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THE INFLUENCE OF NON-COLIFORM BACTERIAL POPULATIONS ON POTABLE WATER QUALITY IN SOUTHERN ARIZONA

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4/12/82
Date

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

Microbiological quality of the groundwater public water supply of the Tucson, Arizona area was surveyed over a ten month period. Samples from 201 wells and 248 distribution sites were analyzed for coliforms, numbers of non-coliform "background" bacteria, pH, and turbidity. Coliforms were detected in 7.3% of the samples, with a higher incidence during July and August. Sample holding time and temperature prior to analysis had a pronounced effect on the microbiological parameters. The effects of high non-coliform populations on the coliform detection methods were investigated. Instances of coliform inhibition were observed, but were not predictable on the basis of high background numbers alone. During the survey, 2.6% of individual most-probable-number and 14% of membrane filtration tests were falsely negative, indicative of coliform suppression.

INTRODUCTION

Ever since the recognition of the water-borne transmission of certain diseases, the microbiological examination of public water supplies to determine sanitary quality has been a major public health concern. Traditionally, microbiological testing for potability consists of the detection and enumeration of indicator organisms, which theoretically are present in detectable quantities in any instance in which a pathogen might be present, rather than testing for the various pathogens directly. Although several groups of organisms have been suggested at one time or another in the search for the "ideal" indicator, the coliform group remains the most widely accepted and most relevant sanitary index for drinking water and has been claimed to be adequate in assuring bacterial safety of water (Allen and Geldreich, 1978). The coliform group is defined as the aerobic and facultative anaerobic gram negative, nonspore-forming, rod-shaped bacteria that ferment lactose in 24-48 h at 35 C. This definition includes the genera Escherichia, Enterobacter, Klebsiella, and Citrobacter. Both the World Health Organization and the U.S. Environmental Protection Agency have based their standards for drinking water quality on coliform testing.

The specific means for the detection and enumeration of coliform bacteria in water as practiced in this country are contained in the "Standard Methods for the Examination of Water and Wastewater" published by the American Public Health Association (14th ed., 1975). There are two methods currently accepted for monitoring drinking water supplies. The most-probable-number (MPN) test utilizes a tubed broth medium containing lactose. A series of these tubes are inoculated with the water sample, and positive tests are indicated by fermentative gas production. Quantification of indicator organisms is based on the combination of positive and negative tubes. The membrane filtration (MF) test provides a direct count of indicator colonies on the surface of a membrane through which the water sample has been filtered. After filtration, the membrane is placed on a selective lactose-containing medium, and the indicator bacteria retained on the membrane produce sheen colonies due to fermentative acid production. The membrane fecal coliform (M-FC) method is a variation of this procedure employing a slightly different medium and a 44.5 C incubation temperature, and is designed to detect coliforms specifically of fecal origin. These procedures have been rigidly standardized to ensure reproducibility and uniformity of interpretation. This system is used as the basis of the U.S. Environmental Protection Agency drinking water standards

(US E.P.A., 1975), which mandate the number of samples to be examined per month relative to population served, and also establish a maximum acceptable quality limit of one coliform per 100 ml of water, a maximum turbidity level of 1.0 nephelometric turbidity unit (NTU), as well as a recommended maximum bacterial count of 500 organisms per ml of finished water as measured by the standard plate count (SPC) procedure.

Since it has been established that one coliform per 100 ml is the limit of sanitary acceptability for drinking water, it is, of course, critical that the standard methods be sensitive enough to detect coliforms at that level. That this may not always be the case has been reported with increasing frequency. Both physical and biological factors have been cited as potentially interfering with coliform detection in water. These can be most aptly considered within the context of microbial ecology, i.e., the interactions among the coliform organisms and the resident aquatic populations, and the effects of the environment on the coliforms. In the first place, the coliform bacteria are transient in water and may be subject to physiological stress, injury, and eventual death in the relatively harsh, nutrient-poor aquatic environment. Several investigators have studied die-off rates of indicator organisms in water, but their results are difficult to generalize because of the varieties and numbers of organisms, water sources, and

conditions used (Granai and Sjogren, 1981). In addition to the question of survival times of coliforms in water, the effects of physiological stress may also be important relative to the detection of coliforms. Bissonnette et al (1975) found that although not necessarily killed, a substantial proportion of a coliform population became injured as a result of exposure to stresses of the aquatic environment, and that this impeded their growth in selective media.

One environmental factor of concern is turbidity, which is a measurable physical quality of water influenced by sand, algae, decaying vegetation, chemicals, and other non-specific components. LeChevallier, Evans, and Seidler (1981) reported that increasing turbidity of chlorinated surface water was directly related to an increased failure rate of the membrane filtration (MF) method to detect coliforms. In their review of large amounts of potable water quality data, Allen and Geldreich (1978) found that interferences with coliform detection methods were generally associated with turbidities >1.0 NTU and background standard plate count (SPC) bacterial populations of >500 organisms/ml.

The interactions among coliform bacteria and the normal resident microflora of water have also been pointed to as potentially interfering with the detection of coliforms in water by the standard methods. The normal flora of water

is highly variable, but typical bacterial genera associated with water include Pseudomonas, Flavobacterium, Achromobacter, Proteus, Klebsiella, Bacillus, Serratia, Corynebacterium, Mycobacterium, Spirillum, Clostridium, Arthrobacter, Gallionella, and Leptothrix (Geldreich et al, 1972). Factors which contribute to high bacterial counts in distribution water include raw water source, reservoir contamination, dosage of disinfection, organic content, seasonal temperature fluctuations, sediment accumulations, and extended retention periods in pipes, reservoirs, and dead-end water mains (Allen, Taylor, and Geldreich, 1976).

The types of interactions bearing on coliform suppression, of course, would be those having a negative effect on the coliforms as a result of competition from the resident flora, i.e., amensalism or antagonism and direct competition for limited essential nutrients, or both. In one of the first studies of its kind Hutchinson, Weaver, and Scherago (1943) isolated microorganisms antagonistic to E. coli from 12 of 79 ground and surface water samples including species of Pseudomonas, Flavobacterium, Sarcina, Micrococcus, actinomycetes, and yeast. These were shown to suppress the detection of E. coli by the most probable number (MPN) procedure by 28-97% when inoculated simultaneously with E. coli in lactose broth in a ratio of 10,000 to 20,000:10. Others have reported that the MPN procedure may result in false-

negative results due to interferences by background bacterial populations (Evans et al, 1981). Recently, Means and Olson (1981) found that 22.1% of potable surface and groundwater flora isolates produced bacteriocin-like substances active against one or more coliform types. Inhibition of coliform recovery by large numbers of Flavobacterium, Acinetobacter, and Arthrobacter has also been reported (Herson and Victoreen, 1980).

A number of other investigators have observed decreased sensitivity of the assay procedures for coliforms with high densities of non-coliform bacteria as measured by the standard plate count (SPC) method. In reviewing the data from a large number of water samples, Geldreich, Allen, and Taylor (1972), Allen and Geldreich (1978), Lamka, LeChevallier, and Seidler (1980), and Clark (1980) found that the incidence of coliforms as determined by the standard methods increased proportionately with increasing SPC numbers, until the SPC population reached 500-1,000 organisms/ml. Above this level, however, the incidence of coliform detection decreased, suggesting possible interference.

In order to evaluate the potential for coliform suppression in the groundwater providing the potable water supplies of Southern Arizona, this study was initiated as part of a cooperative agreement between the Municipal Environmental Research Laboratory of the U.S. Environmental

Protection Agency and Dr. Norval A. Sinclair. Of primary interest was the influence of non-coliform populations on the detection of coliforms in potable water. During the course of the research, the well and distribution water water supply of Tucson was extensively sampled and characterized as to microbiological quality. Finally, the effects of the time and conditions of water sample transport to the laboratory following collection in the field on coliform detection and on non-coliform bacterial populations was investigated.

MATERIALS AND METHODS

Water Sources and Sampling Sites

A total of 201 wells and 248 distribution sites was sampled and analyzed over the ten month period from December 1980 to September 1981. The sampling points were those used by the city of Tucson Water Department for their routine microbiological sampling. The distribution water sampling sites were located throughout Tucson. The wells, which provide the sole public water supply, were located throughout the Tucson Basin and the Avra Valley west of Tucson. In addition, 19 well samples were collected from the communities of Benson, St. David, Tombstone, and Douglas in southern Arizona. Water from the wells, which are typically 300 feet deep, is mixed in the inter-connected distribution system as it is pumped. In most cases, the water is neither treated nor disinfected prior to distribution.

Sample Collection

Approximately 12 water samples per week were collected in sterile 1 liter plastic screw-cap containers. Water temperature and time of collection were recorded, and samples were packed in an iced styrofoam container for

transport. Samples were returned to the laboratory and analyzed, usually within four hours of collection.

Sample Analysis

All samples were analyzed for total coliforms by a five-tube MPN technique employing a 10 ml sample volume in double strength lauryl sulfate broth (BBL, Cockeysville, Md.) and a 100 ml MF procedure with direct placement and incubation of the filter (Gelman GN-6, 0.45 μm, 47 mm; Ann Arbor, Mich.) on m-Endo medium (BBL). All positive MPN tubes were subject to confirmation and completion tests, and all typical and suspect MF colonies were verified according to established procedures (American Public Health Association, 1975). The initial 165 samples were also analyzed by the M-FC procedure, but this was discontinued since no fecal coliforms were ever recovered. Background bacterial density of all samples was monitored by the standard plate count (SPC) pour plate procedure using duplicate 1.0 and 0.1 ml inocula and Standard Methods Agar (BBL). Finally, the pH and turbidity, as measured by the Hach Model 2100A Turbidimeter, were recorded. Those samples having a turbidity >1.0 NTU were sent to the Analytical Chemistry Laboratory of the University of Arizona for total organic carbon (TOC) analysis. All procedure were performed in accordance with the 14th edition of the "Standard Methods for the Examination of Water and Wastewater" (1975) and the Microbiological Methods for

Monitoring the Environment--Water and Wastes" (U.S. Environmental Protection Agency, 1978).

Identification of Isolates

Following verification in Brilliant Green Lactose Bile (BGLB) broth (BBL) and lauryl sulfate broth (LSB), all coliform isolates were identified by means of the API 20E system (Analytab Products, Plainview, New York).

Methods to Detect Coliform Suppression

An additional effort was made to recover coliforms from those samples which, although negative for coliforms by routine methods, gave evidence of potential interference by large populations of non-coliform background bacteria. Specifically, these included samples showing turbidity but no gas in MPN tubes and samples which yielded confluent growth on MF plates.

To uncover masking of coliforms by turbidity in the MPN test, three schemes were used. As a matter of routine, all gas negative turbid MPN tubes were confirmed by transferring 1.0 ml into BGLB medium and inspecting for gas production following a 48 h, 35 C incubation. Those positive, indicating suppression in the original MPN tube, were followed up with the regular completion tests. Secondly, for 82 samples, a modification of the coliform unmasking protocol developed by Evans, et al (1981) was performed. In addition

to the use of BGLB, this procedure entailed subculturing turbid, gas negative MPN tubes onto Eosin-methylene blue (EMB) agar (BBL) and reinoculating multiple and individual coliform-type colonies from the EMB into MPN medium. Finally, for 13 samples, transfer of turbid MPN tubes was made to fresh LSB subsequently capped with an agar seal to preclude interference by aerobes.

Potential coliform inhibition by high numbers of background colonies on m-Endo medium used in the MF technique was also investigated. Filters negative for coliforms after 24 h but which showed confluent growth were rolled and aseptically transferred to LSB, incubated, and observed for gas production.

Effects of Water Sample Holding Time and Temperature on the Detection of Coliforms

Two types of experiments were devised to study the effects of water sample transport conditions on the sensitivity of the two standard methods to detect coliforms and on non-coliform bacterial populations. For the first, a Tucson distribution point with a known high resident background population was selected and 30 liters of water were collected in two large sterile containers. In the laboratory, the sample was aseptically dispensed in 600 ml aliquots to 27 sterile 1 liter containers. Nine of these 27 samples were then inoculated with low levels of *E. coli* (ATCC 25922)

in the following manner. Growth from an 8 h, 35 C Trypticase soy (T-soy) agar slant culture was suspended in 1.0 ml T-soy broth (BBL), and 0.1 ml of the suspension was transferred to 25 ml of sterile T-soy broth in a 125 ml erlenmeyer flask. Following a 16 h, 35 C shaker incubation, the culture was diluted in sterile distilled water and inoculated into the 9 sample bottles to yield a level of 30 to 100 cells/ 100 ml. A second group of 9 samples was inoculated in an identical fashion with Klebsiella pneumoniae (ATCC 13833). The third group of 9 served as an uninoculated control. One sample from each group was assayed immediately (T_0) and the remaining samples in each group divided equally. One-half were refrigerated at 5 C and one-half were incubated at 25 C. Microbiological assay procedures included MPN, MF, and SPC and were performed in duplicate. Assays were repeated on a refrigerated and unrefrigerated sample from each of the three groups after 24, 30, 48, and 54 h of storage. Turbidity and pH were determined for each sample, and the coliform-suppression detection methods previously described were performed on individual test results where applicable.

In a second experiment, five Tucson well sites representing a wide range of bacterial parameters, including: (1) coliform positive, high SPC density; (2) coliform positive, low SPC density; (3) coliform negative, high SPC density; and (4) coliform negative, low SPC density, were

selected. Ten 1 liter samples were collected from each well. Five were cooled immediately in a container with ice-packs and 5 were left at ambient temperature. All samples were returned to the laboratory within two hours and an iced and uniced sample pair from each well were analyzed by the MPN, MF, and SPC methods in duplicate. The remaining samples were incubated at 5 C and 27 C, respectively. Paired samples were assayed as above after 24, 30, 48, and 54 h of storage.

Effects of Non-Coliform Background Bacteria on the Detection of Coliforms

In order to ascertain more specifically the effects of the non-coliform flora on the sensitivity of the detection methods for coliforms, a modification of the previous experiments was undertaken. Thirty-six liters of water from a low and a high SPC level distribution source was collected in a large sterile container, transported to the laboratory, and twenty 600 ml portions were placed in sterile 1 liter bottles. The remaining water was filtered through 0.22 μm membrane filters (Millipore Corp., Bedford, Mass.) and dispensed in 600 ml portions into 16 sample bottles. Replicate filtered and non-filtered samples were in turn inoculated with *E. coli*, *Enterobacter agglomerans*, *Klebsiella pneumoniae*, and *Citrobacter freundii* previously isolated from Tucson water and sewage effluent. Each coliform was grown as

described above and seeded into 4 filtered and 4 non-filtered samples. The experimental protocol is outlined in Figure 1. After inoculation, the samples were divided equally and either refrigerated or incubated at 25 C. One set from each temperature was assayed immediately (T_0) and the remaining samples were assayed after 24 h incubation. Duplicate MPN, MF, and SPC analyses were performed for each sample. The M-FC procedure was included for those samples inoculated with E. coli. Turbidity and pH were also determined for each sample. All turbid, gas negative MPN tubes were confirmed in BGLB and atypical colonies on MF plates were verified by inoculating LSB and BGLB.

Quality Control

The quality control measures recommended by the U.S. Environmental Protection Agency (1978) were conducted to ensure the efficacy of all media, glassware, and equipment used throughout the course of the research.

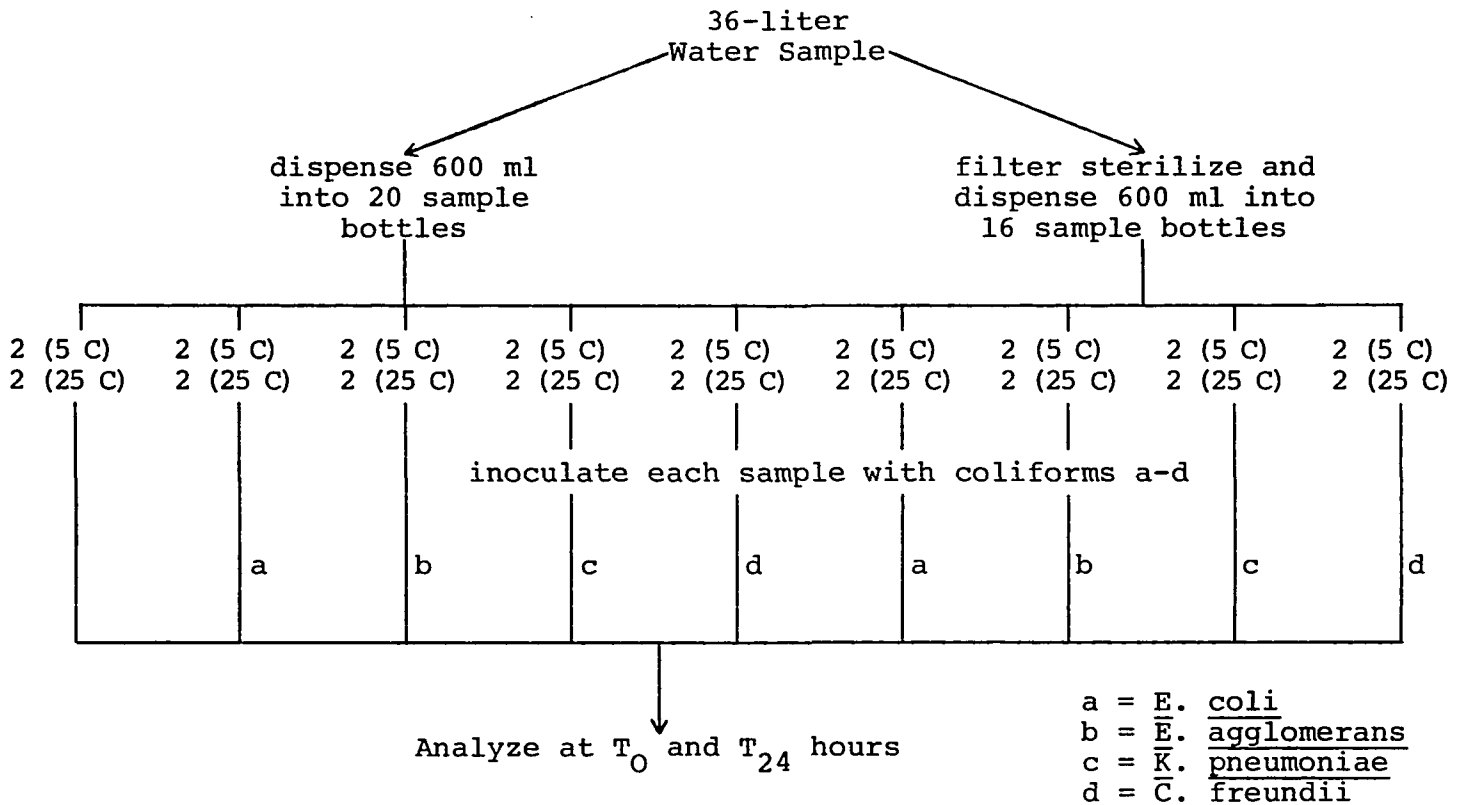

Figure 1. Outline of Experiment to Determine Effects of Non-Coliform Background Bacteria on the Detection of Coliforms.

RESULTS

Southern Arizona Water Supply Survey

Over the ten month period, 201 well samples and 248 distribution samples were examined. Coliforms were found in 23 of the 201 well samples (11.4%) and 9 of the 248 distribution samples (3.6%) by one or both of the methods used. The average water temperature of both well and distribution samples during the survey was 26 C. However, a greater seasonal variation was observed with the distribution samples. Average temperature of distribution samples during January was 18 C and during August was 32 C. Average temperatures of well samples during the same two months were 25 C and 27 C, respectively. The pH values of all samples ranged from 7.0 to 8.2, with an average of 7.7.

Turbidity measurements, results of coliform analyses, and coliform identification for the 23 positive well sites are listed in Table 1 and comparable data for the 9 distribution sites are given in Table 2. The majority of positive well samples were collected during July and August. The data demonstrate a wide range of turbidities and microbiological parameters among the samples. Coliforms were detected in 3 wells (D-35, C-26, and D-30) on more than one occasion, and

Table 1. Summary of Results from Coliform Positive Well Samples.

Date	Source	Turbidity (NTU)	SPC/ml	MF[a]	Total MF[b]	MPN[c]	MPN Index	Identification
12/17/80	Benson	0.2	49	0/11/39	11	2/5	5.1	E. cloacae
12/17/80	St. David	0.2	74	2/0/25	2	2/4	5.1	K. oxytoca
1/7/81	Tucson D-35	1.5	1,085	2/8/TN	2	2/5	5.1	C. amalonaticus
								E. aerogenes
7/7/81	" D-35	0.1	162	9/0/TN	2	0/5	<2.2	C. freundii
7/21/81	" D-35	0.3	54	0/1/CG	1	0/5	<2.2	E. cloacae
9/8/81	" D-35	0.2	74	1/2/12	1	0/5	<2.2	C. freundii
6/30/81	" C-26	4.7	445	1/0/30	0	0/4	2.2	E. agglomerans
								K. pneumoniae
7/21/81	" C-26	0.4	1,280	0/0/CG	0	1/5	2.2	C. cloacae
7/7/81	" D-30	0.2	87	6/30/TN	35	5/5	>16	C. freundii
8/24/81	" D-30	0.2	269	2/10/63	4	5/5	>16	C. amalonaticus
7/14/81	" D-2	4.5	239	0/0/48	0	4/5	16	C. freundii
								E. aerogenes
7/14/81	" E-7	0.1	258	8/0/7	8	4/5	16	K. pneumoniae
								E. cloacae
7/14/81	" D-40	0.2	760	0/6/TN	6	2/3	5.1	E. cloacae
7/21/81	" SC-8	0.3	206	0/1/CG	1	0/5	2.2	E. cloacae
7/21/81	" C-33	9.7	2,275	1/0/CG	1	5/5	>16	K. oxytoca
7/29/81	" B-108	0.6	>3,000	0/2/CG	2	5/5	>16	E. cloacae
7/29/81	" A-57	0.4	33	0/4/39	0	1/2	2.2	E. cloacae
7/29/81	" Z-13	0.4	>3,000	0/12/214	12	2/5	5.1	API group II
7/29/81	" Z-5	0.3	2,205	0/82/CG	66	5/5	>16	API group II
8/24/81	" D-7	5.5	>3,000	2/CG/CG	2	5/5	>16	E. coli
8/24/81	" D-31	0.4	14	1/5/7	4	4/5	16	C. freundii
8/24/81	" E-8	9.3	272	0/0/CG	0	5/5	>16	E. cloacae
8/24/81	" E-14	0.8	3,050	1/13/23	4	5/5	>16	E. aerogenes

a. No. typical (sheen) colonies/no. atypical suspect colonies/no. background colonies TN = too numerous to count CG = confluent growth

b. Total no. verified colonies/100 ml

c. No. tubes gas positive/no. tubes turbid.

Table 2. Summary of Results from Coliform Positive Distribution Samples.

	Source	Turbidity (NTU)	SPC/ml	MF[a]	Total MF[b]	MPN[c]	MPN Index	Identification
2/3/81	104	0.1	36	1/3/TN	1	0/5	<2.2	E. coli
2/3/81	103	0.1	9	1/5/0	1	0/5	<2.2	C. amalonaticus
3/3/81	157	0.3	2,100	0/0/0	0	1/1	2.2	unidentified
3/10/81	206	0.1	575	0/0/0	0	1/4	2.2	K. pneumoniae
3/31/81	317	0.2	4	0/0/0	0	0/1	2.2	unidentified
5/5/81	553	0.1	2	1/2/21	1	0/0	<2.2	E. cloacae
5/5/81	559	0.1	10	6/0/0	6	0/0	<2.2	E. agglomerans
5/5/81	652	0.1	0	1/0/0	1	0/0	<2.2	E. agglomerans
6/16/81	815	0.2	5	2/0/0	2	1/2	2.2	C. freundii E. agglomerans

a. No. typical (sheen) colonies/no. atypical suspect colonies/no. background colonies TN = too numerous to count

b. Total no. verified colonies/100 ml

c. No. tubes gas positive/no. tubes turbid

different SPC densities, turbidities, and coliform types were found on the different sampling dates. Seven well samples (D-30, D-2, C-33, B-108, D-7, E-8, and E-14) had a significantly greater quantification of coliforms by the MPN method than by the MF method. In 5 of these, confluent growth of background colonies was produced on MF plates or very high numbers of SPC bacteria (>3,000/ml) were present, or both. Two of these samples also had turbidities >1.0 NTU. Others have observed the influence of high levels of background growth and turbidity on the MF procedure, and the MPN method has been recommended by the U.S. Environmental Protection Agency (1978) for use with such samples. The importance of verification of atypical colonies on MF medium is apparent from Table 1, as non-sheen producing colonies were verified as coliforms in 11 samples. The coliform positive distribution samples (Table 2) were fewer, and presented lower ranges of turbidity, SPC, and number of coliforms. Five of these samples were positive only by the MF method, in which 100 ml of water was analyzed, as opposed to the MPN method, in which a total of 50 ml was used. With the exception of two E. coli isolates, the indicator organisms identified from both well and distribution sites were not necessarily of fecal origin. Four of the well samples and one distribution sample contained two different coliform species.

Turbidity for all samples ranged from 0.1 to >10 NTU, with an average of 0.5. Twenty-seven samples (22 well and 5 distribution) exhibited turbidity >1.0 NTU, the quality limit set by the U.S. Environmental Protection Agency for drinking water (1975). Total organic carbon (TOC) analyses performed on a subsample of these as well as coliform and SPC data are listed in Table 3. No consistent relationship was observed between high turbidity and high TOC, SPC, confluent growth on membrane filters, or presence of coliforms, although 8 of the 27 had very high (>1,000/ml) SPC numbers. It is notable that in 5 turbid samples in which coliforms were detected (Tucson well samples C-26, D-2, C-33, D-7, and E-8), the MPN method was much more effective than the MF method. In only 2 of these 5 samples were coliforms detected by the MF method, and in quantities markedly less than those indicated by the MPN Index/100 ml. Confluent growth on the MF plates was a factor in 3 of these samples.

The relationship between background flora levels and the detection of coliforms by the two standard methods is analyzed in Table 4.

The percentage of samples positive for coliforms by both methods increased directly with increasing SPC. A general inhibitory effect by high SPC densities on coliform detection is not apparent. A similar direct relationship with coliform detection was observed when the sample data

Table 3. Summary of Results from Turbid Well and Distribution Samples.

Date	Source[a]	Turbidity (NTU)	SPC/ml	MF[b]	MF Count	MPN[c]	MPN Index	TOC[d]
12/17/80	Tombstone	6.1	180	0/0/1	0	0/5	<2.2	-
12/29/80	TW E-23	1.6	8	0/0/2	0	0/1	<2.2	-
4/28/81	TD 515	4.7	0	0/0/0	0	0/0	<2.2	-
5/12/81	TD 614	1.2	1	0/0/0	0	0/2	<2.2	<2
5/19/81	TD 653	1.2	79	0/0/0	0	0/0	<2.2	<2
6/2/81	TD 704	4.8	300	0/0/TN	0	0/5	<2.2	-
6/16/81	TD 814	9.3	4	0/0/0	0	0/0	<2.2	2.04
6/16/81	TW A-8	1.0	2,580	0/3/60	0	0/5	<2.2	2.28
6/30/81	TW C-33	3.1	220	0/1/CG	0	0/4	<2.2	<1
6/30/81	TW C-26	4.7	445	1/0/30	0	0/4	2.2	4.3
7/14/81	TW C-25	2.0	84	0/0/0	0	0/0	<2.2	-
7/14/81	TW B-7	9.5	285	0/0/0	0	0/0	<2.2	<2
7/14/81	TW D-2	4.5	239	0/0/48	0	4/5	16	<2
7/14/81	TW D-51	>10	2,960	0/1/CG	0	1/4	<2.2	-
7/14/81	TW E-3	>10	>3,000	0/0/CG	0	0/5	<2.2	16
7/14/81	TW D-18	3.5	795	0/0/0	0	0/0	<2.2	-
7/14/81	TW C-36	3.8	1,030	0/19/TN	0	0/1	<2.2	-
7/14/81	TW D-17	1.7	3,490	0/0/0	0	0/0	<2.2	-
7/14/81	TW D-19	1.5	1,965	0/0/49	0	1/4	<2.2	-
7/21/81	TW C-33	9.7	2,275	1/0/CG	1	5/5	>16	2.9
7/29/81	TW B-107	1.8	17	0/0/0	0	0/0	<2.2	1.9
8/12/81	TW A-50	1.8	55	0/0/3	0	0/0	<2.2	-
8/24/81	TW C-30	>10	136	0/0/0	0	0/4	<2.2	<2
8/24/81	TW D-7	5.5	>3,000	2/CG/CG	2	5/5	>16	<2
8/24/81	TW E-1	3.2	350	0/0/0	0	0/0	<2.2	<2
8/24/81	TW E-8	9.3	272	0/0/CG	0	5/5	5.1	<2
8/24/81	TW H-1	2.0	870	0/3/0	0	0/3	<2.2	<2

a. TD = Tucson Distribution; TW = Tucson Well

b. No. typical (sheen) colonies/no. atypical suspect colonies/no. background colonies TN = Too numerous to count

c. No. tubes gas positive/no. tubes turbid

d. Total Organic Carbon (µg/ml)

Table 4. Distribution of Samples According to Standard Plate Count (SPC).

	Mean SPC/ml			
	<100	100-499	500-1000	>1000
All Samples	345	80	8	19
Samples with Coliforms (MPN)	7	5	2	9
% of Samples	2%	6.25%	25%	47.4%
MPN Range (per 100 ml)	2.2->16	2.2->16	2.2->16	2.2->16
MPN Mean (per 100 ml)	6.2	15.5	3.6	9.2
Samples with Coliforms (MF)	12	4	1	7
% of Samples	3.5%	5%	12.5%	36.8%
MF Range (per 100 ml)	1-35	1-8	6	1-66
MF Mean (per 100 ml)	5.5	3.75	6	12.7
Total Coliform Occurrences[a]	14	7	2	9
Total Coliform Incidence	4.1%	8.75%	25%	47.4%

a. Detected by either or both methods

were segregated according to increasing levels of background colonies on MF plates and to increasing numbers of MPN tubes showing turbidity. Slight differences were observed, however, in comparing the effectiveness of the two methods. Of 54 samples which produced turbidity in all 5 MPN tubes, coliforms were detected in 15 by the MPN method and in 17 by the MF method; and of 13 samples resulting in confluent growth on the MF plate, 3 were positive for coliforms by the MF method and 4 by the MPN method. These differences suggest that coliform suppression may have occurred in those instances.

Evaluation of Methods to Detect Coliform Suppression

Four methods were evaluated for their ability to recover coliforms from MPN tubes and MF plates which were presumptively negative, but for which the potential of coliform inhibition was indicated. Data are shown in Table 5. Coliforms were recovered from only 14 of 542 tubrid, gas negative MPN tubes inoculated into BGLB. The rate using a modified Evans et al (1981) method was much lower, and no coliforms were recovered in agar-capped LSB tubes. Since it was usually impossible to know if the turbid MPN tubes tested contained suppressed coliforms it is difficult to evaluate the data in other than a descriptive manner. There were, however, three instances in the time and temperature

Table 5. Evaluation of Methods to Unmask Coliform Suppression.

Method	Total No. Samples	No. Coliform (+) Samples[a]	Total No. Transfers	No. Confirmed[b]	% Unmasked
Turbid, gas (-) MPN tube → BGLB	215	60	542	14	2.6
Modified Evans Method	82	17	165	1	0.6
Turbid, gas (-) MPN tube → capped LSB[c]	13	2	24	0	0.0
CG on MF → LSB	50	10	50	7	14.0

a. Determined by one or more positive MPN tubes in the five-tube series and/or positive MF test

b. Gas positive

c. LSB tube capped with sterile agar after inoculation
LSB = Lauryl Sulfate Broth CG = Confluent Growth

effects experiments in which an E. coli inoculated sample resulted in an MF count sufficiently high to predict 5 gas positive tubes in the MPN series, but for which one or more MPN tubes were negative. Neither transfer to BGLB nor the modified Evans et al (1981) method was successful in recovering suppressed coliforms from these tubes. Finally, there were two instances during the Tucson water survey in which the transfer of turbid MPN tubes to BGLB was the only means by which coliforms were detected.

A higher recovery rate was seen with transfer of coliform negative membrane filters with confluent growth to LSB. Ten of the membranes transferred were derived from samples which were positive by the MPN procedure, and coliforms were recovered from 70% of these.

Effects of Sample Holding Time and Temperature on the Detection of Coliforms

The effects of the time and temperature of water sample storage on the recovery of coliforms was investigated by two procedures. The first consisted of collecting a large sample from a high SPC distribution source, and dividing it into 3 subsamples, two of which were inoculated with low levels of coliforms. Bacteriological quality was assayed initially and after various times at two different holding temperatures. Marked differences were observed (Table 6). At 5 C, E. coli persisted for 54 h as determined by the MF

Table 6. Effects of Holding Time and Temperature on the Detection of Coliforms Added to a Distribution Water Sample.

	Uninoculated Control				*E. coli* Added				*K. pneumoniae* Added			
	MPN Index[a]		MF Count[b]		MPN Index		MF Count		MPN Index		MF Index	
Holding Temp. (°C):	5	25	5	25	5	25	5	25	5	25	5	25
Holding Time (hrs):												
T_0	-	<2.2	-	0	-	16	-	124	-	16	-	25
T_{24}	<2.2	<2.2	0	0	16	<2.2	102	0	>16	>16	4	0
T_{30}	<2.2	<2.2	0	0	7.2	<2.2	64	0.5	>16	9.2	2	0
T_{48}	<2.2	<2.2	0	0	<2.2	<2.2	40	0	>16	16	3	0
T_{54}	<2.2	<2.2	0	0	<2.2	<2.2	48	0	>16	>16	1	0

a. Mean Index per 100 ml

b. No. Colonies Verified per 100 ml

method but was not detected by the MPN method after 30 h. K. pneumoniae was detected by both methods after 54 h incubation. In contrast, at 25 C, E. coli was not recoverable by either method after 24 h incubation, while K. pneumoniae remained viable for 54 h but was detected only by the MPN method. As shown in Figure 2, numbers of SPC bacteria increased by approximately 2.5 orders of magnitude after 54 h of 25 C incubation.

The second method involved collecting multiple samples from five Tucson wells with differing microbiological characteristics. Bacterial quality was determined as above at various times at two different holding temperatures. Results of the analysis of the three well samples from which coliforms were recovered are summarized in Table 7. The first well contained a significant number of coliforms and a high SPC population. Coliforms were detected by both the MPN and MF methods over the entire incubation period at both 5 and 27 C, although quantification was superior from the 5 C held samples. This well sample was unusual in that it had an initial SPC of 6,000 organisms/ml, a turbidity of 8.0 NTU, and produced confluent growth on MF plates and turbidity in all MPN tubes after each incubation time. It should also be pointed out that of 23 turbid, gas negative MPN tubes inoculated into BGLB during the experiment, 10 confirmed for the

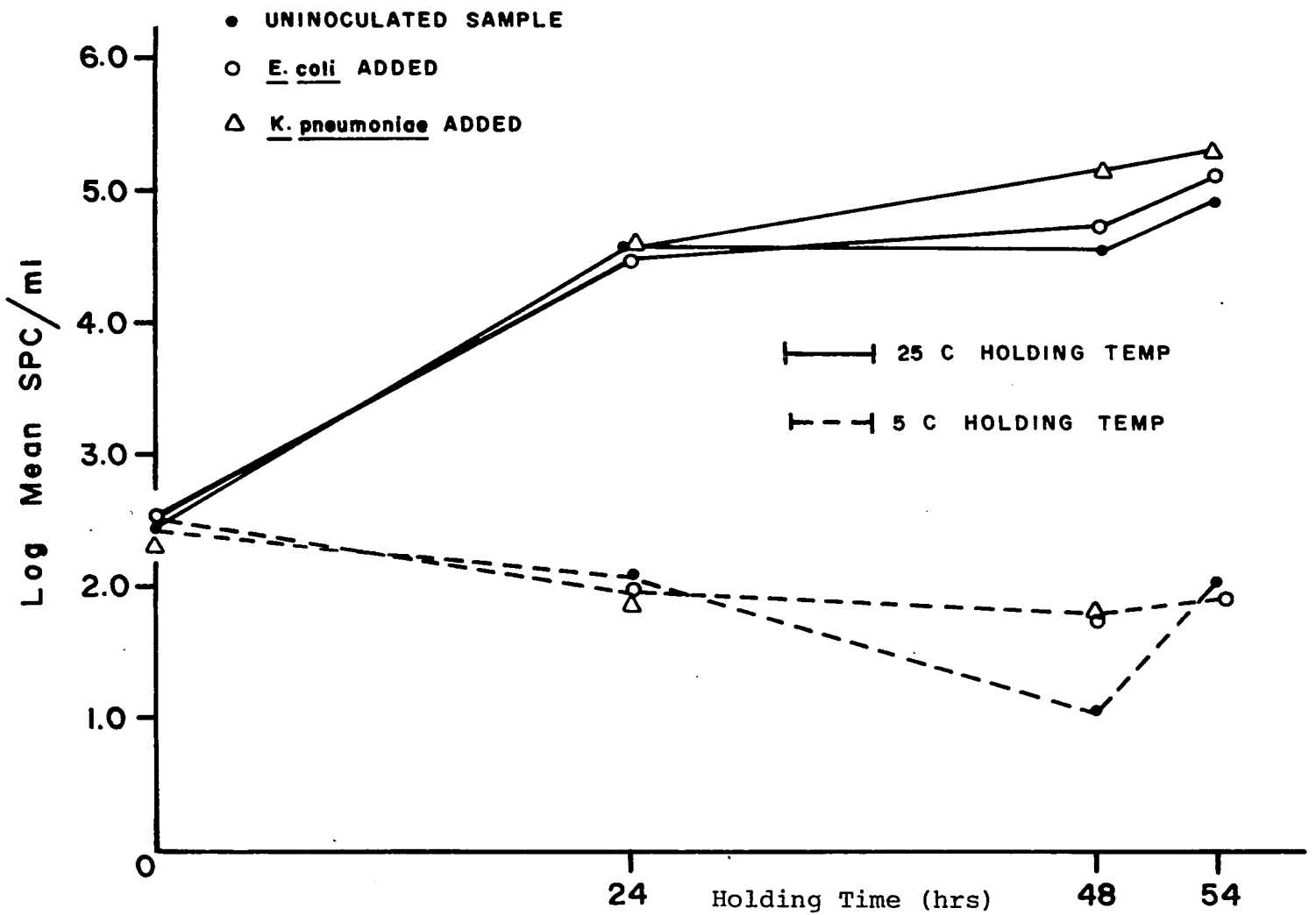

Figure 2. Effect of Distribution Sample Holding Time and Temperature on Standard Plate Count (SPC).

Table 7. Effects of Holding Time and Temperature on the Detection of Coliforms in Well Water Samples.

Holding Temp. (°C):	High SPC Background[a], Coliform (+) Well 1				High SPC Background[b], Coliform (+) Well 2				Low SPC Background[c], Coliform (+) Well 3			
	MPN Index per 100 ml		MF Count per 100 ml		MPN Index per 100 ml		MF Count per 100 ml		MPN Index per 100 ml		MF Count per 100 ml	
	5	27	5	27	5	27	5	27	5	27	5	27
Holding Time (hrs):												
T_0	16	16	9	7	<2.2	<2.2	0	0	<2.2	<2.2	0	2
T_{24}	>16	9.2	10	4	<2.2	<2.2	0	0	<2.2	2.2	2	0
T_{30}	9.2	5.1	6	7	2.2	<2.2	0	0	<2.2	2.2	1	0
T_{48}	>16	>16	10	3	<2.2	<2.2	0	0	<2.2	<2.2	1	1
T_{54}	9.2	2.2	13	4	2.2	<2.2	0	0	5.1	<2.2	0	2

a. Standard Plate Count (T_0) = 6,000/ml

b. Standard Plate Count (T_0) = 2,000/ml

c. Standard Plate Count (T_0) = 70/ml

presence of coliforms, suggesting that suppression had occurred.

The second well had an initial SPC of 2,000 organisms/ml and contained fewer detectable coliforms. Coliforms were detected only in refrigerated samples analyzed after 30 and 54 h by the MPN procedure. The 30 h sample was confirmed only by transfer of a turbid, gas negative MPN tube to BGLB. No coliforms were detected at either temperature by the MF method. Turbidity of this well was 6.0 NTU, and confluent growth occurred on many of the MF plates, which may well have contributed to the failure of the MF procedure.

The third well had a low level of coliforms and an initial SPC of 70 organisms/ml. Coliform detection was sporadic and no pattern was apparent with respect to storage conditions. For example, at 5 C storage, coliforms were recovered by the MPN method only from the sample held 54 h, although they were detected after 24, 30, and 48 h from the samples incubated at 27 C. Coliforms were detected by the MF method after 48 h incubation at 5 C and after 54 h incubation at 27 C. It is notable that confluent growth did not occur on the MF plates, nor was heavy turbidity produced in the MPN tubes by any of the samples from this well, despite the fact that the samples incubated at 27 C contained 10^5 to 10^6 SPC bacteria/ml after 24 h. Finally, Citrobacter

freundii was the only coliform identified from each of the three wells.

The effect of holding time and temperature on SPC populations is shown in Figure 3. Irrespective of the initial count, numbers of bacteria increased 1 to 3 orders of magnitude in samples held at 27 C. In contrast, counts in samples held at 5 C remained unchanged.

Effects of Non-Coliform Background Bacteria on the Detection of Coliforms

The effect of background flora on the sensitivity of the standard methods was investigated by the procedure outlined in Figure 1. Since previous data had shown that the bulk of the increase in SPC occurred within 24 h, bacteriological analyses were performed initially and after 24 h incubation. Two separate situations were examined. The first consisted of a distribution water sample with an initial SPC of 30 bacteria/ml into which coliforms were inoculated at a level of 100-200 cells/100 ml. Results of the MF analyses are displayed in Figure 4. There was a slight but significant decrease in MF counts of E. agglomerans and C. freundii from non-filtered samples incubated 24 h at 25 C. This decrease was not observed with the analogous samples from which the background flora had been removed by filtration nor with the non-filtered samples held at 5 C.

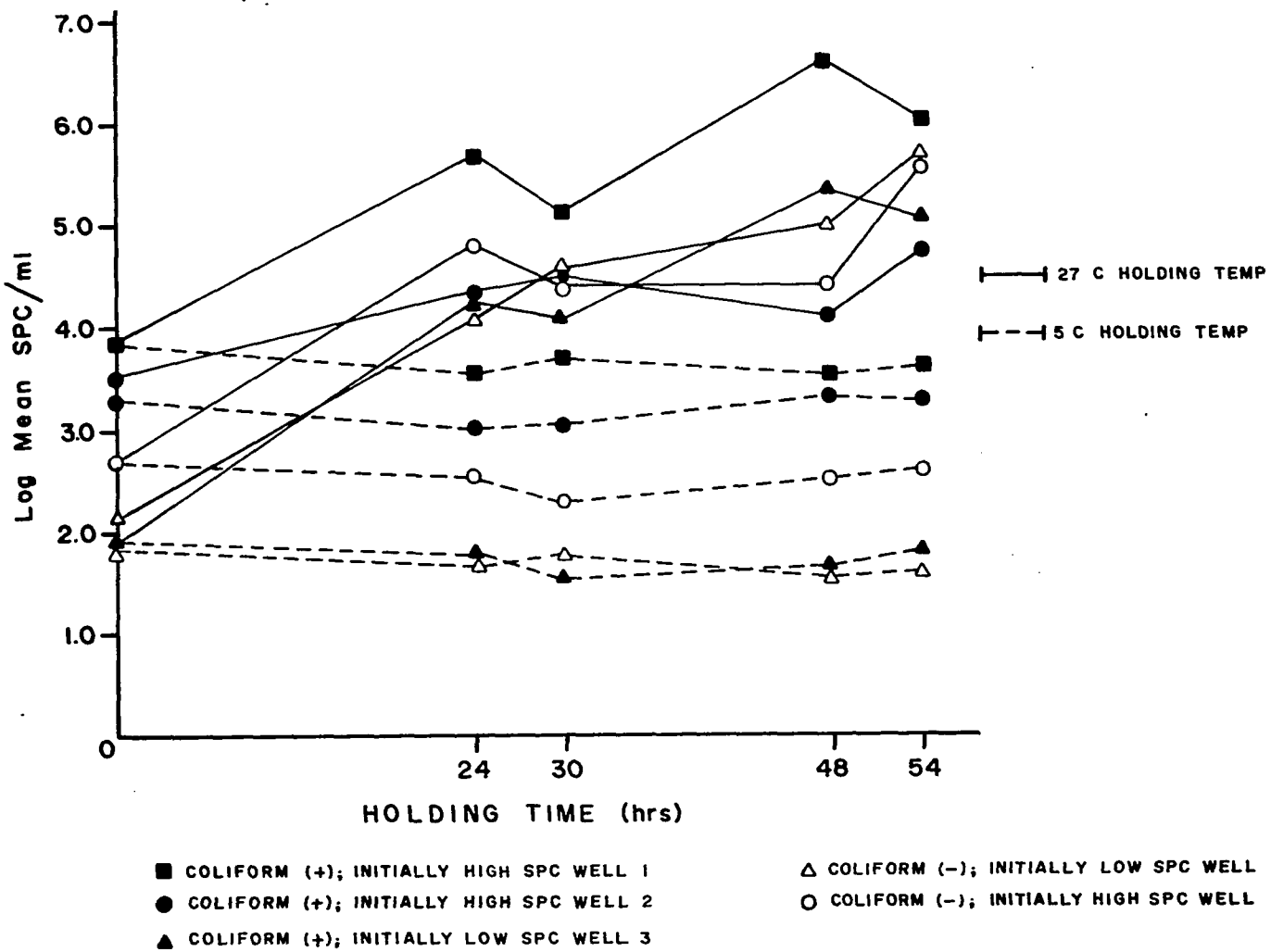

Figure 3. Effect of Well Sample Holding Time and Temperature on Standard Plate Count (SPC).

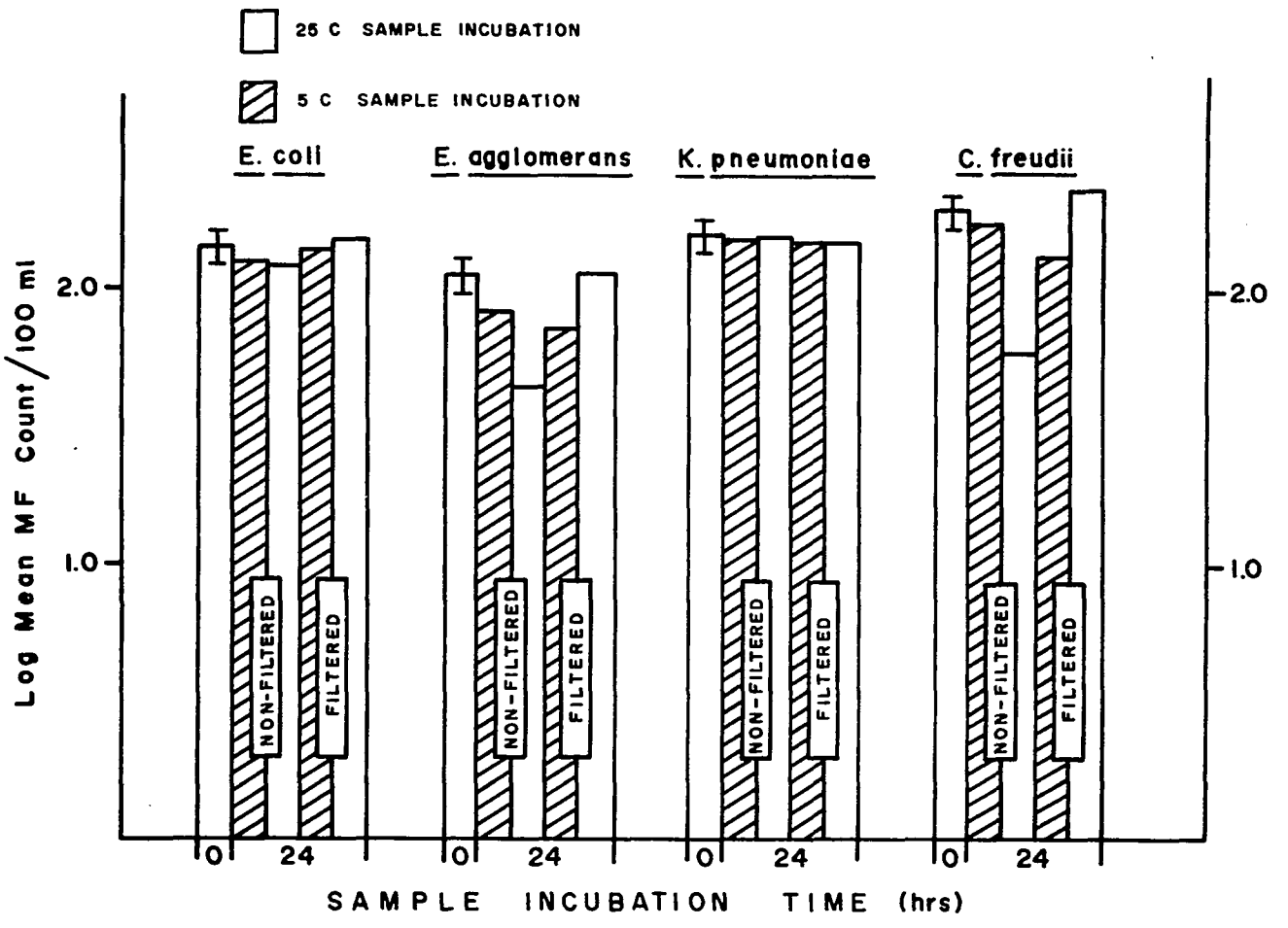

Figure 4. Effects of Non-Coliform Background Populations on the Detection of Coliforms by the Membrane Filtration (MF) Procedure--Trial 1.

The second trial involved a distribution sample with an initial SPC of 1,200 organisms/ml inoculated with approximately 20 coliform cells/100 ml. Results of the MF tests are shown in Figure 5. In this case, K. pneumoniae and C. freundii were recovered in significantly lower quantities from samples containing background flora after 24 h incubation. There was at least as great a decrease, however, in the filtered samples. This suggests that coliform die-off, and not suppression by non-coliforms was responsible for the decreased recovery. A significant decrease was also observed in the filtered E. coli and E. agglomerans samples held at 25 C and 5 C, respectively. This die-off rate was greater than that observed in the first water source, suggesting that chemical and nutrient factors were different in the two water sources used.

Other effects observed in both trials were confluent growth and a high percentage of atypical colonies on MF plates from samples containing large non-coliform populations. For example, 90% of C. freundii in these samples produced no sheen on m-Endo medium. Although these colonies were verifiable using conventional methods, there is a greater likelihood of such colonies being overlooked.

Changes in SPC populations in the non-filtered water samples are represented in Figure 6. As noted previously, an approximately 2-log increase occurred after 24 h incuba-

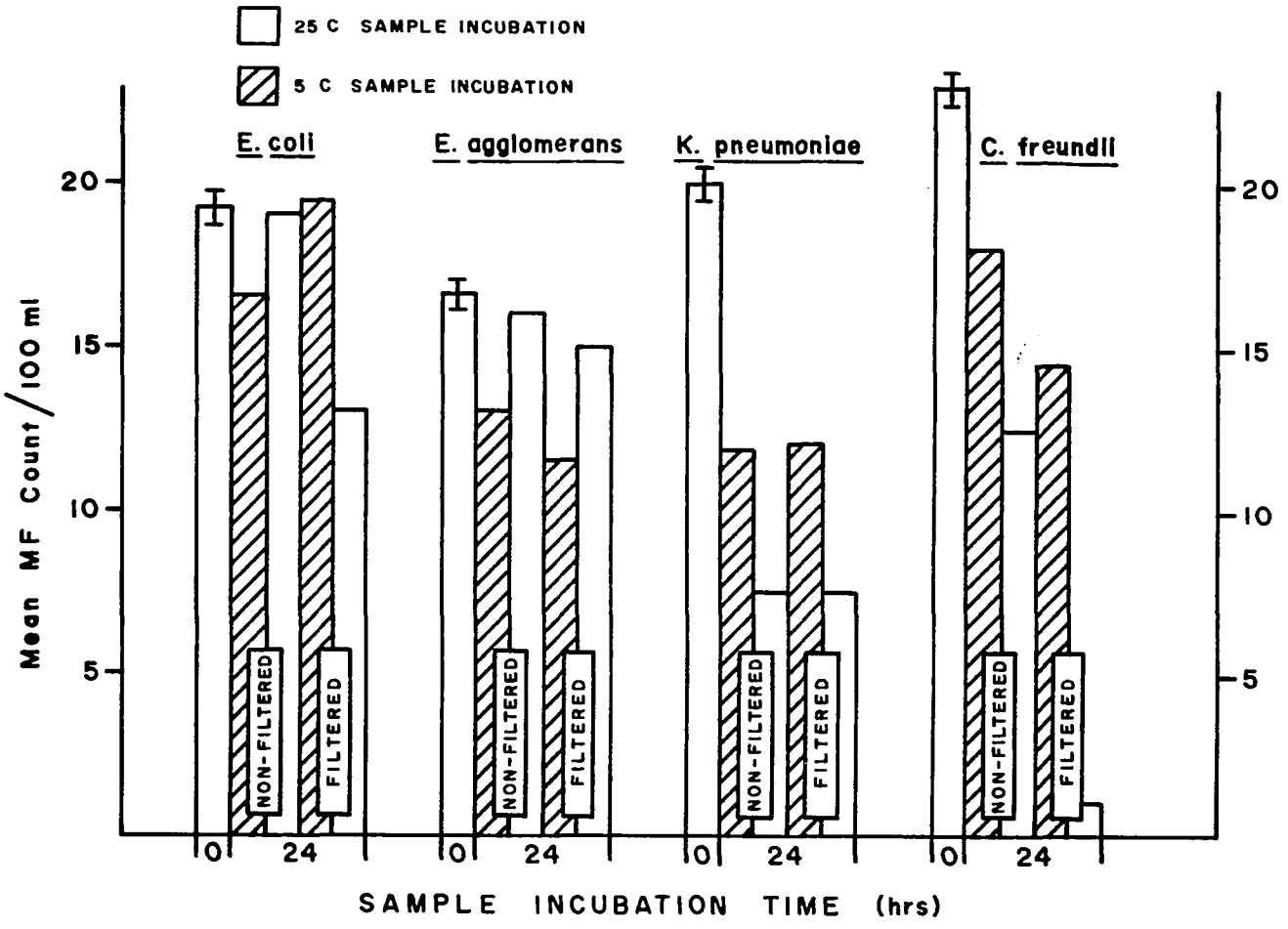

Figure 5. Effect of Non-Coliform Background Populations on the Detection of Coliforms by the Membrane Filtration (MF) Procedure--Trial 2.

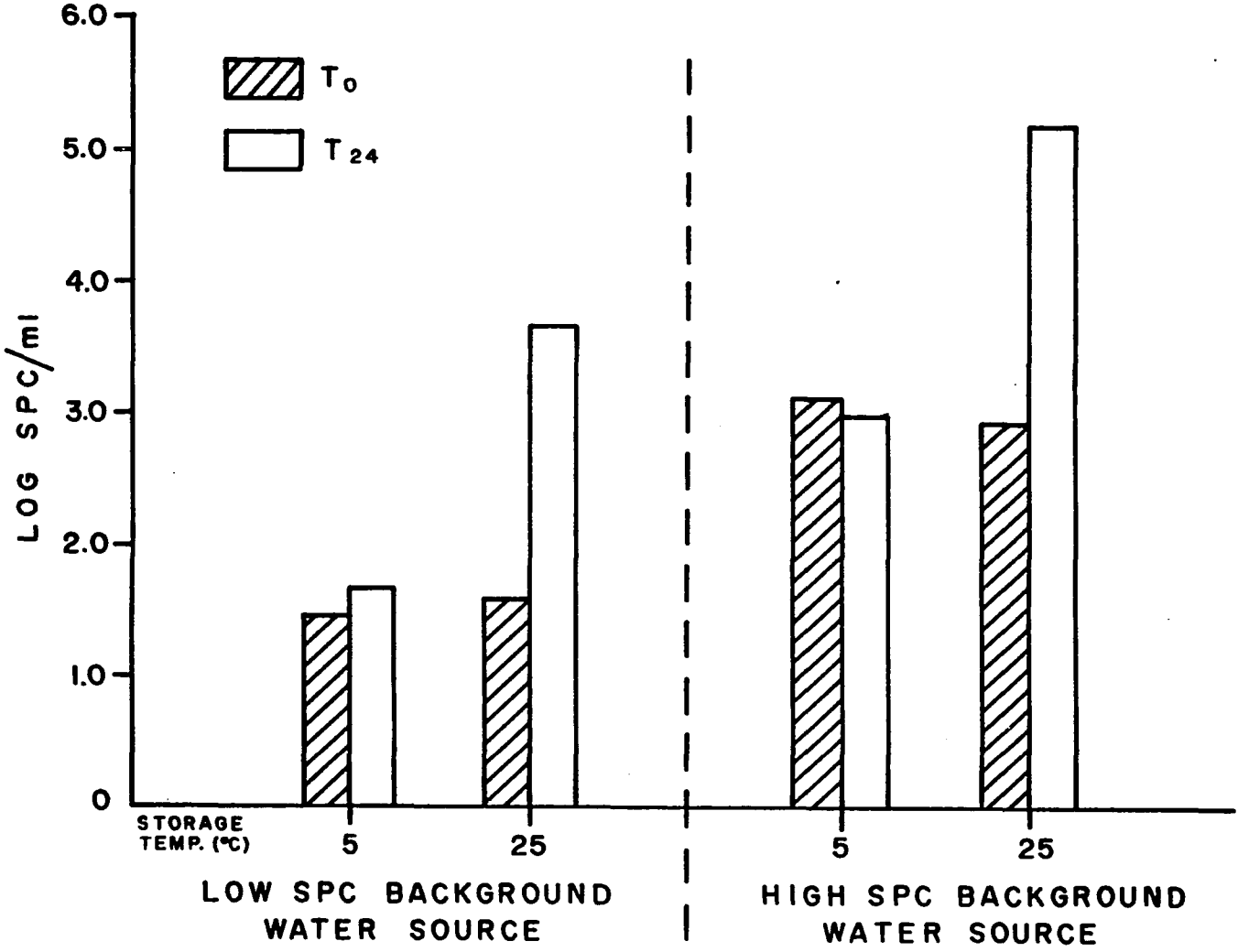

Figure 6. Effects of Time and Temperature of Storage on Non-Coliform Background Populations.

tion at 25 C, whereas counts were stable from samples stored at 5 C.

No significant loss of coliform detection by the MPN method was observed in either trial. Initial MPN Index values were maintained, within 95% probability limits, by all samples incubated 24 h.

The M-FC procedure was also performed on all samples containing E. coli in both trials. As depicted in Table 8, fecal coliform counts were stable or increased in the filtered samples and in the non-filtered samples held at 5 C. In the non-filtered samples held at 25 C, however, counts decreased and in one case were eliminated, suggesting suppression by the background flora.

Table 8. Effect of Non-Coliform Populations on the Detection of a Fecal Coliform.

	Mean M-FC Count per 100 ml							
	100 *E. coli* cells/100 ml Added to Low SPC Background Water[a]				20 *E. coli* cells/100 ml Added to High SPC Background Water[b]			
	Filtered		Non-filtered		Filtered		Non-filtered	
	5	25	5	25	5	25	5	25
Holding Time (hrs)								
T_0	38	81	51	79	14	11	12	15
T_{24}	55	115	58	57	18	11	16	0

a. Standard Plate Count (T_0) = 30/ml

b. Standard Plate Count (T_0) = 1,000/ml

DISCUSSION

The survey of the city of Tucson public groundwater supply indicates that the microbiological quality standards for drinking water set by the E.P.A. are being met. In their nationwide survey of community water supply systems, McCabe et al (1970) found that as a rule, protected wells provided relatively uncontaminated water, with low coliform densities, even though no disinfection treatment is generally practiced prior to distribution. There were, however, several cases in which individual well samples greatly exceeded the minimum acceptable standards. These occurred during the months of July and August, which coincides with the period of heaviest rainfall in Tucson. This suggests that rainfall patterns may directly influence the microbiological quality of the groundwater supply. Studies in other locations have linked periods of heavy rainfall with deteriorating well water quality (Dewalle, Schaff, and Hatlen, 1980; and Barrel and Rowland, 1979). In addition, since demand for water is not as great during rainy periods, water may be subject to prolonged retention in wells and distribution lines, allowing increased bacterial levels to accumulate. The fact that water temperature is highest during this season (approximately

27-31 C) may also be of significance in determining numbers and types of organisms present.

An attempt was made to evaluate the effects of the non-coliform populations on the sensitivity of the accepted methods used to detect coliforms in water. The data presented here, on a limited number of samples, would indicate that a high level of SPC bacteria *per se* does not necessarily adversely affect the detection of indicator organisms. In the two experiments comparing recovery of four coliforms from high SPC background-containing water and the same water from which the background had been removed, no consistent differences attributable to the influence of the background population were found. On the other hand, there were instances of significant detrimental effects on specific coliform type and methodology used. One example of this was the detection of *E. coli* by the M-FC method. For both water sources tested, a significant decline, and in one case elimination, of *E. coli* was recorded from samples containing high background densities. In both, high numbers of background colonies were able to grow on the M-FC plates, suggesting interference by those bacteria. Another example of this has been reported (Lombardo and Canzonier, 1978). It would be expected that antagonistic or competitive effects would be more apparent on the more highly selective procedures such as the M-FC, which employs a 44.5 C incubation temperature,

since it has been postulated that bacteria subjected to one type of stress become hypersensitive to secondary stresses (Postgate, 1967).

Differences with respect to methodology and coliform type were also observed in the experiments dealing with the detection of ATCC strains of E. coli and K. pneumoniae from samples subjected to different storage conditions. The E. coli strain appeared to be inhibited by conditions resulting in high background densities in both MPN and MF media, while adverse effects were manifest in the MF technique but not in the MPN for the K. pneumoniae.

In the experiments in which environmental coliform isolates were inoculated into two water sources, no influence of high SPC background was seen on recovery by the MPN procedure. A slight but significant inhibitory effect was noted on the enumeration of C. freundii and E. agglomerans by the MF procedure in one of the water sources, but this was not duplicated in the other.

These data would suggest that qualitative, as well as quantitative aspects of the resident microflora are important in the suppression of coliforms. To date, few studies have been done concerning the role of specific components of the water microflora in coliform suppression. As with many problems in environmental microbiology, a wide range of studies analyzing the inhibitory effects of specific water

organisms and population levels on the various indicator organisms, relative to the standard methods, needs to be done in order to adequately assess their impact on coliform detection.

In addition to high bacterial densities as measured by the SPC, other factors may be of importance in the successful recovery of indicator organisms, including survival times of coliforms in the water itself, turbidity, amount of background growth on MF plates, and amount of background growth in MPN tubes. The results presented here indicate that, although numbers decrease with time, especially when held at ambient temperatures, viable coliforms were recoverable after 54 hours by one or more methods when the water sample was refrigerated. Turbidity and confluent growth on membrane filters were both implicated in decreasing the effectiveness of the MF procedure. In cases of confluent growth, the "Standard Methods" (American Public Health Association, 1975) require resampling and refiltering of smaller volumes. No provision for resampling is required for samples presumptively negative but showing turbidity in MPN tubes. In the course of this study, 2.6% of turbid, negative MPN tubes were found to contain suppressed coliforms by transfer to BGLB. In two cases, this procedure was the only means by which coliforms were detected. Using their modified MPN procedure, Evans et al (1981) found that 58% of drinking

water samples were falsely negative in the primary MPN series. The much lower number of false negatives found in this study probably reflects a lower coliform incidence. The low success rate of unmasking indicates that it may be worthwhile to concentrate only on heavily turbid tubes which theoretically present a greater potential for coliform suppression. MPN tubes showing any degree of turbidity were followed up in this study. A common problem in all of the unmasking methods evaluated was the frequent heavy growth of pseudomonads in the confirmation media as well as in the primary MPN tubes. In these situations, a method providing anaerobic conditions may be more useful. The use of BGLB or LSB capped with sterile agar after transfer would appear to merit a more adequate evaluation. Anaerobic incubation of the EMB plates used in the Evans modified MPN method might also be warranted to preclude overgrowth by pseudomonads.

The changes in bacterial populations in the individual water sample after collection were also found to be significant, and controllable, depending on transport and storage conditions. In four out of five samples monitored, coliform survival and detection by one or more methods after 24 hours of holding was enhanced when the sample was refrigerated. Two previous studies (Vial and Geoffray, 1980; and Dutka and El-Shaarawi, 1980), have concluded that refriger-

ated samples could be held up to 24 hours without appreciable decreases in coliform counts. Ambient temperature storage was also found to have a pronounced effect on SPC densities. Numbers of SPC bacteria typically increased by a two-log factor in 24 hours, but remained stable if the sample was refrigerated. Other undesirable effects of ambient temperature storage included increased incidences of confluent growth on MF plates and turbidity in MPN tubes. In light of the fact that all of these factors have been implicated in coliform suppression, and that they can be easily stabilized by icing samples after collection, the recommendation for refrigeration of samples given in the 14th edition of Standard Methods (American Public Health Association, 1975) should be followed.

In conclusion, it is recognized that the analysis for indicator of pollution is subject to practical limitations, among them the need for applicability to a wide range of water sources, ease and standardization of interpretation, and cost-benefit ratio. Since the quality limit for drinking water has been established as one coliform per 100 ml, and pathogens have been shown to occur in water with few, if any, coliforms (Seligman and Reitler, 1965), it is important that conditions which have a detrimental effect on coliform detection be recognized and the methodologies improved or supplemented as necessary to counteract those conditions.

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