OWQ WaQI Note

June 7, 2006

Office of Water Quality Water-Quality Information Note 2006.12

Subject: Field Methods—Microbiology: Guidance for use of defined-substrate media and methods (Colilert®, Enterolert™, and related tests) for microbiological monitoring.

Purpose of this memorandum

This memorandum provides guidance on use of defined-substrate tests, such as the IDEXX Laboratories, Inc. products Colilert® and Enterolert™, in USGS water-quality investigations. Specifically, these tests measure fecal-indicator bacteria concentrations (total coliform and *E. coli,* or enterococci) in ground water, fresh surface water, marine surface water, and source water for drinking. A brief overview of the development and validation of these tests is presented, along with example protocols and a description of recommended quality-assurance and quality-control procedures.

Guidance for use of defined-substrate tests

- The quantitative versions of these tests are based on most-probable-number (MPN) estimates of concentration. Comparison tests have shown that results from these methods are not consistently higher or lower than results from colony counts on various media after membrane filtration; however, individual concentration estimates often are different and might lead to different interpretations. As with most other measurements, it is preferred to collect entire data sets using one analytical technique.
    - Use of these tests for detection of total coliforms and *E. coli* in ground water is considered comparable to use of membrane-filtration media such as MI agar and mENDO/NA-MUG agars.
    - Use of these tests for detection of *E. coli* in ambient water is considered comparable to use of membrane-filtration media such as mTEC and modified mTEC agars
    - Use of these tests for detection of enterococci is considered comparable to use of membrane-filtration media such as mEI agar; however, some relevant criteria are near the detection limit.
- Both Enterolert and Colilert support growth by non-target species in undiluted marine water. Marine water must be diluted at least 10:1 in sterile, unbuffered water when analyzed by these methods. Refer to specific instructions by manufacturers of related tests for guidance.
    - Interpretation of Enterolert results in the context of recreational quality of marine waters can be compromised by the requirement to dilute because of the credible interval around the MPN. For example, analysis of a 1:10 dilution that results in a single detect gives an MPN of 10 with a credible interval of 1-55 MPN/100 milliliters (mL) water. This value might be compared to the USEPA 5-sample geometric-mean enterococci swimming beach criterion of 35 MPN/100 mL; thus, this test may be useful to measure when the 5-sample geometric-mean criterion at marine

swimming beaches is exceeded, but cannot be used to indicate that the criterion is met.

- Data summary for concentrations measured by MPN tests includes both the uncertainty in the MPN estimate and analytical variability. As a result, ranges such as 95 percent confidence interval around the geometric mean tend to be broad relative to colony count data. In cases where statistical precision is a priority, membrane-filtration methods are preferred.

General protocols (for use to generate specific Standard Operating Procedures (SOP) for projects)

Summary. To run defined-substrate methods, dehydrated media are added to 100 mL of sample or diluted sample and mixed until dissolved. Presence/absence tests are incubated immediately. MPN test samples are added to a Quanti-Tray®, sealed, and incubated. After incubation, tests are visually inspected for color formation (Colilert) and/or viewed under ultraviolet light for fluorescence (Colilert and Enterolert). Results are recorded as number of positive responses among total number of tests. MPN results are obtained by comparison to a MPN table or by entering the results in an MPN calculator (available from IDEXX; also built into the Microbiology Field Form within PCFF version 5.2.1 and later).

Quality control for both formats:

- Sample collection should be done using sterile equipment as described in the National Field Manual for the Collection of Water-Quality Data (NFM) (TWRI Book 9, chapter 7.1--http://water.usgs.gov/owq/FieldManual/Chapter7/7.1.html).
- Samples should be analyzed within 6 hours of collection unless otherwise indicated (such as total coliform tests to meet the EPA's Total Coliform Rule monitoring requirements).
- Because the MPN calculation assumes an even distribution of bacteria in the sample volume, it is especially important to vigorously shake the sample before decanting into the Quanti-Tray.
- Samples should be held at 4°C between collection and analysis, with headspace in the bottle, protected from sunlight.
- Reagents should be challenged with positive- and negative-control cultures at the highest frequency among the following criteria: 1) once per lot, 2) at the beginning and end of a project, 3) quarterly over the period of sampling.

Product	Target	Positive control	Negative control
Colilert® and Colilert®-18	Total coliforms	*Enterobacter cloacae* or *Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
	Escherichia coli	*Escherichia coli*	*Enterobacter cloacae* or *Klebsiella pneumoniae*
Enterolert™	Enterococci	*Enterococcus faecalis*	*Enterobacter cloacae*

- An equipment blank should be run at least once daily.
- For Colilert products, positive reactions should be compared against the comparator frequently to guard against false-positive detections.

***Presence-absence format*:**

Materials (see Appendix for Sources):

- Reagent capsules (Colilert, Colilert-18, or Enterolert)

- Sterile 100-mL graduated cylinder
- Sterile 100-mL culture bottle with cap
- Water bath set at 35°C ± 0.5 or 44.5°C ± 0.2 (only for Colilert-18, drinking water)
- Incubator capable of maintaining 35°C ± 0.5 (Colilert) or 41°C ± 0.5 (Enterolert), large enough to hold the culture bottle(s).
- Ultraviolet lamp (6-watt, 365 nm)
- If desired, viewing chamber that excludes ambient light.
- Comparator bottle to indicate detection threshold (Colilert)

Procedure:

- Shake the sample well to disperse target bacteria
- Pour 100 mL of sample into the culture bottle by use of a graduated cylinder or graduations imprinted on the culture bottle.
- Holding the reagent packet with the foil toward you, snap the packet open.
- Pour the contents of the reagent packet into the culture bottle.
- Mix well.
- For analysis of drinking water by use of Colilert-18, pre-warm the sample in the 35°C water bath for 20 minutes or in the 44.5°C water bath for 7-10 minutes
- Incubate for 24 ± 2 hours (Colilert and Enterolert) or for the remainder of 18-22 hours (Colilert-18).
- Read total coliform positive (yellow) or negative (colorless) and *E. coli* or enterococci positive (fluoresces under UV light) or negative (does not fluoresce)

***Quanti-Tray® or other MPN format*:**

Materials (see Appendix for Sources):

- Reagent capsules (Colilert, Colilert-18, or Enterolert)
- Sterile 100-mL graduated cylinder
- Sterile 100-mL mixing bottle with cap
- Incubator capable of maintaining 35°C ± 0.5 (Colilert) or 41°C ± 0.5 (Enterolert), large enough to hold the tray(s).
- Tray sealer
- Ultraviolet lamp (6-watt, 365 nm)
- Viewing chamber that excludes ambient light.
- Comparator tray to indicate detection threshold (Colilert)

Procedure:

- Pre-warm sealer and incubators
- Shake the sample well to disperse target bacteria.
- If working with saline water, prepare 100 mL of a 1:10 dilution.
- If dilution is necessary (because of saline water or because of anticipated high concentration), dilution must be made with sterile, unbuffered water such as distilled water.
- Pour 100 mL of sample into the mixing bottle by use of a graduated cylinder or graduations imprinted on the bottle.
- Holding the reagent packet with the foil toward you, snap the packet open.
- Pour the contents of the reagent packet into the mixing bottle.
- Mix well.
- Add mixture to Quanti-Tray® 200 or Quanti-Tray® 2000. With foil side up, hold at an angle and tap lower wells to release air bubbles.
- Place loaded tray into rubber sealer mat and seal.
- Incubate for 24 ± 2 hours (Colilert and Enterolert) or for the remainder of 18-22 hours (Colilert-18).

- Read total coliform positive (yellow) or negative (colorless) and *E. coli* or enterococci positive (fluoresces under UV) or negative (does not fluoresce)
- Record results and obtain MPN concentration by use of the tables provided by IDEXX or and electronic database.

Overview of method development and validation

The defined substrate methods use broth culture media containing carbon and energy sources specific to target bacteria. Bacterial growth is indicated by the enzyme-mediated conversion of a reporter molecule. The reporter molecule sometimes turns from one color to another (such as CPRG, chlorophenol red *β*-D galactopyranoside, which turns from yellow to red), from colorless to colored (such as ONPG, o-nitrophenyl *β*-D galactopyranoside, which turns yellow), or from non-fluorescing to fluorescing (such as MUG, 4-methylumbelliferyl *β*-D glucuronide, which fluoresces bluish-white when activated). Various patented commercial products use different reporters to indicate activity by three enzymes in target bacteria when grown in selective media: *β*-D galactosidase in total coliforms, *β*-D glucuronidase in *E. coli*, and *β*-D glucosidase in enterococci.

These media were developed, in part, to simplify drinking water monitoring under the Total Coliform Rule. This rule requires that detection of total coliforms in drinking water be followed up with a confirmation test for *E. coli*— the defined-substrate media developed in the 1980s and 1990s incorporated simultaneous tests for total coliforms and *E. coli,* allowing a total coliform test and *E. coli* confirmation to be done in one step. The media were adapted from presence-absence format (designed for drinking water) to multi-tube or multi-well most-probable-number (MPN) format to allow quantitative measurements. To date, the IDEXX (Westbrook, Maine) Quanti-Tray® products are the only widely-available commercial products specifically designed to facilitate MPN-format quantification of results.

Media that were made for and tested in both MPN (Quanti-Tray®) format and presence-absence format are the Idexx products Colilert, Colilert-18, and Colisure (for detection of total coliforms and *E. coli*) and Enterolert and Enterolert-E (for detection of enterococci). Other commercially-available defined-substrate media include Readycult®, by EM Science; E*colite™, by Charm Sciences, and Colitag™, by CPI international. These alternate media all measure total coliforms and *E. coli* in a presence/absence format and may be adaptable to the MPN format.

IDEXX products are approved for use in various settings by USEPA and similar agencies internationally, and they are listed in Standard Methods for the Examination of Water and Wastewater (Method 9223). Though not specifically mentioned in the Federal Register, many of the alternate media also are accepted for use in EPA water monitoring regulations (http://www.epa.gov/safewater/methods/rules_micro.html, accessed 23 March 2006). Because of their versatility, only selected IDEXX media are included in the following table of accepted uses:

Target group	Method	Drinking water	Ground water	Fresh surface water	Marine surface water	Waste water
Total coliforms and *E. coli*	Colilert	Yes	Yes	Yes	Yes*	Yes
	Colisure	Yes	No	No	No	No
Enterococci	Enterolert	Yes*	Yes*	Yes	Yes	Yes

* Method results are accepted in these matrices, but there are no applicable criteria for water quality.

Method validation studies and other side-by-side comparisons demonstrate that methods based on various defined-substrate media and membrane-filtration agars tend to yield comparable categorical data (meets or exceeds water-quality criteria) but frequently have different numeric results. The differences in results tend not to be consistently biased high or low—rather, different results are obtained for specific samples. For example, Francy and Darner (2000) compared *E. coli* concentrations recovered from freshwater swimming beaches on four media, including Colilert, in 70 recreational fresh water samples. Though concentrations measured by Colilert MPN frequently were substantially different from the reference method (mTEC agar), Colilert results and mTEC results showed the same classification relative to a water-quality criterion for 86% of samples.

Though Colilert tends to give concentration estimates similar to those measured by mTEC, modified mTEC, and/or MI agar, the same is not always the case for Enterolert (see, for example, the data set reported by Stoeckel and others (2005)). Classification as meeting or exceeding a standard tends to be consistent, as for Budnik and others (1996), which reported that for 43 freshwater samples and 95 marine water samples, Enterolert and mE/EIA membrane filtration methods resulted in similar concentration estimates as well as classification of samples as meeting or exceeding a water-quality criterion (3 percent more samples exceeded the criterion by mE/EIA than by Enterolert). However, a comparison between Enterolert and mEI agar in fresh recreational water demonstrated the wide disparity in concentrations that can be measured by these methods. In one case, a concentration of 2,400 MPN/100 mL was detected on Enterolert when the mEI recovered 50 colony-forming units (CFU)/100 mL (Kinzelman and others, 2003). In fact, reanalysis of the reported data indicated that, when concentrations by both methods were at or above an arbitrary cutoff of 50 per 100 mL (14 of 64 samples), Enterolert concentrations exceeded mEI concentrations in every case (with an average ratio of 6, average ratio of log-transformed values 1.26). Nevertheless, results of both methods tended to classify samples into the same categories when compared against a single standard (94 percent concordance of classification as meeting or exceeding the criterion).

In a separate study (Noble and others, 2003), difference in recoveries among media was addressed by recruiting >20 laboratories to run replicate analyses of reference samples by use of multiple methods. Median concentration estimates were not significantly different between Colilert and various membrane-filtration media or between Enterolert and various membrane-filtration media. However, the variability in replicate analyses tended to be higher for the MPN methods than for the membrane filtration methods.

For both Enterolert and Colilert it is important to remember to dilute marine water. IDEXX recommends diluting marine waters for Enterolert to prevent false-positive reactions caused by non-fecal ambient bacteria. Piscotta and others (2002) demonstrated that a consortium of native marine bacteria could cause false-positive reactions in the Colilert-18 media prepared with undiluted marine water.

Data reporting

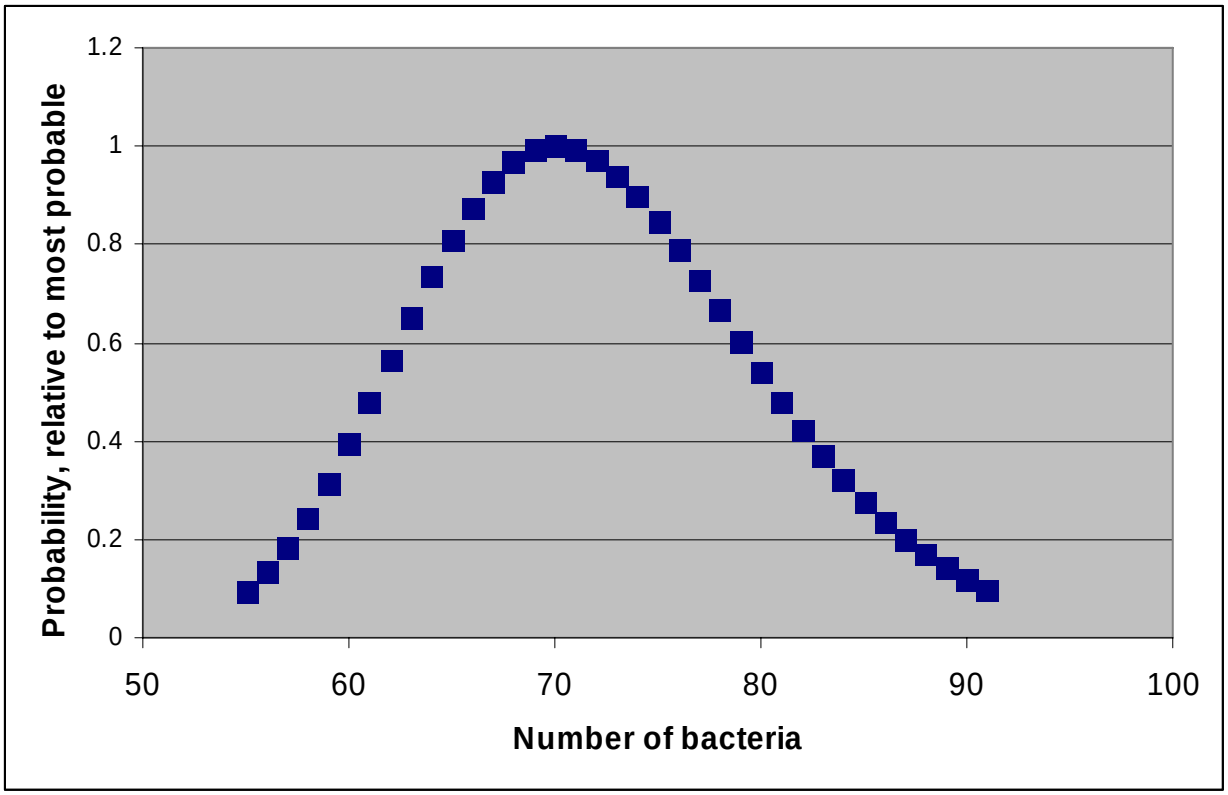
The following parameter codes are used to report results by use of protocols using IDEXX products.

Analyte	Format	Product	Parameter code	Units
Total coliforms in water	Quanti-Tray 200 or Quanti-Tray 2000	Colilert or Colilert 18	50569	MPN per 100 mL
Total coliforms in water	Presence or absence	Colilert or Colilert 18	99595	Present (2) or absent (1) in 100 mL
E. coli in water	Quanti-Tray 200 or Quanti-Tray 2000	Colilert or Colilert 18	50468	MPN per 100 mL
E. coli in water	Presence or absence	Colilert or Colilert 18	99596	Present (2) or absent (1) in 100 mL
E. coli in sediment	Quanti-Tray 200 or Quanti-Tray 2000	Colilert or Colilert 18	50467	MPN per gram dry weight sediment
Enterococci in water	Quanti-Tray 200 or Quanti-Tray 2000	Enterolert	99601	MPN per 100 mL
Enterococci in sediment	Quanti-Tray 200 or Quanti-Tray 2000	Enterolert	N/A	MPN per gram dry weight sediment

There is a fundamental difference between results obtained from a MPN format and those obtained from membrane filtration. In the case of membrane filtration, there is little dispute about the value of a colony count—the rule of thumb is that colony counts by two analysts are expected to agree within 5 percent. In the case of MPN data, however, there is no way to know how many cells are present in each well of the Quanti-Tray. The MPN is the value with the highest probability of being true, and other values have nearly the same probability of being true. For example, see figure 1, which depicts the relative probabilities of various results for which the MPN is 70. Values between about 65 and 75 also are credible; in fact, the 95 percent credible interval for this example is 50-95 MPN/100 mL.

Because of the relative uncertainty associated with MPN values, the confidence interval calculated from multiple measurements tends to be wider than for membrane filtration values. When evaluating single values, it is important to consider the credible interval about the value. When summarizing multiple values, the credible interval may be ignored provided that sufficient replication is done to obtain an estimate of measurement precision. In the MPN methods, measurement precision is a function of three factors: (1) extent to which the sample taken from the sample bottle represents the contents of either the bottle or the water body (depending on whether it is a sequential or split replicate), (2) analytical error, and (3) the uncertainty inherent in the MPN. It is prudent to include more replicate analyses in the study design when using MPN methods compared with membrane filtration methods to better measure the precision of the analytical method.

Figure 1. Probability density function for the number of cells originally in a sample analyzed by Quanti-Tray 2000 in which 36 of 49 large wells were positive and 5 of 48 small wells were positive, plotted relative to the probability associated with the most-probable number (70 MPN/100 mL).



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Budnick, G.E., Howard, R.T., and Mayo, D.R., 1996, Evaluation of Enterolert for enumeration of enterococci in recreational waters: Applied and Environmental Microbiology, v. 62, no. 10, p. 3881-3884.

Francy, D.S., and Darner, R.A., 2000, Comparison of methods for determining *Escherichia coli* concentrations in recreational waters: Water Research, v. 34, no. 10, p. 2770-2778.

Kinzelman, J., Ng, C., Jackson, E., Gradus, S., and Bagley, R., 2003, Enterococci as indicators of Lake Michigan recreational water quality: comparison of two methodologies and their impacts on public health regulatory events: Applied and Environmental Microbiology, v. 69, no. 1, p. 92-96.

Noble, R.T., Weisberg, S.B., Leecaster, M.K., McGee, C.D., Ritter, K., Walker, K.O., and Vainik, P.M., 2003, Comparison of beach bacterial water quality indicator measurement methods: Environmental monitoring and assessment, v. 81, no. 1-3, p. 301-312.

Pisciotta, J.M., Rath, D.F., Stanek, P.A., Flanery, D.M., and Harwood, V.J., 2002, Marine bacteria cause false-positive results in the Colilert-18 rapid identification test for Escherichia coli in Florida waters: Applied and Environmental Microbiology, v. 68, no. 2, p. 539-544.

Stoeckel, D.M., Bushon, R.N., Demcheck, D.K., Skrobialowski, S.C., Kephart, C.M., Bertke, E.E., Mailot, B.E., Mize, S.V., and Fendick, Robert B., Jr., 2005, Bacteriological water quality in the

Lake Pontchartrain Basin, Louisiana, following Hurricanes Katrina and Rita, September 2005: Data Series 143 [variably paginated, interactive map].

Appendix

Sources:
Information is given for a single commercial source of these supplies. In some cases, alternate sources may be available for supplies of comparable quality.

Item	Vendor	Catalog #
Comparator, Colilert and Colilert-18, Presence-absence format	IDEXX	WP104
Comparator, Colilert and Colilert-18, Quanti-Tray 200 format	IDEXX	WQTC
Comparator, Colilert and Colilert-18, Quanti-Tray 2000 format	IDEXX	WQT2KC
Long-wave ultraviolet source, 365 nm, 6 watt	IDEXX	WL160
Viewing cabinet	IDEXX	WCM10
Sterile 100-mL bottle, graduated	IDEXX	various formats, including WV120-20
Quanti-Tray 200	IDEXX	WQT100
Quanti-Tray 2000	IDEXX	WQT-2K
Quanti-Tray sealer	IDEXX	WQTS2X-115
Quanticult QC kit strains for Colilert (*E. coli*, *K. pneumoniae*, *Ps. aeruginosa*)	IDEXX	WKIT1001

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