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Confirmation of the Single-Step Membrane Filtration Procedure for Estimating *Pseudomonas aeruginosa* Densities in Water

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The efficiency of two procedures, membrane filtration and most probable number, to resuscitate and enumerate *Pseudomonas aeruginosa* have been compared at two temperatures and varying incubation periods. Data indicate that the membrane filtration procedure using mPA or mPA medium B is more efficient than the most-probable-number procedure in estimating *P. aeruginosa* populations. It was also found that the specificity of the membrane filtration procedure was such that 92 to 99% of the colonies counted as *P. aeruginosa* were confirmed, whereas only 2.7 to 10% of the nontypical colonies were confirmed as *P. aeruginosa*. Furthermore, the data indicate that mPA medium B combined with a 3- to 4-day incubation period at 41.5°C is slightly more specific than mPA medium and is a valid single-step procedure for the resuscitation and enumeration of *P. aeruginosa* from water or sewage effluent.

Historically, the quality of recreational waters has been monitored and regulated by bacteriological tests. Invariably these tests have been related to the presence and specific densities of "coli," coliforms, fecal coliforms, bacillus coli, or *Escherichia coli*, most of which are pseudonyms for lactose-fermenting *Enterobacteriaceae*, with emphasis on the genus *Escherichia*. The presence of these organisms in water was assumed to indicate a potential health hazard because of their association in excrements with a variety of pathogenic microorganisms: *Salmonella, Shigella, Vibrio, Mycobacterium, Pasteurella, Leptospira*, and enteric viruses.

There is, however, an increasing awareness that the majority of infections acquired through bodily contact with recreational waters are upper respiratory in nature rather than gastrointestinal (9, 15, 18). *Pseudomonas aeruginosa*, having been increasingly implicated in these infections (3, 4), have not been shown to be directly related to coliform levels and, therefore, cannot be indexed by the commonly used enteric indicator systems. Furthermore, there is increasing evidence of a direct relationship between man's activities and the incidence of *P. aeruginosa* in water (3, 8).

Recognition of the increasing importance of *P. aeruginosa* both as an indicator of pollution and as a pathogen has led to the development of a variety of enumeration-isolation procedures (3, 4, 9, 11, 13). These procedures are basically of two types: (i) one based on the production of fluorescent pigment, either in a liquid or on a solid medium, with or without confirmation on a variety of media (1, 4, 8, 14); and (ii) the other based on colonial morphology and color, with or without confirmation (13).

Two membrane filter procedures have shown great promise for estimating *P. aeruginosa* populations. One of these procedures, based on fluorescence, although specifically proposed for swimming pool studies (4), may also have application in routine water testing, whereas the other procedure, based on colonial morphology and proposed by Levin and Cabelli (13), has been evaluated in both fresh- and saltwaters and with sewage samples.

In this report, two membrane filter procedures and the most-probable-number (MPN) procedure using one of the formulations of Drake (8) were compared for their relative efficiency in enumerating *P. aeruginosa* from water and sewage samples collected from within the Canadian Great Lakes Basin. In addition, a modified version of the Levin and Cabelli (13) medium was evaluated for its ability to resuscitate and enumerate stressed *P. aeruginosa* cells.

MATERIALS AND METHODS

Field samples. Five samples per week over a 4-week period were collected from each of the following sites for estimation of *P. aeruginosa* populations: raw sewage inlet, unchlorinated effluent (secondary

treatment), Burlington Canal, and offshore Lake Ontario. Samples were collected approximately 2 to 3 dm below the surface, iced, and processed within 2 to 5 h of collection.

Field sample analysis and culture media. Serially diluted samples were filtered through membrane filters (Gelman GN-6, 0.45 μm). Six filtrations were performed on each dilution. Membranes were placed on two plates of each of the following media: mPA (13), mPA medium B (mPA medium modified by the addition of 1.5 g of magnesium sulfate and reduction of the sodium thiosulfate concentration to 5 g/liter of medium), and MacConkey agar (Difco). One plate of each medium was incubated at 38°C, whereas the other was incubated at 41.5°C. All plates were incubated in saturated humidity for 96 h. Only those membranes that contained less than 100 suspected *P. aeruginosa* colonies were used for density estimates.

MPN five-tube tests (1), using Drake medium (asparagine, 2.0 g; proline, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4$, 0.5 g; K_2SO_4, 10.0 g; ethanol, 26 ml; distilled water, 1 liter), were also performed on the same samples in duplicate; one MPN set was incubated at 38°C and the other at 41.5°C. Tubes exhibiting fluorescence under ultraviolet light or green or blue pigmentation after 4 days of incubation were considered presumptive positives for *P. aeruginosa*.

Confirmation and identification procedures. The *P. aeruginosa* confirmation and identification procedures shown in Fig. 1 were used to confirm a maximum of 10 fluorescing colonies from each MacConkey agar-membrane filter plate. From mPA and mPA medium B, a maximum of 10 typical *P. aeruginosa* colonies (dark brown or greenish black centers with pale outer edges) and 10 doubtful *P. aeruginosa*-like colonies were subcultured to MacConkey agar. After 24 h of incubation at 35°C, all non-lactose-fermenting colonies were subcultured to nutrient agar and subjected to procedures shown in Fig. 1.

All MPN tubes that exhibited green-blue pigment or fluorescence or both under ultraviolet light were subcultured to acetamide agar (6) and incubated up to 96 h at 41.5°C. Positive cultures were subcultured to nutrient agar and treated as shown in Fig. 1.

Sensitivity of enumeration procedures. To evaluate the sensitivity of the membrane filtration (MF) and MPN techniques and media to enumerate actively growing and stressed *P. aeruginosa*, the following test was carried out. Samples of sewage, unchlorinated effluent, and lake water were dispensed in duplicate in 250-ml portions into stoppered flasks and autoclave sterilized for 15 min at 121°C. Approximately 2,000 washed cells (phosphate buffer [1]) of a *P. aeruginosa* isolate (from Lake Ontario) were inoculated into each flask from an overnight tryptic soy broth culture. Flasks were then shaken by hand, and a portion was removed and tested to determine the population at 0 h. One set of flasks (sewage, effluent, and lake water) was incubated at 4°C on a shaker (150 rpm) and the other set of flasks was placed on a similar shaker at 20°C. Density estimates in triplicate were made at 1, 2, 7, and 14 days

by the spread plate technique with nutrient agar, the MF technique with mPA and mPA medium B, and the MPN procedure with Drake medium.

RESULTS

Although the MacConkey agar-membrane filter procedure for enumeration of *P. aeruginosa* proposed by Brodsky and Nixon (4) was designed for the rapid detection of *P. aeruginosa* in swimming pools, the procedure was evaluated to determine whether it would be equally effective in natural waters and sewage samples. Unfortunately, in dilutions in which countable colonies appeared on mPA and mPA medium B membranes, the MacConkey agar-membrane filter combination was always completely overgrown with lactose- and non-lactose-fermenting colonies and fungi. By use of various media combinations, it was found that in several canal water samples there were approximately 100 coliform organisms to each *P. aeruginosa*. It was impossible, therefore, to obtain a *P. aeruginosa* count by the MacConkey agar-membrane filter procedure from any of the samples tested, since the samples had to be diluted beyond the density of *P. aeruginosa* in the sample.
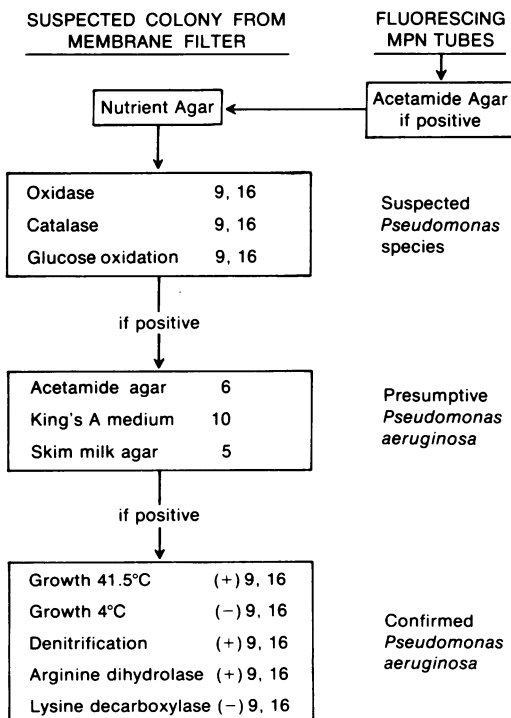

FIG. 1. *Pseudomonas confirmation and P. aeruginosa confirmation scheme. Numbers indicate the references in Literature Cited.*

A variety of mPA formula modifications were evaluated by using a strain of *P. aeruginosa* freshly isolated from lake water. From these pure culture studies, it was found that the addition of 0.15 g of magnesium sulfate per 100 ml and reduction of the sodium thiosulfate content to 0.5 g/100 ml in the Levin and Cabelli (13) formula resulted in slightly better recovery and colony definition.

Studies by Brodsky and Nixon (4) and Levin and Cabelli (13) indicated that satisfactory incubation periods varied from 24 to 48 h. Earlier studies by Drake (8) indicated that a 3-day incubation for MF procedures and a 4-day incubation period for the MPN procedure at 38°C provided maximum population estimations. In preliminary laboratory studies, it had been noted that population estimates by the MPN procedure increased with increased incubation and that incubation temperatures also affected recovery rates. Therefore, two short, independent but related studies were performed to determine ideal incubation periods.

In one study, the two membrane filter media, mPA and mPA medium B, were used to evaluate the effect of incubation from 1 to 5 days on the recovery of *P. aeruginosa* from 20 lake water samples. One-day MF results were uncountable due to insufficient typical colonies, and 5-day counts were uncountable due to colony confluence. Thus, only 2-, 3-, and 4-day data are presented in Table 1. Data are presented as percent changes since the geometric mean of the 20 samples for both media after 2 days of incubation varied by 0.1 and were thus accepted as equivalents and equal to 100%. It can be seen that, at 41.5°C, mPA medium B recovers over four times the *P. aeruginosa* population seen after 2 days of incubation and approximately double the population enumerated by mPA medium after 3 or 4 days of incubation. In the other study, incubation period versus MPN, *P. aeruginosa* densities were assessed in four sets of samples: sewage, unchlorinated effluent, and canal and lake waters. From Table 2, it can be seen that the maximum confirmed

TABLE 1. *Effect of incubation period on percentage of population resuscitated and enumerated by MF procedures from lake water samples*

Day of incuba-tion	% of population resuscitated and enumerated	
	mPA ($n = 20$)	mPA medium B ($n = 20$)
2	100	100
3	152	292
4	182	412

TABLE 2. *Effect of incubation period on percentage of population resuscitated and enumerated by MPN procedure from sewage, effluent, canal, and lake samples at 41.5°C*

Day of incu-bation	% of population resuscitated and enumerated			
	Sew-age ($n = 20$)	Unchlori-nated ef-fluent ($n = 20$)	Canal ($n = 20$)	Lake[a] ($n = 20$)
2	100	100	100	100
4	193	218	265	148
6	225	289	375	180

[a] Samples and data are comparable to Table 1.

P. aeruginosa populations were observed after 6 days of incubation at 41.5°C. However, for practical applications, the 4-day incubation period was used in field study comparisons.

Field study data are presented as geometric mean and medians of confirmed *P. aeruginosa* in Table 3. From these 4-day incubation data it can readily be seen that both MF procedures resuscitated and enumerated more confirmed *P. aeruginosa* than did the MPN procedure. Population estimates from raw sewage by the MF techniques were favored by the 38°C incubation temperature. For the other samples, 41.5°C population estimates tended to be slightly higher.

Table 4 shows the reliability of each enumeration procedure in resuscitating and enumerating confirmed *P. aeruginosa* and the percentage of atypical colonies that would have been missed by the MF procedures. When data from Tables 3 and 4 are compared, it can be seen that MF-confirmed *P. aeruginosa* are 2 to 80 times higher than those estimated by MPN procedures.

There was little difference in the confirmation rate between the two membrane filter media and incubation temperatures, except that slightly more confirmed *P. aeruginosa* colonies were recovered on mPA medium B. This medium also produced slightly fewer atypical *P. aeruginosa* colonies.

The mPA medium B data also show that at 41.5°C there were slightly more confirmed isolates and slightly fewer confirmed atypical isolates than at 38°C.

In the studies to evaluate the various *P. aeruginosa* enumeration procedures with stressed and unstressed organisms, it can be seen (Tables 5 and 6) that mPA medium B offers a slight advantage over the mPA medium and usually a greater advantage over the MPN procedure.

TABLE 3. *Comparison of the efficiency of MF and MPN procedures to enumerate P. aeruginosa from sewage, unchlorinated effluent, and canal and lake waters*

Medium and procedure	Temp (°C)	Sewage $n = 20$		Effluent $n = 20$		Canal $n = 20$		Lake $n = 15$	
		Geometric mean (per ml)	Median (per ml)	Geometric mean (per ml)	Median (per ml)	Geometric mean (per ml)	Median (per ml)	Geometric mean (per ml)	Median (per ml)
mPA, MF	38	1,200	1,800	140	100	11	10	8.1	10
	41.5	290	400	83	100	16	10	12.9	10
mPA medium B, MF	38	2,700	2,200	110	100	14	15	11	19
	41.5	1,100	1,600	240	110	15	10	22	14
Drake medium MPN	38	780	920	5.6	5.4	0.50	0.37	0.15	0.20
	41.5	830	1,200	8.3	9.2	0.85	0.11	0.31	0.22

TABLE 4. *Percentage of typical colonies and fluorescing and/or green-blue-pigmented MPN tubes and percentage of atypical and suspicious colonies that were confirmed as P. aeruginosa* [a]

Medium and procedure	Temp (°C)	Sewage		Effluent		Canal		Lake		Summary				
		% TC	% SC	% TC	% SC	% TC	% SC	% TC	% SC	n [b]	% Typical	n [c]	% Suspicious
mPA, MF	38	97.0	10.0	95.0	7.5	95.0	8.0	96.0	8.0	750	95.5	415	8.5
	41.5	95.0	8.3	94.0	8.3	93.0	6.0	93.3	6.7	750	93.8	415	7.5
mPA medium B, MF	38	98.0	5.0	96.5	3.0	95.3	4.0	92.0	2.7	700	95.5	375	3.8
	41.5	99.0	4.0	98.5	3.0	98.7	3.0	97.3	2.7	700	98.5	375	3.2
Drake medium, MPN	38	80.4		72.0		79.2		64.0		351	76.0		
	41.5	87.0		85.1		88.5		77.8		322	86.1		

[a] TC, Typical colonies confirmed; SC, suspicious colonies confirmed; n, number of bacteria or tubes tested.
[b] Typical colonies.
[c] Atypical colonies.

TABLE 5. *Relative abilities of P. aeruginosa media to resuscitate and enumerate organisms incubated at 4°C in autoclaved sewage, unchlorinated effluent, and lake water*

Sample	Time	%[a] of 0-h counts			
		Nutrient agar spread plate	mPA MF	mPA medium B MF	Drake MPN
Sewage	0 h	100	100	100	100
	1 day	75.7	78	65.8	54.1
	2 days	54.3	64	54.3	31.8
	7 days	8.6	24	22.9	20.6
	14 days	0.0	2.5	1.1	1.9
Unchlorinated effluent	1 h	100	100	100	100
	1 day	47.3	49.8	41.3	78
	2 days	39.8	43.2	40	69.6
	7 days	7.7	10.0	8.8	23.5
	14 days	2.2	4.7	3.1	13.5
Lake water	0 h	100	100	100	100
	1 day	17.5	50	75	21.5
	2 days	3.8	10	30	8.5
	7 days	0.0	0.0	0.0	0.0
	14 days	0.0	0.0	0.0	0.0

[a] Percentage based on median of three replicates.

TABLE 6. *Relative abilities of P. aeruginosa media to enumerate organisms incubated at 20°C in autoclaved sewage, unchlorinated effluent, and lake waters*

Sample	Time (day)	Density[a] (per ml of sample)			
		Nutrient agar spread plate	mPA MF	mPA medium B MF	Drake MPN[b]
Sewage	0	8.0×10^1	1.2×10^2	1.2×10^2	1.7×10^2
	1	8.2×10^2	4.0×10^2	5.0×10^2	2.8×10^2
	2	1.0×10^3	8.0×10^2	9.0×10^2	5.4×10^2
	7	2.1×10^6	1.0×10^6	2.0×10^6	2.4×10^3
	14	8.9×10^7	7.8×10^7	8.0×10^7	1.6×10^5
Unchlorinated effluent	0	1.1×10^2	2.0×10^1	7.0×10^1	7.9×10^1
	1	2.2×10^3	2.0×10^2	3.0×10^2	1.3×10^2
	2	5.4×10^3	1.0×10^3	2.0×10^3	1.6×10^3
	7	2.0×10^6	9.0×10^5	1.5×10^6	2.4×10^3
	14	4.2×10^6	7.7×10^6	7.5×10^6	9.2×10^4
Lake water	0	1.5×10^2	2.0×10^2	3.2×10^2	3.5×10^1
	1	3.8×10^2	4.3×10^2	4.9×10^2	1.6×10^2
	2	8.0×10^2	8.1×10^2	8.2×10^2	1.7×10^2
	7	1.1×10^4	9.2×10^3	9.6×10^3	2.8×10^3
	14	3.4×10^4	2.9×10^4	3.2×10^4	1.6×10^4

[a] Median of three replicates.
[b] Five-tube series.

DISCUSSION

At the initiation of this study, we intended to compare four procedures as to their ability to resuscitate and enumerate *P. aeruginosa* from a variety of samples. One of the procedures proposed was the Brodsky-Nixon (4) Mac-Conkey agar-membrane filtration procedure for swimming pool studies. The procedure as proposed had much merit. It was a 24-h procedure, using a commercially available medium, and density estimates were based on the presence or absence of fluorescence. However, in waters containing moderate to large coliform populations, coliform colonies interfered with the development of *P. aeruginosa* colonies. Therefore, although the procedure may be applicable for population estimates under conditions in which *P. aeruginosa* populations may be unrelated to and in excess of coliform populations, the procedure cannot be applied to natural and effluent waters.

In carrying out the MPN procedure, the formulation by Drake (8) containing proline and ethanol was used. This medium, according to Drake, was more successful than the basal medium that was proposed in the 13th edition of *Standard Methods* (1) as the aspargine (with glycerol)-acetamide combination. However, there is another version (2) of the aspargine-acetamide medium that has a different formulation from the Drake (proline-ethanol) medium used in this study and that appearing in

the 13th edition of *Standard Methods* (1).

Table 4 shows that the specificity of the MF procedures was such that 92 to 99% of the organisms were confirmed as *P. aeruginosa*, whereas the verification rate of the MPN procedure varied from 64 to 86%. Furthermore, except in sewage samples, incubation at 41.5°C invariably produced higher density estimates with greater specificity than incubation at 38°C. The data tend to confirm the findings of Levin and Cabelli (13) that approximately 98% of typical colonies enumerated by membrane filter-mPA agar from recreational waters were confirmed as *P. aeruginosa*.

From the data presented in this study, it can be seen that the longer the *P. aeruginosa* medium is incubated, the greater the population that is resuscitated. However, with the MF technique, 4 days appears to be the maximum incubation period, after which problems with confluent growth arise. Levin and Cabelli (13) found a 48-h incubation period at 41.5°C to be satisfactory, yet for the data it can be seen that a 2-day period produced only one-half of the population resuscitated by the 4-day incubation. Thus, it can be seen that when the incubation period is extended, recovery is optimized.

What is probably more important and relevant is to decide the lowest percentage of test organism recovery one is prepared to accept in favor of a quick, one-step procedure. Perhaps an estimate of 80 to 85% of a population, estimated by a reliable reference technique, would

more than adequately satisfy the requirements of most water microbiologists. However, the quandary one faces is whether to use pure culture studies or field studies to establish this population estimation. Both procedures have their limitations (7); nevertheless, a standardized, acceptable means of evaluating the efficiency of one medium or procedure against another must somehow be established. Perhaps as shown in Tables 1 and 2, by using natural populations and extending the incubation periods, more realistic appraisals of the specificity and resuscitation ability of media and procedures may be ascertained.

Data from this study confirm and support the findings of Levin and Cabelli (13) of the greater efficiency of the MF procedure over the MPN procedure to resuscitate and enumerate *P. aeruginosa* from moderately polluted waters. Furthermore, if a 4-day incubation period is accepted as a minimal period for primary isolation and enumeration, the MPN procedure would take up to 4 days longer before confirmation is completed.

The specificity criteria proposed by Levin and Cabelli (13) "that in the hands of a trained operator, no more than 10% false positives and 10% false negatives will be obtained" was easily met by both MF procedures. Tables 1, 2, and 4 show that, although it may have been possible to obtain similar verification rates of typical colonies after 48-h of incubation, it would also have greatly increased the incidence of false negatives, regardless of which MF procedure was used. Therefore, we chose to work with 4-day incubation periods, although if the enumeration of 80 to 85% of a population is acceptable, a 3-day incubation period could be used.

Because some of the data of Levin and Cabelli (13) were obtained from moderately polluted saltwater (Narragansett Bay) containing populations of *P. aeruginosa* similar to those obtained from the lake and canal waters of this study, these studies substantiate the validity of the MF procedure in both fresh- and saltwaters.

In summary, the MF procedure, utilizing mPA or mPA medium B, as a method for enumerating and isolating *P. aeruginosa* populations represents a considerable advance in sanitary microbiology population estimations. The procedure is simple and relatively rapid and eliminates the extended procedures required by the MPN technique. Another benefit of the MF

procedure is the resuscitation and estimation of a greater proportion of the endemic or stressed *P. aeruginosa* population in a variety of waters as compared with the MPN technique assessed.

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