APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1977, p. 398-402
Vol. 34, No. 4

Printed in U.S.A.

Comparison of Four-Hour and Twenty-Four-Hour Refrigerated Storage of Nonpotable Water for Fecal Coliform Analysis

JON H. STANDRIDGE* AND DAVID J. LESAR

Wisconsin State Laboratory of Hygiene, Wisconsin Department of Natural Resources, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 18 April 1977

The problem of extending the storage time of water samples for fecal coliform analysis was addressed. Included in this report is a literature review of the storage problem. Twenty-eight samples were analyzed in replicate to determine the effect of 24-h storage of water samples at 4°C. A new statistical approach to data analysis, coupled with the concept of practical acceptability, is presented. According to our results, many samples can successfully be stored at 4°C for 24 h.

The 14th edition of *Standard Methods* (1) places stringent requirements on preservation and storage of all types of water samples submitted for bacteriological analysis. Samples submitted for fecal coliform analysis must be stored at less than 10°C for no more than 8 h before analysis is performed. An additional 22-h leeway is allowed on potable waters to be tested for the total coliform group.

If a 24-h storage period could be allowed for the fecal coliform test, the U.S. mail and other parcel services could be used for submission of samples. This, in turn, would greatly expand the geographical area that a laboratory could serve. In Wisconsin, we have been successful mailing these samples in Styrofoam coolers at the approximate cost of 25 cents per sample. This is a substantial savings over the cost of delivering samples by special messenger. The end result of increased storage time would be a large laboratory performing a high volume of analysis at a substantially lower cost per analysis than several small, regional laboratories running the same tests. The experienced personnel, equipment, and quality control programs of a large laboratory insure the generation of high-quality, uniform data. Small laboratories with minimal equipment and only a few samples a day cannot be expected to keep abreast of details important to microbiological testing. The obvious advantages of large laboratories gained through allowing 24-h storage time must be weighed against the advantages gained from the present 8-h storage limit.

A review of the literature shows that the strongly worded 8-h time limit is based on a paucity of evidence. In fact, rumor has it that originally the 8-hour limit was based on the time it took to travel by horse and buggy from the British Minister of Health's laboratories to the Thames River and back again. In 1955, Geldreich et al. (6) completed an extensive literature review in connection with development of a delayed-incubation, membrane filter coliform test. From this review, they concluded that storage of samples had unpredictable results. They also determined that when using the most-probable-number coliform technique on a total of 18 samples, the mean of results from samples held at 5°C for 24 h was 72% of the mean of results from samples analyzed at 2 h.

An English Subcommittee (12), in 1953, concluded that samples must be iced and stored for no more than 6 hours before analysis. This conclusion was based on the fact that 24% of all samples tested in duplicate had a significant increase or decrease in a 70-tube most-probable-number procedure when stored on ice for 24 h. A significant difference was defined as a two-fold change.

In 1933, Caldwell and Parr (3) determined that samples stored on ice at 17°C had different coliform recoveries than samples stored at 10°C for 6 h. In a 1950 article, Jones et al. (9) cited the conflicting data of previous workers and attempted to clarify the storage issue. They found on analyses performed with no replicates that the safety of the water for drinking based on the direct presumptive *Bacterium coli* content at 44°C was significantly different on refrigerated samples tested at both 8 and 24 h.

Alternatively, several workers have looked at storage of various types of water samples and shown that storage has little or no effect on coliform counts. In 1926, Berry determined

that the lactose-fermenting bacteria were unaffected by storage at room temperature for 48 h (2). Hoather, in 1952, looked at storage of 140 samples of various levels of contamination (7). Using basic statistics, he determined there was no significant difference in coliform counts in samples stored for 8 or 24 h. In 1949, Cox and Clairborne concluded that samples stored at refrigerator temperatures for no more than 24 h gave acceptable results (4). They also addressed the problems confronted in running these comparisons that result from the poor precision of the test.

Several workers have tried to pinpoint what factors determine whether or not coliforms can be recovered after storage. McCarthy, in a study of 45 samples, determined that the more polluted a water sample is, the more likely a sample count is to decrease after 24-h storage (10). The British Public Health Sub-Committee determined that the ability of the coliforms to withstand storage depended on the sample source (11). As early as 1923, Hotchkiss showed that cations can exhibit an inhibitive effect on growth of *Escherichia coli* (8). Shipe and Feilds demonstrated that ethylenediaminetetraacetic acid can be used as an effective preservative in water sample storage (14).

This literature review shows that not only are the data limited, but they are often in conflict. We feel that there are several explanations for this conflict. The most prevalent was procedural errors. Examples include not mixing the samples, doing presumptive tests only, not running enough samples, or filling sample bottles completely full, which results in incomplete mixing when shaking.

Many of the conflicts in the literature can be explained by problems in the statistical analysis of data. The most common problem is misuse of methods such as Student's *t* test. When comparing two microbiological methods on data from a variety of sampling sites, one must take two basic assumptions about the data. First, the replicate data must approximate a normal distribution when transformed to logarithms. Secondly, the log transformation must equalize the standard deviations. The *t* test is not valid for data from various samples sites if standard deviations are not similar. We have found that these conditions often cannot be met when dealing with varied water sources.

Another statistical problem found in the literature lies in the definition of "statistically significant" differences. Some authors used percentages (6), whereas others used a twofold increase (12) or Student's *t* test (9) to form final opinions on statistical difference.

Failure to consider the inherent lack of precision in coliform testing was yet another problem encountered in the literature. This lack of precision must be overcome by performing many replicate analyses on each sample. In most of the works cited above, the best that can be found is duplicate analysis.

It is the purpose of this paper to make an extensive comparison of fecal coliform analyses results on samples stored for 4 h at 4°C with results of fecal coliform analyses made from the same bottle after 24-h storage at 4°C. We will present a viable statistical method for comparing enumeration techniques on varied sample sources, using a sufficient number of replicates to result in a sound definition of practical, statistically significant differences.

MATERIALS AND METHODS

Samples and sampling sites. The sampling sites were divided into three categories: (i) secondary sewage treatment plants receiving only domestic wastes, (ii) secondary sewage treatment plants receiving a substantial flow of industrial wastes, and (iii) rivers and streams receiving a mixture of municipal and agricultural wastes. All sample sites chosen were within 75 miles (ca. 120.67 km) of the laboratory to facilitate meeting the given time limits. Care was taken to insure samples represented a true cross section of sample types usually analyzed in a large laboratory. Samples for microbiological analysis were grab samples collected in sterile, single wide-mouth polypropylene bottles with polypropylene-lined caps. Sample bottles contained sodium thiosulfate, to neutralize any residual chlorine present, and were filled to within 1 inch (ca. 2.54 cm) of the top to facilitate mixing of the sample. Samples for chemical analysis were collected simultaneously in separate bottles. Samples were iced immediately and transported to the laboratory in Styrofoam ice chests. Immediately upon arrival at the laboratory, samples were stored under refrigeration at 2 to 4°C. This choice of temperature was based on work done by Shaw et al., which showed that coliform organisms could not carry out metabolic processes at temperatures less than 7.5°C (13).

Analyses. All fecal coliform analyses were performed by the membrane filter technique according to *Standard Methods* (1). Thirty replicates were set up on each sample dilution at both 4 and 24 h. Samples were mixed continuously with a magnetic stir bar during the time it took to perform the replicates. Ten colonies from one replicate plate from each sample were submitted to verification procedures outlined in *Standard Methods* (1). Five-day biochemical oxygen demand analyses were done according to *Standard Methods* (1).

RESULTS AND DISCUSSION

The results of the study are presented in Table 1. Under the first column, sample source, the first group includes secondary sewage treatment plants (STPs) treating a mixture of mu-

TABLE 1. *Comparison of fecal coliform analysis reliability*

Sample description	BOD_5[a] (mg/liter)	Verified (%)	Log $\bar{x}_1$ (4 h)	SD[b] $\bar{x}_1$	Log $\bar{x}_2$ (24 h)	SD $\bar{x}_2$	95% CI[c]	
Group 1								
Beloit, 1976	35	100	2.48	0.078	2.69	0.064	0.67	0.55
Beloit, 1977	28	90	2.21	0.094	2.46	0.090	0.51	0.41
Monroe	10		3.50	0.033	3.50	0.028	0.96	1.04
Fort Atkinson	46	100	2.96	0.034	2.96	0.045	0.95	1.05
Jefferson	62	100	2.51	0.097	2.63	0.182	0.84	0.68
Cross Plains	43	100	4.96	0.054	4.96	0.063	1.04	0.91
Madison, winter	–[d]		2.77	0.046	2.81	0.125	1.02	0.81
Madison, summer	22		3.25	0.047	3.25	0.043	1.06	0.95
Stoughton, 1976	49	90	3.48	0.076	3.63	0.062	0.78	0.65
Stoughton, 1977	125	100	2.85	0.143	2.96	0.138	0.91	0.66
Group 2								
Black Earth	37		4.16	0.075	4.19	0.064	1.01	0.86
Mazomanie	47	90	3.65	0.063	3.60	0.070	1.21	1.04
Sun Prairie	45		4.78	0.156	4.74	0.087	1.27	0.99
Brooklyn	–		6.70	0.065	6.67	0.069	1.16	0.99
Marshall	100		3.81	0.076	3.90	0.055	0.88	0.75
Oregon	39		6.54	0.12	6.57	0.083	1.06	0.83
Verona Co. Home	126	100	5.46	0.086	5.48	0.076	1.05	0.87
Mt. Horeb, 1976	64		4.42	0.11	4.57	0.11	0.81	0.62
Mt. Horeb, 1977	>144	100	4.16	0.147	4.63	0.088	0.39	0.29
Verona	125	90	6.36	0.026	6.35	0.027	1.05	0.99
Group 3								
Blue Mounds Creek	15	100	3.27	0.066	3.26	0.058	1.10	0.95
Spring Creek, Summer	21		3.63	0.053	3.59	0.060	1.17	1.03
Spring Creek, 1976	–		4.56	0.049	4.46	0.054	1.34	1.18
Spring Creek, 1977	95	100	4.42	0.068	4.47	0.070	0.96	0.82
Black Earth Creek	43	100	2.78	0.054	2.74	0.051	1.17	1.03
Starkweather Creek	24	100	2.54	0.076	2.44	0.070	1.12	0.94
Murphy's Creek	53		3.87	0.044	3.82	0.030	1.07	0.98
Willows Creek	35	100	4.45	0.059	4.45	0.093	0.913	1.09

[a] BOD_5, 5-day biochemical oxygen demand test.
[b] SD, Standard deviation.
[c] CI, Confidence interval.
[d] –, Not analyzed.

nicipal and industrial wastes. The second group includes STPs receiving municipal wastes only. The third group includes rivers and streams receiving mixtures of agricultural runoff and STP effluents. The reader should note that several sites showing the largest difference between methods were sampled twice.

The second column, containing results of a 5-day biochemical oxygen demand test done on most samples, measures the dissolved oxygen consumed by microbiological life while assimilating and oxidizing organic matter. This, in turn, gives the worker an inference as to the amount of biodegradable organic material present. The third column contains the results of verification tests performed. Column four lists the arithmetic mean of the 30 replicates transformed to logarithms for testing done after 4-h refrigeration. The fifth column contains the standard deviation for the means of column four. Column six contains the arithmetic mean of the 30 replicates, in log form, for analysis after 24 h of refrigeration. The seventh column contains the standard deviations for means in column six.

In the last column, we have combined the results of columns four thru seven using our statistical methodology. The two numbers in the column represent a range of the results at a 95% confidence level. These numbers were determined by using the formula:

$$95\%\ \text{CI} = \bar{x}_1 - \bar{x}_2 \pm 1.96 \sqrt{\frac{SD_1^2 + SD_2^2}{30}}$$

That is, $\bar{x}_1$ (mean at 4 h) minus $\bar{x}_2$ (the mean at 24 h) added to or substracted from 1.96 (the 95% confidence level factor) multiplied by the combined standard deviations (SD) gives the 95% confidence interval (CI). The antilogs_{10} of

these two numbers are then reported in column eight. An interpretation of column eight would be that the results at 24 h would fall within this range 95% of the time. The range is expressed as the percentage of 4-h results in which the 24-h results would be 95% of the time. For example, for the Monroe STP, the results at 24 h would be between 95 and 104% of the 4-h results. The statistical expression indicates that only those ranges crossing unity would be valid. However, the U.S. Environmental Protection Agency has determined that an 80% agreement between proposed methods is acceptable in similar circumstances (5). The inherent lack of precision in coliform testing has dictated this wider range of acceptability. We, therefore, agree that the 80% agreement is an acceptable degree of variation. Examples of possible situations that may arise in interpretation of coliform data serve to illustrate this point. The sanitary significance of a sewage treatment plant effluent showing a fecal coliform count of 800,000 would be identical to the significance of a count of 1,000,000. Similarly a swimming beach sample showing a count of 800 would be dealt with in the same fashion as a beach showing a count of 1,000. The only time the data could be questioned from a practical standpoint is when the value falls within 20% of the coliform standard that is to be met.

If the data in column eight are looked at from this practical acceptability level and not a statistical standpoint, their use can be greatly expanded. We can see that if the 20% variability is added around unity and the range is then looked at, in 24 of the 28 samples we could be 95% cofident that the results at 24 h would fall within the acceptable limit. For example, Spring Creek 1977, from a statistical point of view, does not cross unity and, therefore, would not be valid. However, if the 20% variation is added, the range would fall within these limits and, from a practical standpoint, would be valid.

Looking at the data in Table 1 pragmatically, the reader can see that the 95% CI falls within the 20% variation requirement in 24 of the 28 samples. Three sampling sites failed to fall within the range. Of these three, the Beloit plant can be characterized as a highly efficient STP utilizing vacuum filters after the final clarifiers. The Stoughton and Mt. Horeb plants can be characterized as grossly overloaded STPs applying large amounts of chlorine on a poor final effluent. In the case of the Beloit plant, the storage of samples for 24 h could affect their ability to meet permit requirements. For the Stoughton and Mt. Horeb plants, the permit limits are substantially exceeded by either method of storage.

The 1970s have been a time of increased fiscal responsibility in government forced by an increasing taxpayer desire to know where money is being spent. The laboratory is not exempt from these financial pressures. Therefore, we feel it is imperative that laboratories determine whether cost-saving alternatives, such as 24-h storage of samples, can be incorporated. Any changes in procedure such as this must be done with continued careful attention to detail and quality assurance programs. In the introduction to this paper, the economical and quality assurance advantages of being able to store samples for 24 h were emphasized. This study indicates that, from the practical standpoint defined above, there are many types of samples that can be successfully stored at 4°C for 24 h. We believe that we have presented an acceptable means for comparing alternative methods. It is the responsibility of the various laboratories to use studies such as this in elevating money-saving alternative procedures.

ACKNOWLEDGMENTS

We acknowledge the financial support of the Wisconsin Department of Natural Resources and the Environmental Sciences Section of the Wisconsin State Laboratory of Hygiene in carrying out this study.

We also gratefully acknowledge the assistance of Camil Fuchs of the University of Wisconsin Department of Computer Science and Statistics, in performing the statistical analyses.

LITERATURE CITED

1. **American Public Health Association.** 1976. Standard methods for the examination of water and wastewater, 14th ed. American Public Health Association, Inc., Washington, D.C.
2. **Berry, F.** 1926. Studies on lactose fermenting bacteria. Am. J. Public Health 16:700.
3. **Caldwell, E. I., and L. W. Parr.** 1933. Present status of handling water samples—comparison of bacteriological analyses under varying temperature and holding conditions with special reference to the direct method. Am. J. Public Health 23:467.
4. **Cox, K. E., and F. B. Clairborne.** 1949. Effect of age and storage temperature on bacteriological water samples. J. Am. Water Works Assoc. 41:948–952.
5. **Geldreich, E. E.** 1975. Evaluating water bacteriological laboratories, p. 119–120. Environmental Protection Agency, Cincinnati.
6. **Geldreich, E. E., P. W. Kabler, H. L. Jeter, and H. F. Clark.** 1955. A delayed incubation test for coliform bacteria in water. Am. J. Public Health 45:1462.
7. **Hoather, R. C.** 1952. The bacteriological examination of water. J. Inst. Water Eng. 6:426–442.
8. **Hotchkiss, M.** 1923. Studies on salt action stimulating an inhibitive effect on certain cations upon bacterial growth. J. Bacteriol. 8:141–162.
9. **Jones, J. E., P. M. Franklin, and S. E. Thomas.** 1950. The effect of overnight refrigeration on the results of the bacteriological examination of farm water sup-

plies. Proc. Soc. Appl. Bacteriol. 43:108.
10. **McCarthy, J. A.** 1957. Storage of water samples for bacteriological examinations. Am. J. Public Health 47:971.
11. **Public Health Laboratory Service Water Sub-Committee.** 1952. The effect of storage on the coliform and *Bacterium coli* counts of water samples. J. Hyg. 50:107.
12. **Public Health Laboratory Service Water Sub-Committee.** 1953. The effect of storage on the coliform and *Bacterium coli* counts of water samples. Storage for six hours at room and refrigerator temperatures. J. Hyg. 51:559.
13. **Shaw, M. K., A. G. Marr, and J. L. Ingraham.** 1971. Determination of minimal temperature for growth of *Escherichia coli*. J. Bacteriol. 105:683–684.
14. **Shipe, E. L., and A. Feilds.** 1956. Chelation as a method for maintaining the coliform index in water samples. U.S. Public Health Rep. 71:974.