as specified by Executive Order 13175 (65 FR 67249, November 9, 2000), nor will it have substantial direct effects on the States, on the relationship between the national government and the States, or on the distribution of power and responsibilities among the various levels of government, as specified in Executive Order 13132 (64 FR 43255, August 10, 1999), because it merely proposes to approve a state rule implementing a federal standard, and does not alter the relationship or the distribution of power and responsibilities established in the Clean Air Act. This proposed rule also is not subject to Executive Order 13045 (62 FR 19885, April 23, 1997), because it is not economically significant.

In reviewing SIP submissions, EPA's role is to approve state choices, provided that they meet the criteria of the Clean Air Act. In this context, in the absence of a prior existing requirement for the state to use voluntary consensus standards (VCS), EPA has no authority to disapprove a SIP submission for failure to use VCS. It would thus be inconsistent with applicable law for EPA, when it reviews a SIP submission, to use VCS in place of a SIP submission that otherwise satisfies the provisions of the Clean Air Act. Thus, the requirements of section 12(d) of the National Technology Transfer and Advancement Act of 1995 (15 U.S.C. 272 note) do not apply. As required by section 3 of Executive Order 12988 (61 FR 4729, February 7, 1996), in issuing this proposed rule, EPA has taken the necessary steps to eliminate drafting errors and ambiguity, minimize potential litigation, and provide a clear legal standard for affected conduct. EPA has complied with Executive Order 12630 (53 FR 8859, March 15, 1988) by examining the takings implications of the rule in accordance with the ''Attorney General's Supplemental Guidelines for the Evaluation of Risk and Avoidance of Unanticipated Takings'' issued under the executive order. This proposed rule does not impose an information collection burden under the provisions of the Paperwork Reduction Act of 1995 (44 U.S.C. 3501 *et seq.*).

List of Subjects in 40 CFR Part 52

Environmental protection, Air pollution control, Carbon monoxide, Intergovernmental relations.

Authority: 42.U.S.C. 7401 *et seq.*

Dated: August 20, 2001.

William J. Muszynski,

Acting Regional Administrator, Region 2.

[FR Doc. 01–21933 Filed 8–29–01; 8:45 am]

BILLING CODE 6560–01–P

ENVIRONMENTAL PROTECTION AGENCY

40 CFR Part 136

[FRL–7045–6]

RIN 2040–AD08

Guidelines Establishing Test Procedures for the Analysis of Pollutants; Analytical Methods for Biological Pollutants in Ambient Water; Proposed Rule

AGENCY: Environmental Protection Agency (EPA).

ACTION: Proposed rule.

SUMMARY: This proposed regulation would amend the ''Guidelines Establishing Test Procedures for the Analysis of Pollutants'' under section 304(h) of the Clean Water Act (CWA), by adding several analytical test procedures for enumerating the bacteria, Escherichia coli (E. coli) and enterococci, and the protozoans, Cryptosporidium and Giardia, in ambient water to the list of Agency-approved methods.

This proposal would make available a suite of Most Probable Number (MPN) (i.e. multiple-tube, multiple-well) and membrane filter (MF) methods for enumerating E. coli and enteroccoci bacteria in ambient water. Both culture-based and enzyme-substrate techniques are included. Some test methods are also applicable to total coliform determinations when these are the preliminary or concurrent steps for E. coli enumeration. Similarly, this document proposes new methods for detecting Cryptosporidium and Giardia in ambient water. Regulators may use these test procedures to assess Cryptosporidium and Giardia concentrations in ambient waters.

DATES: Comments must be postmarked, delivered by hand, or electronically mailed on or before October 29, 2001. Comments provided electronically will be considered timely if they are submitted electronically by 11:59 p.m. Eastern Time (ET) on October 29, 2001.

ADDRESSES: Send written comments on the proposed rule to ''Part 136 Biological Methods'' Comment Clerk (W–99–14); Water Docket (4101); U. S. Environmental Protection Agency; Ariel Rios Building; 1200 Pennsylvania Avenue, NW., Washington, DC 20460. Hand deliveries should be delivered to: EPA's Water Docket at 401 M Street, SW., East Tower Basement (Room EB 57), Washington, DC 20460. If you wish to hand-deliver your comments, please call (202) 260–3027 between 9 a.m. and 4 p.m., Monday through Friday, excluding Federal holidays, to obtain the room location for the Docket. Comments also may be submitted electronically to: *OW-Docket@epa.gov.*

FOR FURTHER INFORMATION CONTACT: For regulatory information regarding this proposal, contact Maria Gomez-Taylor, Ph.D.; Engineering and Analysis Division (4303); Office of Science and Technology; Office of Water; U.S. Environmental Protection Agency; Ariel Rios Building; 1200 Pennsylvania Avenue, NW.; Washington, DC 20460, or call (202) 260–1639.

For technical information regarding analytical methods proposed in today's rule, contact Robin Oshiro; Office of Science and Technology (4304); Office of Water; U.S. Environmental Protection Agency; Ariel Rios Building; 1200 Pennsylvania Avenue, NW.; Washington, DC 20460, or call (202) 260–7278.

SUPPLEMENTARY INFORMATION:

Potentially Affected/Regulated Entities

EPA Regions, as well as States, Territories, and Tribes are authorized to implement the water quality standards program and the National Pollutant Discharge Elimination System (NPDES) program, and to issue permits that comply with the technology-based and water quality-based requirements of the Clean Water Act (CWA). In doing so, permitting authorities, including authorized States, Territories, and Tribes, make discretionary choices when writing permits, including the selection of pollutants to be measured and monitoring requirements. If EPA has ''approved'' (i.e., promulgated through rulemaking) standardized testing procedures for a given pollutant, the permit must specify one of the approved testing procedures or an approved alternate test procedure. Although EPA proposes to include test methods for four biological pollutants in section 136.3, it recommends their use only for ambient water quality monitoring. EPA does not propose to approve these test methods for effluent matrices.

EPA has developed ambient water quality criteria for E. coli and enteroccoci bacteria and is considering criteria for Cryptosporidium and Giardia. The States, Territories, and Tribes may adopt these criteria into their water quality standards and may issue water quality-based permits that require monitoring for these pollutants in ambient waters. Therefore, discharges with water quality-based permits could be affected by the standardization of testing procedures in this rulemaking in instances where the permitting

authority requires that such permits incorporate ambient water monitoring. EPA does not require inclusion of ambient water monitoring for NPDES permits. In addition, when a State, Territory, or authorized Tribe provides certification of Federal licenses under the CWA section 401, and when such certification requires measurement of waste constituents specified in 40 CFR 136, then such measurements must be in accordance with approved testing procedures if such procedures are available. 40 CFR 136.1(c). Categories and entities that ultimately may be affected/regulated include:

Category	Examples of potentially affected/regulated entities
Regional, State, and Territorial Governments and Indian Tribes.	States, Territories, and Tribes authorized to administer the water quality standards programs; States, Territories, and Tribes providing certification under Clean Water Act section 401; Governmental permittees.
Municipalities	Publicly-owned treatment works with water quality-based permits.
Industry	Industrial facilities with water quality-based permits.

This table is not intended to be exhaustive, but rather provides guidance for readers regarding entities likely to be affected/regulated by this action. This table lists the types of entities that EPA is now aware could potentially be affected/regulated by this action. Other types of entities not listed in the table also could be affected/regulated. If you have questions regarding the applicability of this action to a particular entity, consult one of the persons listed in the **FOR FURTHER INFORMATION CONTACT** section.

Record and Commenting Procedures

The record for this rulemaking has been established under docket number W–99–14. A copy of the supporting documents cited in this proposal are available for review at EPA's Water Docket. The record is available for inspection from 9 a.m. to 4 p.m. EST, Monday through Friday, excluding Federal holidays at EPA's Water Docket, 401 M Street SW., East Tower Basement (Room EB 57), Washington, DC 20460. For access to docket materials, please call (202) 260–3027 to schedule an appointment.

Commenters are requested to submit any references cited in their comments. Commenters also are requested to submit an original and three copies of their written comments and enclosures, and to clearly identify the specific pollutant and method to which the comment applies. Commenters that want a confirmed receipt of their comments should include a self-addressed, stamped envelope. All comments must be postmarked or delivered by hand. No facsimiles (faxes) will be accepted.

Electronic comments must be submitted as a Word Perfect for Windows 5/6/7/8 file or an ASCII file, avoiding the use of special characters and any form of encryption. Comments and data also will be accepted on disks in Word Perfect 5/6/7/8 or ASCII file format. Electronic comments on this notice may be filed online at many Federal Depository Libraries. All electronic comments must be identified by docket number. Electronic comments will be transferred into a paper version for the official record. EPA will attempt to clarify electronic comments if there is an apparent error in transmission.

Information on Internet Access

This **Federal Register** document has been placed on the Internet for public review and downloading at the following location: http//www.epa.gov/fedrgstr.

Availability and Sources for Methods

Copies of analytical methods published by EPA are available for a nominal cost through the National Technical Information Service (NTIS); U.S. Department of Commerce; 5285 Port Royal Road; Springfield, VA 22161, or call (800) 553–6847. Copies of the EPA methods cited in this proposal may be obtained from Robin Oshiro; Office of Science and Technology (4304); Office of Water; U.S. Environmental Protection Agency; Ariel Rios Building; 1200 Pennsylvania Avenue, NW.; Washington, DC 20460, or call (202) 260–7278. Copies of several of the EPA methods cited in this proposal may also be downloaded from the EPA Office of Research and Development; National Exposure Research Laboratory (NERL)-Cincinnati Microbiology home page at www.epa.gov/microbes/. Copies of published journal articles for selected EPA methods are available in the public domain. All other methods must be obtained from the publisher. Publishers (with contact information) for all methods are included in the References section of today's rule. Copies of all methods are also available in the public record for this proposal.

Outline of Preamble

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I. Statutory Authority

Today's proposal is pursuant to the authority of sections 303(c), 304(a), 304(h) and 501(a) of the Clean Water Act (CWA), 33 U.S.C. 1313(c), 1314(a), 1314(h), 1361(a) (the ''Act''). Section 303(c) of the Act establishes the basis for the current water quality standards program. This section requires EPA to review and approve or disapprove State-adopted water quality standards. Section 304(a) of the Act requires the EPA Administrator to conduct non-regulatory scientific assessments of ecological and public health effects to support the development of water quality criteria associated with specific ambient water uses. When these criteria are adopted as State water quality standards under section 303, they become the enforceable maximum acceptable levels of pollutants in ambient waters. Section 304(h) of the Act requires the EPA Administrator to ''promulgate guidelines establishing test procedures for the analysis of pollutants that shall include the factors which must be provided in any certification pursuant to section 401 of this Act or permit applications pursuant to section 402 of this Act.'' Section 501(a) of the Act authorizes the Administrator to ''prescribe such regulations as are necessary to carry out this function under this Act.''

II. Regulatory Background

To fulfill the CWA's mandate to maintain ''fishable and swimmable'' waters, EPA is required to develop ambient water quality criteria based on a scientific assessment of the relationship between pollutant concentrations and environmental and human health effects. Ambient water refers to any fresh, marine, or estuarine surface water used for recreation; propagation of fish, shellfish, or wildlife; agriculture; industry; navigation; or as source water for drinking water facilities. These ambient water quality criteria become enforceable water quality standards when adopted by State, Territorial, Tribal, and local governments implementing a water-quality based approach to pollution control. For bacterial pollution in ambient water, EPA has developed bacteriological ambient water quality criteria recommendations for E. coli in freshwater and enterococci in freshwater and marine waters (51 FR 8012, March 7, 1986). There are a number of zoonotic diseases of concern to humans (diseases transferred from animals to humans) if recreational or other waters are contaminated with fecal material from non-human animal species. E. coli species are a subset of the coliform bacteria group that is part of the normal intestinal flora of humans and animals and is, therefore, a direct indicator of fecal contamination from these sources in water. Enterococci, which include Enterococcus faecalis and Enterococcus faecium, are enteric bacteria used to indicate fecal contamination and the possible presence of pathogens, in water. Based on previous EPA guidance, total and fecal coliform bacteria currently have been included in many water quality standards as indicators of bacterial contamination (USEPA, 1976). However, more recent epidemiological studies described in Ambient Water Quality Criteria for Bacteria—1986 (USEPA, 1986a), indicate that E. coli and enterococci show a direct correlation with swimming-associated gastrointestinal illness rates, while fecal coliforms do not. As the concentration of E. coli and/or enterococci increase(s), the illness rates also increase. These indicators are used as part of the bacterial water quality criteria and standards to enhance the protection of human health and the environment.

In addition to bacterial pollution, EPA is concerned about waterborne parasites and has developed test methods for Cryptosporidium and Giardia. These waterborne parasites are responsible for cases of severe and widespread human illness when present in drinking water supplies as a result of contamination of source waters. To support future regulation of these organisms in drinking water, the Safe Drinking Water Act Amendments of 1996 required the EPA to evaluate the risk to public health associated with Cryptosporidium and Giardia contamination. To implement these requirements, EPA plans to assess Cryptosporidium and Giardia occurrence in freshwater surface water bodies. Because one of the designated uses of some ambient waters may include the use of the waterbody as a drinking water source, EPA may develop ambient water quality criteria for Cryptosporidium and Giardia in the future. EPA plans to use the test methods discussed in this notice to support these assessments. By doing so, EPA desires to promote consistency on the methods used for these assessments to ensure that the data collected are of good quality and comparable. EPA also wishes to make these methods available for use by the States and for general use for risk assessments.

In today's notice, EPA is proposing test methods for E. coli, enterococci, Cryptosporidium, and Giardia. Proposal of the bacterial methods supports the use of E. coli and enterococci as indicators in place of the total and fecal coliform indicators in State, Territorial, Tribal, and local water quality-based monitoring programs. Proposal of test methods for Cryptosporidium and Giardia supports the use of these methods in evaluating surface water occurrence of these organisms and the associated watershed vulnerability levels of concern for waterbodies designated as potential drinking water sources under the water quality standards program. EPA proposes to approve the use of test methods for E. coli, enterococci, Cryptosporidium, and Giardia for ambient water quality monitoring only. Although EPA believes that these methods are appropriate for ambient water quality monitoring, the Agency has not determined that these methods are acceptable for application to other matrices.

This proposal was initiated in response to national directives that seek to improve and assist in State, Territorial, Tribal, and local implementation of water quality standards, ambient water monitoring programs, and public notification programs to reduce public health risks posed by biological pollutants in ambient water. The primary initiatives that served as impetus for today's proposal include the Beaches Environmental Assessment Closure and Health (BEACH) Program; the Beach Action Plan (EPA–600–R–98–079); the Beach Watch Program; the Beaches Environmental Monitoring for Public Access and Community Tracking (EMPACT) Program; and the Water Quality Criteria and Standards Plan. Additionally, this rule is expected to satisfy requests by State, Territorial, Tribal, and local governments, regulated entities, and environmental laboratories that EPA publish analytical test procedures for enumerating E. coli, enterococci, Cryptosporidium, and Giardia in ambient water that were evaluated through interlaboratory validation or extensive intralaboratory comparison with previously approved methods.

III. Explanation of Today's Action

A. Methods for Bacterial Pollutants

This proposal would make available a suite of Most Probable Number (MPN) (i.e., multiple-tube, multiple-well), and membrane filter (MF) methods for enumerating (i.e., determining organism density) E. coli and enteroccoci in ambient water as part of State, Territorial, Tribal, and local water quality monitoring programs. Multiple-tube, multiple-well, and MF formats include culture and enzyme-substrate techniques. Culture methods use lactose fermentation (E. coli), presence of turbidity (enterococci), colony formation, or color to detect the target organism. Enzyme-substrate tests use chromogenic (e.g., indoxyl-β-D-glucuronide) or fluorogenic (e.g., 4-methylumbelliferyl-β-D-glucuronide, [MUG]) substrates that react with specific enzymes (generally, β-glucuronidase in E. coli and β-glucosidase in enterococci) to produce color changes or fluorescence to detect the target organism. The methods included in this proposal were developed by EPA, voluntary consensus standards bodies (VCSBs) (i.e., American Public Health Association [APHA], American Water Works Association [AWWA], and Water Environment Foundation [WEF] who jointly publish Standard Methods for the Examination of Water and Wastewater, referred to as ''Standard Methods;'American Society for Testing and Materials [ASTM]; Association of Official Analytical Chemists International [AOAC]), and commercial vendors with methods submitted to the EPA Office of Water (OW) Alternate Test Procedure (ATP) process. For several procedures, an EPA method, VCSB method, and/or a commercially available method (submitted to the ATP program) are proposed.

Although there are several methods (not yet approved by EPA) that are applicable to simultaneous determination of total coliform and E. coli, EPA is proposing to approve methods for analysis of E. coli only. EPA made this choice because at present there are no EPA-approved methods for E. coli, whereas EPA-approved methods are already available for the determination of total coliform. There is a request for comment on the expansion of today's rule to include total coliforms in Section III.A.5. Several of the total coliform test methods (or selected procedural steps) have already been approved by EPA (see Table IA at 40 CFR 136.3) or have been proposed for approval for the Clean Water Act or Safe Drinking Water Act compliance monitoring programs (66 FR 3526, January 16, 2001).

Proposed methods were selected based on data generated by EPA laboratories, submissions to the ATP program and VCSBs, published peer-reviewed journal articles, and/or publicly available study reports that indicate their applicability to quantitative analysis of the target organisms in ambient water. Since data were generated in multiple studies using different method versions and different statistical analyses, the test procedures in today's rule must be evaluated against the end-users' needs based on data quality objectives. End-users should compare any new proposed alternate method with the relevant EPA-recommended method(s) before adopting it for that matrix to ensure that the proposed method generates data of comparable quality. EPA-recommended methods for matrices in which they were tested are summarized in Tables 3 and 5. A media acronym table is provided in Section V. Full citations for methods and data reports are provided in the References section and are included in the docket for today's proposed rulemaking. At the time of final rulemaking, EPA plans to issue a draft protocol for determining the comparability of alternative test methods to those promulgated in the final rule. In addition, EPA will issue draft guidance on acceptable characteristics of methods for determining equivalency (e.g., acceptable range of false positives/false negatives). There is a request for comment in Section III.A.5 inviting suggestions on acceptable characteristics of methods and on method comparability criteria to support the equivalency testing protocol.

1. Most Probable Number (MPN) and Membrane Filtration (MF) Methods

In Most Probable Number tests, the number of tubes/wells producing a positive reaction provides an estimate of the original, undiluted density (i.e., concentration) of target organisms in the sample. This estimate of target organisms, based on probability formulas, is termed the Most Probable Number. MPN tests can be conducted in multiple-tube fermentation (MTF), multiple-tube enzyme substrate, or multiple-well enzyme substrate formats. In multiple-tube tests, serial dilutions may be used to obtain estimates over a range of concentrations, with replicate tubes analyzed at each ten-fold dilution/volume. The numbers of replicate tubes and sample dilutions/volumes are selected based on the expected quality of the water sample. Generally, for non-potable water samples, five replicate tubes at a minimum of three dilutions/volumes are used. Tubes are incubated, and positive results are reported and confirmed. Positive results are determined under specified conditions by the presence of acid and/or the production of gas using MTF tests, or by color change or fluorescence using enzyme substrate tests. Tests also may be conducted in a multiple-well format to determine MPN, using commercially prepared substrate media, multiple-well trays, and MPN tables provided by the manufacturer. Target organism density is estimated by comparing the number of positive tubes or wells with MPN tables. The MPN tables relate the number of positive tubes or wells to an estimate of the mean target organism density based on probability formulas. Results in both types of tests are generally reported as MPN per 100 mL.

The multiple-tube fermentation methodology is useful for detecting low concentrations of organisms (<100/100 mL), particularly in samples containing heavy particulate matter, toxic compounds (e.g. metals), or injured or stressed organisms. Multiple-tube tests are applicable to freshwater, estuarine, and marine ambient waters. Since MPN tables assume a Poisson distribution, samples must be adequately shaken to break up any clumps and provide even distribution of bacteria. If the sample is not gently shaken, the MPN value may underestimate the actual bacterial density. The overall precision of each multiple-tube test depends on the number of tubes used and sample dilutions/volumes tested. Unless a large number of tubes are used (five tubes per dilution/volume or more), the precision of multiple-tube tests can be very poor. Precision is improved when the results from several samples from the same sampling event are processed, estimated separately, and then mathematically combined using the geometric mean. Further background information on multiple-tube tests is available in the 20th Edition of Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

Membrane filtration is a direct-plating method in which sample dilutions/volumes are filtered through 0.45 μm membrane filters that are subsequently transferred to petri plates containing selective primary isolation agar or an absorbent pad saturated with selective broth. A second substrate medium is used in two-step MF procedures to confirm and/or differentiate the target organisms. The total sample volume to be analyzed may be distributed among multiple filters and diluted as needed, based on the anticipated water sample type, quality, and character (e.g., organism density, turbidity). The goal is to obtain plates with counts within the acceptable counting range of the method. The acceptable counting range of membrane filter tests depends on the specific analytical technique and the target organism under study. Plates are incubated and target colonies are counted. A percentage of the target colonies may then be verified as specified by the method. Target colonies are detected by observing the presence of colonies that meet a specific morphology, color, or fluorescence under specified conditions. Colonies may be counted with the aid of a fluorescent light, magnifying lens or dissecting microscope, or long-wavelength (366-nm) ultraviolet (UV) light source. Results generally are reported as colony-forming units (CFU) per 100 mL. Organism density is determined by dividing the number of target CFU by the volume (mL) of undiluted sample that is filtered and multiplying by 100. If verification steps are performed, the initial target colony count is adjusted based upon the percentage of positively verified colonies and reported as a ''verified count per 100 mL'' (APHA, 1998).

Membrane filtration is applicable to most freshwater, estuarine, and marine ambient waters, with limitations where an underestimation of organism density is likely: water samples with high turbidity, toxic compounds, or large numbers of non-coliform (background) bacteria, and organisms damaged by chlorine or toxic compounds. To minimize these interferences, replicates of smaller sample dilutions/volumes may be filtered and the results combined. When the MF method has not been used previously on an

individual water type, parallel tests should be conducted with a MTF to demonstrate applicability, lack of interferences, and at least comparable recovery. For example, colonies from samples containing high-background levels or stressed organisms should be verified. If the MTF results are consistently higher than those obtained in MF tests, or there is an indication of suboptimal recovery, use an appropriate recovery enhancement technique and demonstrate that the recovery enhancement technique is comparable to MTF. Further background information on MF tests is available in Standard Methods for the Examination of Water and Wastewater (APHA,1998).

A statistical comparison of results obtained by the multiple-tube and MF methods showed that the MF method is more precise in enumerating target organisms than the MPN test, but differences in recovery were generally not statistically significant. However, based on susceptibility to interferences, MF tests may underestimate the number of viable bacteria, and the MPN method may overestimate the concentration because of the built-in positive bias of the method (Thomas, 1955). Tables with 95% confidence limits are available for both methods, based on the assumption that bacteria exhibit a Poisson distribution. Because of susceptibility of some MF tests to interferences, verification of some MF results with multiple-tube tests is critical. Additionally, some MPN tests require confirmation tests because of the false positive/false negative rates of the particular media. In general, although numerical results may not be identical, data from each method yield similar water quality information based on performance.

2. Selection of Proposed Methods

A variety of methods for E. coli and enteroccoci are being proposed in today's rule because a range of techniques are routinely used for different applications by regulatory authorities, permitees, laboratories, researchers, and others. The methods presented have been evaluated based on different study designs and statistical analyses. The variety of waters subject to monitoring, the selection of an appropriate method, number of tubes, sample dilutions/volumes, and other analytical design decisions may be made based on the available information on the type, quality, character, consistency of results, anticipated target organism density, and designated use of the water to be monitored.

3. Methods for E. coli

EPA is proposing several methods for enumerating E. coli in ambient water. Brief descriptions of the proposed multiple-tube, multiple-well, and MF methods are provided. Method performance data is summarized in Table 3.

In Table 1, methods in the same row use the same technique, but are published by different entities. For example, ONPG–MUG is published in the ''Standard Methods'' manual and in the Association of Official Analytical Chemists (AOAC) manual, and is also available as a commercial product. Voluntary Consensus Standards (VCS) Methods are those developed or adopted by domestic and international voluntary consensus standard bodies. The American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Foundation (WEF) jointly publish methods approved by a methods approval program in Standard Methods for the Examination of Water and Wastewater (''Standard Methods''). The American Society for Testing and Materials (ASTM) are methods that have met the requirements of the ASTM methods approval program. The Association of Official Analytical Chemists also publishes methods that have met the requirements of the AOAC methods approval program. EPA methods are those that have been developed by the US EPA.

TABLE 1.—PROPOSED METHODS FOR E. COLI ENUMERATION [1,2]

Technique	Method [1,2]	EPA method	VCS methods			Commercial example
			Standard methods	ASTM	AOAC	
Most Probable Number (MPN).	LTB→EC–MUG		9221B.1/ 9221F	.		
	ONPG–MUG		9223B		991.15	Colilert® [3,5]
	ONPG–MUG		9223B			Colilert-18® [3,6]
	CPRG–MUG		9223B			Colisure™ [3,5]
Membrane Filter (MF)	mENDO→NA–MUG		9222B/9222G			
	LES–ENDO→NA–MUG		9222B/9222G			
	mFC→NA–MUG		9222D/9222G			
	mTEC agar	1103.1	9213D	D5392–93		
	Modified mTEC agar	Modified 1103.1				
	MI agar	EPA–600–R–013 [7]				
	m-ColiBlue24 broth					m-ColiBlue24 [4,5]

[1] A media acronym table is provided in Section V.
[2] Tests must be conducted in a format that provides organism enumeration.
[3] Manufactured by IDEXX.
[4] Manufactured by Hach Company.
[5] Method currently approved for determining presence/absence of total coliform and E. coli in drinking water.
[6] Acceptable version of method approved as a drinking water ATP.
[7] Membrane Filter Method for the Simultaneous Detection of Total Coliforms and Escherichia coli in Drinking Water.

Most Probable Number Tests for *E. coli*

a. LTB→EC–MUG (Standard Methods 9221B.1/9221F)

The multiple-tube fermentation method for enumerating E. coli in water uses multiple-tubes and dilutions/volumes in a two-step procedure to determine E. coli concentrations (APHA, 1998). In the first step, or ''presumptive phase,'' a series of tubes containing lauryl tryptose broth (LTB) are inoculated with undiluted samples and/or dilutions/volumes of the samples and mixed. Inoculated tubes are incubated for 24 ± 2 h at 35 ± 0.5 °C. Each tube then is swirled gently and examined for growth (i.e., turbidity) and

production of gas in the inner Durham tube. If there is no growth or gas, tubes are re-incubated for 24 ± 2 h at 35 ± 0.5 °C and re-examined. Production of growth and gas within 48 ± 3 h constitutes a positive presumptive test for coliforms, which include E. coli.

After enrichment in the presumptive medium, positive tubes are subjected to a second step for enumeration of E. coli. Presumptive tubes are agitated, and growth is transferred using a sterile loop or applicator stick to tubes containing EC broth supplemented with 4-methylumbelliferyl-β-D-glucuronide (MUG). Inoculated tubes are incubated at 44.5 ± 0.2 °C for 24 ± 2 h in a water bath. All tubes exhibiting growth and gas production are examined for bright blue fluorescence under long-wavelength UV light (366-nm) indicating a positive test for E. coli. The density of E. coli in MPN/100 mL is then calculated from the number of positive EC–MUG tubes, using MPN tables or formulas.

b. ONPG–MUG (Standard Methods 9223B, AOAC 991.15, Colilert®, Colilert-18®, and Autoanalysis Colilert)

ONPG–MUG tests are chromogenic/fluorogenic enzyme substrate tests for the simultaneous determination of total coliforms and E. coli in water. These tests use commercially available media containing the chromogenic substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG), to detect total coliforms and the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG), to detect E. coli. All tests must be conducted in a format that provides quantitative results for ambient water. Colilert-18® should be used for testing marine waters with a minimum of a 10-fold dilution with sterile freshwater. Media formulations are available in disposable tubes for the multiple-tube procedure or packets for the multiple-well procedure. Appropriate preweighed portions of media for mixing and dispensing into multiple-tubes and wells are also available. The use of commercially prepared media is required for quality assurance and uniformity.

For the multiple-tube procedure, a well-mixed sample and/or sample dilution/volume is added to tubes containing predispensed media. Tubes are then capped and mixed vigorously to dissolve the media. Alternatively, this procedure can be performed by adding appropriate amounts of substrate media to a bulk diluted sample (with appropriate dilutions for enumeration), then mixing and dispensing into multiple-tubes. The number of tubes, and number of dilutions/volumes are determined based on the type, quality, and character of the water sample. A multiple-well procedure may be performed with sterilized disposable packets. The commercially available Quanti-Tray® or Quanti-Tray®/2000 multiple-well tests uses Colilert® or Colilert-18® media to determine E. coli (IDEXX, 1999b,c). In these tests, the packet containing media is added to a 100-mL sample (with appropriate dilutions for enumeration). The sample is then mixed and poured into the tray. A tray sealer separates the sample into 51 wells (Quanti-Tray) or 96 wells (Quanti-tray/2000) and seals the package which is subsequently incubated at 35 ± 0.5 °C for 18 h when using Colilert-18® or 24 h when using Colilert®. If the response is questionable after the specified incubation period, the sample is incubated for up to an additional 4 h at 35 ± 0.5 °C for both Colilert® tests.

After the appropriate incubation period, each tube or well is compared to the reference color ''comparator'' provided with the media. If the sample has a yellow color greater or equal to the comparator, the presence of total coliforms is verified, and the tube or well is then checked for fluorescence under long-wavelength UV light (366-nm). The presence of fluorescence greater than or equal to the comparator is a positive test for E. coli. If water samples contain humic acid or colored substances, inoculated tubes or wells should also be compared to a sample water blank. The concentration in MPN/100 mL is then calculated from the number of positive tubes or wells using MPN tables provided by the manufacturer.

c. CPRG–MUG (Standard Methods 9223B, Colisure™)

CPRG–MUG is a chromogenic/fluorogenic enzyme substrate test for the simultaneous determination of total coliforms and E. coli in water. These tests use a commercially available medium containing the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) to detect total coliforms, and the fluorogen MUG to detect E. coli. The sample is incubated for 24 ± 2 h at 35 ± 0.5 °C. If results are negative after 24 h, the sample is incubated up to an additional 4 h before calculating results. If the sample has changed from a yellow color to a red or magenta color, the presence of total coliforms is verified and the tube or well is then checked for fluorescence. The presence of blue fluorescence under a long-wavelength UV light (366-nm) is a positive test for E. coli. The concentration in MPN/100 mL is then calculated from the number of positive tubes or wells using MPN tables provided by the manufacturer. Colisure™ is a commercially available format of this method and uses the same quantitative formats (multiple-tube and multiple-well) available for the Colilert® tests. Colisure™ is subject to the same interferences and procedural cautions listed for the Colilert® tests.

Membrane Filter (MF) Tests for *E. coli*

a. mEndo, LES–Endo, or mFC followed by transfer to NA–MUG media (Standard Methods 9222B/9222G or 9222D/9222G)

These membrane filter methods for enumerating E. coli are two-step incubation procedures (APHA, 1998). First, a sample is filtered through a 0.45 μm filter, then the filter is placed on a pad saturated with mEndo broth or a plate containing mEndo or LES–Endo agar and incubated for 23 ± 1 h at 35 ± 0.5 °C. Pink to red colonies with a metallic (golden-green) sheen on the filter are considered to be total coliforms. If initial determination of fecal coliforms is desired or non-potable water samples are analyzed, mFC media can be substituted for mEndo/LES–Endo. Following initial isolation of total coliforms (or fecal coliforms), the filter is transferred to nutrient agar containing 4-methylumbelliferyl-β-D-glucuronide (NA–MUG) and incubated for 4 h at 35 ± 0.5 °C. Sheen colonies on mEndo that fluoresce under a long-wavelength UV light (366-nm) are positive for E. coli.

b. mTEC Agar (EPA Method 1103.1, Standard Methods 9212D, ASTM D5392–93)

The mTEC agar method is a two-step procedure that provides a direct count of E. coli in water based on the development of colonies on the surface of a membrane filter when placed on a selective nutrient and substrate media (USEPA, 1985a). This method originally was developed by EPA to monitor the quality of recreational water. This method was also used in health studies to develop the bacteriological ambient water quality criteria for E. coli. In this method, a water sample is filtered through a 0.45μm; membrane filter, the filter is placed on mTEC agar (a selective primary isolation medium), and the plate is incubated first at 35 ± 0.5 °C for 2 h to resuscitate injured or stressed bacteria and then at 44.5 ± 0.2 °C for 23 ± 1 h in a water bath. Following incubation, the filter is transferred to a filter pad saturated with urea substrate medium. After 15 minutes, all yellow or yellow-brown colonies (occasionally yellow-green) are

counted as positive for E. coli using a fluorescent lamp and either a magnifying lens or a stereoscopic microscope.

c. Modified mTEC Agar (Modified EPA Method 1103.1)

The modified mTEC agar method is a single-step MF procedure that provides a direct count of E. coli in water based on the development of colonies on the surface of a filter when placed on selective modified mTEC media (USEPA, 2000a). This is a modification of the standard mTEC media that eliminates bromcresol purple and bromphenol red from the medium, adds the chromogen 5-bromo-6-chloro-3-indoyl-β-D-glucuronide (Magenta Gluc), and eliminates the transfer of the filter to a second substrate medium. In this method, a water sample is filtered through a 0.45μm membrane filter, the filter is placed on modified mTEC agar, incubated at 35 ± 0.5 °C for 2 h to resuscitate injured or stressed bacteria, and then incubated for 23 ± 1 h in a 44.5 ± 0.2 °C water bath. Following incubation, all red or magenta colonies are counted as E. coli.

d. MI Agar

The MI agar method is a single-step procedure used to simultaneously enumerate total coliforms and E. coli (Brenner, 1993). In this EPA-developed method, a water sample is filtered through a 0.45μm membrane filter, the filter is placed on an MI agar plate, and the medium is incubated at 35 ± 0.5 °C for 24 h. As with NA–MUG and modified mTEC, the MI agar MF procedure is based on the ability of E. coli to produce the enzyme β-glucuronidase, which hydrolyzes Indoxyl-β-D-glucuronide (IBDG) to form a blue color (indigo). E. coli colonies exhibit a blue color and may also be fluorescent under a long-wavelength UV light (366-nm). If desired, the plates can also be observed under long-wavelength UV light (366-nm) for the presence of fluorescent total coliform species. Because the blue color from the breakdown of IBDG can mask fluorescence, non-fluorescent blue colonies are included in the total coliform count. Water samples with high turbidity can clog the membrane filter, interfering with filtration and potentially interfering with the identification of target colonies. However, E. coli colonies on MI agar can be counted on plates from waters containing high particulate or background/non-coliform concentrations, chlorine-stressed organisms or nutrient-deprived organisms, temperature-sensitive E. coli, and/or anaerogenic strains that may not be recovered by other multiple-tube or membrane filter tests.

e. m-ColiBlue24 Broth

This broth method is a single-step MF test for enumerating total coliforms and E. coli. As with NA–MUG, modified mTEC, and MI media, the selective identification of E. coli is based on the detection of the β-glucuronidase enzyme. The test medium includes the chromogen 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (BCIG or X-Gluc). The chromogen BCIG is hydrolyzed by β-glucuronidase, releasing an insoluble indoxyl salt that produces blue colonies. M-ColiBlue24 broth is a commercially available format of this method and contains a nutritive lactose-based medium containing inhibitors to eliminate the growth of non-coliforms. With m-ColiBlue24 broth, a water sample is filtered through a 0.45μm membrane filter, and the filter is transferred to a plate containing an absorbent pad saturated with m-ColiBlue24 broth. The filter is incubated at 35 ± 0.5 °C for 24 h and examined for colony growth (Hach, 1999). The presence of E. coli is indicated by blue colonies.

TABLE 2.—ANALYTES DETECTED BY PROPOSED MEDIA

Technique	Media	Total coliform [1]	Fecal coliform [1]	E. coli
Most Probable Number (MPN)	LTB→EC–MUG	X [2]		X
	ONPG–MUG	X		X
	CPRG–MUG	X		X
	mFC→NA–MUG		X	X
	mENDO→NA–MUG	X		X
	LES–ENDO→NA–MUG	X		X
Membrane Filter (MF)	mTEC			X
	Modified mTEC			X
	MI	X		X
	m-ColiBlue24 broth	X		X

[1] Detection of total coliform or fecal coliform are included because their detection may be preliminary steps required for E. coli enumeration and are part of the E. coli method.

[2] LTB is the presumptive test for total coliforms. It is necessary to transfer sample to BGLB for confirmation to determine total coliform density.

Method Comparison Studies

To confirm the applicability and comparability of results obtained with individual methods, parallel quantitative comparison tests with multiple-tube or MF tests, and positive and negative control tests should be conducted for each site-specific sample in accordance with analytical quality control procedures in Standard Methods for the Examination of Water and Wastewater. Performance data for E. coli multiple-tube, multiple-well, and MF methods are provided in Table 3.

TABLE 3.—STUDY COMPARISONS OF E. COLI PROPOSED METHODS

Methods compared or tested	Water type(s) tested	Study design/number of samples	Results [1]	Reference(s) [2]
MI agar compared to mEndo→NA–MUG and/or mTEC agar.	Wastewater, spiked drinking water, and non-potable water.	Two single laboratory studies (23 samples and 51 samples) and an interlaboratory study (19 labs, 6 samples each).	• Overall differences not statistically significant • MI agar: Specificity 95.7%; • MI agar: False Positive (FP) = 4.3%; • MI agar: False Negative (FN) = 4.3%	Brenner, 1993. Brenner, 1996a. Brenner, 1996b.
Colilert® compared to multiple-tube fermentation and membrane filtration.	Surface water	.....	• No significant difference in recovery of E. coli • Correlation Coefficient (r) for Colilert® ranged from 0.706 to 0.89	Cowburn, 1994. Edberg, 1988. Edberg, 1989. Ellgas, undated. Fricker, 1995. Fricker, 1996a. Palmer, 1993.
Colilert® compared to LTB→EC–MUG.	Surface water	47 split samples	Colilert® found to be equally sensitive to LTB→EC–MUG	Edberg, 1990.
mTEC agar compared to modified mTEC agar.	Surface water	Single-laboratory, 43 split-samples.	• E. coli recovery rates were not statistically different • mTEC agar: FP = 6%; FN = 5% • modified mTEC agar: FP = 0%; FN = 4%	EPA, 1999b.
mTEC agar compared to modified mTEC agar, MI agar, and Colilert®.	Beach water (recreational).	70 samples from three Lake Erie beaches.	• No statistically significant difference between MI agar and mTEC agar. Statistically significant differences between modified mTEC agar and/or Colilert and standard method • Modified mTEC agar: r = 0.966*; FP = 0%*; FN = 11%* • MI agar: r = 0.983*; FP = 3%*; FN = 4%* • Colilert: r = 0.946*; FP = 5%*; FN = 9%* • *Based on reference method (mTEC agar)	Francy, 1999.
m-ColiBlue24 broth, mEndo→NA–MUG, and mTEC agar.	Surface water, non-chlorinated wastewater, wastewater spiked drinking water, finished drinking water.	19 surface water samples, 3 non-chlorinated wastewaters, 2 wastewater spiked drinking water, and 1 finished drinking water.	• Overall agreement with the reference methods was 98.8% for m-ColiBlue24 broth and 92.1% for mTEC agar m-ColiBlue24 broth: FP = 2.5%; FN = 0%; Sensitivity = 100%; Specificity = 97.7%	Grant, 1997.
Colilert®, Colilert-18®, and mTEC agar.	Fresh recreational water	204 (Colilert®) samples and 193 (Colilert-18®) samples.	• No statistically significant difference between test results • r = 0.905 and 0.921 respectively	IDEXX, 1999d. IDEXX, 1999e.
Colilert®, most probable number, and membrane filtration.	Marine water, seawater spiked with sewage effluent.	22 laboratories using 13 common samples plus 2 external QC samples.	All three techniques provided comparable results on marine samples	Noble, 1999.
Colilert-18® and membrane filtration.	Untreated surface water	6 rivers draining into drinking water reservoirs.	• Both techniques provided comparable results	Ostensvik, 2000.
Colisure™ compared to EC–MUG (multiple-tube fermentation) and method for detection of chlorine-injured E. coli.	Primary effluent	21 samples from 7 different geographical locations and 31 samples from 6 different locations (for detection of chlorine-injured E. coli).	• Colisure™: FP = 4.3%; FN = 2.4% • Detection of chlorine-injured E. coli: Colisure ™ had an average of 1.76 times more E. coli-positive results after 28 hours than the standard method	59 FR 35891, 1994.

[1] Methods of determining false positive and false negative rates were not standardized for all comparison studies.
[2] Complete reference information is provided in Section VI.

4. Methods for Enterococci

EPA is proposing several methods for enumerating enterococci in water. Brief descriptions of the proposed MPN and MF methods are provided below. In Table 4, methods in the same horizontal row use the same technique, but are published by different entities.

TABLE 4.—PROPOSED METHODS FOR ENTEROCOCCI ENUMERATION.[1, 2]

Methodology	Method[3]	EPA method	VCS method[4]			Commercial example
			Standard Methods	ASTM	AOAC	
Most Probable Number (MPN)	Azide dextrose/PSE/BHI		9230B			
	MUG media			D6503–99		Enterolert™[4]
Membrane Filter (MF)	mE→EIA agar	1106.1	9230C	D5259–92		
	mEI agar	1600				

[1] Complete reference information is provided in Section VI.
[2] A media acronym table is provided in Section V.
[3] Tests must be conducted in a format that provides organism enumeration.
[4] Manufactured by IDEXX.

Most Probable Number (MPN) Tests for Enterococci

a. Azide Dextrose/PSE/BHI (Standard Methods 9230B)

The Azide Dextrose/PSE/BHI technique for enumerating enterococci in water uses multiple-tubes and dilutions/volumes in a three-step procedure (presumptive fecal streptococcus, confirmed fecal streptococcus, and enterococcus) to determine enterococci concentrations (APHA, 1998). In the presumptive phase, multiple-tubes containing azide dextrose are inoculated with sample and mixed with the broth by gentle agitation. Inoculated tubes are incubated for 24 ± 2 h at 35°C ± 0.5 °C. Each tube then is swirled and examined for turbidity. If turbidity is absent, tubes are incubated for an additional 24 h and re-examined. Production of turbidity within 48 ± 3 h constitutes a positive presumptive reaction for fecal streptococci.

After enrichment during the presumptive phase, positive azide dextrose tubes are subjected to a confirmation step for fecal streptococci. A portion of growth from each positive azide dextrose tube is streaked on Pfizer selective Enterococcus (PSE) agar using a sterile loop. Inverted plates are incubated at 35 °C ± 0.5 °C for 24 ± 2 h and observed for the presence of brownish-black colonies with a brown halo. Such colonies are confirmed as fecal streptococci.

Target colonies from the PSE medium can be transferred to a tube of brain-heart infusion (BHI) broth and incubated at 45 °C ± 0.5 °C for 48 h. Simultaneously, these colonies can be transferred to BHI broth containing 6.5% NaCl and incubated at 35 °C ± 0.5 °C for 48 h. Growth at both 45 °C in BHI medium and in BHI medium containing 6.5% NaCl at 35 °C is indicative of the Enterococcus bacterial group. The concentration in MPN/100 mL is then calculated from the number of positive 6.5% NaCl broth tubes using MPN tables or formulas.

b. 4-methylumbelliferyl-β-D-glucoside (MUG) Medium (ASTM D6503–99, Enterolert™)

This method utilizes a medium contaning the fluorogenic substrate 4-methylumbelliferyl-β-D-glucoside (MUG) to determine enterococci concentrations. Enterolert™ is a commercially available test that utilizes this substrate test for the determination of enterococci in water (IDEXX, 1999f). Enterolert™ tests are incubated for 24 h at 41 ± 0.5 °C and may use the same quantitative formats available for the Colilert® tests, cited earlier in Section III–A. After incubation, the presence of blue/white fluorescence is a positive result for enterococci. The concentration in MPN/100 mL is then calculated from the number of positive tubes or wells using MPN tables provided by the manufacturer. Enterolert™ is subject to the same interferences and cautions listed for the Colilert® tests. In addition, marine water samples must be diluted at least tenfold with sterile, non-buffered freshwater (Enterolert™ is already buffered).

Membrane Filter (MF) Tests for Enterococci

a. mE→EIA Agar (EPA 1106.1, Standard Methods 9230C, ASTM D5259–92)

The mEI agar method is a two-step MF procedure that provides a direct count of bacteria in water, based on the development of colonies on the surface of a membrane filter when placed on a selective nutrient medium (USEPA, 1985b). A water sample is filtered through a 0.45µm membrane filter, and the filter is placed on a plate containing selective mE agar. After the plate is incubated at 41 ± 0.5 °C for 48 h, the filter is transferred to an Esculin iron agar (EIA) plate and incubated at 41 ± 0.5 °C for 20–30 min. After incubation, all pink to red colonies on mE agar that form a black or reddish-brown precipitate on the underside of the filter when placed on EIA are counted as enterococci. Organism density is reported as enterococci per 100 mL.

b. mEI Agar (EPA Method 1600)

The mEI agar method is a single-step MF procedure that provides a direct count of bacteria in water, based on the development of colonies on the surface of a filter when placed on selective mEI agar (USEPA, 1997). This medium, a modification of the mE agar in EPA Method 1106.1, contains a reduced amount of 2-3-5-triphenyltetrazolium chloride, and an added chromogen, Indoxyl-β-D-glucoside. The transfer of the filter to EIA is eliminated, thereby providing results within 24 h. In this method, a water sample is filtered, and the filter is placed on mEI agar and incubated at 41 ± 0.5 °C for 24 h. Following incubation, all colonies with a blue halo, regardless of colony color, are counted as enterococci. Results are reported as enterococci per 100 mL.

Method Comparison Studies

To confirm the applicability and comparability of results obtained with individual methods, parallel quantitative comparison tests with multiple-tube or MF tests, and positive and negative control tests should be conducted for each site-specific sample in accordance with analytical quality control procedures in Standard Methods for the Examination of Water and Wastewater. Performance data for enterococci multiple-tube, multiple-well, and MF methods are provided in Table 5.

TABLE 5.—STUDY COMPARISONS OF ENTEROCOCCI PROPOSED METHODS

Methods compared or tested	Water type(s) tested	Study design/Number of samples	Results [1]	Reference(s) [2]
Enterolert™ compared to mE→EIA agar.	Recreational bathing water, tidal lagoons, water from marinas, untreated effluents, and marine water from stormwater-drainage sites.	343 samples	• Data indicated a strong linear correlation (r = 0.927) and no significant difference between the two methods (p = 0.39). • Enterolert™: False Positive (FP) = 2.4%; False Negative (FN) = 0.3%; Sensitivity = 99.8%; Specificity = 97.0%.	Abbott, 1998.
Enterolert™ compared to mE→EIA agar.	Marine and freshwater recreational bathing samples.	138 samples Connecticut Department of Public Health.	• When analyzing the entire sample population, there were no significant differences between the two methods. • Results classified by sample type (freshwater v. marine) showed a greater difference between the two methods. • Enterolert™ FP = 5.1%; FN = 0.4%.	Budnick, 1996.
Enterolert™ compared to mE→EIA agar.	Drinking water, freshwater, marine water, and untreated effluents.	821 samples	• Correlation coefficient (r) of 0.91 between the two methods. • Enterolert™: FP = 4.9%; FN = 0.6%.	Chen, 1996.
Enterolert™ compared to mE→EIA agar.	River water (323), partially treated effluent (516), treated effluents (620), and finished drinking water (1012).	2471 samples Thames Water Utilities	• r = 0.91 • Overall Enterolert™ detected enterococci in more samples and had fewer false positives, but these differences were not statistically significant. • Enterolert™: FP = 4.5% • mE-EIA: FP = 6.2%	Fricker, 1996.
mE→EIA agar compared to mEI agar.	Freshwater and marine water.	176 samples (including 44 duplicates). Single-laboratory study EPA Region 1	• No significant difference between the two methods. • mE→EIA agar: FP = 4%; FN = 8%; RPD= 38.7%. • mEI agar: FP = 2%; FN = 7%; RPD = 45.2%.	Liebman, 1999.
mE→EIA agar compared to mEI agar.	Surface water, non-chlorinated primary effluent, chlorinated secondary effluent, and marine waters.	Single-laboratory study Samples analyzed in duplicate	• No significant difference between the two methods. • mEI agar: FP = 6%; FN = 6.5%	Messer, 1998.
Azide Dextrose/PSE/BHI, mE→EIA agar, and Enterolert™.	Marine water, seawater spiked with sewage effluent.	22 laboratories using 13 common samples plus 2 external QC samples.	• Methods provide comparable results. • Average difference among methods was less than 6%.	Noble, 1999.
Azide Dextrose/PSE/BHI, mE→EIA agar, mEI agar, and Enterolert™.	Seawater samples from randomly selected sites.	7 labs performed side-by-side analyses on approximately 280 samples.	• Idexx vs. Standard Method: r = 0.1; correspondence = 88%*. • mEI agar vs. Standard Method: r = 0.9 correspondence = 99%. • mEI agar vs. Enterolert™: r = 0.89 correspondence = 97%. • Enterolert™ produced concentrations above the State threshold while standard methods produced results below for all samples with contradictory results.	Noble, 2000a.
mE→EIA agar, mEI agar, and Enterolert™.	Seawater samples from 79 randomly selected sites (31 open beach sites and 48 sites within 100 meters of a freshwater outlet).	6 labs performed side-by-side split sample analyses on approximately 48 samples.	• Enterolert™“ vs. mEI agar: r = 0.93. • Enterolert™ vs. mE→EIA agar: r = 0.94.	Noble, 2000b.

[1] Methods of determining false positive and false negative rates were not standardized for all comparison studies.
[2] Complete reference information is provided in Section VI.

5. Request for Comment and Available Data

EPA requests public comments on the proposed methods for the bacterial indicators of fecal contamination. EPA invites comments on the technical merit, applicability, and implementation of the proposed *E. coli* and *enterococci* methods for ambient water monitoring. EPA also requests public comments on whether E. coli methods that are also applicable to total coliforms should be approved for determination of total coliforms in the final rule. Commenters should specify the method and bacteria/organisms to which the comment applies. EPA encourages commenters to provide copies of supporting data or references cited in comments. EPA also requests public comments on acceptable characteristics of these test methods for specific matrix applications, on comparability criteria to determine equivalency of alternative test methods, supporting data, and examples of any available alternative equivalency testing protocols. Additionally, EPA requests comments on any other applicable methods for analyzing *E. coli* and *enterococci* in ambient water not included in today's proposal. Method descriptions and supporting data may be submitted for additional test procedures that are applicable to enumerating these bacteria in ambient water.

B. Methods for Protozoa

EPA developed and validated two methods for determination of protozoan concentrations in ambient waters to support ongoing voluntary monitoring of ambient waters used as source waters for drinking water treatment plants. EPA validated Method 1622 for the determination of *Cryptosporidium* in ambient water in August 1998 and issued a validated draft method in January 1999. EPA validated Method 1623 for the simultaneous determination of *Cryptosporidium* and *Giardia* in ambient water in February 1999 and issued a validated draft method in April 1999. Methods 1622 and 1623 were revised and updated as a result of revised quality control criteria and the development of equivalent filters for use with the methods (USEPA, 2001c). The updates to Method 1622 (EPA–821–R–01–026) and Method 1623 (EPA–821–R–01–025) are proposed in today's rule.

1. Cryptosporidium and Giardia

Discussions of Methods 1622 and 1623 are combined for today's rule since all use essentially the same methodology: filtration, concentration, immunomagnetic separation of oocysts and cysts from captured material, immunofluorescence assay to determine presumptive concentrations, and confirmation through vital dye staining and differential interference contrast (DIC) microscopy for the detection of *Cryptosporidium* oocysts and *Giardia* cysts.

A 10- to 50-L volume of water is filtered and the oocysts, cysts, and extraneous materials are retained on the filter. Elution of the materials on the filter is accomplished with an aqueous buffered salt and detergent solution. The oocysts and cysts are concentrated through centrifugation, and the supernatant fluid is aspirated. Oocysts and cysts are captured by the attachment of magnetic beads conjugated to anti-*Cryptosporidium* and anti-*Giardia* antibodies. The oocysts and cysts are magnetically separated from the extraneous materials, and the extraneous materials are discarded. The magnetic beads are then detached from the oocysts and cysts. The oocysts and cysts are prepared on well slides and stained with fluorescently-labeled monoclonal antibodies and 4′,6-diamidino-2-phenylindole (DAPI). The stained sample is examined using fluorescence and differential interference contrast (DIC) microscopy. Qualitative analysis is performed by carefully scanning each slide well for objects that have the size, shape, and fluorescence characteristics of *Cryptosporidium* oocysts or *Giardia* cysts. Potential oocysts or cysts are confirmed through DAPI staining characteristics and DIC microscopy. Oocysts and cysts are identified when the size, shape, color, and morphology agree with specified criteria and examples in a photographic library. Quantitative analysis is performed by counting the total number of objects confirmed as oocysts or cysts on the slide.

The Method 1622 interlaboratory validation study (EPA–821–R–01–027) was conducted in August 1998 and involved 12 laboratories that analyzed spiked reagent water and raw surface water samples. Eleven laboratories participated in the Method 1623 interlaboratory validation study (EPA–821–R–028) conducted in 1999. Both the interlaboratory validation studies for Methods 1622 and 1623 followed the same approach for preparing spiked suspensions for single-blind test samples. The *Cryptosporidium* results obtained during the Method 1623 study were not statistically different from the *Cryptosporidium* results obtained during the Method 1622 interlaboratory validation study.

2. Request for Comment and Available Data

EPA requests public comments on the proposed methods for the protozoan pollutants. EPA invites comments on the technical merit, applicability, and implementation of the proposed *Cryptosporidium* and *Giardia* methods for ambient water monitoring. Commenters should specify the method and pollutant to which the comment specifies. EPA encourages commenters to provide copies of supporting data or references cited in comments. Additionally, EPA requests comments on any other applicable methods for analyzing for *Cryptosporidium* and *Giardia* in ambient water not included in today's proposal. Method descriptions and supporting data may be submitted for additional test procedures that are applicable to enumerating these protozoa in water.

IV. Administrative Requirements

A. Executive Order 12866: Regulatory Planning and Review

Under Executive Order 12866, [58 FR 51735; (October 4, 1993)], the Agency must determine whether a regulatory action is ''significant'' and therefore subject to Office of Management and Budget (OMB) review and the requirements of the Executive Order. The Executive Order defines ''significant regulatory action'' as one that is likely to result in a rule that may:

(1) Have an annual effect on the economy of $100 million or more or adversely affect in a material way the economy, a sector of the economy, productivity, competition, jobs, the environment, public health or safety, or State, local, or tribal governments or communities.

(2) Create a serious inconsistency or otherwise interfere with an action taken or planned by another agency.

(3) Materially alter the budgetary impact of entitlements, grants, user fees, or loan programs or the rights and obligations of recipients thereof.

(4) Raise novel legal or policy issues arising out of legal mandates, the President's priorities, or the principles set forth in the Executive Order.''

It has been determined that this rule is not a ''significant regulatory action'' under the terms of Executive Order 12866 and is therefore not subject to OMB review.

B. Unfunded Mandates Reform Act

Title II of the Unfunded Mandates Reform Act of 1995 (UMRA), Pub. L.

104–4, establishes requirements for Federal agencies to assess the effects of their regulatory actions on State, Tribal, and local governments and the private sector. Under section 202 of the UMRA, EPA generally must prepare a written statement, including a cost-benefit analysis, for proposed and final rules with ''Federal mandates'' that may result in expenditures to State, Tribal, and local governments, in the aggregate, or to the private sector, of $100 million or more in any one year.

Before promulgating an EPA rule for which a written statement is needed, section 205 of the UMRA generally requires EPA to identify and consider a reasonable number of regulatory alternatives and adopt the least costly, most cost-effective or least burdensome alternative that achieves the objectives of the rule. The provisions of section 205 do not apply when they are inconsistent with applicable law. Moreover, section 205 allows EPA to adopt an alternative other than the least costly, most cost-effective or least burdensome alternative if the Administrator publishes with the final rule an explanation of why that alternative was not adopted. Before EPA establishes any regulatory requirements that may significantly or uniquely affect small governments, including Tribal governments, it must have developed under section 203 of the UMRA a small government agency plan. The plan must provide for the notification of potentially affected small governments, enabling officials of affected small governments to have meaningful and timely input in the development of EPA regulatory proposals with significant Federal intergovernmental mandates, and informing, educating, and advising small governments on compliance with the regulatory requirements.

Today's proposed rule contains no Federal mandates (under the regulatory provisions of title II of the UMRA) for State, Tribal, or local governments or the private sector that may result in expenditures of $100 million or more in any one year. This rule makes available testing procedures for E. coli, enterococci, Cryptosporidium, and Giardia that may be used by a State, Territorial, Tribal or local authority for compliance with water quality standards or ambient monitoring requirements when testing is otherwise required by these regulatory authorities. Thus, today's rule is not subject to the requirements of sections 202 and 205 of the UMRA.

EPA has also determined that this rule contains no regulatory requirements that might significantly or uniquely affect small governments. As discussed below, under the Regulatory Flexibility Act, the economic impact on small entities is anticipated to be small. It would not significantly affect them because any incremental costs incurred are small and it would not uniquely affect them because it would affect entities of all sizes depending upon whether testing for these bacteria or protozoa is otherwise required by a regulatory authority. Further, monitoring for small entities is generally expected to be less frequent than monitoring for larger entities. Thus, today's rule is not subject to the requirements of sections 203 of UMRA.

C. Regulatory Flexibility Act (RFA),as amended by the Small Business Regulatory Enforcement Act of 1996 (SBREFA), 5 U.S.C. 601 et seq.

The RFA generally requires an agency to prepare a regulatory flexibility analysis of any rule subject to notice and comment rulemaking requirements under the Administrative Procedure Act or any other statute unless the agency certifies that the rule will not have a significant economic impact on a substantial number of small entities. Small entities include small businesses, small organizations, and small governmental jurisdictions.

For purposes of assessing the impacts of today's rule on small entities, small entity is defined as: (1) A small business as defined by the Small Business Administration definition of small business; (2) a small governmental jurisdiction that is a government of a city, county, town, school district or special district with a population of less that 50,000; and (3) a small organization that is any not-for-profit enterprise which is independently owned and operated and is not dominant in its field.

After considering the economic impacts of today's proposed rule on small entities, I certify that this action will not have a significant economic impact on a substantial number of small entities. This proposed regulation would approve testing procedures for the measurement of E. coli and enterococci bacteria, and Cryptosporidium and Giardia protozoa in ambient water. EPA anticipates that the methods will be used by State regulatory authorities for evaluating attainment of water quality standards or ambient monitoring requirements. EPA NPDES regulations do not require monitoring of ambient water conditions in NPDES permits. In a few instances, ambient water monitoring requirements may be included in an EPA-issued permit where site-specific circumstances warrant. EPA regulations do, require NPDES permittees to use EPA-approved test methods for all monitoring data reported to the Agency (40 CFR 122.21). Consequently, to the extent that an NPDES permit requires monitoring and reporting of ambient water for E. coli, enterococci, Cryptosporidium, or Giardia (and NPDES regulations require the use of EPA-approved methods for all monitoring), EPA approval of these test methods arguably may impose costs on NPDES permit holders, including small entities. EPA is unaware, however, of any EPA-issued NPDES permits that currently require monitoring of ambient water for such pollutants. Hence, EPA does not expect approval of these methods to impose any additional costs as a result of their applicability to EPA-issued permits. As noted above, EPA's NPDES regulations do not require monitoring of ambient water conditions. Consequently, to the extent that a State requires such monitoring, those requirements are imposed under State, rather than federal, authority. Because States have the discretion *not* to require such monitoring, any increased costs to small entities arising from use of the methods proposed for approval by EPA today that are imposed as a result of State law are not attributable to this regulation.

Nonetheless, EPA evaluated these potential costs to determine whether EPA approval of the methods will have a significant impact on a substantial number of small entities. As previously noted, States may require ambient water monitoring to evaluate attainment of water quality standards. A few States currently require NPDES permit holders to monitor ambient water. Thus, some NPDES permittees are already testing ambient water for these parameters. Hence, the impact of using EPA-approved methods for such dischargers may represent little or no increased burden.

The small entities that might be affected by this rule include small governmental jurisdictions that have publically-owned treatment works (POTWs) and small businesses with water quality-based discharge permits. EPA looked first at the potential cost of the E. coli and enterococci methods proposed today. EPA conducted a survey of State, municipal, and commercial laboratories that routinely conduct bacterial analysis of water to compare the incremental analytical costs for existing total and fecal coliform methods already employed by many water quality monitoring programs with the methods proposed here. The mean analytical costs for total and fecal coliform were $22 ($15–48) and $21

($15–$35), respectively. The mean analytical costs for E. coli and enterococci were $22 ($10–$35) and $32 ($25–$50), respectively. The similarity of costs for total and fecal coliform versus E. coli and enterococci methods is expected since the analytical procedures used to determine these pollutants generally employ similar techniques, media, equipment, and require comparable laboratory time and effort to complete analysis. Some States are already using the proposed test methods for E. coli and enterococci in State ambient water quality monitoring programs (indeed, EPA is proposing to approve consensus methods for enumerating E. coli and enterococci in ambient waters. See section IV.E, below) and thus this rule would formalize current practice in those States. Furthermore, EPA expects that any modest potential increase in costs for enterococci analyses will be reduced once the proposed methods are broadly implemented by environmental laboratories and State water quality monitoring programs.

Next, EPA looked at the costs for testing for Cryptosporidium and Giardia. The range in cost for Methods 1622 and 1623 analysis of Cryptosporidium and Giardia is between $400–$500 for each method. As stated in section IV.E. below, EPA is not aware of any other acceptable test methods currently available for monitoring these pollutants. Methods 1622 and 1623 have been previously used for monitoring of various drinking water plant source waters to establish a national estimate of Cryptosporidium and Giardia occurrence. Because of the relatively high costs, EPA does not anticipate that these test methods will be used for daily or ongoing monitoring, but may be used program-specific occurrence assessments.

The purpose of this rule is only to make these methods available to States, Tribal and municipalities that may want to use them for ambient water monitoring. As noted above, the costs associated with Cryptosporidium and Giardia analysis would not be a Federally-mandated cost, but rather would flow from a State's adoption of ambient monitoring requirements. The inclusion of these test methods in section 136.3 is intended to make these test methods available to States and others for use in water quality monitoring programs. EPA is not establishing any compliance monitoring requirements for these pollutants.

D. Paperwork Reduction Act

This action does not impose an information collection burden under the provisions of the Paperwork Reduction Act, 44 U.S.C. 3501 *et seq*. This rule proposes to make available new test methods for E. coli, enterococci, Cryptosporidium and Giardia for use in ambient water monitoring programs but EPA would not require the use of these test methods.

Burden means the total time, effort, or financial resources expended by persons to generate, maintain, retain, or disclose or provide information to or for a Federal agency. This includes the time needed to review instructions; develop, acquire, install, and utilize technology and systems for the purposes of collecting, validating, and verifying information, processing and maintaining information, and disclosing and providing information; adjust the existing ways to comply with any previously applicable instructions and requirements; train personnel to be able to respond to a collection of information; search data sources; complete and review the collection of information; and transmit or otherwise disclose the information.

An Agency may not conduct or sponsor, and a person is not required to respond to a collection of information unless it displays a currently valid OMB control number. The OMB control numbers for EPA's regulations are listed in 40 CFR part 9 and 48 CFR chapter 15.

E. National Technology Transfer and Advancement Act

Section 12(d) of the National Technology Transfer and Advancement Act (NTTAA) of 1995, Public Law 104–113, section 12(d) (15 U.S.C. 272 note), directs EPA to use voluntary consensus standards in its regulatory activities unless to do so would be inconsistent with applicable law or otherwise impractical. Voluntary consensus standards are technical standards (e.g., material specifications, test methods, sampling procedures, business practices) that are developed or adopted by voluntary consensus standard bodies. The NTTAA directs EPA to provide Congress, through the Office of Management and Budget (OMB), explanations when the EPA decides not to use available and applicable voluntary consensus standards. This rulemaking involves technical standards. Therefore, the Agency conducted a search to identify potentially applicable voluntary consensus standards. EPA's search of the technical literature revealed several consensus methods appropriate for enumerating E. coli and enterococci in ambient waters. Accordingly, methods for E. coli and enterococci published by Standard Methods for the Examination of Water and Wastewater, ASTM, and AOAC are included in this proposal and are listed in Table IA at the end of this notice (see footnotes 4,10, and 11, respectively, for the complete citations). No voluntary consensus standards were found for Cryptosporidium or Giardia. EPA welcomes comments on this aspect of the proposed rulemaking and, specifically, invites the public to identify potentially applicable voluntary consensus standards for enumerating E. coli, enterococci, Cryptosporidium, and Giardia in ambient waters, and to explain why such standards should be used in this regulation.

F. Executive Order 13045—Protection of Children From Environmental Health Risks and Safety Risks

Executive Order 13045, (62 FR 19885, April 23, 1997) applies to any rule that: (1) Is determined to be ''economically significant'' as defined under Executive Order 12866, and (2) concerns an environmental health or safety risk that EPA has reason to believe may have a disproportionate effect on children. If the regulatory action meets both criteria, the Agency must evaluate the environmental health or safety effects of the planned rule on children, and explain why the planned regulation is preferable to other potentially effective and reasonably feasible alternatives considered by the Agency.

This proposed rule is not subject to the Executive Order because it is neither economically significant as defined in Executive Order 12866, nor does it concern an environmental health or safety risk that EPA has reason to believe may have a disproportionate effect on children.

G. Executive Order 13175: Consultation and Coordination With Indian Tribal Governments

Executive Order 13175, entitled ''Consultation and Coordination with Indian Tribal Governments'' (65 FR 67249, November 6, 2000), requires EPA to develop an accountable process to ensure ''meaningful and timely input by tribal officials in the development of regulatory policies that have tribal implications.'' ''Policies that have tribal implications'' is defined in the Executive Order to include regulations that have ''substantial direct effects on one or more Indian tribes, on the relationship between the Federal government and the Indian tribes, or on the distribution of power and responsibilities between the Federal government and Indian tribes.''

This proposed rule does not have tribal implications. It will not have substantial direct effects on tribal

governments, on the relationship between the Federal government and Indian tribes, or on the distribution of power and responsibilities between the Federal government and Indian tribes, as specified in Executive Order 13175. This proposed rule makes available test methods that may be used by a regulatory authority to demonstrate compliance with ambient water quality monitoring or water quality standards. However, Federal regulations do not require the use of these test methods. Thus, Executive Order 13175 does not apply to this rule.

In the spirit of Executive Order 13175, and consistent with EPA policy to promote communications between EPA and tribal governments, EPA specifically solicits additional comment on this proposed rule from tribal officials.

H. Executive Order 13132: Federalism

Executive Order 13132, entitled ''Federalism'' (64 FR 43255, August 10, 1999), requires EPA to develop an accountable process to ensure ''meaningful and timely input by State and local officials in the development of regulatory policies that have federalism implications.'' ''Policies that have federalism implications'' is defined in the Executive Order to include regulations that have ''substantial direct effects on the States, on the relationship between the national government and the States, or on the distribution of power and responsibilities among the various levels of government.''

This proposed rule does not have federalism implications. It will not have substantial direct effects on the States, on the relationship between the national government and the States, or on the distribution of power and responsibilities among the various levels of government, as specified in Executive Order 13132. This proposed rule makes new analytical methods available for conducting analysis of ambient water for enumeration of E.coli, enterococci, Cryptosporidium, or Giardia. EPA does not, however, propose to require use of these methods under this rule. Thus, Executive Order 13132 does not apply to this rule.

Although Section 6 of Executive Order 13132 does not apply to this rule, EPA did consult with representatives of State and local governments in developing the proposed regulation. In fact, it was State representatives who requested that EPA include test methods for these biological pollutants in section 136.3 because they want to use EPA-approved test methods for ambient water monitoring. EPA is proposing this action in response to these requests. EPA included a number of test methods currently being used by States for these pollutants in today's proposed rulemaking. No significant concerns were raised about these methods.

In the spirit of Executive Order 13132, and consistent with EPA policy to promote communications between EPA ans State and local governments, EPA specifically solicits comment on this proposed rule from State and local officials.

I. Executive Order 13211: Actions Concerning Regulations That Significantly Affect Energy Supply, Distribution, or Use

Executive Order 13211, ''Actions Concerning Regulations That Significantly Affect Energy Supply, Distribution, or Use'' (66 FR 28355 (May 22, 2001)), provides that agencies shall prepare and submit to the Administrator of the Office of Information and Regulatory Affairs, Office of Management and Budget, a Statement of Energy Effects for certain actions identified as ''significant energy actions.'' Section 4(b) of Executive Order 13211 defines ''significant energy actions'' as ''any action by an agency (normally published in the **Federal Register**) that promulgates or is expected to lead to the promulgation of a final rule or regulation, including notices of inquiry, advance notices of proposed rulemaking, and notices of proposed rulemaking: (1)(i) that is a significant regulatory action under Executive Order 12866 or any successor order, and (ii) is likely to have a significant adverse effect on the supply, distribution, or use of energy; or (2) that is designated by the Administrator of the Office of Information and Regulatory Affairs as a significant energy action.''

We have not prepared a Statement of Energy Effects for this proposed rule because this rule is not a significant energy action, as defined in Executive Order 13211. This is not a significant regulatory action under Executive Order 12866, and it does not have a significant adverse effect on the supply, distribution, or use of energy.

V. Media Acronyms

BHI—brain-heart infusion agar
BGLB—brilliant green lactose bile broth
CPRG—chlorophenol red-β-D-galactopyranoside
DAPI—4′,6-diamidino-2-phenylindole
DIC—differential interference contrast
EC—E. coli
EIA—esculin iron agar
LES-Endo—Lawrence Experimental Station—Endo Agar
LTB—lauryl tryptose broth
mEI—membrane-Enterococcus iron agar
mFC—membrane-Fecal coliform agar
mTEC—membrane-Thermotolerant E. coli agar
MUG—4-methylumbelliferyl-β-D-glucuronide
NA—nutrient agar
ONPG—ortho-nitorphenyl-β-D-galactopyranoside
PSE—Pfizer selective Enterococcus agar

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List of Subjects in 40 CFR Part 136

Environmental protection, Reporting and recordkeeping requirements, Water pollution control.

Dated: August 23, 2001.

Christine Todd Whitman,

Administrator.

For the reasons set out in the preamble, title 40, chapter I of the Code of Federal Regulations, is proposed to be amended as follows:

PART 136—GUIDELINES ESTABLISHING TEST PROCEDURES FOR THE ANALYSIS OF POLLUTANTS

1. The authority citation for Part 136 continues to read as follows:

Authority: Secs. 301, 304(h), 307, and 501(a) Pub. L. 95–217, 91 Stat. 1566, *et seq.* (33 U.S.C. 1251, *et seq.*) (The Federal Water Pollution Control Act Amendments of 1972 as amended by the Clean Water Act of 1977.)

2. Section 136.3 is amended:

a. In paragraph (a) by revising Table IA.

b. By revising paragraphs (b)(10) and (b)(11), adding and reserving paragraphs (b)(44) to (b)(50), and adding paragraphs (b)(51) through (b)(60).

c. In paragraph (e) by revising the entries in Table II for Table IA and adding a new footnote 17.

§ 136.3 Identification of test procedures.

(a) * * *

TABLE IA.—LIST OF APPROVED BIOLOGICAL METHODS

Parameter and units	Method [1]	EPA	Standard methods 18th, 19th, 20th Ed.	ASTM	AOAC	USGS	Other
Bacteria:							
1. Coliform (fecal), number per 100 mL.	Most Probable Number (MPN), 5 tube. 3 dilution, or Membrane filter (MF) [2], single step.	p.132 [3]	9221C E [4]				
		p. 124 [3]	9222D [4]			B–0050–85 [5]	
2. Coliform (fecal) in presence of chlorine, number per 100 mL.	MPN, 5 tube, 3 dilution, or. MF, single step [6]	p. 132 [3]	9221C E [4]				
		p. 124 [3]	9222D [4].				
3. Coliform (total), number per 100 mL	MPN, 5 tube, 3 dilution, or. MF [2], single step or two step.	p. 114 [3]	9221B [4]				
		p. 108 [3]	9222B [4]			B–0025–85 [5]	
4. Coliform (total), in presence of chlorine, number per 100 mL	MPN, 5 tube, 3 dilution, or. MF2 with enrichment	p. 114 [3]	9221B [4]				
		p. 111 [3]	9222(B+B.5c) [4].				
5. E. coli, number per 100 mL [29]	MPN [7,9,15]	.....	9221B.1/ 9221F [4,12,14].				
			9223B [4,13]	.....	[11] 991.15	.....	Colilert® [13,18] Colilert-® [13,16,18] Colisure ™ [13,17,18]
	MF [2,6,7,8,9]	.....	9222B/ 9222G [4,20].				
		1103.1 [21] ..... 1103.1M [22]. MI agar [23].	921D [4]	53592–93 [10]			
		.....	.....			.....	mColiBlue24 [19]
6. Fecal streptococci, number per 100 mL	MPN, 5 tube, 3 dilution, mf [2], or Plate count	p. 139 [3]	9230B [4]				
		p. 136 [3]	9230C [4]			B–0055–85 [5]	
		p. 143 [3]					
7. Enterococci, number per 100 mL [29]	MPN [7,9]	.....	9230B [4].				
				D6503–99 [10]		.....	Enterolert ™ [13,24]
	MF [2,6,7,8,9]	1106.1 [25] 1600 [26]	9230C [4]	D5259–92 [10]			
	Plate count	p. 14 [33]					
Protozoa:							
8. Cryptosporidium [29]	Filtration/IMS/FA	1622 [27] 1623 [28]					
9. Giardia [29]	Filtration/IMS/FA	1623 [28]					
Aquatic Toxicity:							

TABLE IA.—LIST OF APPROVED BIOLOGICAL METHODS—Continued

Parameter and units	Method [1]	EPA	Standard methods 18th, 19th, 20th Ed.	ASTM	AOAC	USGS	Other
10. Toxicity, acute, fresh water organisms, LC50, percent effluent.	Daphnia, Ceriodaphnia, Fathead Minnow, Rainbow Trout, Brook Trout, or Bannerfish Shiner mortality.	Sec. 9 [30].					
11. Toxicity, acute, estuarine and marine organisms, LC50, percent effluent.	Mysid, Sheepshead Minnow, or Menidia spp. mortality.	Sec. 9 [30].					
12. Toxicity, chronic, fresh water organisms, NOEC or IC25, percent effluent.	Fathead minnow larval survival and growth.	1000.0 [31]					
	Fathead minnow embryo-larval survival and teratogenicity.	1001.0 [31]					
	Ceriodaphnia survival and reproduction.	1002.0 [31]					
	Selenastrum growth.	1000.0 [32].					
13. Toxicity, chronic, estuarine and marine organisms, NOEC or IC25, percent effluent.	Sheepshead minnow larval survival and growth.	1004.0 [31]					
	Sheepshead minnow embryo-larval survival and teratogenicity.	1005.0 [32]					
	Menidia beryllina larval survival and growth.	1006.0 [32]					
	Mysidopsis bahia survival, growth, and fecundity.	1007.0 [32]					
	Arbacia punctulata fertilization.	1008.0 [32]					
	Champia parvula reproduction 1004.032.	1009.0 [32]					

Notes to Table IA:

[1] The method must be specified when results are reported.

[2] A 0.45 μm membrane filter (MF) or other pore size certified by the manufacturer to fully retain organisms to be cultivated and to be free of extractables which could interfere with their growth.

[3] USEPA. 1978. Microbiological Methods for Monitoring the Environment, Water, and Wastes. Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio. EPA/600/8–78/017.

[4] APHA. 1998, 1995, 1992. Standard Methods for the Examination of Water and Wastewater. American Public Health Association. 20th, 19th, and 18th Editions. Amer. Publ. Hlth. Assoc., Washington, DC.

[5] USGS. 1989. U.S. Geological Survey Techniques of Water-Resource Investigations, Book 5, Laboratory Analysis, Chapter A4, Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples, U.S. Geological Survey, U.S. Department of Interior, Reston, Virginia.

[6] Because the MF technique usually yields low and variable recovery from chlorinated wastewaters, the Most Probable Number method will be required to resolve any controversies.

[7] Tests must be conducted to provide organism enumeration (density). Select the appropriate configuration of tubes/filtrations and dilutions/volumes to account for the quality, character, consistency, and anticipated organism density of the water sample.

[8] When the MF method has not been used previously to test ambient waters with high turbidity, large number of noncoliform bacteria, or samples that may contain organisms stressed by chlorine, a parallel test should be conducted with a multiple-tube technique to demonstrate applicability and comparability of results.

[9] To assess the comparability of results obtained with individual methods, it is suggested that side-by-side tests be conducted across seasons of the year with the water samples routinely tested in accordance with the most current Standard Methods for the Examination of Water and Wastewater or EPA alternate test procedure (ATP) guidelines.

[10] ASTM. 2000, 1999, 1998. Annual Book of ASTM Standards—Water and Environmental Technology. Section 11.02. ASTM. 100 Barr Harbor Drive, West Conshohocken, PA 19428.

[11] AOAC. 1995. Official Methods of Analysis of AOAC International, 16th Edition, Volume I, Chapter 17. AOAC International. 481 North Frederick Avenue, Suite 500, Gaithersburg, Maryland 20877–2417.

[12] The multiple-tube fermentation test is used in 9221B.1. Lactose broth may be used in lieu of lauryl tryptose broth (LTB), if at least 25 parallel tests are conducted between this broth and LTB using the water samples normally tested, and this comparison demonstrates that the false-positive rate and false-negative rate for total coliform using lactose broth is less than 10 percent. No requirement exists to run the completed phase on 10 percent of all total coliform-positive tubes on a seasonal basis.

[13] These tests are collectively known as defined enzyme substrate tests, where a substrate is used to detect the enzyme β-glucuronidase produced by E. coli.

[14] After prior enrichment in a presumptive medium for total coliform using 9221B.1, all presumptive tubes or bottles showing any amount of gas, growth or acidity within 48 h ± 3 h of incubation shall be submitted to 9221F. Commercially available EC-MUG media or EC media supplemented in the laboratory with 50 μg/mL of MUG may be used.

[15] Samples shall be enumerated by the multiple-tube or multiple-well procedure. Using multiple-tube procedures, employ an appropriate tube and dilution configuration of the sample as needed and report the Most Probable Number (MPN). Samples tested with Colilert® and Colisure™ tests may be enumerated with the multiple-well procedures, Quanti-Tray® or Quanti-Tray® 2000, and the MPN calculated from the table provided by the manufacturer.

[16] Colilert-18'' is an optimized formulation of the Colilert'' for the determination of total coliforms and E. coli that provides results within 18 h of incubation at 35 °C rather than the 24 h required for the Colilert'' test and is recommended for marine water samples.

[17] Colisure® must be incubated for 28 h before examining the results. If an examination of the results at 28 h is not convenient, then results may be examined at any time between 28 h and 48 h.

[18] Descriptions of the Colilert®, Colilert-18®, Quanti-Tray®, Quanti-Tray®/2000, and Colisure™ may be obtained from IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine 04092.

[19] A description of the mColiBlue24 test is available from Hach Company, 100 Dayton Ave., Ames, IA 50010.

[20] Subject total coliform positive samples determined by 9222B or other membrane filter procedure to 9222G using NA-MUG media.

[21] USEPA. 1985. Test Method 1103.1: Escherichia coli In Water By The Membrane Filter Procedure included in: Test Methods For Escherichia coli and Enterococci In Water By the Membrane Filter Procedure. U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring Support Laboratory, Cincinnati, OH. EPA–600–4–85–076.

[22] USEPA. 2000. Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli. U.S. Environmental Protection Agency. Office of Science and Technology. Washington, DC. EPA/821/R–91/004.

[23] Preparation and use of MI agar with a standard membrane filter procedure is set forth in the article, Brenner et al. 1993. ''New Medium for the Simultaneous Detection of Total Coliform and Escherichia coli in Water.'' Appl. Environ. Microbiol. 59:3534–3544 and electronic document, EPA–600-R–00–013.

[24] A description of the Enterolert® test may be obtained from IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine 04092.

[25] USEPA. 1985. Test Method 1106.1: Enterococci In Water By The Membrane Filter Procedure included in: Test Methods For Escherichia coli and Enterococci In Water By the Membrane Filter Procedure. U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring Support Laboratory, Cincinnati, OH. EPA–600–4–85–076.

[26] USEPA. 1997. Method 1600: Membrane Filter Test Method for Enterococci in Water. U.S. Environmental Protection Agency. Office of Water. Washington, DC. EPA–821-R–97–004.

[27] Method 1622 uses filtration, concentration, immunomagnetic separation of oocysts from captured material, immunofluorescence assay to determine concentrations, and confirmation through vital dye staining and differential interference contrast microscopy for the detection of Cryptosporidium.

[28] Method 1623 uses filtration, concentration, immunomagnetic separation of oocysts and cysts from captured material, immunofluorescence assay to determine concentrations, and confirmation through vital dye staining and differential interference contrast microscopy for the simultaneous detection of Cryptosporidium and Giardia oocysts and cysts.

[29] Recommended for enumeration of target organism in ambient water only. Applicability to other matrices must be demonstrated.

[30] USEPA. 1993. Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms. Fourth Edition. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio. EPA/600/4–90/027F.

[31] USEPA. 1994. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Third Edition. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio. EPA/600/4–91/002.

[32] Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. Second Edition. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio. EPA/600/4–91/003. These methods do not apply to marine waters of the Pacific Ocean.

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(b) * * *

(10) Annual Book of ASTM Standards, Water, and Environmental Technology, Section 11, Volumes 11.01 and 11.02, 1994, 1999, and 2000 in 40 CFR 136.3, Table IA.

(11) AOAC. 1995. Official Methods of Analysis of AOAC International, 16th Edition, Volume I, Chapter 17. AOAC International. 481 North Frederick Avenue, Suite 500, Gaithersburg, Maryland 20877–2417. Table IA.

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(51) IDEXX Laboratories, Inc. 1999. Description of Colilert®, Colilert-18®, Quanti-Tray®, Quanti-Tray®/2000, Colisure™, and Enterolert™ methods are available from IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine 04092. Table IA, Notes 18 and 24.

(52) Hach Company, Inc. 1999. m-ColiBlue24 Method is available from Hach Company, 100 Dayton Ave., Ames, IA 50010. Table IA, Note 19.

(53) USEPA. 1985. Test Method 1103.1: Escherichia coli In Water By The Membrane Filter Procedure included in: Test Methods For Escherichia coli and Enterococci In Water By the Membrane Filter Procedure. U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring Support Laboratory, Cincinnati, OH. EPA–600–4–85–076. Table IA, Note 21.

(54) USEPA. 1985. Test Method 1106.1: Enterococci In Water By The Membrane Filter Procedure included in: Test Methods For Escherichia coli and Enterococci In Water By the Membrane Filter Procedure. U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring Support Laboratory, Cincinnati, OH. EPA–600–4–85–076. Table IA, Note 25.

(55) USEPA. 2000. ''Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli.'' U.S. Environmental Protection Agency, Office of Science and Technology, Washington, DC. EPA/821/R–91/004. Table IA, Note 22.

(56) Brenner et al. 1993. ''New Medium for the Simultaneous Detection of Total Coliform and Escherichia coli in Water.'' Appl. Environ. Microbiol. 59:3534–3544. Table IA, Note 23.

(57) USEPA 2000. ''Membrane Filter Method for the Simultaneous Detection of Total Coliforms and Escherichia coli in Drinking Water.'' February 2000. U.S. Environmental Protection Agency, Office of Research and Development, Cincinnati, OH 45268. EPA 600–R–00–013. Table IA, Note 23.

(58) USEPA. 1997. ''Method 1600: Membrane Filter Test Method for Enterococci in Water.'' U.S. Environmental Protection Agency, Office of Water, Washington, DC. EPA–821–R–97–004. Table IA, Note 26.

(59) USEPA. 2001. ''Method 1622: Cryptosporidium in Water by Filtration/IMS/FA.'' U.S. Environmental Protection Agency, Office of Water, Washington, DC. EPA–821–R–01–026. Table IA, Note 27.

(60) USEPA. 2001. ''Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA.'' U.S. Environmental Protection Agency, Office of Water, Washington, DC. EPA–821–R–01–025. Table IA, Note 28.

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TABLE II.—REQUIRED CONTAINERS, PRESERVATION TECHNIQUES, AND HOLDING TIMES

Parameter No./name	Container [1]	Preservation [2,3]	Maximum holding time [4] (in hours)
Table IA—Bacteria Tests:			
1–5 Coliform, total, fecal, and E. coli	PP, G	Cool, <10 °C, 0.0008% $Na_2S_2O_3$ [5]	6
6 Fecal streptococci	PP, G	Cool, <10 °C, 0.0008% $Na_2S_2O_3$ [5]	6
7 Enterococci	PP, G	Cool, <10 °C, 0.0008% $Na_2S_2O_3$ [5]	6

TABLE II.—REQUIRED CONTAINERS, PRESERVATION TECHNIQUES, AND HOLDING TIMES—Continued

Parameter No./name	Container [1]	Preservation [2,3]	Maximum holding time [4] (in hours)
Table IA—Protozoa Tests:			
8 Cryptosporidium	LDPE	0–8 °C	[17] 72
9 Giardia	LDPE	0–8 °C	[17] 72
Table IA—Aquatic Toxicity Tests:			
10–13 Toxicity, acute and chronic	P, G	Cool, 4 °C [16]	36
* * * * * * *			

[1] Polyethylene (P) or glass (G). For bacteria, plastic sample containers must be made of sterilizable materials (polypropylene [PP] or other autoclavable plastic). For protozoa, plastic sample containers must be made of low-density polyethylene (LDPE).

[2] Sample preservation should be performed immediately upon sample collection. For composite chemical samples, each aliquot should be preserved at the time of collection. When use of an automated sampler makes it impossible to preserve each aliquot, then chemical samples may be preserved by maintaining at 4 °C until compositing and sample splitting is completed.

[3] When any sample is to be shipped by common carrier or sent through the United States Mails, it must comply with the Department of Transportation Hazardous Materials Regulations (49 CFR part 172). The person offering such material for transportation is responsible for ensuring such compliance. For the preservation requirements of Table II, the Office of Hazardous Materials, Transportation Bureau, Department of Transportation, has determined that the Hazardous Materials Regulations do not apply to the following materials: Hydrochloric acid (HCl) in water solutions at concentrations of 0.04% by weight or less (pH about 1.96 or greater); Nitric acid (HNO_3) in water solutions of 0.15% by weight or less (pH about 1.62 or greater); Sulfuric acid (H_2SO_4) in water solutions of concentrations of 0.35% by weight or less (pH about 1.15 or greater); and Sodium hydroxide (NaOH) in water solutions at concentrations of 0.080% by weight or less (pH about 12.30 or less).

[4] Samples should be analyzed as soon as possible after collection. The times listed are the maximum times that samples may be held before analyses and still be considered valid. Samples may be held for longer periods only if the permittee, or monitoring laboratory, has data on file to show that for the specific types of samples under study, the analytes are stable for the longer time, and has received a variance from the Regional Administrator under § 136.3(e). Some samples may not be stable for the maximum time period given in the table. A permittee or monitoring laboratory is obligated to hold the samples for a shorter time if knowledge exists to show that this is necessary to maintain sample stability. See § 136.3(e) for details. The term ''analyze immediately'' usually means within 15 minutes or less of sample collection.

[5] Should only be used in the presence of residual chlorine.

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[16] Sufficient ice should be placed with the samples in the shipping container to ensure that ice is still present when samples arrive at the laboratory. However, even if ice is present when the samples arrive, it is necessary to immediately measure the temperature of the samples and confirm that the 4 °C temperature maximum has not been exceeded. In the isolated cases where it can be documented that this holding temperature can not be met, the permittee can be given the option of on-site testing or can request a variance. The request for a variance should include supportive data which show that the toxicity of the effluent samples is not reduced because of the increased holding temperature.

[17] Holding time is calculated from time of sample collection to the completion of centrifugation.

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DEPARTMENT OF THE INTERIOR

Fish and Wildlife Service

50 CFR Part 17

RIN 1018–AG10

Endangered and Threatened Wildlife and Plants; Proposed Special Regulations for the Preble's Meadow Jumping Mouse

AGENCY: Fish and Wildlife Service, Interior.

ACTION: Proposed rule.

SUMMARY: On May 22, 2001, the U.S. Fish and Wildlife Service adopted special regulations governing take of the Preble's meadow jumping mouse (*Zapus hudsonius preblei*). This notice proposes to amend those regulations, which provide exemption from take provisions under section 9 of the Endangered Species Act for certain activities related to rodent control, ongoing agricultural activities, landscape maintenance, and perfected water rights. This action would provide exemption from the section 9 take prohibitions for certain noxious weed control and ditch maintenance activities. We believe this action would provide further relief for landowners while ensuring conservation of the Preble's meadow jumping mouse.

DATES: Comments must be received on or before October 1, 2001 to receive consideration.

ADDRESSES: Comments concerning this proposal should be sent to LeRoy Carlson, Field Supervisor, Colorado Field Office, Ecological Services, 755 Parfet Street, Suite 361, Lakewood, Colorado 80215. Comments and materials received will be available for public inspection, by appointment, during normal business hours at the above address.

FOR FURTHER INFORMATION CONTACT: LeRoy W. Carlson at the above address or telephone 303/275–2370.

SUPPLEMENTARY INFORMATION:

Background

The final rule listing the Preble's meadow jumping mouse (*Zapus hudsonius preblei*) (Preble's) as a threatened species under the Endangered Species Act (Act) of 1973 (16 U.S.C. 1531 *et seq.*) was published in the **Federal Register** on May 13, 1998 (63 FR 26517). Section 9 of the Act prohibits take of endangered wildlife. The Act defines take to mean harass, harm, pursue, hunt, shoot, wound, kill, trap, capture, or collect or to attempt to engage in any such conduct. However, the Act also provides for the authorization of take and exceptions to the take prohibitions. Take of listed species by non-Federal property owners can be permitted through the process set forth in section 10 of the Act. For federally funded or permitted activities, take of listed species may be allowed through the consultation process of section 7 of the Act. We, the Fish and Wildlife Service, have issued regulations (50 CFR 17.31) that generally apply to threatened wildlife the prohibitions that section 9 of the Act establishes with respect to endangered wildlife. Our regulations for threatened wildlife also provide that a ''special rule'' under section 4(d) of the Act can be tailored for a particular threatened species. In that case, the general regulations for some section 9 prohibitions do not apply to that species, and the special rule contains the prohibitions (and exemptions) necessary and appropriate to conserve that species.