9213

Recreational Waters

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9213 A. Introduction

1. Definition of Recreational Waters and Use of Appropriate Microbiological Indicators

Recreational waters are those in which primary contact occurs. *Primary contact recreation activities* are those that could be expected to result in the ingestion of, or immersion in, water (e.g., swimming, water skiing, and kayaking). There are two general types of recreational waters. The first type includes natural sources of water, such as lakes, streams, and coastal waters, where the water source cannot be disinfected and where these sources are at risk for contamination via point sources, such as sewage and industrial waste, and nonpoint sources, such as streams, storm drains, and animals (e.g., birds and bathers). Natural sources of water are used primarily for swimming, wading, and surfing. The second type of recreational water is found in facilities using municipally treated water; it should be disinfected continuously. The designated uses of this type of water may be recreation, such as swimming pools, or therapy, such as whirlpools. Ideally, the quality of recreational water is suitable for its designated use.

Historically, concentrations of selected microorganisms have been used to determine the suitability of recreational water for its designated use. Recreational water quality indicators are microorganisms for which densities in the water can be related quantitatively to the degree of potential contamination with pathogens and thus health hazards resulting from recreational use, particularly where upper body orifices are exposed to water. These indicators are used to determine whether recreating in the water may be detrimental to human health. The ideal indicator has the best correlation between density and the health hazards associated with a given type of pollution. It also is consistently and exclusively present when the contaminant is present, occurs in higher densities than the contaminant, cannot proliferate more than the contaminant, is more resistant to environmental stresses and more persistent than the contaminant, and has characteristic, simple reactions for easy, simple identification.[1]

Correlations between microbial density in water and predictable disease have been established for exposure to natural sources of recreational water but not for recreational waters that are routinely disinfected. Epidemiological research shows a correlation between gastroenteritis and concentrations of *Escherichia coli* and enterococci in natural fresh water sources used for swimming. Further, results showed that gastroenteritis positively correlated with concentrations of enterococci in fresh and marine waters used for swimming. This research involved water from beaches contaminated by known point sources.[1] Water quality standards have been established using these indicators of fecal contamination. National Pollutant Discharge Elimination system (NPDEs) permits and state permits authorized by the NPDEs program require dischargers to monitor such waters for the parameters included in their permits. For discharges into coastal recreational waters, any permits including fecal indicators likely require monitoring for enterococci or *E. coli.* For continuously disinfected water, such as water in swimming pools and whirlpools, concentrations of disinfectant (e.g., free and total chlorine, bromine) are frequently monitored. In many US communities, suitability of such waters is based primarily on hourly (pH, temperature, and disinfectant) or daily (hardness and alkalinity) water-chemistry measurements rather than frequent monitoring for microorganisms or correlation with predictable health effects. The frequency and type of parameters measured depend on the individual state, county, or municipal swimming pool codes. Although most swimming pools are disinfected with chlorine, bromine or a nonhalogen-based system may be used. If chlorine is used, free chlorine typically is monitored hourly (occasionally total chlorine also is monitored).

2. Transmission of Enteric and Nonenteric Pathogens by Recreational Use of Waters

In general, infections and disease associated with recreational water contact fall into two categories: those due to enteric pathogens and those due to nonenteric pathogens. Infections in the first category typically manifest gastroenteritis symptoms and result from the unintentional ingestion of water contaminated with feces. Enteric microorganisms known to cause gastroenteritis associated with recreational water contact include *Giardia, Cryptosporidium, Shigella, Salmonella, E. coli* O157:H7, *Vibrio,* hepatitis A virus, coxsackie A and B viruses, and noroviruses. An exception are adenoviruses, which cause conjunctivitis in those using swimming pools.[2]

Infections in the second category are caused by nonenteric pathogens that contaminate water and are transmitted by contact with water. These pathogens generally cause nonenteric diseases and may be transmitted via inhalation, contact with mucosal membranes or abraded skin. The sources of the pathogens differ. For example, the bacteria that cause leptospirosis have been found in the urine of infected animals.[3] The source may be from human bodies (e.g., *S. aureus)* or bacteria indigenous to that water (e.g., *Pseudomonas aeruginosa, Legionella* spp., *Naegleria fowleri, Mycobacterium* spp., and *Vibrio* spp). The illnesses or diseases caused by these nonenteric organisms include dermatitis

or folliculitis, otitis externa (inflammation of the external ear), leptospirosis, Pontiac fever, granulomas (nodules of chronically inflamed tissue with granulations), primary amebic meningoencephalitis (PAM), conjunctivitis, and potentially fatal wound infections *(Vibrio vulnificus).*

3. Microbiological Monitoring Approaches and Limitations

The following sections describe recommended methods for measuring pathogens or microbial indicators of recreational water quality. Several key monitoring factors should be considered, including disinfectant residual, indicator organisms, and various physical and chemical characteristics. When selecting the microbiological method or indicator to be used, consider the type of water examined. No single procedure is adequate to isolate all microorganisms from contaminated water. Although bacterial indicators may not adequately reflect the risk of viral, fungal, or parasitic infection from recreational waters, a lack of feasible analytical methods limits monitoring for such organisms in routine laboratory operations.

Routine examination for pathogenic microorganisms is not recommended except for investigations of water-related illness and special studies. Certain pathogenic organisms, such as *Giardia, Cryptosporidium, Mycobacterium, Naegleria,* hepatitis A virus, and noroviruses are more resistant to changes in environmental conditions than indicator bacteria; therefore, routine monitoring may not always reflect the risk of infection from these organisms. In the case of water-related illness, focus the microbiological analyses on the known or suspected pathogen and on the venue. For instance, if the venue is a disinfected swimming pool and symptoms include gastrointestinal distress, the initial focus might be on such organisms as *Cryptosporidium, Giardia,* and noroviruses. If the venue is heated whirlpools or spas where the issue is folliculitis or respiratory illness, the focus might be on *P. aeruginosa*, mycobacteria, and *Legionella,* which are more likely to be found. Methods for detecting nonbacterial pathogens are given in Section 9510 Detection of Enteric Viruses, Section 9711 Pathogenic Protozoa, and Section 9750 Detection of *Naegleria fowleri*.

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9213 B. Swimming Pools

1. General Discussion

a. Characteristics: A *swimming pool* is a body of water of limited size contained within a holding structure.[1] Most public swimming pools use potable fresh water and are designed to be filtered and disinfected continually to maintain high water quality. However, some public swimming pools use natural sources of water, including thermal springs and coastal marine waters, and rely on continuous circulation to maintain water quality. These natural water sources generally are not disinfected.

b. Monitoring requirements:

1) General—Monitor water quality in pools for changes in chemical and physical characteristics that may irritate a bather's skin, eyes, and mucosal barriers or adversely affect disinfection. Microorganisms of concern typically are those from the bather's body and its orifices and include those causing infections of the eye, ear, upper respiratory tract, skin, and intestinal or genitourinary tracts. Accidental release of feces into swimming pools is a serious source of contamination because the volume of water is restricted and exposure of other swimmers is highly likely. This kind of contamination in a swimming pool has been reported to be responsible for transmission of such microorganisms as *Cryptosporidium, Giardia,* noroviruses, and *E. coli* O157:H7.[2] In a pool, all fecal-borne pathogens may be transmitted to other swimmers. Water quality depends on the disinfection efficacy, sanitary conditions, filtration efficacy, the number of bathers in the pool at any one time, and the total number of bathers per day. While the following describes examples of various monitoring and sampling protocols, individual localities and states have their own pool codes that must be complied with.

2) Disinfected indoor pools—Swimming pools must be disinfected continuously when in use. Test swimming pool water for total and free chlorine (to calculate combined) and pH when the pool is initially opened and at least 3 times per day. Collect samples from at least 2 locations for these determinations. Evaluate the clarity of the swimming pool water before opening for the day and during periods of heavy use.[3] A heterotrophic plate count is the primary indicator of disinfection efficacy. Indicators of health risk include normal skin flora that are shed, such as *Pseudomonas* and *Staphylococcus*.[4–7] These organisms account for a large percentage of bacterial swimming-pool-associated illnesses.

3) Disinfected outdoor pools—In addition to the disinfectant levels (if chlorine, total and free) and pool chemistry, the level of cyanuric acid or stabilizer (if used and required by appropriate pool code) may be monitored. Thermotolerant coliforms (formerly known as fecal coliforms) and *Pseudomonas* species are the primary indicators of contamination from pets, rodents, stormwater runoff, and humans. Supporting indicators include coliform bacteria, heterotrophic plate count, and staphylococci.

4) Untreated pools—The primary indicator may be thermotolerant coliforms. Supporting indicators are those described for disinfected pools. Untreated pools are not recommended for recreational use because of increased health risks.

2. Samples

a. Containers: Collect samples for bacteriological examination of swimming pool water as directed in Section 9060 A.1. Use containers with capacities of 120 to 480 mL, depending on analyses to be made. Add sufficient sterile sodium thiosulfate ($Na_2S_2O_3$) to the sample to provide a concentration of approximately 100 mg/L in the sample (see Section 9060 A.2).

b. Sampling procedure: Collect samples during periods of maximum bather load. Information on the number of bathers may be helpful in subsequent interpretation of laboratory results. Use a sampling frequency consistent with state and local health regulations.

Collect samples by carefully removing the cap of a sterile sample bottle and holding the bottle near the base at an angle of 45°. Do not rinse the bottle. Fill in one slow sweep down through the water, with the mouth of the bottle always ahead of the hand. Avoid collecting floating debris. Add sodium thiosulfate, if necessary, and replace the cap. For pools equipped with a filter, samples may be collected from sampling ports provided in the filter's return and discharge lines.

Most bacteria shed by bathers are in body oils, saliva, and mucus discharges that occur near the surface. Collect additional samples of the surface microlayer from the area in 1-m-deep water by plunging a sterile glass plate (approximately 20 × 20 cm) vertically through the water surface and withdrawing it upward at a rate of approximately 6 cm/s. Remove the surface film and water layer adhering to both sides of the plate with a sterile silicone rubber scraper and collect them in a sterile glass bottle. Repeat until the desired volume is obtained. To minimize microbial contamination, wrap the glass plate and scraper in metal foil and sterilize via autoclaving before use. Wear sterile rubber or plastic gloves during sampling or hold the glass plate with forceps, clips, or tongs.

Determine pH, temperature, total and free chlorine or other disinfectant levels at pool side at the time of sample collection (see Section 4500-Cl G). Disinfectant levels and the chemical and physical quality of pool water must be consistent with local and state standards. Verify that the permissible bathing load adheres to local or state pool codes.

c. Sample storage (holding time): Analyze microbiological samples as soon as possible after collection (see Section 9060 B).

d. Sample storage temperature: Less than 10 °C, but not frozen.

e. Sample volume: See Section 9222 B.4*a* and Table 9222:1.

f. Sample dilution: If sample dilutions are required, use 0.1% peptone water or buffered dilution water as a diluent to optimize recovery of stressed organisms (see Section 9050 C.1).

3. Heterotrophic Plate Count

Determine the heterotrophic plate count as directed in Section 9215. Use at least 2 plates per dilution.

4. Tests for Total Coliforms

Determine total coliform bacteria as directed in Sections 9221, 9222, or 9223.

5. Tests for Thermotolerant Coliforms

Test for thermotolerant coliforms according to the multiple-tube fermentation technique (Section 9221), the membrane filter technique (Section 9222), or rapid methods (Section 9211), or as permitted under state and local regulations.

6. Tests for Staphylococci or *Staphylococcus aureus*

a. Single-plate membrane filtration procedure[8]*:*

1) Baird-Parker agar base:

Tryptone	10.0 g
Beef extract	5.0 g
Yeast extract	1.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g
Lithium chloride	5.0 g
Agar	20.0 g
Reagent-grade water	1 L

Sterilize via autoclaving. Cool to 50 °C and aseptically add 50 mL commercial egg yolk tellurite enrichment per liter. Mix well. Final pH is 7.0 ± 0.2.

2) Procedure—This medium is used with the membrane filter procedure. It is important to use 0.22-µm filters as directed in 9222 B because it has been reported that chlorine-stressed *S. aureus* can contract and slip through 0.45-µm membranes.[9] Place the membrane filter on Baird-Parker agar and incubate at 35 ± 0.5 °C for 48 ± 4 h. Staphylococci typically form slate-gray to jet-black, smooth, entire colonies. If *S. aureus* is present, egg yolk clearing due to lecithinase activity may be observed if the membrane filter is raised from the medium. Verify some typical and atypical colonies with a commercial multi-test system or for such key characteristics as positive catalase and coagulase production, aerobic and anaerobic acid production from certain carbohydrates, and typical microscopic staphylococcal morphology.

b. Two-plate membrane filtration procedure: Alternatively, a two-plate method may be used, which will also help recover chlorine-stressed *S. aureus*. The membrane filter procedure is used to prepare samples (see Section 9222 B). After filtration, place membrane filters on R2A agar (Section 9215 A.6*c*) and incubate at 37 ± 0.5 °C for 24 h. Then, aseptically transfer the membrane to Baird-Parker agar and incubate at 35 ± 0.5 °C for another 24 h. Typical and atypical colonies can be verified as described in paragraph 2 above.

c. Modified multiple-tube procedure:

1) m-Staphylococcus broth:

Tryptone	10.0 g
Yeast extract	2.0 g
Lactose	2.0 g
Mannitol	10.0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	5.0 g
Sodium chloride (NaCl)	75.0 g
Sodium azide (NaN_3)	0.049 g
Reagent-grade water	1 L

Mix all ingredients and sterilize by boiling for 4 min; pH must be 7.0 ± 0.2. For 10-mL inocula, prepare and use double-strength medium.

2) Lipovitellenin-salt-mannitol agar—This medium may not be available in dehydrated form and may require preparation from the basic ingredients or by adding egg yolk to a dehydrated base.

Beef extract	1.0 g
Polypeptone	10.0 g
Sodium chloride (NaCl)	75.0 g
D-Mannitol	10.0 g
Agar	15.0 g
Phenol red	0.025 g
Egg yolk	20.0 g
Reagent-grade water	1 L

Sterilize via autoclaving; pH must be 7.4 ± 0.2.

3) Procedure—Inoculate tubes of m-staphylococcus broth as directed in Section 9221 B.3. Place tubes in an incubator within 30 minutes of inoculation. Incubate at 35 ± 1 °C for 24 h. Check tubes for turbidity (growth). The presence of turbidity is a presumptive positive result.

Streak from presumptively positive tubes onto plates of lipovitellenin-salt-mannitol agar and incubate at 35 ± 1 °C for 24 h. (Retain presumptively positive tubes refrigerated for further testing). Opaque (24 h), yellow (48 h) zones around the colonies are positive evidence of lipovitellenin-lipase activity (opaque) and mannitol fermentation (yellow).

If the plate is negative (no opaque or yellow zones), streak another plate from the original enrichment tube before discarding it. Lipovitellenin-lipase activity has a 95% positive correlation with coagulase production. If necessary, confirm positive isolates as catalase-positive, coagulase-positive, Gram positive, fermenting mannitol, fermenting glucose anaerobically, and yielding microscopic morphology typical of staphylococci.

7. Tests for Pseudomonas aeruginosa

Tests for *P. aeruginosa* are presented in 9213 E, F and G.

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9213 C. Whirlpools

1. General Discussion

a. Characteristics: A *whirlpool* is a shallow pool with a maximum water depth of 1.2 m. It has a closed-cycle water system, a heated water supply, and usually a hydrojet recirculation system. It may be constructed of plastic, fiberglass, redwood, or epoxy-lined surfaces. Whirlpools are designed for recreational and therapeutic use and may accommodate one or more bathers. These pools usually are not cleaned, drained, and refilled after each use. They are located in homes, apartments, hotels, athletic facilities, rehabilitation centers, and hospitals.

b. Monitoring: Whirlpool-associated infections are common because of the whirlpool's inherent design and characteristics, which include high temperature, reduced disinfection efficacy, and increased organic material. All these factors contribute to favorable conditions for the growth of microorganisms, especially *Pseudomonas aeruginosa.* Thus, frequent testing for disinfectant levels and pH, along with scheduled maintenance, is necessary for safe whirlpool water quality.[1–5] Studies have also shown that whirlpools can serve as a reservoir of *Legionella pneumophila*. This organism is often detected in water and filter samples during outbreaks; also check spray nozzles because they can aerosolize the organism. Methods to detect *P. aeruginosa* are presented in 9213 E and F and G. They include a membrane filter procedure, a multiwell tray procedure, and a multiple-tube technique. Methods to detect *Legionella* can be found in Section 9268. Refer to state and local guidelines for monitoring guidance.

c. Microbiological indicators: The primary indicator of disinfection efficacy is *P. aeruginosa*, with total coliforms, heterotrophic plate count, and staphylococci as supporting indicators of water quality. The standard index of water quality (i.e., total coliforms) may be insufficient to judge the microbiological quality of whirlpool water. *Pseudomonas aeruginosa* is frequently isolated from whirlpool water that is coliform-negative.[6] In the event of a whirlpool-associated outbreak, collect samples as soon as possible. Analyze for the suspected pathogen and *P. aeruginosa*.

d. Sample preservation: Examine samples as soon as possible after collection. See Section 9060 B for preservation information.

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9213 D. Natural Bathing Beaches

1. General Discussion

a. Characteristics: A *natural bathing beach* is any area of a stream, lake, ocean, impoundment, natural pool, or hot spring that is used for recreation. A wide variety of pathogens can be transmitted to humans through use of natural fresh and marine recreational waters contaminated by point sources, such as sewage and industrial wastes, and nonpoint sources, such as streams, storm drains, and animals (e.g., birds and bathers).[1,2] Contaminating microorganisms may include enteric pathogens (e.g., *Salmonella, Shigella,* enteroviruses, and protozoa) and opportunistic pathogens (e.g., *P. aeruginosa, Klebsiella* spp., and *Aeromonas hydrophila*) which can multiply in recreational waters with sufficient nutrients. Other organisms of concern are those associated with the skin, mouth, or nose of bathers, such as *S. aureus* and other naturally occurring organisms (e.g., nontuberculous mycobacteria, *Vibrio* spp., and *Naegleria*).[3–9]

b. Monitoring requirements: Historically, thermotolerant coliforms have been recommended as the indicator of choice for evaluating the microbiological quality of recreational waters. Many states use this indicator in their water quality standards. Studies have demonstrated that *E. coli* and enterococci showed a stronger correlation with swimming-associated gastroenteritis than do thermotolerant coliforms, and that both indicators were equally acceptable for monitoring fresh-water quality. For marine waters, *E. coli* and other enteric bacteria have now been documented in numerous studies to enter the viable but nonculturable state in response to such stresses as elevated osmotic levels, so the inability to isolate such indicator organisms may not prove a lack of fecal pollution in such waters. In marine waters, enterococci concentrations had the strongest relationship to the gastroenteritis incident rate. The recommended densities of these indicator organisms were calculated to approximate the degree of protection previously accepted for thermotolerant coliforms. EPA-recommended water quality criteria are based on these findings.[10] While the primary indicators of water quality are *E. coli* and enterococci, the enumeration of *P. aeruginosa, A. hydrophila, Klebsiella* spp., and *S. aureus* in recreational waters may be useful in cases that involve discharge of pulp and paper wastes and effluents from textile finishing plants into receiving waters or in waters with higher bather densities.

2. Samples

a. Containers: Collect samples as directed in Section 9060 A. Select container size according to the number and variety of tests to be performed. Adding $Na_2S_2O_3$ to the bottle is unnecessary.

b. Sampling procedure: Collect samples 0.3 m below the water surface in the areas of greatest bather load. Samples may be taken ankle deep (at ~0.075 m below water surface). In deeper waters, if desired, take another sample approximately 0.075 m below the water surface. This area may be somewhere between the knees and the chest, depending on how deep the water is where the sample is taken. Take samples over the range of environmental and climatic conditions, especially during times when maximal pollution can be expected (i.e., periods of tidal, current, and wind influences; stormwater runoff; and wastewater treatment bypasses). See Section 9213 B.2*b* and Section 9060 A.3*d* for methods of sample collection and Section 9222 B.4*a* and Table 9222:1 for suggested sample volumes.

c. Sample storage: See Section 9060 B.

3. Tests for *Escherichia coli*

a. Media: Media described in this section are available commercially. Use commercially prepared dehydrated media when available for uniformity and quality assurance. Ensure that the formulations match those included in this section.

mTEC agar[2] is used in the membrane filtration procedure (see Section 9222 B) for the detection of thermotolerant *E. coli* in recreational waters. Colonies growing on the filter are then tested for the presence of urease; *E. coli* is urease negative.

1) *mTEC agar:*

Proteose peptone No. 3	5.0 g
Yeast extract	3.0 g
Lactose	10.0 g
Sodium chloride (NaCl)	7.5 g
Dipotassium phosphate (K_2HPO_4)	3.3 g
Monopotassium phosphate (KH_2PO_4)	1.0 g
Sodium lauryl sulfate	0.2 g
Sodium desoxycholate	0.1 g
Bromocresol purple	0.08 g
Bromophenol red	0.08 g
Agar	15.0 g
Reagent-grade water	1 L

Mix thoroughly and sterilize via autoclaving; the final pH must be 7.3 ± 0.2. Pour 4 to 5 mL liquefied agar into culture dishes (50 × 100 mm) and solidify at room temperature. Store refrigerated.

2) Urea substrate

Urea	2.0 g
Phenol red	10 mg
Reagent-grade water	100 mL

Adjust pH to 5.0 ± 0.2. Store refrigerated and use within 1 week.

3) Procedure—Filter the sample through a membrane filter (see Section 9222 B), place the membrane on mTEC agar, incubate at 35 ± 0.5 °C for 2 h to rejuvenate injured or stressed bacteria, and then immediately transfer to 44.5 ± 0.2 °C and incubate for 22 h. Transfer the filter to a filter pad saturated with urea substrate. After 15 min incubation at room temperature, count yellow or yellow-brown colonies using a fluorescent lamp and a magnifying lens. Verify or identify a portion of these colonies by streaking a well-isolated colony for isolation and inoculating a pure colony into a commercial multi-test system for *Enterobacteriaceae* that includes lactose fermentation or β-galactosidase activity and CO tests (see Section 9222 B.4*g*2b).

b. Modified mTEC method:

1) Modified mTEC agar[11]—Prepare in the same manner using the same ingredients as mTEC agar, but omit bromocresol purple and bromophenol red and add 0.05 g 5-bromo-6-chloro-3-indoxyl-β-D-glucuronide chromogen. Sterilize via autoclaving. The final pH must be 7.3 ± 0.2. Pour 4 to 5 mL liquefied agar into culture dishes (50 × 40 mm) and solidify at room temperature. Store refrigerated for up to 2 weeks.

2) Procedure—Filter the sample through a membrane filter (see Sections 9222 B.1 and 9222 B.4*c*), place the filter on modified mTEC agar, and incubate at 35 ± 0.5 °C for 2 h to rejuvenate injured or stressed bacteria. Remove plates from the incubator and immediately transfer them to a 44.5 ± 0.2 °C incubator and incubate for 22 h. After incubation, count red or magenta colonies (*E. coli*) using a fluorescent lamp and a magnifying lens. Verify a portion of these differentiated colonies via a commercial multi-test system (see Section 9222 B.4*g*2b).

c. Enzyme substrate test: See Section 9223 B.

4. Tests for Enterococci

Perform tests for enterococci using the multiple-tube technique (Section 9230 B), membrane filter technique (Section 9230 C), or fluorogenic substrate technique (Section 9230 D).

5. Tests for *Pseudomonas aeruginosa*

Perform tests for *P. aeruginosa* as directed in Section 9213 E, F or G. Note that the multiple tube procedure may not be applicable to marine samples.

6. Tests for *Aeromonas hydrophila*

See Section 9262.

7. Tests for *Klebsiella* spp.

See Section 9222 F.

8. Tests for *Staphylococcus aureus*

Another method for enumerating total staphylococci and *S. aureus* in marine waters and swimming pool water (but not natural freshwater) is a modification of the membrane filtration method using Baird-Parker medium [9213 B.6*a*1)].[12] This method adds 0.005% sodium azide to Tellurite Glycine Agar (TGA) or Vogel-Johnson Agar (VJA) as an additional inhibitor to increase the selectivity of these media to enumerate concentrations of total staphylococci and *S. aureus* from natural coastal marine waters. TGA and VJA use the same basic ingredients (lithium chloride, glycine, and tellurite) as selective agents against nonstaphylococci bacteria, which form black target colonies on these media. However, supplementing TGA or VJA with 0.005% sodium azide was required so they could be used to enumerate total staphylococci and *S. aureus* from marine waters but not from natural freshwater streams. Limited data show that this method also works with swimming pool water. Sodium azide inhibited the low percentages of nonstaphylococci bacteria in marine waters from forming black colonies, but not the much higher concentrations of nonstaphylococci bacteria found in freshwater streams. All black colonies are presumptive counts of total staphylococci colonies and must be verified as total staphylococci or *S. aureus* by further testing Gram staining (Gram-positive cocci), catalase (positive), or lysostaphin (positive) to confirm as total staphylococci and via coagulase (positive) to confirm as *S. aureus*.

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9213 E. Membrane Filter Technique for *Pseudomonas aeruginosa*

1. Laboratory Apparatus

See Section 9222 B.1.

2. Culture Media

a. M-PA agar. This agar may not be available in dehydrated form and may require preparation from the basic ingredients.

L-Lysine HCl	5.0 g
Sodium chloride (NaCl)	5.0 g
Yeast extract	2.0 g
Xylose	2.5 g
Sucrose	1.25 g
Lactose	1.25 g
Phenol red	0.08 g
Ferric ammonium citrate	0.8 g
Sodium thiosulfate ($Na_2S_2O_3$)	6.8 g
Agar	15.0 g
Reagent-grade water	1 L

Add ingredients to water and mix thoroughly. Adjust pH to 6.5 ± 0.1 and sterilize via autoclaving. Cool to 55 to 60 °C; adjust the pH to 7.1 ± 0.2 and add the following dry antibiotics per liter of agar base: sulfapyridine, 176 mg; kanamycin, 8.5 mg; nalidixic acid, 37.0 mg; and cycloheximide, 150 mg. After mixing, dispense in 3-mL quantities in 50 × 12 mm petri plates. Store poured plates at 2 to 8 °C. Discard unused medium after 1 month.

b. Modified M-PA agar (Commercially available as M-PA-C agar. Contains magnesium, sulfate, kanamycin, and nalidixic acid.)

c. Milk agar (Brown and Scott Foster Modification):

1) Mixture A:

Instant nonfat milk	100 g
Reagent-grade water	500 mL

2) Mixture B:

Nutrient broth	12.5 g
Sodium chloride (NaCl)	2.5 g
Agar	15.0 g
Reagent-grade water	500 mL

Separately prepare and sterilize Mixtures A and B; cool rapidly to 55 °C; aseptically combine mixtures and dispense 20 to 25 mL per petri dish.

3. Procedure

a. Presumptive tests: Filter 200 mL or less of natural waters or up to 500 mL of swimming pool waters through sterile membrane filters. Place each membrane on a poured plate of modified M-PA agar so there is no air space between the membrane and the agar surface.

Invert plates and incubate at 41.5 ± 0.5 °C for 72 h. Place in an incubator within 30 minutes of being inoculated.

Typically, *P. aeruginosa* colonies are 0.8 to 2.2 mm in diameter and appear flat with light outer rims and brownish to greenish-black centers. Count typical colonies, preferably from filters containing 20 to 80 colonies. Use a 10× to 15× magnifier as an aid in colony counting.

A two-plate method also may be used, which helps recover chlorine-stressed *P. aeruginosa* (e.g., from swimming pools and whirlpools). Use the membrane filter technique to prepare samples. Place membrane filters on R2A agar (Section 9215 A.6*c*) and incubate at 37 ± 0.5 °C for 24 h. Aseptically transfer the membranes to M-PA agar and incubate at 35 ± 0.5 °C for another 24 h.

b. Confirmation tests: Use milk agar to confirm a number of typical and atypical colonies. Make a single streak (2 to 4 cm long) from an isolated colony on a milk agar plate and incubate at 35 ± 1.0 °C for 24 h. *P. aeruginosa* hydrolyzes casein and produces a yellowish to green diffusible pigment.

4. Interpretation and Calculation of Density

Confirmation is not routinely required. In the absence of confirmation, report results as the number of presumptive *P. aeruginosa* per 100 mL.

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9213 F. Multiple-Tube Technique for *Pseudomonas aeruginosa*

1. Laboratory Apparatus and Materials

a. Sample bottles containing sodium thiosulfate: See Section 9060 A.
b. Test tubes, capped, sterile.
c. Incubator or water bath capable of maintaining 35 to 37 °C.
d. Ultraviolet lamp, 365-366 nm, 6 Watt.

2. Culture Media

a. Asparagine broth: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Asparagine, DL	3.0 g
Anhydrous dipotassium hydrogen phosphate (K_2HPO_4)	1.0 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5 g
Reagent-grade water	1 L

Adjust pH to between 6.9 and 7.2 and dispense 10 mL into tubes before sterilization. To prepare double-strength broth, add the above ingredients to a final volume of 500 mL reagent-grade water.

b. Acetamide broth: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Acetamide	10.0 g
Sodium chloride (NaCl)	5.0 g
Anhydrous dipotassium hydrogen phosphate (K_2HPO_4)	1.39 g
Anhydrous potassium dihydrogen phosphate (KH_2PO_4)	0.73 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5 g

Dissolve 1.2 g phenol red in 100 mL 0.01 M NaOH and add 1 mL/L of acetamide broth. Use phenol red stock solution within 1 year. Adjust pH to between 7.1 and 7.3 before sterilization. Final pH must be 7.0 ± 0.2. If agar slants are preferred, prepare as described above but add 15 g/L agar, heat to dissolve agar, and dispense 8-mL quantities in 16-mm tubes. After autoclaving, incline tubes while cooling to provide a large slant surface.

3. Procedure

a. Presumptive test: Perform a five-tube test. Use 10 mL single-strength asparagine broth for inocula of 1 mL or less and 10 mL double-strength asparagine broth for 10-mL inocula. Higher dilutions may be necessary for swimming pools. Incubate inoculated tubes at 35 to 37 °C. After 24 h and again after 48 h of incubation, examine tubes under long-wave ultraviolet light (365-366 nm) in a darkened room. Production of a green fluorescent pigment constitutes a presumptive positive test.

b. Confirmed test: Confirm positive tubes by inoculating 0.1 mL of culture into acetamide broth or onto the surface of acetamide agar slants. Development of purple color (alkaline pH) within 24 to 36 h of incubation at 35 to 37 °C is a positive confirmed test for *Pseudomonas aeruginosa.*

c. Computing and reporting results: To determine MPN results, see Section 9221 C and Table 9221:2.

9213 G. Enzyme Substrate Test for *Pseudomonas aeruginosa*

1. Introduction

This section describes the use of an enzyme substrate test, Pseudalert, for the detection and enumeration of *Pseudomonas aeruginosa* in water. In general, enzyme substrate tests use hydrolyzable chromogenic or fluorogenic substrates to detect specific enzymes produced by the target organism.

The medium specific for this test contains a fluorogenic aminopeptidase substrate that detects an aminopeptidase produced by *P. aeruginosa*. The bacterial aminopeptidase hydrolyzes the fluorogenic substrate to produce 7-amino-4-methylcoumarin, which exhibits a bluish fluorescence when viewed under long-wavelength (365–366 nm) UV light (thereby indicating the presence of *P. aeruginosa)*. Confirmation procedures are not required. Multiwell, multiple-tube and presence–absence (single 100-mL or 250-mL sample size) formats are described.

2. Application

This enzyme substrate test can be used for the analysis of many water matrices including drinking water,[1] hospital waters,[2] source water,[3] groundwater,[3] cooling tower waters,[4] and swimming and spa pool water samples.[1,5,6,7] This test is not for use with marine or carbonated waters. Depending on the water type and regulatory requirements, either a 100-mL or a 250-mL sample typically is analyzed.

If a laboratory has not used this method before, conduct parallel testing, including seasonal variations, with the existing method to assess site-specific effectiveness and to compare results (Section 9020 B.11). Carefully select the medium and procedure that best fits the purpose and quality control requirements. See Section 9020 B.11 for guidance on validating new methods. Although other organisms may produce the aminopeptidase enzyme that hydrolyzes this substrate, the growth of nontarget organisms is generally suppressed thereby minimizing false-positive results.

3. Samples

Collect samples as directed in Section 9060 A, using sample containers specified in Section 9030 B.19. When collecting chlorinated water samples, use sodium thiosulfate as described in Section 9060 A.2. Follow the guidelines for sample bottles described in Section 9020 B.5*d,* sample preparation described in Section 9060 A.3, and sample incubation in Section 9020 B.5*j*8. Adhere to sample holding times and conditions as described in Section 9060 B or required by regulations. Take care to ensure that samples are held at the appropriate temperature and analyzed as soon as possible after sample collection because failure to do so could compromise results. Ensure that samples meet the laboratory's acceptance criteria upon receipt.

4. Laboratory Apparatus, Reagents, and Supplies

a. Vessels, sterile, transparent, nonfluorescent, wide-mouthed (for presence-absence testing); either 100 mL or 250 mL, with or without Antifoam B pre-added.

b. Water, reagent-grade, sterile, for blanks and dilutions if needed.

c. Sodium thiosulfate ($Na_2S_2O_3$).

d. Test tubes, capped, sterile.

e. Cylinder, graduated, 100 mL capacity, sterile.

f. Incubator or water bath capable of maintaining 38 ± 0.5 °C.

g. *Ultraviolet lamp,* 365-366 nm, 6 Watt.

h. Multiwell tray sealer.

i. Multiwell trays, 51-well or 97-well with associated most probable number (MPN) table.

5. Quality Control

Method users must adhere to the quality assurance QA/QC guidelines in Section 9020, including, but not limited to, analytical QC (Section 9020 B.9), instrumentation/equipment (Sections 9020 B.4 and 9030 B), and supplies (Section 9020 B.5). Refer to Table 9020:1 for key QC procedures.

Before using each lot of new media, verify its performance via positive and negative control organisms. Recommended quality control strains are:

Organism	ATCC Number	Purpose
Pseudomonas aeruginosa	ATCC 27853	Positive control
Pseudomonas aeruginosa	ATCC 10145	Positive control
Pseudomonas fluorescens	ATCC 13525	Negative control

Also, analyze a blank (uninoculated sterile water) and test the medium and vessels (bottles, multiwell trays, tubes) to confirm sterility and the lack of autofluorescence.

6. Medium

This method uses a commercially available medium, Pseudalert or Pseudalert 250 (IDEXX Laboratories, Inc.), which is a ready-to-use powder dispensed in premeasured packets.

Commercially prepared medium must be purchased from the manufacturer. Manufacturing quality control incorporated in commercially prepared medium is essential for consistency of performance. The approximate formulation below is for informational purposes only. Do not attempt to prepare this medium in the laboratory.

Component	Formulation (approximate g/L)
Yeast extract	2.50
Milk protein	2.50
Sodium chloride	5.00
Magnesium sulfate, $MgSO_4$	1.00
Potassium nitrate, KNO_3	0.20
Ammonium sulfate, $(NH_4)_2SO_4$	1.00
Buffer	12.15
Vitamins	0.002
Selective agents (proprietary)	0.0671
7-amino-4-methylcoumarin aminopeptidase substrate	0.080

The medium is a light to dark tan, free flowing, granulated powder, free of foreign particles. Store it at 2 to 30 °C out of direct sunlight and use before the expiration date printed on the packet. Discard media that have changed color, appearance, or texture (the medium is hygroscopic and clumps and darkens if exposed to moisture). The medium pH range is 7.0 to 7.6.

Pseudalert medium is available commercially in premeasured packets for presence–absence testing or for use in multiple tube or multiple-well formats. Each packet contains sufficient medium for either one 100 mL or one 250 mL water sample, respectively. Pseudalert 250 is for use with 250-mL water samples for presence–absence testing. Multiwell formats may be used with the premeasured packets for 100-mL water samples to quantitate *P. aeruginosa* present in a sample using most probable number (MPN) tables supplied by the manufacturer.

7. Procedure

Begin the analysis by mixing the sample properly to promote an even distribution of bacteria. For proper mixing to occur, samples ideally have a headspace of 1 inch or more and are shaken vigorously for 7 s (back and forth in a 1 ft arc approximately 25 times).

For each format used, place samples in the incubator within 30 minutes after the medium is added to sample. No matter which format (qualitative [single bottle] or enumerative [tubes or multiwell tray]) is used, the medium is incubated at 38 ± 0.5 °C for 24 to 28 hours.

High mineral content (especially magnesium or calcium) can cause a sample to become cloudy after the medium is added, but this generally does not affect the results.

a. Presence-absence procedure: For presence-absence testing, use water samples of either 100 mL or 250 mL with the corresponding premeasured packets of media.

Aseptically add the premeasured medium to a 100- or 250-mL water sample in a sterile, transparent, nonfluorescent container. Aseptically cap and shake the container vigorously to dissolve the medium. Some medium may remain undissolved, but this does not affect test performance.

b. Multiple tube procedure:

1) Multiple-tube procedure using a 5- or 10-tube MPN test—A 5-tube series (with each tube containing 20 mL sample) or a 10-tube series (with each tube containing 10 mL sample) can be used when levels of bacteria are anticipated to be fairly low or where a fixed 100-mL sample volume must be analyzed (e.g., for regulatory compliance).

To perform the procedure, add a single packet containing the premeasured medium to a well-mixed 100-mL water sample and shake vigorously to dissolve the medium. Arrange the tubes in rows of 5 or 10 in a test tube rack, and label each set of tubes. Aseptically dispense 20 mL of sample into each of 5 sterile tubes or 10 mL into each of 10 sterile tubes, cap tightly, and incubate. After incubation, refer to Table 9221:2 or Table 9221:3 to determine the MPN.

2) Multiple-tube procedure using 15-tube MPN test—A 15-tube test usually consists of using 3 serial dilutions of a sample, with each dilution inoculated into 5 tubes. Typically, 5 tubes contain undiluted sample, 5 tubes contain a 1:10 dilution, and 5 tubes contain a 1:100 dilution. However, other dilutions may be used depending on the numbers of *P. aeruginosa* anticipated to be present in the sample.

Use this technique when a water sample may contain higher bacteria levels and there is no requirement to analyze a fixed volume. The number of tubes and the sample volumes selected depend upon the quality and characteristics of the water to be examined. To preclude any unwanted interaction with the medium, use only sterile, nonbuffered, reagent-grade water to prepare dilutions. The use of buffered, saline, or peptone-containing diluents interferes with the performance of the test.

When working with diluted samples, best laboratory practice is to ensure that all tubes are in place and labeled before analysis begins. Additionally, use clean, sterile pipettes to pipet each dilution because bacterial carry-over from dirty pipettes may make test results inaccurate.

a) Sample preparation for the undiluted series—Add 1 packet of premeasured medium to a sterile vessel containing 100 mL of well-mixed sample and mix vigorously to dissolve the medium. Aseptically pipet 10 mL of the sample-medium mixture into each of 5 sterile, nonfluorescing tubes.

b) Medium preparation for the 1:10 and 1:100 dilutions—Add 1 packet of premeasured medium to 100 mL sterile reagent-grade water in a sterile container, and mix vigorously to dissolve the medium. Aseptically pipet 9 mL of prepared medium into 10 sterile, nonfluorescing tubes. The preparation of enzyme substrate medium must be completed within 1 hour of adding the sample to the prepared medium.

c) Inoculating tubes for the 1:10 dilution—Aseptically pipet 1 mL of well mixed sample into each of 5 tubes containing the 9 mL of prepared medium. Cap and mix well.

d) Inoculating tubes for the 1:100 dilution—Pipet 10 mL of well-mixed sample into a vessel containing 90 mL sterile reagent-grade water. Close and mix well. Aseptically pipet 1.0 mL of this diluted sample into 5 tubes containing 9 mL of prepared medium (above). Cap and mix well.

For any additional dilutions needed, continue with the dilution process as described above.

After incubation, determine the MPN using Table 9221:4. If further dilutions were performed, the MPN value must be multiplied by the dilution factor to obtain the proper quantitative results.

c. *Multiwell procedure:*

1) 100-mL procedure—This procedure is performed with sterilized disposable 51-well multiwell trays. Aseptically add the contents of a single packet containing the premeasured medium to a 100-mL water sample in a container and shake vigorously to dissolve the medium. When the medium has completely dissolved, aseptically transfer it to the tray. To open the tray, use one hand to hold the unit upright (with the well side facing the palm) and squeeze the upper part of the tray so it bends toward the palm. Using the other hand, gently pull the foil tab to separate the foil from the tray, being careful not to touch the inside of either foil or tray. Add the medium-water sample mixture directly into the tray, avoiding contact with the foil tab. Gently tap the bottom wells to release any air bubbles that may be trapped. Allow the foam to settle, although some foam is acceptable. Place the tray into the appropriate rubber insert with the well (plastic) side facing down and feed it into the sealer. The sealer disperses the sample into the wells and individually seals each well in the tray. For added stability, incubate stacked trays paper side down in the incubator.

To minimize air bubbles in wells, samples can be prepared in presterilized vessels containing antifoam B. Antifoam B solution is a sterile 1% active, water soluble suspension of silicone that does not interfere with microbial growth or metabolism and does not affect the results of the test. Alternatively, 2 drops of antifoam B can be added to each vessel using a dropper bottle.

2) 250-mL procedure—This procedure is performed with 51-well multiwell trays and the medium premeasured for a 100-mL sample. To perform this procedure, divide the 250-mL water sample by aseptically dispensing two 100-mL aliquots into 2 sterile, transparent bottles and dispense the remaining 50-mL sample volume into a third sterile, transparent bottle. Aseptically add 50 mL sterile reagent-grade water to the 50-mL sample aliquot to bring the volume up to 100 mL. Aseptically add the contents of a packet containing the premeasured medium to each of the 3 bottles containing 100 mL and mix each well. When the medium has completely dissolved, aseptically pour the 3 volumes of sample-medium mixture individually into 3 separate trays (see above). Appropriate labeling of the 3 trays is essential for correct calculation of the final count.

8. Interpretation

After incubation at 38 ± 0.5 °C for 24 h, examine the samples for fluorescence using a 6 W longwave (365 to 366 nm) UV light. All samples, regardless of test format, are examined for blue fluorescence in a dark room or in a chamber that obscures ambient light. Hold the UV light within 5 in. (12 cm) from UV light, facing away from your eyes and toward the sample.

Any wells, tubes, or vessels that exhibit any degree of blue fluorescence are considered positive for *P. aeruginosa*. For interpretation purposes, incubated samples can be compared to a prepared negative blank (sterile reagent-grade water with medium added that is incubated alongside the test sample). If fluorescence in a

well, tube, or vessel at 24 h is not discernible, return the container to the incubator for further incubation to allow the fluorescence to intensify, but do not exceed the maximum incubation time of 28 h. Placing the rubber insert over the tray may facilitate identification of individual fluorescent wells.

Positive results for *P. aeruginosa* observed before 24 hours and negative results observed after 28 hours are valid.

9. Reporting

For the presence-absence procedure, report results as present or absent in a 100 mL or 250 mL sample. For the multiple tube procedure, calculate the MPN value from the number of positive tubes as described in Section 9221 C.

For the multiwell procedure, determine the MPN from the appropriate MPN tables obtained from the manufacturer. For the 250 mL MPN determination, count the number of positive wells on each of the 3 trays and determine the MPN per 250 mL for *P. aeruginosa* using the 250 MPN table obtained from the manufacturer.

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