APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1988, p. 2118–2122
0099-2240/88/082118-05$02.00/0

Vol. 54, No. 8

Rapid Detection of Total and Fecal Coliforms in Water by Enzymatic Hydrolysis of 4-Methylumbelliferone-β-D-Galactoside

JAMES D. BERG[1]* AND LIV FIKSDAL[2]

Aquateam-Norwegian Water Technology Centre A/S, P. O. Box 6326 Etterstad, 0604 Oslo 6,[1] and University of Trondheim, Norwegian Institute of Technology, N-7034 Trondheim,[2] Norway

Received 15 December 1987/Accepted 31 May 1988

Three fluorogenic methylumbelliferone (MU) substrates were evaluated for rapid detection of total and fecal coliform bacteria (TC and FC) in drinking water. 4-MU-β-D-galactoside, MU-heptanoate, and MU-glucuronide were used to determine enzyme activity as a surrogate measure of coliform concentration. Coliforms occurring in river water and in potable water artificially contaminated with raw sewage were tested. The initial rate of hydrolysis (ΔF) of MU-β-D-galactoside showed promise as an indicator of TC and FC within 15 min. ΔF of MU-glucuronide was insufficient in the 15-min assay, and combinations of the MU substrates did not enhance ΔF. A direct membrane filter method incorporating MU-β-D-galactoside into an agar medium allowed the detection of as few as 1 FC per 100 ml within 6 h.

Conventional microbiological analyses of drinking water for coliform bacteria require 24 to 72 h to complete. The elapsed time is too great to provide useful information about a decrease in quality that may require immediate remedial measures. Hence, the first reason for the development of a rapid method is to protect the public health, especially in cases in which contaminated sources or interrupted treatment can occur. Lippy and Waltrip (9) have reported that of the 38 waterborne disease outbreaks or 10,000 illnesses per year in the United States, 80% were attributable to water system deficiencies. Furthermore, rapid methods have the potential to be less costly than conventional methods by reducing labor costs, especially if they can be automated. Lastly, rapid methods could be used for monitoring and controlling treatment processes such as disinfection and sand filtration (6, 8).

The objective of the present study was to evaluate the use of the enzymatic hydrolysis of fluorogenic substrates as a rapid method for detecting fecal contamination of water. Guidelines for the research were (i) to improve upon the time and/or sensitivity limits given for the rapid methods reported in Part 919 of *Standard Methods for the Examination of Water and Wastewater* (1); (ii) to use common and relatively inexpensive instruments and reagents; and (iii) to develop procedures that ultimately can be automated.

Three fluorogenic substrates were chosen for evaluation, 4-methylumbelliferone (MU)-β-D-galactoside, 4-MU-heptanoate, and 4-MU-glucuronide. A large group of 4-MU compounds exists and has been used in clinical enzyme assays (4, 10, 16). Also, MU-glucuronide has been used to specifically identify *Escherichia coli* in water samples (3, 13).

MATERIALS AND METHODS

Sources of bacteria. Natural populations of coliforms were obtained from a river (Nidelva, Trondheim, Norway) receiving sewage effluent and from fresh raw domestic sewage. Samples were collected daily for experiments and diluted in tap water in various concentrations to model drinking water with fecal and nonspecific heterotrophic bacterial contamination. Concentrations of the total coliforms ranged from 1/100 to 1,000/100 ml as determined by the membrane filtration technique (see below).

Routine microbiological assays. Heterotrophic bacteria were recovered on Standard Plate Count agar (Difco Laboratories) or R2A agar and counted after 3 days of incubation at 20°C. Total coliform (TC) bacteria were assayed by the membrane filtration technique with m-Endo agar (Difco) and incubated for 24 h at 35°C. The standard M 7h FC membrane filtration method (1) was used to recover fecal coliforms (FC) at 41.5°C. Peptone (0.1%) was used for all serial dilutions. All assays were done according to *Standard Methods for the Examination of Water and Wastewater* (1) unless otherwise noted.

Rapid-detection methods. (i) Initial fluorescence velocity. A water sample (500 ml) was filtered through a 0.45-μm-pore-size, 47-mm-diameter membrane filter (Millipore Corp.) and aseptically placed in a 250-ml flask containing 18 ml of sterile buffered medium. (The 500-ml sample size was chosen as the maximum volume of surface water of turbidity <5 nephelometric turbidity units that could be filtered in a short period.) The medium consisted of phosphate-buffered saline (pH 7.3), 0.02% sodium lauryl sulfate (Sigma Chemical Co.), 0.56% nutrient broth (Difco), and 0.35% lactose. Individual fluorogenic substrates (described below) or combinations of them were added to each flask and to a sterile control flask containing the sterile medium and then placed in a shaking water bath. The samples were incubated at 35°C for determining TC activity and 41.5°C for determining FC activity. Flasks were removed from the bath and placed in a fluorimeter (Turner model 111) every 5 min for 30 min to measure the fluorescence intensity. The excitation and emission wavelengths for MU are 365 and 465 nm with light filters 7-60 and 2A+47B, respectively. The initial fluorescence velocity (ΔF, per minute) was determined by least squares linear regression.

(ii) Direct counting of FC. Concentrations of FC of <100/100 ml were analyzed by direct counting. Water samples were filtered and placed on M 7h FC agar (1) lacking the pH indicators and D-mannitol but containing 4-MU-β-D-galactoside. Fluorescent galactosidase-positive microcolonies were visible after 6 h of incubation at 41.5°C with a 366-nm long-wave UV light source (UVL-56; Ultraviolet Products). The addition of 0.2 ml of 0.1 N NaOH after the incubation

* Corresponding author.

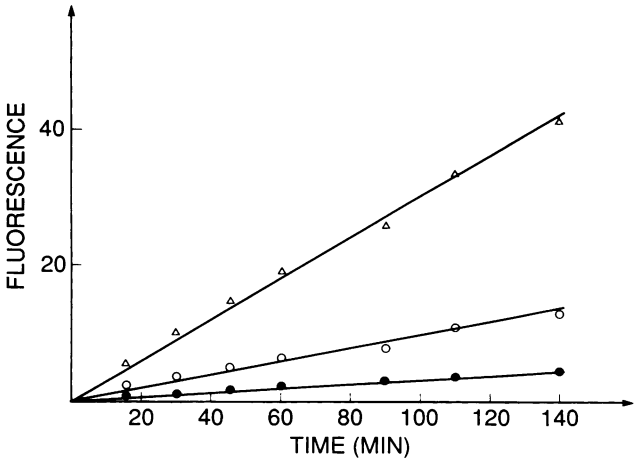

FIG. 1. Production of MU from 4-MU-β-D-galactoside by coliform bacteria in drinking water contaminated with raw sewage (●). Addition of lactose enhanced induction of β-D-galactosidase (○). Addition of sodium lauryl sulfate, a common selective ingredient for coliforms, further enhanced activity (△). Enzyme assay temperature was 41.5°C.

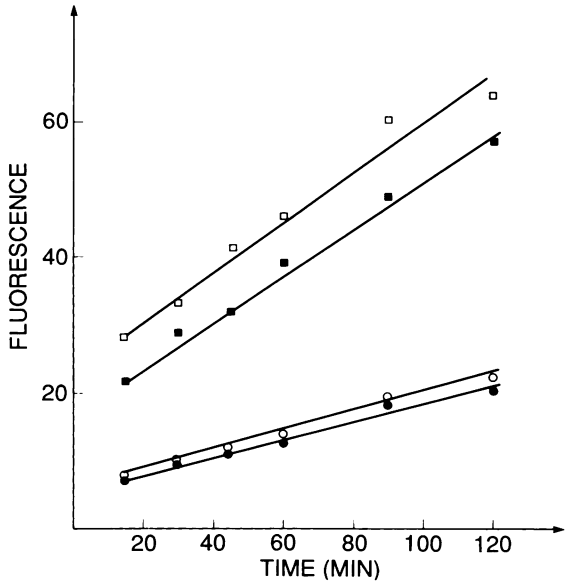

FIG. 2. Effect of pretreatment of drinking water for 60 min with crystal violet (1 mg/liter), bile salts (1.5 g/liter), and sodium lauryl sulfate (0.2 g/liter) (common noncoliform inhibitors) plus NaCl (1.5 g/liter). Coliform concentrations were 7.35×10^2 TC per 100 ml (■ and □) and 1.47×10^2 TC per 100 ml (○ and ●). Samples were untreated (□ and ○) or pretreated (■ and ●) before addition of fluorogenic substrate. Enzyme assay temperature was 41.5°C.

period increased the fluorescence (10) and facilitated counting.

Fluorogenic substrates. 4-MU-heptanoate, 4-MU-β-D-galactoside, and 4-MU-glucuronide (Sigma Chemical Co.) were prepared fresh daily and added to samples at a final concentration equal to 0.05 mg/ml. (The methods described herein have been filed with the U.S. Patent Office.)

RESULTS

Coliform detection by ΔF. MU-β-D-galactoside was selected to detect the coliform group by measuring galactosidase activity. A second fluorochrome, MU-β-D-glucuronide, although specific for *E. coli* under the assay conditions, was rejected because ΔF was deemed too low for a rapid method. Nor did fluorogenic substrate combinations (MU-β-D-galactoside-MU-glucuronide-MU-heptanoate or MU-β-D-galactoside-MU-glucuronide) yield increased sensitivity by virtue of additive fluorescent product formation (data not shown). The results of experiments to enhance ΔF for galactosidase activity and to assure specificity for the coliform group are shown in Fig. 1 and 2. The fluorescence scale (y axis) (Fig. 1 and 2) depicts the relative fluorescence intensity (unitless) ranging from 0 to 100 on this instrument.

The addition of lactose and sodium lauryl sulfate to the medium enhanced galactosidase activity (Fig. 1). The specificity of MU-galactoside for members of the coliform group was tested by pretreating water samples with common inhibitors used in coliform media (1). Most of the activity was retained after pretreatment, suggesting that the galactosidase activity was attributable to the coliform group as defined by the conditions of the assay (Fig. 2). Replacement of sodium lauryl sulfate by the selective agents used in m T7 medium, polyethylene ether W-1 and Tergitol 7 (7), did not enhance activity (data not shown). Linearity of the rate of product formation was generally high ($r = 0.99$) for most assays. Therefore, the duration of the assay in the remaining experiments was decreased from 120 to 30 min, although 15 min was sufficient.

Initial attempts to maximize enzyme activity of TC included raising the rapid-assay temperature to 41.5°C while retaining the 35°C incubation temperature for recovery of TC colonies in the parallel standard assay. In Fig. 1 and 2, FC enzyme activity at 41.5°C is compared with TC colony growth at 35°C. Thereafter, the same rapid-assay and standard-assay incubation temperatures were used to correlate ΔF with the corresponding coliform group (Fig. 3 to 5); colonies incubated at 41.5°C are reported as FC, while those incubated at 35°C are reported as TC. The parameter ΔF, derived from the slope of fluorescence intensity versus time experiments (Fig. 1), is proportional to cell density.

The relation between ΔF (41.5°C incubation) and FC from natural populations of coliforms in river water is shown in Fig. 3. The sensitivity limit as defined by the rate of autohydrolysis ($\Delta F = 0.02$/min) would correspond to 10 coliforms per 100 ml. ΔF (35°C incubation) versus TC is shown in Fig. 4, and ΔF (41.5°C incubation) versus FC is shown in Fig. 5; both experiments used drinking water artificially contaminated with raw sewage.

Direct coliform counting. A direct membrane filter technique was developed in which MU-galactoside, incorporated directly into an agar medium, permitted direct counting of fluorescing colonies within 6 h (41.5°C incubation) (Fig. 6). The results are compared with those obtained by the M 7h FC method in Fig. 7. The average ratio of MU 6h MF colonies to M 7h FC colonies was 1.21:1 for 34 samples. A total of 62 fluorescing colonies randomly selected from three experiments showed 100% confirmation as FC by gas production in lauryl tryptose broth (35°C, 24 h) and EC broth (44°C, 24 h). In all experiments, heterotrophic bacterial concentrations ranged from 300/ml to 50,000/ml.

DISCUSSION

Two approaches were taken to develop a rapid indicator of fecal contamination of drinking water, an indirect estimation of the coliform group by comparing ΔF with coliform con-

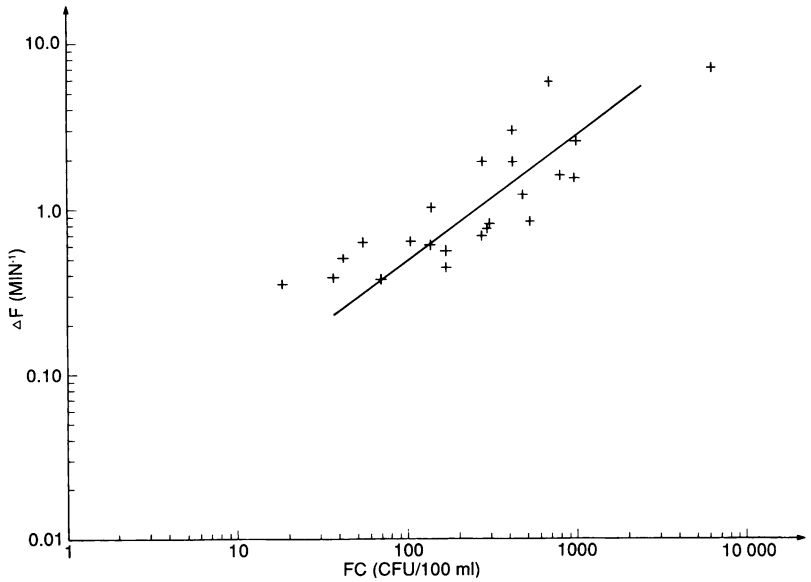

FIG. 3. Initial rate of hydrolysis of 4-MU-β-D-galactoside by FC bacteria in river water. The theoretical sensitivity limit of $\Delta F = 0.02$/min was determined by autohydrolysis of the substrate in a sterile sample. Enzyme assay temperature was 41.5°C.

centration and direct counting of coliform colonies. ΔF has been shown to correlate well with bacterium concentration (15), although much higher cell concentrations (10^5 to 10^8 CFU/ml) were used than in the present study and no extra nutrients had been added to increase enzyme activity. We found a good correlation between ΔF and TC by using MU-β-D-galactoside (correlation coefficient [r] = 0.87) and a detection limit of ca. 60 to 100 TC per 100 ml. The addition of the nutrient lactose enhanced the activity of the inducible galactosidase to much higher values than those obtained by addition of the fluorogenic substrate alone; this may partially explain the greater sensitivity attained in the present study, compared with the sensitivity reported for *E. coli* in other work in which much higher cell concentrations (10^5 to 10^8 CFU/ml) and no extra nutrients were used to increase enzyme activity (15). It has been reported that the enzyme concentration can increase by as much as 1,000-fold, beginning several minutes after lactose addition, confirming our

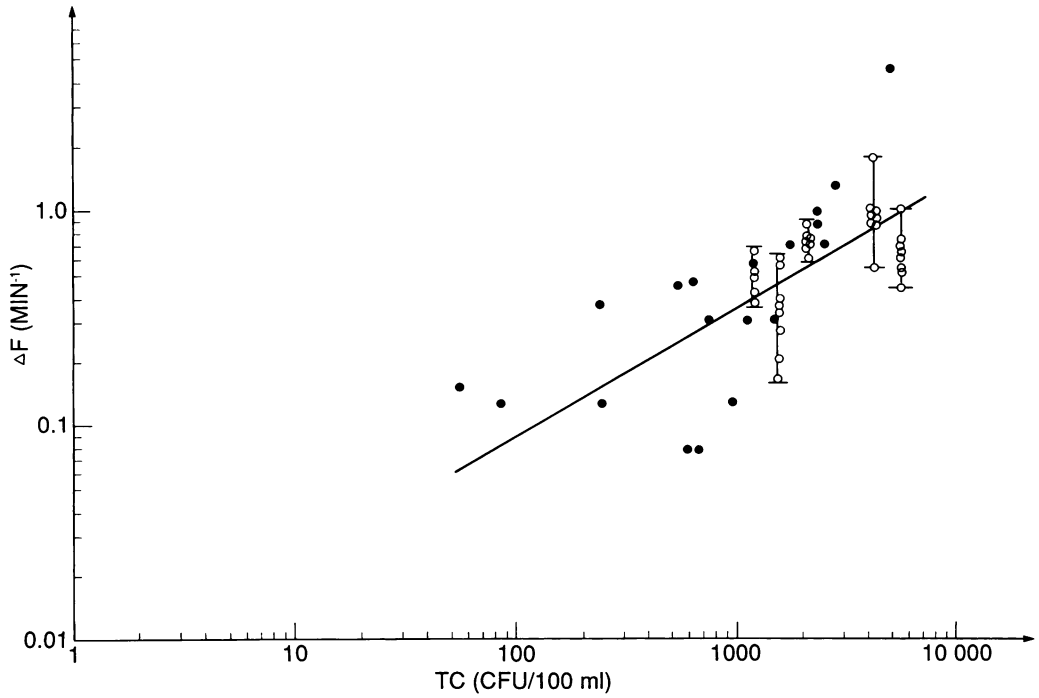

FIG. 4. Initial rate of hydrolysis of 4-MU-β-D-galactoside by TC bacteria derived from raw sewage and mixed in drinking water (●). Enzyme assay temperature was 35°C. Error bars represent the range of ΔF obtained when replicate samples ($5 < n < 8$) were incubated from one original sample (○).

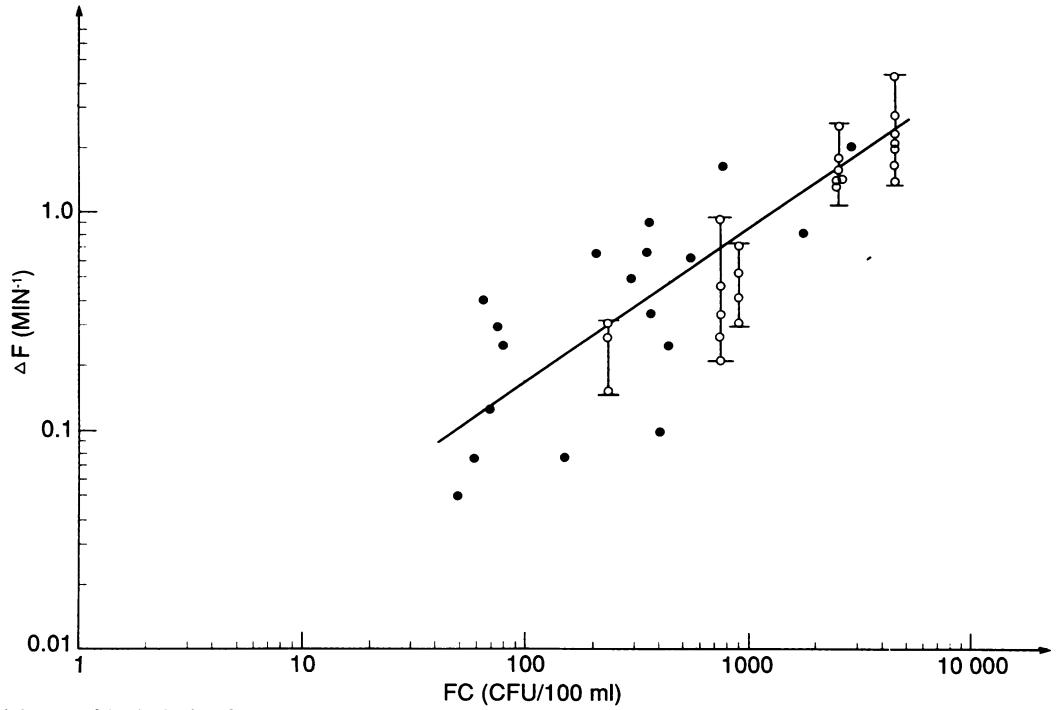

FIG. 5. Initial rate of hydrolysis of 4-MU-β-D-galactoside by FC bacteria derived from raw sewage and mixed in drinking water (●). Enzyme assay temperature was 41.5°C. Error bars represent the range of ΔF obtained when replicate samples ($5 < n < 8$) were incubated from one original sample (○).

observation (5). The other important constituent in the test solution was sodium lauryl sulfate. The detergent, in addition to providing selectivity to coliform bacteria, appeared to enhance activity by improving the transfer of the substrate and/or enzyme across the outer membrane. The detection limit of the fluorometric galactosidase assay was not determined by autohydrolysis (ΔF = 0.02/min), which was generally insignificant. There was, however, a background level of fluorescence corresponding to 0 TC per 100 ml that could be detected within the 15-min assay, which yielded a practical limit detection of ca. 60 to 100 CFU/100 ml. The detection limit is insufficient for finished drinking water, since an impractical volume of water would need to be

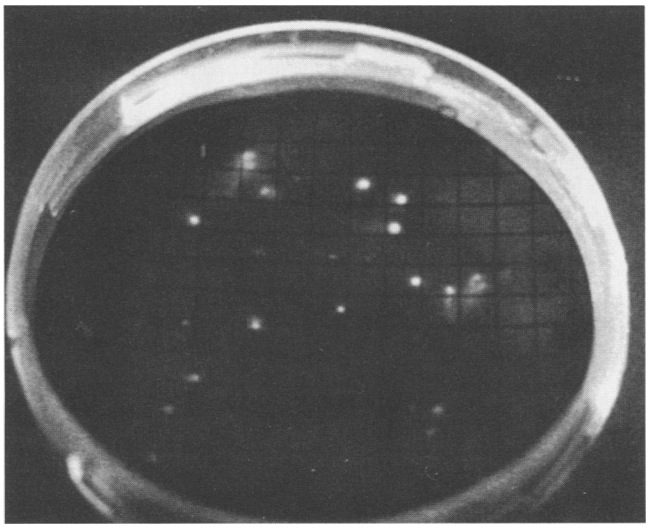

FIG. 6. Fluorescing, galactosidase-positive colonies seen with a 366-nm light source after 6 h of incubation. Some "bleeding" of the fluorescence is an artifact of the delay associated with photographing the sample. Selected fluorescing colonies were verified as FC (see text).

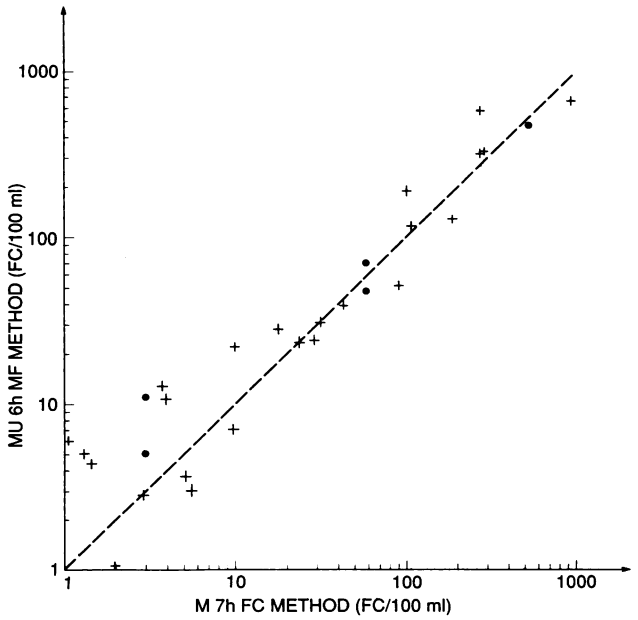

FIG. 7. Comparison of FC counts after 6 h by the direct counting of fluorescing colonies (MU 6h MF) and M 7h FC methods (1). Symbols: +, raw sewage-contaminated drinking water stored at 15°C and sampled after 24 to 72 h for coliforms; ●, river water samples; – – –, ideal correlation, i.e., not matched to actual data.

concentrated to increase the sensitivity. However, recent research (2) suggests that more sophisticated techniques, such as high-pressure liquid chromatography, can be used to separate and concentrate the fluorescent products, thereby increasing sensitivity.

Several sources of error and hence limits on the precision of the rapid assay are possible. The enzyme activity, of course, is subject to the physiological status of the bacteria, and a highly variable fraction (10 to 90%) of the coliforms may be "stressed" (1). The same phenomena, as well as suppression of activity by the higher concentrations of heterotrophic bacteria, can also introduce error into the standard recovery procedures with which the enzyme activities are compared. Galactosidase-negative coliforms and galactosidase-positive noncoliforms, although generally present in insignificant numbers, may also affect the assay (14).

Some notion of the precision of the indirect 15-min assay is obtained by comparing the results with the 95% confidence interval of the most probable number (MPN) test (1). For an MPN index equal to 110 MPN per 100 ml, the approximate lower detection limit presently attainable, the 95% confidence interval equals 40 to 300. For the TC data shown in Fig. 3 and 4, the estimated 95% confidence intervals for 100 MPN per 100 ml equal 30 to 300 and 20 to 500, respectively; for the FC data shown in Fig. 5, the same MPN value yields a 95% confidence interval equal to 25 to 450. The precision is therefore comparable to that of the MPN test, yet improvements in both sensitivity limit and precision are being sought in ongoing research.

The MU 6h MF method, the second approach for rapid coliform detection, was conceived to increase sensitivity and precision by incorporating the MU-galactoside into an agar medium and directly counting FC colonies. Our results correlated quite well with those obtained by the M 7h FC method (1), and the same level of sensitivity is achievable, i.e., 1 FC per 100 ml. Incorporation of MU-glucuronide into agar media has been reported (3, 11) to detect ca. 1,000 *E. coli* per 100 ml after overnight incubation. Since the M 7h FC method has been reported to correlate well with the 24h FC membrane filter method (ratio of M 7h FC colonies to 24h FC colonies equals 1.08:1 for surface waters [12]), it is expected that the proposed MU 6h MF rapid method would also behave similarly.

The increase in sensitivity requires 6 h instead of 15 min as for the indirect determination by fluorometry and therefore would not be suitable for on-line process control. However, the method is promising for rapidly monitoring water quality, especially in emergency situations or situations in which results are required within one working day (6 h).

The results suggest that coliforms in the range of 60 to 100 CFU/100 ml can be detected by fluorometry within 15 min. The method uses relatively inexpensive equipment, i.e., a water bath and a fluorometer, and it can be automated. The direct MU 6h MF method permits counting as few as 1 FC per 100 ml in 6 h and requires only a hand-held long-wave UV lamp to visualize colonies.

ACKNOWLEDGMENTS

We are extremely grateful for the financial support provided by The Royal Norwegian Council for Scientific and Industrial Research and the McNamara Fellowship from The World Bank.

We thank Hanna Wasilewski for her technical assistance.

LITERATURE CITED

1. **American Public Health Association.** 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
2. **Colin, F., and M. J. Jourdain.** 1986. A new method of evaluating the biological activity of drinking water distribution network surfaces. Report RH 86-174. Institut de Recherches Hydrologiques Groupement de'Intérêt Public STELOR, Nancy, France.
3. **Feng, P. C. S., and P. A. Hartman.** 1982. Fluorogenic assays for immediate confirmation of *Escherichia coli*. Appl. Environ. Microbiol. **43**:1320–1329.
4. **Godsey, J. H., M. R. Matteo, D. Shen, G. Tolman, and J. R. Gohlke.** 1981. Rapid identification of *Enterobacteriaceae* with microbial enzyme activity profiles. J. Clin. Microbiol. **13**:483–490.
5. **Gottschalk, G. (ed.).** 1986. Bacterial metabolism, 2nd ed., p. 142–166. Springer-Verlag, New York.
6. **Lange, K. P., W. D. Bellamy, D. W. Hendricks, and G. S. Logdon.** 1986. Diatomaceous earth filtration of giardia cysts. J. Am. Water Works Assoc. **78**:76–84.
7. **LeChevallier, M. W., S. C. Cameron, and G. A. McFeters.** 1983. New medium for improved recovery of coliform bacteria from drinking water. Appl. Environ. Microbiol. **45**:484–492.
8. **Lippy, E. C.** 1986. Chlorination to prevent and control waterborne diseases. J. Am. Water Works Assoc. **78**:49–52.
9. **Lippy, E. C., and S. C. Waltrip.** 1984. Waterborne disease outbreaks. J. Am. Water Works Assoc. **76**:60–67.
10. **Maddocks, J. L., and M. J. Greenan.** 1975. A rapid method for identifying bacterial enzymes. J. Clin. Pathol. **28**:686–687.
11. **Petzel, J. P., and P. A. Hartman.** 1985. Monensin-based medium for determination of total gram-negative bacteria and *Escherichia coli*. Appl. Environ. Microbiol. **49**:925–933.
12. **Reasoner, D. J., J. C. Blannon, and E. E. Geldreich.** 1979. Rapid seven-hour fecal coliform test. Appl. Environ. Microbiol. **38**: 229–236.
13. **Robison, B. J.** 1984. Evaluation of a fluorogenic assay for detection of *Escherichia coli* in foods. Appl. Environ. Microbiol. **48**:285–288.
14. **Rychert, R. C., and G. R. Stephenson.** 1986. Lactose negative *Escherichia coli* from rangeland streams: source, antibiotic resistance, and colicinogenicity. Water Resour. Bull. **22**:39–42.
15. **Snyder, A. P., T. T. Wang, and D. B. Greenberg.** 1986. Pattern recognition analysis of in vivo enzyme-substrate fluorescence velocities in microorganism detection and identification. Appl. Environ. Microbiol. **51**:969–977.
16. **Trepeta, R. W., and S. C. Edberg.** 1984. Methylumbelliferyl-β-D-glucuronide-based medium for rapid isolation and identification of *Escherichia coli*. J. Clin. Microbiol. **19**:172–174.