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Selective Detection and Enumeration of Fecal Coliforms in Water by Potentiometric Measurement of Lipoic Acid Reduction

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Water samples of various origins were inoculated into a specific coliform-selective lactose broth provided with lipoic (thioctic) acid, and the time evolution of the redox potential of the cultures was monitored during incubation at 41°C by use of gold versus reference electrodes. Positive potential-time responses, i.e., 100-mV potential shifts recorded within 20 h of inoculation, were related to the initial number of fecal coliforms in the broth determined by control enumeration techniques, and the organisms responsible were isolated and identified by conventional procedures. A total of 30 samples of wastewater, 38 of surface water, 553 of groundwater, and 110 of drinking water were tested successively. A total of 240 natural water samples, including 172 groundwater samples, and 1 drinking water sample were found to be positive in the potentiometric test. The majority (i.e., 92.5%) of the relevant potentiometric detection times were shorter than 15 h, and 96% of these could be attributed to *Escherichia coli*. Fifteen hours corresponded to the limit for detecting 1 *E. coli* cell per 100 ml of water. About 78% of the potentiometric responses occurring after 15 h were induced by fecal coliforms other than *E. coli* (*Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Citrobacter freundii*). Calibration curves relating detection times shorter than 15 h to fecal coliform (i.e., *E. coli*) concentrations were constructed for the natural water samples tested. There were minor variations in the average growth rate of the organisms in relation to the contamination level of the water tested. The number of false-positive samples in the potentiometric test was equivalent to that of false-negative samples (groundwater or drinking water).

The potentiometric measurement of lipoic acid reduction by microbial cultures with gold versus reference electrodes provides simple and original data suitable for investigations of the antibacterial effects of drugs and for the enumeration of bacteria (see reference 3 for a review). In this latter field of application, the useful experimental parameter is t(100 mV), the time that elapses between the inoculation of the culture medium and the appearance of a 100-mV shift in the gold electrode potential; t(100 mV) values decrease linearly with the logarithm of the initial cell concentration in the broth.

In an earlier work (4), we standardized this logarithmic relationship with a laboratory strain of *Escherichia coli* grown under standard nonselective incubation conditions that allowed various species of microorganisms to reduce exogenous lipoic acid. Then, in a recent paper (1), we showed that the selective incubation conditions used in conventional methods for coliform analysis were sufficient to inhibit the potentiometric responses of most organisms belonging to the usual aquatic flora; among the few noninhibited organisms, *E. coli* exhibited the highest reductive activity, i.e., the shortest t(100 mV) values. These results, obtained with laboratory strains, were further confirmed by potentiometric measurements in samples collected from naturally contaminated water; *E. coli* was the main organism detected and enumerated among the bacterial flora of this environmental water (1). In this paper, we extend the potentiometric analysis of bacterial contamination to various natural waters, in particular weakly contaminated waters, and to disinfected water suitable for drinking. The potentiometric data are systematically related to the fecal coliform analysis by standard methods.

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MATERIALS AND METHODS

Sample sources and inoculum preparation. Water samples of various origins and contamination levels were tested by the potentiometric technique: unchlorinated wastewater (secondary effluent from an activated sludge treatment plant), surface water (collected from a river in a rural area), groundwater (37 sources, wells or springs issuing from highly karstic limestone aquifers lying at different depths), and drinking water (collected from a supply network).

Successive 10-fold dilutions of wastewater samples in sterile distilled water were used as inocula (inoculum volume, 0.2 ml), and samples of surface water, groundwater, or drinking water were inoculated undiluted into selective broth (30- or 100-ml inocula).

Conventional methods. (i) Enumeration of fecal coliforms in samples. The number of fecal coliforms in highly contaminated samples (i.e., wastewater) was determined by control coliform selective plate counts as described previously (1). Fecal coliforms in surface water, groundwater, or drinking water samples were enumerated by the membrane filtration procedure; water sample volumes of 10 or 100 ml were filtered through 0.45-μm-pore-size, 47-mm-diameter membrane filters (HAWG; Millipore Corp., Bedford, Mass.), the membrane filters were placed on Tergitol 7 Agar plates (Institut Pasteur Production, Paris, France), and colonies were enumerated after incubation of the plates for 24 h at 44 or 37°C (drinking water).

(ii) Isolation and identification procedures. Isolation and identification of bacteria in flasks with positive potential-time responses were carried out by the conventional methods used routinely in the Le Havre water quality testing laboratory and described elsewhere (1). In the case of drinking water samples inducing false-negative potentiometric responses, colonies of different types on Tergitol 7 Agar

were picked off and tested by the same conventional procedures.

Potentiometric technique. (i) Medium and growth conditions. Potential-time measurements were performed in the minimal medium described earlier (4) and supplemented with yeast extract (2 g/liter; Difco Laboratories, Detroit, Mich.), lactose (4 g/liter); lipoic acid (2 mg/liter; E. Merck AG, Darmstadt, Federal Republic of Germany), and sodium deoxycholate (0.5 g/liter; Merck).

Cultures were incubated at 41°C in 50- or 150-ml Erlenmeyer flasks containing, respectively, 35- or 125-ml final volumes of medium. Wastewater or surface water samples were inoculated into 50-ml flasks containing either 35 ml of single-strength medium (0.2-ml inocula) or 5 ml of 7×-strength medium (30-ml inocula) for the lowest contamination levels (surface water). One-hundred-milliliter volumes of groundwater or drinking water were inoculated into 150-ml flasks containing 25 ml of 5×-strength medium. At the beginning of any potential-time measurement, the concentration of dissolved oxygen in the culture broth, standardized as described elsewhere (1), was equal to 6.5 mg/liter.

The flasks were placed in a reciprocal water bath shaker (type S.B.K. 25; Salvis) (agitation speed, 85 rpm, stroke, 15 mm). During the potentiometric measurements, the kinetics of oxygen transfer from residual air in 150-ml flasks to the culture broth did not significantly differ from those determined previously in 35-ml flasks (5; see also T. Jouenne, 3rd Cycle Thesis, University of Rouen, Rouen, France, 1984).

(ii) Potential-time measurements. The potentiometric apparatus has been described elsewhere (4).

RESULTS

Highly contaminated water samples. (i) Wastewater. A total of 112 potential-time measurements, corresponding to 30 samples and including replicates and 10-fold dilutions, were carried out in the selective medium inoculated with wastewater samples. The relevant potential-time data, i.e., detection times [t(100 mV)], were grouped along a straight line as a function of the initial concentration of fecal coliforms in the broth determined by conventional methods (Fig. 1). The least-squares line had the following equation: $t(100\ mV) = -71.3 \log x_0 + 758$, where t(100 mV) is given in minutes and x_0 is the initial concentration of fecal coliforms in the broth (cells per liter). The r value was 0.949.

E. coli was found in all the flasks that were kept for the isolation and identification of the responsible organisms (i.e., 30 flasks, 1 per water sample), either alone (20 flasks) or accompanied by *Klebsiella pneumoniae* (10 flasks). In Fig. 1, t(100 mV) values attributable to *E. coli* alone are distinguished from those corresponding to mixed cultures of *E. coli* and *K. pneumoniae*.

(ii) Surface water. A total of 38 samples of surface water were submitted to the potentiometric test. The values of log x_0, as determined by the control filtration procedure, ranged from 2.2 to 4.4, and all the flasks had positive potentiometric responses, i.e., detection times [t(100 mV)] shorter than 20 h.

Of the 38 flasks submitted to standard isolation and identification procedures, 21 contained *E. coli* alone and 12 contained *E. coli* accompanied by other species: *K. pneumoniae* (9 flasks), *Acinetobacter calcoaceticus* (2 flasks), and *Enterobacter cloacae* and *A. calcoaceticus* (1 flask). Four flasks contained *K. pneumoniae* alone, and one flask contained *K. pneumoniae* accompanied by *Citrobacter freundii*; two of these flasks had detection times longer than 15 h (one containing *K. pneumoniae* and one containing *K. pneumoniae* and *C. freundii*).

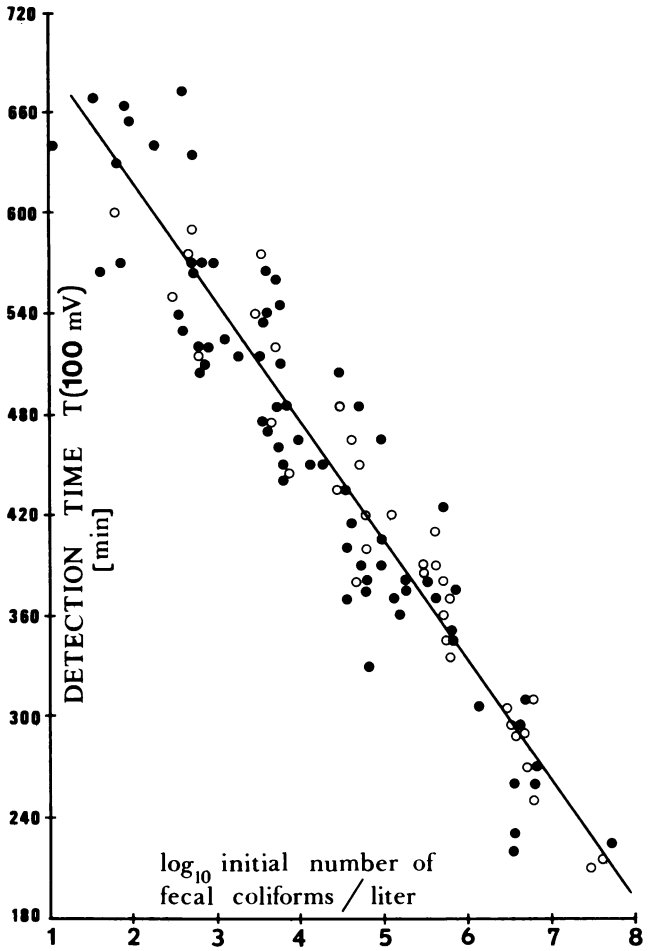

FIG. 1. Potentiometric detection times [t(100 mV)] as a function of the initial concentration of fecal coliform organisms in flasks inoculated with wastewater samples. Symbols: ●, experimental data attributable to *E. coli* alone; ○, experimental data attributable to *E. coli* accompanied by *K. pneumoniae*; ——, correlation line obtained by least-squares analysis of experimental data attributed to *E. coli* alone or accompanied by *K. pneumoniae*.

The detection times [t(100 mV)] were more dispersed than those corresponding to wastewater samples when related to log x_0. The least-squares analysis of experimental data attributable to *E. coli*, either alone or accompanied (60 pieces of data, including replicates), led to a correlation line that followed the equation $t(100\ mV) = -73.7 \log x_0 + 804$. The r value was 0.711.

Weakly and inconstantly contaminated water samples. (i) Groundwater. A total of 553 groundwater samples were analyzed by the potential-time technique and by the conventional enumeration method. A total of 36 samples that the control coliform selective filtration procedure had judged to be uncontaminated induced positive potential-time responses when inoculated into selective medium; 32 samples, however, appeared to be false-negative in the potentiometric test (Table 1).

The results of identification of the organisms present in flasks with positive potentiometric responses (172 water

TABLE 1. General results of analysis of groundwater samples by the potential-time technique and by the conventional membrane filtration procedure

Membrane filtration procedure	Potential-time technique	
	No. of positive samples	No. of negative samples
No. of positive samples	136	32
No. of negative samples	36	349

samples) are shown in Table 2. The bacterial species found in most of the positive flasks were fecal coliforms, mainly *E. coli* and *K. pneumoniae*; a limited number of flasks contained nonfecal coliforms or other gram-negative bacteria.

The results of 210 potential-time measurements, corresponding to the positive samples (including replicates and excluding false-positives), are shown in Fig. 2 as a function of log x_0. The least-squares analysis of 186 pieces of data attributable to *E. coli*, either alone or accompanied (150 water samples), led to a correlation line that followed the equation $t(100\ \text{mV}) = -77.5 \log x_0 + 776$. The r value was 0.715.

The detection times [t(100 mV)] recorded in flasks that did not contain *E. coli* were greatly delayed compared with those attributable to the presence of this organism. Thus, all flasks contaminated by samples containing *E. coli* (150 samples) had potentiometric detection times shorter than 15 h. Only 6 of the 22 water samples free of *E. coli* (Table 2) had detection times shorter than 15 h; 2 contained *K. pneumoniae*, 2 contained *E. cloacae*, 1 contained *K. pneumoniae* and *E. cloacae*, and 1 contained *K. pneumoniae* and *A. calcoaceticus*.

(ii) Drinking water. Of 110 samples of disinfected water suitable for drinking, 106 were found to be negative and 1 was found to be positive by the potentiometric technique as well as by the standard membrane filter procedure. Three samples which were found to be positive by the standard enumeration procedure induced negative potential-time responses when inoculated into selective broth; the organisms enumerated by membrane filtration were identified as noncoliform (oxidase-positive) species or as nonfecal coliforms (*Buttiauxella agrestis* or nonfecal *Citrobacter* spp.).

The potentiometric detection time recorded in the positive flask was 810 min, and the corresponding initial concentration of fecal coliforms was 125 cells per liter (log x_0 = 2.10). *E. coli* was the only organism isolated from this flask.

TABLE 2. Organisms inducing positive potentiometric responses in selective broth inoculated with groundwater samples

Species	No. of samples with indicated species
E. coli	119
E. cloacae	8
P. mirabilis	4
C. freundii	3
K. pneumoniae	3
E. coli + *K. pneumoniae*	16
E. coli + *A. calcoaceticus*	5
E. coli + *C. freundii*	3
E. coli + *K. oxytoca*	3
E. coli + *E. agglomerans*	1
E. coli + *P. aeruginosa*	1
E. coli + *K. pneumoniae* + *K. oxytoca*	1
E. coli + *K. pneumoniae* + *K. oxytoca* + *A. calcoaceticus*	1
K. pneumoniae + *C. freundii*	2
K. pneumoniae + *E. cloacae*	1
K. pneumoniae + *A. calcoaceticus*	1

DISCUSSION

In preliminary experiments with natural water samples (1), we showed that the potentiometric test mainly detected *E. coli* among the microbial flora of the water tested. This selectivity of the potentiometric technique for *E. coli* was confirmed by the more detailed results reported here; the positive potential-time responses obtained by inoculating selective broth with water samples of various origins were in

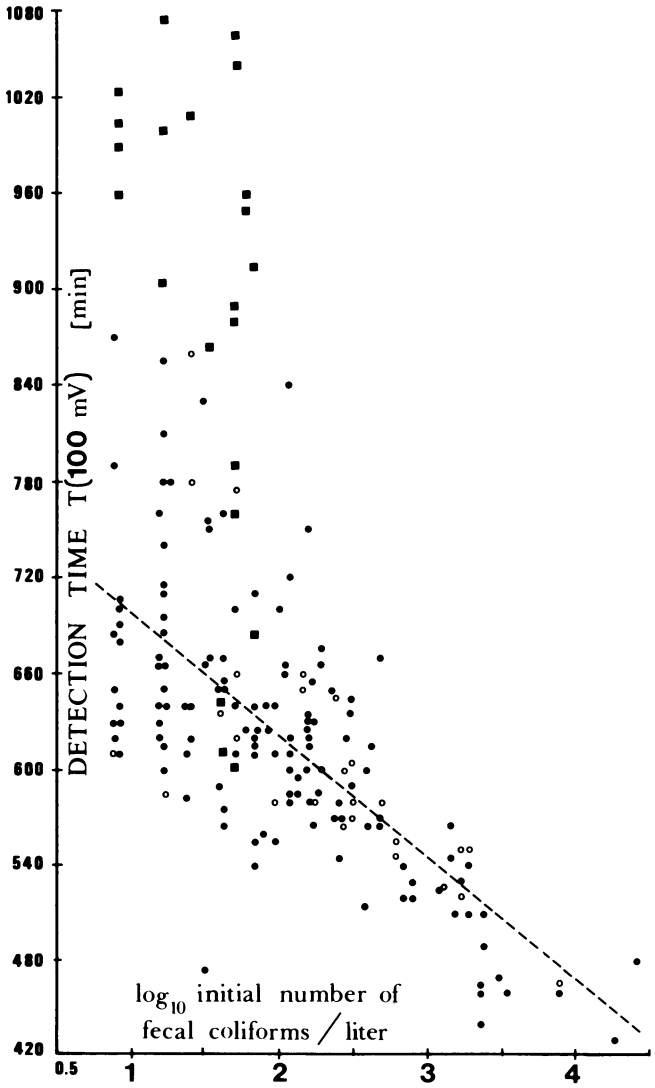

FIG. 2. Potentiometric detection times [t(100 mV)] as a function of the initial concentration of fecal coliform organisms in flasks inoculated with groundwater samples. Symbols: ●, experimental values attributable to *E. coli* alone; ○, experimental values attributable to *E. coli* accompanied by other species; ■, experimental values attributable to species other than *E. coli*; -----, correlation line obtained by least-squares analysis of experimental data attributable to *E. coli* (alone or accompanied).

TABLE 3. Distribution of organisms in culture flasks inoculated with natural water samples of various origins and having positive potentiometric responses

Organism	Presence (+) or absence (−) of other organisms[a]	No. of flasks from which organisms were isolated and identified			
		Wastewater (30)[b]	Surface water (38)	Ground water (172)	Total (240)
E. coli	−	20	21	119	160
	+	10 (100)[c]	12 (86.8)	31 (87.2)	53 (88.75)
K. pneumoniae	+	10	9	18	37
	−	0 (33.3)	5 (36.8)	7 (14.5)	12 (20.4)
A. calcoaceticus	+	0	3	6	9
	−	0	0 (7.9)	1 (4.1)	1 (4.2)
E. cloacae	+	0	1	0	1
	−	0	0 (2.6)	9 (5.2)	9 (4.2)
C. freundii	+	0	0	3	3
	−	0	1 (2.6)	5 (4.65)	6 (3.75)
K. oxytoca	+	0	0	5	5
	−	0	0	0 (2.9)	0 (2.1)
P. mirabilis	+	0	0	0	0
	−	0	0	4 (2.3)	4 (1.7)
E. agglomerans	+	0	0	1	1
	−	0	0	0 (0.6)	0 (0.4)
P. aeruginosa	+	0	0	1	1
	−	0	0	0 (0.6)	0 (0.4)

[a] For organisms other than *E. coli*, the accompanying organism was *E. coli*.
[b] Total number of flasks submitted to standard isolation and identification procedures (one flask per water sample).
[c] Percentage of flasks containing the organism (alone or accompanied).

their great majority imputable to *E. coli*, alone or accompanied by other organisms (Table 3). The number of bacterial species isolated from positive flasks increased when the contamination level of the tested water decreased; these included fecal coliforms other than *E. coli* (*K. pneumoniae*, *E. cloacae*, *C. freundii*, and *Klebsiella oxytoca*), of which *K. pneumoniae* was the most frequently isolated, nonfecal coliforms (*Enterobacter agglomerans*), noncoliform *Enterobacteriaceae* sp. (*Proteus mirabilis*), and other gram-negative bacteria (*A. calcoaceticus* and *Pseudomonas aeruginosa*). A striking element of this set of results was the proportion of positive potentiometric responses induced by fecal coliform organisms; only 4 (1.7%) of the 240 flasks submitted to standard identification procedures did not contain coliforms of fecal origin; they contained *P. mirabilis* alone (groundwater).

Our previous experiments on various laboratory strains have elicited the possibility of detecting *K. pneumoniae* and *A. calcoaceticus* by potential-time measurements, and we have already discussed the significance of such detections at the ecological and sanitary levels (1). In the same work (1), we reported that pure laboratory cultures of *E. cloacae* and *K. oxytoca* induced positive potentiometric responses within 20 h, whereas *C. freundii* and *P. aeruginosa* did not develop significant reductive activity under selective incubation conditions (pure cultures of *E. agglomerans* and *P. mirabilis* were not tested in these preliminary assays). Most bacterial species that we have isolated and identified in positive flasks were also detected by Warren et al. with a colorimetric β-galactosidase assay in EC broth at 44.5°C (8) or by Reasoner et al. with a rapid fecal coliform test in a lactose-base medium at 41.5°C (6).

E. agglomerans and *P. aeruginosa*, which are not considered pollution indicator organisms, were isolated infrequently and were always accompanied by *E. coli*. Like *A. calcoaceticus*, *P. aeruginosa* is an opportunistic pathogen, and its detection might indicate a more general epidemiological risk which could be significant for public health. The problem is the same as for the detection of the occasionally pathogenic *K. pneumoniae*, the presence of which in environmental waters does not necessarily imply fecal contamination, as its habitat is both intestinal and aquatic. The isolation of *P. mirabilis* is surprising; this organism is devoid of β-galactosidase and could exhibit significant reductive activity without metabolizing lactose, i.e., by developing a proteolytic metabolism that degrades the nitrogenous organic compounds in yeast extract. *P. mirabilis*, which is generally nonpathogenic, occasionally is present in the human intestinal flora and may be found in a number of wastewaters (2); its thermotolerance could signify a fecal origin.

In our previous paper (1), we assumed that the potentiometric technique would detect 1 *E. coli* cell per 100 ml of water within 15 h. This assumption has been strengthened by our investigations of the distribution of bacterial species identified in positive flasks as a function of the detection times (Table 4). A total of 95.95% of the detection times

TABLE 4. Distribution of bacterial species isolated from positive flasks as a function of the detection times (natural water samples)

Species isolated	No. (%) of flasks with indicated species at:	
	t (100 mV) ≤ 15 h[a]	15 h < t (100 mV) ≤ 20 h[b]
E. coli	213 (95.95)	0
E. cloacae	2 (0.9)	6 (33.3)
E. cloacae + *K. pneumoniae*	1 (0.45)	0
K. pneumoniae	5 (2.25)	2 (11.1)
K. pneumoniae + *A. calcoaceticus*	1 (0.45)	0
K. pneumoniae + *C. freundii*	0	3 (16.7)
C. freundii	0	3 (16.7)
P. mirabilis	0	4 (22.2)

[a] 222 of 240 flasks (92.5%).
[b] 18 of 240 flasks (7.5%).

shorter than 15 h were associated with the presence of *E. coli* (alone or accompanied), whereas *E. coli* was absent from all positive flasks with detection times longer than 15 h; 77.8% of t(100 mV) values over 15 h corresponded to the detection of other fecal coliforms (*K. pneumoniae*, *E. cloacae*, and *C. freundii*). The minimal concentration of *E. coli* in the flasks at the beginning of the potential-time measurements was 1 bacterium per 125 ml of broth (100-ml inocula), i.e., in the range of the maximal concentration permitted in drinking water (1 *E. coli* cell per 100 ml). Thus, the potential-time technique may be used to test the potability of water designed for human drinking; 20-h analyses would be required for the detection of other fecal coliforms, whereas 15-h analyses would be sufficient for detecting *E. coli* (this was confirmed by our positive potentiometric analysis of a drinking water sample). Such application to weakly and inconstantly contaminated water raises the question of the sensitivity (i.e., detection threshold) of the potentiometric technique as compared with that of the standard enumeration procedure. Our results for groundwater or drinking water showed that false-positive samples in the potentiometric test balanced false-negative samples (Table 1); this statistical equivalence between false-positives and false-negatives reflected the random distribution of cells in water samples with low bacterial concentrations and indicated a satisfactory sensitivity of the potentiometric test, similar to that of the standard technique.

Besides acting as an emergency device, the potentiometric technique aims at evaluating quantitatively the contamination level of a water sample, i.e., the concentration of fecal coliform organisms in the tested sample. This enumeration function is based on the construction of calibration curves relating detection times [t(100 mV)] to initial concentrations of fecal coliforms determined by conventional methods. In the case of highly contaminated waters, such constructions were easily made by testing a wide range of inocula obtained by successive dilutions of the original samples and enumerated with satisfactory precision by standard procedures; like inocula prepared from highly polluted surface water samples (1), inocula obtained from wastewater samples had detection times that were grouped with a good correlation (r close to 1) along a straight line as a function of log x_0 (Fig. 1). Since no preliminary ultrafiltration step was used to gain higher x_0 values, the range of the experimental x_0 values was more limited when the tested water was weakly contaminated. The standardization of x_0 for each potential-time measurement depended on conventional membrane filtration enumeration. Thus, the t(100 mV) values were somewhat dispersed around the correlation line when related to log x_0; the dispersion increased when x_0 decreased (Fig. 2).

In the present work, we constructed the different calibration curves by least-squares analysis of the experimental data corresponding to the presence of *E. coli* in positive culture flasks. The relevant correlation lines had quite similar equations, whatever the water sample origins; the slope, which was proportional to the generation time of the organisms (i.e., *E. coli*), increased slightly when the contamination level of the tested water decreased. In our preliminary assays with pure *E. coli* cultures, we showed that the growth rate of *E. coli* determined by potential-time measurements varied from one strain to another (1). Here, the generation times calculated from the slopes were, respectively, 21.5 min (wastewater), 22.2 min (surface water), and 23.3 min (groundwater); these values were in the range of the generation times obtained with laboratory strains of *E. coli* (1). Such variations in the average growth rate of *E. coli* may have been due to the diversity of strains present in the different natural waters tested and to variations in the stresses to which *E. coli* was subjected in the different waters. As reported by Silverman and Munoz, using impedance measurements (7), variations in the average growth rate of *E. coli* in the same water may occur according to the moment when potential-time measurements are performed. The practical counting of *E. coli* in any given environmental water sample will thus require the construction of a specific calibration curve that will perhaps have to be reconsidered periodically; the precision of the enumeration will depend on that of the preliminary conventional counts and will decrease with the degree of contamination of the natural water tested.

In conclusion, our present results show that the potentiometric technique may serve as an emergency test for detecting fecal contamination of drinking water and may also be applied to the counting of *E. coli* in highly contaminated water. An important step in the practical development of these applications would be the automation of the potential-time measurements. The potentiometric method, which is technologically simple, has been patented both in the United States and Europe, and we are now looking for industrial support for the construction of an automated detector prototype.

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