UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
THE ADMINISTRATOR

EPA-SAB-DWC-91-014

August 16, 1991

Honorable William K. Reilly
Administrator
U.S. Environmental Protection Agency
401 M Street, S.W.
Washington, D.C. 20460

Subject: Review of Protocol for Microbiological Testing of Drinking Water

Dear Mr. Reilly:

The Science Advisory Board's Drinking Water Committee met in Washington, D.C. February 7-8, 1991 to review the Office of Drinking Water's and Office of Research and Development's proposed protocol for microbiological testing of drinking water.

The specific charge to the Committee addressed the adequacy of the protocols for a study in which detection of low densities of chlorine-injured E. coli will be compared among several analytical methods, including the Colilert test; methods for subculture (transfer) of E. coli from Colilert tubes to EPA-approved media; needed incubation times for such subcultures; and the use of sewage as a "spike" for drinking water samples.

The Committee generally agrees that additional testing is necessary to validate the usefulness of the Colilert and similar tests for the detection of low levels of chlorine injured coliforms. The Committee feels that the protocols reviewed at this meeting are a correct approach and has only some minor suggestions for improving them. Among these suggestions are: evaluating the effect of the centrifugation procedure in possibly inducing collateral injury to the E. coli organisms; assessing the effect of holding time on bacterial detection; measuring the concentrations and species of chlorine at several time intervals; and using a minimum of 40 tubes inoculated per dilution.

The Committee appreciates the opportunity to review this protocol and looks forward to receiving your response to the recommendations contained in the attached report.

Sincerely,

Raymond C. Loehr

Dr. Raymond C. Loehr
Chairman
Science Advisory Board

Verne R. Ray

Dr. Verne Ray
Acting Chairman
Drinking Water Committee

ENCLOSURE

United States
Environmental
Protection Agency

Science Advisory
Board (A-101)

EPA-SAB-DWC-91-014
August 1991

EPA

MICROBIOLOGICAL TESTING OF DRINKING WATER

REVIEW OF THE OFFICE OF RESEARCH AND DEVELOPMENT'S AND OFFICE OF WATER'S DRAFT PROTOCOLS FOR MICROBIOLOGICAL TESTING OF DRINKING WATER BY THE DRINKING WATER COMMITTEE

ABSTRACT

The Science Advisory Board's Drinking Water Committee met in Washington, D.C. February 7-8, 1991 to discuss the Office of Drinking Water's and Office of Research and Development's proposed protocol for microbiological testing of drinking water.

The specific charge to the Committee addressed the adequacy of the protocols for a study in which detection of low densities of chlorine-injured E. coli is being compared among several analytical methods, including the Colilert test; methods for subculture (transfer) of E. coli from Colilert tubes to EPA-approved media; needed incubation times for such subcultures; and the use of sewage as a "spike" for drinking water samples.

The Committee found that additional testing is necessary to validate the usefulness of the Colilert and similar tests for the detection of low levels of chlorine injured coliforms. The Committee feels that the protocols reviewed at this meeting are a correct approach and have only some minor suggestions for improving them. Among these suggestions are: evaluating the effect of the centrifugation procedure in possibly inducing collateral injury; assessing the effect of holding time on bacterial detection; measuring the concentrations and species of chlorine at several time intervals; and using a minimum of 40 tubes inoculated per dilution.

KEYWORDS: Colilert; E. coli; chlorine-injured; subculture; microbiology.

U. S. ENVIRONMENTAL PROTECTION AGENCY

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ENVIRONMENTAL PROTECTION AGENCY
SCIENCE ADVISORY BOARD
DRINKING WATER COMMITTEE
FEBRUARY 1991

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1. EXECUTIVE SUMMARY

Several specific issues concerning EPA protocols designed to evaluate the detection of low numbers of chlorine exposed E. coli in water were reviewed. The review of these protocols was brought about by scientific controversies concerning the ability of new methods proposed for quantification of E. coli in drinking water. These methods are needed for compliance testing under drinking water regulations.

The specific charge to the Committee addressed the adequacy of the protocols for a study in which detection of low densities of chlorine-injured E. coli is being compared among several analytical methods, including the Colilert test; methods for subculture (transfer) of E. coli from Colilert tubes to EPA-approved media; needed incubation times for such subcultures; and the use of sewage as a "spike" for drinking water samples

The Committee did not believe that the previous studies presented for our review were adequate to document the effects of low level contamination of chlorine-stressed E. coli in drinking water on the performance of the Colilert MMO-MUG[1] test and to demonstrate Colilert's compatibility to reference method(s). After reviewing the EPA test protocol designed to address these concerns the Committee recommended that:

1. The preferred source or organisms for evaluating the tests should be primary sewage effluent, as it is likely to provide the widest range of E. coli strains in a population of bacteria that includes substantial numbers of other coliforms and non-coliforms already suspended in a water matrix.

2. Holding times be kept to a minimum in order minimize potential injury to the test bacteria during sample preparation. In addition, the effect of centrifugation should be evaluated to see if this process induces injury.

3. Actual concentrations and forms of chlorine present during the test procedures should be measured.

4. The chlorine treated samples should be diluted to contain 3-5 bacterial cells per volume, since it is important that any test be capable of detecting low numbers of organisms in drinking water.

[1] One of a series of tests based on the ability of E. coli to hydrolyze the 4-methylumbelliferyl-beta-D-glucuronide (MUG) in the culture medium to form 4-methylumbelliferone, which fluoresces when exposed to ultraviolet light.

The Committee agrees with EPA that, without further testing, there is insufficient information to support the position that coliform-positive Colilert tubes initially containing 1-4 chlorine-injured cells can be successfully transferred to currently approved EPA medium.

2. INTRODUCTION

The Science Advisory Board's Drinking Water Committee (SAB DWC) met in Washington, D.C. on February 7-8, 1991 to review four specific issues concerning EPA protocols designed to evaluate the detection of low numbers of disinfectant- (chlorine-) exposed E. coli in water. The issues are:

1. EPA is conducting a study in which detection of low densities of chlorine-injured E. coli is being compared among several analytical methods, including the Colilert test. Will the protocol being used for this study be sufficient to provide a reasonable answer to the question of how effective the Colilert test is for detection of low densities (1-4 cells per 100 ml) of chlorine-stressed E. coli: If not, what changes are needed?

2. If EPA does not approve the Colilert test for E. coli detection, then the Agency might allow laboratories to subculture (transfer) total coliforms from the Colilert tube to an EPA-approved E. coli medium. EPA, however, does not yet have sufficient confidence that the specified incubation time (24-28 hours) and medium for the Colilert test is sufficient to allow the growth of 1-4 chlorine-stressed E. coli to a density where a laboratory could subculture successfully with a standard loop or applicator stick. Are the existing data sufficient to answer whether the density of such cultures after 28 hours of incubation is sufficiently high (10^4/ml) to allow subculturing.

3. If EPA does not approve the Colilert test for E. coli detection, then the Agency might allow laboratories to subculture total coliforms from the Colilert tube to an EPA-approved E. coli medium. If the SAB does not believe that existing data are sufficient to answer this question then what short-term tests need to be conducted? Will this protocol answer the question of whether EPA should allow laboratories to subculture from the Colilert medium?

4. Some studies have spiked drinking water samples with dilute sewage to collect data on low levels of environmentally stressed E. coli. Can such data simulate the conditions in drinking water for E. coli, i.e., are the results of such studies applicable to E. coli in drinking water, especially disinfected drinking water?

The review of these issues was brought about by scientific controversies concerning the reliability and effectiveness of one of the new methods proposed for quantitation of E. coli in drinking waters under conditions where the organisms are present in low concentration and have been exposed to chlorine. This new method (Colilert) and similar "defined substrate" (Minimal Medium ONPG-MUG) methods are being considered as alternative methods for E. coli detection

in the Coliform Rule.[2] In this review the term "chlorine-injured" is used to imply that exposure of the bacteria (E. coli) to chlorine produces injury or damage that affects the detectability of the organisms by a culture method. Although injury of E. coli and other bacteria by chlorine has been documented in the scientific literature, the specific nature of such injury has not been adequately defined at the cellular, biochemical and molecular levels, nor has it been compared on this basis to injury induced by other agents.

Reliable analytical methods are essential for compliance testing of drinking water for the presence of total coliforms and the determination of whether or not the total coliforms detected are either fecal coliforms or E. coli. The choice of further testing total coliform-positive samples for either fecal coliforms or E. coli is another aspect of the Coliform Rule that deserves scientific consideration, but this issue will not be specifically addressed in this report. The issues before the DWC concern the analytical methods for the option of E. coli testing. Under the Coliform Rule effective December 31, 1990, EPA proposed three alternative analytical methods for detecting E. coli in drinking water. One of the three methods, the Minimal Medium ONPG-MUG (MMO-MUG) test, was previously approved for the detection of total coliforms but not specifically approved for the detection of E. coli.[3] On January 8, 1991, the EPA published a Final Rule on National Primary Drinking Water Regulations; Analytical Techniques; Coliform Bacteria[4] in which two of three previously proposed methods for E. coli detection in drinking water were approved. However, the third method, the MMO-MUG test, was not approved at this time because of uncertainties about its ability to detect low levels of chlorine-injured E. coli in drinking water. The MMO-MUG test offers some major advantages in time and cost over the other methods because both coliforms and E. coli can be determined in a single test in 24 hours without culture transfer or other confirmation steps.

EPA has criteria and protocols for obtaining approval of new methods for detection of specific microbes and other analyses in water. However, these criteria and protocols are now considered inadequate and are being revised. These revisions (including a recent scientific workshop for them held in Cincinnati, Ohio, February 26-28, 1991) are still in progress. There are serious concerns about the abilities of the existing criteria and protocols for approving new methods to adequately address the specific issues at hand, namely, the ability of a new or alternative method to detect and quantify low concentrations of chlorine-injured E. coli in drinking water. The EPA has recently proposed specific test protocols for this purpose. Members of the

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[2] Fed. Reg.,56(5):636-643, Jan. 8, 1991; Fed. Reg., 55(106):22752-22756, June 1, 1990; Fed. Reg., 54(124):27544-27568, June 29, 1989

[3] Fed. Reg., 54(124):27544-27568, June 29, 1989

[4] Fed. Reg, 56(5):636-643

SAB DWC reviewed in detail these protocols and the four issues presented on them. The reviews of these four issues are presented below.

3. ISSUE 1. REVIEW OF PROTOCOLS FOR DETECTION OF CHLORINE-INJURED E. COLI

In order to evaluate the ability of an alternative analytical method (specifically the method known as Colilert) for its ability to detect low numbers of chlorine-injured E. coli in drinking water, the EPA prepared a specific test protocol. This protocol was presented to the DWC for review and comment. The protocol involves three main steps: (i) the selection of the source of test organisms, (ii) the method of chlorine treatment of the test organisms, and (iii) the evaluation of the alternative method for detecting low numbers of these chlorine-injured test organisms.

3.1 Source of Test Organisms

The EPA protocol proposes three possible sources of E. coli: (a) contaminated raw source water, (b) primary or secondary sewage treatment plant effluent and (c) fecal specimens. Of these three sources, the Committee considers primary sewage effluent to be the preferred choice. This is because primary sewage effluent is likely to provide the widest range of E. coli strains in a population of bacteria that includes substantial numbers other coliforms and non-coliforms already suspended in a water matrix. Raw source water is considered a less desirable and reliable source of E. coli because it may contain few target organisms and be subject to considerable variability in E. coli presence, both over time and by location. Fecal specimens are also less desirable as a source because they may contain fewer E. coli strains and fewer and less diversified non-coliform populations compared to natural waters or wastewaters. If fecal specimens are used as the source, it is recommended that they be a composite from various animal sources so that a wider variety of E. coli strains are represented.

3.2 Sample Preparation and Chlorine Treatment

The Committee is concerned about the potential for collateral injury to test bacteria during sample preparation and chlorine treatment. That is, the procedures for sample preparation and chlorine treatment include steps or treatments that may cause bacterial injury unrelated to the specific injury(s) caused by chlorine treatment. Also, the sample preparation and chlorine treatment procedures may also allow for repair of chlorine-induced injury. The concerns are further explained below. Centrifugation is used to recover the bacteria from the initial suspension when sewage effluent is the sample source. Because centrifugation may cause injury of E. coli and because the mechanism(s) of centrifugation-induced injury may be different from chlorine-induced injury, it is recommended that the centrifugation procedure be evaluated for its ability to induce collateral injury in the context of the test.

In addition to possible centrifugation-induced injury, the Committee is concerned that the samples are held for a total of two 24-hour periods at 4° C during sample preparation and chlorine treatment and prior to analysis. These holding times at 4° C could result in additional changes in the bacteria, such as cold-induced injury or, alternatively, repair of chlorine injury. Therefore, it would be preferable to eliminate the holding time after chlorination, but this may require another approach to estimating the concentration of viable test bacteria prior to the methods evaluation phase of the protocol. It is recognized that the proposed holding times have a purpose: namely, to allow for estimation of bacterial concentration in the prepared samples prior to analysis by the test and reference methods. Furthermore, the holding times and temperature are not inconsistent with the exposures the organisms might experience in waters of the real world, such as during distribution of treated water or transport of a collected water sample to an analytical laboratory. Nevertheless, it must be recognized that these holding times are a potential additional source of alteration to the test bacteria. Consequently, it is recommended that their effects on bacterial detection and analysis be determined by further experimentation. It is specifically recommended that EPA determine if there is a difference in the detectability of the bacteria by the methods being evaluated as a consequence of the holding period after chlorine treatment.

3.3 Chlorine Treatment

A critical part of the test protocol is the treatment of the organisms with chlorine. We believe that it is essential to measure analytically the actual concentrations and forms (molecular species) of chlorine in the treated samples during chlorine exposure. Because the concentrations and species of chlorine may change over time, it is recommended that these measurements be made at several intervals in (at least initial, middle and end of) the exposure period.

3.4 Methods Evaluation

3.4.1 Concentration of Test Bacteria in Sample Volumes

The methods evaluation phase of the protocol specifies that the test and reference methods be applied to samples containing 1-10 cells per sample volume and that the inoculations of media be done simultaneously (alternate inoculations into each medium until all cultures are inoculated). The Committee concurs with this inoculation scheme but recommends that efforts be made to dilute the chlorine treated samples to contain 3-5 cells per volume. This is because greater than 5 cells per sample volume may "mask" the potential of a method to give false negative results. That is, a method may fail to detect bacteria only when there are very low numbers in the sample volume. Also, greater than 5 cells per sample volume does not

adequately represent the low numbers of E. coli typically found in treated drinking water. If there are less than 3 cells per sample volume, difficulties arise in obtaining statistical reliability when comparing test and reference methods because of the increased probabilities that sample volumes will contain no cells. Having too many sample volumes with no cells makes it more difficult to compare the test and reference methods because there must be bacteria present for detection.

3.4.2 Number of Cultures (Tubes) per Sample and Number of Sample Dilutions

There is an apparent discrepancy between the text version and the flow diagrams of the proposed protocol in terms of the number of replicate sample tubes per method. If the procedure is conducted according to the text protocol (which specifies 10 tubes per dilution per medium), then the protocol would have to be repeated four times to obtain a total of 40 tubes. The Committee recommends that there be a minimum of 40 tubes inoculated per dilution and that one of dilutions provide test organisms at the target concentration of 3-5 cells per sample (inoculum) volume. This is recommended in order to obtain adequate statistical power in comparing the results of the test and reference methods.

4. Issues 2 and 3. Transferability (Subculture) of Total Coliform-Positive Colilert Tubes to EPA-approved Media

Two issues of the Charge (numbers 2 and 3) concerned the transferability of samples positive in the Colilert (MMO-MUG) test to an EPA approved E. coli medium, EC-MUG. Based on existing data, EPA is not sufficiently confident that the specified incubation time of 24-28 hours in Colilert (MMO-MUG) medium allows the growth of 1-4 initial, chlorine-stressed E. coli cells to a high enough density for successful subculture using a standard loop or applicator stick. Therefore, they have requested the Committee's scientific opinion on this matter (Issue 2). The Committee agrees with EPA that there insufficient scientific information (data and analyses) to support the position that coliform-positive Colilert tubes initially containing 1-4 chlorine-injured cells can be successfully transferred to an EPA approved E. coli medium. Because of concerns about the lack of scientific data to support the position that Colilert tubes initially containing 1-4 chlorine-stressed cells will grow to a sufficiently high density to allow successful transfer to an EPA approved E. coli medium for their detection, the EPA has developed a draft testing protocol for the purpose of addressing this issue. EPA has requested the Committee's scientific opinion of the ability of the protocol to resolve this question (issue 3). The Committee believes that the draft test protocol to determine transferability (transferability evaluation protocol) is generally adequate to address this issue. However, the Committee recommends that all MMO-MUG tubes be read (scored) for both coliforms and E. coli when doing these studies. This will provide additional direct comparative data of the test method (MMO-MUG) to a reference method (EC-MUG).

5. ISSUE 4. VALIDITY OF PROCEDURES IN PREVIOUS STUDIES ON DETECTABILITY OF LOW LEVELS OF STRESSED E. COLI, ACCEPTABILITY OF EPA'S DRAFT PROTOCOL FOR SUCH TESTING AND RECOMMENDATIONS FOR ITS MODIFICATION.

The Committee does not believe that the previous studies presented for our review (Edberg and Edberg, 1988; Edberg et al, 1988; National Primary Drinking Water Standards, 1991; Covert et al, 1989; Clark et al, 1991) were adequate to document the effects of low level contamination of chlorine-stressed E. coli in drinking water on the performance characteristics of the Colilert MMO-MUG test and to demonstrate Colilert's comparability (equivalency) to the reference method(s) for such samples. In part, this is because previous studies did not obtain sufficient documentation on the concentrations of viable, chlorine-stressed E. coli, the concentration of residual chlorine, or the chlorine species in the samples tested. Also, previous studies did not adequately document the quality of the test waters with respect to parameters that could influence chlorine disinfection, such as temperature, Ph, turbidity, ammonia, organic carbon, etc. The Committee believes that, with modification, the draft test protocols are generally adequate to provide results that will allow the resolution of these issues concerning the ability of the Colilert MMO-MUG test to detect low levels of chlorine-stressed E. coli in drinking water. In addition to the other recommended modifications already provided above, the Committee recommends that future evaluations of the comparability of test methods to reference methods include adequate documentation (water quality analyses) of the test waters for chlorine species and their concentrations and for other water quality parameters that could influence chlorine disinfection and the response of test organisms to chlorine injury.

6. DISCUSSION AND CONCLUSIONS

The Committee was asked to respond to a set of laboratory protocols designed to evaluate the acceptability of new methods for the detection of E. coli in water. The Committee made a number of specific recommendations which it believed would enhance the experimental design of these protocols to answer the specific issues presented to the Committee. However, the Committee cautions against relying solely on laboratory test protocols and the data derived therefrom for such an important matter concerning bacterial indicators designed to ensure the sanitary quality of our drinking water. Any model laboratory test system for evaluating the response of microorganisms to environmental conditions in drinking water, including treatment processes, has limitations. The range of environmental conditions and microbial traits that may be encountered in the "real world" of drinking water is unknown, and hence, can not be faithfully simulated in a laboratory experiment. Therefore, the Committee encourages scientists concerned with the issues at hand to also conduct carefully designed field studies in actual drinking water in a effort to determine the comparability of Colilert and similar MMO-MUG tests to standard (reference) method, for detection of low levels of chlorine-injured E. coli. A substantial data base from field studies would be invaluable in assessing the validity and reliability of these methods.

It is also obvious that the EPA needs to develop and evaluate standard protocols for the validation of any microbial test procedure(s). Such protocols have been lacking for microorganisms. Given the rapid development of new technology for the detection of microorganisms in water, such protocols are necessary to ensure the application of rapid and low cost methods for ensuring the microbial quality of drinking water.

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