Appendix G
Kenner and Clark (1974) Analytical Method for *Salmonella* sp. Bacteria*

Detection and enumeration of *Salmonella* and *Pseudomonas aeruginosa*

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THE FEDERAL WATER POLLUTION CONTROL AMENDMENTS of 1972 [1-4] may well require the quantification and enumeration of pathogens such as *Salmonella* species in all classes of waters. The requirements are described by Shedroff. [5]

One of the continuing programs of the Environmental Protection Agency (EPA) is a research project concerned with the development of practical laboratory methods for the isolation, quantification, and enumeration of pathogens from polluted waters. This paper reports a monitoring method developed for the simultaneous isolation and enumeration of *Salmonella* species and *Pseudomonas aeruginosa* from potable waters, reuse waters, treatment plant effluents, receiving waters, and sludges.

The method described herein, and developed by Kenner,[6] is practical because readily available bacteriological media, chemicals, and equipment are all that are required to obtain the desired results. These results are the establishment of the absence or presence of *Salmonella* species (pathogenic hazardous bacteria) and/or *Pseudomonas aeruginosa* (potential pathogens) that affect persons who are in a debilitated condition and are very common as infectious agents in hospitals because of their resistance to antibiotic therapy.[7-9] Potable waters have also been shown to contain *Ps. aeruginosa.*[6,10] The sources of these potential pathogens are human and animal feces and wastewaters.[11,12]

When the monitoring method was used, it was found that 100 percent of municipal wastewaters and treatment plant sludges contained both of these potential pathogens. *Ps. aeruginosa* has been found in potable water supplies of large and small municipalities where insufficient residual chlorine is evident. Also important is the fact that these organisms may be found in the absence of fecal coliforms, whereas negative indicator tests may give a false sense of security. It is believed by the authors that these organisms may be better indicators than fecal coliforms of pollution in potable, direct reuse, bathing, and recreational waters.

MATERIALS AND METHODS

The monitoring method uses a multiple tube (MPN) procedure in which dulcitol selenite broth (DSE) [13] is used for primary enrichment medium, and is modified by the use of sodium acid selenite (BBL). The formula is proteose peptone (Bacto), 0.4 percent; yeast extract (Bacto), 0.15 percent; dulcitol, 0.4 percent; BBL, 0.5 percent; Na_2HPO_4, 0.125 percent; and KH_2PO_4, 0.125 percent in distilled water. The constituents are dissolved in a sterile flask, covered with foil, and heated to 88°C in a water bath to obtain a clear sterile medium that does not require adjustment of pH. Productivity for *Salmonella* species is enhanced by the addition of an 18-hr, 37°C culture of *Salmonella paratyphi* A (10 percent by volume) in single-strength DSE broth, killed by heating to 88°C.

Concentration of bacteria from large volumes of water is necessary when potable, direct reuse, receiving waters, and treatment effluents are being monitored.

-Vol. 46, No. 9, September 1974 2163

TABLE I.-Retentive Characteristics of SeveralGlass Fiber Filter Papers* Compared with Membrane Filters

Filter	Total Bacteria † Filtered	Number Passing Filter	Percentage Retention
Millipore (MF) HAWG 047 HA 0.45 μ, white, grid, 47 mm, Millipore Filter Corp.	1,376	0	100
984H Ultra Glass Fiber Filter, 47 mm, Reeve Angel Corp.	1,229	25	98
GF/F Glass Paper Whatman, ‡47 mm, Reeve Angel Corp.	2,698	6	99.8
GF/D Glass Paper Whatman,‡ 47 mm, Reeve Angel Corp.	2,622	2,166	17.4
934AH Glass Fiber Filter, 47 mm, Reeve Angel Corp.	1,049	198	81
GF/A Glass Paper Whatman, 47 mm, Reeve Angel Corp.	1,066	680	36

* The 984H Ultra Glass Fiber Filter is flexible when wet, readily allows filtration of large volumes of water, can readily be bent double with forceps, and, when placed into primary enrichment broth, disintegrates when tube is shaken and releases entrapped bacteria.

† Enteric bacteria. *E. coli*, 0.5 X 1-3 μ

‡ A new paper filter GF/F has better retentive properties than the 984H, and has same properties (tested Oct. 1973).

Concentration is attained by filtration through glass fiber filters* in a membrane filter apparatus. After the desired volume of water is filtered through the ultra filter, the flexible filter is folded double with sterile forceps and inserted into a suitable volume of single-strength DSE medium contained in a test tube located in the first row of the multiple tube setup. The tube should then be shaken to cause filter to disintegrate (Table I and Figure 1). To obtain MPN results per one l or per 10 l, 100 ml or 1,000 ml of sample, respectively, are filtered for each tube of DSE medium in the first row of the five-tube MPN setup. Additional dilutions are made by transferring material from tubes in the first row to tubes farther back in the setup.

Obtaining results on a per 1-gal (3.8-l) basis requires filtration of 380 ml, and on a per l0-gal (38-1) basis requires filtration of 3,600 ml for each tube in the first row. Where concentration of bacteria is not usually required, as in municipal wastewaters, sludges, or primary effluents, the regular transfer of 10 ml of sample to each tube in the first row of the setup into 10 ml of double-strength DSE is made, 1 ml of sample in 9 ml of single-strength DSE in the second row, and so on. The MPN table in "Standard Methods" [14] is used to read directly the results per volume of sample.

Incubation temperature of 40° ± 0.2°C for 1 and 2 days is critical to obtain optimum recovery of *Salmonella* sp. and *Pseudomonas aeruginosa* when DSE broth is used for primary enrichment. After primary incubation at 40°C surface loopfuls (scum) (7 mm platinum or nichrome wire loop) are removed from each multiple-tube culture and streaked on each of two sections of a divided plate of Xylose lysine desoxycholate agar (XLD) [15] in order to isolate colonial growth. The numbered plates are inverted and incubated at 37°C for a period not to exceed 24 hr.

Commercial dehydrated XLD agars (BBL and Difco) are satisfactory if they are reconstituted in distilled water in sterile foil-covered flasks and heated to 88° or 92°C, respectively. The agar is then cooled to 55° to 60°C and distributed in sterile petri dishes. This laboratory prefers l0-ml portions in each section of a divided sterile disposable plastic dish (Figure 1).

* Reeve Angel 984H ultra glass fiber filter, 47 mm Reeve Angel & Co., Inc., Clifton, N. J. Mention of trade names does not constitute endorsement or recommendation by EPA.

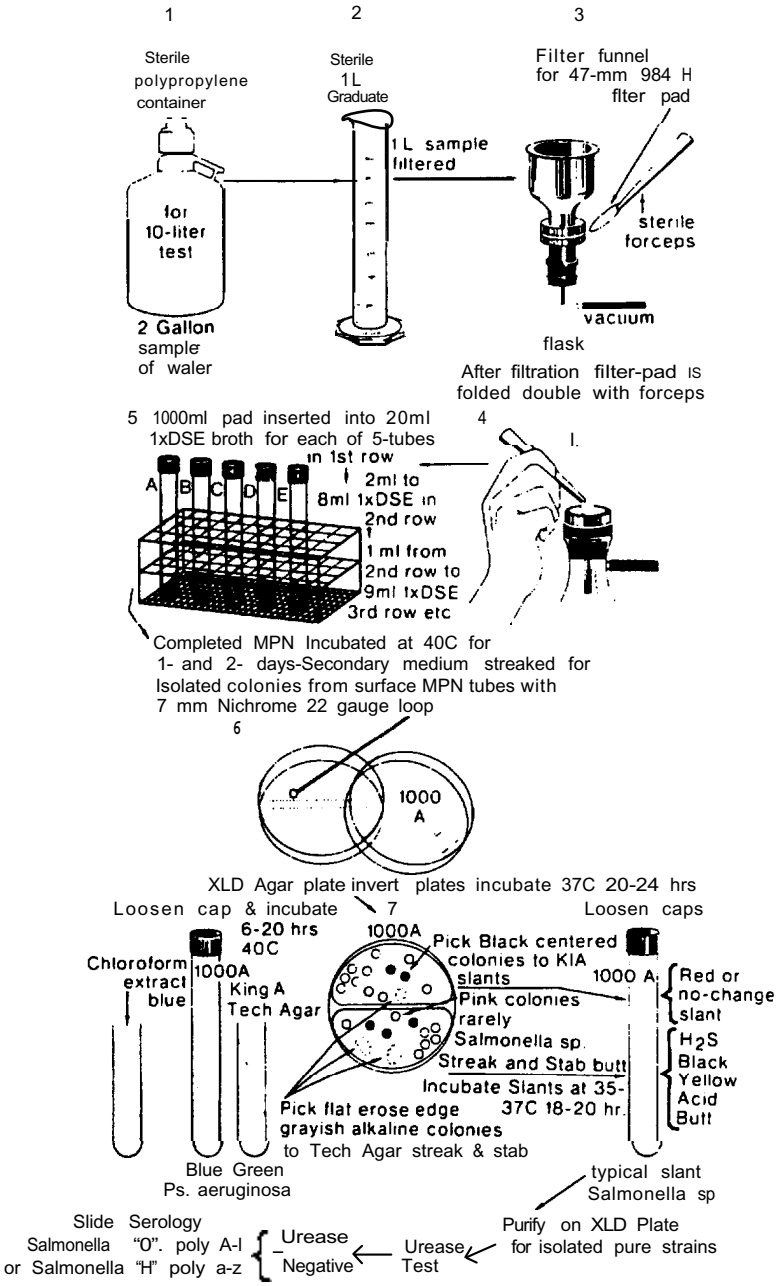

FIGURE I.--Procedure for isolation of pathogens.

Positive incubated XLD plate cultures contain typical clear, pink-edged, black-centered *Salmonella* colonies, and flat, mucoid, grayish alkaline, pink erose-edged *Ps. aeruginosa.* The *Salmonella* colonies are picked to Kligler iron agar (KIA) or Triple sugar iron agar slants for typical appearance, purification, and identity tests. *Ps. aeruginosa* colonies are picked to King A agar slants (Tech agar BBL) for ohtaining the bluegreen pyocyanin confirmation at 40°C (Figure 1).

Typically, *Salmonella* sp. slant cultures (streaked and stabbed), incubated over-

TABLE II.-Advantage of Ultra-filter 984H Use in Monitoring Suspected Waters for *Salmonella* species

Type of Sample	*Salmonella* (no./100 ml)	Serotypes Found (no./100ml)	*Salmonella* (no./gal)	Serotypes Found (no./gal)
Stormwater runoff	4.5	S. bareilly[7]	210	*S. kottbus*[10] *S. bareilly*[11]
Stormwater runoff	<3.0	none	7.3	S. *java*[4] *S. muenchen*[2]
Activated sludge effluent	<3.0	none	3.6	S. group G[4]
Municipal wastewater	6.2	Arizona[3]	1,500	Arizona[4] *S. anatum*[2] *S. newport*[4] *S. san diego*[7] *S. worthington*[2]
Municipal wastewater	<3.0	none	110	*S. anatum*[3] *S. derby*[1] *S. newport*[3]
Activated sludge effluent	<3.0	none	28	*S. blockley*[7] *S. newport*[3]
Mississippi River water, mile 403.1	43	*S. ohio*[10]	>11,000	*S. ohio*[19] *S. derby*[2] *S. meleagridis*[6]
Municipal wastewater	3.0	*S. cholerasuis* var. kunzendorf[2]	21	*S. cholerasuis* var. kunzendorf[5] *S. newport*[6]

night at 37°C, give an unchanged or alkaline red-appearing slant; the butt is blackened by H_2S, is acid-yellow, and has gas bubbles, except for rare species. Typical-appearing slant cultures are purified by transferring them to **XLD** agar plates for the development of isolated colonies. The flat or umbonated-appearing colonies with large black centers and clear pink edges then are picked to **KIA** slants (streaked and stabbed), incubated at 37°C, and urease tested before the identification procedure (Figure 1). Urease-negative tubes are retained for presumptive serological tests and serotype identification.

Typical Tech agar slant cultures for *Ps. aeruginosa* that are incubated at 40°C overnight turn a bluegreen color from pyocyanin, a pigment produced only by this species. A reddish-blue color is caused by the additional presence of pyorubin. The blue pigment is extractable in chloroform and is light blue in color after a few hours at room temperature. No further tests are necessary. The count is read directly from the MPN table.

JUSTIFICATION FOR PROCEDURES

Choice of primary enrichment medium and secondary isolation agar. Most of the enrichment media described in contemporary literature were designed for the isolation of pathogens from clinical specimens from ill persons or from samples of suspected foods, and they work quite well for those types of samples. When they are used, however, for the isolation of pathogens from polluted waters and other types of environmental samples, such as soils, they do not prove adequate. Enrichment media that were tested and found wanting in regard to detection and selectivity were tetrathionate broth (**TT**), with and without brilliant green at 41.5°C; selenite cystine broth at 37°C; selenite F broth at 37°C; selenite brilliant green, with and without sulfa, at 37° and 41.5°C; and Gram-negative broth (**GN**) at 40°C and 41.5°C.

None of the media named worked well at 37°C for the isolation of *Salmonella* sp., and isolation from wastewaters only oc-

TABLE III.-Percentage of Colony Picks from DSE-XLD Combination Positive for *Salmonella* species

Liquid Samples	No.	Total Picks from XLD	No. Positive	No. Negative	Percentage Positive	Range of *Salmonella* counts/100 ml
Municipal wastewater	15	315	250	65	79	3.0-1,500
Stockyard wastewater	1	36	36	0	100	2,100
Rivers						
Mississippi						
Ohio	8	110	84	26	76	1.5->300
Stormwater runoffs	2	18	14	4	78	0.2-1.5
Activated sludge biological effluent	20	386	306	80	79	0.1-1,100
Trickling filter effluent	7	103	78	25	76	0.35-140
Package plant effluent	6	83	55	28	66	1.8-620
Package plant sludge	2	41	37	4	90	43-240
Chlorinated primary outfall	2	17	13	4	76	3-43
Creek 1 mile (1.6 km) below package plant outfall	2	37	16	21	43	4.5-12
Home cisterns	2	17	10	7	59	0.26-1.1
Dupont R-O						
Feed	1	20	14	6	70	4.3
Reject	1	16	8	8	50	0.91
Product-negative						
Raw primary sludge	4	80	66	14	83	13-700
Primary activated sludge	1	15	13	2	87	23
Anaerobic digester sludge	3	78	65	13	83	79-170
Anaerobic digester sludge (28 days)	1	9	3	6	33	2
Activated secondary sludge	6	189	155	34	82	11->11,000
Total	84	1,570	1,223	347	average 78	

curred by chance and was purely qualitative. Of the above-named media used in preliminary tests, selenite brilliant green sulfa broth (SBGS) at 41.5°C gave the best isolation of *Salmonella* sp. from wastewaters (with and without the addition of S. *typhimurium* in known numbers). Of thirteen wastewater samples tested in SBGS at 41.5°C, six contained *Salmonella* or 46 percent were positive. With DSE broth at 40°C, 28 of 28, or 100 percent of wastewater samples, gave positive results.

Studies were not continued on SBGS medium when it was noted that some lots of commercially available SBGS seemed to be selective for *Salmonella* sp. while others were not. The medium was then prepared according to the original formula [16] with six different lots of brilliant green (certified), only one of which was selective. The use of brilliant green agar as a selective medium is subject to the same variability, according to Read and Reyes.[17]

The main reasons for rejection of TT, with and without brilliant green, and for selenite broth's using brilliant green agar and XLD agar as secondary media are not only fewer isolations of *Salmonella* sp., but also the poor selectivity of these combinations when they are used for monitoring polluted waters. These combinations' poor selectivity at 41.5°C is apparent in the results of Dutka and Bell,[18] where the TT broth-XLD combination yielded 26 percent confirmation of colonial picks, and selenite broth-BGA and selenite broth-XLD gave 55 and 56 percent confirmations, respectively. The authors had similar results. The GN-XLD combination was poorest for water samples at 40° and 41.5°C yielding less than 10 percent isolations from wastewaters.

Effect of incubation temperature on isolation of *Salmonella* sp. In a study of 26 wastewater samples that was conducted with the DSE multiple tube setups at three

TABLE IV.-Serotypes of *Salmonella* Found in Polluted Waters

Serotype	No. of Strains	Rank in Water Isolations	Rank in Human Occurrence*
1 *typhimurium**	375	1	1 and 6†
2 *derby*	287	2	12
3 *cubana*	223	3	20
4 *chester*	203	4	19
5 *newport*	188	5	4
6 *kottbus*	158	6	—‡
7 *blockley*	157	7	11
8 *infantis*	141	8	9
9 *enteritidis*	128	9	5
10 *anatum*	127	10	13
11 *heidelberg*	110	11	6
12 *manhattan*	97	12	14
13 *paratyphi* B	91	13	17
14 *illinois*	77	14	—‡
15 *thompson*	63	15	3
16 *livingstone*	52	16	—‡
17 *montevideo*	47	17	16
18 *muenchen*	45	18	18
19 *oranienberg*	44	19	15
20 *san diego*	44	19	18
21 *barielly*	42	20	23
22 *tshiongwe*	41	21	—
23 *orion*	41	21	—‡
24 *senftenberg*	39	22	25
25 *schwarzengrund*	37	23	—‡
26 *lexington*	33	24	—‡
27 *cholerasuis*	30	25	—‡
28 *binza*	29	26	24
29 *cholerasuis* var. kunzendorf	29	26	—‡
Other serotypes:			
30 *albany*	20	31	—‡
31 *benfica*	10	40	—‡
32 *braenderup*	13	38	—‡
33 *brancaster*	1		—‡
34 *bredeney*	8		23
35 *california*	2		—‡
36 *drypool*	14	37	—‡
37 *friedenau*	4		—
38 *give*	25	29	22
39 *grumpensis*	10	40	—‡
40 *haifa*	2		—‡
41 *hartford*	2		—‡
42 *havana*	16	35	—‡
43 *indiana*	10	40	—‡
44 *java*	15	36	7
45 *javiana*	13	38	8
46 *litchfield*	17	34	21
47 *lomita*	26	28	—‡
48 *meleagridis*	18	33	25
49 *mission*	14	37	—‡
50 *newington*	2		—‡
51 *newlands*	8		—‡
52 *norwich*	14	37	—‡
53 *ohio*	19	32	—‡
54 *preston*	2		—
55 *reading*	26	28	—‡
56 *rubislaw*	15	36	—‡
57 *saint paul*	21	30	10
58 *schleissheim*	12	39	—
59 *simsbury*	9		—‡
60 *taksony*	16	35	—‡
61 *tennessee*	12	39	20
62 *typhi-suis* var. voldagsen	2		—
63 *uzaramo*	28	27	—‡
64 *wil*	3		—
65 *worthington*	10	40	24
Sub total	3,417		
Arizona	151		
Incomplete serology	232		
Total	3,800		

* Rank in human occurrence Table I, Martin and Ewing.[19]

† Separation of *S. typhimurium* and var. copenhagen not done after initial identifications.

‡ Serotypes occurring in humans, 1965-1971, Center for Disease Control, Salmonella Surveillance, Annual Summary 1971, Table IX, U. S. DHEW, PHS DHEW Publ. No. (HSM) 73-8184 (Oct. 1972).

different temperatures, it was found that 100 percent of the samples contained *Salmonella* sp. and ***Ps. aeruginosa*** at 40°C. At 41.5°C, however, only 50 percent or 13 of the samples yielded ***Salmonella*** sp., and at 37°C only 8 percent or 2 of the samples yielded ***Salmonella*** sp.

Effect of enhancement of DSE broth with a killed culture of S. *paratyphi* A. In a study of 84 samples of activated sludge effluents, trickling filter effluents, package plant effluents, and stream waters, **DSE** broth enhanced with a killed culture of *S. paratyphi* A in **DSE** broth (10 percent by volume) yielded isolations in 64 samples or 74 percent isolated *Salmonella* sp., compared with 48 samples or 57 percent isolations when the **DSE** broth was used without enhancement. An improved isolation of 17 percent was achieved with enhanced **DSE** broth.

Ultra-filter. The advantages of ultrafilter use in testing water samples are illustrated in Table II.

RESULTS AND DISCUSSION

Of importance to those who must use bacteriological tests to obtain *Salmonella* sp. and ***Ps. aeruginosa*** counts from waters is the amount of work that must be done to secure accurate results. Table III presents the percentage of colony picks made with the described method that proved to be *Salmonella* sp. If there are black-centered colonies on the **XLD** plates, more than 75 percent of the picks will prove to be *Salmonella* sp.; thus, the method leads to less unproductive work. When other methods were used, the authors have at times had to pick 50 black-centered colonies to obtain only 5 *Salmonella* sp. strains. This type of unproductive work has given the search for pathogens in the environment an undeserved bad reputation, and it has caused some to give up.

In Table II it may readily be seen that in many cases the fault with many tests has been the testing of an insufficient volume of sample. Many people think that it involves too much work, and that only expensive fluorescent antibody techniques will work. The problem is, however, to

TABLE V.-Percentage of Various Types of Water Samples Positive for *Salmonella* species

Type of Sample	Number of Samples	Number Positive	Number Negative	Percentage Positives
Municipal wastewaters	28	28	0	100.0
Municipal primary efflucnts (chlorinated)	9	5	4	56.0
Activated sludge effluents (clarified)	40	29	11	72.5
Activated sludge effluents Before chlorination	5	4	1	80.0
After chlorination, 1.4-2.0 mg/l residual, 5 min contact	8	0	8	0.0
Trickling filter effluents	26	15	11	57.7
Package plant effluents	15	7	8	46.7
Creek 1 mile (1.6 km) below package plant	3	3	0	100.0
Ohio River above Cincinnati public landing	20	9	11	45.0
Wabash River	4	3	1	75.0
Mississippi River	4	3	1*	75.0
Streams collective	31	18	13	58.0
Stormwater runoff after heavy rain	6	3†	3‡	50.0
Farm wells	4	0	4	0.0
Home cisterns suburban	5	2	3	40.0
Septic tank sludges	6	3	3	50.0
Totals	183	114	69	

* Municipal intake.
† Positive by per-gallon technique.
‡ Negative by per-100 ml technique.

concentrate the bacteria in a 10-gal (38-l) sample or a l00-gal (380-l) sample of potable or reuse water to obtain results, and still not require even more expensive filtration or centrifugation equipment. It also seems unrealistic to test only extremely small samples of the water being examined, because they may not be representative.

Table IV contains a list of *Salmonella* serotypes isolated from polluted waters and ranked according to the frequency of serotype isolations. It will be noted that all of the serotypes except S. ***typhi*** were isolated from environmental samples by the monitoring method, and that only 6 of the 65 serotypes reported were not reported as occurring in humans in the U. S. over the period from 1965 to 1971.

Table V summarizes the percentage of various types of water samples positive for *Salmonella* sp. Of interest is the fact that 100 percent of the municipal wastewaters tested contain *Salmonella* sp., that 56 percent of chlorinated primary effluents tested contain the pathogens, and that 100 percent of chlorinated secondary effluents are negative for pathogens. There are more studies scheduled for testing of secondary and tertiary effluents to obtain minimal chlorine residuals. Calabro et al.[20] reported that more than 50 attempts at isolating *Salmonella* sp. from septic tank samples using SBGS-BGSA combinations were unsuccessful.

Table VI summarizes the isolation of ***Ps. aeruginosa*** from potable water supply, that is, wells, cisterns, and small municipal water supply. It should be noted that fecal coliforms were not detected in most of these samples. Fecal streptococci counts were higher than fecal coliform counts where both tests were used. ***Ps. aeruginosa*** were present in all but three of the tests, and *Salmonella* sp. were isolated from two different cistern samples.

It is of importance to the user of pathogen tests that the test be quantitative. In initial studies on the DSE-XLD combination, it was important to know if the enrichment broth would support the growth of a wide

TABLE VI.-Isolation of *Pseudomonas aeruginosa* from Potable Water Supply

Type of Sample	*Ps. aeruginosa* Isolation	Indicators/100 ml		
		Total Coliforms	Fecal Coliforms	Fecal Streptococci
Well 8/16/71	+	4	—	—
Well 8/25/71	+	22	—	—
Well 3/27/72	+	—	<1	—
Well 3/27/72 (chlorinated)	+	—	<1	—
Well 8/23/72	+	—	0.25	62
Well 10/ 4/72	+	—	<2	46
Suburban cisterns				
8/ 4/72	+	—	180	—
10/ 9/72*	+	—	15	156
11/ 6/72*	+	—	<2	22
11/ 6/72	+	—	<2	2
11/26/72	+	—	3	28
Municipal supplies				
Population served 54,700				
3/17/71	+	—	<1	—
6/21/71	+	—	<1	—
7/19/71	+	—	<1	—
6/19/72	+	—	0.26	—
10/ 9/72	+	—	<1	<1
5/ 8/72	0	—	<1	—
Population served 14,000				
5/ 8/72	0	—	<1	—
10/24/72	+	—	<1	18
Population served < 10,000				
11/27/72	0	—	<1	<1

* *Salmonella* sp. also present in samples.

range of ***Salmonella*** serotypes. Laboratory cultures of ***S. paratyphi*** A, ***S. typhimurium, S. bredeney, S. oranienberg, S. pullorum, S. anatum, S. give,*** and S. ***worthington*** were tested in three enrichment broths. The time required to isolate each of the above cultures from an estimated 10 to 20 organisms/l00 ml in buffer water was 48 to 72 hr for ***S. paratyphi*** A in TT broth, 24 hr for DSE broth, and 36 to 48 hr for SBGS broth. The rest of the cultures were isolated in estimated numbers in 14 to 24 hr in TT and DSE broths. In SBGS broth, ***S. typhimurium, S. bredeney, S. anatum, S.*** give, and S. ***worthington*** required 36 to 48 hr incubation, and ***S. pullorum*** and S. ***oranienberg*** required 48 to 72 hr incubation.

It is impossible to know if 100 percent of ***Salmonella*** sp. in a polluted water sample are isolated. In tests where laboratory cultures have been added in low numbers to wastewater and treatment effluent samples, all of the numbers added were detected, as well as the ***Salmonella*** sp. that were naturally occurring. The higher the quality of the water (for example, secondary or tertiary treatment effluent, or even potable waters), the better the possibility of isolation of all the *Salmonella* serotypes present, as well as Ps. ***aeruginosa,*** a potential pathogen.

SUMMARY

A practical laboratory method is presented for the simultaneous isolation and enumeration of ***Salmonella*** sp. and *Pseudomonas* ***aeruginosa*** from all classes of waters, including potable water supplies, with a minimum of interfering false positive isolations. The method allows for the testing

of large volumes of high quality waters, wherein the absence of indicator bacteria (that is, total coliforms, fecal coliforms, and fecal streptococci), may give a false sense of security because of the low volumes of water usually tested. Justification for each step of the procedural method is presented.

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Authors. Bernard A. Kenner is supervisory research microbiologist, and Harold P. Clark is biological technician, Waste Identification and Analysis Activity of the Advanced Waste Treatment Research Laboratory, Natonal Environmental Research Center, U. S. Environmental Protection Agency, Cincinnati, Ohio.

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