material from

these plaques is further passaged in cell cultures have been reported.^{27,28} Whether this problem is due to nonviral, plaque-like areas of cytotoxicity from the sample or to technical inability to passage viruses successfully from the initial plaques remains uncertain.^{27,28}

The use of specific plaque assay conditions for optimizing the recovery of certain enteric virus groups may preclude efficient recovery of other enteric groups requiring different plaque assay conditions. Furthermore, some viruses, such as adenoviruses, do not form plaques efficiently under any conditions. Cytotoxicity due to water or wastewater constituents in sample concentrates is difficult to control in plaque assay systems because the agar overlay medium is difficult to remove and replace.

A potential limitation of quantal assays is the possibility that two or more different virus types will be inoculated into the same cell culture and thus produce a simple positive culture. This not only results in an underestimation of virus concentration but also requires separation of the individual virus types by further passage in cell culture. Such mixed cultures may go undetected unless virus isolates are identified serologically. Recent results indicate that mixed positive cultures are encountered rarely when samples are divided into small portions for inoculation into a series of replicate cell cultures.^{7,25}

Cytotoxicity due to constituents of sample concentrates usually can be controlled in quantal assay cell cultures by replacing the culture medium before the cells die.

b. Cell culture procedures for virus isolation and assay: To assay sample concentrates in cell cultures by quantal or plaque methods, drain the medium from newly confluent cultures. To reduce toxicity, rinsing with buffered saline solution may be helpful. Inoculate with unit volumes of sample. Use no more than 0.06 mL sample/cm² of cell layer surface, e.g., maximum volumes of 1.0, 3.0, and 6.0 mL in cell culture flasks with areas of 25, 75, and 150 cm², respectively.²⁹ If samples are expected to contain such large quantities of viruses that it would be difficult to make reliable estimates of concentration, inoculate cell cultures with small sample volumes or dilutions of concentrates. Allow viruses to adsorb to cells for 2 h at 37 ± 0.5°C. Redistribute inoculum over the cell layer manually every 15 min or keep cultures on a mechanical rocker during the adsorption period. Add liquid maintenance medium to cultures for quantal assays or agar-containing medium for plaque assays. Invert plaque assay cultures so that cell (agar) side of culture faces up and incubate at 37°C.

Microscopically examine quantal assay cultures for the appearance of cytopathic effects (CPE) daily during the first 3 d and then periodically for a total of at least 14 d. Do not change cell culture medium unless cytotoxicity or cell deterioration occurs. Freeze cultures developing CPE at -70°C when more than 75% of the cells become involved. After 14 or more days, freeze at -70°C all remaining cultures, including those remaining negative for CPE as well as controls. Thaw cultures and clarify culture fluid-cell lysate by slow-speed centrifugation or filtration through sterile 0.22- or 0.45-μm porosity filters. Inoculate clarified material from each initial (first-passage) culture into a second (second-passage) culture by transferring 20% of the total initial culture into newly confluent cell cultures of the same type. Microscopically examine second-passage cultures for development of CPE periodically over a period of 14 or more days. Consider second-passage cultures developing CPE as con-

firmed virus-positive. Freeze and store at -70°C for virus identification. Discard as negative any virus cultures negative for CPE after this second incubation period of 14 or more days.

Periodically examine plaque assay cultures for appearance of plaques over a 14-d period. Mark and tally plaques as they appear. Transfer viruses from each plaque directly to at least two newly confluent, liquid-medium cell cultures of the same type²⁷ before plaques become too large and grow together or before the entire cell layer deteriorates. Do not store material obtained from plaques before transfer to new cell cultures, as this may result in loss of virus titer and unsuccessful transfers. Microscopically examine these second-passage cultures periodically over 14 d for development of CPE. Freeze cultures developing CPE at -70°C for virus identification.

c. Virus isolation and assay in mice: To detect group A and B coxsackieviruses in mice, inoculate samples into animals no older than 24 h using standard procedures.^{2,3,8} Use either the intracerebral or intraperitoneal route, inoculating 0.02 and 0.05 mL, respectively. Observe mice daily over a 14-d period for development of weakness, tremors, and either flaccid (due to group A coxsackieviruses) or spastic (due to group B coxsackieviruses) paralysis. Sacrifice animals developing symptoms, and using sterile technique, prepare 20% tissue suspensions in Hanks balanced salt solution of the entire skinned, eviscerated torso or just the brain and legs. Store suspensions at -70°C until used for further passage and identification. For second passage in mice, follow general procedures used for the initial inoculations. However, making a second passage in cell cultures is preferable to making a second passage in mice because it is easier to do subsequent virus identification by neutralization tests.

d. Estimating virus concentration: Determining the amount of virus in a sample concentrate depends on the assay used. If a sample concentrate is assayed in cell cultures by the plaque technique, count all plaques and calculate the virus concentration, expressed as plaque-forming units (PFU).

If a sample concentrate is assayed by the quantal method, estimate the virus concentration by the most probable number (MPN) method and express as most probable number of infectious units (MPNIU), or by a 50% end point method and express as 50% infectious or lethal dose (ID₅₀ or LD₅₀).^{2,4,30-32} If the undiluted sample concentrate or a single sample dilution was inoculated into a series of replicate cell cultures (or mice), calculate the MPNIU from the number of confirmed CPE-negative cultures (or mice), q , per total number of cultures (or mice) inoculated, n , according to the formula

$$\text{MPN} = -\ln(q/n)$$

If more than one sample dilution was inoculated into cell cultures (or mice), calculate the MPNIU from the formula developed by Thomas:³³

$$\text{MPN/mL} = \frac{P}{\sqrt{NQ}}$$

where:

- P = total number of positive cultures (or mice) from all dilutions,
- N = total mL sample inoculated for all dilutions, and
- Q = total mL sample in all negative cultures (or mice).

In using this formula, exclude from the computation all dilutions containing only positive cultures (or mice).

For MPN values obtained from a single sample dilution, the 95% confidence interval is based on the standard error of the binomial distribution when more than 30 cultures (or mice) are inoculated or from the confidence coefficient table of Crow^{32,34} when 30 or fewer cultures (or mice) are inoculated.

Make 50% end-point estimates arithmetically by either the Reed-Muench or Karber method.^{2,4} These methods require results from several equally spaced sample dilutions, preferably with about the same number of dilutions above and below the 50% end point, and may not be useful for sample concentrates containing relatively low virus levels.

e. Identification of virus isolates: Identify enteric viruses isolated from sample concentrates by standard serological techniques, although preliminary identification of genus (enterovirus, reovirus, or adenovirus) sometimes can be made on the basis of information obtained from the isolation procedure. Enteric viruses recovered in suckling mice are likely to be either group A or B coxsackieviruses. For enteric viruses isolated in cell cultures, preliminary identification of genus often can be made from the characteristic appearance of cytopathic effects (CPE) in infected cell cultures.

Confirm preliminary identification of suspected adenovirus and reovirus isolates by detecting their respective group specific antigens by complement fixation tests using clarified, second-passage cell-culture lysate as the antigen. Identify specific reovirus serotypes by hemagglutination-inhibition (HI) or neutralization (Nt) tests. Adenovirus serotypes can be separated into four groups on the basis of their ability (or inability) to hemagglutinate rhesus monkey or rat erythrocytes.^{2,3,8} Except for type 18, the first 28 numbered adenoviruses can be identified as to specific serotype by HI. Alternatively, identify all adenovirus serotypes by Nt tests using either individual type-specific antisera or intersecting antisera pools. Also identify specific enterovirus serotypes by neutralization tests in cell cultures using intersecting pools of hyperimmune sera.^{2,3,8,35} Use mice for Nt tests for group A and B coxsackieviruses only if the virus isolates fail to propagate in cell cultures.³⁶ Because polioviruses often are the most prevalent enteroviruses in water and wastewater, test enterovirus isolates for neutralization by an antisera pool against the three types of poliovirus before making neutralization tests with intersecting antisera pools.

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9610 DETECTION OF FUNGI*

9610 A. Introduction

1. Significance

Fungi, including yeasts and filamentous species or molds, are ubiquitously distributed, achlorophyllous, heterotrophic organisms with organized nuclei and usually with rigid walls. They may be found wherever nonliving organic matter occurs, although some species are pathogenic and others are parasitic. In spring water near the source, the number of fungus spores usually is minimal. Unpolluted stream water has relatively large numbers of species representing the true aquatic fungi (species possessing flagellated zoospores and gametes), aquatic Hyphomycetes, and soil fungi. Moderately polluted water may carry cells or spores of the three types; however, it has fewer true aquatic fungi and aquatic Hyphomycetes, and soil fungi are more numerous. Heavily polluted water has large numbers of soil fungi. The group designated as soil fungi includes yeast-like fungi, many species of which have been isolated from polluted waters.

The association between fungal densities and organic loading suggests that fungi may be useful indicators of pollution. Unfortunately, no single species or group of fungi has been identified as important in this role. There may be some exceptional special cases; for example, the principal distinction between the yeasts *Candida lambica* and *C. krusei* is the ability to use pentose sugars. Because the former species grows well on pentoses, it could be used as an indicator of pulp and paper mill wastes, which are high in such sugars. Certain species of yeasts and filamentous fungi are characteristic of warmer waters and may be useful indicators of thermal pollution.

Because fungi possess broad enzymatic capabilities, they can degrade actively most complex natural substances and certain

synthetic compounds, including some pesticides. Most fungi are aerobic or microaerophilic, although a few species show limited anaerobic metabolism and a very few are capable of totally anaerobic growth.¹ Some species do not require light.

2. Occurrence and Survival

Fungi are present in, and have been recovered from, diverse, remote, and extreme aquatic habitats including lakes, ponds, rivers, streams, estuaries, marine environments, wastewaters, sludge, rural and urban stormwater runoff, well waters, acid mine drainage, asphalt refineries, jet fuel systems, and aquatic sediments.

a. Fungi in potable water: Fungi have been found in potable water²⁻⁷ and on the inner surface of distribution system pipes.⁸ Either they survive water treatment or they enter the system after treatment and remain viable. Tuberculate macroconidia of *Histoplasma capsulatum*⁹ can pass through a 0.75-m rapid sand filter. Plain sedimentation or alum flocculation and settling removed 80 to 99% of the spores. If these relatively large (8- to 14- μ m) globose, tuberculate macroconidia pass through treatment, it is not surprising that other fungi, typically with smaller spores, are found in treated water.

Having survived treatment or having been introduced after treatment, fungal spores can remain viable for extended periods of time. Pathogenic fungi have been stored effectively in sterile distilled water for relatively long periods.¹⁰ Spores of *H. capsulatum*, stored in raw Ohio River water and sterile tap water, remained highly infective for mice after 400 d.¹¹

Tastes and odors in potable water are associated with the presence of procaryotic organisms such as bacteria, actinomycetes, and cyanobacteria. However, fungi may be involved.^{6,7}

Propagules from 19 genera of filamentous fungi have been isolated from a chlorinated surface water system and a nonchlor-

* Approved by Standard Methods Committee, 2000.

minated groundwater distribution system.² The mean number of colony-forming units (CFU) was 18/100 mL in the groundwater system and 34/100 mL in the surface system.

In Finland,³ fungi were isolated from rivers, lakes, and ponds supplying nine communities with sand-filtered water, three with artificially recharged groundwater, of which two used chemical coagulation, and three with chemically coagulated and disinfected water. Mesophilic fungi were common in all raw water samples; however, thermotolerant fungi were more abundant in river than in lake water. Chemical coagulation and disinfection proved far more efficient in removing fungi than sand filtration and disinfection. *Aspergillus fumigatus* was the most common fungus.

Five chlorinated groundwater systems⁴ in the U.S. yielded an average count per positive sample of about 5.5 CFU/100 mL. In France⁵ yeasts were recovered from 50% of 38 samples and filamentous fungi from 81%.

Except for *Aspergillus fumigatus*,^{3,4} *A. flavus*,^{4,5} and *A. niger*,⁴ the fungi isolated from potable water usually are not considered to be medically important. Fungus infections may be significant for individuals with compromised immune systems. Most of the fungi are common soil saprobes.

b. Fungi in recreation waters: Some fungi pathogenic to humans may be expected in recreational waters such as pools and beaches and in accompanying washing facilities such as shower stalls.

Trichophyton mentagrophytes, the cause of tinea pedis or athlete's foot, has been isolated¹² from the wooden flooring of a shower stall. Seven species of pathogenic and potentially pathogenic fungi were isolated from 361 samples of beach sand in Hawaii.¹³ Beach sand on the German Baltic coast, in Portugal, and on the Adriatic coast yielded *Epidermophyton*.¹⁴

c. Survival on chlorination: An unidentified yeast was isolated from other organisms that survived chlorination of wastewater effluents.¹⁵ This yeast survived 1 mg free chlorine/L for 20 min in contrast with *E. coli*, which failed to survive 5 min contact with 0.03 mg free chlorine/L. The amount of chlorine for fungus control is known at least for *C. albicans*. It has been shown¹⁶ that cells of *C. albicans* were inactivated effectively with 4 mg chlorine/L in 30 min when the initial cell count was 10⁵ cells/mL. In an Illinois study with *C. parapsilosis*,^{17,18} a commonly isolated yeast known to cause health problems in the tropics, greater amounts of chlorine were required to inactivate the organism than coliform bacteria. Mechanisms of inactivation by chlorine on assimilative stages of yeasts and other microorganisms have been suggested.¹⁹ Fungal cells, especially conidia, can survive much higher doses of chlorine than coliform bacteria,²⁰ including a 10-min exposure to 10 mg chlorine/L when the initial spore count was approximately 10⁶/mL.

3. Growth Patterns and Identification

In water there are two basic patterns of fungal growth. True aquatic fungi produce zoospores or gametes that are motile by means of flagella, either of the whiplash or tinsel type. Some fungi, particularly the Trichomycetes (fungi that inhabit the hind gut of certain worms, mosquito larvae, etc.), have amoeboid stages. Aquatic fungi typically are collected by exposing suitable baits (solid foodstuffs) in the habitat being examined or in a sample within the laboratory. Relatively little work on these

fungi in polluted water has been done in the U.S. They have been studied more extensively in polluted waters in England, Germany, and Japan.

The second fungal growth form is nonmotile in all stages of the life cycle. Growth and reproduction usually are asexual (anamorphic). Three growth processes have been recognized: (a) filamentous growth with blastic spores or spores produced in special structures; (b) filamentous growth with the filaments breaking up in an arthric (fragmenting) manner to form separate spores called arthroconidia as in *Geotrichum* and related genera; and (c) single-celled growth produced on each parent cell, called budding, typical of the yeasts.

Identification of fungi, which are considerably larger than bacteria, is dependent on colonial morphology on a solid medium, growth and reproduction morphology, and, for yeasts, physiological activity in laboratory cultures. Increasing numbers of fungi usually indicate increasing organic loadings in water or soil. Large numbers of similar fungi suggest excessive organic load while a highly diversified mycobiota indicates populations adjusted to the environmental organics. Despite their wide occurrence, little attention has been given to the presence and ecological significance of fungi in aquatic habitats. The relevance of fungi and their activities in water is emphasized by increasing knowledge of their pathogenicity for humans, animals, and plants; their role as food or energy sources; their activity in natural purification processes; and their function in sediment formation.

A survey of the literature of fungi occurring in water, wastewater, and related organically polluted substrata listed 984 species²¹; 133 species were assigned to the Mastigomycotina (fungi with flagellated zoospores); 79 to the Zygomycotina, mostly mucoraceous fungi; 161 to the Ascomycotina, including perfect (teleomorphic) states of some of those assigned to the Fungi Imperfecti; 18 to the Basidiomycotina, including perfect states of several yeasts; and 593 to the Deuteromycotina or Fungi Imperfecti. Of the total, 133 species were zoosporic, 131 species were yeast-like, and 718 species were filamentous. Most zoosporic species were recovered from mildly polluted or unpolluted waters; of the remaining species fewer than half were recovered in numbers large enough to indicate membership in a population for even a brief period of time. The significance of fungi in both aquatic and terrestrial environments has been discussed in detail.²²⁻³⁸

Quantitative enumeration of fungi is not equivalent to that of unicellular bacteria because a fungal colony may develop from a single cell (spore), an aggregate of cells (a cluster of spores or a single multi-celled spore), or from a mycelial or pseudomycelial fragment (containing more than one viable cell). It is assumed that each fungal colony developing in laboratory culture originates from a single colony-forming unit (CFU), which may or may not be a single cell.

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9610 B. Pour Plate Technique

1. Samples

a. Containers: Collect samples as directed in Section 9060A. Alternatively, use cylindrical plastic vials with snap-on caps. These vials usually are sterile as received. Transport them in an upright position to minimize the chance of leakage and discard after use.

b. Storage: Hold samples for not more than 24 h. If analysis is not begun promptly after sample collection, refrigerate.

2. Media

For counting, neopeptone-glucose-rose bengal-aureomycin® agar is the usual medium of choice, although experience may indicate that Czapek agar (for *Aspergillus*, *Penicillium*, and related fungi) and yeast extract-malt extract-glucose agar or Diamalt agar (for yeasts) may be preferable. For inventory, use neopeptone-glucose agar.

a. Neopeptone-glucose-rose bengal aureomycin® agar: Add 5.0 g neopeptone, 10.0 g glucose, 3.5 mL rose bengal solution (1 g/100 mL reagent-grade water), and 20.0 g agar to 1 L reagent-grade water. Because this medium is used for making pour plates, prepare and store basal agar either in bulk, or more conveniently, in tubes in 10-mL amounts. Sterilize by autoclaving; the final pH should be about 6.5.

Separately prepare a solution of chlortetracycline or tetracycline (1.0 g water-soluble antibiotic/150 mL reagent-grade water) and refrigerate. Before use, sterilize by filtration. To complete the medium, add 0.05 mL sterile solution to 10 mL melted basal agar at about 45°C.

This medium may not be available in dehydrated form and may require preparation from the basic ingredients. Dehydrated Cooke's rose bengal agar may be used in place of the agar base.

This medium is useful for isolating a broad spectrum of fungal species.

b. Czapek (or Czapek-Dox) agar: Dissolve 30.0 g sucrose, 3.0 g sodium nitrate (NaNO₃), 1.0 g dipotassium hydrogen phosphate (K₂HPO₄), 0.5 g magnesium sulfate (MgSO₄), 0.5 g potassium chloride (KCl), 0.01 g ferrous sulfate (FeSO₄), and 15.0 g agar in 1 L reagent-grade water. The pH should be 7.3 after sterilization.

This medium is useful for isolating species of *Aspergillus*, *Penicillium*, *Paecilomyces*, and some other fungi with similar physiological requirements.

c. Yeast extract-malt extract-glucose agar: Dissolve 3.0 g yeast extract, 3.0 g malt extract, 5.0 g neopeptone (or equivalent), 10.0 g glucose, and 20.0 g agar in 1 L reagent-grade water. No adjustment of pH is required.

This medium is useful for isolating yeasts.

d. Diamalt agar: Dissolve 150 g diamalt and 20.0 g agar in 1 L reagent-grade water. No adjustment of pH is required. The medium will be turbid but filtration is unnecessary.

This medium is useful in the purification of yeast isolates and for study of yeast species in various specified tests.

e. Neopeptone-glucose agar: Dissolve 5.0 g neopeptone (or equivalent), 10.0 g glucose, and 20.0 g agar in 1 L reagent-grade water. The pH should be about 6.5 after sterilization. (This medium is known also as Emmons' Sabouraud Agar or Emmons' Sabouraud Dextrose Agar.)

This medium is useful for maintaining stock cultures. It is comparable to neopeptone-glucose-rose bengal aureomycin® agar but contains neither rose bengal nor an antibiotic.

3. Procedure

As many as 40 samples can be analyzed simultaneously by a single analyst by the following procedure; however, 20 samples represents the optimum number.

a. Preparation and dilution: To a sterile 250-mL erlenmeyer flask add 135 mL sterile reagent-grade water and 15 mL sample to obtain a 1:10 sample dilution. Use a sterile measuring device for each sample, or, less preferably, rinse the measure with sterile reagent-grade water between samples. Mix sample well before withdrawing the 15-mL portion. Shake flask on a rotary shaker at about 120 to 150 oscillations/min for about 30 min or transfer flask contents to a blender jar, cover and blend at low speed for 1 min or high speed for 30 s. Preferably use a sterile blender jar and appurtenances for each sample or wash jar thoroughly between samples and rinse with sterile water. Further dilutions may be made by adding 45 mL sterile water to 5 mL of a 1:10 diluted suspension.

For stream water samples a dilution of 1:10 usually is adequate. Dilute samples with large amounts of organic material, such as sediments, to 1:100 or 1:1000. Dilute stream bank or soil samples to 1:1000 or 1:10 000.

b. Plating: Prepare five plates for each dilution to be examined. To use neopeptone-glucose-rose bengal-aureomycin® agar, aseptically transfer 10 mL of medium at 45°C to a 9-cm petri dish. Add 1 mL of appropriate sample dilution and mix thoroughly by tilting and rotating dish (see plating procedure under heterotrophic plate count, Section 9215). Alternatively add to petri dish 1 mL sample, 0.05 mL antibiotic solution, and 10 mL liquefied agar medium at 43 to 45°C. Solidify agar as rapidly as possible. (In arid areas use more medium to prevent dehydration during incubation.)

c. Incubation: Stack plates but do not invert. Incubate at room conditions of temperature (20 to 24°C) and lighting but avoid direct sunlight. Examine and count plates after 3, 5, and 7 d.

d. Counting and inventory: The fungus plate count will provide the basis for rough quantitative comparisons among sam-

ples; the inventory will give relative importance of at least the more readily identifiable species or genera.

In preparing plates, use sample portions that will give about 50 to 60 colonies on a plate. Determine this volume by trial and error. When first examining a new habitat, plate at least two sample dilutions. Estimates of up to 300 colonies may be made, but discard more crowded plates. The medium containing rose bengal tends to produce discrete colonies and permits slow-growing organisms to develop.

The inventory includes the direct identification of fungi based on colonial morphology and the counting of colonies assignable to various species or genera. When discrete colonies cannot be

identified and identification is important, use a nichrome wire with its tip bent in an L-shape to pick from each selected colony and streak on a slant of neopeptone-glucose agar (§ 2e). If five plates are used per sample, the average number of colonies on all plates (total number of colonies counted/5) times the reciprocal of the dilution (10/1, 100/1, 1000/1, etc.) equals the fungus colony count per milliliter of original sample. For solid or semisolid samples, use a correction for the water content to report fungus colonies per gram dry weight. Determine water content by drying paired 15-mL portions of original sample at 100°C overnight; the difference between wet and dry weights is the amount of water lost from the sample.

9610 C. Spread Plate Technique

The spread plate technique is an alternative procedure for obtaining quantitative data on colony-forming units.

1. Samples

See Section 9610B.1.

2. Media

Use any of the following media. Aureomycin®-rose bengal-glucose-peptone agar (ARGPA) (e) and streptomycin-terramycin®-malt extract agar (STMEA) (f) are useful in analyzing sewage and polluted waters.¹

a. *Neopeptone-glucose-rose bengal aureomycin® agar*: See Section 9610B.2a.

b. *Czapek (or Czapek-Dox) agar*: See Section 9610B.2b.

c. *Yeast extract-malt extract-glucose agar*: See Section 9610B.2c.

d. *Diamalt agar*: See Section 9610B.2d.

e. *Aureomycin®-rose bengal-glucose-peptone agar*: Dissolve 10.0 g glucose, 5.0 g peptone, 1.0 g potassium dihydrogen phosphate (KH_2PO_4), 0.5 g magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 0.035 g rose bengal, and 20.0 g agar in 800 mL reagent-grade water and sterilize. Dissolve 70.0 mg aureomycin® hydrochloride in 200 mL reagent-grade water, sterilize by filtration, and add to the cooled (42 to 45°C) agar base. The pH should be 5.4. Pour 25-mL portions into sterile petri dishes (100 × 15 mm) and let agar harden. Poured plates may be held up to 4 weeks at 4°C.

f. *Streptomycin-terramycin®-malt extract agar*: Dissolve 30.0 g malt extract, 5.0 g peptone, and 15.0 g agar in 800 mL reagent-grade water and sterilize. Dissolve 70.0 mg each of streptomycin and terramycin® in separate 100-mL portions reagent-grade water, sterilize by filtration, and add to the cooled (42 to 45°C) agar base. The pH should be 5.4. Pour about 20-mL portions into sterile petri dishes (60 × 15 mm) and let agar harden. Poured plates may be held up to 4 weeks at 4°C.

3. Procedure

a. *Preparation and dilution*: See Sections 9215A.5 and 9610B.3a. Make dilutions with buffered water (Section

9050C.1) and select dilutions that yield 20 to 150 colonies per plate.

b. *Plating*: Pre-dry plates separately with lids off in a laminar-flow hood at room temperature and about 30% relative humidity for 1 to 1.5 h. Prepare at least three plates, or five plates if data are to be analyzed statistically, per sample or dilution. Using a sterile pipet, transfer 1.0 mL of sample or dilution onto surface of a pre-dried agar plate. Spread sample over entire agar surface using a sterile L-shaped glass rod or use a mechanical device to rotate plate and ensure proper sample distribution.

c. *Incubation*: With dish covers on, let plates dry at room temperature, invert plates, and incubate at 15°C for 7 d in an atmosphere of high humidity (90 to 95%). Alternatively incubate at 20°C for 5 to 7 d. Slow-growing fungi may not produce noticeable colonies until 6 or 7 d.

d. *Counting and recording*: Using a Quebec colony counter, count all colonies on each selected plate. If counting must be delayed temporarily, hold plates at 4°C for not longer than 24 h. Depending on colony size, plates with as many as 150 colonies can be counted but the optimal maximum number is 100 colonies.

Record results as colony-forming units (CFU)/100 mL original sample. For solid or semisolid samples report CFU/g wet or dry, preferably dry. If three or more plates are used per sample, use average number of colonies times the reciprocal of the dilution (see 9610B) to give colony count. If no plates have colonies, record count as < 1 for the highest dilution. If there are more than 150/plate, record as Too Numerous To Count (TNTC) but indicate a count of > 150 for the appropriate dilution. If colonies are crowded and overlapping with spreaders, record as "obscured" (OBSC) and repeat analysis with higher dilution or earlier observations.

4. Reference

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9610 D. Membrane Filter Technique

For general information on the membrane filter technique and apparatus needed see Section 9222. However, except for comparisons of different manufacturers' membranes, no critical tests have been reported for membrane filters for fungal isolation efficiency. Media components, pH levels, and antibiotics have been used in routine plating procedures. It appears that the reported procedures are satisfactory.

1. Samples

See Sections 9610B.1 and 9060A.

2. Media

Use unmodified or modified aureomycin®-rose bengal-glucose-peptone agar (MARGPA) or modified streptomycin-terramycin®-malt extract agar (MSTMEA).¹ These media are prepared identically to the unmodified media described in 9610C.2e and f except that the concentration of each antibiotic is increased from 70 mg/L to 200 mg/L. Dispense media in portions of 5 to 7 mL in glass or plastic petri dishes (60 × 15 mm); plastic dishes with tight-fitting lids are preferred.

3. Procedure

a. Preparation and dilution: See Sections 9215A.5 and 9610B.3a. Select dilutions to yield 20 to 100 colonies per membrane.

b. Filtration: Filter appropriate volumes of well-shaken sample or dilution, in triplicate, through membrane filters with pore diameter of 0.45 or 0.8 µm. See Section 9222.

c. Incubation: Transfer filters to dishes, invert dishes or not, and incubate at 15°C for 5 d in a humid atmosphere. Alternatively incubate at 20°C for 3 d, or longer depending on fungi present.

d. Counting and recording: Using a binocular dissecting microscope at a magnification of 10×, count all colonies on each selected plate. If counting must be delayed temporarily, hold plates at 4°C for not longer than 24 h. Ideal plates have 20 to 80 colonies per filter. See Section 9610C.

4. Reference

1. QURESHI, A.A. & B.J. DUTKA. 1978. Comparison of various brands of membrane filter for their ability to recover fungi from water. *Appl. Environ. Microbiol.* 32:445.

9610 E. Technique for Yeasts

Of the total number of fungal colonies obtained from polluted waters, as many as 50% may be yeast colonies. Solid media such as those described above do not permit growth of all yeasts; thus, a quantitative enrichment technique may be useful in addition to the plate count (see also Fungi Pathogenic to Humans, Section 9610H).

1. Media

For enrichment, use yeast nitrogen base-glucose broth; for isolation, use yeast extract-malt extract-glucose agar or diamalt agar.

a. Yeast nitrogen base-glucose broth: Dissolve 13.4 g yeast nitrogen base in 1 L reagent-grade water; sterilize by filtration. Prepare 500 mL each of 2% and 40% aqueous glucose solutions and sterilize separately by filtration. To make final medium, aseptically add to a sterile 250-mL erlenmeyer flask 25 mL yeast nitrogen base solution and 25 mL of either 2% or 40% glucose solutions to make 1% or 20% final glucose concentrations. Stopper flask with a gauze-wrapped cotton stopper and store until used.

b. Yeast extract-malt extract-glucose agar: See Section 9610B.2c.

c. Diamalt agar: See Section 9610B.2d.

2. Procedure

a. Sample preparation and dilution: Prepare as directed in Section 9610B.

b. Enrichment: In 250-mL erlenmeyer flasks prepare one flask each of yeast nitrogen base medium containing 1% and 20% glucose. Inoculate with 1 mL of appropriate sample dilution and incubate at room temperature on a rotary shaker operating at 120 to 150 oscillations/min for at least 64 h. Shaken cultures are necessary to prevent overgrowth by filamentous fungi.

c. Isolation: Remove flasks from shaker and let settle 4 to 5 h. Yeast cells, if present, will settle to the bottom, bacteria and filamentous fungi will remain in suspension, and filamentous fungi will float on the surface or will be attached to the glass surface at or above the meniscus. With a nichrome wire loop remove a loopful of sediment at the sediment-supernatant interface from a tilted flask and smear-streak on yeast extract-malt extract-glucose agar. Use three plates per flask. Incubate at room temperature but out of direct sunlight for 2 to 3 d. It is not necessary to invert dishes. To obtain pure cultures, pick from reasonably isolated colonies and restreak on the same medium or on diamalt agar plates. Obtain pure cultures of as many different colonies as can be recognized.

d. Counting: It is impossible to obtain a meaningful plate count after this type of enrichment isolation. If it is assumed that one cell in the original sample will produce one or more colonies on the plates after enrichment, it can be stated that yeasts, or specific types of yeasts, occur at a minimal number dependent on the highest positive dilution. The reciprocal of this dilution is the indicated number of yeasts in the sample.

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9610 F. Zoosporic Fungi

1. Occurrence and Significance

Most fungi found in lacustrine (lake) and lotic (river) habitats that reproduce asexually by motile, uniflagellate spores and have determinate growth of the fungal body belong to the class Chytridiomycetes. Fungi with indeterminate growth, asexual reproduction by motile, biflagellate spores, and sexual reproduction involving oogonia and antheridia, are members of the class Oomycetes. A reduction in numbers of species of both classes tends to occur in polluted areas of rivers but more species of Oomycetes than of Chytridiomycetes can be found in polluted situations. Species of the Oomycete genera *Saprolegnia* (notably *S. ferax*) and *Leptomit* appear to be more tolerant than other forms. Bioassay studies indicate that Oomycetes are more tolerant to zinc, cyanide, and mannitol than are Chytridiomycetes. The latter appear to be more tolerant to treatment with surfactants than do the Oomycetes.

Some Chytridiomycetes may parasitize planktonic and other algae. In the case of epidemic fungal infections of phytoplankton species, the activities of fungi may affect the composition of phytoplankton communities by delaying the time of algal maxima and by reducing the population of certain algae so that other phytoplankters will replace the infected algal populations. In the case of nonepidemic infections, fungi may not influence algal populations; instead, they may infect only phytoplankters during periods of decline and thus only hasten decomposition of the algae.

Filamentous Oomycetes, particularly members of the Saprolegniaceae and Pythiaceae, are found in virtually all types of freshwater habitats and damp-to-wet soils. Most of the nearly 250 species involved occur as saprobes on dead and decaying organic matter such as insect exuviae, algae, and submerged vascular plant remains. A few occur as parasites of algae, aquatic invertebrates, fish, and vascular plants; none are associated with human disease.

Rarely do any of these fungi develop in sufficient numbers to be observed or collected directly. Consequently, various techniques have been devised for their collection and isolation.

2. Sampling and Baiting

Collect samples in sterile 35-mL plastic vials, refrigerate, and start analysis within 6 to 8 h. Place each sample in a sterile plate (20 × 100 mm) and dilute with 10 to 15 mL sterile reagent-grade water. Add three to four split hemp seed halves (*Cannabis sativa*), or whole seeds of mustard (*Brassica*) or sesame (*Sesamum*) as bait to each culture. Incubate at 18 to 23°C and examine

daily for fungal growth on the bait. As growth becomes evident, usually within 72 h, remove the infected bait, wash it thoroughly with water from a wash bottle, and transfer to a fresh plate of water containing two to three halves of hemp or other seed. Genera may be identified from spore arrangement within the sporangium and the manner in which spores are released. Specific determination requires microscopic examination of the sexual reproductive structures.

To collect the few naturally occurring parasites or pathogens, place the host organisms in a plate containing sterile water and hemp seed.

3. Isolation

Although most filamentous Oomycetes can be cultivated on plain cornmeal agar, selective media for isolating *Saprolegnia* from fresh water have been developed.¹

Obtain axenic cultures by drawing spores into a micropipet as they emerge from the sporangium. Less preferably, use hyphal tips, but note that several different genera and species frequently occur on a single piece of bait. Transfer the spore suspension or hyphal tip to a plate of cornmeal agar. When growth on the agar has occurred, remove bacteria-free hyphal tips aseptically by cutting out a small block of agar. Transfer to fresh medium or water. If growth is not free from contamination after one transfer, make additional transfers to insure pure cultures. Other methods have been outlined.²

4. Dilution Plating

Make serial dilutions with sterile reagent-grade water (1:100 000 to 1:700 000) and spread 1 mL over surface of a freshly prepared cornmeal agar plate. Remove each developing colony and transfer to water for identification. This method permits numerical estimation and determination of composition of the Oomycete community but requires at least 10 plates.

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9610 G. Aquatic Hyphomycetes

1. Occurrence and Significance

Freshwater Hyphomycetes are a very specialized group of conidial fungi that usually occur on partially decayed, submerged leaves and occasionally wood of angiosperms. The mycelium, which is branched and septate, ramifies through the leaf tissue, especially in petioles and veins. The conidiophores project into the water and the conidia that usually develop are liberated under water. Mature conidia also can be found in the surface foam of most rivers, streams, and lakes. The conidia of the majority of these fungi are hyaline, thin-walled, and either tetra- or polyradially branched, that is, with four divergent arms, or sigmoid (S-shaped) with the curvature in more than one plane. A special feature of the conidia is that while suspended in water, even for long periods, they do not germinate. However, if they come to rest on a solid surface, germ tubes are produced within a few hours. The size and morphology of these spores make them potentially more prominent in plankton analysis work than the spores of other fungi.

Ecological investigations of freshwater Hyphomycetes have been limited to substrate, habitat, dispersal, and their role in the enhancement of leaf substrates as food for aquatic invertebrates. The most common substrates of these organisms are submerged, decaying leaves of angiosperms such as alder (*Alnus*), oak (*Quercus*), hazelnut (*Corylus*), elm (*Ulmus*), maple (*Acer*), chestnut (*Castanea*), blackberry (*Rubus*), ash (*Fraxinus*), and willow (*Salix*). Submerged gymnosperm leaves usually are free of aquatic Hyphomycetes. The usual habitat of these fungi is well-oxygenated water, such as alpine brooks, mountain streams, and fast-flowing rivers. However, they also have been found in slow-running, often contaminated, rivers, stagnant or temporary pools, melting snow, and soil. There is often an increase in the

numbers of species and individuals of aquatic Hyphomycetes from autumn until spring, with a decline between April and June.

2. Sample Collection and Storage

For most freshwater environments, collect foam or partially decayed, submerged, angiosperm leaves in sterile bottles. Refrigerate sample until it is examined.

3. Sample Treatment and Analysis

Wash the leaf samples in sterile distilled water and place one to three leaves in a sterile petri dish about 1 cm deep containing sterile pond, river, or lake water. Incubate at room temperature. Within 1 to 2 d, the mycelium and conidia develop. Conidiophores and conidia can be observed with a dissecting microscope on any portion of a leaf surface, but most frequently are seen on petioles and veins. When released, the conidia either remain suspended in the water or settle to the dish bottom. Using a dissecting microscope, pick up single conidia with a micropipet. Transfer each conidium to a microscope slide in a drop of water for identification. The conidium may be transferred with a sterile needle to a plate of 2% malt extract agar for colony production. Search for conidia in foam samples with a dissecting microscope and isolate single conidia as described above. Submerge mycelial plugs from stock culture isolates of aquatic Hyphomycetes in autoclaved pond water in deep petri dishes; conidiogenesis usually occurs within 2 to 10 d.

Conidia in all stages of development can be preserved on slides with lactophenol mounting medium in which either acid fuchsin or cotton blue is dissolved and sealed with clear fingernail polish. To permit good adherence of the nail polish, avoid excessive amounts of mounting medium.

9610 H. Fungi Pathogenic to Humans

1. Occurrence and Significance

Routine isolations of fungi from polluted streams and wastewater treatment plants usually have yielded relatively few species pathogenic to human and other higher animals. *Geotrichum candidum*, an arthroconidium-producing fungus, for which there is, in the United States, presumptive evidence of an association with disease, is isolated almost universally. When its teleomorphic stage (an ascus) develops, it is known as *Endomyces candidus*. *Rhinochlamydia mansonii*, now called *Exophiala mansonii*, a causal agent of one form of chromomycosis, usually in the tropics, is equally widespread. It has been listed also as *Phialophora jeanselmei* and *Trichosporium heteromorphum*. *Aspergillus fumigatus*, a causal agent of pulmonary aspergillosis, is commonly isolated. *Pseudallescheria* (*Petriellidium*, *Allescheria*) *boydii* is a causal agent of eumycotic mycetomas and other eumycotic conditions grouped¹ under the heading "Pseudallescheriasis." Infection may follow from a puncture wound with

contaminated materials or breathing such contaminated materials as sprays at wastewater treatment plants or contaminated air. It usually is recovered in its anamorphic state, *Scedosporium* (*Monosporium*) *apiospermum*. The presence of these fungi in stream water probably represents soil runoff because virtually all zoopathogenic fungi exist saprobially in soil as their natural reservoir. Other zoopathogenic fungi occasionally are recovered in low frequencies from streams, polluted or not. Another fungus, the yeast *Candida albicans*, can be recovered in varying numbers from wastewater treatment plant effluents, streams receiving such effluents, and recreational waters. In humans this fungus is usually a commensal organism, like *Geotrichum candidum*, coexisting in harmony with its host organism; up to 80% of normal, healthy adults have detectable levels of *C. albicans* in their feces, while about 35% harbor it in their oral cavities in the absence of any overt disease. A very large proportion of the female population has vaginal candidiasis in varying degrees of severity. The presence of *C. albicans* in raw wastewater, waste-

water treatment plant effluent, or contaminated water is not surprising. *C. albicans* has been isolated from these habitats on routine media heavily supplemented with antibacterial drugs, but not on media or with techniques described in Sections 9610B or E. It also has been isolated from estuarine and marine habitats on a maltose-yeast nitrogen base-chloramphenicol-cycloheximide medium.

2. Identification of *C. albicans*

C. albicans can be identified among the white and pink yeasts growing on an 0.8- μ m black membrane filter on maltose-yeast nitrogen base-chloramphenicol-cycloheximide medium. From each colony, inoculate a 0.5-mL portion of calf or human blood serum, incubate at 37°C for 2 to 3 h, transfer a drop or two to a slide, and examine microscopically for the production of germ

tubes from a majority of the cells. Of the white yeasts, only *C. albicans* produces these short hyphae from the parent cell within 2 to 3 h incubation.²

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9711 PATHOGENIC PROTOZOA*

9711A. Introduction

1. Significance

Pathogenic protozoa are widely distributed in water supplies, are relatively resistant to inactivation by chemical disinfectants, and have caused numerous waterborne outbreaks. These organisms generally cause diarrhea or gastroenteritis of varying severity although more serious consequences, including death, can occur. The protozoan genera that have been associated with waterborne infections include *Giardia*, *Cryptosporidium*, *Toxoplasma*, *Cyclospora*, *Entamoeba*, *Acanthamoeba*, and the microsporidia, *Enterocytozoon* and *Encephalitozoon*. All of these organisms produce environmentally resistant cysts, oocysts, or spores, and except for the free-living *Acanthamoeba*, they are shed in the feces of infected individuals. *Cryptosporidium* can be zoonotic but *Toxoplasma* always is. While it is probable that *Giardia* can cause zoonotic infections, the available evidence is circumstantial.

During the 1970s, waterborne outbreaks due to *Giardia duodenalis* (syn. *G. intestinalis*, *G. lamblia*) were noted with increasing frequency, especially in communities using unfiltered surface water sources.¹ More than 100 outbreaks of disease in private and municipal drinking water supplies and in recreational water have been reported worldwide.² *Giardia* probably causes between 100 000 and 2.5 million infections a year in the United States and it is the most commonly detected protozoan parasite in the world.³ The relative contribution of waterborne, foodborne, and person-to-person transmission to nonepidemic giardiasis is unknown, but the seasonal peak of cases in late summer

and fall with the highest number of cases in 0 to 5-year-olds, suggests that many cases may be due to heavy use by diapered children of communal swimming areas (lakes, rivers, swimming pools and water parks).³ Symptomatic infection (giardiasis) can result in acute, chronic, or recurring bouts of diarrhea of varying severity. Mortality has been reported rarely and effective drugs are available.

Waterborne outbreaks due to *Cryptosporidium parvum* oocysts were first recognized in the mid 1980s, and in 1993, this organism was responsible for the largest waterborne outbreak in U.S. history.⁴ Both drinking water and recreational waterborne outbreaks have been reported.^{5,6} Infection with *Cryptosporidium* (cryptosporidiosis) can cause a severe diarrhea that is self-limiting in immunocompetent individuals but may be prolonged and life-threatening in the immunocompromised.⁷

Two waterborne outbreaks due to *Toxoplasma gondii* have been reported: one in U.S. soldiers due to contaminated creek water in Panama⁸ and a second associated with municipal drinking water in British Columbia, Canada.⁹ *Toxoplasma* requires intermediate and final (definitive) hosts to complete its life cycle.¹⁰ Felines are the only known animals to act as definitive hosts and to produce oocysts that are excreted in the feces. The oocysts require an external maturation period of 1 to 5 d to sporulate and become infective. Infection (toxoplasmosis) is often asymptomatic but may result in flu-like symptoms or swollen glands. Pregnant women can pass the infection to the fetus, resulting in birth defects. Disease in the immunocompromised can be severe, widely disseminated, and result in brain lesions.

Cyclospora cayentanensis has been more commonly associated with consumption of contaminated produce (berries, mesclun, and basil) in North America, but two waterborne outbreaks have been reported.¹⁰ This organism also produces oocysts that are

* Approved by Standard Methods Committee, 2001.

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shed in the feces and require an external maturation period (5 to 14 d at 22 to 32°C) to become infective. *Cyclospora cayentanensis* infection (cyclosporiasis) can result in diarrheal illness that may persist for several weeks and is normally self-limiting in immunocompetent individuals. The disease can be treated effectively with sulfur-containing antibiotics.

Several genera of organisms in the microsporidia group have been identified as causing human disease.¹⁰ Although the potential for waterborne transmission exists, only one water-associated outbreak has been reported.¹¹ Infection with microsporidia (microsporidiosis) may result in diarrhea and self-limiting illness in immunocompetent individuals. Infection can be more problematic in the elderly and in the immunocompromised. Drug treatment is available.

Acanthamoeba is discussed in Section 9711C.

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9711 B. *Giardia* and *Cryptosporidium* Methods

1. Overview of Detection and Identification Methods^{1,2}

Methods for detecting and identifying *Giardia* and *Cryptosporidium* in environmental samples generally consist of three steps: (a) sampling and concentration; (b) purification or separation of the target organisms from other particulates in the sample, and (c) assay. These organisms will replicate and produce infectious stages only in a suitable host. Consequently, sample collection usually incorporates a procedure, e.g., filtration or centrifugation, to concentrate the organisms in the sample. The concentrate may contain inorganic and organic debris and other organisms in addition to the target organisms. Purification procedures such as density gradient centrifugation (flotation) and immunomagnetic separation may be used to minimize the quantity of interfering material in the concentrate. Assay procedures may involve colorimetric determination, microscopic examination using dyes or fluorescent antibodies, molecular techniques (e.g., polymerase chain reaction), or studies of infectivity in animal models or cell cultures.

Selection of appropriate concentration, purification, and assay procedures will depend on the particular matrix being sampled and on the objectives of the sampling program. The precision and sensitivity of the method, as well as the level of detail required (e.g., presence/absence vs. quantitative results; identification to the genus or species level; whether or not detected organisms are infective) may dictate selection of the optimal

procedure for a given application. For example, a method that can produce rapid results might be more important for investigation of a suspected waterborne disease outbreak than it would be for determining the occurrence and distribution of organisms in a watershed.

This section identifies procedures used successfully by analysts in environmental laboratories. Where possible, guidance is offered on procedures suitable for particular types of matrices and applications. However, research is continuing on improving the concentration, purification, and assay procedures; consult the current literature for the latest methodology.

2. Sampling and Concentration

Guidance for selection of sampling and concentration procedures is given in Table 9711:I. In choosing a sampling method and volume include matrix characteristics, filter characteristics, and objectives of the study.

a. Sampling and storage procedures: If parasite levels are expected to be high, as in wastewater, a grab sample as small as 500 mL may be sufficient. In a clean matrix, such as a finished water sample, a volume of 1000 L or more may be collected. In general, the lower the expected density of cysts or oocysts, the greater the sample volume or the frequency of sampling. Grab samples are appropriate for highly contaminated samples or when field filtration is not practical. While grab samples are easy

TABLE 9711:I. GUIDANCE FOR SELECTION OF SAMPLING OR CONCENTRATION PROCEDURES FOR *GIARDIA* AND *CRYPTOSPORIDIUM*

Procedure	Application			
	Sample Volume L	Water Turbidity*	Advantages	Disadvantages
Grab samples ^{3,4}	≤ 20	Low to high	Easy to collect No filter costs	Larger volumes may be difficult and costly to cool and ship
Flocculation ⁵	≤ 20	Low to high	Simple to perform	Not compatible with viability assays or sucrose flotation
Continuous-flow centrifugation ⁶	≤200	Low to high	High recovery rate	High initial equipment cost and weight Power source required for field concentration
Wound filters ⁷	≤100 raw ≥1000 finished	Low to high	High filtration rate Low cost Easy field concentration	Poor retention and recovery of cysts and oocysts Time-consuming elution process
Pleated membrane filters ^{3,4}	≤100 raw ≥1000 finished	Low to moderate	Good retention and recovery of cysts and oocysts Easy field concentration Easy elution	High cost Slow filtration rate
Disk membrane filters ^{3,4}	≤10	Low	Lower cost than pleated filters Good retention and recovery of cysts and oocysts	Low sample volumes and turbidities Low filtration rate
Hollow fiber filters ⁸	≤100	Low to moderate	Good retention and recovery of cysts and oocysts Filters can be re-used Moderate cost Collect other pathogens	Method not fully developed and evaluated Filters need cleaning and testing before reuse
Compressed foam filters ^{3,4,9,10}	≤50 raw ≥1000 finished	Low to high	Excellent retention and recovery of cysts and oocysts Moderate cost; good precision	Special equipment for elution and concentration must be decontaminated

* Low = <1 NTU; moderate = 1–10 NTU; high = >10 NTU

to collect, larger volumes may be difficult to handle as well as costly to cool and ship to the laboratory. Grab samples up to a practical maximum limit of 20 L can be shipped in carboys to the laboratory where the sample may be concentrated.

Dechlorinate chlorinated samples. Transport samples to the laboratory and store at specified temperatures. While it is desirable to receive samples at the laboratory and process them as quickly as possible, the holding and processing times for some methods can be difficult to meet. If extension of holding times/temperatures is unavoidable, conduct experiments with known additions to ascertain the impact of the changes on recovery.¹¹

Most methods were developed to sample source waters or finished drinking water; validate use with saline waters or wastewater effluents for a particular method. Make known cyst and oocyst additions periodically with all matrices and methods to ascertain that there are no interfering conditions.

b. Concentration methods: Basic information about these methods is given in Table 9711:I.

1) Flocculation—Calcium carbonate flocculation of 10-L samples produces a dense and stable precipitate that can be easily dissolved in sulfamic acid. This method¹² is simple and robust, and has been shown to be reproducible in a number of laboratories. Flocculation reduces oocysts viability; do not use this method if assessing viability.⁵

2) Centrifugation—Direct centrifugation is an option for small sample volumes. Recommended centrifugation speeds and times for concentration vary for specific methods.^{3,4,7,9,13} Increasing centrifugal force to 5000g for 10 min, coupled with staining in suspension, has been found to increase the overall efficiency of recovery of both cysts and oocysts.⁵

A differential continuous-flow centrifugation device, originally developed for blood cell separation, has been modified for

TABLE 9711:II. GUIDANCE FOR SELECTION OF SEPARATION/PURIFICATION TECHNIQUES

Technique	Advantages	Disadvantages
Flotation ^{1,3}	Inexpensive Quick	Poor recovery of organisms Background debris often excessive High interlaboratory variability Efficiency highly dependent on matrix type Retention of target organisms is not selective
Immunomagnetic separation ^{14,15}	Improved recovery of organisms (60 – > 95 %) Relatively inexpensive Reduced intra- and inter-laboratory variability Minimal background debris Selective retention of target organisms	Interference from matrices containing iron and other magnetic substances High-turbidity samples may interfere and result in retention of background debris
Fluorescence activated cell sorting ^{16–18}	Excellent separation of cysts and oocysts from background debris High sample throughput Relatively rapid	High initial equipment cost High maintenance costs Requires highly trained analyst

field collection of large-volume water samples. The equipment, though costly, is compact, mobile, and flexible, produces high recovery, and has minimum requirements for post-concentration of the initial concentrate.

3) Filtration—Options include wound filters, membrane filters, and compressed foam filters (see Table 9711:I).

Wound filters, most commonly 1- μ m nominal-porosity polypropylene yarn filter cartridges, have been used for many years to collect parasite samples in a wide variety of waters.

Flat membranes of various materials and pore sizes can be used in filter holders ranging from 90 to 293 mm in diameter.³ Comparative studies suggest that cellulose acetate with a 1.2- μ m pore size provides the best recovery of *Cryptosporidium* oocysts, and cellulose nitrate with a 3.0- μ m pore size the best recovery of *Giardia* cysts.⁵ The amount of sample that can be collected in a particular water is difficult to predict and is related to membrane pore size and the matrix characteristics of the water. Elution procedures are simple.^{3,9} While the cost of sheet membranes is low, the cost of the filter holder can be substantial.

Pleated 1- μ m-porosity membranes are easy to use, but elution of capsule filters requires a laboratory shaker with side arms for agitation of capsules during the elution step while other filters with reusable filter holders are eluted by backwashing.^{3,4}

Hollow fiber filters⁸ theoretically can capture all enteric pathogens simultaneously, including bacteria and viruses as well as protozoans. The filters may offer potential savings in time and effort, but require further evaluation.

Compressed foam filters consist of multiple layers of compressed foam disks.¹⁰ The filter modules do not suffer from the breakthrough problems normally associated with conventional cartridge/membrane filters. The filter module and housing are compact, allowing for easy transport and use in the field. During elution the compressed disks are expanded and washed in an elution module; concentration then takes place across a membrane in a concentrator module rather than by centrifugation. The elution and concentration modules must be washed and decontaminated before reuse with a subsequent sample.

3. Purification /Separation

Purification or separation procedures attempt to isolate the target protozoa from all of the extraneous debris that may interfere with detection. Information on advantages and disadvantages of commonly used purification techniques is given in Table 9711:II.

a. Density gradient centrifugation (flotation): The flotation process utilizes the specific gravity of the target organisms to assist in their separation and purification. Various chemicals have been used to prepare density gradients, including solutions of sodium chloride, cesium chloride, sucrose, magnesium sulfate, zinc sulfate, and a colloidal suspension of silica particles.^{8,19–21} The flotation procedure most commonly used in environmental testing is a continuous density gradient. The use of a continuous gradient combined with centrifugation forces the heavier particles to the bottom and the lighter particles or objects to an area within the continuous gradient that matches their specific gravity. The portion of the gradient expected to contain the cysts or oocysts is removed, immediately diluted to reduce osmotic stresses, and then further concentrated. The most common flotation material used in *Giardia/Cryptosporidium* analyses of drinking water is a 1.09 to 1.10 specific gravity solution composed of 2.5 *M* sucrose, silica suspension, and particle-free deionized water.⁷ This solution causes less osmotic stress than other chemical gradients and therefore, less damage to cyst or oocyst morphology.

b. Immunomagnetic separation (IMS): The IMS method uses specific (at the genus level) high-affinity monoclonal antibodies linked to the surface of paramagnetic particles to attach to cysts or oocysts. A magnet is then used to separate the target organisms from the debris. A high-affinity monoclonal anti-cyst or oocyst antibody is attached to a paramagnetic bead about 4.5 μ m in diameter. These antibody-bead units are added, in excess, to a concentrate from a sample suspected to contain the target organisms. After rotation and incubation at room temperature, during

* Percoll® or equivalent

TABLE 9711:III. GUIDANCE FOR SELECTION OF *GIARDIA* AND *CRYPTOSPORIDIUM* ASSAY PROCEDURES

Procedure	Advantages	Disadvantages
Immunofluorescence (IFA) ^{3,4,7}	Established methods Quantitative Relatively easy Viability assessment (when combined with vital dyes) Capable of detecting dead and empty cysts and oocysts	Expensive equipment (microscope) Labor-intensive Antibody cross-reactivity Limited species specificity and no genotype specificity Staining does not always correlate well with viability
Polymerase chain reaction (PCR) ^{25,26}	Very specific High throughput Inexpensive equipment Viability determination (RT-PCR) Genotyping to determine animal host Standard PCR detects viable and nonviable intact cysts and oocysts	Inhibition by materials in environmental samples Non-quantitative (potentially semi-quantitative) Some RNA targets are not good indicators of viability Cannot detect empty cysts and oocysts

which the specific antibody complexes bind to the target organisms, the suspension is exposed to a magnetic field. The magnetic bead-antibody units and organisms attached to these units will be separated from nonmagnetic particles and can easily be removed from suspension and concentrated. A disassociation step to remove the magnetic bead-antibody unit from the protozoa is used to allow for microscopic examination of the protozoa and confirm presence of internal structures. Recovery efficiency ranges between 70 and 97 % for both seeded tap water samples and seeded river water samples.²² Commercially available high affinity monoclonal antibody kits† demonstrate recovery efficiencies ranging from 60 to 100%, depending on matrix type.^{15,23,24}

c. *Fluorescence activated cell sorting (FACS)*: Flow cytometry coupled with cell sorting has been used for the isolation and analysis of *Cryptosporidium*, *Giardia*, and other protozoa in water sample concentrates.^{16,17} In this procedure, the target specimens are tagged with fluorescein isothiocyanate or phycoerythrin labeled antibodies, and then passed through a flow cytometer equipped with a suitable fluorescence excitation system such as the argon-ion laser beam. When a labeled organism passes through the laser beam, a fluorescence signal is generated. Analysis of the 90° light scatter and the fluorescence intensity is used to discriminate a positive signal from nontarget debris. When an electrical charge is applied to the droplet containing the fluorescing organism, the specimen is deflected and separated from the rest of the sample.

High recovery efficiencies (90 to 94%) can be achieved for water samples with known additions.¹⁸ Although it is technically possible to count organisms using the flow cytometer, in practice samples isolated by the instrument are analyzed subsequently by microscopy. Problems with nonspecific autofluorescence, antibody cross-reactivity, or poor fluorescence due to loss of antigenic determinants limit flow cytometry as a method of direct counting.^{16,18}

4. Assay

Guidance for the selection of assay procedures is given in Table 9711:III.

a. *Immunofluorescence assay (IFA)*: The most common method for the detection of *Cryptosporidium* oocysts and *Giardia* cysts in purified environmental water concentrates is an immunofluorescence assay (IFA). IFA uses monoclonal antibodies directed against oocyst and cyst antigens and fluorescence microscopy to detect and enumerate oocysts and cysts. Indirect staining⁷ involves the use of unlabeled primary antibodies that bind to oocyst and cyst antigens followed by application of a fluorophore- (commonly fluorescein) labeled secondary antibody to detect the bound primary antibodies. Direct staining^{3,4} involves the application of only primary antibodies that are fluorophore-labeled. Protozoa purified using the methods described above can be stained directly following application of cysts or oocysts to slides or membrane filters. Upon microscopic examination, organisms are identified on the basis of their size, shape, color, and intensity of fluorescence. Presumptive cysts or oocysts are confirmed by the presence of internal structures identified by using differential interference contrast (DIC) microscopy.

Comparisons of the performance (specificity, avidity, fluorescence intensity) of a variety of commercially available and new antibodies have been published.²⁷ The procedure has been modified to include the uptake or exclusion of dyes such as DAPI, propidium iodide, hexidium, SYTO-9, and SYTO-59 as measures of viability^{28,29} but the correlation between these staining procedures and cyst or oocyst viability has not been conclusively demonstrated.

b. *Polymerase chain reaction (PCR)*: PCR is a sensitive and specific amplification process for generating easily detectable amounts of DNA from very minute quantities. Reverse transcriptase (RT)-PCR allows amplification of RNA targets. Numerous PCR-based methods for the detection of *Cryptosporidium* and *Giardia* have been described and compared.^{25,26} The various PCR assays have different nucleic acid targets and include modifications for viability determination by detecting mRNA (RT-PCR), different specificities (e.g. all *Cryptosporidium* spp. or only *C. parvum*) and sensitivities as low as one to five cysts or oocysts. Amplification targets for *Giardia* include genes encoding giardin, triose phosphate isomerase, and a heat shock protein. *C. parvum* DNA has been amplified by using PCR primers targeting genes for 18S ribosomal RNA, β -tubulin, heat shock, and oocyst wall proteins, among others. DNA and RNA extraction methods differ greatly among the various published assays. Cyst and oocyst purification methods such as IMS and

† Dynal Inc., 5 Delaware Dr., Lake Success, NY 11042; Immucell Corp., 56 Evergreen Drive, Portland, ME 04103; or equivalent.

advances in nucleic acid purification have reduced, but not eliminated, interference by PCR inhibitory substances, which are often present in environmental samples. Currently most PCR detection assays for protozoa in environmental samples are only qualitative (i.e., presence or absence); however, quantitative methods are under development. Possibly the greatest challenge is the selection of a single, standardized PCR assay for the detection of *Cryptosporidium* and *Giardia* in water. Recently the integration of cell culture and PCR for the infectivity determination of waterborne oocysts has been reported.^{30,31} PCR kits requiring just the addition of DNA and primers are available as is a complete kit for the detection of *C. parvum*.[‡]

Genotyping—Recent advances in the molecular taxonomy of *Cryptosporidium* using PCR-based tools have led to the identification of several genotypes of *C. parvum*. The species *C. parvum* comprises at least two distinct genotypes (or sub-species), one isolated almost exclusively from human beings, the other from both human beings and other animals. A review of several PCR-based *Cryptosporidium* genotyping assays has been published.³² *G. duodenalis* is the only species of *Giardia* known to cause infection in humans, but the organism is currently recognized as a heterogeneous complex of organisms comprising at least two distinct groups. Cysts representing both primary genotypes have been isolated from water.³³

Although there are reports on variations in the level of infectivity between different isolates and genotypes of both *C. parvum* and *G. duodenalis*, there is no information concerning the relative recovery efficiencies or detection sensitivities of the assay methods described above for the separate genotypes.

5. Viability/Infectivity

Three in-vitro methods—excystation,^{28,34} fluorogenic dye inclusion/exclusion, and reverse transcriptase polymerase chain reaction (RT-PCR)³⁵—and two in-vivo or infectivity methods—animal systems³⁶ and cell culture—have been used to evaluate the viability of the enteric protozoa. Fluorogenic dyes and cell culture have the greatest application to evaluating the significance of cysts or oocysts detected in environmental samples, and are discussed in more detail below.

a. Fluorogenic dyes: The inclusion and/or exclusion of certain dyes can serve as a marker of intact membranes in cysts and oocysts and also can indicate the presence of internal features such as nuclei. The dyes are fluorogenic and can be used with the IFA assay (§ 4a above) and microscopic procedures. This approach has been used for both *Cryptosporidium* and *Giardia* as a potential test for viability and is particularly useful for application to small numbers of cysts and oocysts found in environmental samples.^{28,37–40}

For *Giardia* cysts, the hydrolysis of fluorescein diacetate (FDA) by intracellular membranes cleaving the fluorescein can be visualized by fluorescence microscopy under excitation of 450- to 490-nm wavelength as green fluorescence. Propidium iodide (PI) cannot transverse an intact lipid bilayer in the membrane because it is a large molecule. However, if the membrane is compromised, the PI enters the cyst and can be visualized

microscopically as a red fluorescence using 500-nm wavelength excitation. The inclusion of FDA and exclusion of PI as a marker of viability has been related to animal infectivity, excystation, and cyst morphology.^{38,39} This method has been used to assess the potential viability of cysts isolated from contaminated water.⁴⁰

For *Cryptosporidium* oocysts, exclusion of PI or inclusion of 4',6-diamidino-2-phenylindole (DAPI) is used to indicate a live oocyst.²⁸ Viewed using epifluorescent microscopy equipped with filters for 350-nm wavelength excitation and 450-nm wavelength emission, the nuclei inside the four sporozoites fluoresce a bright blue. The DAPI/PI test has been highly correlated with oocyst excystation²⁸ and used to study oocysts in environmental waters.⁴⁰

Since the dye tests and excystation are known to overestimate viability and the staining can be variable with a portion of the cysts and oocysts not staining with either dye,^{28,37–41} caution should be exercised in interpreting results.

b. Cell culture: A cell culture method has been used for *Cryptosporidium* infectivity but has not been used for *Giardia*. A high level of sensitivity of cell culture to low levels of *Cryptosporidium* oocysts has been demonstrated.^{30,31,42} While numerous cell lines⁴³ and detection schemes have been used, most investigators use a human ileocecal adeno-carcinoma (HCT-8) cell line. Filtration and IMS procedures have been used to prepare environmental concentrates for inoculation onto cell monolayers, where the oocysts undergo excystation and initiate infection in the cells. After incubation in a CO₂ incubator at 37°C for about 48 h, the cells are fixed and infection is observed using labeled antibodies and microscopy.⁴² Alternatively the cells are extracted and PCR or RT-PCR is used to detect infection.^{30,31} Cell culture has been used for detecting naturally occurring oocysts.³⁰

An advantage of using molecular assays is that the products of PCR and RT-PCR can be used to determine the genotype of the parasite. It is possible to use genotype information to determine the specific strain or outbreak source, and sequences can be compared. The ability to quantify the infection is an advantage of microscopic-based methods such as the use of immunofluorescence where visible confirmation of the life stages present is possible. PCR-based assays are semiquantitative and may detect nonexcysted and dead oocysts on the monolayer.

The benefits of using cell culture to study *Cryptosporidium* infectivity include the following: the method is applicable to environmental oocysts from treated and untreated waters, both genotypes will infect the HCT-8 cells, results are available in 48 h, and the method is less labor-intensive than animal infectivity and fairly easy to perform.

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9711 C. *Acanthamoeba*

1. Significance

Acanthamoeba are the most prevalent of free-living amoebae. Some can cause chronic encephalitis and keratitis,^{1,2} but not all species or strains are pathogenic for humans. *A. castellanii*, *A. culbertsoni*, *A. hatchetti*, *A. healyi*, *A. polyphaga*, *A. rhyodes*, *A. astronyxis*, and *A. divionensis* have all been implicated in human infections.³

Acanthamoeba are found worldwide in animal intestines, vegetative material, soil, dust, air, fresh water, marine water, sediments, sewage, compost, tap water, and bottled water.^{4,5} High numbers are found in sediments and surface layers of fresh-water lakes, corresponding to high-density bacterial populations. *Acanthamoeba* colonize hot tubs, chemical showers, eyewash fountains, drinking water fountains, dental units, dialysis units, swimming pools, air conditioning systems, humidifiers, and hot-water systems.⁶ Chlorinated swimming pools may contain up to 10⁴ *Acanthamoeba*/L, since their cysts are highly resistant to chlorine. The presence of *Acanthamoeba* in swimming pools bears no correlation to bacteriological indicators of water quality. *Acanthamoeba* reach high numbers where water temperatures are warm and nutrients are abundant. While the optimal growth temperature is between 30 and 40°C, thermotolerant strains withstand exposure to temperatures up to 43°C.⁶

Some *Acanthamoeba* spp. are opportunistic pathogens of humans and can cause encephalitis in the immunosuppressed and in those with chronic debilitating diseases.⁷ Portals of entry leading to human infection include the respiratory tract, skin ulceration, and wounds. Amoebae disseminate to various organs and the brain by the bloodstream. No treatment has been successful, prognosis is poor, and death is the usual outcome. Ocular infections are acquired by direct contact of amoebae with corneal tissue, and they occur most frequently after abrasion or injury, followed by exposure to contaminated water. Contact lenses and contaminated contact lens solutions are highly associated with *Acanthamoeba* keratitis.⁸

Water treatment does not prevent cysts from entering the distribution system, where they excyst and proliferate in bio-

films. *Legionella* and *Mycobacterium* spp. replicate inside *Acanthamoeba*, and their ability to thrive intracellularly in amoebae may facilitate their movement within water distribution systems. Routine monitoring of drinking water for amoebae is not practical because of technical difficulties associated with recovery and identification of amoebae, and because no practical method is available to differentiate pathogenic from nonpathogenic strains.

Because non-water exposures far exceed drinking-water exposures, and because ingestion is not associated with encephalitis, enhanced water treatment rules are unlikely to reduce the incidence of disease.

2. Laboratory Methods

The trophozoite form of *Acanthamoeba* is between 2 and 40 µm in diam and is characterized by granular vacuolated cytoplasm, with thin, spine-like protrusions called filopodia or acanthopodia, giving the cell a sea-urchin-like appearance.⁹ Cysts of *Acanthamoeba* are spherical, averaging 18 µm in diam (range 15 to 25 µm), and consist of a double-walled outer membrane enclosing a star-like central structure.

For isolation of amoebae from the environment, a water sample of 10 mL to 1 L is passed through a 0.45-µm membrane filter with vacuum source adjusted to produce a flow rate of not more than 1.3 mL/min. The filter is inverted onto a plate of non-nutritive agar seeded with a lawn of *E. coli** prepared from a washed cell suspension containing approximately 10⁴ to 10⁶ bacteria/mL.¹⁰ Plates are incubated at 30° C for 3 to 4 d. The membranes are removed and discarded, and the plates are examined for trails of amoebae. Plates are reincubated at 30° C and examined periodically for up to 2 weeks before being considered negative. Examinations are conducted at 10× magnification. Microscopic examination of saline wet mounts facilitates genus identification. The genus can be identified by morphological

* Other bacteria have been used, including *Enterobacter* spp. and *Pseudomonas* spp; however, pseudomonads may depress growth and survival of *A. castellanii*.

characteristics, but species identification requires specialized techniques such as immunofluorescence staining, isoenzyme electrophoresis, restriction fragment length polymorphism analysis, or polymerase chain reaction.¹¹

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PART 10000

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