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Assessment of the Effects of Holding Time and Temperature on *Escherichia coli* Densities in Surface Water Samples

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Escherichia coli **is a routinely used microbiological indicator of water quality. To determine whether holding time and storage conditions had an effect on** ***E. coli*** **densities in surface water, studies were conducted in three phases, encompassing 24 sites across the United States and four commonly used monitoring methods. During all three phases of the study,** ***E. coli*** **samples were analyzed at time 0 and at 8, 24, 30, and 48 h after sample collection. During phase 1, when 4°C samples were evaluated by Colilert or by placing a membrane onto mFC medium followed by transfer to nutrient agar containing 4-methylumbelliferyl-β-D-glucuronide (mFC/NA-MUG), three of four sites showed no significant differences throughout the 48-h study. During phase 2, five of seven sites showed no significant difference between time 0 and 24 h by membrane filtration (mFC/NA-MUG). When evaluated by the Colilert method, five of seven sites showed no significant difference in** ***E. coli*** **density between time 0 and 48 h. During phase 3, 8 of 13 sites showed no significant differences in** ***E. coli*** **densities between time 0 and the 48-h holding time, regardless of method. Based on the results of these studies, it appears that if samples are held below 10°C and are not allowed to freeze, most surface water** ***E. coli*** **samples analyzed by commonly used methods beyond 8 h after sample collection can generate** ***E. coli*** **data comparable to those generated within 8 h of sample collection. Notwithstanding this conclusion,** ***E. coli*** **samples collected from surface waters should always be analyzed as soon as possible.**

Escherichia coli testing is an important tool used by public health experts for the prevention of waterborne disease. The detection of *E. coli* in a water sample from an environmental source provides direct evidence of fecal contamination. Regulatory agencies are increasingly requiring more emphasis on *E. coli* testing as part of programs aimed at curtailing waterborne disease. Holding time and temperature can have a significant impact on the density of microbiological indicators at the time of sample analysis (4, 5, 7). Recommendations for *E. coli* holding times range from 8 h (2, 3, 9) to 24 h (8), and holding temperatures below 10°C are generally considered acceptable (2, 3, 8, 9). It is also recommended that when transport conditions result in delays longer than 6 h, the use of field laboratory facilities located at the site of collection or delayed incubation procedures be considered (2). The Surface Water Treatment Rule requirements of the U.S. Environmental Protection Agency (USEPA) for total coliform and fecal coliform monitoring of surface water used as drinking water sources (3) specify that the time from sample collection to initiation of analysis is not to exceed 8 h; the regulations also encourage (but do not require) drinking water system personnel to hold samples at below 10°C during transit.

Unfortunately, data from evaluations of microbiological indicator density that support current holding time recommendations are limited, particularly for *E. coli*. In a study comparing fecal coliform levels in samples collected from municipal wastewater and stored for 4 and 24 h at 2 to 4°C (7), results indicated that the means of the 4- and 24-h replicates were within 20% of each other for 24 of 28 sampling events. The researchers considered this range acceptable on the basis of inherent method variability. Researchers evaluating total coliforms in drinking water observed that when the samples were stored at 5°C, densities were 34% lower at 24 than at 0 h (4). In the same study, when samples were stored at 22°C total coliform densities were 87% lower after 24 h, indicating that both storage time and temperature can impact total coliform densities. In another study evaluating total coliforms in drinking water (8), researchers observed that total coliform densities in some samples stored for 24 h at 22 and 5°C were 47% and 23% lower than densities at 6 h. Total coliform densities were 62 and 33% lower, respectively, after 30 h of storage.

For the USEPA's upcoming Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR), a Federal Advisory Committee recommended that large drinking water treatment systems (serving ≥10,000 people) monitor their source waters for *Cryptosporidium* oocysts (10) to determine the need for additional treatment. The Committee recommended that large systems also monitor for *E. coli* to provide additional data on the use of *E. coli* as a surrogate to determine a source water's vulnerability to *Cryptosporidium*.

To reduce monitoring costs, the Committee recommended that small systems (serving <10,000 people) initially monitor their source water for *E. coli* in lieu of *Cryptosporidium* monitoring and only monitor for *Cryptosporidium* when *E. coli*

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TABLE 1. Summary of factors evaluated during each phase of analysis[a]

Phase	Temp (°C) or coolant conditions	Method	No. of sites tested	Site location
1	Maintained under controlled conditions at 4, 10, 20, or 35 °C	Colilert (Quanti-Tray 2000) or membrane filtration (mFC/NA-MUG)	4	Within 2 h driving distance of the Madison, Wis., laboratory
2	Stored to simulate real-world storage conditions by using wet ice or Utek ice packs	Colilert (Quanti-Tray 2000) or membrane filtration (mFC/NA-MUG)	7	Within 2 h driving distance of the Cincinnati, Ohio, laboratory
3	Stored to simulate real-world storage conditions by using wet ice, Utek ice packs, or Blue ice	Colilert (Quanti-Tray 2000) or membrane filtration (mFC/NA-MUG, mEndo/NA-MUG, or mTEC)	13	Throughout the continental United States and within 2 h driving distance of the laboratory

[a] Samples were analyzed at holding times of 0, 8, 24, 30, and 48 h for all phases of analysis.

levels exceed certain trigger values. Under the Committee's recommended approach, small-system monitoring would begin after completion of the large-system monitoring so that large system monitoring data could be used to further assess the *E. coli* values that would trigger small system *Cryptosporidium* monitoring.

Large systems can typically analyze *E. coli* samples within an 8-h holding time either through on-site analyses at the utility's laboratory or by using off-site laboratories within driving distance. However, small systems that do not have onsite *E. coli* analysis capability and are not within driving distance from a certified laboratory are not able to comply with an 8-h holding time. (The use of delayed incubation procedures, such as Standard Methods 9222E [2], which could increase the allowable sample holding time, may not be appropriate for most small systems, as most such systems do not have personnel trained to perform the sterile-transfer techniques that would be required.) Consequently, the potential widespread violation by small systems of an 8-h *E. coli* holding time is a significant concern. Analysis of *E. coli* samples at 24, 30, or 48 h after sample collection might bias results, giving data with values that are lower or higher than those of *E. coli* analyses performed on the same sample within 8 h of sample collection.

To determine whether holding time has an effect on *E. coli* sample results, studies were conducted in three phases. A summary of each phase is provided in Table 1. Figure 1 shows the geographical locations of all sites differentiated by phase. For all phases, *E. coli* density results for each holding time were compared to the initial *E. coli* density at time 0 and also to the 8-h results. Since both comparisons yielded similar results, only the comparisons to time 0 are presented.

The objective of the phase 1 study was to determine whether data from *E. coli* samples collected and held for various time periods and temperatures could be considered comparable to data from samples analyzed within the recommended 8-h holding time. The controlled conditions under which samples were held in the study represented possible conditions that might be encountered when systems ship samples overnight to an off-site laboratory for analysis. During phase 1, to assess the effects of different shipping conditions, *E. coli* sample densities were measured at 8, 24, 30, and 48 h after sample collection for samples stored at 4, 10, 20, and 35°C.

The objective of the phase 2 study was to use real-world sample storage practices (i.e., coolers with wet ice or Utek ice packs) to evaluate additional *E. coli* samples from surface waters used as drinking water sources. During the phase 2 study, *E. coli* samples from seven sites within a 2-h driving distance of the Cincinnati laboratory were analyzed at 8, 24, 30, and 48 h after sample collection.

The objective of the phase 3 study was to evaluate *E. coli* holding times from different geographical regions throughout the United States and to evaluate a greater variety of monitoring methods. During the phase 3 study, volunteer utilities organized by the American Water Works Association evaluated *E. coli* samples from 15 drinking water utility sources at 8, 24, 30, and 48 h after sample collection.

MATERIALS AND METHODS

Phase 1. During the phase 1 study, *E. coli* samples from four sites, representing a high-water-quality lake (Rainbow Lake in King, Wis.), a low-water-quality lake (Lake Winnebago in Oshkosh, Wis.), a high-water-quality flowing stream (Wisconsin River, just below the Wisconsin Dells Dam, Wis.), and a low-water-quality flowing stream (Lincoln Creek in Milwaukee, Wis.), were analyzed. The holding time studies for the Wisconsin River, Rainbow Lake, Lincoln Creek, and Lake Winnebago samples were conducted during the weeks of 24 September, 8 October, 22 October, and 3 December 2001, respectively.

For the sites that serve as drinking water source waters (Lake Winnebago and Rainbow Lake), the utilities provided data from the sample collection points used to collect samples for this study for the following water quality parameters: turbidity, temperature, pH, phosphates, total nitrogen, total organic carbon, and heterotrophic plate counts. When water quality data were not provided by a utility (Lincoln Creek and Wisconsin River), the Wisconsin State Laboratory of Hygiene (WSLH) performed the water quality analyses. WSLH collected water quality samples and *E. coli* samples at the same time.

To determine whether spiking would be necessary, WSLH collected a 1-liter sample 1 day prior to beginning the analysis of samples from each site. To determine ambient *E. coli* densities, upon collection the WSLH immediately placed each sample on wet ice and transported it to the laboratory for *E. coli* analysis in triplicate by each method (Colilert and membrane filtration).

On the day the holding time and temperature experiments were started for each site, a sample collection technician drove to the site and collected a 10-liter sample, placed the sample on wet ice, and immediately transported the sample back to the WSLH. For Lake Winnebago and Rainbow Lake, samples of the untreated water were collected directly into sterile, 10-liter cubitainers at the in-plant raw water collection tap. Using a sterile 1-liter bottle multiple times to fill a 10-liter cubitainer, samples were collected at Lincoln Creek and Wisconsin River as composite grab samples.

After each sample arrived at WSLH, it was continuously stirred while 100-ml sample aliquots were dispensed into sterile, 160-ml plastic sample containers. Samples for assessment of each holding time, temperature, and method were randomly selected throughout the dispensing process. For waters in which (according to the results of the previous day's analyses) ambient densities of *E. coli* were below 100 *E. coli* bacteria per 100 ml, individual 100-ml samples were spiked within 2 h of sample collection. Samples from Lake Winnebago and Rainbow Lake were spiked. Samples from Lincoln Creek and the Wisconsin River had sufficient ambient densities and were not spiked.

The following approach was used to spike samples from Lake Winnebago and Rainbow Lake: a single-passage master culture of an environmental isolate of *E. coli* was verified as *E. coli* via biochemical reactions, aliquoted to multiple

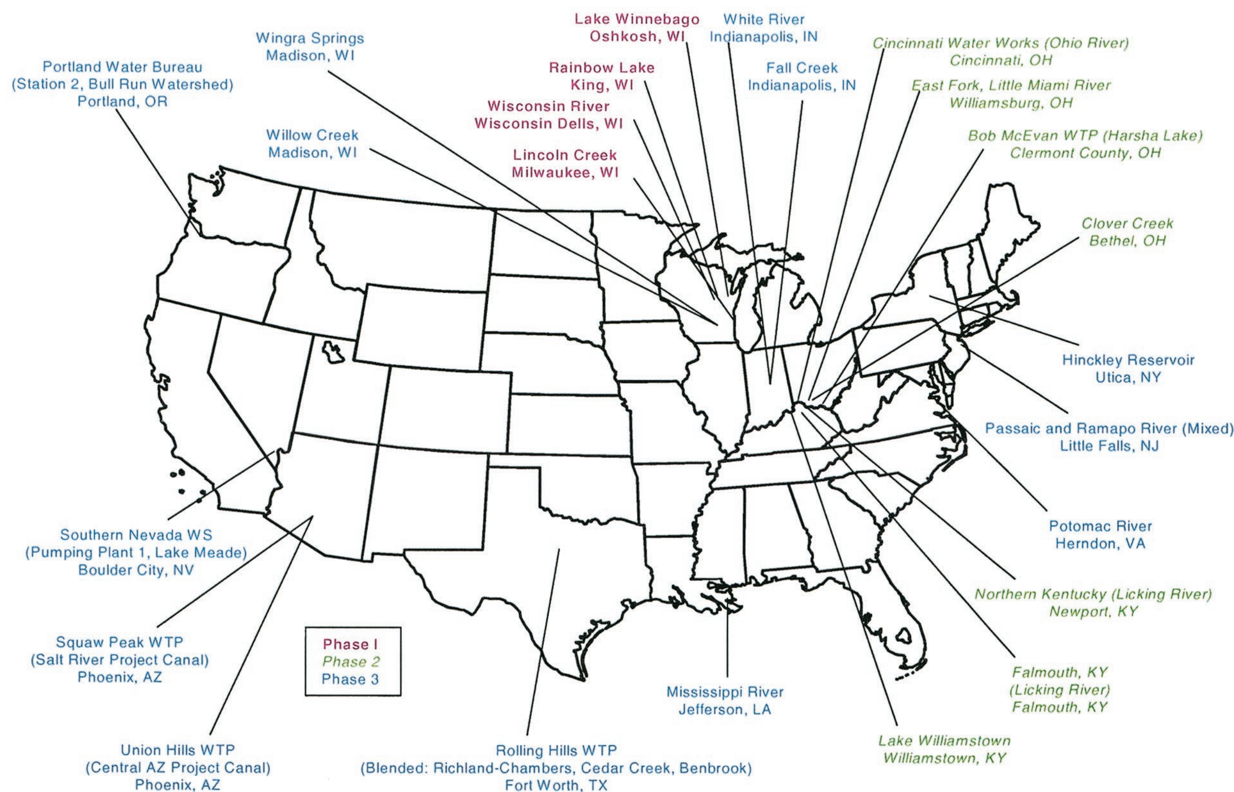

FIG. 1. Geographical locations of all sampling sites (differentiated by phase). WTP, water treatment plant.

cryovials, and frozen at −70°C. For each site, a frozen vial was thawed, inoculated into Trypticase soy broth, and incubated overnight. Using a spectrophotometer, the overnight culture was diluted to a 0.5 McFarland turbidity standard. From this, two sequential serial 1:1,000 dilutions were made using sterile phosphate buffer. Of the second 1:1,000 dilution, 1 ml was used to spike each 100-ml surface water sample, resulting in a spike of approximately 100 *E. coli* per 100 ml.

Storage temperatures, holding times, methods, and time 0 sample analysis. Using two methods, WSLH analyzed *E. coli* samples in triplicate from four sites at four sample storage temperatures and four holding times. The storage temperatures, holding times, methods, and analysis of time 0 samples are discussed below.

(i) Four sample storage temperatures. Temperatures of 4, 10, 20, and 35°C were maintained in a refrigerator or incubator (as appropriate) to meet the target temperatures. These temperatures were selected on the basis of the results of informal sample temperature range-finding studies conducted by DynCorp in preparation for this study. The laboratory recorded sample temperatures using iButtons in temperature sample containers identical to the actual test vials. (A Thermocron iButton is a small instrument [about the size of five dimes] that records temperature at time intervals specified by researchers.) The temperature samples contained unspiked source water collected at the same time as the *E. coli* samples. The iButtons were set to record temperature every 10 min throughout the study.

(ii) Four holding times. Holding times of 8, 24, 30, and 48 h from the time that samples were spiked or dispensed into 100-ml aliquots were used.

(iii) Two methods. Colilert Quanti-Tray/2000 (11) and membrane filtration onto mFC medium followed by transfer to nutrient agar containing 4-methylumbelliferyl-β-D-glucuronide (mFC/NA-MUG) are two of the most widely used methods for *E. coli* analysis. A minimum of two sample volumes were filtered for the mFC/NA-MUG method to ensure that countable plates were obtained.

(iv) Time 0 sample analysis. For all sites (with the exception of Rainbow Lake) and methods, seven time 0 samples were analyzed to determine the initial *E. coli* density. For Rainbow Lake, time 0 samples were analyzed in triplicate for each method. Please note that the initial time 0 samples were not exposed to the holding temperatures because the samples were analyzed prior to being stored.

Phase 2. Seven surface waters presently used as drinking water sources were selected for evaluation during phase 2. To ensure that sample analysis could begin within 3 h, all sites were within a 2-h driving distance of the USEPA's Technical Support Center laboratory in Cincinnati, Ohio. The sites selected for the phase 2 study were as follows: Bethel, Ohio; Cincinnati Water Works, Ohio; Clermont County, Ohio; Falmouth, Ky.; Northern, Newport, Ky.; Williamsburg, Ohio; and Williamstown, Ohio.

The utilities provided the temperature and pH data from the sample collection points used to collect samples for this study. The USEPA analyzed the samples for turbidity, phosphates, total nitrogen, and total organic carbon. Water quality samples were collected at the same time as the *E. coli* samples.

Samples were collected and analyzed between March and June of 2002. To determine whether spiking would be necessary, USEPA personnel collected a 1-liter sample 1 day prior to beginning the analysis of samples from each site. Upon collection, the USEPA personnel immediately placed each sample on wet ice and transported it to the laboratory for *E. coli* analysis in triplicate by each method (Colilert and mFC/NA-MUG) to measure ambient *E. coli* concentrations. On the day that the holding time and storage condition experiments were started for each site, utility personnel collected a 10-liter sample and placed the sample on wet ice and the sample was transported back to the USEPA.

After the sample arrived at the USEPA, it was spiked (if necessary) and continuously stirred. While being stirred, 100-ml sample aliquots were dispensed into sterile sample containers. Samples for each holding time and method were randomly selected throughout the dispensing process. For waters in which (according to the results of the previous day's analyses) ambient densities of *E. coli* were below 100 per 100 ml, the 10-liter sample was spiked within 3 h of sample collection. Samples from Bethel, Cincinnati Water Works, Clermont County, Williamsburg, and Williamstown were spiked. Samples from Falmouth and Northern (Newport, Ky.) had sufficient ambient densities and were not spiked.

The following approach was used to spike samples from Bethel and Williamstown. A master culture of an environmental isolate of *E. coli* was verified as *E. coli* via biochemical reactions and maintained on a nutrient agar slant. For each site, fresh nutrient agar slants were inoculated and incubated for 24 ± 2 h at 35 ± 0.5°C. Dilutions were prepared from the slant with the best growth, and the bulk surface water sample was spiked. The same approach was used to spike samples from Cincinnati Water Works, Clermont County, and Williamsburg, with a laboratory strain of *E. coli* being used instead of an environmental isolate.

TABLE 2. Source water characterization for *E. coli* sample collection points ($n = 1$)

Phase[a]	Site	Temp (°C)	pH	Turbidity (NTU)[b]	Total organic carbon (mg/liter)	Total phosphorus (mg/liter)	Total Kjeldahl nitrogen (mg/liter)	Heterotrophic plate count (CFU/ml)
1	Rainbow Lake, King, Wis.	11.6	NA[d]	1.5	4.3	0.017	0.83	5
	Wisconsin River, Wisconsin Dells, Wis.	14.5	8.1	10.4	8.1	0.085	1.09	160
	Lincoln Creek, Milwaukee, Wis.	19.0	7.6	38.5	6.4	0.149	0.84	21,500
	Lake Winnebago, Oshkosh, Wis.	10.1	8.0	10.2	6.0	0.067	0.57	NA
2	Cincinnati Water Works, Cincinnati, Ohio	5.4	7.6	6.3	9.2	ND[c]	0.74	240
	Clermont County, Ohio	10.7	7.7	NA	5.8	ND	1.07	3,000
	Williamsburg, Ohio	11.0	8.5	14.0	4.9	0.147	1.14	2,300
	Northern, Newport, Ky.	14.7	6.6	54.1	3.8	0.100	0.48	9,100
	Falmouth, Ky.	15.8	6.5	264.0	6.8	0.199	0.77	94,000
	Bethel, Ohio	17.9	8.0	3.0	3.5	ND	0.04	69
	Williamstown, Ky.	17.7	7.3	4.2	4.6	ND	0.37	930

[a] Source water characterization was not available for phase 3.
[b] NTU, nephelometric turbidity units.
[c] ND, not detected.
[d] NA, not analyzed.

Storage conditions, holding times, methods, and time 0 sample analysis. The laboratory analyzed *E. coli* samples in triplicate from seven sites, using two methods, two sample storage conditions (coolants), and four holding times. The storage conditions, holding times, methods, and analysis of time 0 samples are discussed below.

(i) Two sample storage conditions (coolants). To mimic real-world sample storage practices for samples being shipped, samples were stored in coolers with wet ice or Utek ice packs. iButtons in temperature sample containers identical to the actual *E. coli* sample containers were used to record sample temperatures. The iButtons were set to record temperature every 15 min throughout the study.

(ii) Four holding times. Holding times of 8, 24, 30, and 48 h from the time that samples were spiked or dispensed into 100-ml aliquots were used.

(iii) Two methods. Colilert Quanti-Tray/2000 and mFC followed by NA-MUG (membrane filtration) methods were used.

(iv) Time 0 samples. For all sites and methods, three time 0 samples were analyzed to determine the initial *E. coli* density. The initial time 0 samples were not exposed to the holding temperatures, because the samples were analyzed prior to being stored.

Phase 3. Drinking water utilities and laboratories participated in the phase 3 study on a volunteer basis. WSLH samples were analyzed during the week of 2 August 2002. All other samples were analyzed during the week of 24 June 2002. During phase 3, the following reservoir and/or lake drinking water sources were evaluated: Rolling Hills Water Treatment Plant (Fort Worth Water Department, Fort Worth, Tex.), Squaw Peak and Union Hills Water Treatment Plant (Phoenix, Ariz.), Water Bureau Station 2 (Portland, Oreg.), SNWS Pumping Station (Southern Nevada Water System, Boulder City, Nev.), Hinckley Reservoir (Mohawk Valley Water Authority, Utica, N.Y.), and Harwood's Mill (Newport News Waterworks, Newport News, Va.).

The following flowing stream drinking water sources were evaluated: Potomac River (Fairfax County Water Authority, Herndon, Va.), Fall Creek and White River (Indianapolis Water Company, Indianapolis, Ind.), Mississippi River (Jefferson Parish Water Department, Jefferson, La.), mixed source waters of the Passaic and Ramapo Rivers (Passaic Valley Water Commission, Little Falls, N.J.), Las Vegas Wash (Southern Nevada Water System, Boulder City, Nev.), and Wingra Springs and Willow Creek (Madison, Wis.).

To determine whether spiking would be necessary, the utilities collected an *E. coli* sample at 24 h prior to sampling for the holding-time study. On the day that the holding-time and storage condition experiments were started, a 10-liter sample was collected, placed on wet ice, and immediately transported back to the utility laboratory. After the sample arrived at the utility, it was spiked (if necessary) and continuously stirred while 100-ml sample aliquots were dispensed into sterile, plastic sample containers. Samples for each holding time and method were randomly selected throughout the dispensing process. For waters in which (according to the results of the previous day's analyses) ambient densities of *E. coli* were below 100 per 100 ml, the 2-liter sample was spiked within 2 h of sample collection. When necessary, the USEPA provided *E. coli* spiking suspensions prepared from an environmental isolate. With the exception of WSLH, spiking suspensions were shipped to the utilities on Tuesday, 25 June 2002, and samples were collected and spiked (if necessary) on Wednesday, 26 June 2002.

Personnel at each utility analyzed *E. coli* samples in triplicate, stored samples in a cooler using a coolant of their choice, and packed the samples as they would for shipment. Four holding times (8, 24, 30, and 48 h) were evaluated, and holding times began from the time that samples were spiked or dispensed into 100-ml aliquots. For all sites, three time 0 samples were analyzed to determine the initial *E. coli* density. The initial time 0 samples were not exposed to the holding temperatures, because the samples were analyzed prior to being stored. Personnel at each utility evaluated one or more sites using a method(s) of their choice. Methods used to analyze *E. coli* samples during the phase 3 study included Colilert Quanti-Tray/2000, membrane filtration onto mFC followed by transfer to NA-MUG, membrane filtration onto mEndo followed by transfer to NA-MUG, and membrane filtration using mTEC (Standard Methods 9213D.3) (2).

RESULTS AND DISCUSSION

Phase 1. DynCorp used a standardized checklist to evaluate laboratory results against method requirements, study requirements, and quality control (QC) sample results. When judged on the basis of the review of the data, all results from this study were considered valid for use in data analysis. A summary of the site-specific source water characterization results is provided in Table 2.

E. coli results for site, method, holding time, and holding temperature predictor variables were compared using general linear models (GLM) (6) at an alpha = 0.05 level of significance. GLM models are linear models that test for significant linear effects of one or more predictor variables (and interactions between predictor variables) on one or more result variables. In this specific case, where there is a single result variable (*E. coli* density) and the predictor variables are categorical (i.e., have only a few possible values), the GLM model is analogous to an analysis of variance model. Interactions with methods and sites were observed; as a result, all subsequent statistical analyses were stratified by method and site.

Using Dunnett's test (1), *E. coli* density results for each holding time and temperature combination were compared to the initial *E. coli* density at time 0 and the 8-h *E. coli* density results for each method (alpha = 0.05). Dunnett's test is a statistical method of testing for pairwise differences in means

TABLE 3. Summary of phase 1 Dunnett's test results for time 0 comparisons (alpha = 0.05)

Site	Temp (°C)	No. of *E. coli*/100 ml (significant change in density)[a] at indicated time (h) after sample collection by:									
		Colilert method					Membrane filtration (mFC/NA-MUG) method				
		0	8	24	30	48	0	8	24	30	48
Lake Winnebago	4	246	NS	NS	NS	NS	201	NS	NS	NS	NS
	10	246	NS	NS	NS	NS	201	NS	NS	NS	NS
	20	246	NS	NS	134 (D)	128 (D)	201	NS	NS	91 (D)	115 (D)
	35	246	NS	NS	NS	136 (D)	201	NS	NS	NS	NS
Rainbow Lake	4	76	NS	NS	NS	NS	89	NS	53 (D)	61 (D)	63 (D)
	10	76	NS	NS	NS	NS	89	NS	50 (D)	45 (D)	41 (D)
	20	76	NS	45 (D)	40 (D)	15 (D)	89	58 (D)	24 (D)	13 (D)	6 (D)
	35	76	18 (D)	6 (D)	5 (D)	0.3 (D)	89	8 (D)	4 (D)	4 (D)	3 (D)
Lincoln Creek	4	7,949	NS	NS	NS	NS	7,057	NS	NS	NS	NS
	10	7,949	NS	NS	NS	4,581 (D)	7,057	9,400 (I)	NS	NS	4,933 (D)
	20	7,949	NS	NS	NS	3,614 (D)	7,057	NS	NS	NS	2,680 (D)
	35	7,949	NS	NS	2,893 (D)	1,186 (D)	7,057	NS	3,320 (D)	2,560 (D)	1,520 (D)
Wisconsin River	4	79	NS	NS	NS	39 (D)	76	NS	NS	NS	NS
	10	79	53 (D)	NS	52 (D)	33 (D)	76	NS	NS	34 (D)	33 (D)
	20	79	NS	32 (D)	21 (D)	11 (D)	76	NS	39 (D)	9 (D)	10 (D)
	35	79	NS	27 (D)	7 (D)	19 (D)	76	NS	17 (D)	6 (D)	3 (D)

[a] D, significant decrease in *E. coli* density compared to the time 0 results; I, significant increase in *E. coli* density compared to the time 0 results; NS, no significant difference compared to the time 0 results.

in which control group results are compared to the results for each treatment combination. These pairwise comparisons are run after the GLM because even though GLMs determine whether the concentration of at least one level of a given predictor variable differs significantly from the concentration of at least one other level, GLMs do not specify whether these pairwise differences include the control group. By comparing treatment groups to the control group only, Dunnett's test holds the overall type I error rate to 5% and is more powerful than other tests, such as Tukey or Scheffe, for pairwise comparisons. In this study, the controls (represented by the time 0 results for each method and site) were compared to each treatment combination (i.e., results from a specific holding time and temperature for each method and site). It was necessary to compare the control to each time and temperature combination, because the time 0 samples did not have an associated holding temperature. A summary of the phase 1 Dunnett's test results for the time 0 comparison is provided in Table 3.

The following observations were made. (i) Samples stored at 20°C or 35°C. Results from 20 and 35°C holding times exhibited variable results for both methods, with *E. coli* densities being significantly less within 8 to 48 h (depending on the site).

(ii) Colilert samples stored at 4°C or 10°C. *E. coli* densities from samples stored at 4 and 10°C and analyzed by Colilert usually did not decrease significantly compared to time 0 densities until samples had been held for at least 48 h. However, samples from the Wisconsin River showed significant decreases in *E. coli* densities at 8 and 30 h when held at 10°C. For *E. coli* samples held at 4°C, none of the results for any of the sites were significantly different from time 0 results through 30 h and the results for only one of four sites were significantly different at 48 h.

(iii) Membrane filtration (mFC/NA-MUG) samples stored at 10°C. Membrane filtration results for samples held at 10°C were variable, with *E. coli* densities being significantly increased for Lincoln Creek samples at 8 h compared to those at time 0, decreased for Rainbow Lake samples at 24 h, decreased for Wisconsin River samples at 30 h, and not significantly different for Lake Winnebago samples for any holding time.

(iv) Membrane filtration (mFC/NA-MUG) samples stored at 4°C. Samples collected from Rainbow Lake exhibited a significant *E. coli* density decrease by 24 h compared to that observed at time 0. Samples from the other three sites were stable throughout the 48-h duration of the study.

The phase 1 results suggest that *E. coli* samples can be analyzed beyond 8 h after sample collection using some methods (with chilling) while still generating reliable *E. coli* data. However, a wider variety of surface waters from different regions throughout the United States still needed to be evaluated to verify these preliminary observations. Phases 2 and 3 were conducted to address these issues.

Phase 2. The results for most QC checks were acceptable. However, the Clermont 30-h mFC plates were incubated for 48 h and the *Klebsiella* negative control exhibited a positive result. As a result, these data were considered invalid and were not used in subsequent data analyses. It should also be noted that many of the spiked Williamsburg samples were at least partially frozen during sample storage, regardless of type of coolant (wet ice or Utek ice packs) used in the coolers. Since the QC checks for Williamsburg were acceptable, these data were included in subsequent data analyses. However, it is possible that the significant decreases in *E. coli* concentration at the Williamsburg site are related to cells lysing at freezing temperatures. In general, samples from other sites were maintained at <10°C and above freezing. A summary of the site-specific source water characterization results is provided in Table 2.

Mean *E. coli* densities (*E. coli* per 100 ml) for site, method, holding time, and coolant (wet ice or Utek ice packs) were

TABLE 4. Summary of phase 2 Dunnett's test results for time 0 comparisons (alpha = 0.05)

Site	Method	Spiked	Coolant	Mean no. of *E. coli*/100 ml at time 0	No. of *E. coli*/100 ml (significant change in density) at indicated time (h) after sample collection[a]			
					8	24	30	48
Williamstown, Ky.	Colilert	Yes	Wet ice	66	NS	NS	NS	NS
	Colilert	Yes	Utek	66	NS	NS	NS	NS
	mFC/NA-MUG	Yes	Wet ice	57	NS	NS	NS	NS
	mFC/NA-MUG	Yes	Utek	57	NS	NS	NS	NS
Northern, Newport, Ky.	Colilert	No	Wet ice	3,057	NS	NS	NS	NS
	Colilert	No	Utek	3,057	NS	NS	NS	NS
	mFC/NA-MUG	No	Wet ice	2,523	NS	NS	NS	NS
	mFC/NA-MUG	No	Utek	2,523	NS	NS	NS	NS
Bethel, Ohio	Colilert	Yes	Wet ice	102	NS	NS	NS	NS
	Colilert	Yes	Utek	102	NS	NS	NS	NS
	mFC/NA-MUG	Yes	Wet ice	92	NS	NS	NS	69 (D)
	mFC/NA-MUG	Yes	Utek	92	NS	NS	NS	NS
Cincinnati Water Works, Cincinnati, Ohio	Colilert	Yes	Wet ice	89	NS	NS	NS	NS
	Colilert	Yes	Utek	89	NS	NS	NS	NS
	mFC/NA-MUG	Yes	Wet ice	63	NS	NS	NS	NS
	mFC/NA-MUG	Yes	Utek	63	NS	NS	NS	41 (D)
Clermont County, Ohio	Colilert	Yes	Wet ice	78	NS	NS	NS	NS
	Colilert	Yes	Utek	78	NS	NS	NS	NS
	mFC/NA-MUG	Yes	Wet ice	83	NS	NS	INVD	NS
	mFC/NA-MUG	Yes	Utek	83	NS	NS	INVD	55 (D)
Falmouth, Ky.	Colilert	No	Wet ice	9,840	NS	NS	6,087 (D)	4,240 (D)
	Colilert	No	Utek	9,840	6,827 (D)	NS	6,073 (D)	5,700 (D)
	mFC/NA-MUG	No	Wet ice	8,200	NS	5,400 (D)	4,533 (D)	3,600 (D)
	mFC/NA-MUG	No	Utek	8,200	NS	5,200 (D)	NS	4,400 (D)
Williamsburg, Ohio	Colilert	Yes	Wet ice	1,597	2,051 (I)	NS	1,089 (D)	893 (D)
	Colilert	Yes	Utek	1,597	NS	NS	NS	NS
	mFC/NA-MUG	Yes	Wet ice	1,477	NS	1,083 (D)	906 (D)	1,067 (D)
	mFC/NA-MUG	Yes	Utek	1,477	1,117 (D)	1,097 (D)	992 (D)	1,066 (D)

[a] D, significant decrease in *E. coli* density compared to the time 0 results; I, significant increase in *E. coli* density compared to the time 0 results; NS, no significant difference in *E. coli* density compared to the time 0 results; INVD, invalid data.

compared using GLM at an alpha = 0.05 level of significance. Interactions with methods and sites were observed, and as a result, all subsequent statistical analyses were stratified by method and site. Using Dunnett's test, the *E. coli* density results for each holding-time and coolant (wet ice or Utek ice packs) combination were compared to the initial *E. coli* density results at time 0 and to the 8-h *E. coli* density results for each method (alpha = 0.05). A summary of the phase 2 Dunnett's test results for the time 0 comparison is provided in Table 4.

For membrane filtration (mFC/NA-MUG), five of seven sites showed no significant difference between time 0 and 24 h holding time (regardless of the coolant used), four sites showed no significant difference at 30 h of holding time (regardless of coolant), and only two of seven sites showed no significant difference between time 0 and 48 h of holding time (regardless of coolant). For Colilert, five of seven sites showed no significant difference in *E. coli* between time 0 and 48 h (regardless of the coolant used). It should also be noted that as assessed by membrane filtration and Colilert methods, the *E. coli* samples collected from Williamsburg, the only site with partially frozen samples, were significantly different at 8 h.

Phase 3. DynCorp used a standardized checklist to evaluate laboratory results against method requirements, study requirements, and QC sample results. The results for most checks were acceptable. However, some issues were identified; in particular, samples from Southern Nevada Pumping Plant 1 had not been maintained below 10°C after 12 h. While data from this site were evaluated, it should be noted that significant increases in *E. coli* density at this site might be related to holding temperature. Also, some samples from Mohawk Valley were partially frozen. In general, samples from other sites were maintained at <10°C and above freezing. All statistical analyses were stratified by site. Using Dunnett's test, the *E. coli* density results for each holding time were compared to the initial *E. coli* density at time 0 and the 8-h *E. coli* density results (alpha = 0.05). A summary of the phase 3 Dunnett's test results for the time 0 comparison is provided in Table 5.

During phase 3, 8 of 13 sites showed no significant differences in *E. coli* densities between time 0 and the 48-h holding time, regardless of evaluation method and coolant used. Also, five of six sites analyzed by the Colilert method exhibited no significant difference by 48 h.

Conclusions. Based on the results of these studies, which encompassed 24 sites across the United States, 11 laboratories, and four commonly used monitoring methods for *E. coli* in water, it appears that most *E. coli* samples analyzed by com-

TABLE 5. Summary of phase 3 Dunnett's test results for time 0 comparisons (alpha = 0.05)

Laboratory	Site	Method	Spiked	Coolant	Mean no. of *E. coli*/100 ml at time 0	No. of *E. coli*/100 ml (significant change in density) at indicated time (h) after sample collection[a]			
						8	24	30	48
Fairfax County Water	Potomac River	Colilert	Yes	Wet ice	73	NS	51 (D)	NS	NS
Fort Worth Water	Rolling Hills WTP[b]	Colilert	Yes	Utek	63	NS	NS	NS	NS
Indianapolis Water	Fall Creek	Colilert	No	Utek	337	NS	NS	NS	NS
	White River	Colilert	No	Utek	534	NS	NS	NS	NS
City of Phoenix	Squaw Peak WTP	Colilert	No	Wet ice	11	NS	NS	NS	NS
	Union Hills WTP	Colilert	Yes	Wet ice	69	NS	NS	NS	NS
Jefferson Parish	Mississippi River	mTEC	No	Wet ice	310	NS	NS	NS	NS
Southern Nevada	SNWS Pumping Plant 1	mTEC	Yes	Utek	17	30 (I)	32 (I)	34 (I)	44 (I)
Passaic Valley	Passaic & Ramapo Rivers	mFC/NA-MUG	No	Blue ice	193	NS	90 (D)	108 (D)	85 (D)
Portland Water Bureau	Station 2	mEndo/NA-MUG	Yes	Blue ice	44	NS	55 (I)	NS	NS
Mohawk Valley	Hinckley Reservoir	mEndo/NA-MUG	Yes	Utek	42	97 (I)	NS	NS	NS
Wisconsin State Laboratory of Hygiene	Willow Creek	mEndo/NA-MUG	No	Wet	56,000	NS	NS	NS	NS
	Wingra Springs	mEndo/NA-MUG	No	Wet	367	NS	NS	NS	NS

[a] D, significant decrease in *E. coli* density compared to the time 0 results; I, significant increase in *E. coli* density compared to the time 0 results; NS, no significant difference in *E. coli* density compared to the time 0 results.
[b] WTP, water treatment plant.

mon methods can be analyzed beyond 8 h after sample collection while still generating comparable *E. coli* data, provided that the samples are held below 10°C and are not allowed to freeze. Notwithstanding this conclusion, to ensure that the most accurate data are generated, *E. coli* samples collected from surface waters should always be analyzed as soon as possible and within 8 h when on-site facilities are available or when a qualified laboratory is within driving distance. Those involved in *E. coli* monitoring should evaluate how their data will be used—and how rigorous the data quality must be—before deciding to use extended holding times and should consider generating data (with their methods and their water[s]) on the effect of extended holding times to verify that extended holding times meet data quality needs.

Although not one of the objectives of this study, it was observed through continuous sample temperature monitoring that water samples for *E. coli* sometimes froze during storage. Because cell lysis may occur under these conditions, precautions should be taken to prevent samples from freezing during storage.

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