
CURRENT PRACTICES IN WATER MICROBIOLOGY



TRAINING MANUAL

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF WATER PROGRAMS

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CURRENT PRACTICES IN WATER MICROBIOLOGY

This course is designed for engineers, chemists, biologists, bacteriologists, and other administrative personnel responsible for the planning and conduct of water pollution surveys.

BR 105

ENVIRONMENTAL PROTECTION AGENCY
Office of Water Program Operations
TRAINING PROGRAM

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BACTERIOLOGICAL INDICATORS OF WATER POLLUTION

Part 1. General Concepts

I INTRODUCTION

A Bacterial Indication of Pollution

- 1 In the broadest sense, a bacterial indicator of pollution is any organism which, by its presence, would demonstrate that pollution has occurred, and often suggest the source of the pollution.
- 2 In a more restrictive sense, bacterial indicators of pollution are associated primarily with demonstration of contamination of water, originating from excreta of warm-blooded animals (including man, domestic and wild animals, and birds).

B Implications of Pollution of Intestinal Origin

- 1 Intestinal wastes from warm-blooded animals regularly include a wide variety of genera and species of bacteria. Among these the coliform group may be listed, and species of the genera Streptococcus, Lactobacillus, Staphylococcus, Proteus, Pseudomonas, certain spore-forming bacteria, and others.
- 2 In addition, many kinds of pathogenic bacteria and other microorganisms may be released in wastes on an intermittent basis, varying with the geographic area, state of community health, nature and degree of waste treatment, and other factors. These may include the following:
 - a Bacteria: Species of Salmonella, Shigella, Leptospira, Brucella, Mycobacterium, and Vibrio comma.

- b Viruses: A wide variety, including that of infectious hepatitis, Polio-viruses, Coxsackie virus, ECHO viruses (enteric cytopathogenic human orphan -- "viruses in search of a disease"), and unspecified viruses postulated to account for outbreaks of diarrheal and upper respiratory diseases of unknown etiology, apparently infective by the water-borne route.

- c Protozoa: Endamoeba histolytica

- 3 As routinely practiced, bacterial evidence of water pollution is a test for the presence and numbers of bacteria in wastes which, by their presence, indicate that intestinal pollution has occurred. In this context, indicator groups discussed in subsequent parts of this outline are as follows:

- a Coliform group and certain subgroupings

- b Fecal streptococci and certain subgroupings

- c Miscellaneous indicators of pollution

- 4 Evidence of water contamination by intestinal wastes of warm-blooded animals is regarded as evidence of health hazard in the water being tested.

II PROPERTIES OF AN IDEAL INDICATOR OF POLLUTION

- A An "ideal" bacterial indicator of pollution should:

- 1 Be applicable in all types of water

- 2 Always be present in water when pathogenic bacterial constituents of fecal contamination are present. Ramifications of this include --
 - a Its density should have some direct relationship to the degree of fecal pollution.
 - b It should have greater survival time in water than enteric pathogens, throughout its course of natural disappearance from the water body.
 - c It should disappear rapidly from water following the disappearance of pathogens, either through natural or man-made processes.
 - d It always should be absent in a bacteriologically safe water.

3 Lend itself to routine quantitative testing procedures without interference or confusion of results due to extraneous bacteria

4 Be harmless to man and other animals

B In all probability, an "ideal" bacterial indicator does not exist. The discussion of bacterial indicators of pollution in the following parts of this outline include consideration of the merits and limitations of each group, with their applications in evaluating bacterial quality of water.

III APPLICATIONS OF TESTS FOR POLLUTION INDICATORS

A Tests for Compliance with Bacterial Water Quality Standards

- 1 Potability tests on drinking water to meet Interstate Quarantine or other standards of regulatory agencies.
- 2 Determination of bacterial quality of environmental water for which quality standards may exist, such as shellfish waters, recreational waters, water resources for municipal or other supplies.

3 Tests for compliance with established standards in cases involving the protection or prosecution of municipalities, industries, etc.

B Treatment Plant Process Control

- 1 Water treatment plants
- 2 Wastewater treatment plants

C Water Quality and Pollutant Source Monitoring

- 1 Determination of intestinal pollution in surface water to determine type and extent of treatment required for compliance with standards
- 2 Tracing sources of pollution
- 3 Determination of effects on bacterial flora, due to addition of organic or other wastes

D Special Studies, such as

- 1 Tracing sources of intestinal pathogens in epidemiological investigations
- 2 Investigations of problems due to the Sphaerotilus group
- 3 Investigations of bacterial interference to certain industrial processes, with respect to such organisms as Pseudomonas, Achromobacter, or others

IV SANITARY SURVEY

The laboratory bacteriologist is not alone in evaluation of indication of water pollution of intestinal origin. On-site study (Sanitary Survey) of the aquatic environment and adjacent areas, by a qualified person, is a necessary collateral study with the laboratory work and frequently will reveal information regarding potential bacteriological hazard which may or may not be demonstrated through laboratory findings from a single sample or short series of samples.

Part 2. The Coliform Group and Its Constituents

I ORIGINS AND DEFINITION

A Background

- 1 In 1885, Escherich, a pioneer bacteriologist, recovered certain bacteria from human feces, which he found in such numbers and consistency as to lead him to term these organisms "the characteristic organism of human feces."

He named these organisms Bacterium coli-commune and B. lactis aerogenes. In 1895, another bacteriologist, Migula, renamed B. coli commune as Escherichia coli, which today is the official name for the type species.

- 2 Later work has substantiated much of the original concept of Escherich, but has shown that the above species are in fact a heterogeneous complex of bacterial species and species variants.
 - a This heterogeneous group occurs not only in human feces but representatives also are to be found in many environmental media, including sewage, surface freshwaters of all categories, in and on soils, vegetation, etc.
 - b The group may be subdivided into various categories on the basis of numerous biochemical and other differential tests that may be applied.

B Composition of the Coliform Group

1 Current definition

As defined in "Standard Methods for the Examination of Water and Wastewater" (13th ed): "The coliform group includes all of the aerobic and facultative anaerobic, Gram-negative, nonspore-forming rod-shaped bacteria which ferment lactose with gas formation within 48 hours at 35°C."

- 2 The term "coliforms" or "coliform group" is an inclusive one, including the following bacteria which may meet the definition above:

- a Escherichia coli, E. aurescens, E. freundii, E. intermedia
- b Aerobacter aerogenes, A. cloacae
- c Biochemical intermediates between the genera Escherichia and Aerobacter

- * The above terminology is in accordance with the current editions of Standard Methods and Bergey's Manual of Determinative Bacteriology and will be consistent throughout this manual until these sources are modified.
- 3 There is no provision in the definition of coliform bacteria for "atypical" or "aberrant" coliform strains.
 - a An individual strain of any of the above species may fail to meet one of the criteria of the coliform group.
 - b Such an organism, by definition, is not a member of the coliform group, even though a taxonomic bacteriologist may be perfectly correct in classifying the strain in one of the above species.

II SUBDIVISION OF COLIFORMS INTO "FECAL" AND "NONFECAL" CATEGORIES

A Need

Single-test differentiations between coliforms of "fecal" origin and those of "nonfecal" origin are based on the assumption that typical E. coli and closely related strains are of fecal origin while A. aerogenes and its close relatives are not of direct fecal origin. (The latter assumption is not fully borne out by investigations at this Center. See Table 1, IMViC Type ---+++). A number of single differential tests have been proposed to differentiate between "fecal" and "nonfecal" coliforms.

Without discussion of their relative merits, several may be cited here:

B Types of Single-Test Differentiation

1 Determination of gas ratio

Fermentation of glucose by E. coli results in gas production, with hydrogen and carbon dioxide being produced in equal amounts.

Fermentation of glucose by A. aerogenes results in generation of twice as much carbon dioxide as hydrogen.

Further studies suggested absolute correlation between H_2/CO_2 ratios and the terminal pH resulting from glucose fermentation. This led to the substitution of the methyl red test.

2 Methyl red test

Glucose fermentation by E. coli typically results in a culture medium having terminal pH in the range 4.2 - 4.6 (red color a positive test with the addition of methyl red indicator). A. aerogenes typically results in a culture medium having pH 5.6 or greater (yellow color, a negative test).

3 Indole

When tryptophane, an amino acid, is incorporated in a nutrient broth, typical E. coli strains are capable of producing indole (positive test) among the end products, whereas A. aerogenes does not (negative test).

In reviewing technical literature, the worker should be alert to the method used to detect indole formation, as the results may be greatly influenced by the analytical procedure.

4 Voges-Proskauer test (acetylmethyl carbinol test)

The test is for detection of acetylmethyl carbinol, a derivative of 2,3, butylene-

glycol, as a result of glucose fermentation in the presence of peptone. A. aerogenes produces this end product (positive test) whereas E. coli gives a negative test:

- a Experience with coliform cultures giving a positive test has shown a loss of this ability with storage on laboratory media for 6 months to 2½ years, in 20 - 25% of cultures (105 out of 458 cultures).
- b Some workers consider that all coliform bacteria produce acetylmethyl carbinol in glucose metabolism. These workers regard acetylmethyl carbinol-negative cultures as those which have enzyme systems capable of further degradation of acetylmethyl carbinol to other end products which do not give a positive test with the analytical procedure. Cultures giving a positive test for acetylmethyl carbinol lack this enzyme system.
- c This reasoning leads to a hypothesis (not experimentally proven) that the change of reaction noted in certain cultures in 4.a above is due to the activation of a latent enzyme system.

5 Citrate utilization

Cultures of E. coli are unable to use the carbon of citrates (negative test) in their metabolism, whereas cultures of A. aerogenes are capable of using the carbon of citrates in their metabolism (positive test).

Some workers (using Simmons Citrate Agar) incorporate a pH indicator (brom thymol blue) in the culture medium in order to demonstrate the typical alkaline reaction (pH 8.4 - 9.0) resulting with citrate utilization.

6 Elevated temperature (Eijkman) test

- a The test is based on evidence that E. coli and other coliforms of fecal

origin are capable of growing and fermenting carbohydrates (glucose or lactose) at temperatures significantly higher than the body temperature of warm-blooded animals. Organisms not associated with direct fecal origin would give a negative test result, through their inability to grow at the elevated temperature.

- b While many media and techniques have been proposed, EC Broth, a medium developed by Perry and Hajna, used as a confirmatory medium for 24 hours at $44.5 \pm 0.2^\circ\text{C}$ are the current standard medium and method. While the "EC" terminology of the medium suggests "E. coli" the worker should not regard this as a specific procedure for isolation of E. coli.
- c A similar medium, Boric Acid Lactose Broth, has developed by Levine and his associates. This medium gives results virtually identical with those obtained from EC Broth, but requires 48 hours of incubation.
- d Elevated temperature tests require incubation in a water bath. Standard Methods 13th Ed. requires this temperature to be $44.5 \pm 0.2^\circ\text{C}$. Various workers have urged use of temperatures ranging between 43.0°C and 46.0°C . Most of these recommendations have provided a tolerance of $\pm 0.5^\circ\text{C}$ from the recommended levels. However, some workers, notably in the Shellfish Program of the Public Health Service, stipulate a temperature of $44.5 \pm 0.2^\circ\text{C}$. This requires use of a water bath with forced circulation to maintain this close tolerance. This tolerance range was instituted in the 13th Edition of Standard Methods and the laboratory worker should conform to these new limits.

e The reliability of elevated temperature tests is influenced by the time required for the newly-inoculated cultures to reach the designated incubation temperature. Critical workers insist on placement of the cultures in the water bath within 30 minutes, at most, after inoculation.

7 Other tests

Numerous other tests for differentiation between coliforms of fecal vs. nonfecal origin have been proposed. Current studies suggest little promise for the following tests in this application: uric acid test, cellobiose fermentation, gelatin liquefaction, production of hydrogen sulfide, sucrose fermentation, and others.

C IMViC Classification

- 1 In 1938, Parr reported on a review of a literature survey on biochemical tests used to differentiate between coliforms of fecal vs. nonfecal origin. A summary follows:

Test	No. of times used for differentiation
Voges-Proskauer reaction	22
Methyl red test	20
Citrate utilization	20
Indole test	15
Uric acid test	6
Cellobiose fermentation	4
Gelatin liquefaction	3
Eijkman test	2
Hydrogen sulfide production	1
Sucrose fermentation	1
a-Methyl-d-glucoside fermentation	1

- 2 Based on this summary and on his own studies, Parr recommended utilization of a combination of tests, the indole, methyl red, Voges-Proskauer, and the citrate utilization tests for this differentiation. This series of reactions is designated by the mnemonic "IMViC". Using this scheme, any coliform culture can be described by an "IMViC Code" according to the reactions for each culture. Thus, a typical culture of E. coli would have a code ++--, and a typical A. aerogenes culture would have a code --++.
- 3 Groupings of coliforms into fecal, non-fecal, and intermediate groups, as shown in "Standard Methods for the Examination of Water and Wastewater" are shown at the bottom of this page.

D Need for Study of Multiple Cultures

All the systems used for differentiation between coliforms of fecal vs. those of nonfecal origin require isolation and study of numerous pure cultures. Many workers prefer to study at least 100 cultures from any environmental source before attempting to categorize the probable source of the coliforms.

III NATURAL DISTRIBUTION OF COLIFORM BACTERIA

A Sources of Background Information

Details of the voluminous background of technical information on coliform bacteria recovered from one or more environmental media are beyond the scope of this discussion. References of this outline are suggested routes of entry for workers seeking to explore this topic.

B Studies on Coliform Distribution

- 1 Since 1960 numerous workers have engaged in a continuing study of the natural distribution of coliform bacteria and an evaluation of procedures for differentiation between coliforms of fecal vs. probable non-fecal origin. Results of this work have special significance because:

- a Rigid uniformity of laboratory methods have been applied throughout the series of studies
- b Studies are based on massive numbers of cultures, far beyond any similar studies heretofore reported

Groupings of Coliforms into Fecal, Nonfecal and Intermediate Groups

Organism	Indole	Methyl red	Voges-Proskauer	Citrate
<u>E. coli</u> , Variety I	+	+	-	-
Variety II	-	+	-	-
<u>E. freundii</u> (Intermediates)				
Variety I	-	+	-	±
Variety II	+	+	-	+
<u>A. aerogenes</u>				
Variety I	-	-	+	±
Variety II	±	-	+	+

- c A wider variety of environmental and biological sources is being studied than in any previous series of reports.
- d All studies are based on freshly recovered pure culture isolates from the designated sources.
- e All studies are based on cultures recovered from the widest feasible geographic range, collected at all seasons of the year. It is believed that no more representative series of studies has been made or is in progress.

2 Distribution of coliform types

Table 1 shows the consolidated results of coliform distributions from various biological and environmental sources.

- a The results of these studies show a high order of correlation between known or probable fecal origin and the typical E. coli IMViC code (++--). On the other hand, human feces also includes numbers of A. aerogenes and other IMViC types, which some regard as "nonfecal" segments of the coliform group. (Figure 1)
- b The majority of coliforms attributable to excretal origin tend to be limited to a relatively small number of the possible IMViC codes; on the other hand, coliform bacteria recovered from undisturbed soil, vegetation, and insect life represent a wider range of IMViC codes than fecal sources, without clear dominance of any one type. (Figure 2)
- c The most prominent IMViC code from nonfecal sources is the intermediate type, -+--, which accounts for almost half the coliform cultures recovered from soils, and a high percentage of those recovered from vegetation and from insects. It would appear that if any coliform segment could be termed a "soil type" it would be IMViC code -+--.

- d It should not be surprising that cultures of typical E. coli are recovered in relatively smaller numbers from sources judged, on the basis of sanitary survey, to be unpolluted. There is no known way to exclude the influence of limited fecal pollution from small animals and birds in such environments.
- e The distribution of coliform types from human sources should be regarded as a representative value for large numbers of sources. Investigations have shown that there can be large differences in the distribution of IMViC types from person to person, or even from an individual.

3 Differentiation between coliforms of fecal vs. nonfecal origin

Table 2 is a summary of findings based on a number of different criteria for differentiating between coliforms of fecal origin and those from other sources.

- a IMViC type ++-- is a measurement of E. coli, Variety I, and appears to give reasonably good correlation between known or highly probable fecal origin and doubtful fecal origin.
- b The combination of IMViC types, ++--, +---, and -+--, gives improved identification of probable fecal origin, and appears also to exclude most of the coliforms not found in excreta of warm-blooded animals in large numbers.
- c While the indole, methyl red, Voges Proskauer, and citrate utilization tests, each used alone, appear to give useful answers when applied only to samples of known pollution from fecal sources, the interpretation is not as clear when applied to coliforms from sources believed to be remote from direct fecal pollution.

Table 1. COLIFORM DISTRIBUTION BY IMViC TYPES AND ELEVATED TEMPERATURE TEST FROM ENVIRONMENTAL AND BIOLOGICAL SOURCES

IMViC type	Vegetation		Insects		Soil				Fecal sources				Poultry	
	No. strains	% of total	No. strains	% of total	Undisturbed		Polluted		Human		Livestock		No. strains	% of total
					No. strains	% of total	No. strains	% of total	No. strains	% of total	No. strains	% of total		
++--	128	10.6	134	12.4	131	5.6	536	80.6	3932	87.2	2237	95.6	1857	97.9
---+	237	19.7	113	10.4	443	18.8	13	2.0	245	5.4	0	<0.1	1	0.1
-+--	23	1.9	0	<0.1	78	3.3	1	0.2	99	2.2	14	0.6	20	1.1
+++--	2	0.2	0	<0.1	7	0.3	0	<0.1	106	2.4	59	2.5	0	<0.1
----+	168	14.0	332	30.6	1131	48.1	87	13.0	50	1.1	1	<0.1	5	0.3
++-+	116	9.6	118	10.9	87	3.7	22	3.3	35	0.8	27	1.2	11	0.6
-+++	32	2.7	28	2.6	181	7.7	5	0.7	21	0.5	0	<0.1	0	<0.1
++++	291	24.2	254	23.4	159	6.8	0	<0.1	6	0.1	0	<0.1	0	<0.1
+-++	88	7.3	46	4.2	67	2.9	0	<0.1	14	0.2	0	<0.1	0	<0.1
----+	87	7.2	42	3.9	4	0.2	1	0.2	2	<0.1	0	<0.1	0	<0.1
-+--	5	0.4	0	<0.1	1	<0.1	0	<0.1	0	<0.1	0	<0.1	0	<0.1
---+	19	1.6	0	<0.1	53	2.3	0	<0.1	0	<0.1	0	<0.1	0	<0.1
+-+	2	0.2	0	<0.1	6	0.3	0	<0.1	0	<0.1	0	<0.1	0	<0.1
----+	5	0.4	8	0.7	0	<0.1	0	<0.1	0	<0.1	0	<0.1	0	<0.1
----	0	<0.1	9	0.8	0	<0.1	0	<0.1	2	<0.1	0	<0.1	2	<0.1
Total	1203		1084		2348		665		4512		2339		1896	
No. EC +	169*		162*		216		551		4349		2309		1765	
% EC +	14.1*		14.9*		9.2		82.9		96.4		98.7		93.0	

*120 of these
were ++--,
15 ---+,
11 ----

*129 of these
were ++--,
27 ---+,
5 ----

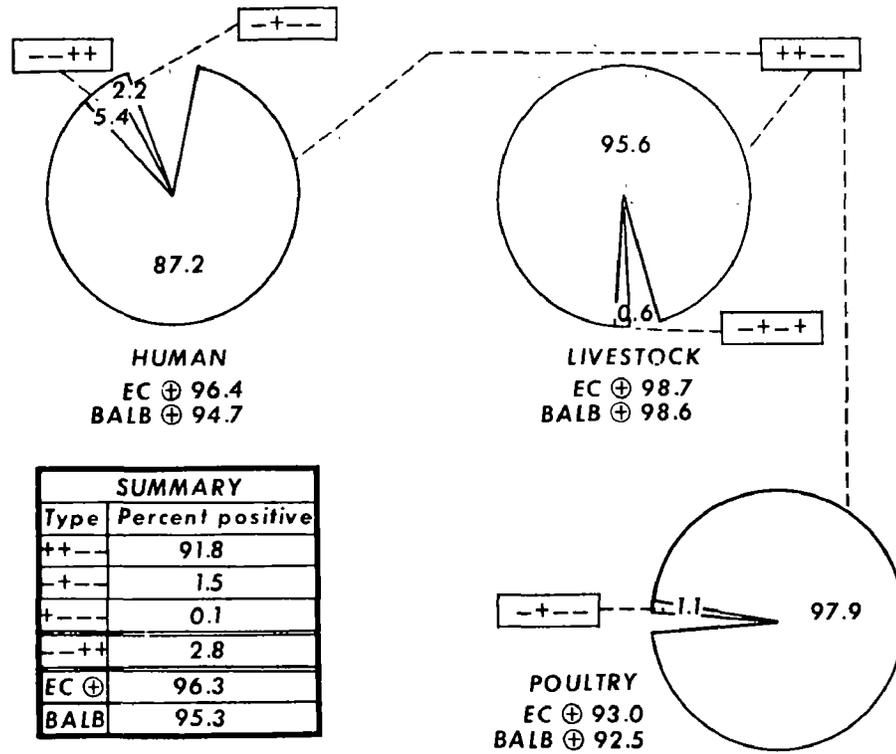


FIGURE 1

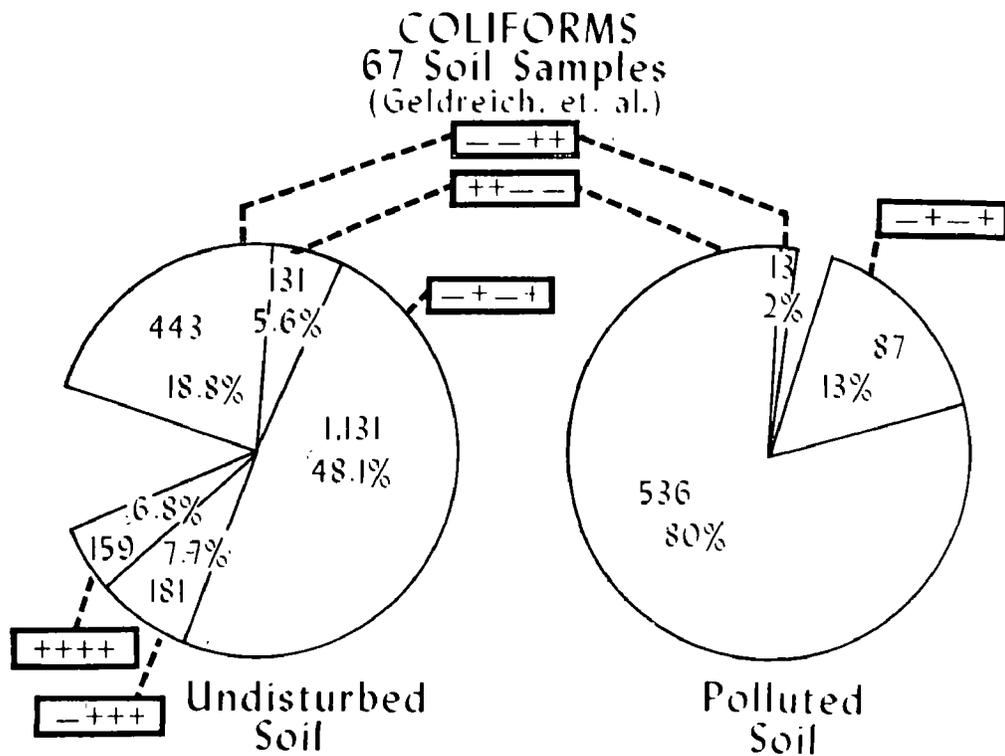


FIGURE 2

Table 2. COMPARISON OF COLIFORM STRAINS ISOLATED FROM WARM-BLOODED ANIMAL FECES, FROM UNPOLLUTED SOILS AND POLLUTED SOILS WITH USE OF THE IMViC REACTIONS AND THE ELEVATED TEMPERATURE TEST IN EC MEDIUM AT 44.5° C ($\pm 0.5^\circ$) (12th ed. 1965: Standard Methods for the Examination of Water and Wastewater)

Test	Warm-blooded animal feces	Soil. Unpolluted	Soil. Polluted	Vegetation	Insects
++--	91.8%	5.6%	80.6%	10.6%	12.4%
+- - -, - - - - and - - - -	93.3%	8.9%	80.7%	12.5%	13.2%
Indole positive	94.0%	19.4%	82.7%	52.5%	52.4%
Methyl red positive	96.9%	75.6%	97.9%	63.6%	79.9%
Voges-Proskauer positive	5.1%	40.7%	97.3%	56.3%	40.6%
Citrate utilizers	3.6%	88.2%	19.2%	85.1%	86.7%
Elevated temperature (EC) positive	96.4%	9.2%	82.9%	14.1%	14.9%
Number of cultures studied	8,747	2,348	665	1,203	1,084

Total Pure Cultures Studied: 14,047

- d The elevated temperature test gives excellent correlation with samples of known or highly probable fecal origin. The presence of smaller, but demonstrable, percentages of such organisms in environmental sources not interpreted as being polluted could be attributed largely to the warm-blooded wildlife in the area, including birds, rodents, and other small mammals.
- e The elevated temperature test yields results equal to those obtained from the total IMViC code. It has marked advantages in speed; ease and simplicity of performance, and yields quantitative results for each water sample. Therefore, it is the official standard method for differentiation coliforms of probable direct fecal origin from those which may have become established in the bacterial flora of the aquatic or terrestrial habitat.

IV EVALUATION OF COLIFORMS AS POLLUTION INDICATORS

A The Coliform Group as a Whole

1 Merits

- a The absence of coliform bacteria is evidence of a bacteriologically safe water.
- b The density of coliforms is roughly proportional to the amount of excretal pollution present.
- c If pathogenic bacteria of intestinal origin are present, coliform bacteria also are present, in much greater numbers.
- d Coliforms are always present in the intestines of humans and other warm-blooded animals, and are eliminated in large numbers in fecal wastes.

- e Coliforms are more persistent in the aquatic environment than are pathogenic bacteria of intestinal origin.
- f Coliforms are generally harmless to humans and can be determined quantitatively by routine laboratory procedures.

2 Limitations

- a Some of the constituents of the coliform group have a wide environmental distribution in addition to their occurrence in the intestines of warm-blooded animals.
- b Some strains of the coliform group may multiply in certain polluted waters ("aftergrowth"), of high nutritive values thereby adding to the difficulty of evaluating a pollution situation in the aquatic environment. Members of the A. aerogenes section of the coliform are commonly involved in this kind of problem.
- c Because of occasional aftergrowth problems, the age of the pollution may be difficult to evaluate under some circumstances.
- d Tests for coliforms are subject to interferences due to other kinds of bacteria. False negative results sometimes occur when species of Pseudomonas are present. False positive results sometimes occur when two or more kinds of non-coliforms produce gas from lactose, when neither can do so alone (synergism).

B The Fecal Coliform Component of the Coliform Group (as determined by elevated temperature test)

1 Merits

- a The majority (over 95% of the coliform bacteria from intestines of warm-blooded animals grow at the elevated temperature.

- b These organisms are of relatively infrequent occurrence except in association with fecal pollution.
- c Survival of the fecal coliform group is shorter in environmental waters than for the coliform group as whole. It follows, then, that high densities of fecal coliforms is indicative of relatively recent pollution.

- d Fecal coliforms generally do not multiply outside the intestines of warm-blooded animals. In certain high-carbohydrate wastes, such as from the sugar beet refineries, exceptions have been noted.
- e In some wastes, notably those from pulp and paper mills, Klebsiella has been found in large numbers utilizing the elevated temperature test. There has been much controversy about whether the occurrence of Klebsiella is due to aftergrowth due to soluble carbohydrates in such wastes. The significance of Klebsiella as an indicator of direct discharge of intestinal wastes thus is under challenge. The issue is still further complicated by questions over whether Klebsiella is in and of itself a pathogenic organism or is potentially pathogenic. This is a serious problem which is the subject of current intensive research by this Agency.

2 Limitations

- a Feces from warm-blooded animals include some (though proportionately low) numbers of coliforms which do not yield a positive fecal coliform test when the elevated temperature test is used as the criterion of differentiation. These organisms are E. coli varieties by present taxonomic classification.
- b There is at present no established and consistent correlation between

ratios of total coliforms/fecal coliforms in interpreting sanitary quality of environmental waters.

In domestic sewage, the fecal coliform density commonly is greater than 90% of the total coliform density. In environmental waters relatively free from recent pollution, the fecal coliform density may range from 10-30% of the total coliforms. There are, however, too many variables relating to water-borne wastes and surface water runoff to permit sweeping generalization on the numerical relationships between fecal- and total coliforms.

- c Studies have been made regarding the survival of fecal coliforms in polluted waters compared with that of enteric pathogenic bacteria. In recent pollution studies, species of *Salmonella* have been found in the presence of 220 fecal coliforms per 100 ml (Spino), and 110 fecal coliforms per 100 ml (Brezenski, Raritan Bay Project).
- 3 The issue of the *Klebsiella* problem described in an earlier paragraph may ultimately be resolved as a merit or as a limitation of the value of the fecal coliform test.

V APPLICATIONS OF COLIFORM TESTS

A Current Status in Official Tests

- 1 The coliform group is designated, in "Standard Methods for the Examination of Water and Wastewater" (13th ed., 1971), through the Completed Test MPN procedure as the official test for bacteriological potability of water.

The Confirmed Test MPN procedure is accepted where it has been demonstrated, through comparative tests, to yield results equivalent to the Completed Test. The membrane filter method also is accepted for examination of waters subject to interstate regulation.

- 2 The 12th edition of Standard Methods introduced a standard test for fecal coliform bacteria. It is emphasized that this is to be used in pollution studies, and does not apply to the evaluation of water for potability. This procedure has been continued in the 13th Edition.

B Applications

- 1 Tests for the coliform group as a whole are used in official tests to comply with interstate drinking water standards, state standards for shellfish waters, and in most, if not all, cases where bacterial standards of water quality have been established for such use as in recreational or bathing waters, water supplies, or industrial supplies. Laboratory personnel should be aware of possible implementation of the fecal coliform group as the official test for recreational and bathing waters.
- 2 The fecal coliform test has application in water quality surveys, as an adjunct to determination of total coliform density. The fecal coliform test is being used increasingly in all water quality surveys.
- 3 It is emphasized that no responsible worker advocates substitution of a fecal coliform test for total coliforms in evaluating drinking water quality.

Part 3. The Fecal Streptococci

I INTRODUCTION

Investigations regarding streptococci progressed from the streptococci of medical concern to those which were distributed in differing environmental conditions which, again, related to the welfare of man. The streptococci were originally reported by Laws and Andrews (1894), and Houston (1899, 1900) considered those streptococci, which we now call "fecal streptococci," as . . . "indicative of dangerous pollution, since they are readily demonstrable in waters recently polluted and seemingly altogether absent from waters above suspicion of contamination.

From their discovery to the present time the fecal streptococci appear characteristic of fecal pollution, being consistently present in both the feces of all warm-blooded animals and in the environment associated with animal discharges. As early as 1910 fecal streptococci were proposed as indicators to the Metropolitan Water Board of London. However, little progress resulted in the United States until improved methods of detection and enumeration appeared after World War II.

Renewed interest in the group as indicators began with the introduction of azide dextrose broth in 1950, (Mallmann & Seligmann, 1950). The method which is in the current edition of Standard Methods appeared soon after. (Litsky, et al. 1955).

With the advent of improved methods for detection and enumeration of fecal streptococci, significant body of technical literature has appeared.

This outline will consider the findings of various investigators regarding the fecal streptococci and the significance of discharges of these organisms into the aquatic environment.

II FECAL MATERIALS

A Definition

The terms "enterococci," "fecal streptococci," "Group D streptococci," "*Streptococcus fecalis*," and even "streptococci" have been used in a loose and interchangeable manner to indicate the streptococci present in the enteric tract of warm-blooded animals or of the fresh fecal material excreted therefrom.

Enterococci are characterized by specific taxonomic biochemistry. Serological procedures differentiate the Group D streptococci from the various groups. Although they overlap, the three groups, fecal streptococcus, enterococcus, and Group D streptococcus, are not synonymous. Because our emphasis is on indicators of unsanitary origin, fecal streptococcus is the more appropriate term and will include the enterococcus as well as other groups.

A rigid definition of the fecal streptococcus group is not possible with our present knowledge. The British Ministry of Health (1956) defines the organisms as "Gram-positive" cocci, generally occurring in pairs or short chains, growing in the presence of bile salt, usually capable of development at 45° C, producing acid but not gas in mannitol and lactose, failing to attack raffinose, failing to reduce nitrate to nitrite, producing acid in litmus milk and precipitating the casein in the form of a loose, but solid curd, and exhibiting a greater resistance to heat, to alkaline conditions and to high concentrations of salt than most vegetative bacteria." However, it is pointed out that "streptococci departing in one or more particulars from the type species cannot be disregarded in water."

For the purposes of this outline, and in line with the consensus of most water microbiologists in this country, the definition of the fecal streptococci is:

. . . "The group composed of Group D species consistently present in significant numbers in fresh fecal excreta of warm-blooded animals, which includes all of the enterococcus group in addition to other groups of streptococci."

B Species Isolated

1 Findings

a Human feces

Examination of human fecal specimens yields a high percentage of the enterococcus group and usually demonstration of the S. salivarius which is generally considered a member of the human throat flora and to be surviving in human fecal materials rather than actively multiplying in the enteric tract. Also present would be a small percentage of variants or biotypes of the enterococcus group.

b Nonhuman Feces

1) Fecal material which are from nonhuman or not from fowl will yield high percentages of the S. bovis and/or S. equinus organisms with a concomitantly reduced percentage of the enterococcus group.

2) Fowl excreta

Excrement from fowl characteristically yields a large percentage of enterococcal biotypes as well as a significant percentage of enterococcus group.

2 Significance

Species associations with particular animal hosts is an established fact and leads to the important laboratory technique of partition counting of colonies from the membrane filter or agar pour plates in order to establish or confirm the source of excretal pollution in certain aquatic investigations.

It is important to realize that a suitable medium is necessary in order to allow all of the streptococci which we consider to be fecal streptococci

to grow in order to give credence to the derived opinions. Use of liquid growth media into which direct inoculations from the sample are made have not proven to be successful for partition counting due to the differing growth rates of the various species of streptococci altering the original percentage relationships. Due to the limited survival capabilities of some of the fecal streptococci it is necessary to sample fresh fecal material or water samples in close proximity to the pollution source especially when multiple sources are contributing to a reach of water. Also the pH range must be within the range of 4.0-9.0.

III FECAL STREPTOCOCCI IN THE AQUATIC ENVIRONMENT

A General

From the foregoing it appears that the preponderant human fecal streptococci are composed of the enterococcus group and, as this is the case, several media are presently available which will detect only the enterococcal group will be suitable for use with aquatic samples which are known to be contaminated or potentially contaminated with purely domestic (human) wastes. On the other hand, when it is known or suspected that other-than-human wastes have potential egress to the aquatic environment under investigation, it is necessary to utilize those media which are capable of quantitating the whole of the fecal streptococci group.

B Stormwaters and Combined Sewers

1 General

Storm sewers are a series of pipes and conduits which receive surface runoffs from the action of rainstorms and do not include sewage which are borne by a system of sanitary sewers. Combined sewers receive both the storm runoff and the water-borne wastes of the sanitary system.

Table 1

Both storm water and combined sewer flows have been found to usually contain large quantities of fecal streptococci in numbers which generally are larger than those of the fecal coliform indicator organisms.

DISTRIBUTION OF FECAL STREPTOCOCCI IN DOMESTIC SEWAGES AND STORMWATER RUNOFFS

Water Source	Fecal Streptococci	Ratio FC/FS
	per 100 ml median values	
<u>Domestic Sewage</u>		
Preston, ID	64,000	5.3
Fargo, ND	290,000	4.5
Moorehead, MN	330,000	4.9
Cincinnati, OH	2,470,000	4.4
Lawrence, MA	4,500,000	4.0
Monroe, MI	700,000	27.9
Denver, CO	2,900,000	16.9
<u>Stormwater</u>		
Business District	51,000	0.26
Residential	150,000	0.04
Rural	58,000	0.05

2 Bacteriological Findings

Table 1 represents, in a modified form, some of the findings of Geldreich and Kenner (1969) with respect to the densities of fecal streptococci when considering Domestic sewage in contrast to Stormwaters:

The Ratio FC/FS is that of the Fecal coliform and Fecal streptococci and it will be noted that in each case, when considering the Domestic Sewage, it is 4.0 or greater while it is less than 0.7 for stormwaters. The use of this ratio is useful to identify the source of pollution as

Table 2. ESTIMATED PER CAPITA CONTRIBUTION OF INDICATOR MICROORGANISMS. FROM SOME ANIMALS*

Animals	Avg wt of Feces/24 hr, wet wt, g	Average indicator density per gram of feces		Average contribution per capita per 24 hr		Ratio FC/FS
		Fecal coliform, million	Fecal streptococci, million	Fecal coliform, million	Fecal streptococci, million	
Man	150	13.0	3.0	2,000	450	4.4
Duck	336	33.0	54.0	11,000	18,000	0.6
Sheep	1,130	16.0	38.0	18,000	43,000	0.4
Chicken	182	1.3	3.4	240	620	0.4
Cow	23,600	0.23	1.3	5,400	31,000	0.2
Turkey	448	0.29	2.8	130	1,300	0.1
Pig	2,700	3.3	84.0	8,900	230,000	0.04

*Publication WP-20-3, P. 102

being human or nonhuman warm-blooded animal polluted. When the ratio is greater than 4.0 it is considered to be human waste contaminated while a ratio of less than 0.7 is considered to be nonhuman. It is evident that the storm-waters have been primarily polluted by excreta of rats and other rodents and possibly domestic and/or farm animals.

Species differences are the main cause of different fecal coliform-fecal streptococci ratios. Table 2 compares fecal streptococcus and fecal coliform counts for different species. Even though individuals vary widely, masses of individuals in a species have characteristic proportion of indicators.

C Surface Waters

In general, the occurrence of fecal streptococci indicates fecal pollution and its absence indicates that little or no warm-blooded fecal contribution. In studies of remote surface waters the fecal streptococci are infrequently isolated and occurrences of small numbers can be attributed to wild life and/or snow melts and resultant drainage flows.

Various examples of fecal streptococcal occurrences are shown in Table 3 in relation to surface waters of widely varying quality. (Geldreich and Kenner 1969).

IV FECAL STREPTOCOCCI: ADVANTAGES AND LIMITATIONS

A General

Serious studies concerning the streptococci were instituted when it became apparent that they were the agents responsible or suspected for a wide variety of human diseases. Natural priority then focused itself to the taxonomy of these organisms and this study is still causing consternation as more and more microbiological techniques have been brought to bear on these questions. The sanitary microbiologist is concerned with those streptococci which inhabit the enteric tract of warm-blooded animals, their detection, and utilization in developing a criterium for water quality standards.

Table 3

Water Source	INDICATOR ORGANISMS IN SURFACE WATERS	
	Densities/100 ml	
	Fecal coliform	Fecal streptococci
<u>Prairie Watersheds</u>		
Cherry Creek, WY	90	83
Saline River, KS	95	180
Cub River, ID	110	160
Clear Creek, CO	170	110
<u>Recreational Waters</u>		
Lake Mead	2	444
Lake Moovalaya	9	170
Colorado River	4	256
Whitman River	32	88
Merrimack River	100	96
<u>Public Water Intakes</u>		
Missouri River (1959)		
Mile 470.5	11,500	39,500
Mile 434.5	22,000	79,000
Mile 408.8	14,000	59,000

Kabler (1962) discussed the slow acceptance of the fecal streptococci as indicators of pollution resulting from:

- 1 Multiplicity and difficulty of laboratory procedures
- 2 Poor agreement between methods of quantitative enumeration
- 3 Lack of systematic studies of
 - a sources
 - b survival, and
 - c interpretations, and
- 4 Undue attention to the S. faecalis group.

Increased attention to the fecal streptococci, especially during the last decade, have clarified many of the earlier cloudy issues and have elevated the stature of these organisms as indicators of pollution. Court precedents establishing legal status and recommendations of various technical advisory boards have placed the fecal coliform group in a position of primacy in many water quality applications. The fecal streptococci have evolved from a position of a theoretically useful indicator to one which was ancillary to the coliforms to one which was useful when discrepancies or questions evolved as to the validity of the coliform data to one where an equality status was achieved in certain applications. In the future it is anticipated that, for certain applications, the fecal streptococci will achieve a position of primacy for useful data, and, as indicated by Litsky (1955) "be taken out of the realm of step-children and given their legitimate place in the field of sanitary bacteriology as indicators of sewage pollution."

B Advantages and Limitations

1 Survival

In general, the fecal streptococci have been observed to have a more limited survival time in the aquatic environment when compared to the coliform group. They are rivaled in this respect only by the fecal coliforms. Except for cases of persistence in waters of high electrolytic content, as may be common to irrigation waters, the fecal streptococci have not been observed to multiply in polluted waters as may sometimes be observed for some of the coliforms. Fecal streptococci usually require a greater abundance of nutrients for survival as compared to the coliforms and the coliforms are more dependent upon the oxygen tension in the waterbody. In a number of situations it was concluded that the fecal streptococci reached an extinction point more rapidly in warmer waters while the reverse was true in the colder situations as the coliforms now were totally eliminated sooner.

2 Resistance to Disinfection

In artificial pools the source of contamination by the bathers is usually limited to throat and skin flora and thus increasing attention has been paid to indicators other than those traditionally from the enteric tract. Thus, one of the organisms considered to be a fecal streptococci, namely, S. salivarius, can be a more reliable indicator when detected along with the other fecal streptococci especially since studies have confirmed the greater resistance of the fecal streptococci to chlorination. This greater resistance to chlorination, when compared to the fecal coliforms, is important since the dieoff curve differences are insignificant when the curves of the fecal coliforms are compared to various Gram negative pathogenic bacteria which reduces their effectiveness as indicators.

3 Ubiquitous Strains

Among the fecal streptococcus are two organisms, one a biotype and the other a variety of the S. faecalis, which, being ubiquitous (omnipresent) have limited sanitary significance. The biotype, or atypical, S. faecalis is characterized by its ability to hydrolyze starch while the varietal form, liquefaciens, is nonbeta haemolytic and capable of liquefying gelatin. Quantitation of these organisms in anomalous conditions is due to their capability of survival in soil or high electrolytic waters and in waters with a temperature of less than 12 Degrees C.

Samples have been encountered which have been devoid of fecal coliforms and yet contain a substantial number of "fecal streptococci" of which these ubiquitous strains constitute the majority or all of the isolations when analyzed biochemically.

V STANDARDS AND CRITERIA

Acceptance and utilization of Total Coliform criteria, which must now be considered a pioneering effort, has largely been supplanted in concept and in fact by the fecal coliforms in establishing standards for recreational waters.

The first significant approach to the utilization of the fecal streptococci as a criterium for recreational water standards occurred in 1966 when a technical committee recommended the utilization of the fecal streptococci with the total coliforms as criteria for standards pertaining to the Calumet River and lower Lake Michigan waters. Several sets of criteria were established to fit the intended uses for this area. The use of the fecal streptococci as a criterium is indicated to be tentative pending the accumulation of existing densities and could be modified in future standards.

With the existing state-of-the-art knowledge of the presence of the fecal streptococci in waters containing low numbers of fecal coliforms it is difficult to establish a specific fecal streptococcus density limit of below 100 organisms/100 ml when used alone or in conjunction with the total coliforms.

Part 4. Other Bacterial Indicators of Pollution

I TOTAL BACTERIAL COUNTS

A Historical

- 1 The early studies of Robert Koch led him to develop tentative standards of water quality based on a limitation of not more than 100 bacterial colonies per ml on a gelatin plating medium incubated 3 days at 20° C.
- 2 Later developments led to inoculation of samples on duplicate plating media, with one set incubated at 37° C and the other at 20° C.
 - a Results were used to develop a ratio between the 37° C counts and the 20° C counts.
 - b Waters having a predominant count at 37° C were regarded as being of probable sanitary significance, while those giving predominant counts at 20° C were considered to be of probable soil origin, or natural inhabitants of the water being examined.

B Groups Tested

There is no such thing as "total" bacterial count in terms of a laboratory determination.

- 1 Direct microscopic counts do not differentiate between living and dead cells.
- 2 Plate counting methods enumerate only the bacteria which are capable of using the culture medium provided, under the temperature and other growth conditions used as a standard procedure. No one culture medium and set of growth conditions can provide, simultaneously, an acceptable environment for all the heterogeneous, often conflicting, requirements of the total range of bacteria which may be recovered from waters.

C Utilization of Total Counts

- 1 Total bacterial counts, using plating methods, are useful for:
 - a Detection of changes in the bacterial composition of a water source
 - b Process control procedures in treatment plant operations
 - c Determination of sanitary conditions in plant equipment or distributional systems
- 2 Serious limitations in total bacterial counts exist because:
 - a No information is given regarding possible or probable fecal origin of bacterial changes. Large numbers of bacteria can sometimes be cultivated from waters known to be free of fecal pollution.
 - b No information of any kind is given about the species of bacteria cultivated.
 - c There is no differentiation between harmless or potentially dangerous forms.
- 3 Status of total counts
 - a There is no total bacterial count standard for any of the following:

Interstate Quarantine Drinking Water Standards

PHS regulations for water potability (as shown in "Standard Methods" Public Health Service Drinking Water Standards of 1962.)
 - b The most widely used current application of total bacterial counts in water bacteriology today is in

water treatment plants, where some workers use standard plate counts for process control and for determination of the bacterial quality of distribution systems and equipment.

- c Total bacterial counts are not used in PHS water quality studies, though extensively used until the 1940's.

B Spore-Forming Bacteria (Clostridium perfringens, or C. welchii)

1 Distribution

This is one of the most widely distributed species of bacteria. It is regularly present in the intestinal tract of warm-blooded animals.

2 Nature of organism

C. perfringens is a Gram-positive, spore-forming rod. The spores cause a distinct swelling of the cell when formed. The organism is extremely active in fermentation of carbohydrates, and produces the well-known "stormy fermentation" of milk.

3 Status

The organism, when present, indicates that pollution has occurred at some time. However, because of the extremely extended viability of the spores, it is impossible to obtain even an approximation of the recency of pollution based only on the presence of C. perfringens.

The presence of the organism does not necessarily indicate an unsafe water.

C Tests for Pathogenic Bacteria of Intestinal Origin

- 1 Groups considered include Salmonella sp, Shigella sp, Vibrio comma, Mycobacterium sp, Pasteurella sp, Leptospira sp, and others.

2 Merits of direct tests

Demonstration of any pathogenic species would demonstrate an unsatisfactory water quality, hazardous to persons consuming or coming into contact with that water.

3 Limitations

- a There is no available routine procedure for detection of the full range of pathogenic bacteria cited above.
- b Quantitative methods are not available for routine application to any of the above.
- c The intermittent release of these pathogens makes it impossible to regard water as safe, even in the absence of pathogens.
- d After detection, the public already would have been exposed to the organism; thus, there is no built-in margin of safety, as exists with tests for the coliform group.

4 Applications

- a In tracing the source of pathogenic bacteria in epidemiological investigations
- b In special research projects
- c In water quality studies concerned with enforcement actions against pollution, increasing attention is being given to the demonstration of enteric pathogenic bacteria in the presence of the bacterial indicators of pollution.

D Miscellaneous Indicators

It is beyond this discussion to explore the total range of microbiological indicators of pollution that have been proposed and

investigated to some extent. Mention can be made, however, of consideration of tests for the following.

- 1 Bacteriophages specific for any of a number of kinds of bacteria
- 2 Serological procedures for detection of coliforms and other indicators, a certain amount of recent attention has been given to applications of fluorescent antibodies in such tests
- 3 Tests for Pseudomonas aeruginosa
- 4 Tests for viruses, which may persist in waters even longer than members of the coliform group.

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Descriptors: Coliforms, Escherichia coli, Fecal Coliforms, Fecal Streptococci, Indicator Bacteria, Microbiology, Sewage Bacteria, Water Pollution

BACTERIA AND THEIR SURVIVAL IN THE AQUATIC ENVIRONMENT

I INTRODUCTION

The bacteriology of the aquatic environment may be considered from two viewpoints, the first dealing with natural history and the second concerned with man's welfare. The first consideration concerns itself with the naturally occurring or true water bacteria while the second considers the bacteria commonly found but not indigenous to the aquatic environment. This outline will primarily deal with the second consideration and place emphasis upon the bacterial indicators of pollution.

II BACTERIA IN THE AQUATIC ENVIRONMENT

A Bacterial Content of Various Waters

1 Precipitation

The part of the hydrologic cycle which ultimately supplies us with freshwater is rain, snow, hail, sleet or dew. These sources are by no means free from bacterial contamination and there is a direct relationship between the dust content and the total bacterial

count. Miquel (1886) observed counts of 4.3 bacteria per milliliter in the country and 19 bacteria per milliliter in the city of Paris. Tissandier later observed that the dust in the air amounted to 4 mg/cu m. in the open country and 23 mg/cu m. in Paris. Pollution indicator counts for rainfall are generally less than 1/100 milliliters.

The bacterial content of natural ice is usually very low unless it is formed from heavily polluted water trapped on the surface of formed ice. Instances of disease caused by polluted ice are a matter of record. Samples of ice from Quebec rivers have given the results shown in Table 1 and these are typical of those found in the average marketed product.

Table 1. BACTERIA IN NATURAL ICE

Ice			Ice		
Sample	Bacteria/ml	Coliforms/100 ml	Sample	Bacteria/ml	Coliforms/100 ml
1	1	0	9	16	0
2	2	0	10	16	0
3	4	0	11	16	0
4	4	1	12	18	0
5	10	1	13	18	0
6	14	0	14	19	0
7	14	0	15	19	0
8	16	0	16	26	0

2 Land runoff

Bacteriological numbers increase tremendously as the waters of precipitation contact the earth's surface and flow over the varied topography. Total counts from rivulets in roadways or ploughed land can exceed several hundred thousand per milliliter. Availability of nutrients and attendant physical and chemical conditions provide a complex "medium" in which bacterial counts can vary in an unpredictable manner. An example of these complexities are provided by

studies of stormwater pollution. In an abridged form Table 2 shows differences between pollution indicators from sewer overflows from the cities of Detroit and Ann Arbor during monthly monitoring. Significant differences between the two cities can be attributed mainly to the differences in populations, the type of systems (Detroit: Combined; Ann Arbor: Separate), amounts of rainfall, and the species of warm-blooded animal wastes predominating in the sewage contribution (human vs non-human).

Table 2. DETROIT AND ANN ARBOR OVERFLOWS

Month	Overflows Successfully Monitored		Analysis	Range of Geometric Means Count/100 ml	
	Ann Arbor	Detroit		Ann Arbor	Detroit
March	0	4	Tot. Col.	-	1,100,000- 8,500,000
			Fec. Col.	-	-
			Fec. Strep.	-	-
April	3	11	Tot. Col.	120,000- 350,000	570,000- 8,200,000
			Fec. Col.	7,400- 17,000	190,000- 2,600,000
			Fec. Strep.	12,000- 31,000	-
May	2	7	Tot. Col.	139,000- 880,000	1,900,000- 24,000,000
			Fec. Col.	29,000- 73,000	410,000- 3,600,000
			Fec. Strep.	73,000- 320,000	280,000- 1,500,000
June	5	9	Tot. Col.	190,000-10,000,000	1,300,000- 41,000,000
			Fec. Col.	24,000- 130,000	470,000- 8,700,000
			Fec. Strep.	42,000- 330,000	660,000- 980,000
July	5	11	Tot. Col.	390,000-15,000,000	12,000,000- 45,000,000
			Fec. Col.	60,000- 560,000	1,900,000- 10,500,000
			Fec. Strep.	180,000- 670,000	180,000- 490,000
August	7	10	Tot. Col.	220,000-34,000,000	12,000,000- 45,000,000
			Fec. Col.	160,000- 750,000	1,400,000- 16,000,000
			Fec. Strep.	120,000- 480,000	410,000- 790,000
Sept.	0	11	Tot. Col.	-	3,200,000-110,000,000
			Fec. Col.	-	3,200,000- 20,000,000
			Fec. Strep.	-	200,000- 900,000
Oct.	0	2	Tot. Col.	-	900,000- 85,000,000
			Fec. Col.	-	900,000- 5,300,000
			Fec. Strep.	-	1,100,000

In addition to the differences previously observed from the overflows of two large communities it is common to observe seasonal variations. Table 3 indicates the seasonal variations in stormwater. It is well at this point to compare these pollution indicator counts with those obtained from domestic sewage. The following typical median values have been noted by the same author:¹

<u>Source</u>	<u>Total coliform</u>	<u>Fecal coliform</u>	<u>Fecal streptococci</u>
Domestic Sewage	33,000,000	10,900,000	2,470,000

Table 3. Seasonal Variations (Median Values) for Bacterial Discharges in Stormwater and Rainwater from Suburban Areas, Cincinnati, Ohio, and in Agricultural Land Drainage, Coshocton, Ohio (Count/100 ml)

Source	Date	Total Samples	Season	Total Coliform	Fecal Coliform	Fecal Streptococcus
Wooded hillside	Feb. 62 to Dec. 64	278	Spring	2,400	190	940
			Summer	79,000	1,900	27,000
			Autumn	180,000	430	13,000
			Winter	260	20	950
Street gutters	Jan. 62 to Jan. 64	177	Spring	1,400	230	3,100
			Summer	90,000	6,400	150,000
			Autumn	290,000	47,000	140,000
			Winter	1,600	50	2,200
Business district	Apr. 62 to Jul. 66	294	Spring	22,000	2,500	13,000
			Summer	172,000	13,000	51,000
			Autumn	190,000	40,000	56,000
			Winter	46,000	4,300	28,000
Rural	Jan. 63 to Aug. 64	94	Spring	4,400	55	3,600
			Summer	29,000	2,700	58,000
			Autumn	18,000	210	2,100
			Winter	58,000	9,000	790,000
Rainwater	Jun. 65 to Feb. 67	49	Spring	<1.0	<0.3	<1.0
			Summer	<1.0	<0.7	<1.0
			Autumn	<0.4	<0.4	<0.4
			Winter	<0.8	<0.5	<0.5

¹ References for each outline table and figure are provided at end of outline.

3 Surface waters

Rivers in inhabited regions contain several hundreds to thousands of total bacteria per milliliter. These bacterial contents are likely to show sudden fluctuations due to a variety of factors such as stream flow and rainfall. An example of this striking fluctuation was observed by Gage (1906) where the bacterial content of the Merrimac was highest when the stream was lowest and, therefore, when its sewage content was less subject to dilution. (Table 4)

When a stream of fair quality is compared to a highly polluted one the effects of surface contamination due to rainfall are informative. A stream study by Kiskalt (Table 5) compares the Lahn (fair quality) with the Wieseck (highly polluted). In the Lahn, as is a general rule with streams of fair and high quality, fluctuations are most pronounced as runoff contamination enters the stream. On the other hand, the Wieseck shows less pronounced fluctuations since the constant influx of sewage damps out the surface runoff contributions.

Table 4. MERRIMAC RIVER

flow of stream (cu ft/sec/sq mi of watershed)	Bacteria/ml		<u>B. coli</u> */ml	
	Canal	Intake	Canal	Intake
less than 1	7,500	10,800	66	88
1 - 2	6,800	6,200	50	51
2 - 4	3,600	5,600	29	39
over 4	3,400	3,100	16	29

*The Bacillus coli of the earlier water bacteriologists corresponds approximately to our present species Escherichia coli.

Table 5
Monthly Variations of Bacteria in a Normal and in a Polluted Stream
bacteria per milliliter (1904-1905)

	<u>Lahn</u>	<u>Wieseck</u>
July	318	104,000
July	132	156,000
Aug	840	98,400
Oct*	1,235	28,400
Oct*	420	58,000
Nov	2,340	39,200
Nov*	1,740	52,000
Dec*	780	28,600
Dec*	1,220	21,200
Jan*	3,668	29,920
Feb*	5,380	11,900
Mar*	1,210	8,250
Apr*	4,925	5,910
May	570	14,800
June	686	50,180

*Rain or high water due to previous thaw.

The total bacterial content of large reservoirs, lakes, or ponds which do not have sewage or nutrient contamination ordinarily have only a few hundred per milliliter and in many cases are less than one hundred. The 20^o counts and the coliform counts of Western Canadian Lakes were examined by the Quebec Ministry of Health in the 1930's and these are tabulated in Table 6. Note that the total bacterial count is per milliliter while the coliform count is per 100 milliliters.

Table 6. Bacteria in Eastern Canadian Lakes

Lake	Bacteria per ml	Coliforms per 100 ml
1	9	0
2	13	0
3	31	2
4	39	0
5	46	0
6	55	15
7	80	8
8	110	3
9	110	6
10	120	0
11	130	2
12	200	9
13	240	27
14	300	7
15	350	5
16	500	7
17	550	1
18	650	0
19	650	11
20	850	2

4 Estuarine and marine waters

It has been estimated that the number of viable bacteria occurring in the sea range from 10⁰ to 10⁶ cells/ml. In general the amount of bacterial life decreases as we proceed outward from the shore and downward from the surface. Table 7 indicates this trend as one samples to a greater depth.

Table 7. Bacteria in the Atlantic Ocean
Bacteria per milliliter
(Otto and Neumann, 1904)

Nearest Land	Depth in Meters			
	5	50	100	200
Canary Islands	120	76	20	1
Cape Verde Islands	58	16	64	6
St. Paul Island	20	480	54	4
Pernambuco	48	168	83	14

In the estuarine and marine environment, as well as in fresh waters, the ability and/or disposition of bacteria to migrate to the sediment layers has been well recognized. Table 8 indicated the extremely high counts which may be found in these environments as well as their biological capabilities for life in the sea.

This tendency to sediment can be seen in Figure 1 which is a profile of densities of coliform bacteria in the Hyperion outfall (city of Los Angeles, California). It is important to note the coliform "sag" in the left of the figure and the current flow is from right to left. The fresh water effluent has a tendency to rise in the saline waters of the bay which further emphasizes this "sedimentation" sag.

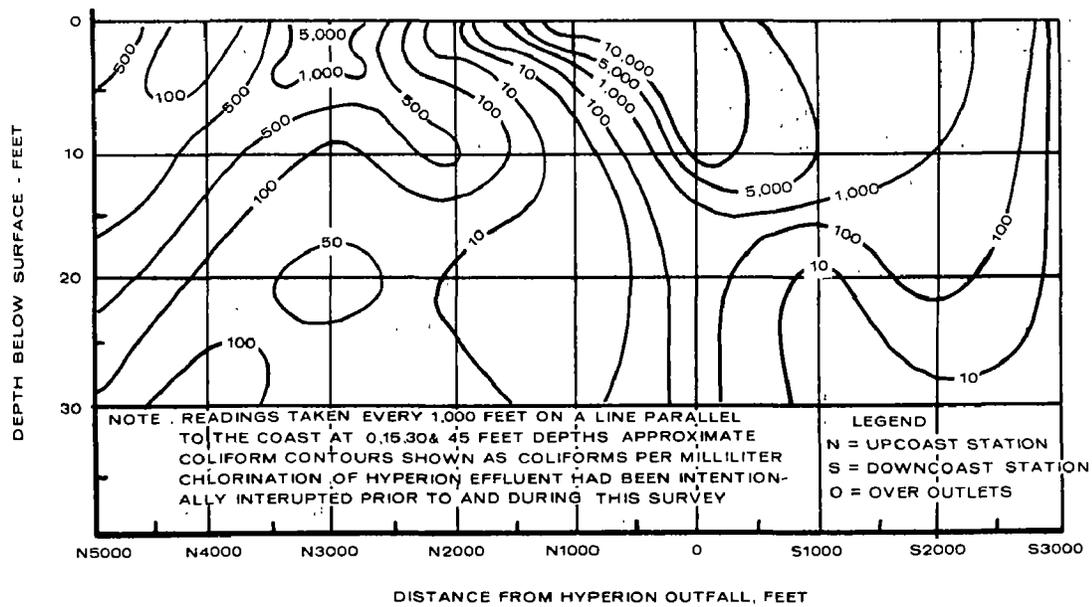


FIGURE 1 PROFILE OF DENSITIES OF COLIFORM ORGANISMS ABOUT ONE MILE OFF SHORE, VICINITY OF HYPERION OUTFALL ON JAN 12, 1956

Table 8

Sediment Sample	8160	8330	9309
Station Location	32° 51.2'N 117° 28.3'W	33° 25.9'N 118° 06.5'W	33° 44.2'N 118° 46.1'W
Depth of overlying water (meters)	780	505	1,322
Bacteria per gram of sediment (wet basis)			
Total aerobes (plate count)	930,000	31,000,000	8,800,000
Total anaerobes (oval-tube count)	190,000	2,600,000	1,070,000
Ammonification (peptone → NH ₄)	100,000	1,000,000	1,000,000
Ammonification (nutrose → NH ₄)	10,000	1,000,000	100,000
Urea fermentation (urea → NH ₄)	100	+	1,000
Proteolysis (gelatin liquefaction)	100,000	10,000,000	1,000,000
Proteolysis (peptone → H ₂ S)	10,000	1,000,000	100,000
Denitrification (NO ₃ → N ₂)	100	10,000	10,000
Nitrate reduction (NO ₃ → NO ₂)	100,000	10,000,000	10,000
Nitrogen fixation	0	0	0
Nitrification (NH ₄ → NO ₂)	0	0	0
Sulfate reduction (SO ₄ → H ₂ S)	1,000	1,000	10,000
Dextrose fermentation	10,000	100,000	1,000
Xylose fermentation	10,000	+	10,000
Starch hydrolysis	10,000	100,000	10,000
Cellulose decomposition	1,000	+	1,000
Fat hydrolysis (lipoclastic)	1,000	+	+
Chitin digestion	100	+	+

From C. E. Zobell, J. Sedimentary Petrology, 8:10, 1938

B Factors Influencing Reduction of Bacteria Density

The forces which tend to decrease the numbers in stored samples are important. Some of these forces have been extensively studied and much is known of their actions. Others are more obscure and as little as mere postulations have been advanced for their actions of presence. The following discussion will deal with some of these forces and, it will be evident from the individual topic, that some of these will only be found in a particular environment as, for instance, salinity in ocean waters.

1 Natural self-purification

Natural self-purification can be described as the result of the combined effects of all of the forces which tend to diminish the numbers of bacteria in a given time interval. These combined influence include physical, chemical, and biological factors. The following two tables (Table 9 and 10) show the bacterial reductions which occur with the passage of time. In the first case the reductions occur as one obtains counts at successive points away from the highly polluted mouth of the River Brathay. In the second case successive passages to three reservoirs from a polluted river show a marked decrease in bacteria.

Table 9. Numbers of Bacteria at Various Distances from the Mouth of the River Brathay, Windermere, at Depth of 1 Meter
(Taylor, 1940)

Distance from River Mouth (meters)	Bacteria* per ml	Distance from River Mouth (meters)	Bacteria* per ml
22 July, 1938		2 November, 1939	
0	14,300	River	20,200
67	15,260	0	18,500
133	12,120	191	620
536	4,720	351	1,060
757	4,400	542	960
30 November, 1938		670	960
		861	650
2,800	4,300	990	700
3,200	3,900	1,212	640
4,000	4,150	1,467	570
4,800	4,180	1,690	550
5,300	3,420	4,180	580
6,000	3,260		

*On special agar, 20° C, 15 days

Table 10. Reduction of Bacteria in Washington Reservoirs
Bacteria per milliliter, Monthly Average, 1907

	Potomac River	Dalecarlia Reservoir	Georgetown Reservoir	Washington City Reservoir
January	4,400	2,400	2,200	950
February	1,000	950	1,000	750
March	11,500	8,300	7,200	3,600
April	3,700	2,100	1,400	475
May	750	350	325	130
June	2,300	950	600	100
July	2,700	600	350	160
August	3,000	275	425	80
September	6,200	...	1,900	230

These same forces are naturally in effect when the bacterial indicators of pollution are considered. Figure 2 indicates the curves for coliform reduction in streams in winter. This curve is flatter than that of the summer reductions indicating a greater survival at lower temperatures, which, in general, can be said to be the case for the majority of bacteria.

Figure 3 indicates the survival, or persistence, of these enteric bacteria as compared to the "dotted line" of the pathogen. It is important to note that the apparent longer persistence of the pathogen over two of the three standard indicators does not imply that a number of them will be found after complete dieoff of the indicators - in other words, the percent survival is based upon vastly differing numbers of original inoculum of bacteria.

2 Predation

The predators of bacterial populations belong to the group of microphagic organisms which draw part of their nutrient from ingested microbes which themselves have transformed organic matter. Numerous in vitro studies have confirmed the voracity of these predators in rapidly reducing microbial populations. At the present time investigators vary in their consensus of the importance of predation in reducing the bacterial populations and claims vary from negligible effect to a first order importance.

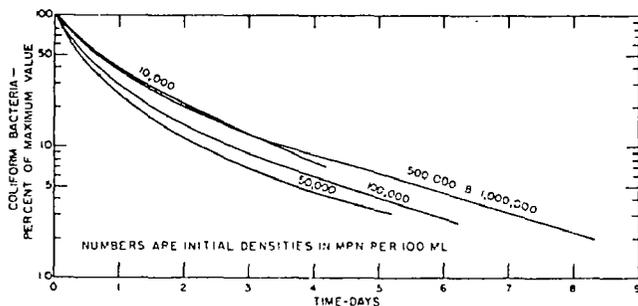


Figure 2. Decrease in coliform bacteria in streams in winter

A study of pollution indicators and their survival when compared to a pathogen, in this case Salmonella typhimurium, was done by Geldreich, et al, utilizing stormwater stored at different temperatures.

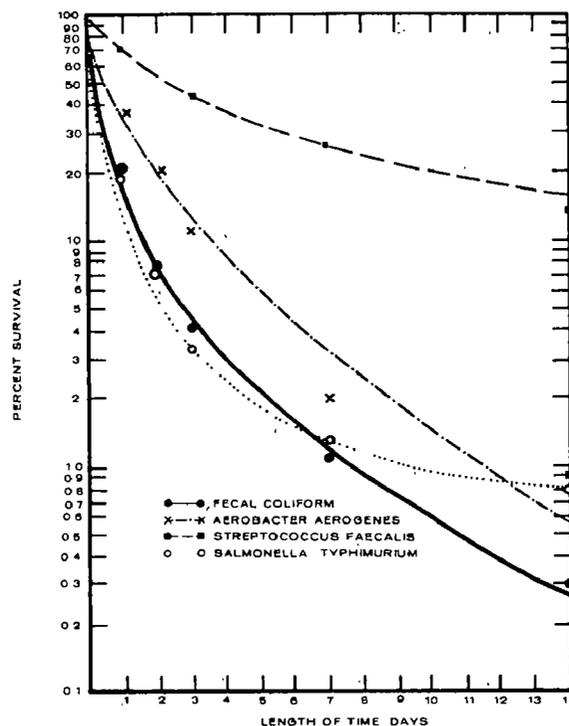


FIGURE 3 PERSISTENCE OF SELECTED ENTERIC BACTERIA IN
STORMWATER STORED AT 20° C

3 Sunlight

Although considerable information regarding the bactericidal properties of sunlight has been generated relatively little has been done regarding this property in relation to water. The major consensus is that it is a contributory factor in bacterial destruction and of secondary importance. It can be readily seen that in waterbodies of shallow depth it assumes greater importance than if deep ocean depths are considered. Figure 4 illustrates this thought as it can be seen that the coliform count diminishes at a greater rate for the container closer to the surface (0.18 meter) than for the seawater sample at the greater depth (4 meters).

4 Adsorption and sedimentation

Although a few investigators have discounted the effects of adsorption and sedimentation upon removal of bacteria from the aqueous environment the majority of studies have shown varying degrees of removal and deposition of bacteria to the bottom stratum. Bacterial adsorption is found to have a direct relationship to particle size and physiochemical nature of the particle. Marine muds have been found to be highly adsorptive while sand particles are only slightly adsorptive. Table 8, previously presented, indicates the high counts which can be found in the sediments.

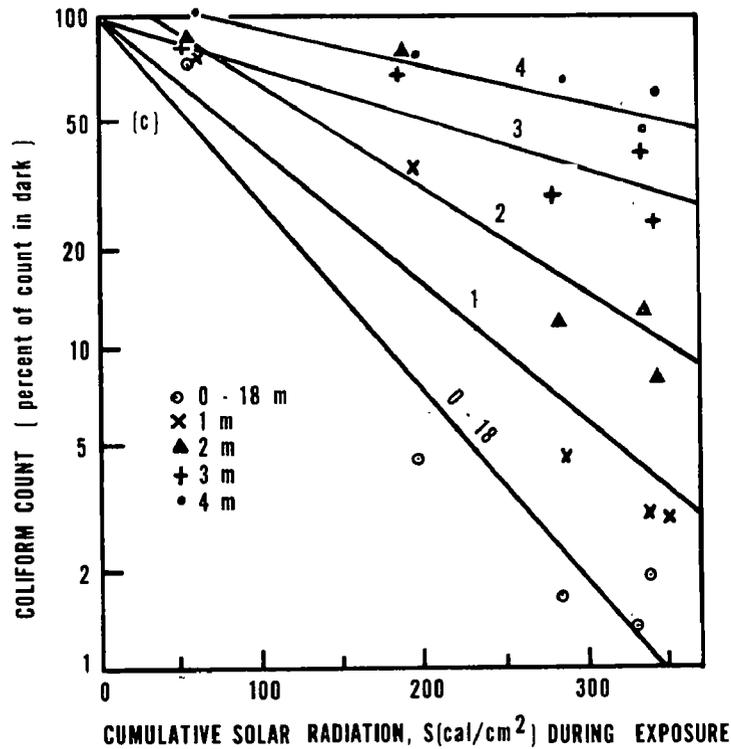


FIGURE 4

5 Antibiotics and toxins

These factors are ordinarily considered as agents of the marine environment but one cannot restrict them from the fresh water environment since some common bacteria have this property. Numerous studies have been conducted in estuarine and oceanic environments in which the presence of an antibiotic or toxic principle has been apparent. One method of measuring the bactericidal action of seawater is to compare the differences in survival when using untreated and treated portions of seawater.

An example of this type of study is provided in Table 11 where the survival of *E. coli* is compared when untreated, filtered, and autoclaved seawater from the

same sample point are used as 48 hour diluents. Notice that the untreated seawater has the greatest bactericidal activity which has been appreciably diminished or eliminated by the treatment.

Table 11. Survival of *Escherichia coli* in untreated, filtered, and autoclaved portions of six seawater samples collected during July and August, 1957

Treatment	Survival after 48 hr in seawater sample no.					
	1	2	3	4	5	6
None	2.2	0.7	4.6	22.8	4.4	2.7
Filtered	8.3	3.8	4.8	30.1	0.6	39.6
Autoclaved	8.4	30.9	64.6	53.6	69.6	38.5

It is interesting to compare the same treatment given to waters having a subsequent inoculum of coliphage (Table 12). Again the untreated seawater is viricidal (phage are virus particles which are capable of infecting and/or killing bacteria of a specific species) and the phage are rapidly eliminated while the treatments allow for a greater persistence of the coliphage (phage active against coliforms).

Table 12
Persistence of coliphage in various waters

Water	Survival		
	10 days	20 days	30 days
	%	%	%
Untreated seawater	2.0	<0.01	<0.01
Filter sterilized seawater	56.0	37.0	6.4
Autoclaved seawater	102.0	68.0	58.0
0.85% NaCl in deionized water	1.2	0.4	0.05

6 Salinity

The salinity of surface seawater averages about 3.5 percent and this concentration may be considerably reduced in areas where interactions with fresh water occur. These inorganic salts may adversely affect the survival of bacteria by either specific ion toxicity or osmotic effect. Although some investigators have reported that sea waters do not appreciably affect enteric bacteria in this environment there is general agreement that toxic effects are exerted to enteric bacteria in ocean environments but not to a degree of primary importance. Tables 13 and 14, in an abridged form, indicate this effect. Note that at a 25 percent concentration of sea water the affect is stimulatory when compared to a 0 percent or concentrations above the 25 percent sea water. Table 12 enforces

this concept as sea water salinities are compared with NaCl solutions of equal salinities. (Note: the 0.85 percent roughly equates with the 25 percent sea water concentrations.)

Table 13
Influence of concentration of sea water on the survival of Escherichia coli

Concentration of Sea Water	Survival after 48 Hr
%	%
0	59.9
25	74.5
50	34.6
75	22.5
100	8.2

Table 14
Survival of Escherichia coli in sea water and NaCl solutions of equal salinity

Solution	Salinity	Survival after 48 Hr
	%	%
Sea water	0.85	40.4
	2.50	7.6
	5.00	<0.01
NaCl	0.85	41.3
	2.50	2.1
	5.00	<0.01

7 Temperature

It is a general rule that bacteria survive somewhat longer at lower temperatures. An example of this generality can be seen in Figure 5 which shows the effect of temperature on coliform bacteria in Pacific Ocean water.

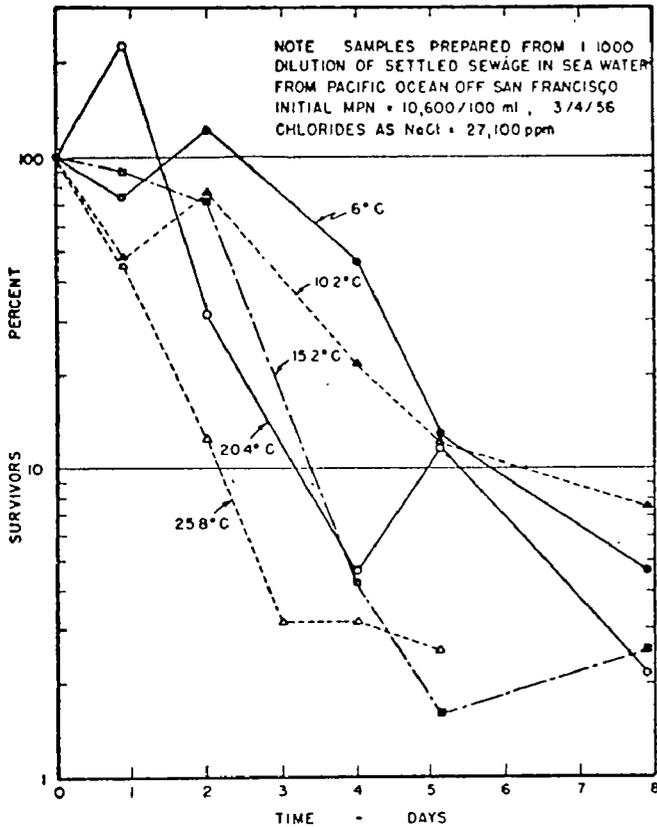


Figure 5. Effect of temperature on coliform survival in Pacific Ocean water

As an example to confirm that forces acting on bacterial survival are complex and in unison affect the population viable at each specific time interval, Figure 6 is presented. Here the variable is still the temperature (as was Figure 5) but to each test sample was added an equal amount of organic matter (120 ppm lactose broth) and this drastically altered the survival times for the different temperatures. In many instances, an adequate food supply can overcome detrimental conditions to the bacterial populations.

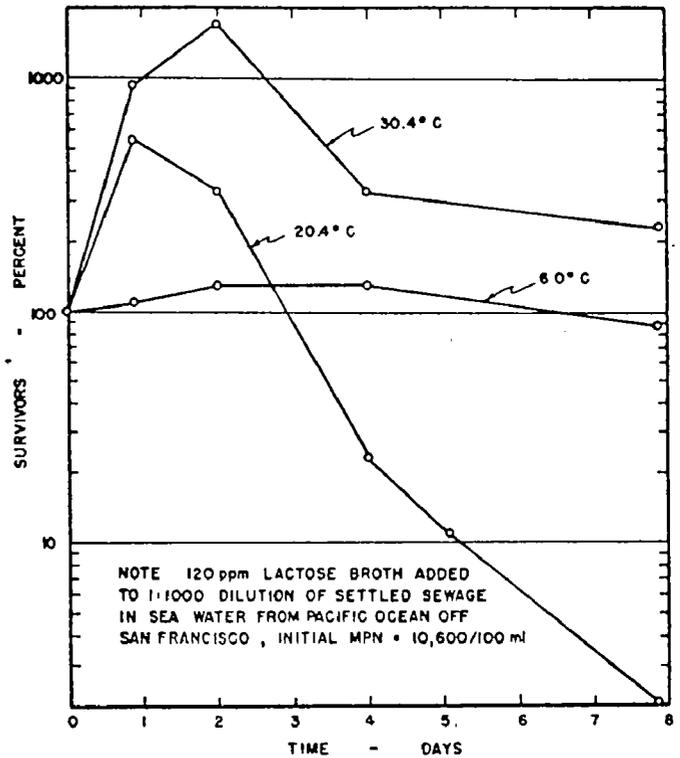


Figure 6. Effect of temperature on coliform survival in sea water containing organic matter.

8 Other factors

There are a multitude of other biological, physical, and chemical factors which affect the bacterial populations. Such other factors are pH, turbidity, chlorination, industrial wastes, etc. Some factors are still obscure and others are postulations. Factors in bacterial survival may be simply overlooked as may be the case shown in Figure 7 where channel characteristics can profoundly effect survival rates.

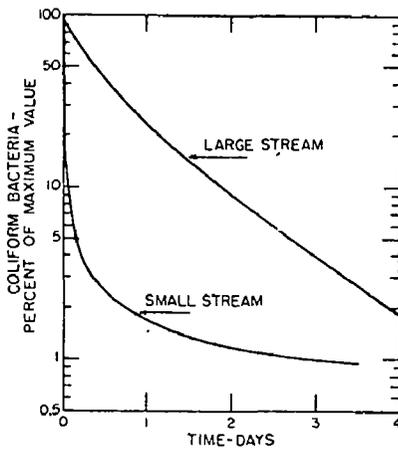


Figure 7. Effect of channel characteristics on the rate of coliform decrease

III RATES OF BACTERIAL DISAPPEARANCE FROM RECEIVING WATERS

A Orlob (1956) graphically summarized data from several investigations of bacterial survivals in sea waters. (See Figure 8)

Based on these observations, Orlob expressed bacterial survivals on several different equations, shown and plotted on Figure 9.

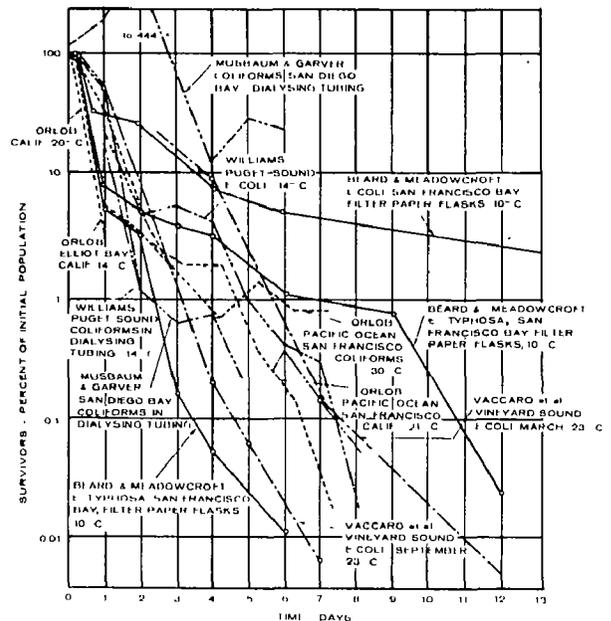


FIGURE 8 SURVIVAL OF BACTERIA IN SEA WATER DATA REPORTED BY VARIOUS INVESTIGATORS

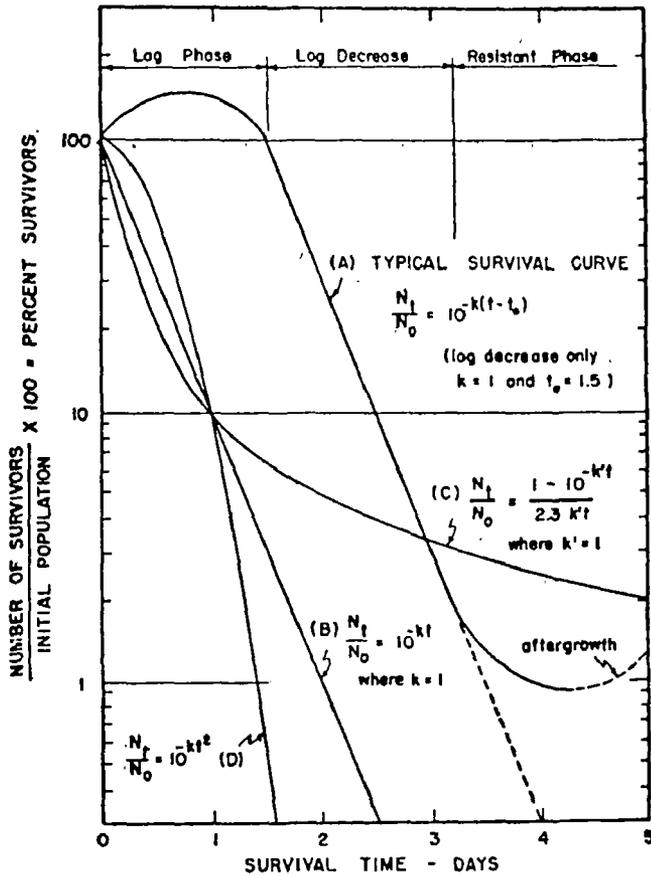


Figure 9. Typical survival curves for bacteria in sea water

Explanation of symbols:

N_t = number of survivors at time t (days)

N_0 = initial population at $t = 0$

k = rate constant (usually in the range 0.4 - 1.6)

k' = special rate constant for equation C

t_0 = lag period in days before onset of logarithmic decrease

The value of k is found as follows:

$$k = \frac{\text{Log} \frac{N_1}{N_2}}{t_2 - t_1}$$

N_1 = bacterial population at time t_1 at end of lag period

N_2 = bacterial population at time t_2

t_1 = time in days at end of lag period (at onset of logarithmic rate of decrease)

t_2 = subsequent time t_2 in days

1 Curve A is a "typical" survival curve when the receiving environmental water permits extended survival, or even limited growth, prior to onset of rapid disappearance of the sewage organisms. This lag period is not included in the rate calculation. Results of this type are not uncommon, particularly with pure cultures in restricted environmental laboratory studies or in containers placed in the marine environment.

2 Curve B is a pattern of bacterial disappearance when the receiving environment is totally unfavorable to the introduced bacteria. Many of the curves in Figure 9 begin in this type of pattern. Curves of this type can be expected in many laboratory studies of bacterial survival in marine water. Pure cultures often are used in such studies. With all cells representing the same species, and being at approximately the same level of physiological activity, the rate of disappearance should be uniform under unfavorable environmental conditions.

3 Curve C is to be expected when multiple species are introduced into the marine environment. Coliform bacteria, representing several species and levels of physiological activity, can be expected to represent different susceptibilities to the adverse factors in a marine environment. Similar overall patterns are apparent in Figure 9, some of which represent pure culture studies.

4 Curve D represents an increasing rate of bacterial disappearance with time, and has been interpreted by some as indicative of increasing susceptibility of the bacteria to an unfavorable environmental factor such as a toxic agent. Such a pattern of bacterial disappearance might also occur with increasing populations of predatory biota.

B Evidence of bacterial disappearance from saline waters is not restricted to that environment. Freshwater also is not a suitable habitat for extended survival of sewage bacteria. See Figure 10 (Kittrell and Furfari). Comparison with Figure 9, with respect to bacterial disappearance from sea waters, reveals a generally similar pattern, with the differences being largely a matter of degree.

IV SUMMARY AND CONCLUDING REMARKS

A Sea waters do not represent a favorable environment for extended survival of sewage bacteria. Factors bringing about bacterial disappearance are numerous, interrelated, and many also are applicable to bacterial removals from the freshwater environment. These common factors include dilution, sedimentation, predation, and some chemical factors.

B Some factors influencing bacterial disappearance appear to be more specific to sea waters. This includes salinity and the presence of soluble toxic substances.

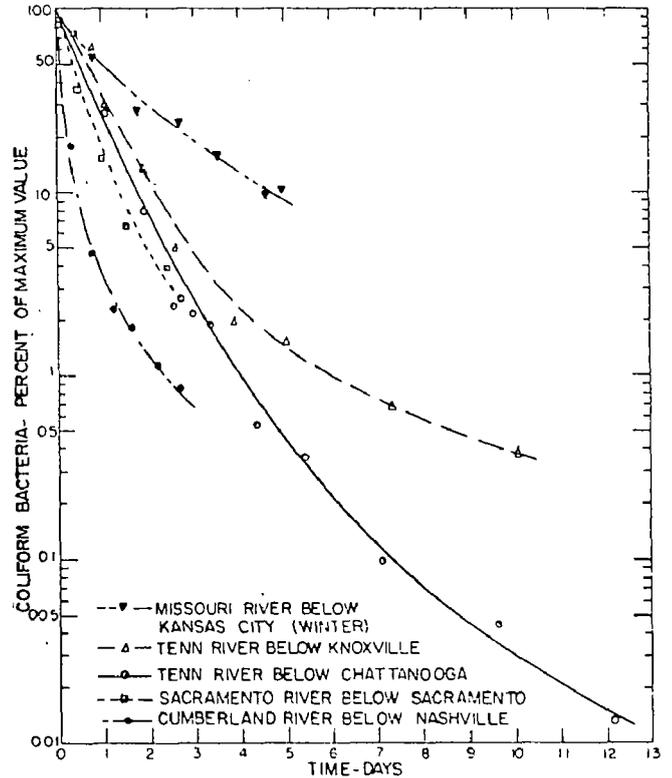


Figure 10. Rates of coliform decrease below five selected cities

C Because each factor which influence bacterial survival is a variable, in any given situation in which the fate of sewage organisms is in question, it will be necessary to conduct special investigations to determine the overall effects of the factors leading to bacterial disappearance in that particular environment.

D Of primary interest to the water microbiologist is that the concept of the indicator-pathogen dieoff curve (i. e., gradual dieoff of indicator bacteria but yet at a slower rate and for a greater period than any pathogen) is maintained in the aquatic environment. As yet, there have been no observable deviations from this concept which would seriously challenge established criteria.

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BACTERIOLOGICAL PATHOGENS IN THE AQUATIC ENVIRONMENT

I INTRODUCTION

Of the large group of microorganisms which have been implicated with outbreaks by organisms in the aquatic environment this outline will deal with only those of a bacteriological nature.

It must be realized that any of the pathogenic bacteria are capable of initiating a disease process in an individual or group when waterborne and viable. This outline, however, will emphasize only those which through the history of mankind have been implicated epidemiologically and clinically with waterborne outbreaks.

II BASIC DEFINITIONS

In order to follow the descriptions of the disease process it is necessary to be knowledgeable of some basic definitions which follow:

CARRIER. . . . An individual who harbors a pathogenic organism which may manifest itself either as an observable clinical disease or be undetectable. In both cases the individual can initiate disease outbreaks by continual or sporadic passing of the organisms to the environment.

ENDEMIC. . . . The continual occurrence of a disease entity to a specific geographical area which serves as a focus for future invasions to widespread areas.

EPIDEMIC. . . . The occurrence of an illness clearly in excess of the normal expectancy and arising from a common source.

INCUBATION PERIOD. . . . The time interval between the acquisition of the disease producing organism and when the first observable signs of the disease are evident.

RESERVOIR. . . . Reservoirs for the infectious diseases may be man, animal, plant, soil, etc., which are hosts upon which the disease agent lives and multiplies. Man is the most frequent reservoir for these organisms which are transmitted to man.

III DESCRIPTIONS OF COMMUNICABLE DISEASES

The following descriptions of the various diseases are written with an overview of the aquatic environment. In many cases the individual diseases can be spread just as well by person-to-person contact, arthropod transfer, inanimate object contamination, etc. This must be kept in mind when observing morbidity and mortality data in this outline as the source of infection may be any of the above.

A Cholera

1 General

Cholera is a severe acute intestinal infection which is usually characterized by a sudden onset, vomiting, profuse watery diarrhea, dehydration, and possible collapse. An epidemic is usually explosive and fatalities can vary from 5 to 75% with deaths occurring within a few hours of disease onset. It is endemic in parts of India and although absent in epidemic proportions in Europe and the Western Hemisphere for many years it has repeatedly invaded. Since 1962 two Americans, both tourists to foreign countries, (1) developed Cholera while overseas.

One can see the explosive nature of the disease by referring to Table 1 (Abridged from G.I. Forbes)⁽²⁾ of the incidence of Cholera since it first appeared in Hong Kong in 1961 and is inclusive to the year 1966. (2)

TABLE 1

Year	1961	1962	1963	1964	1965	1966
Cases.	77	11	115a	34b	-	1
Contacts ...	748	120	2210c	391	-	4
Carriers...	53	20	119d	29e	-	-
Deaths	15	1	6	4	-	-

a....includes 17 cases from a common source

b....includes 16 cases from a common source

c....includes 231 contacts of 10 "nightsoil" carriers

d....includes 14 "nightsoil" carriers and 34 carriers from a restaurant

e....includes 3 "nightsoil" carriers and 18 carriers from a restaurant

2 Infectious agent

Vibrio cholerae.... A gram-negative "comma" shaped bacteria.

3 Reservoir

The reservoir is an infected person and the source is infected feces and vomitus of patients.

4 Transmission

In the initial wave of an epidemic the spread is regularly by the contaminated water route. The carrier has not been a significant factor in this disease. This transmission period lasts as long as viable organisms are being excreted and this is usually about 7 to 14 days for feces.

5 Incubation period

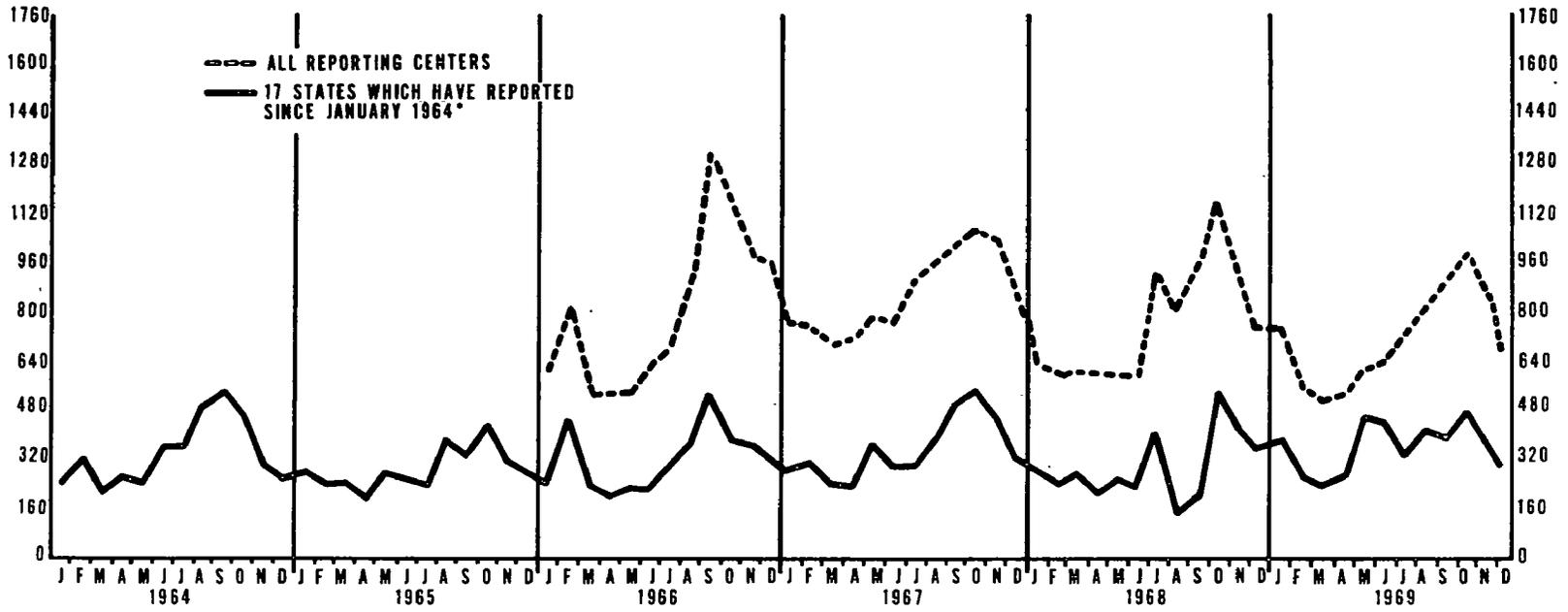
This can vary from a few hours to 5 days with the usual period of 3 days.

B Dysentery, Bacillary (Shigellosis)

1 General

Shigellosis (this is now the preferred nomenclature for reporting to the National Communicable Disease Center) is an acute infection of the intestinal tract and is characterized in severe cases by bloody stools with mucous and pus and accompanied by malaise, fever, toxemia, and cramps. Some cases may be inapparent or accompanied by mild symptomology. This disease is rarely fatal and in the cases which are, it is usually of the very young, very old, or debilitated. In 1969 there were a total of 9,054 isolations of Shigella reported to NCDC⁽³⁾ and this released total does not include 1,943 clinical cases reported from California during 1969. Figure 1 includes the reported isolations of Shigella in the United States during the periods 1964 to 1969.

REPORTED ISOLATIONS OF SHIGELLA IN THE UNITED STATES



*ALASKA, ARIZONA, HAWAII, ILLINOIS, KANSAS, MARYLAND, NEW JERSEY, NEW MEXICO, NORTH CAROLINA, NORTH DAKOTA, OHIO, OKLAHOMA, OREGON, SOUTH DAKOTA, TENNESSEE, TEXAS AND VERMONT

**ADJUSTED TO FOUR-WEEK MONTHS
MMWR Vol. 19, No. 14 NCDC

Figure 1

2 Infectious agent

Various species of the genus *Shigella*: *Sh. dysenteriae*, *Sh. sonnei*, *Sh. flexneri*, *Sh. boydii*, and others. Table 2 lists the six most frequently reported serotypes of *Shigella* from humans in 1969.

Unless the carrier state is present the infectious agent is present for only a few weeks. Carriers have been known to harbor the bacteria for a year or two, rarely longer.

3 Reservoir

The reservoir is man and the source of infection is feces from an infected person.

C *Leptospira*

1 General

Leptospirosis is an acute systemic infection characterized by fever, headache, malaise, chills, vomiting, muscular aches and less frequently jaundice, renal insufficiency and hemorrhage in the skin and mucous membranes. The acute illness lasts from 1 to 3 weeks and relapses may occur. Case fatalities are generally low but increase with advancing age levels and may reach 20% or more in the severe cases.

4 Incubation period

One to 7 days but usually less than 4 days.

5 Period of communicability

The infected person is able to transmit the disease during the acute stages of the affliction and whenever the bacteria is present in the feces.

Table 2
The Six Most Frequently Reported Serotypes of *Shigella* from Humans, 1969

Rank	Serotype	Reported Number	Calculated** Number	Calculated Percent	Rank in 1968
1	<i>S. sonnei</i>	5,584	5,513	60.9	1
2	<i>S. flexneri</i> 2a	668	1,308	14.4	2
3	<i>S. flexneri</i> 3a	303	768	8.5	3
4	<i>S. flexneri</i> 6	309	386	4.3	4
5	<i>S. flexneri</i> 2b	170	333	3.7	5
6	<i>S. flexneri</i> 4a	154	295	3.3	6

**Calculated number is derived by distributing the unspecified isolations in each group to their subgroups in the same proportions as the distribution of the specified isolations of that group.

Table 3 lists the numbers of cases and deaths attributed to this disease for the years indicated:

Table 3
Reported Cases and Deaths from Leptospirosis: United States

	1968	1967	1966	1965	1964	1963	1962	1961	1960
No. of Cases Reported.....	69	67	72	84	142	89	79	71	53
No. of Deaths....		4	9	11	14	14	10	9	14

Source: National Center for Health Statistics, Vital Statistics of the United States

2 Infectious agent

Many species of the Genus *Leptospira*, of which there are at least 21 (serogroups containing 59 serotypes), have been isolated from the aquatic environment. Some nonpathogenic leptospires have been found in water.

3 Reservoir

Reservoirs include cattle, dogs, and swine. The wide spectrum of animals and birds implicated with infection also include rats and other rodents, foxes, skunks, racoons, opossums, and a variety of water birds. The source of infection is the urine of the infected animals.

4 Incubation period

Four to 19 days with the usual period of 10 days.

D Paratyphoid fever

1 General

Paratyphoid fever is a generalized infection which is characterized by an abrupt onset, with continued fever and usually accompanied by diarrhea. Fatality rates are much lower than for typhoid fever and its incidence in the United States has dropped along with that of typhoid fever.

2 Infectious agent

Salmonella paratyphi, S. schottmuelleri, S. hirshfeldii (paratyphoid bacilli A, B, and C).

3 Reservoir

The reservoir for paratyphoid fever is man either as a patient or a carrier. The source of the infectious agent is urine or feces.

4 Incubation period

One to 10 days and usually somewhat longer for paratyphoid A than for B and C.

Preventative measures, epidemic measures and control of patients contacts, and the immediate environment closely parallel that for typhoid fever.

E Salmonellosis

1 General

Of the many clinical syndromes for salmonellosis the three main types are enteric fever, acute gastroenteritis, and septicemia. Every Salmonella strain is capable of initiating a disease process. Table 4 lists the number of cases and deaths attributed to Salmonellosis during the years indicated.

2 Infectious agent

Numerous species of the genus Salmonella of that group pathogenic for man and animal. For reporting purposes the primary human pathogens (typhoid and paratyphoid fevers) are excluded from this group by the national reporting agencies although they are also taxonomically part of the salmonellae. The more common species in the United States are S. typhimurium, S. choleraesuis, S. newport, S. oranienburg, S. montevideo, S. panama, and S. anatum. On a world evaluation S. typhimurium is the most prevalent and this trend is evident in Table 5 as reported by the Salmonellosis Unit, NCDC.

3 Incubation period

In epidemics this is from 6 to 48 hours and usually about 12 hours. In sporadic cases this time is believed to be about 1 to 7 days.

Table 4
Reported Cases and Deaths Attributed to Salmonellosis
(excluding typhoid fever) in the United States

	1968	1967	1966	1965	1964	1963	1962	1961	1960
No. of Cases Reported.....	16514	18120	16841	17161	17144	15390	9680	8542	6929
No. of deaths....		63	73	87	67	72	62	64	82

Table 5

10 Most Frequently Reported Salmonella Serotypes Isolated from Humans and Nonhumans
October, November, and December 1969

Serotype	Number	Percent
Human		
typhi-murium*	1,590	25.1
enteritidis	534	8.4
newport	498	7.9
heidelberg	422	6.7
thompson	306	4.8
saint-paul	303	4.8
infantis	246	3.9
javiana	176	2.8
typhi	165	2.6
	153	2.4
Subtotal	4,393	69.5
Total all serotypes	6,324	
Nonhuman		
typhi-murium**	354	12.3
heidelberg	300	10.5
saint-paul	192	6.7
anatum	188	6.6
cholera-suis K	172	6.0
thompson	101	3.5
derby	92	3.2
senftenberg	91	3.2
montevideo	87	3.0
infantis	85	3.0
Subtotal	1,662	57.9
Total all serotypes	2,868	
* Includes var. copenhagen 73		1.2
** Includes var. copenhagen 46		1.6

F Typhoid Fever

1 General

A systemic infection characterized by continued fever, involvement of lymphoid tissue, and constipation more often than diarrhea. Many mild inapparent infections remain unrecognized and this group contributes to the carrier population. A fatality rate of 10% can be reduced to 2 to 3% by proper antibiotic therapy. An unusual feature of this disease is that the typhoid bacilli are found in the blood during the first two weeks and in the feces and urine after the second week. Table 6 lists the number of cases and deaths attributed to typhoid fever in the United States for the intervals shown.

2 Infectious agent

Salmonella typhi is the infectious bacilli for typhoid fever. About 50 types are distinguishable by phage typing and this procedure has proved invaluable in the epidemiology of this disease.

Table 6
Reported Cases and Deaths attributed to Typhoid Fever in the United States

	1968	1967	1966	1965	1964	1963	1962	1961	1960	1959
No. of Cases Reported.....	395	396	378	454	501	566	608	814	816	859
No. of Deaths		12	15	6	14	21	15	17	21	22

3 Reservoir

The reservoir is man in the patient or carrier state. Infected urine and feces are the sources of infection. The carrier state is more prevalent in persons over 40 years of age and females are afflicted to a greater extent. About 10% of patients will excrete the bacilli as long as 3 months after onset and about 2 to 5% will become permanent carriers.

contact, mechanical transfer by insects, etc. The following information is given to provide a means of comparison for reported outbreaks. It will be noted that in some cases the infectious agent is unknown and it is common to record this fact in several ways: "unknown etiology"; gastroenteritis"; "unknown", and "other".

4 Incubation period

The usual range is from 1 to 3 weeks and averages about 2 weeks.

The following three tables (tables 7, 8, and 9) are taken from Weibel, et al (6) and show the incidence of water-borne disease, types of illness and its relationship to various water systems, and the principal causes of water-borne disease outbreaks in the United States. It will be noted that some of the infectious agents are not bacterial but are either viral or protozoan.

IV RELATIONSHIPS OF PATHOGENS TO WATER-BORNE OUTBREAKS

As was previously mentioned disease processes cannot be strictly categorized as solely water-borne. Typhoid fever can just as well, for instance, be transmitted via food contamination, person-to person

Table 7

Average Annual Number of Reported Disease Outbreaks in the United States, 1938-60

Years	Water-Borne Disease		All Other Sources of Disease	
	Outbreaks	Cases per Outbreak	Outbreaks	Cases per Outbreak
1938-40	45	583	201	34
1941-45	39	201	330	45
1946-50	23	121	360	33
1951-55	8	139	211	44
1956-60	7	121	254	41

Table 8

Water-Borne Disease Outbreaks in the United States, 1946-60, by Type of Illness and System

Illness	Private or Semi-Public Systems		Public Utilities		All Systems	
	Outbreaks	Cases	Outbreaks	Cases	Outbreaks	Cases
Gastroenteritis	92	4,233	34*	9,397	126	13,630
Typhoid	33	403	6	103	39	506
Infectious hepatitis	14	430	9	500	23	930
Diarrhea	7	320	9	4,840	16	5,160
Shigellosis	4	596	7	5,057	11	5,653
Salmonellosis	3	22	1	2	4	24
Amebiasis	2	36	0	0	2	36
Other	3	16	4	29	7	45
TOTAL	158	6,056	70	19,928	228	25,984

* One gastroenteritis outbreak also included a typhoid case.

Table 9

Principal Causes of Water-Borne Disease Outbreaks in the United States, 1920-36

Causes	No. of Outbreaks	Percentage of Outbreaks*	No. of Cases	Percentage of Cases**
Surface pollution of shallow wells	52	13	3,403	3
Cross-connection with polluted supply	40	10	10,636	9
Contamination of spring or infiltration gallery by pollution on watershed	31	8	1,185	1
Contamination of stream by pollution on watershed	27	7	3,283	3
Untreated water from river or irrigation ditch	25	6	668	1
Inadequate chlorination when the only treatment	23	6	4,500	4
Inadequate control of filtration and allied treatment processes	22	5	49,410	42
Seepage of surface water into gravity conduits	13	3	11,354	10
TOTAL (principal causes)	233	58	84,439	73
TOTAL (all causes)	399	100	116,000	100

* All outbreaks (399) from all causes (31).

** All cases (116,000) from all causes.

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TRANSMISSION OF VIRUSES BY WATER

I INTRODUCTION

- A Certain viruses which are capable of producing human disease are excreted into domestic sewage in large quantities with the feces of numerous individuals, many of whom are not ill. With the demand for water becoming increasingly acute in many sections of the country, the reuse of sewage is becoming a common practice and presents an imposing problem in public health.
- B Only limited data are available regarding the survival of viruses in various waters and little more is known about their resistance to disinfectants. Under certain conditions, it may be possible for water to be safe in terms of coliform density and residual chlorine level and yet be capable of initiating an enormous epidemic of a viral disease.
- C In rural areas small explosive outbreaks of infectious hepatitis occur from time to time which are traced to the consumption of accidentally contaminated small water supplies (e. g., wells, ponds, streams).
- D The risk of transmitting viral diseases through water under field conditions is real. For this reason, disinfectants prepared for use in drinking water and wash water must be capable of quickly destroying viruses.

II DEFINITION AND CHARACTERIZATION OF VIRUSES

- A Size - About 10 - 300 μ .
- B Structure - Nucleic acid core surrounded by a protein coat (capsid) and sometimes by an envelope, sperm-like (phage).
- C All viruses are obligate, intracellular parasites, many of which multiply only in certain cell types of certain hosts. They do not reproduce by themselves. Instead, they channel the cells complex biochemical systems into the production of new viruses.

- D Chemical composition - Some smaller viruses may be composed of nothing but nucleic acid and protein while some of the larger viruses may contain more complex substances in addition.
- E Sensitivity to antibiotics - Except for the larger more complex viruses of the parrot fever group, viruses are completely insensitive to all known antibiotics. In the laboratory large quantities of antibiotics are added to viral suspensions in order to minimize bacterial contamination.

III TECHNICS AND PROBLEMS IN GROWING VIRUSES IN THE LABORATORY

A Cell Culture

- 1 Simple to obtain
- 2 Various types are highly susceptible to many viruses
- 3 Relatively inexpensive

B Animals

- 1 Some species are more susceptible to some viral infections than known types of cell cultures.
- 2 Much is known about animal susceptibility to certain viral infections which have not yet been studied to any great extent in cell cultures.

C Chick Embryos

This is an extensively used and very sensitive host especially for members of the influenza-mumps group of viruses.

D Human Volunteers

The obvious risk involved precludes the use of this host except in very special circumstances.

E Detection of Virus in an Infected Host

- 1 Cell culture - Characteristic cell destruction is produced by viruses and is observable microscopically and sometimes macroscopically.
- 2 Animals - Typical disease is produced by viruses. Also an antibody rise usually occurs.
- 3 Chick embryos - Death of the embryo may result. Certain viruses can be detected by mixing embryonic fluids with red blood cells. The presence of certain viruses causes a typical agglutination of the red blood cells. Some viruses produce pocks on the embryonic membranes which are easily detectable and countable.
- 4 Man - Same as for animals

F Problem of Latency

In cell cultures and in animals, latent virus infections may occur. In this circumstance, the presence of the virus is not detected because cell destruction and disease do not occur. Under some conditions, however, the virus may suddenly multiply actively and produce disease. If this occurs subsequent to the inoculation into cell culture or animals of a specimen suspected of containing virus, the previously latent virus present in the host may appear to have been present in the specimen.

Other viruses, such as influenza, mumps, and Herpes simplex (cold or fever sore virus) are found occasionally in the feces as a result of being swallowed, and are probably not significant.

B Epidemiology of Enteric Virus Infections

- 1 Infections with these agents are widespread in the normal population especially during the summer and early fall.

Infection rates in any given area often are greatly dependent on hygienic conditions. Thus, infants and young children of the lowest socioeconomic groups usually suffer the highest attack rate. This may range from under 5 to 60% or higher. In only a very few cases does overt disease accompany infection. Illness, when it occurs, may be as mild as a "slight cold" or gastrointestinal upset, or as severe as paralytic poliomyelitis. Factors such as virulence of the virus and individual resistance play important roles in determining the course of infections. Large quantities of virus usually are shed in the feces of infected individuals and are detectable in domestic sewage.

- 2 Viruses have never been isolated from adequately treated drinking water. It is possible that small amounts of certain viruses survive treatment and produce sporadic cases or endemic foci of infection. Usually an explosive water-borne disease outbreak occurs only when a breakdown of good sanitary practices occurs.

IV THE ENTERIC VIRUSES

A Definition of Enteric Viruses

For our purposes, enteric viruses may be defined as those which may be demonstrable in large quantities in the feces of infected individuals and in sewage. They include infectious hepatitis virus(es), the enteroviruses (polioviruses, coxsackieviruses, echoviruses), the reoviruses, and the adenoviruses. Most of these viruses probably multiply in the gastrointestinal tract.

C Character of the Enteric Viruses

- 1 Infectious hepatitis virus

a Significance

This virus has not yet been propagated demonstrably in laboratory animals or in cell cultures. It can be demonstrated only by producing disease in human volunteers after oral inoculation.

Infectious hepatitis was a major medical problem in World War II. In recent years, tens of thousands of cases have been reported annually in the United States.

b The role of water in the transmission of infectious hepatitis.

Infectious hepatitis appears to be transmitted by the fecal-oral route, usually by personal contact though occasionally through the ingestion of water contaminated by infectious feces. Numerous documented waterborne outbreaks of infectious hepatitis have occurred in the U. S. and Alaska, none of which involved more than 350 people. In one case, the presence of the virus in the drinking water was demonstrated. These drinking water supplies were either unchlorinated or inadequately chlorinated. The infectious hepatitis outbreak in New Delhi, India, which occurred in December 1955 and January 1956, involved tens of thousands of cases. The raw water source, during the period of infection, was heavily contaminated with sewage. Contamination of the raw water was known to have occurred and was compensated for at the water treatment plant. It is significant that during the epidemic of infectious hepatitis there was no concomitant increase in any enteric bacterial disease. This suggests that treatment of drinking water adequate for eliminating the enteric bacterial pathogens is not necessarily adequate for destroying the virus of infectious hepatitis.

2 Poliovirus

There are three serologically distinct types of poliovirus, all of which are easily propagated in cell cultures. The intensive study of these viruses during the last two decades has resulted in the Salk formalinized vaccine and in the Sabin live-virus vaccine.

The polioviruses are responsible primarily for paralytic poliomyelitis, some aseptic meningitis (nonparalytic poliomyelitis), and possibly for some cases of mild gastrointestinal upsets which occur in the summer and fall.

Poliovirus infection apparently is transmitted by the fecal-oral route primarily by personal contact. Only two suspected water-borne outbreaks have been reported to date. In both cases, fecal contamination of the water and inadequate chlorination were believed responsible.

3 Coxsackieviruses

The coxsackieviruses are classified into Group A (32 human serotypes) and Group B (6 human serotypes) depending upon the lesions they produce in suckling mice. Some of the Group A strains cause herpangina and aseptic meningitis. The Group B strains are responsible for pleurodynia, aseptic meningitis and infantile myocarditis. Both groups possibly cause diarrheal disease in infants and young children during the summer and fall. Transmission of these viruses is probably by the fecal-oral and respiratory routes. No water-borne outbreaks due to these agents have been reported.

4 Echoviruses (enteric cytopathogenic human orphan viruses)

This is a group now composed of 32 serologically distinct agents which by original definition are found in the feces of infected individuals, produce cytopathic changes in cultures of human and simian renal tissues, and are not associated with known human disease (orphans in search of a disease). Since the original classification, however, several members of this group have been clearly associated with many cases of aseptic meningitis and "summer rash". Members of this group, too, may be responsible for cases of diarrheal disease in the summer and early fall. These viruses are transmitted

by the fecal-oral route but no known water-borne outbreaks of disease due to the echoviruses have been reported.

5 Adenoviruses

The classification of these 30 odd (human strains) viruses is based on their common soluble complement fixing antigen. Agents of this group produce a number of acute respiratory diseases of the cold-influenza type and at least two types of eye infection. These viruses appear to be responsible also for some of the upper respiratory disease in infants and young children. Transmission of these viruses is probably by the respiratory route. Several outbreaks of a disease now called pharyngoconjunctival fever, caused by adenovirus 3, have occurred with which chlorinated swimming pools have been associated.

6 Diarrheas and upper respiratory disease of unknown etiology.

Apparently water-borne outbreaks of diarrheal diseases have occurred in tourists visiting summer resorts. While not proved responsible, viruses have been suggested as a possible cause. It is probable that viruses produce many sporadic and epidemic outbreaks of gastrointestinal and upper respiratory illnesses, and it is difficult to tell presently how many of these outbreaks are water-borne.

V ISOLATION OF VIRUSES FROM SEWAGE

A Isolation

1 Grab samples

Sampling bottle is immersed in sewage and sample is removed. Because a definite sample volume is collected, this method is useful in quantitative studies.

2 Gauze pad method

Gauze pad is immersed in flowing sewage for varying lengths of time. Virus is trapped in cotton and can be eluted. Controlled tests have demonstrated that this method yields more positive samples than the grab sample technic but because it is impossible to estimate accurately the amount of sewage that flows through the pad, the gauze pad method cannot be used for quantitative determinations.

B Concentration Technics

1 Cliver technic (membrane filtration)

2 Shuval technic (two-phase separation)

3 Bier technic (forced-flow electrophoresis)

See References for details

C Results of Isolation Studies

Polioviruses, coxsackieviruses, echoviruses, reoviruses and adenoviruses have been isolated from sewage. Peak isolations occur during the summer and early fall. The seasonal peak corresponds well with the peak isolations of viruses from healthy children and with the peak occurrences of illnesses apparently caused by viruses of these groups.

D Problems in Technics of Isolation and Concentration

1 Technics are not sufficiently sensitive and quantitative

2 Concentration technics may be specific for certain viruses.

VI VIRUS SURVIVAL STUDIES

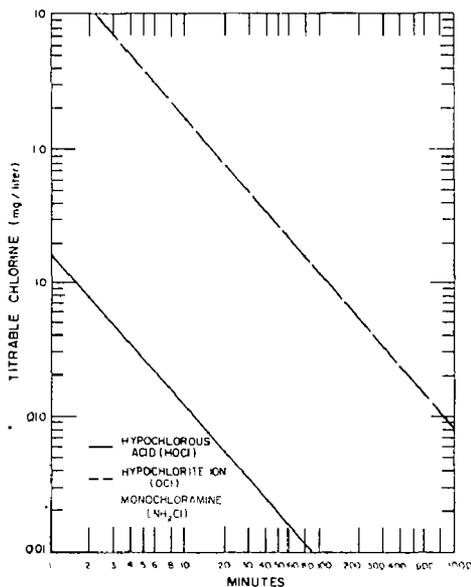
A A number of laboratory studies have demonstrated that several viruses of the enteric group survive storage in certain cool waters for as long as 6 to 7 months.

B Laboratory studies have shown that flocculation, sedimentation, and filtration do not completely eliminate several enteric viruses from processed water.

C Chlorine

Chlorine, in the form of hypochlorous acid (HOCl), is one of the fastest disinfectants available. However, chlorine exists as HOCl only at relatively low pH and in the absence of ammonia and organic materials. As the alkalinity of a solution increases beyond 7, HOCl ionizes to form hypochlorite ion (OCl⁻) which possesses relatively little germicidal activity. At pH 9 almost all free chlorine is present as OCl⁻.

Furthermore, when ammonia is present, chlorine exists as some form of chloramine. Figure 1 demonstrates the relative bactericidal efficiencies of HOCl, OCl⁻, and monochloramine. These data were obtained by us from an analysis of available literature. At 2 - 6°C, at a given concentration, OCl⁻ requires about 66 times as much time as HOCl to destroy the same amount of *Escherichia coli*. Monochloramine requires about 300 times as much time as HOCl to achieve the same amount of destruction.



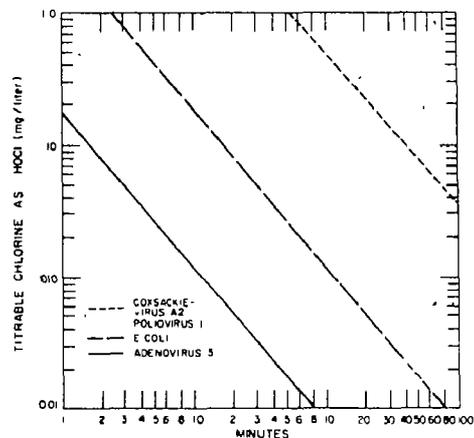
RELATIONSHIP BETWEEN CONCENTRATION AND TIME FOR 99% DESTRUCTION OF *E. COLI* BY 3 FORMS OF CHLORINE AT 2-6° C

FROM BUTTERFIELD ET AL., WATER, WERKIDIAN, & CHAMBERS, C. W. AND BUTTERFIELD ET AL., WATER, WERKIDIAN, & CHAMBERS, C. W.

Figure 1

While the rate of destruction increases considerably with rising temperature, data at precise temperatures are not available.

Figure 2 demonstrates the relative rates of destruction of several viruses and *E. coli*. These data were obtained by us from an analysis of the studies of several investigators who worked under dissimilar conditions. The different data can be compared directly with each other only on a crude approximation basis.



RELATIONSHIP BETWEEN CONCENTRATION AND TIME FOR 99% DESTRUCTION OF *E. COLI* AND 3 VIRUSES BY HYPOCHLOROUS ACID (HOCl) AT 0-6° C

FROM BUTTERFIELD ET AL., WATER, WERKIDIAN, & CHAMBERS, C. W. AND BUTTERFIELD ET AL., WATER, WERKIDIAN, & CHAMBERS, C. W.

Figure 2

The data with *E. coli* suggest that this bacterium is destroyed more slowly than adenovirus 3 by HOCl at 0 - 6°C. However, the poliovirus took more than 5 times longer to destroy than the *E. coli* and the coxsackievirus took more than 24 times longer to destroy than the *E. coli*.

D Iodine

Elemental iodine (I₂), too, is a very rapid disinfectant though not as rapid as HOCl. However, iodine, under most conditions, does not react with ammonia to form iodamines and is generally less reactive than HOCl with organic material. In addition, the presence of color, in the absence of excess iodide, is a good indicator of the presence of disinfecting potential.

Care must be taken in the preparation of iodine solutions not to use any more iodide

ion (I^- , usually in the form of KI or NaI) than necessary to solubilize elemental iodine which is soluble only to the extent of about 300 ppm at room temperature. Both I^- and triiodide ion (I_3^- , which results from the interaction of I_2 and I^-) have little, if any, virucidal capacity.

As the pH of iodine solution increase from 6 to 8, I_2 hydrolyzes (reacts with water) progressively to form hypoiodous acid (HOI) which is more virucidal than I_2 . However, while more HOI forms as the pH increases beyond 8, it also decomposes to form non-virucidal iodate ion (IO_3^-). Figure 3 demonstrates the relative rates of destruction of several viruses by elemental iodine.

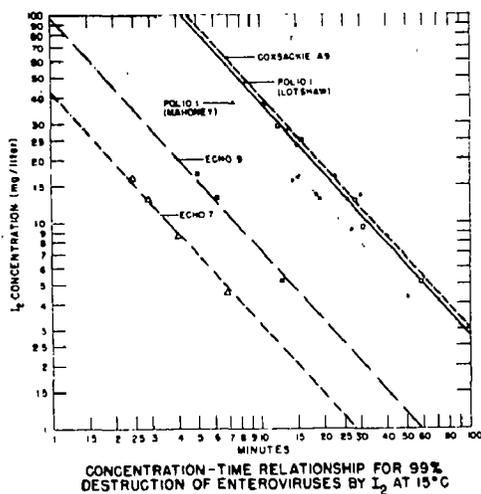


Figure 3

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FILAMENTOUS BACTERIA

I INTRODUCTION

There are a number of types of filamentous bacteria that occur in the aquatic environment. They include the sheathed sulfur and iron bacteria such as Beggiatoa, Crenothrix and Sphaerotilus, the actinomycetes which are unicellular microorganisms that form chains of cells with special branchings, and Gallionella, a unicellular organism that secretes a long twisted ribbon-like stalk. These filamentous forms have at times created serious problems in rivers, reservoirs, wells, and water distribution systems.

II BEGGIATOA

Beggiatoa is a sheathed bacterium that grows as a long filamentous form. The flexible filaments may be as large as 25 microns wide and 100 microns long. (Figure 1)

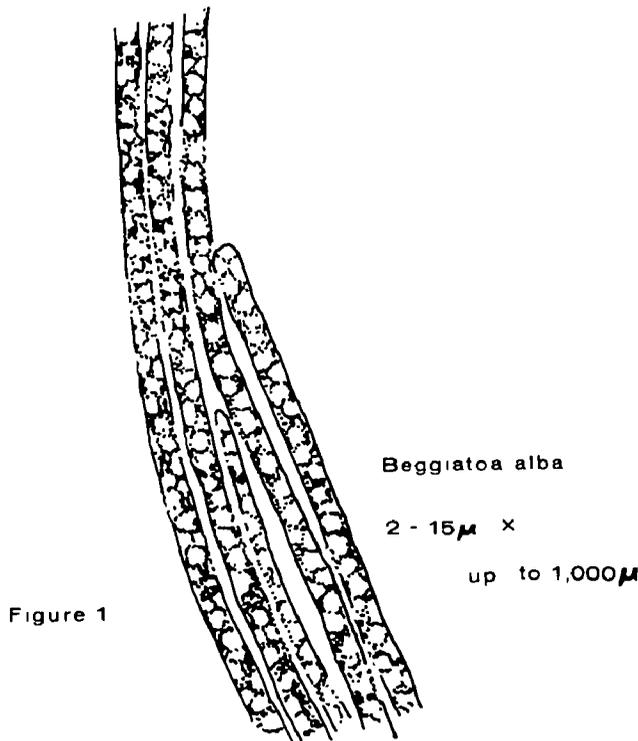
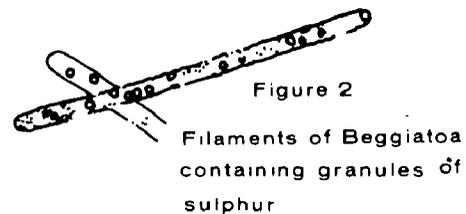


Figure 1

Transverse separations within the sheath indicate that a row of cells is included in one sheath. The sheath may be clearly visible or so slight that only special staining will indicate that it is present.

The organism grows as a white slimy or felted cover on the surface of various objects undergoing decomposition or on the surface of stagnant areas of a stream receiving sewage. It has also been observed on the base of a trickling filter and in contact aerators.

It is most commonly found in sulfur springs or polluted waters where H_2S is present. Beggiatoa is distinguished by its ability to deposit sulfur within its cells; the sulfur deposits appear as large refractile globules. (Figure 2)



When H_2S is no longer present in the environment, the sulfur deposits disappear. Dr. Pringsheim of Germany has recently proved that the organism can grow as a true autotroph obtaining all its energy from the oxidation of H_2S and using this energy to fix CO_2 into all material. It can also use certain organic materials if they are present along with the H_2S .

Faust and Wolfe, and Scotten and Stokes have grown the organism in pure culture in this country. Beggiatoa exhibits a motility that is quite different from the typical flagellated motility of most bacteria, the filaments have a flexible gliding motion.

The only major nuisance effect of Beggiatoa known has been overgrowth on trickling filters receiving waste waters rich in H_2S . The normal microflora of the filter was suppressed and the filter failed to give good treatment. Removal of the H_2S from the water by blowing air through the water before it reached the filters caused the slow decline of the Beggiatoa and a recovery of the normal microflora. Beggiatoa usually indicates polluted conditions with the presence of H_2S rather than being a direct nuisance.

III ACTINOMYCETES AND EARTHY ODORS IN WATER

Actinomycetes are unicellular microorganisms, 1 micron in diameter, filamentous, non-sheathed, branching monopodially, and reproduced by fission or by means of special conidia. (Figure 3)

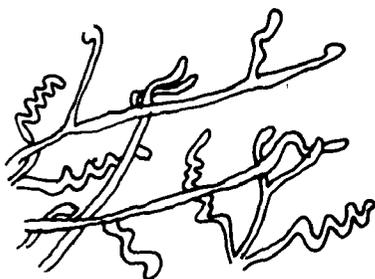


Figure 3 Filaments of Actinomycetes

Their filamentous habit and method of sporulation is reminiscent of fungi. However, their size, chemical composition, and other characteristics are more similar to bacteria. (Figure 4)

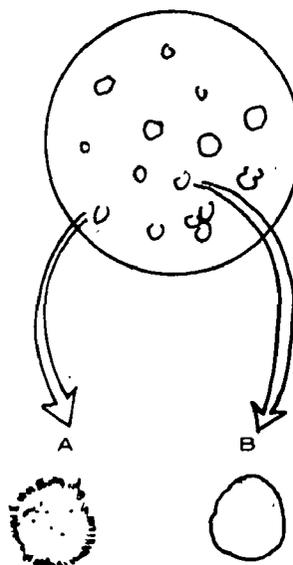


Figure 4
Egg albumin isolation plate
'A' an actinomycete colony,
and 'B' a bacterial colony

Appearance: dull and powdery Appearance: smooth and mucoid

These organisms may be considered as a group intermediate between the fungi and the bacteria. They require organic matter for growth but can use a wide variety of substances and are widely distributed.

Actinomycetes have been implicated as the cause of earthy odors in some drinking waters (Romano and Safferman, Silvey and Roach) and in earthy smelling substance has been isolated from several members of the group by Gerber and Lechevalier. Safferman and Morris have reported on a method for the "Isolation and Enumeration of Actinomycetes Related to Water Supplies." But the actinomycetes are primarily soil microorganisms and often grow in fields or on the banks of a river or lake used for the water supply. Although residual chlorination will kill the organisms in the treatment plant or distribution

system, the odors often are present before the water enters the plant. Use of permanganate oxidation and activated carbon filters have been most successful of the methods tried to remove the odors from the water. Control procedures to prevent the odorous material from being washed into the water supply by rains or to prevent possible development of the actinomycetes in water rich in decaying organic matter is still needed.

IV FILAMENTOUS IRON BACTERIA

The filamentous iron bacteria of the Sphaerotilus-Leptothrix group, Crenothrix, and Gallionella have the ability to either oxidize manganous or ferrous ions to manganic or ferric salts or are able to accumulate precipitates of these compounds within the sheaths of the organisms. Extensive growths or accumulations of the empty, metallic encrusted sheaths devoid of cells, have created much trouble in wells or water distribution systems. Pumps and back surge valves have been clogged with masses of material, taste and odor problems have occurred, and rust colored masses of material have spoiled products in contact with water.

Crenothrix polyspora has only been examined under the microscope as we have never been able to grow it in the laboratory. The organism is easily recognized by its special morphology. Dr. Wolfe of the University of Illinois has published photomicrographs of the organism. (Figure 5)

Organisms of the Sphaerotilus-Leptothrix group have been extensively studied by many investigators (Dondero *et. al.*, Dondero, Stokes, Waitz and Lackey, Mulder and van Veen, and Amberg and Cormack.) Under different environmental conditions the morphological appearance of the organism varies. The usual form found in polluted streams or bulked activated sludge is Sphaerotilus natans. (Figure 6)

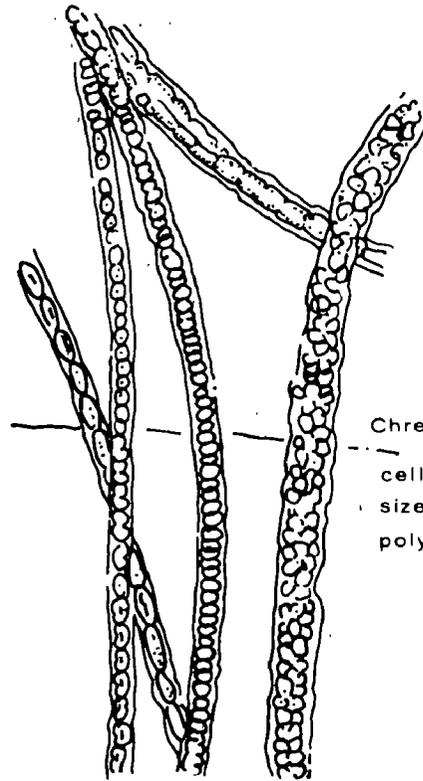


Figure 5
Crenothrix polyspora
cells are very variable in size from small cocci or polyspores to cells $3 \times 12 \mu$

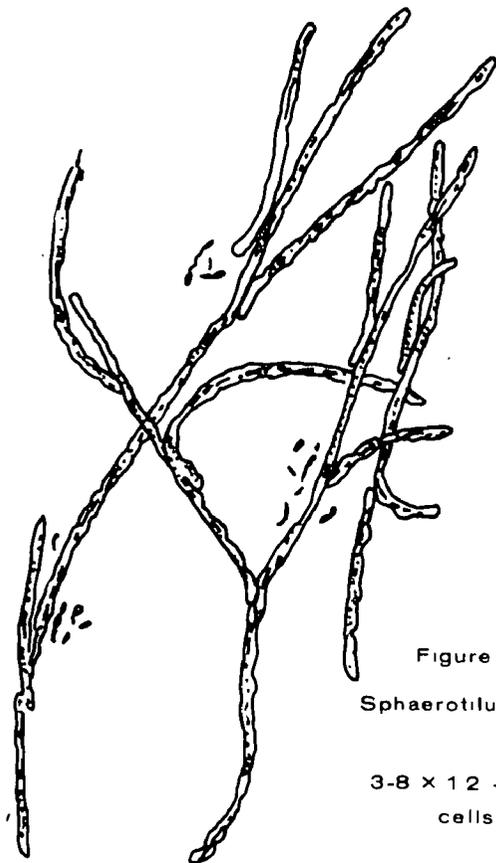


Figure 6
Sphaerotilus natans
 $3-8 \times 12 - 18 \mu$
cells

This is a sheathed bacterium consisting of long, unbranched filaments, whereby individual rod-shaped bacterial cells are enclosed in a linear order within the sheath. The individual cells are 3-8 microns long and 1.2-1.8 microns wide. Sphaerotilus grows in great masses; at times in streams or rivers that receive wastes from pulp mills, sugar refineries, distilleries, slaughterhouses, or milk processing plants. In these conditions, it appears as large masses or tufts attached to rocks, twigs, or other projections and the masses may vary in color from light grey to reddish brown. In some rivers large masses of Sphaerotilus break loose and clog water intake pipes or foul fishing nets. When the cells die, taste and odor problems may also occur in the water.

Amberg, Cormack, and Rivers and McKeown have reported on methods to try to limit the development of Sphaerotilus in rivers by intermittent discharge of wastes. Adequate control will probably only be achieved once the wastes are treated before discharge to such an extent that the growth of Sphaerotilus is no longer favored in the river. Sphaerotilus grows well at cool temperatures and slightly low DO levels in streams receiving these wastes and domestic sewage. Growth is slow where the only nitrogen present is inorganic nitrogen; peptones and proteins are utilized preferentially.

Gallionella is an iron bacterium which appears as a kidney-shaped cell with a twisted ribbon-like stalk emanating from the concavity of the cell. Gallionella obtains its energy by oxidizing ferrous iron to ferric iron and uses only CO₂ and inorganic salts to form all of the cell material; it is an autotroph. Large masses of Gallionella may cause problems in wells or accumulate in low-flow low-pressure water mains. Super chlorination (up to 100 ppm of sodium hypochlorite for 48 hours) followed by flushing will often remove the masses of growth and periodic treatment will prevent the nuisance effects of the extensive masses of Gallionella. (Figure 7)

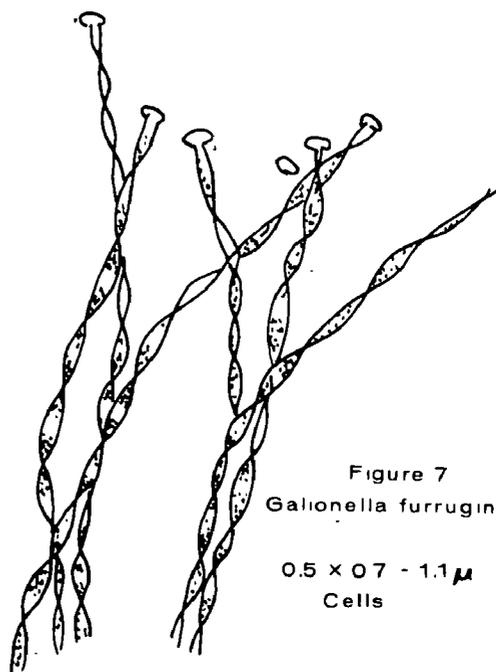


Figure 7
Gallionella furruginea
0.5 x 0.7 - 1.1 μ
Cells

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WATER QUALITY CRITERIA FOR RECREATION AND AESTHETICS

I INTRODUCTION

A Legal Aspects

The following discussion is based upon the Water Quality Act of 1965 wherein a partial statement of Paragraph 3, Section 10 states:

"Standards of quality established pursuant to this subsection shall be such as to protect the public health or welfare, enhance the quality of water and serve the purposes of this act. . ."

Recommended criteria in relation to the above is stated in this outline from the Report of the Committee on Water Quality Criteria of the Federal Water Pollution Control Administration dated April 1, 1968.

B Definitions

For a knowledgeable evaluation of these recommendations, it is necessary to be aware of the use of the following terms:

- 1 STANDARD - A plan that is established by governmental authority as a program for water pollution prevention and abatement.
- 2 CRITERIA - A scientific requirement on which a decision or judgment may be based concerning the suitability of water quality to support a designated use.
- 3 PRIMARY CONTACT RECREATION - Activities in which there is a prolonged and intimate contact with the water involving considerable risk of ingesting water in quantities sufficient to pose a significant health hazard.
- 4 SECONDARY CONTACT RECREATION - Activities not involving significant risks of ingestion.

C Preliminary Objectives

The subcommittee recommendations were based upon the preliminary objectives of:

- 1 Recommend water quality criteria for recreation and aesthetic uses, and
- 2 Identify research needs and priorities relating to water quality for recreation and aesthetic uses.

D Microbiological Considerations

The basis for the microbiological considerations for the Water Quality criteria have been due to:

- 1 Careful consideration of the lengthy historical background of the total coliforms and its relationships to the fecal coliform group of organisms.
- 2 Epidemiological studies of
 - a Great Lakes (Michigan), and
 - b Inland River (Ohio)

These studies have indicated an epidemiologically detectable health effect at levels of 2,300 - 2,400 coliforms per 100 ml and a later study of the Inland River established the fecal coliform load at 18% of the total coliform. Based upon these findings the indications are that the detectable health effects may occur at a fecal coliform level of about 400 per 100 ml and a factor of safety would use this level as a starting point

c Santee project

This work correlated the prevalence of virus with fecal coliform concentrations following sewage treatment. Following secondary treatment

the virus levels can be expected to be 1 PFU (plaque forming unit) per ml with a ratio of one virus particle per 10,000 fecal coliforms.

are designed to enhance the quality of water and consequently the aesthetic values are enhanced by continuing improvement in quality conditions in microbiological, chemical, and physical terms.

II CRITERIA DEVELOPMENT

A Aesthetics

Since natural conditions vary widely, recommendations are based upon a series of descriptive rather than numerical criteria. The following requirements have been recommended:

1 General requirements

- a All surface waters should be capable of supporting life forms of aesthetic value
- b Surface waters should be free of substances attributable to discharges or wastes as follows:
 - 1) Materials that will settle to form objectionable deposits
 - 2) Floating debris, oil, scum, and other matter.
 - 3) Substances producing objectionable color, odor, taste, or turbidity
 - 4) Materials, including radionuclides, in concentrations or combinations which are toxic or which produce undesirable physiological responses in human, fish, and other animal life and plants.
 - 5) Substances and conditions or combinations thereof in concentrations which produce undesirable aquatic life.

2 Desirable additional requirements

- a The positive aesthetic values of water should be attained through continuous enhancement of water quality. This is based upon the concept that water quality standards

- b The aesthetic values of unique or outstanding waters should be recognized and protected by development of appropriate criteria for each individual case.

Aesthetic considerations should be continually desired objective and the recommended standards should be borne in mind when waters are examined for recreation as they are not a separate entity from the overall desired water quality

3 Research needs

- a Greater evaluation of key factors for limited waters within restricted areas. Extensive research on a national level would be unwarranted due to the variance in numerical values from community to community
- b Improved control over discharges of materials producing objectionable changes.
- c Studies on the economical and sociological aspects as they relate to land values and human acceptance of water for various usage.

B Recreation

1 Considerations

Historical developments and sub-committee recommendations have been guided by the precepts of health and safety for the recreation user. Criteria have been recommended to:

- a Provide for and enhance general recreation use of surface waters,
- b Enhance recreation value of waters designated for recreation use, and

c Provide special protection for the recreation user where significant body contact with water is involved.

water quality Table 1 lists the recommended criteria for general recreational use of surface waters.

2 Surface waters

Surface waters should be suitable for use in secondary contact recreation without reference to official designation of recreation as a water use. It should be noted that this consideration will provide an impetus for the enhancement of water quality in many waterbodies which fall below this desired surface

It will be noted, from previous discussion of microbiological considerations, that the microbiological criteria is based upon fecal coliform densities which further consider that these surface waters are devoid of primary contact recreation and therefore have a risk considered to be one-tenth that which has primary contact recreation.

TABLE 1
GENERAL RECREATIONAL USE OF SURFACE WATERS
(Without reference to specific designation of recreation as a water use)

Recommended Criteria	Microbiological	Enjoyment of Recreation Activities	Harvest Species
		Fecal Coliform: Average not to exceed 2000 per 100 ml Maximum Value (except in specific mixing zones adjacent to outfalls) 4000 per 100 ml	Recommendations of the National Technical Advisory Subcommittee on Fish, Other Aquatic Life, and Wildlife

3 Waters designated for recreation uses
 Waters within this category are those which, for water quality management purposes, have been placed in an area designated for recreation uses but where primary recreation does not occur.

This category is deemed necessary to keep water quality at a high level and yet not have the stringent criteria which is necessary to those waters providing primary recreation contact. Table 2 lists the recommended criteria for Waters Designated for Recreation Uses.

TABLE 2
WATERS DESIGNATED FOR RECREATION USES
(Other than primary contact recreation)

Recommended Criteria	Microbiological	Enjoyment of Recreation Activities
		Fecal Coliform: Should not exceed a log mean of 1000 per 100 ml Never equal or exceed 2000 per 100 ml in more than 10% of the samples

4 Waters designated for primary contact recreation

Based upon the Microbiological Considerations previously mentioned the following recommendations in Table 3 apply to Waters Designated for Primary Contact Recreation.

Among the additional criteria for these waters, which are deemed to be desirable but not mandatory, are the ones relating to clarity and temperature.

- a Clarity - The basis for this recommendation is that of visual appeal, recreational enjoyment, and safety. The clarity should be such that a Secchi disc is visible at a minimum depth of 4 feet except in the learn-to-swim areas where it should be visible when resting on the bottom.
- b Temperature - Experience with military personnel indicates that exposure to warm water continuously for several hours can be safely tolerated with a maximum temperature of 30°C (85°F) and these limits are recommended.

TABLE 3
WATERS DESIGNATED FOR PRIMARY CONTACT RECREATION

	Microbiological	pH
Recommended Criteria	<p>Fecal Coliform:</p> <p>Shall not exceed a log mean of 200 per 100 ml (based on a minimum of not less than 5 samples taken over not more than a 30-day period)</p> <p>Not more than 10% of total samples during any 30-day period shall exceed 400 per 100 ml.</p>	<p>Within the range of 6.5-8.3 except when due to natural causes and in no case shall be less than 5.0 nor more than 9.0</p> <p>When the extended ranges of the natural causes are considered, any discharges which increase the buffering capacities are to be limited</p>

5 Research needs

- a Additional research on indicator organisms from specific sources to more effectively monitor water quality control programs.
- b Development of techniques that will indicate potential presence of pathogenic viruses.
- c Studies of the marine or estuarine environment associated with indicator organisms.
- d Work on pathogenic organisms in the applied research area.

- e Degree to which the various pathogens are waterborn and, as such, the infective doses which will initiate the disease processes.
- f Temperature and injuries as they relate to recreation users.
- g Managing water quality for secondary contact recreation users as related to its impact on health
- h Treatment and control of wastes affecting recreation uses of receiving waters.

- i Development of improved methodology for disinfection as these effluents effect recreational waters.

III CONCLUSIONS

A Criteria

It must be realized that the problem facing the establishment of any criteria relating to water quality must be that of balancing reasonable safeguards for public health while guarding against placing undue restrictions upon the available water-source in present or future planning. The most pressing problem which directly effects the setting of such standards is the lack of precise knowledge regarding microbiological pollution and its relationship to public health. Since this is the present situation and much remains to be ascertained in future epidemiological studies the criteria is based on the three previously mentioned studies and, in the light of future developments, the standards can be revised to reflect more complete knowledge.

B Subcommittee Membership

The conclusions relating to the establishment of Criteria for Water Quality in Aesthetics and Recreation were drawn by the following group of distinguished individuals:

- Mr. R. Frank Gregg, Chairman, New England River Basins Commission, Boston, Mass.
- Dr. Leonard Duhl, Special Assistant to the Secretary of Housing and Urban Development, Washington, D. C.
- Mr. Clarence W. Klassen, Chief Sanitary Engineer, Illinois Department of Public Health, Springfield, Ill.
- Mr. William J. Lucas, Assistant Director, Division of Recreation, National Forest System, Forest Service, U. S. Department of Agriculture, Washington, D. C.

Mr. Leland J. McCabe, Assistant Program Chief for Disease Studies and Basic Data, Water Supply and Sea Resources Program, Public Health Service, U. S. Department of Health, Education, and Welfare, Cincinnati, Ohio.

Mr. John C. Merrell, Jr., Chief, Southern California Field Station, Federal Water Pollution Control Administration, U. S. Department of the Interior, Garden Grove, California.

Mr. Eric W. Mood, Assistant Professor of Public Health, Chief, Environmental Health Section, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Conn.

Mr. Harold Romer, Director, Department of Air Pollution Control, City of New York, and Professor of Environmental Pollution Control, Long Island University, Brooklyn, New York.

Mr. Roy K. Wood, Chief, Division of Water Resources Studies, Bureau of Outdoor Recreation, U. S. Department of the Interior, Washington, D. C.

Dr. Richard T. Gregg, Technical Executive Secretary, Federal Water Pollution Control Administration, U. S. Department of the Interior, Washington, D. C.

C Bibliography

The following lists a selected bibliography which have been instrumental in the conclusions rendered by the subcommittee:

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ACKNOWLEDGMENT:

The majority of this outline must be considered an abridgement of the Report of the Subcommittee on Water Quality Criteria, FWPCA, USDI, April 1, 1968, Section 1, Recreation and Aesthetics, which should be consulted for more complete rationale.

This outline was prepared by R. Russomanno, Microbiologist, National Training Center, WPO, EPA, Cincinnati, OH 45268.

WATER QUALITY SURVEYS
ORGANIZING THE STREAM SURVEY

I INTRODUCTION

A Theoretical Approach

- 1 This discussion will present as a base, the procedures for the conduct of an ideal water quality survey.

B Practical Limitations

- 1 In practice, limitations of personnel, facilities, time and money, always will require compromise between the ideal and the possible.

C Objectives of Quality Studies

- 1 The four basic objectives behind the almost limitless number of possible reasons for water quality studies are:
 - a Determination of the natural water quality of the stream
 - b Measurement, in a selected and limited period of time, of the existing effects of wastes on water quality and uses
 - c Procurement of data on waste loads, water quality and stream characteristics that will permit projection of the data to describe probable water quality and effects on uses under a variety of conditions other than those that prevailed during the study.
 - d Determination of corrective measures needed to protect the stream water quality for proper uses.

II PRELIMINARY PLANNING

A Available Information

Assemble and review all readily available maps, information, and reports bearing on the stream under consideration.

B Problem and Objectives of Study

- 1 Define the problem requiring study as completely as possible on the basis of available information.

C Tentative Plan of Study

- 1 Prepare a brief preliminary plan of study for guidance in the subsequent field reconnaissance.
- 2 Include in the preliminary plan:
 - a Locations and strengths of known sources of waste
 - b Locations of areas of water use and a list of legitimate water uses
 - c Section of major stream and locations of important tributaries and unique features such as dams and points of major diversions
 - d Possible sampling stations
 - e Sampling frequency and number of samples at each station

- f Types of laboratory determinations
- g Existing stream gauging stations and additional points where stream flow data are needed.
- h Other hydrological data needed and possible means of procurement
- i Potential laboratory locations
- j Special supplies and equipment
- k Personnel requirements
- l Approximate cost of field operations

III. PRELIMINARY FIELD OPERATIONS

A Importance

This step is often neglected, but a small advance party of two or three experienced field men can obtain information and make preparations that will save time and money and avoid much confusion and possible error when the subsequent field study starts.

B Local Contacts

Contact local agencies and individuals who use or have knowledge of the stream, and assemble available maps, reports, operating records, and verbal information.

C Sources of Wastes

1 Sewage

- a Obtain best available estimate of sewered population.
- b Examine sewage treatment plant, if any, and obtain copies of operating records on volumes and characteristics of sewage.
- c Locate all points of discharge and make any installations necessary for sampling and gauging.

2 Industrial wastes

- a Investigate and prepare flow diagram of process with especial reference to points of water use and waste discharge, and quantities of raw materials and finished products.
- b Obtain information on plant operating schedule, with especial attention to daily or seasonal variations, and to any anticipated changes in process or increase in production.
- c Obtain data on water consumption, numbers of plant employees by work shifts, and disposal of domestic sewage.
- d Investigate waste treatment facilities, if any, and obtain operating records on volumes and characteristics of wastes.
- e Locate all points of waste discharge, trace origins in plant process, and make any installations necessary for sampling and gauging.
- f Collect samples for preliminary examination in laboratory to permit selection of proper analytical procedures.

D Stream

- 1 Observe entire reach of stream involved by wading, walking bank, or boat.
- 2 Note especially points of waste discharge and dispersion patterns, visual evidences of pollution confluences and mixing of tributaries, stream flow characteristics, approximate widths and depths, and locations of prospective sampling stations, and of water uses.
- 3 Collect bottom samples of biological organisms and bottom deposits, and observe evidences of algae.

- a Bottom organisms serve as an index of degree of pollution, and of length of river that should be sampled.
 - b Bottom deposits indicate extent of sludge deposits.
 - c Algae indicates probable significance of photosynthesis and possible need for night sampling or light-dark bottle technique for full evaluation of dissolved oxygen variations.
- 4 Determine times of water travel between pertinent points on stream.
- a Knowledge of times of water travel not only will permit the most intelligent conduct of study, but also is essential to the complete analysis, interpretation, and projection of data.
 - b Time and effort involved are a minor portion of the totals devoted to the stream study, and once established, need not be repeated in subsequent studies.
 - c Observations should be made at three or more stream flows to develop a time of travel versus stream discharge curve, which will permit interpolation for any desired stream flow.
 - d Methods include: tracing an inherent or added variable stream constituent, timing surface or submerged floats; and determining stream cross sections at selected intervals.
- 5 Select and identify sampling stations
- a For DO evaluation on the majority of streams, there should be: one station just above each major waste source; stations at about half day intervals for two days time of travel below waste sources; and stations at about one day intervals for an additional three to four days time of travel.
 - b For coliform evaluation, the stations should be similar to those for DO except that the downstream stations at one day intervals should extend to a total of 10 to 20 days time of water travel below the source of wastes.
 - c In very small or very turbulent streams, natural purification may be well advanced in as little as one to two days time of water travel and stations should be at much closer intervals than suggested above.
 - d If tributary streams are important, there should be one station near the mouth of each tributary and one on the main stream just above each confluence.
 - e Stations should be established as close as practicable to points or areas of important water uses.
 - f At stations where wastes and tributary waters are well mixed, one sampling point near mid-channel usually is adequate.
 - g At stations where mixing is inadequate, common practice is to sample at the quarter points of the stream.
 - h Identify stations by adequate description and marking, and make any necessary preparations for use by sampler.
 - i Select best access routes to sampling stations and make a round-trip to stations to determine time required for sampling.
- E Preliminary Stream Samples
- 1 Collect one or more sets of stream samples for preliminary examination.
 - a Dissolved oxygen results will be useful in establishing sampling stations and length of stream that should be studied

- b Determinations of coliform organisms and BOD will assist in selection of proper dilutions for subsequent use when study is started.
- c Determinations of other constituents will enable analyst to select most acceptable laboratory procedures and to determine sizes of sample portions necessary for those analyses in which concentrations of constituents govern choice of portion sizes.

F Laboratory Location

Examine potential laboratory locations in relation to convenience to the field operations, adequacy of space and utilities, storage space, and revisions or additional facilities that will be needed.

G Miscellaneous

- 1 Locate local sources of supplies that will be purchased during study.
- 2 Determine transportation routes and schedules for shipments of samples, supplies, and materials.
- 3 Arrange for local personnel and special facilities that will be used.
- 4 Investigate rooming facilities convenient to the field operations.

IV FINAL PLANNING

A Revision of Preliminary Plan

With the preliminary plan as a guide, and with the knowledge gained from the preliminary field operations it will be possible to prepare an intelligent, workable plan for the field study.

B Objectives of Study

Redefine, add to, or delete from, the initial list of objectives, and prepare a set of specific objectives which will include provisions for all essential

answers to the problem at hand, but will also eliminate needless expenditure, on nonessential matters, of effort which might better be devoted to the principal problem.

C Period of Field Operations

- 1 The overall period selected for the study should be at the time of year when past experience indicates that stream-flows usually are relatively stable.
- 2 For evaluation of DO depletion; a period of warm weather and low streamflow is desirable.
- 3 For evaluation of coliform contamination, a period of intermediate flow may be desirable.
- 4 A period of intensive round-the-clock sampling for a few days generally is preferable to a period of a month of sampling daily or on alternate days, but a combination of both methods may be better than either one.

D Sampling

1 General

- a If sources of wastes do not vary significantly during period of study, the wastes may be sampled in advance of the stream study.
- b Sampling procedures and preservation of samples should follow "Standard Methods for the Examination of Water and Wastewater" and/or procedures recommended by the investigators agency.

2 Sewage

- a Collect samples around-the-clock at 15 minute to 1 hour intervals for three to seven days.
- b Composite sample portions in portion to flow for three to four equal time periods of each day.

3 Industrial wastes

- a In general, the better known the process is, and the less variable the waste discharge, the less detailed need the sampling be.
- b For well known processes with little variation, equal sample portions collected at one-half to one hour intervals and composited for eight hours or for a complete cycle of operation on three to five days should be adequate.
- c For little known and variable processes, detailed and prolonged sampling may be necessary, with samples collected at 5 to 15 minute intervals, and composited in proportion to flow for six to 24 hour periods for seven or more days or complete cycles of operation.

4 Stream

- a If streamflow, waste discharge, or oxygen production by algae vary widely throughout the day, sample around-the-clock at several stations for at least one or two days to establish the daily cycle of variation in waste constituents and effects.
- b In most streams, grab samples each day or on alternate days for two weeks to a month is adequate if conditions remain stable.
- c The total number of samples collected from a single station during a field study generally ranges between 15 and 20. In the absence of wide variations in streamflow, 12 to 15 sets of samples usually yield adequate, usable results.
- d If sampling is not around the clock, sample collection should be varied throughout the day as much as is feasible, and as a minimum, the direction of the sampling trip should be reversed on alternate days.
- e When more than one point is sampled at a station, individual field deter-

minations are made and a separate bacteriological sample is taken at each point, but samples for other determinations may be combined in a single composite. Bacteriological samples from two or more points at the same station may be composited in the laboratory.

E Gauging

- 1 Methods of waste and stream gauging are covered in another reference outline.
- 2 Generally stream discharge records are obtained from existing U. S. Geological Survey stations, or from special stations established and rated by them upon request.
- 3 Streamflow data at each main stream sampling station generally can be computed with sufficient accuracy from records for one or two main stream gauging stations and one for each major tributary.

F Laboratory Operations

- 1 Select principal determinations that will measure pertinent waste constituents and their effects, and auxiliary determinations that will contribute to interpretation of principal results.
- 2 Reject determinations that would be "interesting" but would not contribute to solution of the problem.
- 3 Generally useful principal determinations common to many stream studies are those for coliform bacteria, bottom organisms, temperature, DO, and BOD, and auxiliary determinations frequently are for pH, alkalinity, and turbidity.

Other determinations must be selected for special purposes and for special types of wastes.
- 5 Laboratory methods should follow procedures recommended in "Standard Methods for the Examination of Water and Wastewater."
- 6 Avoid excessive overload of the laboratory by working closely with the

laboratory supervisor during the planning stage and accepting his estimate of the volume of work that can be handled.

7 In establishing the laboratory work load, allow time for calculation of analytical results at the end of each day.

8 Ship samples to the headquarters laboratory for all determinations of stable or preserved constituents that do not have to be made in the field.

G Personnel

1 The field crew should possess competencies for the specific tasks involved, or should be trained in advance if necessary.

2 Personnel needed commonly will include:

a Sanitary engineers

b Chemists

c Bacteriologists

d Biologists

e Sampling and laboratory aides

H Supplies and Equipment

1 Prepare and use a check list of needed field supplies and equipment.

2 All operations that can be performed in advance at headquarters, such as training, purchases, equipment repair, and reagent preparation will save money and valuable time in the field.

I Cost Estimate

1 Revise the initial estimate of cost of field operations in light of the final plan adopted.

2 Adjust the final plan to fit the money available for the study, or arrange for the additional money needed.

V FIELD OPERATIONS

A Briefing

1 Assemble the field crew and explain the problem, objectives, and details of field operations.

2 Take at least key personnel on a tour of pertinent physical features of the study.

3 Specify responsibilities of individuals for the various phases of the operation.

B Communications

Arrange a system of communication by which any member of the crew can be contacted within a reasonable length of time.

C Records

1 A few simple field and laboratory forms will serve to systematize the maintenance of basic records.

2 Keep all permanent field and laboratory records in bound volumes.

3 Encourage the recording of other than routine observations by both sampling and laboratory personnel.

4 Write down at once, and do not depend on memory for all significant observations.

D Revision of Operations

1 Review all daily data accumulated at the end of each day, and note especially any omissions or unusual results.

- 2 On the basis of the daily review, consider the need to:
 - a Add new determinations if needed.
 - b Omit determinations that are not showing significant results.
 - c Revise analytical methods.
 - d Change sampling stations or scheduled times of sampling.
 - e Investigate causes of apparently abnormal or erroneous data.
- 3 Do not revise operations unless the data or other new information indicates changes are essential to achieve the desired objectives.

VI USE OF DATA

A Analysis and Interpretation

- 1 The raw data are of only limited value until submitted to analysis and interpretation by an experienced person who is familiar with the problem involved.
- 2 Methods of analysis and interpretation constitute a major subject in themselves and are covered by other lectures.
- 3 No amount of statistical manipulation of the data can produce sound conclusions unless the stream study is soundly conceived, and carefully and conscientiously executed.

B Report

A report should be prepared if the data are to achieve their maximum usefulness, since raw data in dead files only rarely benefit anyone.

C Ultimate Disposal

After use, the data should be filed, indexed, and described so that they can be available and understandable years later to others not directly associated with the study

D Maximum Usefulness

The results of a stream pollution study yield their maximum benefit and return their greatest satisfaction to those who worked to obtain them only when the results serve as a basis for actual correction of an abuse of stream water quality.

REFERENCE

Kittrell, F. W. A Practical Guide to Water Quality Studies of Streams. USDI, FWPCA, CWR-5. 1969.

This outline was prepared by Francis W. Kittrell, former Special Consultant, National Field Investigations Center, -- Cincinnati Office of Enforcement and General Counsel, EPA, Cincinnati, OH 45268

Descriptors: Baseline Studies, Data Collections, Hydrologic Data, On-Site Data Collections, On-Site Investigations, Streams, Surveys, Planning, Sampling, Stream Pollution, Water Quality

WATER QUALITY SURVEYS
ROLE OF BACTERIOLOGIST

I INTRODUCTION

The determination of bacteriological quality of water should include active participation of the bacterial analyst in all phases of the survey, from the planning stages through and including the performance of the survey and the final preparation and presentation of the finished report.

The principal bacteriologist may be a professional bacteriologist serving as the principal survey bacteriologist, a bacteriological consultant, or, in practice, these duties sometimes may be assumed by a qualified engineer or scientist nominally representing some other professional discipline.

Chief responsibilities of the principal bacteriologist in a survey are summarized in the following paragraphs.

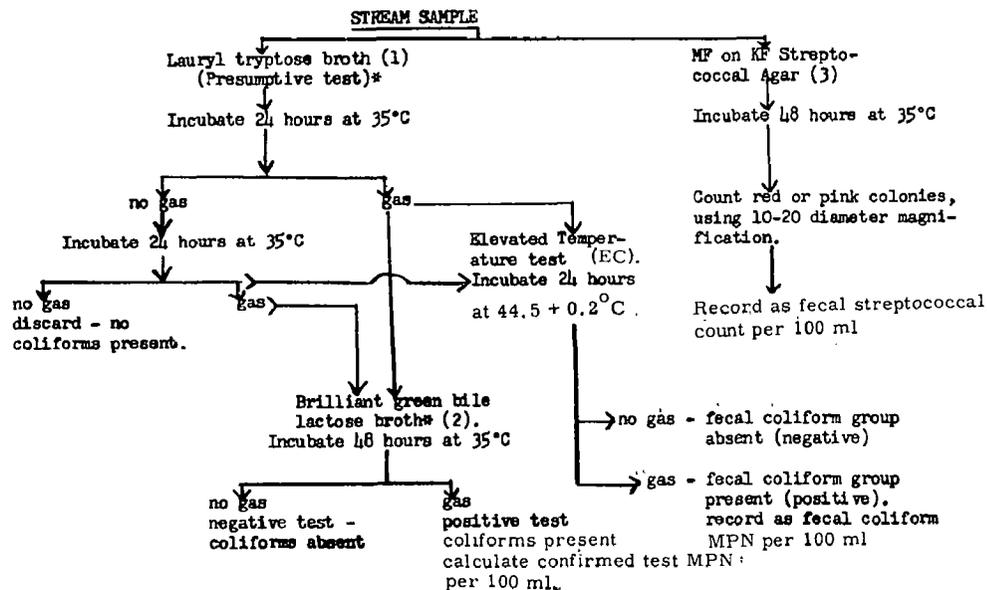
II IDENTIFICATION OF BACTERIOLOGICAL IMPLICATIONS OF SURVEY OBJECTIVES

- A Through consultation with survey management and review of survey objectives, he determines what specific pollution problems are related to bacteria.
- B A determination is made of the kind of bacteriological information which must be developed in order to meet survey objectives.
- C The related chemical and physical tests are identified in terms of their expected need with respect to interpretation of bacteriological data.

III DETERMINATION OF PROCEDURES

In order to develop the kind of bacteriological information required to meet survey objectives, the following decisions must be made, and pertinent information obtained

- A The specific indicator organisms used to detect and evaluate pollution must be selected.
- B The methods used for their quantitative measurement must be determined.
- C Consultations must be included with respect to bacteriological sampling.
 - 1 The sample points must be established.
 - 2 The frequency of bacteriological testing at each point must be determined.
 - 3 The location of proposed sampling stations with reference to assumed influences on bacterial levels must be reviewed. These influences may be alleged pollution sources, entrance of diluting waters, drainage areas, or, in some cases, industrial drainage or outfalls.
- D Any existing data on bacterial densities, as well as water flow velocity and volume should be obtained and studied.
- E Based on the sampling plan and determinations to be made, a protocol of bacteriological sample examination is developed. Figure 1 is an example of such a protocol, used in a recent water pollution survey.



* Indicates procedure described as standard test in Standard Methods for the Examination of Water and Wastewater, 13th ed. (1971)

- (1). Lactose broth may be substituted for lauryl tryptose broth.
- (2). Eosin Methylene blue or Endo agar may be substituted for the brilliant green bile lactose broth.
- (3). The KF Agar pour plate count may be used interchangeably with the MF colony counting technique.

Figure 1

IV REVIEW OF BACTERIOLOGICAL PLANS

The bacteriological plan is reviewed for adequacy to accomplish the objectives of the survey, based on a "paper review" and on preliminary samples which should be examined at the selected sampling stations if at all feasible.

- A Sufficient qualified personnel for the laboratory load should be provided. Estimates of work loads are shown in Part 2 of this chapter.
- B The detailed methods for all procedures are reviewed with all personnel responsible for collection and handling, transmission to the laboratory, and with the laboratory personnel responsible for sample testing procedures.
- C The duties assigned to all personnel must be reviewed with all concerned, work scheduled, reviewed and understood by all. Care must be taken to assure that assigned

work is consistent with training, ability, and experience of individuals concerned.

- D Location and quality of the laboratory facilities must be reviewed to ascertain that sample testing procedures will be in accordance with established plan, standard procedures, and to assure that adequate work space is available.
- E Laboratory equipment and supplies must be adequate for the expected work loads. Glassware and other supplies which must be used on a rotational basis should, in general, be $3\frac{1}{2}$ - 4 times the expected daily need.

V REVIEW OF LABORATORY OPERATIONS

After the established daily work of the survey has gotten well underway, the principal bacteriologist should review operations based on the plan established as the final plan for survey operations. Typical check points include:

- A Review of sample collection and handling procedures and assurance that there is minimum delay between collection and starting testing procedures on individual samples.
- B Evaluation of Laboratory Work
 - 1 Determination of whether the methods actually used are in conformance with plans.
 - 2 Elimination or justification of deviations from planned procedures.
 - 3 Problems which have arisen during the operations are discussed, particularly with reference to matters not anticipated when planning the survey.
 - 4 Data are reviewed to determine validity for use in the survey, in terms of reliability of results.
 - a When membrane filter or plate count methods are used, check the correlation between occasional duplicate or triplicate plate count for reproducibility.
 - b When multiple dilution tube methods are used, check the percentage of the usual codes, according to the method described by Woodward and Walton (JAWWA, 1957, 49:1060-1068, "How Probable is the Most Probable Number?")

Table 1. STATISTICAL EXPECTANCY OF POSITIVE TUBE COMBINATIONS FOR A SERIES OF 5 PORTIONS PLANTED IN EACH OF 3 DECIMAL DILUTIONS

Expectancy group	Positive tube combinations constituting group						Frequency of occurrence	
							By group %	Cumulative %
Group I (Frequent)	100	200	300	400	500	550	67.5	67.5
					510	551		
					520	552		
					530	553		
					540	554		
Group II (Common)	110	210	310	410	511		23.6	91.1
				420	521			
					531			
					541			
Group III (Uncommon)	001	101	201	301	401	501	7.9	99.0
	010	120	211	311	411	502		
	020		220	320	421			
				330	430	522		
					431	532		
					440	533		
						543		
					544			
Group IV (Rare)	All other positive tube combinations						1.0	100.0

Ref.: Richard L. Woodward and Graham Walton, 1957.

Table 2. ACTUAL POSITIVE TUBE COMBINATIONS
 CLASSIFIED BY EXPECTANCY GROUP
 PLANT A. RAW WATER COLIFORM TEST

Positive tube combination (5 portions planted in each of 3 decimal dilutions)	Number of times found	I Frequent	II Common	III Uncommon	IV Rare
100	1	1			
110	1		1		
200	2	2			
220	1			1	
300	6	6			
301	1			1	
310	3		3		
400	8	8			
401	1			1	
410	12		12		
411	2			2	
412	1				1
420	4		4		
430	2			2	
440	1			1	
500	13	13			
510	11	11			
511	2		2		
512	1			1	
520	9	9			
521	2		2		
530	9	9			
531	3		3		
540	3	3			
541	5		5		
542	1		1		
550	2	2			
551	1	1			
Total No.	108	65	33	9	1
Percent of total	100.0	60.3	30.5	8.3	0.9
Cumulative percent	100	60.3	90.8	99.1	100.0

Ref.: Richard L. Woodward and Graham Walton. 1957.

- c Data sometimes show apparent anomalies which necessitate additional test procedures or special tests on selected samples in order to account for seemingly unusual results or to establish the validity of the results being obtained.

VI INTERIM REVIEW OF DATA

It is essential that periodic review of data be conducted during the survey. It is suggested that such reviews be conducted daily during the initial stages of the survey and thereafter at weekly or biweekly intervals.

- A Review can reveal the need for changes in procedures to meet the objectives of the survey.
- B Changes needed in sampling plan can be revealed. According to the type of results being obtained, it may be necessary to add further sampling stations, to delete certain

stations, or to modify the schedule of sample collection.

- C Need for additional test procedures may be demonstrated.
- D Interim data may show that certain test procedures are producing data of no value in meeting survey objectives, such procedures can be dropped.

VII FINAL REVIEW OF DATA AND PREPARATION OF REPORT

Development of the survey plan, performance of the planned operations to meet survey objectives, and the final data analyses and preparation of report are interdependent activities.

There are many techniques available for summarizing and presenting data for interpretation of findings. This topic is discussed at greater length in the outline titled "Presentation and Interpretation of Bacteriological Data "

Laboratory Operations

I INTRODUCTION

Part 1 of this outline has been concerned with planning the bacteriological aspects of a survey. With this part of the outline, certain specific recommendations are presented with respect to personnel qualifications and work schedules, desired location and features of laboratory facilities, and the care and handling of samples prior to starting test procedures.

II PERSONNEL

A Skill Levels

- 1 The principal bacteriologist should have at least 3 - 5 years of professional experience in the sanitary bacteriology of water. The duties of the principal bacteriologist, who may or may not personally perform the laboratory procedures, have been identified in Part 1 of this outline.
- 2 Subordinate bacteriologists preferably should have at least 1 - 2 years of working experience in water bacteriology.

The laboratory bacteriologist is responsible for the correct testing of samples according to predetermined plans and for preparation of accurate records of results, in an orderly manner. Nonprofessional laboratory assistants, if employed, must be closely supervised in all duties undertaken.

- 3 Nonprofessional laboratory assistants may be needed. In a short-term, highly intensive survey, the writer is reluctant to employ any but experienced laboratory helpers. Such experience can best be gained in a fixed laboratory where close supervision and direction are available for routine operations than would be possible with the short-term intensive survey operations.

Duties of nonprofessional laboratory assistants include washing and sterilization of glassware, preparation of sample bottles and related supplies, preparation and maintenance of culture media supplies, and related duties.

B Work Loads

- 1 Based on the assumption of utilization of highly skilled, fast, laboratory workers, the bacteriologist-consultants of many water quality surveys have recommended a maximum of 20 coliform determinations per man-day of bacteriology laboratory service. This includes media preparation and other laboratory support service.
- 2 Additional tests may require adjustment of work loads.
 - a Fecal coliform tests require a slight addition of time. For planning purposes however, coliform tests plus fecal coliform tests have been considered to permit the 20-samples per man-day. Probably 16 samples per man-day is more realistic.
 - b The addition of fecal streptococcus tests to the coliform and fecal coliform tests requires major readjustment in the number of daily samples. With this series of tests, a maximum of 10-14 samples can be examined per man-day of laboratory service.
- 3 When considering above work loads, the customary 8-hour day may not be possible.

For a short-term, highly intensive, survey there usually is little difficulty in operating on this basis, provided that personnel are motivated to the task at hand. With long-term investigations, personnel levels should be held at such levels as to permit normal work-days and work-weeks with off-duty days provided at periodic intervals.

III LABORATORY FACILITIES

A Location

The requirement for prompt examination of samples after collection demands attention to the location of laboratory facilities.

- 1 With suitable transportation arrangements it may be possible to perform examinations in established water bacteriology laboratories. This is to be preferred if at all possible. Recently, increasing attention has been given to air transport of samples as a means of resolving this problem.
- 2 With limitations on available transportation it more commonly is necessary to establish a temporary laboratory. This may be temporary space in established laboratories, as in local or hospital laboratories, or it may be necessary to use a mobile trailer laboratory.
- 3 It is not practiced to conduct the entire bacteriological examination of water in stream surveys under field conditions, even with membrane filter methods. In some cases, however, it may be necessary to inoculate samples into primary tube media or on membrane filters in the field. In such samples, temporary incubation must be provided (no icing of inoculated samples) for transport of the sample to the laboratory where the remainder of the incubation and subsequent laboratory procedures are performed.

B Space

The bacteriology laboratory requires provision for several functions, each of which requires a significant amount of space. To give assurance that adequate space is provided, the following functions must be considered:

1 Bench space

Bench space of the type used for chemical determinations is suitable for bacteriological work. Unless the number of bacteriological samples is quite small, the bench space should be reserved for this function. About six lineal feet of bench space per worker is a minimum allowance.

2 Preparation area

If the number of daily samples is low, it may be feasible to make and dispense culture media, prepare sample bottles, etc., at the laboratory bench. If the work load is large, one general area should be reserved for media preparation, washing and sterilization of glassware, and other supporting functions.

3 Incubation and sterilization

Space must be provided for certain fixed laboratory equipment, such as washing equipment, dry sterilizer and autoclave, water bath, and incubator, according to the work load anticipated for the survey.

IV SUPPLIES

A Source

In survey planning, consideration should be given to preparing all culture media and reusable supplies in a central laboratory, and transporting such supplies to the field laboratory site. The practice frequently is more economical than efforts to provide such support at a field site.

B Sample Bottles

- 1 All sample bottles must be clean, sterile, and free of substances unfavorable to bacterial survival. Although a wide variety of containers is acceptable, the preferred form is a glass-stoppered, wide-mouth borosilicate glass bottle, having about 250 ml capacity.
- 2 Some samples, such as effluent from some waste treatment plants, may contain residual chlorine. In such cases, enough sodium thiosulfate is introduced into the clean sample before sterilization to give 100 mg/l thiosulfate in the sample. For example, add 0.2 ml of a 10% solution of sodium thiosulfate to a 250 ml bottle such as described above. This will dechlorinate samples containing up to about 23 mg chlorine per liter. The bottle is dry or moist heat sterilized following introduction of the sodium thiosulfate.

Laboratory tests have shown that this amount of sodium thiosulfate has no adverse effect on the survival and growth of coliform bacteria or fecal streptococci.

V SAMPLE COLLECTION AND HANDLING

A Collection

- 1 In many cases, sampling is limited to surface grab samples. The opened bottle is plunged into the water to a depth of about one foot. The sample bottle never should be filled more than one-half to three-fourths full. This is to facilitate mixing by shaking when laboratory tests are started.
- 2 If but one sample is taken from each station at the selected intervals, it usually is collected from near the center of the main channel of flow. Preliminary tests may demonstrate need for multiple samples from each station. In this case, it may be necessary to collect samples from predetermined points across the body of water

and/or at designated depths. Such samples are not composited and retain their identity as single samples throughout the laboratory testing procedure.

- 3 The sample collection is made in such a manner as to insure obtaining a sample representative of the source. Thus, if sampling is from a boat, the bottle should be dipped into the upstream side of the boat, with the mouth of the bottle also directed upstream. If there is no discernible current, then the bottle is moved through the water in a direction away from the hand holding the bottle.
- 4 If depth sampling is indicated, it is necessary to use special depth samplers, and open them at the desired depth.

B Identification

- 1 All samples must be immediately and fully identified at the time of collection, including at least the sample location, and the date and time of sampling. Many workers use a supplemental sheet to record temperature, pH and other data.
- 2 The sample tag should be affixed to the sample bottle. If the bottle has permanent identifying marks, a supplemental sheet may be used to identify the sample.
- 3 Wax pencils are not satisfactory. Indelible pencils are preferred. Any marking material that will run if wetted will be unsatisfactory.

C Care in Transit

- 1 Time between sample collection and starting tests
 - a The test procedures should ideally be run immediately, or if this is not feasible, should preferably be started within 1 hour after sample collection; it never should exceed 8 hours (six hours transit time and two hours laboratory).

- b With some samples, such as those from heavily polluted streams, even greater limitation on this interval is necessary.

2 Temporary storage

With temporary storage of samples, "Standard Methods" now recommends the practice of icing samples prior to examination.

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Descriptors: Personnel, Planning, Laboratory Equipment, Laboratory Tests, Microbiology, Sampling, Surveys, Water Quality

WATER QUALITY SURVEYS
PRESENTATION AND INTERPRETATION OF BACTERIOLOGICAL DATA

I OBJECTIVES

Bacteriological data analysis consists of an orderly assembly and summary of the data obtained in the investigation, in the simplest available manner, leading to demonstration and explanation of the observed results in terms of the initial survey objectives. The topics of summary of data and interpretation of data are discussed separately in this presentation; however these are mutually interdependent activities.

A There are many ways in which data can be summarized and analyzed. The most commonly used procedures are described in this outline.

B A number of criteria apply to the selection of procedures for data analysis:

The analysis must be -

- 1 Understandable by readers of the report;
- 2 Consistent with survey objectives,
- 3 Accurate summary of data, and
- 4 *Representative of technically sound reasoning.*

II PRESENTATION OF QUANTITATIVE DATA

A Data on coliform bacteria, fecal coliforms, and fecal streptococci must be presented on a quantitative basis if useful interpretations are to be drawn. Partition counting of coliforms (IMViC typing) or streptococci (biochemical characterization or species identification) should be done on a quantitative basis, through study of a representative number of (100 for example) pure cultures, with determination of percentage of occurrence of each identified variant.

B Development of Expression of Central Tendency

1 Single central value

a Median

The median is determined by assembling all data in an array of ascending or descending order. The median value is the central value, a positional value in the array.

b Arithmetic mean, or average. This is much influenced by individual extremes.

c Geometric mean. (Logarithmic mean)

Many workers prefer use of this value, as it includes all determinate values but minimizes the effects of extremely high or extremely low values.

d In selecting the form of expression of central value, it is necessary to know the methods of calculation required in meeting established standards. For example, geometric mean values often are lower than arithmetic mean values on identical data. If the standards of quality are based on use of arithmetic mean values, it would be necessary to use the arithmetic mean value in the data analysis.

2 Distributions of values

a Data may be calculated and arranged on a distribution table. The method has particular usefulness when the investigation is related to designated standards of water quality.

- b The central value may be obtained by one of the above methods, plus showing the maximum value at the designated sampling point.
- c The central value may be obtained, plus a percentage range for the data from a single location. Some investigators recommend use of the geometric mean value, plus the range of the middle 80 percent of all values from the data being consolidated. The method permits expression of average water quality (geometric mean) and the maximum and minimum quality demonstrated. Exclusion of the extreme 10 percent of high and low values reduces the danger of misinterpretation by ascribing particular significance to the sporadic extremes of values which may not be truly representative of quality of the water.

C Values Used to Describe Numbers of Organisms

- 1 Number per 100 ml of water. This is direct use of data obtained in laboratory work, and may be a Most Probable Number value; or it may be a value obtained by membrane filter methods or plate count methods in which a simple calculation is made to relate the number of colonies to the volume of sample tested, computing the number of test organisms per 100 ml.

This form of data expression appears to be most applicable to investigations for compliance with established water quality standards, or to trace pollution indicators when there is a relatively fixed volume of water.

- 2 In stream surveys where there may be multiple sources of pollution with changes in volume of the receiving

water through waste outfalls, or juncture with other streams, it may be necessary to determine the total number of bacteria and trace their fate in the investigation.

Because the numbers of organisms may reach nearly astronomical proportions, and to simplify presentation, consolidated values often are used.

- a Bacterial Quantity Unit (QU) is the number of bacteria passing a given point in one day if the concentration is 1000 organisms per milliliter, and the stream flow is 1 cubic foot per second. One QU is equivalent to 2.45×10^{12} bacteria.
- b The Bacterial Population Equivalent (BPE) is another useful tool for consolidating data and representing the total number of organisms in the water being studied. This value does not represent the number of coliform bacteria discharged by an individual but, instead, represents a value obtained by determining the relationship between the number of coliform bacteria passing some downstream point and the number of individuals in the community discharging wastes into the flowing waterbody. The BPE (calculated sewered populations) are derived by converting flows of cfs to flows of 100 ml/day, multiplying by density of coliforms (MPN/100 ml) and dividing the total numbers by 400 billion/day (summer data) or 125 billion/day (winter data).

Summer (15 C. or more):

$$BPE = Q \text{ (cfs)} \times \text{MPN}/100 \text{ ml} \times (6.1 \times 10^{-5})$$

Winter (less than 15 C.)

$$BPE = Q \text{ (cfs)} \times \text{MPN}/100 \text{ ml} \times (1.95 \times 10^{-4})$$

D Methods of Presentation of Data

1 Distribution tables (Table 1)

Table 1. Data Presentation by Showing Distribution of Values

MPN over	2	10	50	100	250	500	750	1000	2500
no. tests	50	49	49	36	31	6	1	1	0
percentage	100	98	98	72	62	12	2	2	0

2 Circles, histograms, or other geometric figures superimposed on maps to show, by relative dimensions, relative values obtained.

3 Graphs

4 Development of mathematical expressions to describe death rate of the organisms.

a The method involved determination of a peak value for coliforms at some point immediately downstream (10 - 15 hours flow time) of the point of discharge.

b The decreasing numbers of bacteria often can be described by the equation

$$Y = A \times 10^{-bt} + C \times 10^{-dt}$$

Where:

Y = the fraction of the peak number of bacteria remaining after the time, t ;

A = the fraction of the bacteria out of the peak number which decrease at a rapid rate;

b = the rate at which the "A" fraction of bacteria decrease;

C = the fraction of the bacteria out of the peak number which decrease at a different (lesser) rate; and

d = the rate at which the "C" fraction of bacteria decrease.

The values of the rate coefficient b and d are determined on the basis of the coliform data, using techniques similar to those used for development of the rate coefficient k in the BOD equation. (See Figure 1)

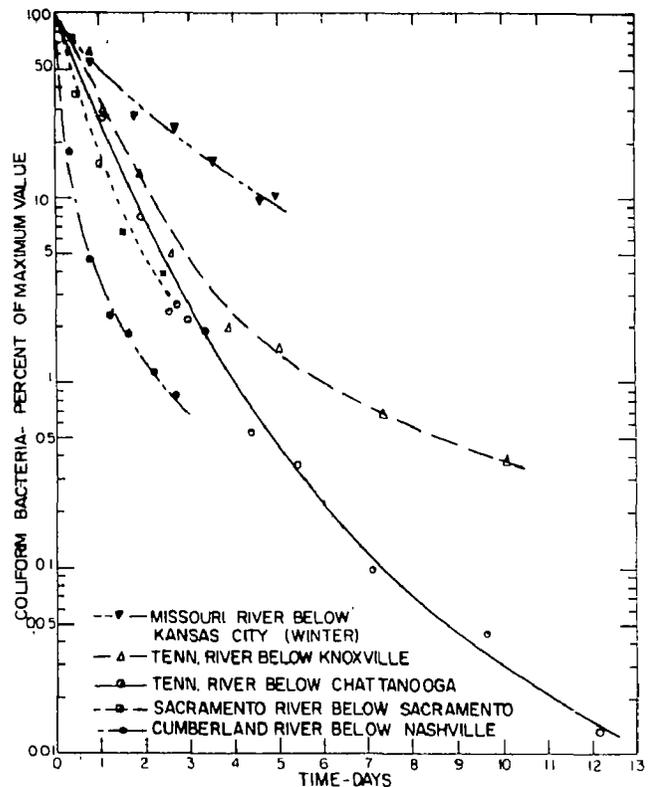


Figure 1. —Rates of coliform decrease below five selected cities.

III INTERPRETATION OF FECAL COLIFORM - FECAL STREPTOCOCCUS RELATIONSHIPS

A Development of Ratios

Using the single central-tendency values from each sampling station in a survey, it often is useful to determine the ratio of fecal coliforms to fecal streptococci. If, for example, at a given station the fecal coliform value is given as 72,000 per 100 ml and the fecal streptococcus value is 16,000, then the fecal coliform/fecal streptococcus ratio is 4.5.

B Interpretation of Ratios

- 1 When the fecal coliform/fecal streptococcus ratio is greater than 4.0, this is regarded as overwhelming evidence of pollution derived from human origin; or that if the pollution is of mixed origin, the majority of such pollution is of human origin.
- 2 When the fecal coliform/fecal streptococcus ratio is less than 0.7, this suggests pollution derived predominantly or entirely from livestock or poultry wastes. Feedlots, stockyards, and even stormwater runoff usually produce such ratios.
- 3 Ratios falling between 4.0 - 0.7 are not quite so certain. To be sure, a ratio of 3.5, for example, would be more suggestive of pollution representing predominantly human origin; and a ratio of 0.9 would be more suggestive of animal origin. A truly "gray-area" of interpretation of these ratios is in the range 2.0 to 1.0.
 - a When the ratio is in this range, it frequently represents significant mixtures of both human and animal contribution, or

- b The source of pollution may be somewhat remote, and due to differences in the rates of disappearance of the two bacterial groups, the original numerical relationships have been obscured.

4 Limitations on interpretation of fecal coliform-fecal streptococcus ratios.

- a The ratios have greatest reliability for samples taken not more than 24 hours flow time (or distance) from the origin of the pollution, and
- b The ratios must be based on waters in pH range between 4.0 - 9.0.
- c Total coliform counts cannot be used in determination or interpretation of ratios with fecal streptococci.

IV PRESENTATION OF QUALITATIVE DATA

- A For some determinations, notably demonstrations of pathogenic microorganisms, quantitative methodology either is lacking or is so expensive and time-consuming as to make sure tests completely impractical.
- B In surveys involving pollution of intestinal origin, much attention is being given currently to the demonstration of pathogenic bacteria, notably bacteria of the genus Salmonella. Such organisms, when found, are interpreted to represent positive proof of deleterious bacteriological quality of the water, since all members of the genus are disease-causing bacteria.
- C The FWQA survey report of the Red River of the North presented data on occurrence of Salmonella as shown in Table 2 and Figure 2.

Table 2. SALMONELLA ISOLATIONS - RED RIVER OF THE NORTH

Date	Station	River mile	Distance from waste source (miles)	Flow time (days)	Total coli/100ml	Fecal coli/100ml	Salmonella isolated
Sept. 1964	Moorhead sewage treatment plant	448	0		Not done	Not done	S. kentucky
	RR 11	436	12	0.5	250,000	64,500	S. kentucky
	RR 12	426	22	1.0	47,600	2,850	S. saint paul
Nov. 1964	RR 9	462	-14		314	49	Absent
	RR 10	441	7	.3	432,000	155,000	S. typhimurium S. braenderup S. reading
	RR 11	436	12	.4	249,000	85,600	S. braenderup S. heidelberg S. reading
	RR 12	426	22	.8	68,000	16,800	S. blockley S. braenderup
	RR 16	386	62	3.0	6,630	1,610	S. heidelberg
	RR 28	292	7	.3	39,800	2,970	S. reading
	RR 29	286	13	.7	27,800	1,030	S. reading S. infantis
	Jan. 1965	RR 10	441	7	.2	162,000	61,000
RR 11		436	12	.4	83,500	34,000	S. chester S. heidelberg S. st. paul S. enteritidis S. typhimurium
SH 13		428-1			650	218	Absent
RR 14		416	32	1.2	18,600	9,170	S. enteritidis S. chester S. st. paul S. thompson
RR 16		386	62	3.3	7,800	5,160	S. st. paul S. enteritidis
RR 18		375	73	4.0	6,140	2,950	S. thompson S. st. paul S. thompson

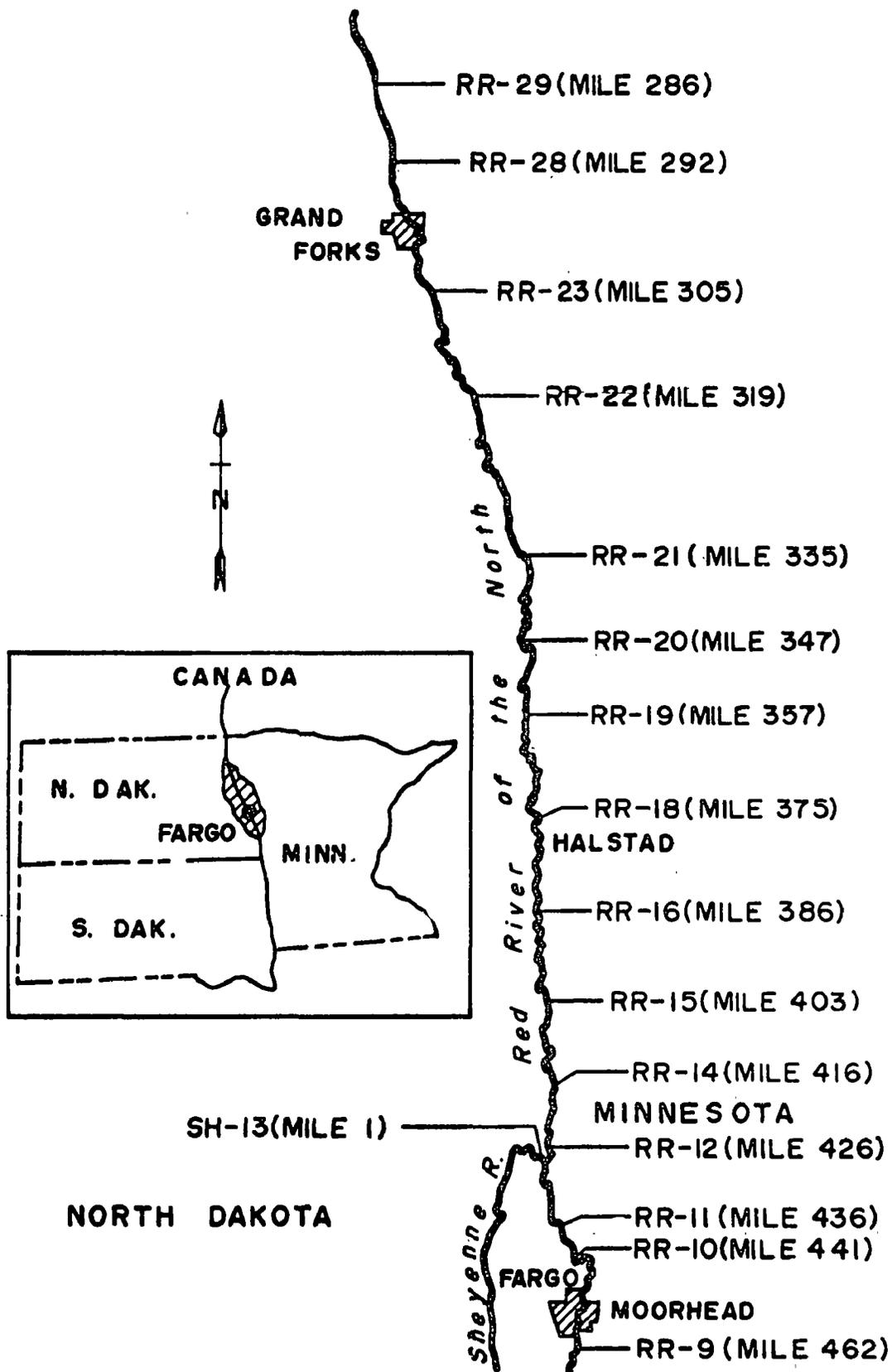


Figure 2. Location of Sampling Stations and Station Numbers (River Miles) of Samples from the Red River of the North, North Dakota and Minnesota

V CASE EXAMPLES

The following studies illustrate the ways in which certain pollution surveys became needed, the examinations made, and the ways in which data were summarized and presented, leading to the final conclusion.

A Case Study #1

1 Need

To determine whether the outfalls discharging raw sewage from a sewer population of 3820 persons in Villageville to River A between miles 20 and 18, causes a deterioration of water quality, thus constituting a hazard to the city water plant of Statesville with intake located at river mile 15.2 and the County Water Plant with the intake located at river mile 13.0.

2 Survey and procedures

A survey was established for the study of the reach of the river which included the pollution in the river above the alleged polluting city, pollution contributed from a tributary creek, the pollution contributed by Villageville and the effect on water quality at the water plant intakes. Samples were collected and examined every six hours for seven consecutive days. Bacteriological procedures were the Standard Methods multiple tube procedure by the confirmed test for the coliform group using three acceptable dilutions of 5/5/5; the test for coliforms of fecal origin; and the tentative plate count procedures for fecal streptococcal group and their confirmation by supporting biochemical tests.

3 Results

These data are presented in Table 3 and Figure 3.

4 Interpretation of the data

- a The bacteriological data obtained at sample points located at river miles 24 to 21 inclusive, established the water in the river as being of relatively good quality, with total coliform density of approximately 600, 20 to 30 fecal coliforms and 10 to 16 fecal streptococci per 100 ml. This is a relatively high-quality raw-water river supply in this area.
- b The Clear Creek tributary was an overflow from a lake with a coliform density of approximately 20 per 100 ml and both fecal coliforms and fecal streptococci were absent. In the absence of data on the cfs of Clear Creek and of River A, it is not possible to partition the bacterial densities at river mile 20 but it is evident that there is an apparent improvement in water quality due to the dilution factor.
- c Between river mile 20 and 18, a marked increase in pollution occurred with 25,000, 10,000 and 4,000, respectively, being the densities of total coliform, fecal coliform and the fecal streptococcal groups. Periodic sampling of the sewer outfalls indicated the domestic waste from Villageville was the source of the bacterial densities.
- d The bacterial data at river miles 16 to 10 inclusive demonstrated the poor quality of the water due to the pollution originating from the untreated wastes entering the river from Villageville.

- e The presence of the fecal coliform group proves that this portion of the total coliform group originated from the gut of warm-blooded animals and is present in large quantities.
- f The streptococcal group was proven by a series of biochemical reactions to be identical with the fecal streptococcal group found in the gut of warm-blooded animals and therefore confirmed the interpretation of the fecal coliform group.
- g The presence of fecal pollution may be at any time and frequently is associated with enteric pathogenic bacteria, viral agents and parasitic organisms and by these associations, is a hazard to health.
- h The presence of fecal pollution, as indicated by the data in Table 3, constitutes an unnecessary and remedial hazard and risk in the raw water supplies to the water plants.

Table 3. INDICATOR MICROORGANISMS PER 100 ml IN RIVER A

River mile	Total Coliform	Fecal Coliform	Fecal Streptococci	Remarks
24	610	30	26	
23	620	23	16	
22	600	28	10	
21	590	23	10	
20	310	9	13	Clear Creek enters at river mile 20.4.
19	2,000	600	180	Four sewer outfalls between river miles 19.7 and 18.5 from Villageville.
18	25,000	10,000	4,000	
17				
16	20,000	10,000	4,200	
15				Statesville water intake at mile 15.2
14	17,000	9,000	3,900	
13				County water plant intake at mile 13.0
12	14,000	9,200	3,940	
11				
10	10,000	8,900	3,700	

EXPLANATION OF DATA

Bacterial densities calculated as geometric mean value per 100 ml. Sample collected and examined every six hours for seven consecutive days.

Population (sewered) of Villageville, 3,820.

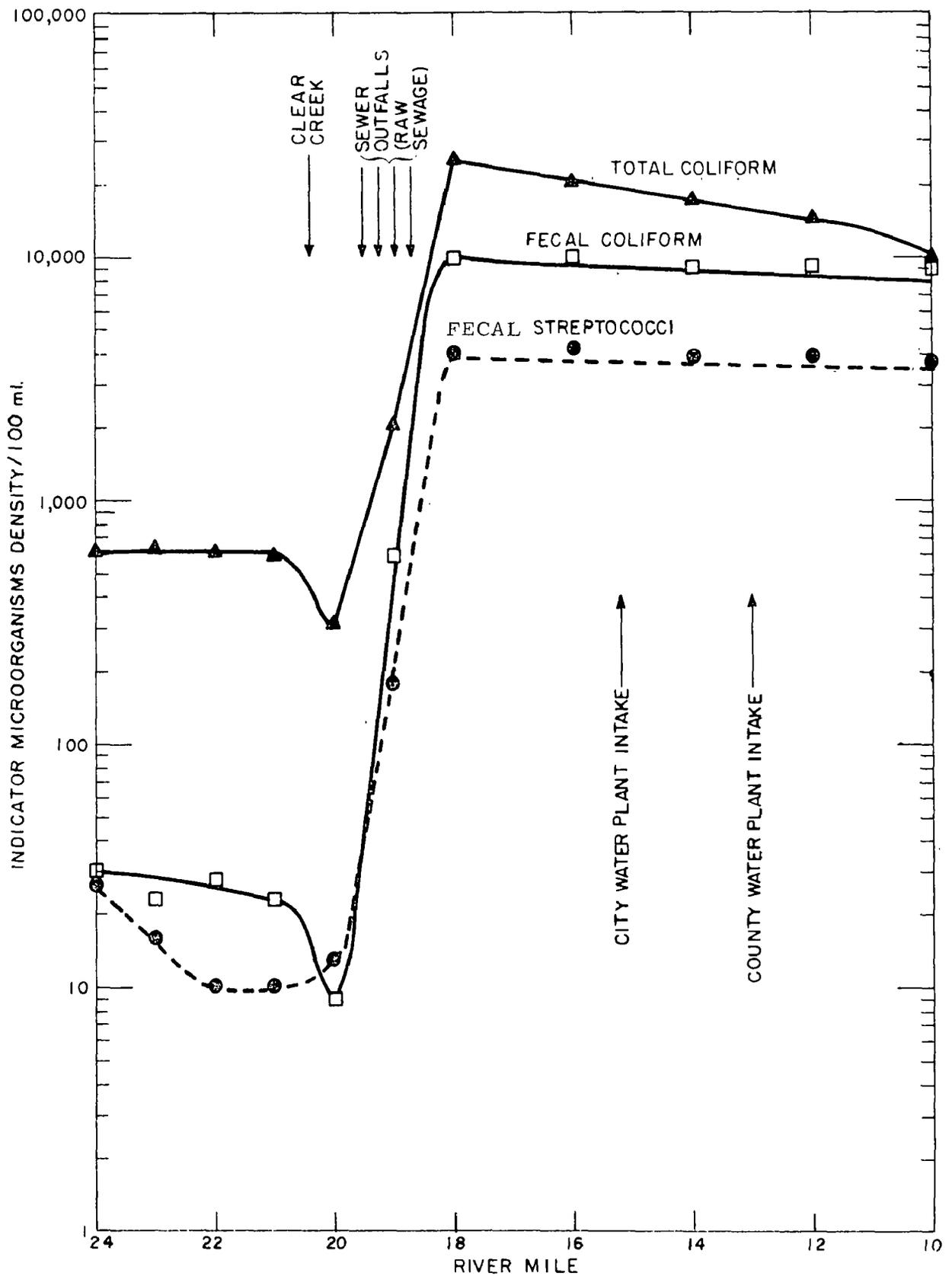
Consumers of water from city water plant, 70,000.

Consumers from county water plant, 205,000.

Velocity of river flow, 0.5 miles per hour.

Volume in cfs, information not available.

Report of sanitary survey by engineers: no known sources of pollution observed between river mile 24 and 10 except as noted under remarks.



A GRAPHIC PRESENTATION OF BACTERIOLOGICAL DATA

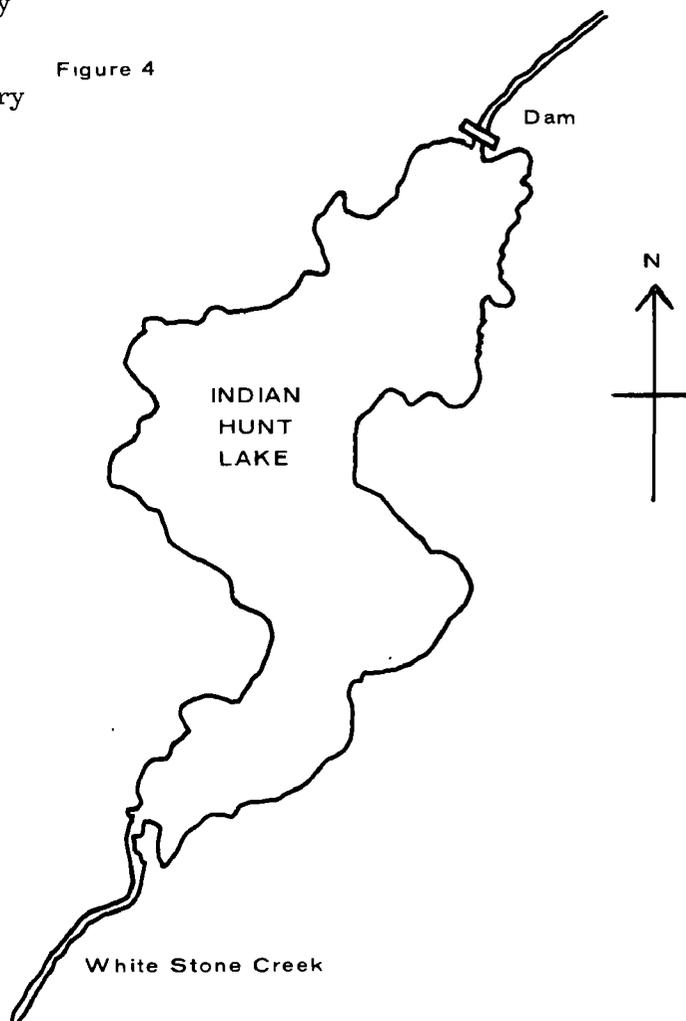
Figure 3

B Case Study #2

1 Need

A study was initiated at the request of a south western state to prepare a supplemental report on the bacteriological quality of a national wildlife refuge lake based upon state gathered data as well as an intensive study by the FWQA conducted during the same year. A massive localized fish kill and observed bacterial pollution indicator encroachments on the southern half of the lake precipitated an urgent need for this report. Figure 4 depicts this lake, Indian Hunt Lake, (here given a fictitious name) and its major pollution tributary source. A closer view of this source in Figure 5 shows White Stone Creek as the major influence with its tributary Freehand Brook.

Figure 4



2 Survey and procedures

Indian Hunt Lake is a man made 2000 acre wildlife refuge and recreational area. Since there is a large population of waterfowl (exceeding 1,025,000 during resting and wintering periods) and contributing polluting sources from cattle feed lots, it was apparent that data would be required during dry periods and during rainfall and subsequent land runoff periods to determine the extent of this pollution effect. The necessity of this runoff data became even more mandatory when it was ascertained that Freehand Brook was not a continual free-running tributary and this effect was only evident during periods of rainfall and the dry periods produced a dry bed and storage in Oxbow Lake. Stream flow determinations were made and from 14 year averages peak flows were determined and in this manner maximum effects upon the Indian Hunt Lake could be more easily determined from current data.

Previous data for this study area was compiled by the state agency and consisted only of the total coliform indicator taken by the MPN method in monthly intervals. A four phase plan was established to accomplish the survey objectives:

- a Acquire bacteriological data for all sample stations.
- b Acquire additional bacteriological data and note the effect of recreational use on the bacteriological quality of Indian Hunt Lake.
- c Evaluate the effect of rainfall on the receiving stream and Indian Hunt Lake.
- d Data analysis and report preparation.

A mobile laboratory study was initiated and samples were analyzed by the membrane filter technique and the indicator organisms included the total coliforms, the fecal coliforms, and the fecal streptococci.

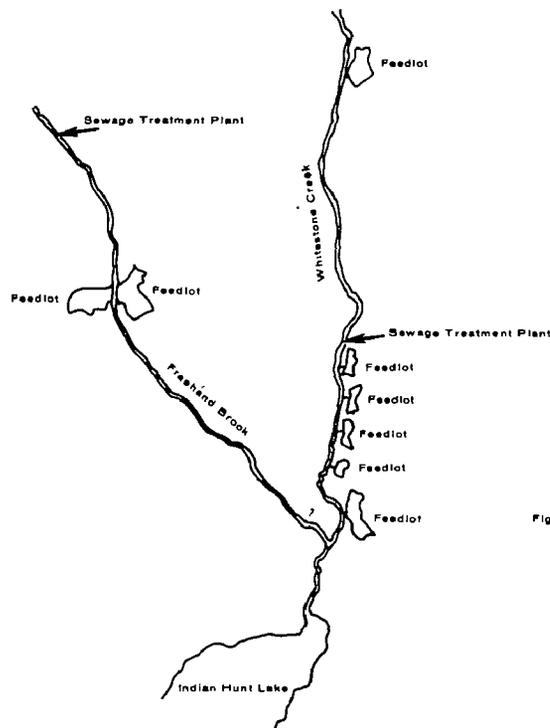


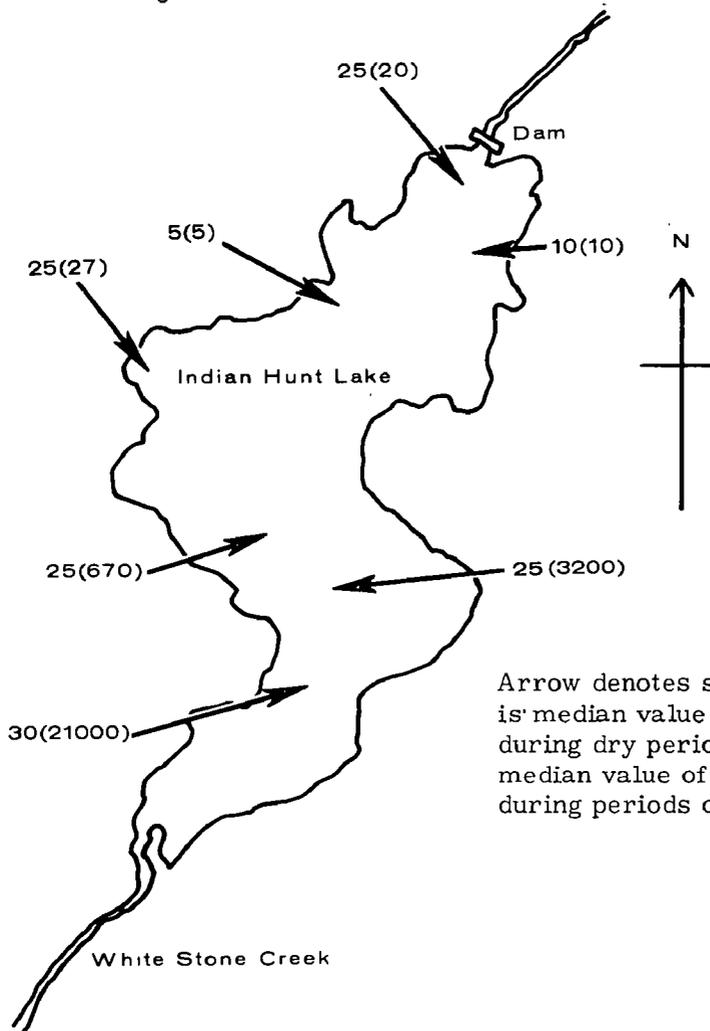
Figure 5

3 Results

After the acquisition of background data from the study area Indian Hunt Lake was sampled to ascertain the effect of recreational activities on the bacteriological quality during dry periods. Some of this data is reflected in Figure 6 which notes the fecal coliform median values per 100 ml for this

period and the numbers in parenthesis gives the median values per 100 ml during periods of rainfall, heavy storm-water runoff, and subsequent encroachment of runoff bacteria into the lake.

Figure 6



Arrow denotes sampling point. First number is median value of fecal coliform per 100 ml during dry periods and second number is median value of fecal coliform per 100 ml during periods of heavy stormwater runoff.

4 Interpretation of data

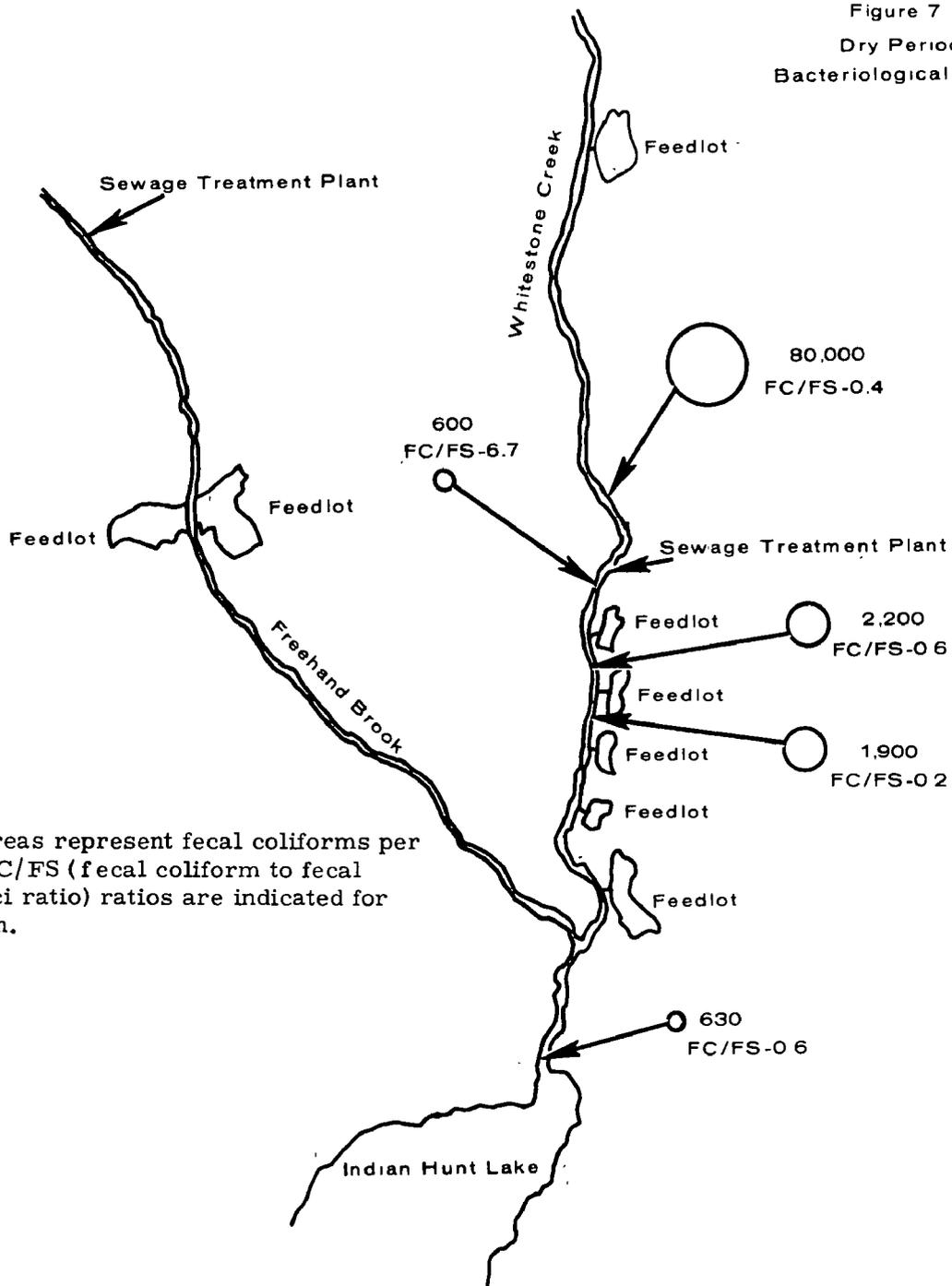
Table 4 indicates the actual recommendations given by the final report and the column designated as Remarks gives the interpretations derived from the data.

TABLE 4
Corrective Measures to More Adequately Control Discharges from Domestic Sewage and Cattle Feedlot Drainage into Indian Hunt Lake

Recommendations	Remarks
1. Development of diversion dykes around all cattle feedlots to channel drainage into waste stabilization ponds.	Influences of the cattle feedlot wastewater effluents are evident in Figure 7 and Figure 8 and this effect is pronounced during heavy stormwater overflows. Fecal coliform/fecal streptococci ratios of less than 0.7 throughout most of the tributary and of White Stone Creek indicate the predominance of animal wastes other than human.
2. Existing sewage treatment facilities should be expanded to produce a better quality effluent with a BOD reduction goal of 85-90%. Post chlorination is desirable especially during the recreation season to further reduce pathogenic hazards during this period.	Enormous bacterial indicator contributions from the sewage treatment plant is evident in Figure 8 and the resultant FC/FS ratio confirms that the effluent is from domestic wastes (greater than 4).
3. Recreational restrictions will be necessary whenever intense rainfall occurs in a magnitude sufficient to produce an inflow of more than 450 acre feet of water from White Stone Creek.	These inflow measurements were made during measurements of flows occurring during periods of intense rainfall and noting the fecal coliform count intrusions to Indian Hunt Lake. With continual facility improvements this value could be revised upwards as effluent values exhibit lower bacterial parameter counts. Current standards of 200 fecal coliforms per 100 ml would determine when restrictions could be lifted for primary contact recreation.
4. Restrictions to be placed upon the horsepower and number of pleasure-boats in the area of inflow from White Stone Creek.	Since shallow areas exist in this lake it is necessary to include this provision to prevent and control the resuspension of indicator and pathogenic bacteria from the muds and deposits.
5. A buffer zone must be continually maintained and enforced between the wildlife refuge area and those areas designated for swimming, wading, and skiing.	Figure 9 depicts these areas and calendar dates. These designated buffer zones will lessen the potential health hazards from pathogens entrapped in the organic muck and lake water sediments. Pathogens gain entrance through inflow of White Stone Creek and to a more limited extent from the waterfowl.

A similar study was made of White Stone Creek and Freehand Brook and this is shown in Figure 7 and Figure 8. In both figures (Figure 7 - dry periods and Figure 8 - after heavy stormwater overflow) the circular areas indicate the fecal coliform values and also the FC/FS ratios (fecal coliform to fecal streptococci) are indicated for this sampling point.

Figure 7
Dry Period
Bacteriological Data



Circular areas represent fecal coliforms per 100 ml. FC/FS (fecal coliform to fecal streptococci ratio) ratios are indicated for each station.

Figure 8
Heavy Stormwater Overflow
Bacteriological Data

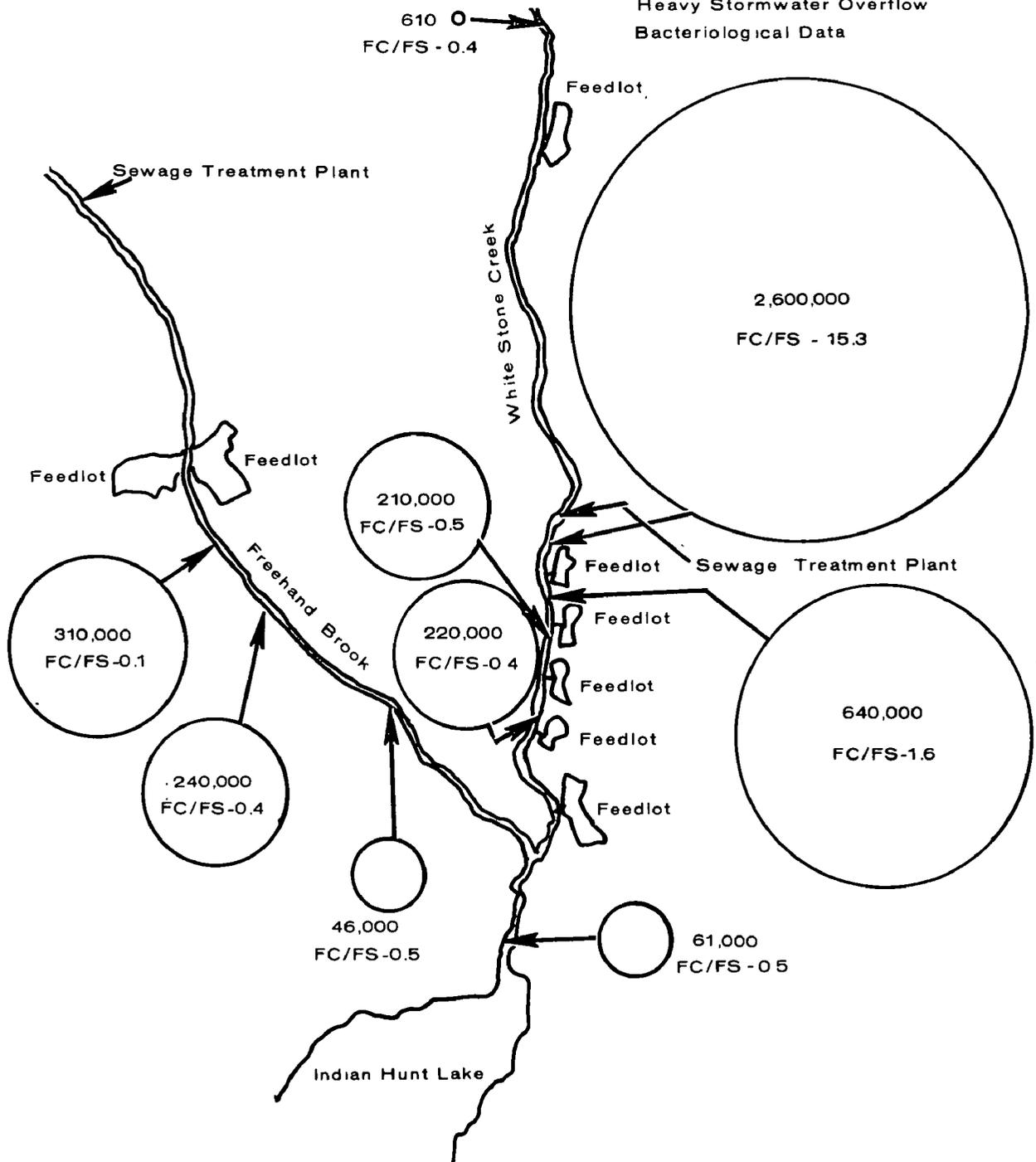
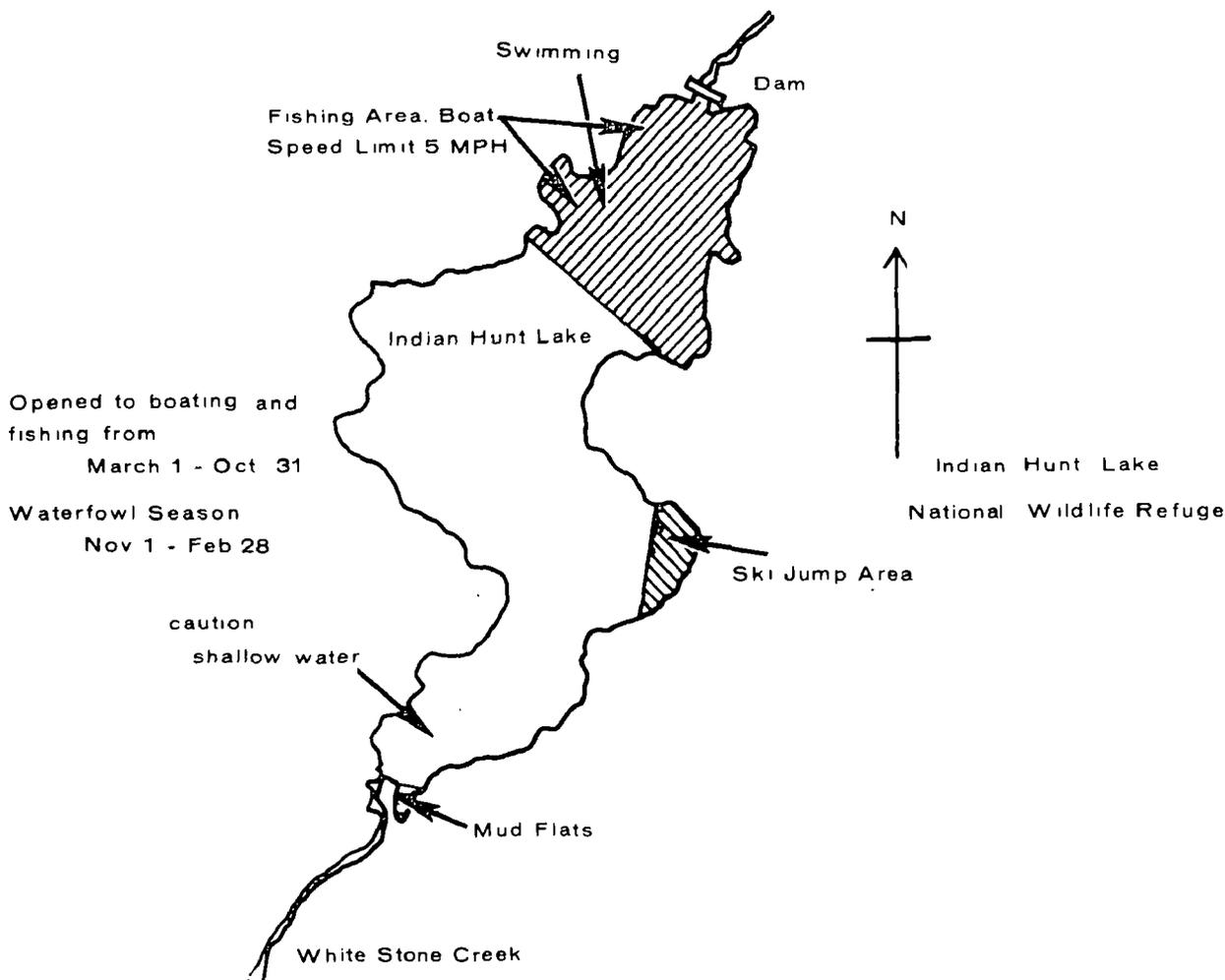


Figure 9



ACKNOWLEDGEMENT:

In the preparation of this outline the author has made extensive use of material made available by Mr. Harold F. Clark (deceased) formerly bacteriological consultant to the Enforcement Branch, DWS & PC by Mr. F. W. Kittrell (retired) formerly Chief, Technical Advisory and Investigations Activities, FWQA and Mr. Edwin Geldreich, Chief Bacteriologist, Bureau of Water Hygiene, Environmental Protection Agency, Cincinnati, OH.

REFERENCES

- 1 Outline, this manual, titled "Bacteriological Indicators of Water Pollution" and related references.

- 2 Kittrell, F.W. and Furfari, S. A. Observations of Coliform Bacteria in Streams. Jour Water Pollution Control Federation 35:1361-85 1963.

This outline was prepared by H. L. Jeter, Director, and R. Russomanno, Microbiologist, National Training Center, WPO, EPA, Cincinnati, OH 45268.

Descriptors: Data Handling, Evaluation, Microbiology, Surveys, Water Pollution, Water Quality

WATER QUALITY SURVEYS
PREPARATION OF SURVEY REPORTS

I TYPE OF REPORT

The type of stream survey report to be prepared depends on two basic factors. These are the purpose and the audience for whom intended.

A The Purpose of the Report

- 1 A report of findings or basic data
- 2 A report of existing causes and effects together with an explanation of how and why.
- 3 An exposition of existing causes and effects and a projection of conditions that reasonably may occur due to natural variations in stream flow and temperature.
- 4 A purpose similar to 3 above plus a prediction of the effects of population growth and industrial change.
- 5 The same purpose as 4 above plus an estimate of the need to protect water uses, and cause reduction in waste loads.

B The Specific Audience for Whom the Report Is Prepared

- 1 For the record - no expository purpose
- 2 Other technical agencies with competencies in the same field
- 3 Other technical agencies in other fields
- 4 Public officials supporting or opposing the recommendations of the report
- 5 The general public

C Both in style and content the report should be adequate to serve as a basis for action to accomplish the recommended objectives.

II ORGANIZATION OF REPORT

A Title, Authors, Contents

B Acknowledgement of Aid and Assistance

- 1 Can be included in a letter of transmittal or submission
- 2 Can be incorporated in a preface or foreword
- 3 Should include names of persons and of corporations, public and private, who assisted or aided the survey

C Authority

- 1 Source of Authority
- 2 Date of authorization

D Report Summary

- 1 A brief summary of the report and its recommendations generally precedes the report proper and should include three topics:
 - a Summary of specific findings
 - b Conclusions drawn from findings
 - c Recommendations in general terms.
- 2 Brevity is essential but not at the expense of clarity.
- 3 A very brief but lucid description of the stream section involved should be included.
- 4 This will be the only part of the report read by many of its audience. Consequently it should be drafted with the utmost care.

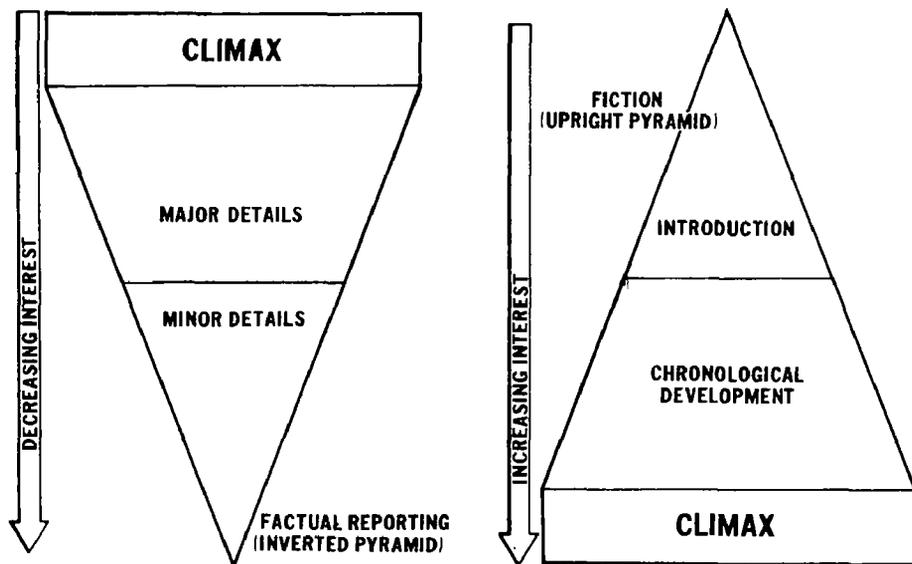


Figure 1

- 5 Figure 1 illustrates the principles of written communication format. It is important that technical reports be presented in factual report arrangement and NOT in one similar to that of fiction.
- 6 Figure 2 shows how the various portions of a survey report relate to the generalized factual report.
- 7 Recommendations, although briefly stated in general terms, should be couched in positive, unexaggerated language.
- 8 Cost estimates of compliance with recommendations is helpful but not essential.
- 9 Both tangible and intangible benefits may be listed briefly under conclusions.

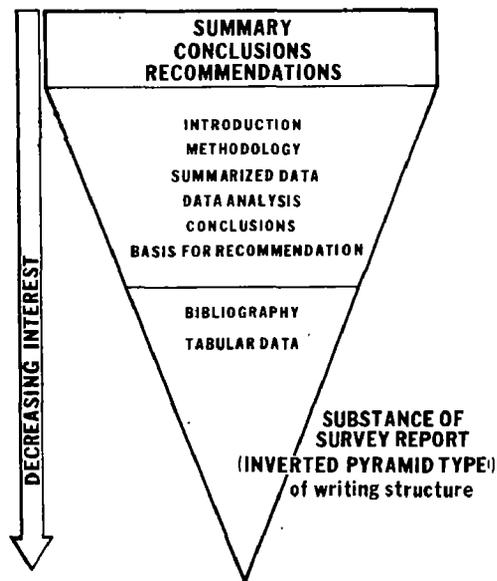


Figure 2

E INTRODUCTION

The body of the report should begin with a statement of the problem and a discussion of the reasons for and the location of the study.

- 1 Statement of the problem
 - a A description of the area, emphasizing pertinent features, should be included.

- 1) The inclusion of pertinent historical data is of value for audience orientation.
 - 2) The relationship of this study to other current water resource studies.
 - 3) Water use and economic data may be important.
- b An area map is an absolute necessity.
- 1) Features included should be carefully selected and section of the stream involved should be emphasized.
 - 2) Do not include unnecessary detail.
 - 3) A general location map usually orients the reader to the area map.
- 2 Objectives of the Survey
- a A statement of the purpose by listing the specific answers sought by the survey to address various aspects of the problem.
 - b The geographical and time scope of the objectives of the survey.
- F Survey Methodology
- A complete description of the methods of study employed is an important part of the record.
- 1 The time period of survey should be noted.
 - 2 All sampling and gauging station locations should be identified by river mile.
 - 3 Sampling and analytical methods
 - a Provide adequate description of all non-standard methods.
 - b An appendix for these descriptions may be required if they are lengthy.
 - 4 Frequency of sampling
 - 5 Description of laboratory types and locations
 - 6 Hydrological methods employed for:
 - a Times of water travel
 - b Stream flow data
 - c Any waste flow measurements
- G Survey Results
- 1 Sources of wastes
 - a Computed waste loads based on known contributing populations and industrial waste strength
 - b Results of sampling and gauging program
 - c Data summaries or displays suffice for text of report.
 - 2 Stream data
 - a Summarized survey in the text
 - b Complete tabulations including time of collection and averages in appendix
 - 3 Hydrological data
 - a Usually tabulated with analytical results both in the text and in appendices
 - b Time of water travel curve or curves
 - c Stream flow frequency charts
 - d Pertinent groundwater data
 - 4 Aesthetic considerations are of real importance.

H Analysis and Interpretation of Data

- 1 Three fundamental procedures are required:
 - a Comparison of survey results and appropriate water quality criteria
 - b Projection of survey data to provide for comparison of stream conditions with water quality criteria under more adverse conditions
 - c Estimates of permissible wastes loads under present and future conditions
- 2 Since this topic is the subject of other outlines it will not be further developed here.
- 3 A description of the methods of analysis and interpretation belong in the report.
 - a Necessary assumptions should be stated
 - b All statistical methods used should be identified.
- 4 Results of analysis and interpretation are best presented in chart form insofar as possible, to support discussion and interpretation rationale.

I Conclusions

- 1 Focus of attention, much of it critical, is on this section of the report.
- 2 Clearness, conciseness, and positiveness are essential.
- 3 This section indicates reasoning that leads from findings to conclusions.

J Recommendations

- 1 This is the crux of the report and should answer the question, "What needs to be done to resolve problems delineated in the report?"

- 2 Cost estimates are highly desirable, if possible, and appropriate

K Bibliography

- 1 Useful to the student and perhaps to future workers
- 2 Entails additional time and effort in assembling references in proper form

III TECHNIQUES AND TOOLS

A Prepare a Detailed Outline

- 1 This is an important step if the coverage of the report is to be completed and its arrangement logical. It is also a time-saver, if properly done
- 2 Topics to be included in the outline will become apparent from the foregoing discussion of report content.

B Words are Your Tools

- 1 Keep dictionary, thesaurus, and glossary available
- 2 Define any vague terms or abbreviations
- 3 Control superlatives and slang.
- 4 Avoid emphatics such as "it is to be noted" or "it is a well-known fact".
- 5 Limit intensive expressions, such as "extremely" or "undoubtedly."
- 6 Use active verbs when possible.

C Regularly review the report organization and development with a colleague.

This outline was prepared by Staff Members, National Training Center, EPA, WPO, Cincinnati, OH 45268

Descriptors: Reports, Surveys, Technical Writing, Water Quality

BACTERIOLOGICAL TESTS IN EPA WATER QUALITY SURVEILLANCE SYSTEMS

I INTRODUCTION

A The Pollution Surveillance Systems

The Water Quality Surveillance Systems Section collects chemical, physical, biological and microbiological data on a continuing basis from selected sampling sites throughout the United States, for the purpose of determining the status of water quality and pollution control. The Surveillance Systems also acquire and publish specific information on problems such as pollution-caused fish kills, the financing and construction of sewage treatment facilities, and inventories of the existing sewage systems, populations served, types of treatment and need for additional facilities.

B Organization

The Technical Data and Information Branch directs the surveillance program and provides data information service, data evaluation and computer operations. The ten regional offices carry out the acquisition of data through their own laboratory and field support facilities and with the cooperation of other agencies such as the USGS, Corps of Engineers, TVA, Bureau of Reclamation, the states and basin authorities (e. g. ORSANCO or the Miami River Conservancy District).

The surveillance program is designed upon the major river basin concept. Regional administration of the systems places EPA personnel in close contact with local problems and the specific sample sites.

C Objectives

The long term objectives of the pollution surveillance program are to identify:

- 1 Compliance or non-compliance with water quality standards.

- 2 Baseline water quality and long term trends.

- 3 Improvement in water quality produced by abatement measures.

- 4 Emerging water quality problems.

The program recognizes that the surveillance systems merely monitor water quality. Additional short term concentrated surveys are necessary, for instance, to acquire enforcement data or pinpoint sources of pollution problems.

D Origin

The present surveillance systems originated with 17 stations under the PHS National Water Quality Network in 1957. The data were published in annual Water Quality Reports through water year 1963. The data are now entered into STORET, the Environmental Protection Agency central system for data storage and retrieval, and can be rapidly retrieved to satisfy the needs of various water quality agencies.

E Scope

Currently in the overall surveillance program there are over 900 sample stations monitored for various parameters by federal agencies and 600 to 800 monitored by the states. The most frequently tested parameters are temperature and pH. Microbiological parameters are performed on the majority of these stations, wherever there is a recreational use or the need to assess the suitability of a potable water supply.

The Pollution Surveillance Section plans to completely integrate the state-federal network by 1975. At that time the program will include 900 stream and 1500 open water federally-funded stations and an additional 7,000 to 10,000 state-funded stations are anticipated.

II BACTERIOLOGICAL SURVEILLANCE

The program utilizes the recognized indicators of bacterial pollution:

A Need

Bacteriological data are used in the detection system:

- 1 Total coliforms
- 2 Fecal coliforms
- 3 Fecal streptococci

- 1 To enforce water quality standards - the computer will rapidly compare current data to bacteriological standards set by the states and previously entered into STORET.
- 2 To monitor water quality for the effectiveness of pollution control.
- 3 To provide an early warning system for bacteriological pollution.
- 4 To establish water quality baselines.
- 5 To reflect long term trends.
- 6 To indicate more sudden changes or seasonal variations.
- 7 To plan and manage comprehensive water quality programs.

The regions are encouraged to use the standard methods whenever practical. The immediate membrane filter tests are the method of choice where applicable. Field methods are necessary in some regional programs. The original PHS Basic Data Network relied upon the delayed MF procedure for total coliform analyses. This procedure is now used when it is impractical to get results by conventional methods.

B Regional administration of Surveillance Systems

D Quality Control

The Chief of Pollution Surveillance in each region with the support of laboratory personnel determines the specific details of the program according to regional and state needs such as:

The following steps are taken to ensure that the bacteriological data acquired by the surveillance system and entered into STORET is acceptable.

- 1 Selection of sampling sites.
- 2 Collection of samples.
- 3 Frequency of sampling.
- 4 Bacteriological parameters required.
- 5 Bacteriological methods.
- 6 Data reporting.
- 7 Entry of data into the STORET system.

- 1 Standard Methods are followed wherever possible. USGS Water Resources Division has written a manual of "Selected Interim Procedures for Biological and Microbiological Investigations" describing the methods to be followed by USGS personnel.
- 2 Agencies and states encourage personnel performing bacteriological surveillance tests to acquire adequate training including the NTC course "Current Practices in Water Microbiology."
- 3 Regional surveillance personnel and Quality Control Officers maintain close contact with the laboratories contributing data to the program.

C Bacteriological parameters and methods

- 4 Intralaboratory quality control measures such as colony confirmation, replicate analyses and repeat counting by more than one technician are recommended.
- 5 Identification of data entered into the STORET system includes the parameter code, method and source of the data and related information.
- 6 If the Delayed Incubation MF Procedure is used, results from the same sampling points should be compared to those performed by the standard immediate test procedure.

III THE DELAYED INCUBATION PROCEDURE (References 1-4)

A Applications

The Delayed Incubation Method is necessary because bacteriological samples must be analyzed as soon as possible after collection before bacterial populations change drastically and no longer represent the count at the time of sampling. This test is useful where:

- 1 It is not possible to maintain the desired sample temperature during transport.
- 2 When the elapsed time between sample collection and analysis would exceed the approved time limit.
- 3 Where the sampling location is remote from laboratory services.
- 4 When it is necessary to monitor streams or waterbodies by a standardized procedure.
- 5 Other reasons which prevent the analysis of the sample at or near the sample site.

B General Test Procedure

The delayed MF Coliform Procedure is a modification of the Standard MF Method for total coliforms and consists of the following steps:

- 1 Sample collection.
- 2 Sample filtration at the collection site or transport of the iced sample to laboratory facilities for filtration as soon after collection as possible.
- 3 Placement of the inoculated membrane on the preservative medium in well-marked tight-fitting plastic petri dishes.
- 4 Shipment in mailing containers at ambient temperatures to Regional laboratories (not to exceed 72 hours).
- 5 Receipt of membranes and transfer to growth medium.
- 6 Incubation at $35^{\circ} \pm 0.5^{\circ} \text{C}$ for 22 - 24 hours.
- 7 Counting of the characteristic coliform colonies.
- 8 Computing the value per 100 ml and recording results.

Steps 1 through 4 are performed in the field or at a permanent laboratory by a cooperator, participant or technician; the remaining steps are completed in the receiving laboratory.

C Recent Research

In a recent evaluation of the Delayed Incubation Test, with reference to sea water sampling, Brezenski and Winter³ arrived at the following conclusions following statistical comparisons of 162 samples of Immediate Incubation (IMF) vs Delayed Incubation (DMF):

There was no apparent difference between recovery of coliform organisms by the two procedures at these salt water stations and reported recoveries at fresh water stations.

Although the data suggest lower $\frac{\text{DMF}}{\text{IMF}}$ ratios at low pollution stations and higher $\frac{\text{DMF}}{\text{IMF}}$ ratios at higher pollution stations, there is no significant difference between

the performance of the immediate and delayed tests at the 95% confidence level.

The delayed incubation procedure can be used to quantitate coliform bacteria in salt water samples when storage of the liquid sample would be necessary; where processing must be done in a central laboratory located a great distance from the sampling site or where immediate field analysis cannot be accomplished.

The authors state that... "the delayed incubation procedure can be expected to provide results far superior to those obtained from samples stored in bottles under various conditions of time and temperature."

IV THE DELAYED MF TEST IN THE SURVEILLANCE PROGRAM (Section III)

A Equipment and Supplies

Filtration equipment used for membrane filter analysis are provided to those participating laboratories which do not have it available. Other laboratory equipment and supplies necessary for membrane filtrations are also provided only if they are not available. Expendable supplies such as m-Endo broth, membrane filters, petri dishes, etc. are provided to participating laboratories and cooperators at six month intervals. A sample data sheet is provided at the end of this outline to indicate microbiological bench data utilizing Form FWPCA-79-12 (4-67) which may be replaced by standard forms utilized by cooperating organizations.

B Formulation

M-Endo Broth MF Code 0749

M-Endo broth contains the following ingredients per liter:

	g
Tryptone	10.0
Thiopeptone	5.0
Casitone	5.0
Yeast extract	1.5
Lactose	12.5
Sodium chloride	5.0
Dipotassium hydrogen phosphate..	4.375
Potassium dihydrogen phosphate..	1.375
Sodium lauryl sulfate	0.050
Sodium desoxycholate	0.10
Sodium sulfite	2.10
Basic fuchsin	1.05

Add 3.84 g per liter sodium benzoate (USP Grade) or 3.2 ml of a 12 percent sodium benzoate solution per 100 ml of M Endo Broth MF.

Add 500 mg per liter Cycloheximide*

*Actidione manufactured by the Upjohn Company, Kalamazoo, Michigan or equivalent.

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- 1 Geldreich, E. E., Kabler, P. W., Jeter, H. L. and Clark, H. F. A Delayed Incubation Membrane Filter Test for Coliform Bacteria in Water. AJPB 45, 1462-1474. 1955.
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- 3 Brezenski, Francis T. and Winter, John A. Use of the Delayed Incubation Membrane Filter Test for Determining Coliform Bacteria in Sea Water. In preparation, North Atlantic Water Quality Management Center, Edison, N. J. - FWPCA, DI. 1968.
- 4 Standard Methods for the Examination of Water and Wastewater, 13th ed American Public Health Association, New York, N. Y. 1971 Part VII, Routine Bacteriologic Examinations of Water to Determine its Sanitary Quality.

This outline was prepared by Robert H. Bordner, Chief, Microbiological Methods Section, Analytical Quality Control Laboratory, National Environmental Research Center, EPA, Cincinnati, OH 45268.

U. S. DEPARTMENT OF THE INTERIOR
 FEDERAL WATER POLLUTION CONTROL ADMINISTRATION
 DIVISION OF POLLUTION SURVEILLANCE
 1014 BROADWAY, CINCINNATI, OHIO 45202

FORM APPROVED
 BUDGET BUREAU NO. 42-R1489

MICROBIOLOGICAL BENCH DATA

STATION NO. AND LOCATION:				FOR DIV OF POLL. SURV. USE ONLY:					
LABORATORY LOCATION:									
SAMPLE COLLECTION BY:		DATE: YR MO DA		TIME:		SAMPLE RECEIVED DPS: YR MO DA TIME:		ELAPSED TIME, HOURS:	
SAMPLE FILTRATION BY:		DATE: YR MO DA		TIME:					
MLS UNDILUTED	MLS 1: ___ DIL	CONTAINER SERIES NO	DPS LAB NUMBER	COLIFORM COLONY COUNT	COLIFORMS PER 100 ML	REMARKS			

REMARKS _____

COMPUTER CODED DATA: (FOR DIVISION OF POLLUTION SURVEILLANCE USE ONLY)

STATION SERIAL NUMBER	YR	MO	DA																																																				
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FWPCA-79-12 (4-67) (Formerly NL-C-3)

EXAMINATION OF WATER FOR COLIFORM AND
FECAL STREPTOCOCCUS GROUPS
(Multiple Dilution Tube (MPN) Methods)

I INTRODUCTION

The subject matter of this outline is contained in three parts, as follows:

A Part 1

- 1 Fundamental aspects of multiple dilution tube ("most probable numbers") tests, both from a qualitative and a quantitative viewpoint.
- 2 Laboratory bench records.
- 3 Useful techniques in multiple dilution tube methods.
- 4 Standard supplies, equipment, and media in multiple dilution tube tests.

B Part 2

Detailed, day-by-day, procedures in tests for the coliform group and subgroups within the coliform group.

C Part.3

Detailed, day-by-day, procedures in tests for members of the fecal streptococci.

D Application of Tests to Routine Examinations

The following considerations (Table 1) apply to the selection of the Presumptive Test, the Confirmed Test, and the Completed Test. Termination of testing at the Presumptive Test level is not practiced by laboratories of this agency. It must be realized that the Presumptive Test alone has limited use when water quality is to be determined.

TABLE 1

Examination Terminated at -			
Type of Receiving Water	Presumptive Test	Confirmed Test	Completed Test
Sewage Receiving	Applicable	Applicable	Important where results are to be used for control of raw or finished water. Application to a statistically valid number of samples from the Confirmed Test to establish its validity in determining the sanitary quality.
Treatment Plant - Raw	Applicable	Applicable	
Chlorinated	Not Done	Applicable	
Bathing	Not Done	Applicable	
Drinking	Not Done	Applicable	
Other Information		Applicable in all cases where Presumptive Test alone is unreliable.	

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the Environmental Protection Agency.

II BASIS OF MULTIPLE TUBE TESTS

A Qualitative Aspects

- 1 For purely qualitative aspects of testing for indicator organisms, it is convenient to consider the tests applied to one sample portion, inoculated into a tube of culture medium, and the follow-up examinations and tests on results of the original inoculation. Results of testing procedures are definite: positive (presence of the organism-group is demonstrated) or negative (presence of the organism-group is not demonstrated.)
- 2 Test procedures are based on certain fundamental assumptions:
 - a First, even if only one living cell of the test organism is present in the sample, it will be able to grow when introduced into the primary inoculation medium;
 - b Second, growth of the test organism in the culture medium will produce a result which indicates presence of the test organism; and,
 - c Third, extraneous organisms will not grow, or if they do grow, they will not limit growth of the test organism; nor will they produce growth effects that will be confused with those of the bacterial group for which the test is designed.
- 3 Meeting these assumptions usually makes it necessary to conduct the tests in a series of stages (for example, the Presumptive, Confirmed, and Completed Test stages, respectively, of standard tests for the coliform group).
- 4 Features of a full, multi-stage test
 - a First stage: The culture medium usually serves primarily as an enrichment medium for the group tested. A good first-stage growth medium should support growth of all the living cells of the group tested, and it should include provision for indicating the presence of the test

organism being studied. A first-stage medium may include some component which inhibits growth of extraneous bacteria, but this feature never should be included if it also inhibits growth of any cells of the group for which the test is designed. The Presumptive Test for the coliform group is a good example. The medium supports growth, presumably, of all living cells of the coliform group; the culture container has a fermentation vial for demonstration of gas production resulting from lactose fermentation by coliform bacteria, if present; and sodium lauryl sulfate may be included in one of the approved media for suppression of growth of certain noncoliform bacteria. This additive apparently has no adverse effect on growth of members of the coliform group in the concentration used. If the result of the first-stage test is negative, the study of the culture is terminated, and the result is recorded as a negative test. No further study is made of negative tests. If the result of the first-stage test is positive, the culture may be subjected to further study to verify the findings of the first stage.

- b Second stage: A transfer is made from positive cultures of the first-stage test to a second culture medium. This test stage emphasizes provision to reduce confusion of results due to growth effects of extraneous bacteria, commonly achieved by addition of selective inhibitory agents. (The Confirmed Test for coliforms meets these requirements. Lactose and fermentation vials are provided for demonstration of coliforms in the medium. Brilliant green dye and bile salts are included as inhibitory agents which tend to suppress growth of practically all kinds of noncoliform bacteria, but do not suppress growth of coliform bacteria when used as directed).

If result of the second-stage test is negative, the study of the culture is terminated, and the result is recorded as a negative test. A negative test here means that the positive results of the first-stage test were "false positive," due to one or more kinds of extraneous bacteria. A positive second-stage test is partial confirmation of the positive results obtained in the first-stage test; the culture may be subjected to final identification through application of still further testing procedures. In routine practice, most sample examinations are terminated at the end of the second stage, on the assumption that the result would be positive if carried to the third, and final stage. This practice should be followed only if adequate testing is done to demonstrate that the assumption is valid. Some workers recommend continuing at least 5% of all sample examinations to the third stage to demonstrate the reliability of the second-stage results.

B Quantitative Aspects of Tests

- 1 These methods for determining bacterial numbers are based on the assumption that the bacteria can be separated from one another (by shaking or other means) resulting in a suspension of individual bacterial cells, uniformly distributed through the original sample when the primary inoculation is made.
- 2 Multiple dilution tube tests for quantitative determinations apply a Most Probable Number (MPN) technique. In this procedure one or more measured portions of each of a stipulated series of decreasing sample volumes is inoculated into the first-stage culture medium. Through decreasing the sample increments, eventually a volume is reached where only one cell is introduced into some tubes, and no cells are introduced into other tubes. Each of the several tubes of sample-inoculated first-stage medium is tested independently, according to the principles previously described, in the qualitative aspects of testing procedures.
- 3 The combination of positive and negative results is used in an application of probability mathematics to secure a single MPN value for the sample.
- 4 To obtain MPN values, the following conditions must be met:
 - a The testing procedure must result in one or more tubes in which the test organism is demonstrated to be present; and
 - b The testing procedure must result in one or more tubes in which the test organism is not demonstrated to be present.
- 5 The MPN value for a given sample is obtained through the use of MPN Tables. It is emphasized that the precision of an individual MPN value is not great when compared with most physical or chemical determinations.
- 6 Standard practice in water pollution surveys conducted by this organization, is to plant five tubes in each of a series of sample increments, in sample volumes decreasing at decimal intervals. For example, in testing known polluted waters, the initial sample inoculations might consist of 5 tubes each in volumes of 0.1, 0.01, 0.001, and 0.0001 ml, respectively. This series of sample volumes will yield determinate results from a low of 200 to a high of 1, 600, 000 organisms per 100 ml.

III LABORATORY BENCH RECORDS

A Features of a Good Bench Record Sheet

- 1 Provides complete identification of the sample.
- 2 Provides for full, day-by-day information about all tests performed on the sample.
- 3 Provides easy step-by-step record applicable to any portion of the sample.
- 4 Provides for recording of the quantitative result which will be transcribed to subsequent reports.
- 5 Minimizes the amount of writing by the analyst.
- 6 Identifies the analyst(s).

B There is no such thing as "standard" bench sheet for multiple tube tests; there are many versions of bench sheets. Some are prescribed by administrative authority (such as the Office of a State Sanitary Engineer); others are devised by laboratory or project personnel to meet specific needs.

C It is not the purpose of this discussion to recommend an "ideal" bench form; however, the form used in this training course manual is essentially similar to that used in certain research laboratories of this organization. The student enrolled in the course for which this manual is written should make himself thoroughly familiar with the bench sheet and its proper use. See Figure 1.

IV NOTES ABOUT WORKING PROCEDURES IN THE LABORATORY

A Each bacteriological examination of water by multiple dilution tube methods requires a considerable amount of manipulation; much is quite repetitious. Laboratory workers must develop and maintain good routine working habits, with constant alertness to guard against lapses into careless, slipshod laboratory procedures and "short cuts" which only can lead to lowered quality of laboratory work.

The student reader is urged to review the form for laboratory surveys (PHS-875, Rev. 1966) used by Public Health Service personnel charged with responsibility for accreditation of laboratories for examination of water under Interstate Quarantine regulations.

B Specific attention is brought to the following by no means exhaustive, critical aspects of laboratory procedures in multiple dilution tube tests:

1 Original sample

- a Follow prescribed care and handling procedures before testing.
- b Maintain absolute identification of sample at all stages in testing.
- c Vigorously shake samples (and sample dilutions) before planting in culture media.

2 Sample measurement into primary culture medium

- a Sample portions must be measured accurately into the culture medium for reliable quantitative tests to be made. Standard Methods prescribes that calibration errors should not exceed $\pm 2.5\%$.

BACTERIOLOGY BENCH SHEET

Multiple Dilution Tube Tests

Project Ohio River Survey

Sample Station Broadway Landing

Collection Data

Analytical Record

Date 2/6/67 Time 8:50 By KJ
 Temperature 8°C pH 7.3
 Other Observations _____

Bench Number of Sample 2
 Analyst Zobel-Jeter
 Test started at 11:45 By HLJ

ml sample	Coliform Test							Fecal coli- form 24	Fecal Streptococcus				Remarks	
	LTB		BGLB		EMB	LSTB			Gram stain	A - D		EVA		
	24	48	24	48	24	24	48			24	48	24		48
10	X													
10	X													
0.1	X													
0.01	X													
0.001	X													
0.001	X													

Coliform MPN/100 ml
 Confirmed:
 Completed:
 Fecal Coliform MPN:

Fecal Streptococcus MPN/100 ml
 A - D - EVA:

Figure 1. SAMPLE BENCH SHEET

Suggested sample measuring practices are as follows: Mohr measuring pipets are recommended. 10 ml samples are delivered at the top of the culture tube, using 10 ml pipets. 1.0 ml samples are delivered down into the culture tube, near the surface of the medium, and "touched off" at the side of the tube when the desired amount of sample has been delivered. 1.0 ml or 2.0 ml pipets are used for measurement of this volume. 0.1 ml samples are delivered in the same manner as 1.0 ml samples, using great care that the sample actually gets into the culture medium. Only 1.0 ml pipets are used for this sample volume. After delivery of all sample increments into the culture tubes, the entire rack of culture tubes may be shaken gently to carry down any of the sample adhering to the wall of the tube above the medium.

Workers should demonstrate by actual tests that the pipets and the technique in use actually delivers the rated volumes within the prescribed limits of error.

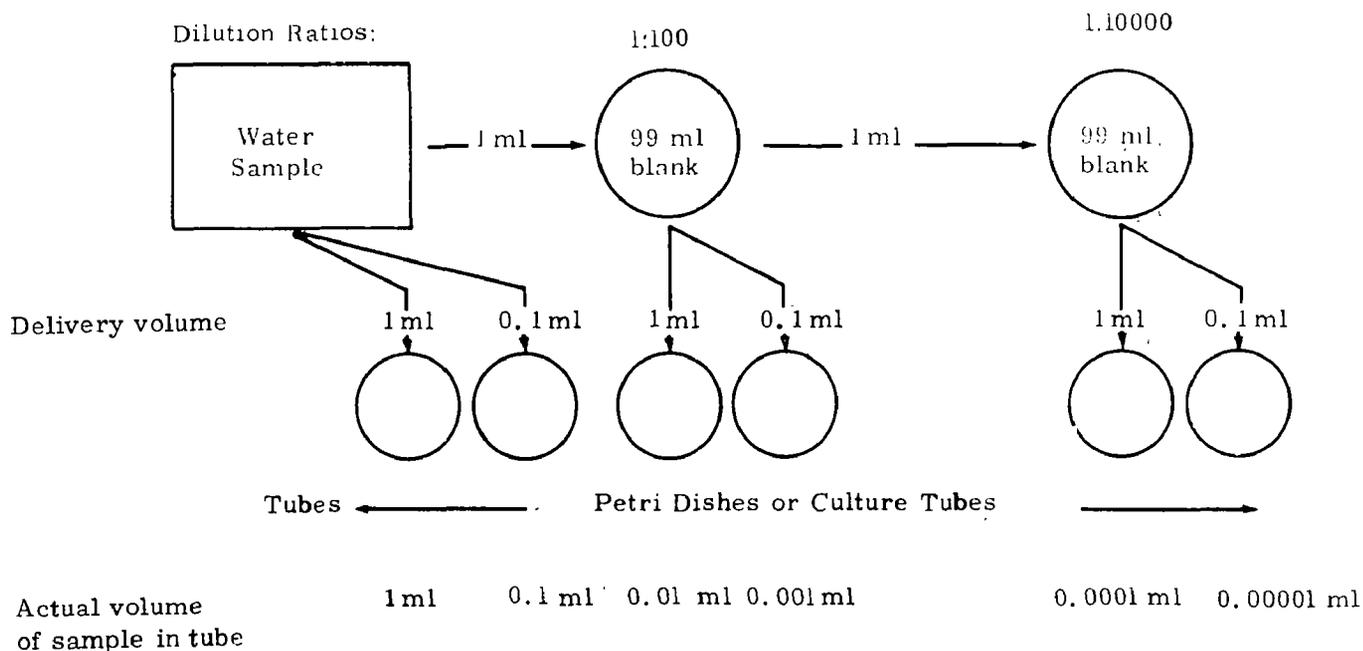
- b Volumes as small as 0.1 ml routinely can be delivered directly from the sample with suitable pipets. Lesser sample volumes first should be diluted, with subsequent delivery of suitable volumes of diluted sample into the culture medium. A diagrammatic scheme for making dilutions is shown in Figure 2.
- 3 Reading of culture tubes for gas production
 - a On removal from the incubator, shake culture rack gently, to encourage release of gas which may be supersaturated in the culture medium.

- b Gas in any quantity is a positive test. It is necessary to work in conditions of suitable lighting for easy recognition of the extremely small amounts of gas inside the tops of some fermentation vials.
- 4 Reading of liquid culture tubes for growth as indication of a positive test requires good lighting. Growth is shown by any amount of increased turbidity or opalescence in the culture medium, with or without deposit of sediment at the bottom of the tube.
 - 5 Transfer of cultures with inoculation loops and needles
 - a Always sterilize inoculation loops and needles in flame immediately before transfer of culture; do not lay it down or touch it to any non-sterile object before making the transfer.
 - b After sterilization, allow sufficient time for cooling, in the air, to avoid heat-killing bacterial cells on the hot wire.
 - c Loops should be 3 mm in inside diameter, with a capability of holding a drop of water or culture.

For routine standard transfers requiring transfer of 3 loopsful of culture, many workers form three 3-mm loops on the same length of wire.

- 6 As an alternative to use of standard inoculation loops, the use of "applicator sticks" have now been sanctioned by the 13th Edition of Standard Methods.

Figure 2. PREPARATION OF DILUTIONS



The applicator sticks are dry heat sterilized (autoclave sterilization is not acceptable because of possible release of phenols if the wood is steamed) and are used on a single-service basis. Thus, for every culture tube transferred, a new applicator stick is used.

This use of applicator sticks is particularly attractive in field situations where it is inconvenient or impossible to provide a gas burner suitable for sterilization of the inoculation loop. In addition, use of applicator sticks is favored in laboratories where room temperatures are significantly elevated by use of gas burners.

7 Streaking cultures on agar surfaces

- a All streak-inoculations should be made without breaking the surface of the agar. Learn to use a light touch with the needle; however, many inoculation needles are so sharp that they are virtually useless in this respect. When the needle is platinum or platinum-iridium wire, it sometimes is beneficial to fuse the working tip into a small sphere. This can be done by momentary insertion of a well-insulated (against electricity) wire into a carbon arc, or some other extremely hot environment. The sphere should not be more than twice the diameter of the wire from which it is formed, otherwise it will be entirely too heat-retentive to be useful.

When the needle is nichrome resistance wire, it cannot be heat-fused; the writer prefers to bend the terminal 1/16 - 1/8" of the wire at a slight angle to the overall axis of the needle. The side of the terminal bent portion of the needle then is used for inoculation of agar surfaces.

- b When streaking for colony isolation, avoid using too much inoculum. The streaking pattern is somewhat variable according to individual preference. The procedure favored by the writer is shown in the accompanying figures. Note particularly that when going from any one stage of the streaking to the next, the inoculation needle is heat-sterilized.
- 8 Preparation of cultures for Gram stain
- a The Gram stain always should be made from a culture grown on a nutrient agar surface (nutrient agar slants are used here) or from nutrient broth.

- b The culture should be young, and should be actively growing. Many workers doubt the validity of the Gram stain made on a culture more than 24 hours old.
- c Prepare a thin smear for the staining procedure. Most beginning workers tend to use too much bacterial suspension in preparing the dried smear for staining. The amount of bacteria should be so small that the dried film is barely visible to the naked eye.

V EQUIPMENT AND SUPPLIES

Consolidated lists of equipment, supplies, and culture media required for all multiple dilution tube tests described in this outline are shown in Table 2. Quantitative information is not presented; this is variable - according to the extent of the testing procedure, the number of dilutions used, and the number of replicate tubes per dilution. It is noted that requirements for alternate procedures are fully listed and choices are made in accordance to laboratory preference.

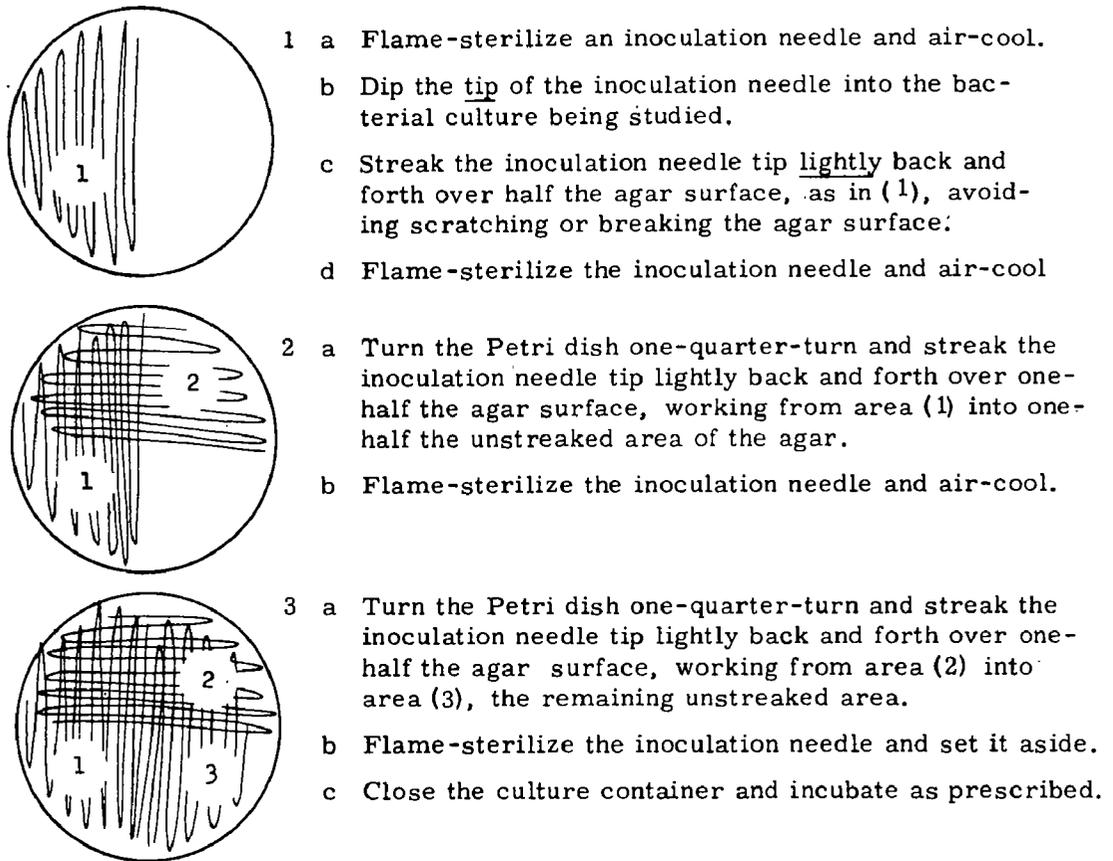


Figure 3. A SUGGESTED PROCEDURE FOR COLONY ISOLATION BY A STREAK-PLATE TECHNIQUE

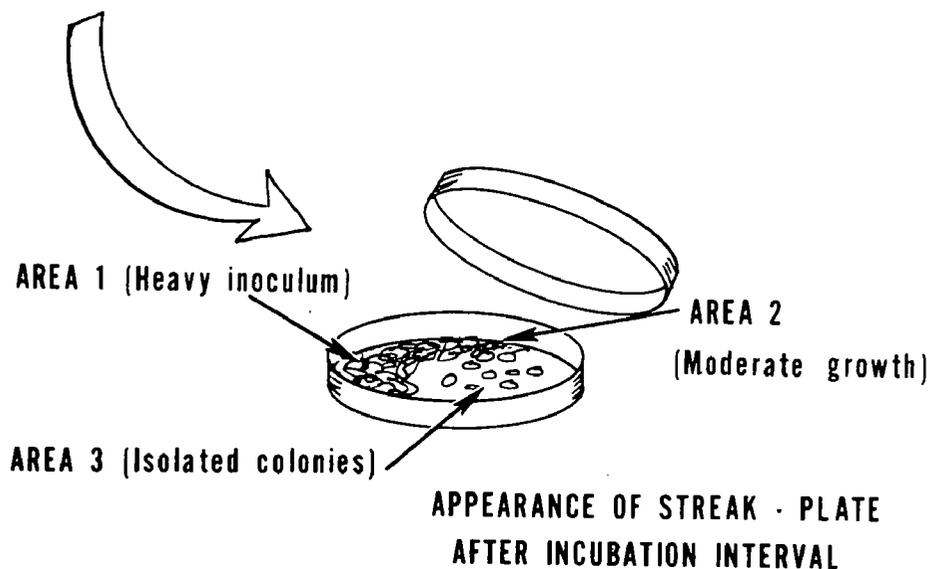


TABLE 2. APPARATUS AND SUPPLIES FOR STANDARD FERMENTATION TUBE TESTS

Description of Item	Total Coliform Group			Fecal Coliforms	
	Presumptive Test	Confirmed Test	Completed Test	(BALB)	(EC broth)
Lauryl tryptose broth or Lactose broth. 20 ml amounts of 1.5 X concentration medium, in 25 X 150 mm culture tubes with inverted fermentation vials, suitable caps.	X				
Lauryl tryptose broth or Lactose broth. 10 ml amounts of single strength medium in 20 X 150 mm culture tubes with inverted fermentation vials, suitable caps.	X		X		
Brilliant green lactose bile broth, 2% in 10 ml amounts, single strength, in 20 X 150 mm culture tubes with inverted fermentation vials, suitable caps.		X	X		
Eosin methylene blue agar, poured in 100 X 15 mm Petri dishes		X	X		
Endo Agar, poured in 100 X 15 mm dishes		X			
Nutrient agar slant, screw cap tube			X		
Boric acid lactose broth, 10 ml amounts of single strength medium in fermentation tubes.				X	
EC Broth, 10 ml amounts of single strength medium in fermentation tubes.					X
Formate ricinoleate broth (provisional)			X		
Culture tube racks, 10 X 5 openings, each opening to accept 25 mm diameter tubes.	X	X	X	X	X
Pipettes, 10 ml, Mohr type, sterile, in suitable cans	X				
Pipettes, 2 ml (optional), Mohr type, sterile, in suitable cans	X				
Pipettes, 1 ml, Mohr type, sterile in metal suitable cans	X				
Standard buffered dilution water, sterile, 99-ml amounts in screw-capped bottles.	X				
Gas burner, Bunsen type		X	X	X	X
Inoculation loop, loop 3mm diameter, of nichrome or platinum-iridium wire, 26 B & S gauge, in suitable holder. (or sterile applicator stick)		X	X	X	X
Inoculation needle, nichrome, or platinum-iridium wire, 26 B & S gauge, in suitable holder.		X	X		
Incubator, adjusted to 35 ± 0.5°C	X	X	X		
Waterbath incubator, adjusted to 43 ± 0.2°C				X	
Waterbath incubator, adjusted to 44.5 ± 0.2°C.					X
Glass microscopic slides, 1" X 3"			X		
Slide racks (optional)			X		
Gram-stain solutions, complete set			X		
Compound microscope, oil immersion lens, Abbe' condenser			X		
Basket for discarded cultures	X	X	X	X	X
Container for discarded pipettes	X				

Part 2

DETAILED TESTING PROCEDURES FOR MEMBERS OF THE
COLIFORM GROUP BY MULTIPLE DILUTION TUBE METHODS

I SCOPE

A Tests Described

- 1 Presumptive Test
- 2 Confirmed Test
- 3 Completed Test
- 4 Fecal Coliform Test

B Form of Presentation

The Presumptive, Confirmed, and Completed Tests are presented as total, independent procedures. It is recognized that this form of presentation is somewhat repetitious, inasmuch as the Presumptive Test is preliminary to the Confirmed Test, and both the Presumptive Test and the Confirmed Test are preliminary to the Completed Test for total coliforms.

In using these procedures, the worker must know at the outset what is to be the stage at which the test is to be ended, and the details of the procedures throughout, in order to prevent the possibility of discarding gas-positive tubes before proper transfer procedures have been followed.

Thus, if the worker knows that the test will be ended at the Confirmed Test, he will turn at once to Section III, TESTING TO THE CONFIRMED TEST STAGE, and will ignore Sections II and IV.

The Fecal Coliform Test is described separately, in Section V, as an adjunct to the Confirmed Test and to the Completed Test.

II TESTING TO PRESUMPTIVE TEST
STAGE

A First-Day Procedures

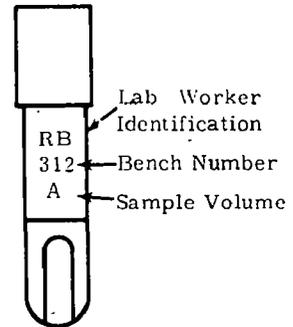
- 1 Prepare a laboratory data sheet for the sample. Record the following information: assigned laboratory number, source of sample, date and time of collection, temperature of the source, name of sample collector, date and time of receipt of sample in the laboratory. Also show the date and time of starting tests in the laboratory, name(s) of worker(s) performing the laboratory tests, and the sample volumes planted.
- 2 Label the tubes of lauryl tryptose broth required for the initial planting of the sample (Table 3). The label should bear three identifying marks. The upper number is the identification of the worker(s) performing the test (applicable to personnel in training courses), the number immediately below is the assigned laboratory number, corresponding with the laboratory record sheet. The lower number is the code to designate the sample volume and which tube of a replicate series is indicated.

NOTE: Be sure to use tubes containing the correct concentrations of culture medium for the inoculum/tube volumes. (See the chapter on media and solutions for multiple dilution tube methods or refer to the current edition of Standard Methods for Water and Wastewater).

Table 3. SUGGESTED LABELING SCHEME FOR ORIGINAL CULTURES AND SUBCULTURES IN MULTIPLE DILUTION TUBE TESTS

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Sample volume represented
Bench number Volume & tube	312 A	312 B	312 C	312 D	312 E	Tubes with 10 ml of sample
Bench number Volume & tube	312 a	312 b	312 c	312 d	312 e	Tubes with 1 ml of sample
Bench number Volume & tube	312 <u>a</u>	312 <u>b</u>	312 <u>c</u>	312 <u>d</u>	312 <u>e</u>	Tubes with 0.1 ml of sample
Bench number Volume & tube	312 1a	312 1b	312 1c	312 1d	312 1e	Tubes with 0.01 ml of sample
Bench number Volume & tube	312 2a	312 2b	312 2c	312 2d	312 2e	Tubes with 0.001 ml of sample

Typical Example



Tube of Culture Medium

The labeling of cultures can be reduced by labeling only the first tube of each series of identical sample volumes in the initial planting of the sample. All subcultures from initial plantings should be labeled completely.

- 3 Place the labeled culture tubes in an orderly arrangement in a culture tube rack, with the tubes intended for the largest sample volumes in the front row, and those intended for smaller volumes in the succeeding rows.
 - 4 Shake the sample vigorously, approximately 25 times, in an arc of one foot within seven seconds and withdraw the sample portion at once.
 - 5 Measure the predetermined sample volumes into the labeled tubes of lauryl tryptose broth, using care to avoid introduction of any bacteria into the culture medium except those in the sample.
 - a Use a 10 ml pipet for 10 ml sample portions, and 1 ml pipets for portions of 1 ml or less. Handle sterile pipets only near the mouthpiece, and protect the delivery end from external contamination. Do not remove the cotton plug in the mouthpiece as this is intended to protect the user from ingesting any sample.
 - b When using the pipet to withdraw sample portions, do not dip the pipet more than 1/2 inch into the sample; otherwise sample running down the outside of the pipet will make measurements inaccurate.
 - 6 After measuring all portions of the sample into their respective tubes of medium, gently shake the rack of inoculated tubes to insure good mixing of sample with the culture medium. Avoid vigorous shaking, as air bubbles may be shaken into the fermentation vials and thereby invalidate the test.
 - 7 Place the rack of inoculated tubes in the incubator at $35^{\circ} \pm 0.5^{\circ} \text{C}$ for 24 ± 2 hours.
- B 24-hour Procedures
- 1 Remove the rack of lauryl tryptose broth cultures from the incubator, and shake gently. If gas is about to appear in the fermentation vials, the shaking will speed the process.

- 2 Examine each tube carefully. Record, in the column "24" under LST on the laboratory data sheet, each tube showing gas in the fermentation vial as a positive (+) test and each tube not showing gas as a negative (-) test. GAS IN ANY QUANTITY IS A POSITIVE TEST.
- 3 Discard all gas-positive tubes of lauryl tryptose broth, and return all the gas-negative tubes to the 35°C incubator for an additional 24 ± 2 hours.

C 48-hour Procedures

- 1 Remove the rack of culture tubes from the incubator, read and record gas production for each tube.
- 2 Be sure to record all results under the 48-hour LTB column on the data sheet. Discard all tubes. The Presumptive Test is concluded at this point, and Presumptive coliforms per 100 ml can be computed according to the methods described elsewhere in this manual.

III TESTING TO CONFIRMED TEST STAGE

Note that the description starts with the sample inoculation and includes the Presumptive Test stage. The Confirmed Test preferred in Laboratories of this agency is accomplished by means of the brilliant green lactose bile broth (BGLB) and the acceptable alternate tests are mentioned in III F. In addition, the Fecal Coliform Test is included as an optional adjunct to the procedure.

A First-Day Procedures

- 1 Prepare a laboratory data sheet for the sample. Record the following information: assigned laboratory number, source of sample, date and time of collection, temperature of the source, name of sample collector, date and time of receipt of sample in the laboratory. Also show the date and

time of starting tests in the laboratory. name(s) of worker(s) performing the laboratory tests, and the sample volumes planted.

- 2 Label the tubes of lauryl tryptose broth required for the initial planting of the sample. The label should bear three identifying marks. The upper number is the identification of the worker(s) performing the test (applicable to personnel in training courses), the number immediately below is the assigned laboratory number, corresponding with the laboratory record sheet. The lower number is the code to designate the sample volume and which tube of a replicate series is indicated.

NOTE: If 10-ml samples are being planted, it is necessary to use tubes containing the correct concentration of culture medium. This has previously been noted in II A-2.

- 3 Place the labeled culture tubes in an orderly arrangement in a culture tube rack, with the tubes intended for the largest sample volumes in the front row, and those intended for smaller volumes in the succeeding rows.
- 4 Shake the sample vigorously, approximately 25 times, in an up-and-down motion.
- 5 Measure the predetermined sample volumes into the labeled tubes of lauryl tryptose broth, using care to avoid introduction of any bacteria into the culture medium except those in the sample.
 - a Use a 10-ml pipet for 10 ml sample portions, and 1-ml pipets for portions of 1 ml or less. Handle sterile pipets only near the mouthpiece, and protect the delivery end from external contamination. Do not remove the cotton plug in the mouthpiece as this is intended to protect the user from ingesting any sample.

- b When using the pipet to withdraw sample portions, do not dip the pipet more than 1/2 inch into the sample; otherwise sample running down the outside of the pipet will make measurements inaccurate.
 - c When delivering the sample into the culture medium, deliver sample portions of 1 ml or less down into the culture tube near the surface of the medium. Do not deliver small sample volumes at the top of the tube and allow them to run down inside the tube; too much of the sample will fail to reach the culture medium.
 - d Prepare preliminary dilutions of samples for portions of 0.01 ml or less before delivery into the culture medium. See Table 1 for preparation of dilutions. NOTE: Always deliver diluted sample portions into the culture medium as soon as possible after preparation. The interval between preparation of dilution and introduction of sample into the medium never should be as much as 30 minutes.
- 6 After measuring all portions of the sample into their respective tubes of medium, gently shake the rack of inoculated tubes to insure good mixing of sample with the culture medium. Avoid vigorous shaking, as air bubbles may be shaken into the fermentation vials and thereby invalidate the test.
 - 7 Place the rack of inoculated tubes in the incubator at $35^{\circ} \pm 0.5^{\circ} \text{C}$ for 24 ± 2 hours.

B 24-hour Procedures

- 1 Remove the rack of lauryl tryptose broth cultures from the incubator, and shake gently. If gas is about to appear in the fermentation vials, the shaking will speed the process.
- 2 Examine each tube carefully. Record, in the column "24" under LST on the laboratory data sheet, each tube showing gas in the fermentation vial as a positive (+) test and each tube not showing gas as a negative (-) test. GAS IN ANY QUANTITY IS A POSITIVE TEST.
- 3 Retain all gas-positive tubes of lauryl tryptose broth culture in their place in the rack, and proceed.
- 4 Select the gas-positive tubes of lauryl tryptose broth culture for Confirmed Test procedures. Confirmed Test procedures may not be required for all gas-positive cultures. If, after 24-hours of incubation, all five replicate cultures are gas-positive for two or more consecutive sample volumes, then select the set of five cultures representing the smallest volume of sample in which all tubes were gas-positive. Apply Confirmed Test procedures to all these cultures and to any other gas-positive cultures representing smaller volumes of sample, in which some tubes were gas-positive and some were gas-negative.
- 5 Label one tube of brilliant green lactose bile broth (BGLB) to correspond with each tube of lauryl tryptose broth selected for Confirmed Test procedures.
- 6 Gently shake the rack of Presumptive Test cultures. With a flame-sterilized inoculation loop transfer one loopful of culture from each gas-positive tube to the corresponding tube of BGLB. Place each newly inoculated culture into BGLB in the position of the original gas-positive tube.
- 7 After making the transfers, the rack should contain some 24-hour gas-negative tubes of lauryl tryptose broth and the newly inoculated BGLB.
- 8 If the Fecal Coliform Test is included in the testing procedures, consult Section V of this part of the outline of testing procedures.

- 9 Incubate the 24-hour gas-negative BGLB tubes and any newly-inoculated tubes of BGLB an additional 24 + 2 hours at 35° + 0.5° C.

C 48-hour Procedures

- 1 Remove the rack of culture tubes from the incubator, read and record gas production for each tube.
- 2 Some tubes will be lauryl tryptose broth and some will be brilliant green lactose bile broth (BGLB). Be sure to record results from LTB under the 48-hour LTB column and the BGLB results under the 24-hour column of the data sheet.
- 3 Label tubes of BGLB to correspond with all (if any) 48-hour gas-positive cultures in lauryl tryptose broth. Transfer one loopful of culture from each gas-positive LTB culture to the correspondingly-labeled tube of BGLB. NOTE: All tubes of LTB culture which were negative at 24 hours and became positive at 48 hours are to be transferred. The option described above for 24-hour cultures does not apply at 48 hours.
- 4 If the Fecal Coliform Test is included in the testing procedure, consult Section V of the part of the outline of testing procedures.
- 5 Incubate the 24-hour gas-negative BGLB tubes and any newly-inoculated tubes of BGLB 24 + 2 hours at 35° + 0.5° C.
- 6 Discard all tubes of LTB and all 24-hour gas-positive BGLB cultures.

D 72-hour Procedures

- 1 If any cultures remain to be examined, all will be BGLB. Some may be 24

hours old and some may be 48 hours old. Remove such cultures from the incubator, examine each tube for gas production, and record results on the data sheet.

- 2 Be sure to record the results of 24-hour BGLB cultures in the "24" column under BGLB and the 48-hour results under the "48" column of the data sheet.
- 3 Return any 24-hour gas-negative cultures for incubation 24 + 2 hours at 35 + 0.5° C.
- 4 Discard all gas-positive BGLB cultures and all 48-hour gas-negative cultures from BGLB.
- 5 It is possible that all cultural work and results for the Confirmed Test have been finished at this point. If so, codify results and determine Confirmed Test coliforms per 100 ml as described in the outline on use of MPN Tables.

E 96-hour Procedures

At most only a few 48-hour cultures in BGLB may be present. Read and record gas production of such cultures in the "48" column under BGLB on the data sheet. Codify results and determine Confirmed Test coliforms per 100 ml.

- F Streak-plate methods for the Confirmed Test, using eosin methylene blue agar or Endo agar plates, are accepted procedures in Standard Methods. The worker who prefers to use one of these media in preference to BGLB (also approved in Standard Methods) is advised to refer to the current edition of "Standard Methods for the Examination of Water and Wastewater" for procedures.

IV TESTING TO COMPLETED TEST STAGE

(Note that this description starts with the sample inoculation and proceeds through the Presumptive and the Confirmed Test stages. In addition, the Fecal Coliform Test is referred to as an optional adjunct to the procedure.)

A First-Day Procedures

- 1 Prepare a laboratory data sheet for the sample. Record the following information: assigned laboratory number, source of sample, date and time of collection, temperature of the source, name of sample collector, date and time of receipt of sample in the laboratory. Also show the date and time of starting tests in the laboratory, name(s) of worker(s) performing the laboratory tests, and the sample volumes planted.
- 2 Label the tubes of lauryl tryptose broth required for the initial planting of the sample. The label should bear three identifying marks. The upper number is the identification of the worker(s) performing the test (applicable to personnel in training courses), the number immediately below is the assigned laboratory number, corresponding with the laboratory record sheet. The lower number is the code to designate the sample volume and which tube of a replicate series is indicated. Guidance on labeling for laboratory data number and identification of individual tubes is described elsewhere in this outline.

NOTE: If 10-ml samples are being plated, it is necessary to use tubes containing the correct concentration of culture medium. This has previously been noted elsewhere in this outline and referral is made to tables.

- 3 Place the labeled culture tubes in an orderly arrangement in a culture tube rack, with the tubes intended for the largest sample volumes in the front row, and those intended for smaller volumes in the succeeding rows.
- 4 Shake the sample vigorously, approximately 25 times, in an up-and-down motion.
- 5 Measure the predetermined sample volumes into the labeled tubes of lauryl tryptose broth, using care to avoid introduction of any bacteria into the culture medium except those in the sample.
 - a Use a 10-ml pipet for 10 ml sample portions, and 1-ml pipets for portions of 1 ml or less. Handle sterile pipets only near the mouthpiece, and protect the delivery end from external contamination. Do not move the cotton plug in the mouthpiece as this is intended to protect the user from ingesting any sample.
 - b When using the pipet to withdraw sample portions, do not dip the pipet more than 1/2 inch into the sample; otherwise sample running down the outside of the pipet will make measurements inaccurate.
 - c When delivering the sample into the culture medium, deliver sample portions of 1 ml or less down into

the culture tube near the surface of the medium. Do not deliver small sample volumes at the top of the tube and allow them to run down inside the tube; too much of the sample will fail to reach the culture medium.

- d Prepare preliminary dilutions of samples for portions of 0.01 ml or less before delivery into the culture medium. See Table 2 for preparation of dilutions. NOTE: Always deliver diluted sample portions into the culture medium as soon as possible after preparation. The interval between preparation of dilution and introduction of sample into the medium never should be as much as 30 minutes.

- 6 After measuring all portions of the sample into their respective tubes of medium, gently shake the rack of inoculated tubes to insure good mixing of sample with the culture medium. Avoid vigorous shaking, as air bubbles may be shaken into the fermentation vials and thereby invalidate the test.
- 7 Place the rack of inoculated tubes in the incubator at $35^{\circ} \pm 0.5^{\circ} \text{C}$ for 24 ± 2 hours.

B 24-hour Procedures

- 1 Remove the rack of lauryl tryptose broth cultures from the incubator, and shake gently. If gas is about to appear in the fermentation vials, the shaking will speed the process.
- 2 Examine each tube carefully. Record, in the column "24" under LST on the laboratory data sheet, each tube showing gas in the fermentation vial as a positive (+) test and each tube not showing gas as a negative (-) test. **GAS IN ANY QUANTITY IS A POSITIVE TEST.**
- 3 Retain all gas-positive tubes of lauryl tryptose broth culture in their place in the rack, and proceed.

- 4 Select the gas-positive tubes of lauryl tryptose broth culture for the Confirmed Test procedures. Confirmed Test procedures may not be required for all gas-positive cultures. If, after 24-hours of incubation, all five replicate cultures are gas-positive for two or more consecutive sample volumes, then select the set of five cultures representing the smallest volume of sample in which all tubes were gas-positive. Apply Confirmed Test procedures to all these cultures and to any other gas-positive cultures representing smaller volumes of sample, in which some tubes were gas-positive and some were gas-negative.

- 5 Label one tube of brilliant green lactose bile broth (BGLB) to correspond with each tube of lauryl tryptose broth selected for Confirmed Test procedures.

- 6 Gently shake the rack of Presumptive Test cultures. With a flame-sterilized inoculation loop transfer one loopful of culture from each gas-positive tube to the corresponding tube of BGLB. Place each newly inoculated culture into BGLB in the position of the original gas-positive tube.

- 7 If the Fecal Coliform Test is included in the testing procedure, consult Section V of this outline for details of the testing procedure.

- 8 After making the transfer, the rack should contain some 24-hour gas-negative tubes of lauryl tryptose broth and the newly inoculated BGLB. Incubate the rack of cultures at $35^{\circ} \text{C} \pm 0.5^{\circ} \text{C}$ for 24 ± 2 hours.

C 48-hour Procedures

- 1 Remove the rack of culture tubes from the incubator, read and record gas production for each tube.
- 2 Some tubes will be lauryl tryptose broth and some will be brilliant green lactose

bile broth (BGLB). Be sure to record results from LTB under the 48-hour LTB column and the BGLB results under the 24-hour column of the data sheet.

3 Label tubes of BGLB to correspond with all (if any) 48-hour gas-positive cultures in lauryl tryptose broth. Transfer one loopful of culture from each gas-positive LTB culture to the correspondingly-labeled tube of BGLB. NOTE: All tubes of LTB culture which were negative at 24 hours and became positive at 48 hours are to be transferred. The Option described above for 24-hour LTB cultures does not apply at 48 hours.

4 Incubate the 24-hour gas-negative BGLB tubes and any newly-inoculated tubes of BGLB 24 + 2 hours at 35° + 0.5° C. Retain all 24-hour gas-positive cultures in BGLB for further test procedures.

5 Label a Petri dish preparation of eosin methylene blue agar (EMB agar) to correspond with each gas-positive culture in BGLB.

6 Prepare a streak plate for colony isolation from each gas-positive culture in BGLB on the correspondingly-labeled EMB agar plate.

Incubate the EMB agar plates 24 + 2 hours at 35° + 0.5° C.

D 72-hour Procedures

1 Remove the cultures from the incubator. Some may be on BGLB; several EMB agar plates also can be expected.

2 Examine and record gas production results for any cultures in BGLB.

3 Retain any gas-positive BGLB cultures and prepare streak plate inoculations for colony isolation in EMB agar. Incubate the EMB agar plates 24 + 2 hours at 35 + 0.5° C. Discard the gas-positive BGLB cultures after transfer.

4 Reincubate any gas-negative BGLB cultures 24 + 2 hours at 35° + 0.5° C.

5 Discard all 48-hour gas-negative BGLB cultures.

6 Examine the EMB agar plates for the type of colonies developed thereon. Well-isolated colonies having a dark center (when viewed from the lower side, held toward a light) are termed "nucleated or fisheye" colonies, and are regarded as "typical" coliform colonies. A surface sheen may or may not be present on "typical" colonies. Colonies which are pink or opaque but are not nucleated are regarded as "atypical colonies." Other colony types are considered "noncoliform." Read and record results as + for "typical" (nucleated) colonies + for "atypical" (non-nucleated pink or opaque colonies), and - for other types of colonies which might develop.

7 With plates bearing "typical" colonies, select at least one well-isolated colony and transfer it to a correspondingly-labeled tube of lactose broth and to an agar slant. As a second choice, select at least two "atypical" colonies (if typical colonies are not present) and transfer them to labeled tubes of lactose broth and to agar slants. As a third choice, in the absence of typical or atypical coliform-like colonies, select at least two well-isolated colonies representative of those appearing on the EMB plate, and transfer them to lactose broth and to agar slants.

8 Incubate all cultures transferred from EMB agar plates 24 + 2 hours at 35 + 0.5° C.

E 96-hour Procedures

1 Subcultures from the samples being studied may include: 48-hour tubes of BGLB, EMB agar plates, lactose broth tubes, and agar slant cultures.

- 2 If any 48-hour tubes of BGLB are present, read and record gas production in the "48" column under BGLB. From any gas-positive BGLB cultures prepare streak plate inoculations for colony isolation on EMB agar. Discard all tubes of BGLB, and incubate EMB agar plates 24 ± 2 hours at $35 \pm 0.5^\circ\text{C}$.
 - 3 If any EMB plates are present, examine and record results in the "EMB" column of the data sheet. Make transfers to agar slants and to lactose broth from all EMB agar plate cultures. In decreasing order of preference, transfer at least one typical colony, or at least two atypical colonies, or at least two colonies representative of those on the plate.
 - 4 Examine and record results from the lactose broth cultures.
 - 5 Prepare a Gram-stained smear from each of the agar slant cultures, as follows:

NOTE: Always prepare Gram stain from an actively growing culture, preferably about 18 hours old, and never more than 24 hours old. Failure to observe this precaution often results in irregular staining reactions.

 - a Thoroughly clean a glass slide to free it of any trace of oily film.
 - b Place one-drop of distilled water on the slide.
 - c Use the inoculation needle to suspend a tiny amount of growth from the nutrient agar slant culture in the drop of water.
 - d Mix the thin suspension of cells with the tip of the inoculation needle, and allow the water to evaporate.
 - e "Fix" the smear by gently warming the slide over a flame.
 - f Stain the smear by flooding it for 1 minute with crystal violet solution.
 - g Flush the excess crystal violet solution off in gently running water, and gently blot dry with filter paper or with other clean absorbent paper.
 - h Flood the smear with Lugol's iodine for 1 minute.
 - i Wash the slide in gently running water and blot dry with filter paper.
 - j Decolorize the smear with 95% alcohol solution with gentle agitation for 10-30 seconds, depending upon extent of removal of crystal violet dye, then blot dry.
 - k Counterstain for 10 seconds with safranin solution, then wash in running water and blot dry.
 - l Examine the slide under the microscope, using the oil immersion lens. Coliform bacteria are Gram-negative, nonspore-forming, rod-shaped cells, occurring singly, in pairs, or rarely in short chains.
 - m If typical coliform staining reaction and morphology are observed, record + in the appropriate space under the "Gram Stain" column of the data sheet. If typical morphology and staining reaction are not observed, then mark it + or -, and make suitable comment in the "remarks" column at the right-hand side of the data sheet.
 - n If spore-forming bacteria are observed, it will be necessary to repurify the culture from which the observations were made. Consult the instructor, or refer to Standard Methods, for procedures.
- At this point, it is possible that all cultural work for the Completed Test has been finished. If so, codify results and determine Completed Test coliforms per 100 ml.

F 120-hour Procedures and following:

- 1 Any procedures to be undertaken from this point are "straggler" cultures on media already described, and requiring step-by-step methodology already given in detail. Such cultures may be on: EMB plates, agar slants, or lactose broth. The same time-and-temperature of incubation required for earlier studies applies to the "stragglers" as do the observations, staining reactions, and interpretation of results. On conclusion of all cultural procedures, codify results and determine Completed Test coliforms per 100 ml.

number of tubes to the Confirmed Test sometimes can be applied here. However, the worker is urged to avoid exercise of this option until he has assured the applicability of the option by preliminary tests on the sample source.

- c Transfer one loopful of culture from each gas-positive culture in lauryl tryptose broth to the correspondingly labeled tube of EC broth.
- d Incubate EC broth tubes 24 ± 2 hours at $44.5 \pm 0.2^{\circ}\text{C}$ in a waterbath with water depth sufficient to come up at least as high as the top of the culture medium in the tubes. Place in waterbath as soon as possible after inoculation and always within 30 minutes after inoculation.

V FECAL COLIFORM TEST

A General Information

- 1 The procedure described is an elevated temperature test for fecal coliform bacteria.
- 2 Equipment required for the tests are those required for the Presumptive Test of Standard Methods, a water-bath incubator, and the appropriate culture media.

B Fecal Coliform Test with EC Broth

- 1 Sample: The test is applied to gas-positive tubes from the Standard Methods Presumptive Test (lauryl tryptose broth), in parallel with Confirmed Test procedures.
- 2 24-hour Operations. Initial procedures are the planting procedures described for the Standard Methods Presumptive Coliform test.
 - a After reading and recording gas-production on lauryl tryptose broth, temporarily retain all gas-positive tubes.
 - b Label a tube of EC broth to correspond with each gas-positive tube of lauryl tryptose broth. The option regarding transfer of only a limited

3 48-hour operations

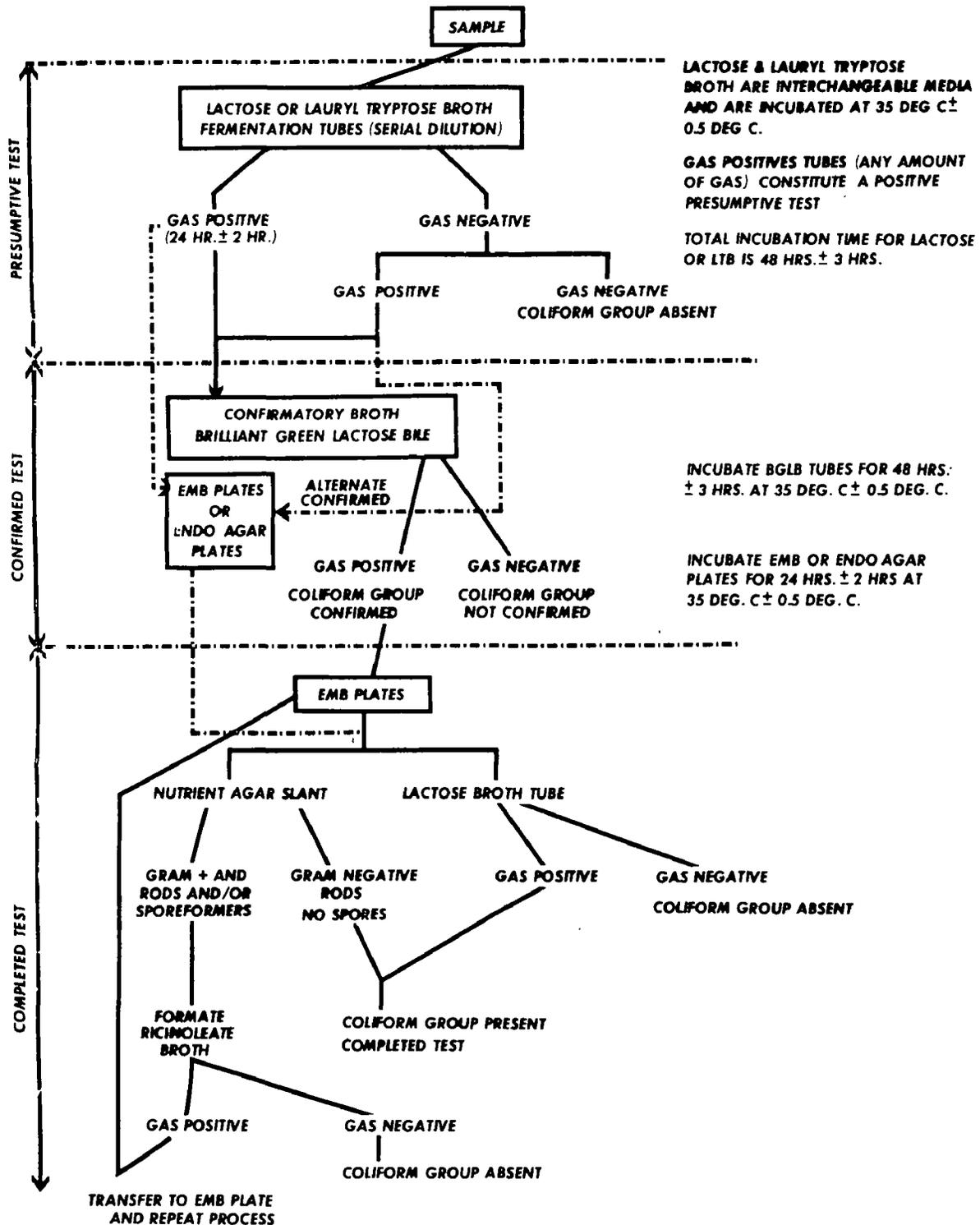
- a Remove the rack of EC cultures from the waterbath, shake gently, and record gas production for each tube. Gas in any quantity is a positive test.
- b As soon as results are recorded, discard all tubes. (This is a 24-hour test for EC broth inoculations and not a 48-hour test.)

- c Transfer any additional 48-hour gas-positive tubes of lauryl tryptose broth to correspondingly labeled tubes of EC broth. Incubate 24 ± 2 hours at $44.5 \pm 0.2^{\circ}\text{C}$.

4 72-hour operations

- a Read and record gas production for each tube. Discard all cultures.
- b Codify results and determine fecal coliform count per 100 ml of sample.

TESTS FOR COLIFORM GROUP



Part 3
LABORATORY METHODS FOR FECAL STREPTOCOCCUS
(Day-By-Day Procedures)

I GENERAL INFORMATION

- A The same sampling and holding procedures apply as for the coliform test.
- B The number of fecal streptococci in water generally is lower than the number of coliform bacteria. It is good practice in multiple dilution tube tests to start the sample planting series with one sample increment larger than for the coliform test. For example: If a sample planting series of 1.0, 0.1, 0.01, and 0.001 ml is planned for the coliform test, it is suggested that a series of 10, 1.0, 0.1, and 0.01 ml be planted for the fecal streptococcus test.
- C Equipment required for the test is the same as required for the Standard Methods Presumptive and Confirmed Tests, except for the differences in culture media.

II STANDARD METHODS (Tentative)
PROCEDURES

A First-Day Operations

- 1 Prepare the sample data sheet and labeled tubes of azide dextrose broth in the same manner as for the Presumptive Test. NOTE: If 10-ml samples are included in the series, be sure to use a special concentration (ordinarily double-strength) of azide dextrose broth for these sample portions.
- 2 Shake the sample vigorously, approximately 25 times, in an up-and-down motion.
- 3 Measure the predetermined sample volumes into the labeled tubes of azide dextrose broth, using the sample measurement and delivery techniques used for the Presumptive Test.

- 4 Shake the rack of tubes of inoculated culture media, to insure good mixing of sample with medium.
- 5 Place the rack of inoculated tubes in the incubator at $35^{\circ} \pm 0.5^{\circ} \text{C}$ for 24 ± 2 hours.

B 24-hour Operations

- 1 Remove the rack of tubes from the incubator. Read and record the results from each tube. Growth is a positive test with this test. Evidence of growth consists either of turbidity of the medium, a "button" of sediment at the bottom of the culture tube, or both.
- 2 Label a tube of ethyl violet azide broth to correspond with each positive culture of azide dextrose broth. It may be permissible to use the same confirmatory option as described for the coliform Confirmed Test, in this outline.
- 3 Shake the rack of cultures gently, to resuspend any living cells which have settled out to the bottom of the culture tubes.
- 4 Transfer three loopfuls of culture from each growth-positive tube of azide dextrose broth to the correspondingly labeled tube of ethyl violet azide broth.
- 5 As transfers are made, place the newly inoculated tubes of ethyl violet azide broth in the positions in the rack formerly occupied by the growth-positive tubes of azide dextrose broth. Discard the tubes of azide dextrose broth culture.
- 6 Return the rack, containing 24-hour growth-negative azide dextrose broth tubes and newly-inoculated tubes of ethyl violet azide broth, to the incubator. Incubate 24 ± 2 hours at $35^{\circ} \pm 0.5^{\circ} \text{C}$.

C 48-hour Operations

- 1 Remove the rack of tubes from the incubator. Read and report results. Growth, either in azide dextrose broth or in ethyl violet azide broth, is a positive test. Be sure to report the results of the azide dextrose broth medium under the "48" column for that medium and the results of the ethyl violet azide broth cultures under the "24" column for that medium.
- 2 Any 48-hour growth-positive cultures of azide dextrose broth are to be transferred (three loopfulls) to ethyl violet azide broth. Discard all 48-hour growth-negative tubes of azide dextrose broth and all 24-hour growth-positive tubes of ethyl violet azide broth.
- 3 Incubate the 24-hour growth-negative and the newly-inoculated tubes of ethyl violet azide broth 24 ± 2 hours at 35° ± 0.5° C.

D 72-hour Operations

- 1 Read and report growth results of all tubes of ethyl violet azide broth.
- 2 Discard all growth-positive cultures and all 48-hour growth-negative cultures.
- 3 Reincubate any 24-hour growth-negative cultures in ethyl violet azide broth 24 ± 2 hours at 35° ± 0.5° C.

E 96-hour Operations

- 1 Read and report growth results of any remaining tubes of ethyl violet azide broth.

- 2 Codify results and determine fecal streptococci per 100 ml.

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Descriptors: Coliforms, Fecal Coliforms, Fecal Streptococci, Indicator Bacteria, Laboratory Equipment, Laboratory Tests, Microbiology, Most Probable Number, MPN, Sewage Bacteria, Water Analysis

MEDIA AND SOLUTIONS FOR MULTIPLE DILUTION TUBE METHODS

I INTRODUCTION

A This chapter is intended to present detailed information on preparation and management of media and solutions needed with the tests and observations described elsewhere in this course manual.

B The preparation and management of supplies of culture media and solutions is one of the most critical aspects of a bacteriological water quality testing program.

- 1 In the same manner that the chemist relies on correctly prepared and standardized reagents for his analytical work, the bacteriologist must depend on satisfactory culture media for the type of analysis with which he is concerned.
- 2 In many laboratories preparation of media is entrusted to subprofessional personnel. Most such personnel, properly trained and guided, are able to perform the required tasks efficiently and reliably.
- 3 The professional supervisor should maintain close attention to all details, however, to guard against gradual introduction of bad habits in preparing and preserving media and other liquid supplies.

II GENERAL INFORMATION

A Use of Commercially Available Dehydrated Media

- 1 The preparation of all media described in this chapter is given in terms of the

individual components, and preparation of the finished medium. This is done, even through commercially available dehydrated media are widely used, to acquaint the worker with the composition of the media and to indicate the required specifications of each medium.

- 2 The use of commercially available dehydrated media, requiring only careful weighing and dissolving of the powder in the proper quantity and quality of distilled water, is strongly recommended. Such media are much more likely to have uniformity at an acceptably high level of quality than are media compounded in the laboratory from the individual constituents.
- 3 It is recommended that the worker, when using commercially prepared dehydrated media, keep a careful record of the lot numbers of media being used. With first use of each new lot number of a given medium, it is suggested that the medium be checked for stability, pH after sterilization, and to see that performance is satisfactory. While rare, an occasional lot of medium will have some unforeseen fault which reduces or destroys its effectiveness. Maintenance of lot number records on medium gives opportunity for communication with the manufacturer to determine whether similar problems are being encountered in other laboratories.

B Quality of General Materials

- 1 Distilled water

Distilled water, or demineralized water, is required. It must be free from

NOTE: Mention of commercial products and manufacturers is for illustration and does not imply endorsement by the Environmental Protection Agency.

dissolved metals or chlorine. Freedom from bactericidal constituents or growth promoting substances should be demonstrated through laboratory tests. A procedure for this test is described elsewhere in this course manual.

2 Beef extract

Any brand of beef extract is acceptable, provided that it is known to give results acceptable to the user. Meat infusion is not acceptable.

3 Peptones

Peptones are sold under a wide variety of trade names. Any peptone shown satisfactory by comparative tests with an acceptable peptone, may be accepted.

4 Sugars

All sugars must be chemically pure, and suitable for bacteriological media.

5 Agar

Any form of bacteriologic grade of agar can be used.

6 General chemicals must be reagent grade or ACS if used in culture media. Chemicals used in the distilled water quality test must be of the highest purity available.

7 Dyes

All dyes used in culture media must be certified by the Biological Stain Commission; they will be so labeled on the container.

C Quality of Equipment and Supplies Used for Preparation of Media

1 Glassware

It is recommended that all glassware be of borosilicate glass. Such glass is not subject to release of soluble products into the culture medium, as with some of the so-called "soft glass."

2 Balance

A balance with sensitivity of ± 2 grams with a load of 150 grams is the minimum acceptable standard for weighing of culture media in dehydrated form.

3 pH meter

An electrometric meter is recommended. While a comparator block with pH indicator solutions is useful for such media as lauryl tryptose broth, it cannot be used satisfactorily with dye-containing media such as brilliant green lactose bile broth. Therefore it is suggested that all pH control work on bacteriological media be done with an electrometric type of pH meter. Accuracy of the meter should be established through calibration against a standard buffer.

4 Autoclave

The autoclave should be of sufficient size to permit loose packing of tubed media when normal load is being sterilized. This is to permit free access of steam to all surfaces.

Operation should be such that sterilizing temperature is reached in not more than 30 minutes.

A pressure gauge should be present. More important, the autoclave should be equipped with at least 1 thermometer, which should be located properly in the exhaust line.

Pressure regulation should permit operation up to and including 121°C . When media containing carbohydrates are present; sterilization should be continued 12 - 15 minutes, in media not containing carbohydrates, normal sterilization time should be a standard 15 minutes.

After sterilization, media should be removed from the autoclave as soon as possible. In no case should an autoclave simply be turned off after

the usual exposure to steam under pressure, and allowed to stand until the following morning before removing media.

5 Utensils for mixing and preparing media

Borosilicate glass is suggested, but other materials, such as stainless steel, porcelain (unchipped) containers, or other containers free of soluble bactericidal or bacteriostatic materials, are acceptable. In any case, the containers must be thoroughly clean.

III CONCENTRATION OF MEDIA

A Basic formulas of all media described in Section IV are presented as single-strength media. Most media are used in the single-strength concentration.

B The concentration of primary inoculation media (media into which the measured portions of the original sample are delivered) requires special consideration.

- 1 When the amount of medium is 10 ml or greater, and the volume of sample or sample dilution is 1 ml or less, then single-strength medium is satisfactory.
- 2 When the sample volume introduced into the primary inoculation medium is greater than 1 ml, then it is necessary to compensate for the diluting effect of the sample on the culture medium. In such cases, it is necessary to increase the initial concentration of the medium so that after sample inoculation the concentration of nutrients in medium-plus-sample is equivalent to the concentration of nutrients in the single strength medium.

IV PREPARATION OF MEDIA AND SOLUTIONS

A Lauryl Tryptose Broth (Lauryl Sulfate Broth)

- 1 Use: Primary inoculation medium in Presumptive Test

2 Composition:

Tryptose (or Trypticase or equivalent)	20.0 g
Lactose	5.0 g
Dibasic Potassium Phosphate (K ₂ HPO ₄)	2.75 g
Monobasic Potassium Phosphate (KH ₂ PO ₄)	2.75 g
Sodium Chloride	5.0 g
Sodium Lauryl Sulfate	0.1 g
(Total Dry Constituents)	35.60 g
Distilled Water	1000 ml

Sterilization: 12 - 15 minutes at 121°C
 Reaction after sterilization: pH 6.8 (approximately)

3 Compensation for diluting effect of samples

No. ml in tube	ml of sample or dilution	Nominal concentration before inoculation	No. grams dehydrated medium per liter
10	0.1 - 1.0	1x	35.6
10	10	2x	71.2
20	10	1.5x	53.4
35	100	4x	137.3

B Brilliant Green Lactose Bile Broth

- 1 Use: Confirmed Test

2 Composition

Peptone (Bacto or equivalent)	10.0 g
Lactose	10.0 g
Oxgall (dehydrated)	20.0 g
Brilliant Green	0.0133 g
(Total weight dry constituents)	40.0133 g
Distilled Water	1000 ml

Sterilization: 12 - 15 minutes at 121°C
 Reaction after sterilization: pH 7.1 to 7.4

C Eosin Methylene Blue Agar

1 Confirmed Test

Use: Isolation of coliform-like colonies as a preliminary to Completed Test procedures.

2 Composition

Peptone (Bacto or equivalent)	10	g
Lactose	10	g
Dipotassium Phosphate (K_2HPO_4)	2	g
Agar	20	g
Eosin Y	0.4	g
Methylene Blue	0.65	g
(Total weight dry constituents	43.05	g)

Distilled Water 1000 ml

Sterilization: 12 - 15 minutes at 121°C

3 Special suggestions on preparation:

- a This medium can be prepared and dispensed into bottles or flasks in portions of 100 ml or 200 ml each. The sterile medium may be stored for extended periods in cool places out of the light.
- b When ready for use of such medium, the medium should be melted by immersion of the bottle of prepared medium in a boiling water bath, after which it is dispensed into sterile Petri dishes in portions of approximately 15 ml. After cooling and solidifying in the Petri dish, the medium is ready for use. It should be used preferably on the day it is poured into Petri dishes, but can be stored for a day or two in the refrigerator.
- c An alternate method of preparing this medium requires preparation the agar base medium which includes all the constituents of the medium except the dyes. When ready to use such a preparation, the agar base medium is melted in a water bath, and to each 100 ml of the melted agar base medium, 2 ml of 2% of aqueous solution of eosin Y and 1.3 ml of 0.5% methylene blue

solution is delivered with a pipet. The medium is mixed thoroughly, poured into Petri dishes, and used as previously described.

D Agar Slants

- 1 Use: This medium is used in the Completed Test, to cultivate pure cultures of strains of bacteria being cultivated in preparation of a Gram-stained smear.
- 2 Composition: The medium is nutrient agar

Peptone	5.0	g
Beef extract	3.0	g
Agar	15.0	g
(Total weight dry constituents	23.0	g)

Distilled Water 1000 ml

Sterilization: 15 minutes at 121°C

Reaction after sterilization: pH 6.8 approximately

- 3 Special instructions: Dissolve the constituents, using heat as needed; dispense in amounts of approximately 8 ml per tube. Screw-capped tubes extend shelf life of the medium. After sterilization, remove the melted medium from the autoclave and place in a slanting position until the medium has become solidified. A routine procedure should be established so that a uniform volume of medium and a uniform surface of slanted medium be present in each tube. While this has no particular bearing on Standard Methods procedures, certain other laboratory procedures do require uniform exposed surface area of the slanted medium.

E Plate Count Agar

- 1 Use: This medium is used in the distilled water test. It is not used in other Standard Methods procedures described in this course manual.

2 Composition: (Tryptone Glucose Yeast Agar)

Peptone-tryptone (or equivalent)	5.0 g
Yeast extract	2.5 g
Glucose (dextrose)	1.0 g
Agar	<u>15.0 g</u>
(Total weight dry constituents)	23.5 g

Distilled Water 1000 ml

Sterilization: 15 minutes at 121°C

Reaction after sterilization: pH 7.0 ± 0.1

3 Special instructions in preparation:

Use heat as needed to dissolve and melt the constituents. Dispense the medium in flasks or bottles in portions of 100 or 200 ml each and sterilize. In this state it can be preserved for many months, provided that it is protected from evaporation of the water.

When ready to use, melt the medium by heating, and cool to 45°C. At this temperature the medium still should be melted, and will be satisfactory for preparation of pour plates for plate counts.

F EC Broth

1 Use: Test for fecal coliform bacteria

2 Composition:

Tryptose (Bacto or equivalent)	20.0 g
Lactose	5.0 g
Bile Salts (Bacto #3 or equivalent)	1.5 g
Dipotassium phosphate (K ₂ HPO ₄)	4.0 g
Monopotassium phosphate (KH ₂ PO ₄)	1.5 g
Sodium chloride	<u>5.0 g</u>
(Total weight dry constituents)	37.0 g

Distilled Water 1000 ml

Sterilization: 12 - 15 minutes at 121°C

Reaction after sterilization: pH 6.9

3 This medium is dispensed into culture tubes with inverted fermentation vials and suitable caps.

G Azide Dextrose Broth

1 Use: Primary inoculation medium for fecal streptococcal presumptive test.

2 Composition:

Beef extract	4.5 g
Tryptone or Polypeptone	15. g
Glucose	7.5 g
Sodium chloride	7.5 g
Sodium azide	<u>0.2 g</u>
(Total dry constituents)	34.7 g

Distilled Water 1000 ml

Sterilization: 12 - 15 minutes at 121°C

Reaction after sterilization: about pH 7.2

3 Fermentation vials are not used with azide dextrose broth.

H Ethyl Violet Azide Broth

1 Use: Confirmed test for fecal streptococci

2 Composition:

Tryptone or Biosate	20 g
Glucose	5 g
Sodium chloride	5 g
Potassium phosphate, dibasic (K ₂ HPO ₄)	2.7 g
Potassium phosphate, monobasic (KH ₂ PO ₄)	2.7 g
Sodium azide	0.4 g
Ethyl violet (certified dye if available)	<u>.00083 g</u>
(Total dry constituents)	35.8 g

Distilled Water 1000 ml

Sterilization: 12 - 15 minutes at 121°C

Reaction after sterilization: about pH 7

- 3 Fermentation vials are not used with ethyl violet azide broth.

I Buffered Dilution Water

- 1 Use: Preparation of sample dilutions preliminary to primary inoculation, in membrane filter work, and in plate counts.

2 Composition

a Stock phosphate buffer solution

Monobasic Potassium Phosphate (KH_2PO_4) 34.0 g

Distilled Water 500 ml

1N NaOH solution (about 175 ml) to give pH 7.2

Distilled water sufficient to bring final volume to 1000 ml

b Working solution of phosphate buffered distilled water

Stock phosphate buffer solution 1.25 ml

Distilled water 1000 ml

3 Preparation and handling:

- a Stock solution: After preparation the stock solution should be stored in the refrigerator until use. If at any time evidence of mold or other contamination appears, the stock solution should be discarded and a fresh solution prepared.
- b Working solution: Dispense the required amount into distilled water, and deliver into screw-capped bottles for dilution water. The amount added should be such that, after sterilization, the bottles will contain 99 ± 2 ml of the dilution water. Ordinarily this requires initial addition of approximately 102 ml of the solution prior to sterilization.

- c Sterilization is 20 minutes at 121°C .

- d Tightly stoppered bottles of the dilution water, protected against evaporation, in suitable containers, appear to last indefinitely.

J Solutions for Gram Stain

i Ammonium oxalate crystal violet solution:

- a Dissolve 2 g crystal violet (approximately 85% dye content) in 20 ml of 95% ethyl alcohol.

- b Dissolve 0.8 grams ammonium oxalate in 80 ml distilled water.

- c Mix solutions a and b.

- d Filter through cheesecloth or coarse filter paper.

- e Problems with the gram stain technique frequently are traceable to the ammonium oxalate crystal violet solution. In the event that decolorization does not seem satisfactory, the amount of crystal violet in the solution can be reduced to as little as 10% of the recommended amount.

- 2 Lugol's iodine: Dissolve 1 g iodine crystals and 2 g potassium iodide in the least amount (usually about 5 ml) of distilled water in which they are soluble. After all crystals are in solution, add sufficient distilled water to bring the final solution to a volume of 300 ml.
- 3 Counterstain: Dissolve 2.5 grams of safranin in 100 ml of 95% ethyl alcohol. For the working solution of counterstain, add 10 ml of this solution of safranin to 100 ml of distilled water.

REFERENCES

1 Standard Methods for the Examination of
Water and Wastewater. 13th ed. 1971 .

2 Public Health Service Bacteriological
Survey Form for Water Laboratories.
PHS Form 875 (Revised 1966).

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USE OF TABLES OF MOST PROBABLE NUMBERS

Part 1

I INTRODUCTION

A Using probability mathematics, it is possible to estimate the number of bacteria producing the observed result for any combination of positive and negative results in dilution tube tests. Because the computations are so repetitious and time-consuming, it is common laboratory practice to use Tables of Most Probable Numbers. These tables are orderly arrangements of the possible cultural results obtainable from inoculating various sample increments in differential culture media. Each possible combination of positive and negative tube results is accompanied by the result (MPN) of the calculated estimate and the 95% confidence limits of the MPN.

B The Tables of Most Probable Numbers used in the current (13th) edition of Standard Methods for the Examination of Water and Wastewater were developed by Swaroop.⁽¹⁾ Previous editions of Standard Methods have used the tables prepared by Hoskins.⁽²⁾

1 Most of the tables are based on using 3 sample volumes in decreasing decimal increments. Thus, the systems are based on using volumes of 10 ml, 1.0 ml, and 0.1 ml, etc. Other quantity relationships can be used, such as 50 ml, 10 ml, and 1.0 ml in a table. Tables of Most Probable Numbers can be prepared for any desired series of sample increments.

2 In addition, tables can be devised for different numbers of replicate inoculations of individual sample volumes. For example, the MPN Table most commonly used in the laboratories of this agency is based on five replicate 10 ml portions, five 1.0 ml portions, and five 0.1 ml portions. A separate table is required for another combination of sample volumes, consisting of five replicate 10 ml portions,

one 1.0 ml portion, and one 0.1 ml portion. This is popular in bacteriological potability tests on water. MPN Tables can be prepared for any desired combinations of replicates of the sample increments used in a dilution tube series.

3 An approximation of the MPN values shown in the Tables can be obtained by a simple calculation, developed by Thomas.⁽³⁾ The formula and application of this calculation is shown on a later page of this chapter.

C The method of using a Table of Most Probable Numbers is described here, based on the table for five 10 ml portions, five 1.0 portions, and five 0.1 portions. The principles apply equally to the other tables presented in the current edition of Standard Methods for the Examination of Water and Wastewater.

II DETERMINING THE MOST PROBABLE NUMBER

A Codifying Results of the Dilution Tube Series

If five 10 ml portions, five 1.0 ml portions, and five 0.1 ml portions are inoculated initially, and positive results are secured from five of the 10 ml portions, three of the 1.0 ml portions and none of the 0.1 ml portions, then the coded result of the test is 5-3-0. The code can be looked up in the MPN Table, and the MPN per 100 ml is recorded directly. If more than the above three sample volumes are to be considered, then the determination of the coded result may be more complex. The examples described in Table 1 are useful guides for selection of the significant series of three sample volumes.

Table 1. EXAMPLES OF CODED RESULTS

No. ml sample per tube → 100 No. tubes per sample vol. → 5	10 5	1.0 5	0.1 5	0.01 5	0.001 5	Code	See Below
No. tubes in sample giving positive results in test	5	4	1			5-4-1	
5	5	4	0	0	0	5-4-0	(1)
	4	1	0	0	0	4-1-0	(2)
5	5	4	1	1	0	5-4-2	(3)
	5	5	5	4		5-5-4	(4)
	5	5	5	5		5-5-5	(5)
	0	0	0	0		0-0-0	(6)
	0	1	0	0		0-1-0	(7)
	1	0	0	0		1-0-0	(8)

Discussion of examples:

- 1 When all the inoculated tubes of more than one of the decimal series give positive results, then it is customary to select the smallest sample volume (here, 10 ml) in which all tubes gave positive results. The results of this volume and the next lesser volumes are used to determine the coded result.
- 2 When none of the sample volumes give positive results in all increments of the series, then the results obtained are used to designate the code. Note that it is not permissible to assume that if the next larger increment had been inoculated, all tubes probably would have given positive results and therefore assign a 5-4-1 code to the results.
- 3 Here the results are spread through four of the sample volumes. In such cases, the number of positive tubes in the smallest sample volume is added to the number of tubes in the third sample volume (counting down from the smallest sample volume in which all tubes gave positive results).

- 4 Here it is necessary to use the 5-5-4 code, because inoculations were not made of 0.001 ml sample volumes; and it is not permissible to assume that if such sample volumes had been inoculated, they would have given negative results, or any other arbitrarily-designated result.
- 5 This is an indeterminate result. Many MPN tables do not give a value for such a result. If the table used does not have the code, then look up the result for code 5-5-4, and report the result "greater than" the value shown for the 5-5-4 code. The first number of the 5-5-4 code is based on the 1.0 ml sample volume.
- 6 Like (5), this is an indeterminate result. If the code does not appear in the table being used, then look up the result for code 1-0-0, and report the MPN as "less than" the value shown for the 1-0-0 code.
- 7 The current edition of Standard Methods stipulates this type of code designation when unusual results such as this occur.

8 Note the difference from (7) above. Inoculations of 100 ml portions were not made, and it cannot be assumed that the result would have called for code 0-1-0.

B Computing and Recording the MPN

When the dilution tube results have been codified, they are read and recorded from the appropriate MPN Table.

- 1 If, as in the first four of the examples shown under (A) the first number in the coded result represents a 10 ml sample volume, then the MPN per 100 ml is read and recorded directly from the appropriate column in the table.
- 2 On the other hand, if the first number in the coded result represents a sample volume other than 10 ml, then a calculation is required to give the corrected MPN. For example (4) under (A) above, the first "5" of the 5-5-4 code represents a sample volume of 1.0 ml. Look up the 5-5-4 code as if the 1.0 ml volume actually were 10 ml, as if the 0.1 ml volume actually were 1.0 ml and as if the 0.01 ml volume actually were 0.1 ml. The MPN obtained (1600) then is multiplied by a factor of 10 to give the corrected value. A simple formula for this type of correction is shown on a later page of this chapter.

III PRECISION OF THE MPN VALUE

- A The current edition of Standard Methods shows for each MPN value, the 95% confidence limits for that value. This draws attention to the fact that a given MPN value is not a precise measurement, but an estimate. The 95% confidence limits means that the observer will be correct 95% of the time when he considers that the actual number of cells producing the observed combination of positive and negative tubes was somewhere between the stated upper limit and the stated lower limit.
- B The greater the number of replicates of each sample volume in a dilution series, the greater the precision (in other words, the narrower the limits of the 95% confidence range) of the test. The precision of results, based on numbers of tubes inoculated per sample volume, is shown in Table 2.
- C Woodward ⁽⁴⁾ and other workers have studied the precision of the MPN in detail. Such reports should be studied by those desiring further information regarding the precision of the MPN test.

Table 2. Approximate Confidence Limits for Bacterial Densities as Per Cent of MPN as Determined from Various Numbers of Tubes in Three Decimal Dilutions*

Number of tubes in each dilution	50%		75%		80%		90%		95%	
	Lower	Upper								
1	33	186	18	340	15	402	10	637	6.5	955
2	47	160	31	246	27	276	20	383	15	511
3	53	150	38	215	34	237	26	311	21	395
5	64	139	49	182	46	196	37	241	31	289
10	76	127	63	152	60	160	52	184	46	208

*The interpretation of these figures is as follows: When MPN estimates are made on the basis of dilution tests using one tube in each of three decimal dilutions, you will be right 50% of the time if you say that the true bacterial density is between 33% and 186% of the MPN. If you had used 5 tubes in each dilution you could reduce this interval to from 64% to 139% of the MPN and still be right 50% of the time. If a greater certainty were desired, say 95%, you would have to widen this interval to from 31% to 289%.

IV OCCURRENCE OF IMPROBABLE TUBE RESULTS

A Many of the theoretically possible tube results are omitted from the MPN Table. For example, codes 0-0-3, 0-0-4, and 0-0-5 are not included as well as many others. These are omitted, because, in the opinion of the authors of the tables, the probability of occurrence of such results is so low as to exclude them from practical consideration.

B The frequency of occurrence of various code results is shown in the Table 2 both on a theoretical basis and on the basis of actual laboratory experience.

C From the MPN tables, it can be inferred that the codes omitted from the MPN Table can be expected to occur up to 1% of the time. If, in reviewing laboratory data, the theoretically unlikely codes occur appreciably more than 1% of the time, there is an indication for inquiry into the causes. Such results can occur (1) as a consequence of faulty laboratory procedures, or (2) as a result of extraneous influences in the samples.

D The current edition of Standard Methods does not include MPN values for many rare combinations listed in previous editions. By pruning out those codes listed as Group IV in Table 3, the table has been considerably condensed. Table 4 suggests maximum permissible numbers of samples for various numbers of samples tested.

Table 3
FIVE-TUBE AND THREE-TUBE CODES THAT
INCLUDE 99 PER CENT OF ALL RESULTS

Group	Theoretically Ex- pected Percentage of Results	Theoretically Ex- pected Cumulative Percentage	Observed Percentage of 360 Samples
Five-Tube-Test			
Class 1 codes 550, 551, 552, 553, 554, 500, 510, 520, 530, 540, 100, 200, 300, 400.	67.5	67.5	68.0
Class 2 codes 511, 521, 531, 541, 542, 110, 210, 310, 410, 420.	23.6	91.1	23.1
Class 3 codes 501, 010, 532, 320, 522, 220, 543, 430, 120, 533, 330, 502, 020, 544, 440, 301, 401, 431, 201, 411, 101, 311, 421, 211, 001.	7.9	99.0	7.5
Improbable codes	1.0	100.0	1.4
Three-Tube Test			
Class 1 codes 330, 331, 332, 300, 310, 320, 100, 200.	81.5	81.5	81.7
Class 2 codes 321, 311, 301, 210, 110, 010.	14.9	96.4	14.1
Class 3 codes 322, 220, 201, 101 312, 120.	2.7	99.1	3.7
Improbable codes	0.9	100.0	0.6

Table 4

MAXIMUM PERMISSIBLE NUMBERS OF IMPROBABLE CODES FOR VARIOUS NUMBERS OF SAMPLES TESTED

Number of Samples	Maximum Number of Improbable Codes
1 - 15	1
16 - 45	2
46 - 83	3
84 - 130	4
131 - 180	5
181 - 233	6
234 - 290	7
291 - 350	8
351 - 413	9
414 - 477	10
478 - 543	11

Example: From a sample of water, 5 out of five 0.01 - ml portions, 2 out of five 0.001 - ml portions, and 0 out of five 0.0001 - ml portions, gave positive reactions.

From the code 5-2-0 in the MPN table, the MPN index is 49

$$\begin{matrix} 49 \\ \text{(from table)} \end{matrix} \times \frac{10}{0.01} = 49,000$$

$$\text{MPN Index} = 49,000$$

A simple approximation of the most probable number may be obtained from the following formula (after Thomas):

$$\text{MPN}/100 \text{ ml} =$$

$$\frac{\text{No. of Positive Tubes} \times 100}{\sqrt{\text{No. of ml in negative tubes} \times (\text{No. of ml in all tubes})}}$$

E Table 5 is from International Standards for Drinking-Water, published by the World Health Organization, Geneva (1958). The last three values, not shown in the WHO publication, are from Woodward, "How Probable is the Most Probable Number."(4)

F Several theoretically possible combinations of positive tube results are omitted in Table 5. These combinations are omitted because the statistical probability of occurrence of any of the missing results is less than 1%. If such theoretically unlikely tube combinations occur in more than 1% of samples, there is need for review of the laboratory procedures and of the nature of the samples being tested.

When the series of decimal dilutions is other than 10, 1.0 and 0.1 ml, use the MPN in Table 5, according to the following formula:

$$\text{MPN (from table)} \times \frac{10}{\text{Largest quantity tested}}$$

$$= \text{MPN}/100 \text{ ml}$$

Example: From a sample of water, 5 out of five 10 - ml portions, 2 out of five 1.0 ml portions, and 0 out of five 0.1 ml portions gave positive results.

$$\text{MPN}/100 \text{ ml} = \frac{7 \times 100}{\sqrt{(3.5) \times (55.5)}} = 50.22$$

$$\text{MPN}/100 \text{ ml} = 50$$

Note that the MPN obtained from the table on the preceding pages with these tube results is 49. "Most probable numbers computed by the above formula deviate from values given by the usual methods by amounts which ordinarily are insignificant. The formula is not restricted as to the number of tubes and dilutions used ---" (Thomas)

Use of Tables of Most Probable Numbers

Table 5. MPN INDEX AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN FIVE 10-ML PORTIONS, FIVE 1-ML PORTIONS AND FIVE 0.1 ML PORTIONS ARE USED

No. of Tubes Giving Positive Reaction out of			MPN Index per 100 ml	95% Confidence Limits		No. of Tubes Giving Positive Reaction out of			MPN Index per 100 ml	95% Confidence Limits	
5 of 10 ml Each	5 of 1 ml Each	5 of 0.1 ml Each		Lower	Upper	5 of 10 ml Each	5 of 1 ml Each	5 of 0.1 ml Each		Lower	Upper
0	0	0	<2								
0	0	1	2	<0.5	7	4	2	1	26	9	78
0	1	0	2	<0.5	7	4	3	0	27	9	80
0	2	0	4	<0.5	11	4	3	1	33	11	93
						4	4	0	34	12	93
1	0	0	2	<0.5	7						
1	0	1	4	<0.5	11	5	0	0	23	7	70
1	1	0	4	<0.5	11	5	0	1	31	11	89
1	1	1	6	<0.5	15	5	0	2	43	15	110
1	2	0	6	<0.5	15	5	1	0	33	11	93
						5	1	1	46	16	120
2	0	0	5	<0.5	13	5	1	2	63	21	150
2	0	1	7	1	17						
2	1	0	7	1	17	5	2	0	49	17	130
2	1	1	9	2	21	5	2	1	70	23	170
2	2	0	9	2	21	5	2	2	94	28	220
2	3	0	12	3	28	5	3	0	79	25	190
						5	3	1	110	31	250
3	0	0	8	1	19	5	3	2	140	37	340
3	0	1	11	2	25	5	3	3	180	44	500
3	1	0	11	2	25	5	4	0	130	35	300
3	1	1	14	4	34	5	4	1	170	43	490
3	2	0	14	4	34	5	4	2	220	57	700
3	2	1	17	5	46	5	4	3	280	90	850
3	3	0	17	5	46	5	4	4	350	120	1,000
4	0	0	13	3	31	5	5	0	240	68	750
4	0	1	17	5	46	5	5	1	350	120	1,000
4	1	0	17	5	46	5	5	2	540	180	1,400
4	1	1	21	7	63	5	5	3	920	300	3,200
4	1	2	26	9	78	5	5	4	1600	640	5,800
4	2	0	22	7	67	5	5	5	2400		

Table 6. MPN AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE RESULTS IN A PLANTING SERIES OF FIVE 10-ml PORTIONS OF SAMPLE

No. of Positive Tubes Out of: Five 10-ml Tubes	MPN per 100 ml	Limits of MPN	
		Lower	Upper
0	2.2	0	6.0
1	2.2	0.1	12.6
2	5.1	0.5	19.2
3	9.2	1.6	29.4
4	16.0	3.3	52.9
5	> 16	8.0	

Table 7. MPN AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE RESULTS IN A PLANTING SERIES OF FIVE 10-ml, ONE 1-ml, AND ONE 0.1-ml PORTIONS OF SAMPLE

No. of Positive Tubes Out of:			MPN per 100 ml	Limits of MPN	
Five 10-ml Tubes	One 1-ml Tube	One 0.1-ml Tube		Lower	Upper
0	0	0	<2		5.9
0	1	0	2	0.050	13
1	0	0	2.2	0.050	13
1	1	0	4.4	0.52	14
2	0	0	5	0.54	19
2	1	0	7.6	1.5	19
3	0	0	8.8	1.6	29
3	1	0	12	3.1	30
4	0	0	15	3.3	46
4	0	1	20	5.9	48
4	1	0	21	6.0	53
5	0	0	38	6.4	330
5	0	1	96	12	370
5	1	0	240	12	3700
5	1	1	>240	88	

IV TABLES OF MOST PROBABLE NUMBERS

These tables consist of the MPN indices and 95% confidence limits, within which the actual number of organisms can lie, for various combinations of positive and negative tubes. Three MPN tables are presented. Table 5 is based on five 10 ml, five 1.0 ml and five 0.1 ml sample portions; Table 6 is based on five 10 ml sample portions; and Table 7 is based on five 10 ml, one 1 ml and one 0.1 ml sample portion.

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This outline was prepared by H. L. Jeter, Director, National Training Center, Water Programs Operations, Environmental Protection Agency, Cincinnati, OH 45268.

MPN THEORY

Part 2

I DERIVATION OF THE MPN

A Assumptions

The validity of the MPN procedure is based upon two principal assumptions.

- 1 In statistical language, the first is that the organisms are distributed randomly throughout the liquid. This means that an organism is equally likely to be found in any part of the liquid, and that there is no tendency for pairs or groups of organisms either to cluster together or to repel one another.
- 2 The second assumption is that each sample from the liquid, when incubated in the culture medium, is certain to exhibit growth whenever the sample contains one or more organisms.

B The Probability Equation

Based upon these assumptions, an equation for the probability of the observed combination of positive and negative tubes can be derived as a function of the true density δ . By solving this equation for different values of δ a curve can be plotted as shown in Figure 1.

Curves of this type always have a single maximum or peak. The value of δ , say d , which corresponds to the peak of the curve is called the most probable number, commonly designated as MPN.

The MPN is "most probable" in the sense that it is the number which maximizes the probability of the observed results. It is interesting to note that although the original derivation of the MPN predates modern statistical estimation, the MPN procedure corresponds to the currently accepted estimation procedure known as the "method of maximum likelihood."

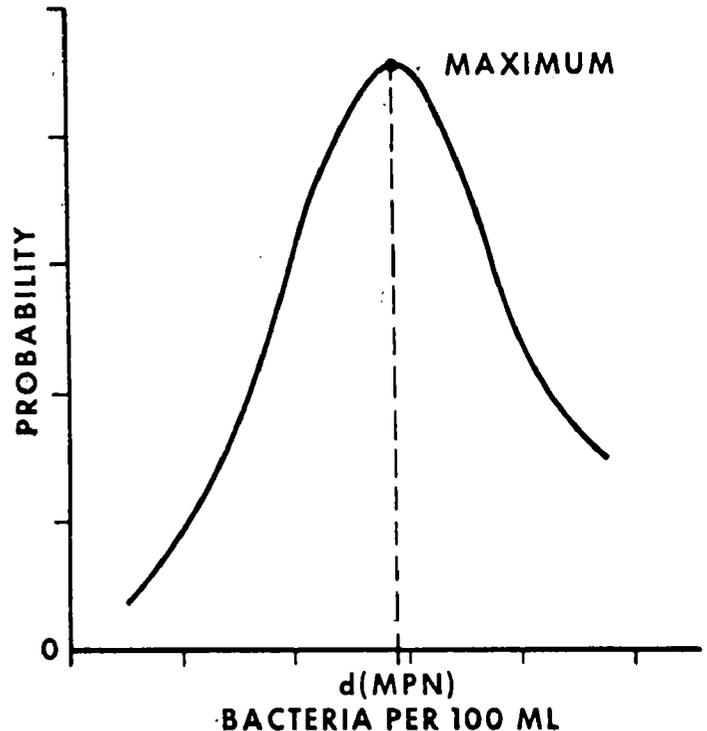


FIGURE 1

C Indeterminant Solutions

The MPN provides a meaningful estimate of δ only if there are both positive and negative tubes in at least one dilution. If all tubes are negative, the maximum of the probability curve occurs when δ is set equal to zero (see Figure 2) and thus the MPN is zero. If all tubes are positive, the maximum of the probability curve occurs when δ is set equal to infinity (see Figure 3) and thus the MPN is infinity.

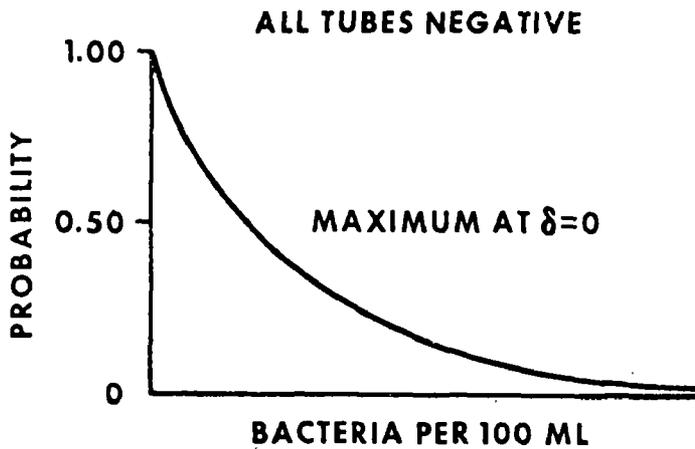


FIGURE 2

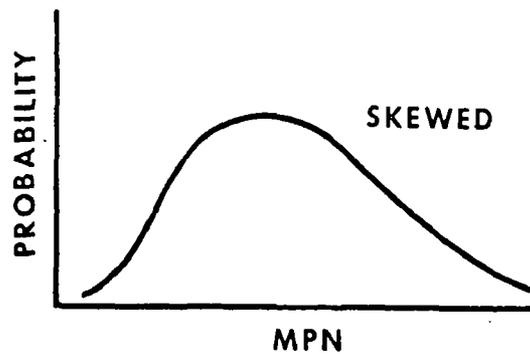


FIGURE 4

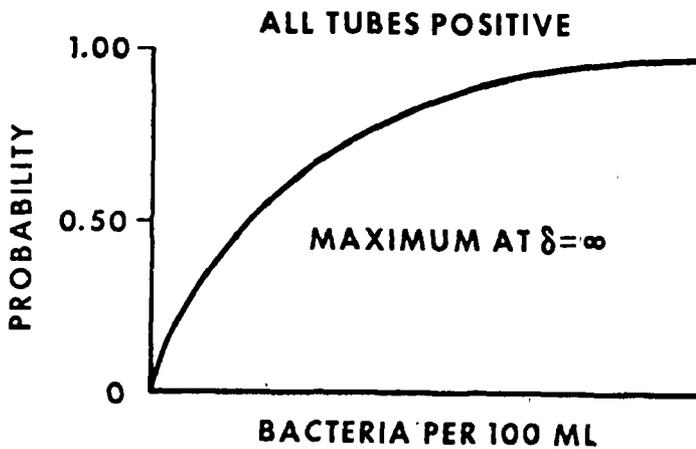


FIGURE 3

II DISTRIBUTION OF MPN VALUES

A Skewed Distribution

If a very large number of independent MPN determinations were made on the same water sample, the distribution of the MPN values would be such that very high values relative to the median value would occur more frequently than very low values. Thus the distribution of MPN values is skewed to the right as shown in Figure 4.

B Logarithmically Normal Distribution

Since it is mathematically inconvenient to work with data distributed asymmetrically, it is desirable to transform the skewed data in such a way that the transformed values have a symmetric distribution resembling the normal. In the case of MPN values the logarithms of the MPN's are approximately normally distributed as shown in Figure 5.

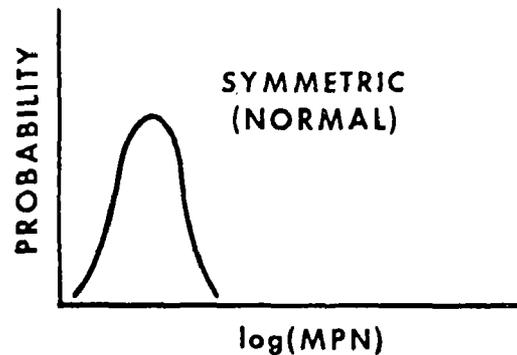


FIGURE 5

C Precision of MPN Estimates

The lack of precision of MPN estimates of bacterial densities is generally recognized. A measure of the precision is given by the confidence limits on the estimate which can be computed on the basis of the normal distribution of the logarithms of the MPN values. It has been verified that three-tube and five-tube MPN estimates are approximately logarithmically normal and the standard deviation of the logarithms of the MPN's is given by the formula:

$$\sigma_{\log} = \frac{0.58}{\sqrt{n}}$$

where σ_{\log} is the standard deviation of the logarithms of the MPN estimates and n is the number of tubes in each dilution.

The upper and lower 95% confidence limits of an MPN estimate are given by the formulas:

$$\text{UCL} = \text{antilog} (\log \text{MPN} + 1.96\sigma_{\log})$$

$$= \text{MPN} \cdot k,$$

$$\text{LCL} = \text{antilog} (\log \text{MPN} - 1.96\sigma_{\log})$$

$$= \text{MPN} \div k,$$

$$\text{where } k = \text{antilog} (1.96\sigma_{\log}).$$

Notice that the confidence limits are not symmetric about the MPN estimate.

The precision of the MPN estimate can be increased by increasing the number of tubes per dilution. Figure 6 shows the width of the 95% confidence interval expressed as a percentage of the MPN estimate for various values of n . Notice that the width of the confidence interval decreases as n increases.

III PLANNING A DILUTION SERIES

A The Rationale

It was mentioned that the MPN procedure provides a reasonable estimate of the true density only if there are both positive and negative tubes in at least one dilution. It follows that in a series of dilutions the expected number of organisms in the highest sample volume (lowest dilution) ν_H should be at least one, otherwise all tubes may be negative and the result will be an indeterminate value.

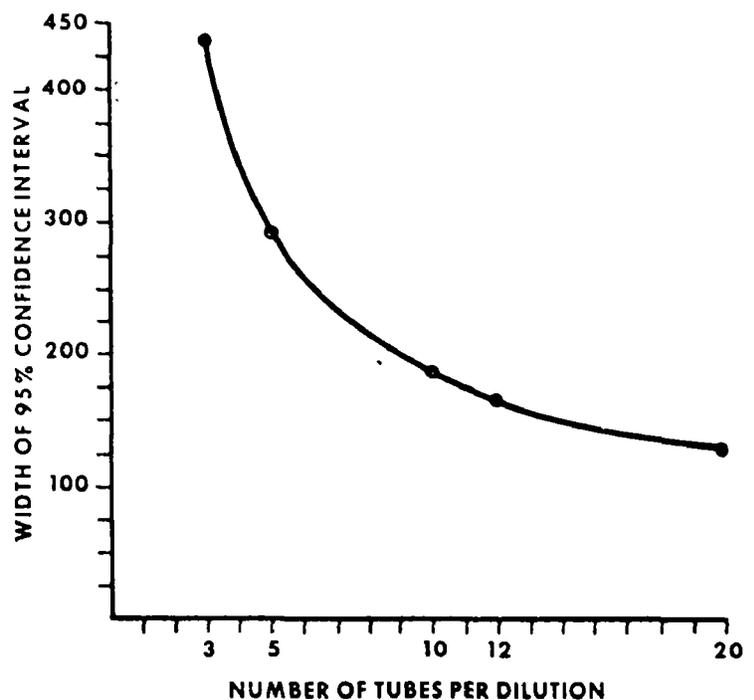


FIGURE 6

Similarly, the expected number of organisms in the lowest sample volume (highest dilution) v_L should not exceed one, to avoid the risk that all tubes will be positive.

density lies. The sample volumes are then chosen to satisfy the rules

$$v_H \geq \frac{1}{\delta_L} \quad ; \quad v_L \leq \frac{1}{\delta_H}$$

B The Rule

The above line of reasoning leads to the rule that a dilution series is capable of estimating any density between $1/v_H$ and $1/v_L$. In practice, we use the rule by first guessing two limits δ_H and δ_L between which we are fairly certain that the actual

Table 1 displays the range of densities covered by various decimal dilution series.

TABLE 1

SAMPLE VOLUME (ML)	RANGE COVERED (COLIFORMS/100 ML)
10^1	$10^1 - 10^3$
10^0	
10^{-1}	$10^2 - 10^4$
10^{-2}	
10^{-3}	$10^3 - 10^5$
10^{-4}	
10^{-5}	$10^4 - 10^6$
10^{-6}	
10^{-7}	$10^5 - 10^7$
	$10^6 - 10^8$
	$10^7 - 10^9$

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THE MEMBRANE FILTER IN WATER BACTERIOLOGY

I HISTORICAL BACKGROUND

There is sometimes a tendency to look upon membrane filters and their bacteriological applications as new developments. Both the filters and many of their present bacteriological applications are derived from earlier work in Europe.

A Some European developments prior to 1947 are as follows:

- 1 Fick is credited with application of collodion membranes in biological investigations in 1855.
- 2 Sanarelli, in 1891, reported development of membrane filters impermeable to bacteria but permeable to their toxins.
- 3 Bechhold, in the early 1900's made a systematic study of the physico-chemical properties of a number of varieties of these membranes. After 1911 numerous investigations were made in several countries with respect to the properties of collodion membranes.
- 4 Zsigmondy and Bachmann, 1916-1918, developed improved production methods which were applicable on a commercial scale. Membrane filters have been produced for many years at the Membranfiltergesellschaft, Sartorius Werke, in Goettingen, Germany. In 1919 Zsigmondy applied for a U.S. patent on his production methods; it was granted in 1922.
- 5 In the 1930's, W. J. Elford in England, and P. Graber in France, made new contributions in developing and teaching methods for making collodion membranes with controlled pore size.
- 6 Before World War II filtration procedures using the Zsigmondy membrane had been suggested for the

determination of bacterial counts, coliform determinations, and isolation of pathogenic bacteria from water and other fluids. Most early interest in developing these techniques seems to have been in Germany and in Russia. During World War II Dr. G. Mueller applied membrane filter techniques to the bacteriological examination of water, following bomb destruction of many of the laboratories.

B Developments in the United States

- 1 In 1947, Dr. A. Goetz reported on a mission to Germany as a scientific consultant to the Technical Industrial Intelligence Branch, U. S. Department of Commerce. He obtained detailed information about the nature, method of preparation, and specific bacteriological applications of the Zsigmondy Membranfilter being manufactured by the Membranfiltergesellschaft in Goettingen.
- 2 After his return to this country, Dr. Goetz developed methods for preparing and improved type of membrane filter from domestic materials. On a small scale he manufactured filters under a government contract; afterward membrane filter manufacture was continued by a commercial organization.
- 3 In 1950, bacteriologists of the Public Health Service began intensive study of the applications of membrane filters in bacteriological examinations of water. Their first report was published in 1951, and was followed by numerous reports of other similar investigations. Such studies have been widely expanded, as indicated in references shown elsewhere in this manual.

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the OWP, Environmental Protection Agency.

- 4 In 1955 the 10th Edition of "Standard Methods for the Examination of Water, Sewage, and Industrial Wastes" included a tentative method for coliforms by membrane filter method. In the 11th and 12th editions, the membrane filter method for coliforms has become official. In addition, methods for enterococcus (fecal streptococci) are included as tentative methods. The 13th edition has given the fecal streptococcus test a standard designation and the tentative method status reserved for the "pour plate" technique of quantitation.
- 5 The membrane filter is an official method for examination of potable waters in interstate commerce. The Public Health Service Drinking Water Standards (1962) state "Organisms of the coliform group... All the details of technique... shall be in accordance with Standard Methods for Examination of Water and Wastewater, current edition..." Thus, acceptance by Standard Methods as official automatically validates a method for use with interstate waters.

II PROPERTIES OF MEMBRANE FILTERS

Membrane filters used in water bacteriology are flat, highly porous, flexible plastic discs about 0.15 millimeters in thickness and usually 47-50 millimeters in diameter.

A Principle of Manufacture

The procedures described below are from FIAT Report 1312. While the methods indicated by Goetz do not necessarily describe the current manufacturing processes, it is assumed that similar principles of manufacture still apply.

- 1 One or more cellulose esters, such as cellulose nitrate, is dissolved in a suitable solvent.
- 2 Water, or some other liquid insoluble in the cellulose solution, is added and mixed, to form an emulsion having great uniformity in size and distribution of droplets of the insoluble liquid.

- 3 The emulsion is cast on plates and dried in an environment rigidly controlled as to humidity and temperature. The droplets of insoluble fluid retain their size and identity in the dried film, eventually becoming the pores of the finished membrane.
- 4 The dried porous film is cut into filter discs of the desired size. Representative discs are subjected to control tests for accurate determination of the pore size obtained.
- 5 Particle retention by membrane filters is at or very near the filter surface, by a mechanical, sieve-like action. (This applies to hydrosols, not to aerosols.) Through manufacturing control it is possible to make membrane filters with controlled pore size, within narrow limits.

B Some Important Characteristics of Membrane Filters

- 1 The membrane filters used in microbiology should be flat, circular, gridded, of uniform thickness and porosity, non-toxic to microorganisms, wettable, able to withstand commonly employed sterilizing conditions, and unaffected by the fluid to be filtered.
- 2 Without reference to specific manufacturers, some particulars of their products have included:
 - a ... Average pore diameter ranging from 5 millimicrons to 10 microns. Thicknesses ranging from 70 to 150 microns. Can be sterilized by autoclaving at 121°C for 10 minutes.
 - b ... mean flow pore size ranging from 7.5 millimicrons to 5 microns. The pore size used in water bacteriology having a standard diameter, has a water flow rate of 70cc/min/cm² and must pass 100 ml of particle-free water within 9 seconds.

- c ... currently produced in more than twenty distinct pore sizes from 14 microns to 10 millimicrons in discs ranging from 13 mm to 293 mm in diameter. The total range of pore size distribution of the type used in water microbiology of 0.45 microns is plus or minus 0.02 micron.
- d ... membranes are offered in graduated pore sizes ranging from 12 microns to 5 millimicrons. The types used in water bacteriology have a distilled water flow rate of 65 ml/min/cm² at 700 mm Hg differential pressure or an air flow rate of 0.4 liters/min/cm² at a differential pressure of 500mm water.
- 4 Membrane filters are wettable. Thus, after sample filtration, when a filter is placed on moist culture medium the medium diffuses through the pores and is available to organisms collected on the opposite surface.
- 5 Membrane filters are free of soluble chemical substances inhibitory to bacterial growth. Water soluble plasticizers are included in one commercially produced filter (glycerol, 2.5%). The cellulose esters themselves have some absorbing tendency illustrated by some dyes and heavy metals. Total ash is very low, less than 0.0001%.
- 6 Membrane filters have a uniform index of refraction. With membrane filters, this index is N_D : 1.5. When wetted with a liquid having refractive index within this range, the filters become transparent. This property permits direct microscopic examination of particulate matter collected on the filter surface.
- 7 Temperature resistance depends on plastics used in the filter. The nitrocellulose membrane filter is stable dry

up to 125°C in air. Membranes of cellulose triacetate are advertised to withstand dry heat to 266°C. In general, however, membranes in current use must be sterilized cautiously. Consult the laboratory equipment discussion for details. Overheating of all types interferes with filtration by blocking pores.

C Nomenclature

Membrane filters used in bacteriological tests on water are known under several names. Though the names are different, the filters are similar in form, properties, and method of use. Names commonly encountered are:

- 1 Membrane filter. This is the general name for filters made according to the general principles and having the properties discussed above. The term "membrane filter" is most used in technical reports on filters of this type.
- 2 Molecular filter. This name used by Goetz for the improved type of filter that he and his associates developed after study of the manufacturing methods at the Membranfiltergesellschaft in Goettingen, Germany.
- 3 Millipore filter is a trade name for membrane filters made by the Millipore Filter Corporation.
- 4 Bac-T-Flex filter is a trade name applied to certain membrane filters made by Carl Schleicher and Schuell Company.
- 5 Oxoid filter is a trade name applied to filters made by Oxo, Ltd., London, England.

- 6 Micropore, Polypore and Metricel have been trade names used by the Gelman Instrument Company.

- 2) A verified membrane filter coliform test can be used when needed as a supplement to the direct membrane filter test. Pure cultures are obtained from individual colonies differentiated on the membrane filter and subjected to further cultural, biochemical, and staining tests to establish the identity of the colonies being studied.

III APPLICATIONS IN WATER BACTERIOLOGY

A The basic cultural procedures for bacteriological tests on membrane filters are:

- 1 A sample is filtered through a membrane filter.
- 2 The filter is placed in a culture container, on an agar medium or a paper pad impregnated with moist culture medium.
- 3 The inoculated filter is incubated under prescribed conditions of time, temperature, and humidity.
- 4 After incubation, the resulting culture is examined and necessary interpretations and/or additional tests are made.

B With variations in such factors as culture media, incubation time, and combinations with other cultural and biochemical tests, several different kinds of tests are available.

- 1 Total bacterial counts are made by cultivation of bacteria on membrane filters using an enriched all-purpose culture medium.
- 2 Tests for bacterial indicators of pollution.

a Coliform tests

- 1) The direct membrane filter tests for coliforms is one in which, after sample filtration, the membrane filter is incubated in contact with one or more special media. At least one of the media is a selective, differential medium including components which permit coliform bacteria to develop colonies easily recognizable by form, color, sheen, or other characteristics.

- 3) The delayed membrane filter coliform test was developed to overcome bacterial changes frequently occurring when there is a delay of one to several days between sample collection and the initiation of laboratory tests. The test consists of sample filtration at or shortly after the time of sample collection. The inoculated filter is placed on a preservative medium and taken or sent to a laboratory, where it is transferred to a growth medium for the differentiation of coliform colonies. After incubation the culture is examined and the results are evaluated as for the direct membrane filter coliform test.

- 4) A medium and technique for detecting and counting fecal coliform bacteria has been developed and is called M-FC Broth. This medium currently is being used increasingly in water pollution studies.

- b Selective, differential, culture media have been developed for direct cultural tests for members of the enterococcus group of bacteria.

3 Tests for pathogenic bacteria

- a Workers are currently testing new media for the differentiation of members of the Salmonella-Shigella group of enteric pathogens. Available information indicates potential usefulness of a screening medium

for differentiation of nonlactose-fermenting, non urease-producing bacteria.

- b One medium has been used for screening tests in detection of Salmonella typhosa.
- c Further confirmatory cultural, biochemical, and serological tests are necessary to establish the identity of bacteria differentiated with these screening media.

C Membrane filter techniques can be applied both in the laboratory and under field conditions. Several varieties of portable membrane filter field units have been developed on a commercial basis.

IV ADVANTAGES AND LIMITATIONS

This evaluation is limited to tests for the coliform group. Similar, but separate evaluations would have to be made for any other bacteriological test.

A Advantages

- 1 Results are obtained in approximately 24 hours, as compared with 48-96 hours required for the standard fermentation tube method.
- 2 Much larger, and hence more representative samples of water can be sampled routinely with membrane filters.
- 3 Numerical results from membrane filters have much greater precision (reproducibility) than is expected with the fermentation tube method.
- 4 The equipment and supplies required are not bulky. A great many samples can be examined with minimum requirements for laboratory space, equipment, and supplies.

B Limitations

- 1 Samples having high numbers of non-coliform bacteria capable of growing on

Endo type culture media sometimes give difficulty. In such cases a high ratio of these noncoliform bacteria to coliforms results in poor sheen production, or even suppression, of the coliform organisms.

- 2 In samples having low coliform counts and relatively great amounts of suspended solids, bacterial growth sometimes develops in a continuous film on the membrane surface. In such cases the typical coliform sheen sometimes fails to develop.
- 3 Some samples containing as much as 1 milligram per liter of copper or zinc, or both, show irregular coliform bacterial results.
- 4 Occasional strains of bacteria growing on membrane filters producing sheen colonies prove, on subsequent testing, to be acid but not gas-producers from lactose. Where this occurs it may give a falsely-high indication of coliform density.

Such limitations as these are not frequent, but they do occur often enough to require consideration. In samples where these difficulties often occur, the best course of action often is to avoid use of membrane filter methods and use the multiple fermentation tube procedures.

V SUMMARY

The development of membrane filters and their bacterial applications has been discussed briefly, from their European origin to their current status in this country. Membrane filters currently available here have been described, and their properties have been considered. Applications of membrane filters in water bacteriology are indicated in general terms. Some of the advantages and limitations of membrane filter methods are presented for coliform tests.

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MEMBRANE FILTER EQUIPMENT AND ITS PREPARATION FOR LABORATORY USE

I Some equipment and supplies used in the bacteriological examination of water with membrane filters are specific for the method. Other items are standard in most well-equipped bacteriological laboratories and are readily adapted to membrane filter work. This chapter describes needed equipment and methods for its preparation for laboratory use. Where more than one kind of item is available or acceptable for a given function, sufficient descriptive information is provided to aid the worker in selecting the one best suited to his own needs.

II EQUIPMENT FOR SAMPLE FILTRATION AND INCUBATION

A Filter Holding Unit

1 The filter holding unit is a device for supporting the membrane filter and for holding the sample until it passes through the filter. During filtration the sample passes through a circular area, usually about 35 mm in diameter, in the center of the filter. The outer part of the filter disk is clamped between the two essential components of the filter holding unit. (See Plate 1)

a The lower element, called the filter base, or receptacle, supports the membrane filter on a plate about 50 mm in diameter. The central part of this plate is a porous disk to allow free passage of liquids. The outer part of the plate is a smooth nonporous surface. The lower element includes fittings for mounting the unit in a suction flask or other container suitable for filtration with vacuum.

b The upper element, usually called the funnel, holds the sample until it is drawn through the filter. Its

lower portion is a flat ring that rests on the outer part of the membrane filter disk, directly over the non-porous part of the filter support plate.

c The assembled filter holding unit is joined by a locking ring or by one or more clamps

2 Characteristics of filter holding units should include:

a The design of filter holding units should provide for filtration with vacuum.

(A) ASSEMBLED FILTER HOLDING UNIT

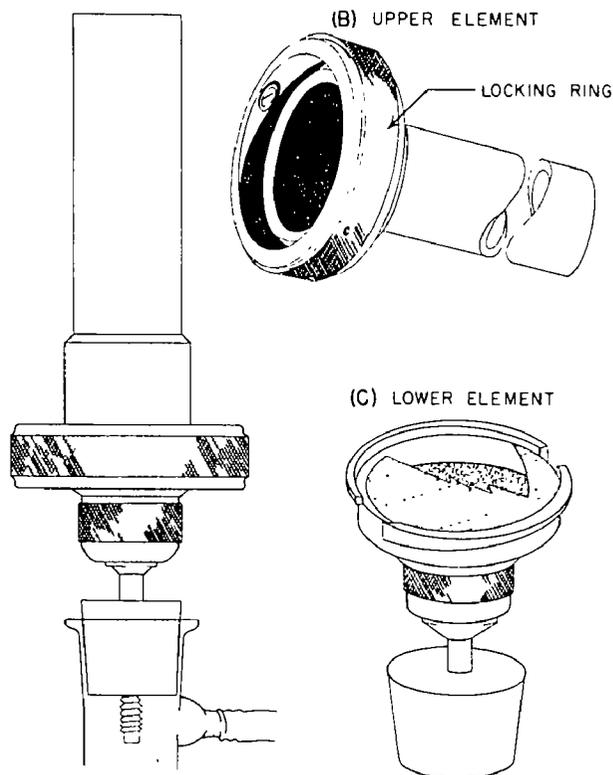


PLATE 1

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the CWP and the Environmental Protection Agency.

- b Filter holding units may be made of glass, porcelain, plastic, non-corrosive metal, or other impervious material.
 - c Filter holding units should be made of bacteriologically inert materials.
 - d All surfaces of the filter holding assembly in contact with the water sample prior to its passage through the membrane filter should be uniformly smooth and free from corrugations, seams, or other surface irregularities that could become lodging places for bacteria.
 - e Filter holding units should be easily sterilized by routine methods.
 - f The filter holding unit should be easily and quickly assembled and disassembled in routine operational use.
 - g Filter holding units should be durable and inexpensive. Maintenance should be simple.
- 3 Several forms of filter holding units have been developed for use with aqueous suspensions.
- a SS 47 Membrane Filter Holder (Plate 2, Figure 1)

Conical-shape funnel with a 500 ml capacity. The base section includes a wirescreen membrane support. Funnel and base section are evenly joined by a locking ring mechanism. This assembly is designed to hold a 47 mm diameter membrane firmly in place allowing an effective filter area of approximately 9.6 square centimeters. The entire filter unit is made of stainless steel with the funnel interior having a mirror-like finish.
 - b The Millipore Pyrex Filter Holder (Plate 2, Figure 2)

The unit is made of pyrex glass with coarse grade fitted support in base for filter. The upper element of early models of glass filter holders had a capacity of 1 liter. Currently available units are supplied with upper elements having 300 ml capacity. The assembled filter holder is joined with a spring clamp which engages on flat surfaces encircling the upper and lower elements.
 - c Millipore Standard Hydrosol Filter Holder (Plate 2, Figure 3)

Most components of this unit are made of stainless metal. The porous membrane support plate is fine-mesh stainless steel screening. The upper element is a straight-sided cylinder 4 to 5 inches in diameter, constricted to a narrow cylinder at the bottom, to fit the lower element. Capacity of the funnel element is about 1 liter. The assembled filter holding unit is joined by a bayonet joint and locking ring. Accessories may be obtained for collection of small amounts of filtrate and for anhydrous sterilization of the filter holding assembly.
 - d Gelman "Parabella Vacuum Funnel" (Plate 2, Figure 5)

The unit is made of spun stainless steel. The locking ring is a bayonet-type fitting, and is spring-loaded. The funnel element has a 1-liter capacity.
 - e The Sabro Membrane Filter Holder (Plate 2, Figure 4)

The unit is mostly of stainless steel construction. The lower element is a combination vacuum chamber, filtrate receiver, and filter supporting element. It consists of a stainless steel cup with a metal cover. The cover is fitted with a rubber gasket permitting airtight fit of the cover into the top of the cup. A porous sintered stainless steel membrane support disk is mounted in the center of the cover. At the side

of the beaker is a valve to which a pumping device can be fitted. The upper element is a stainless steel funnel with about 500 ml capacity. The assembled filter holding unit is joined by a locking ring at the base of the upper element. This engages on three spring clamps on the covering plate of the lower element.

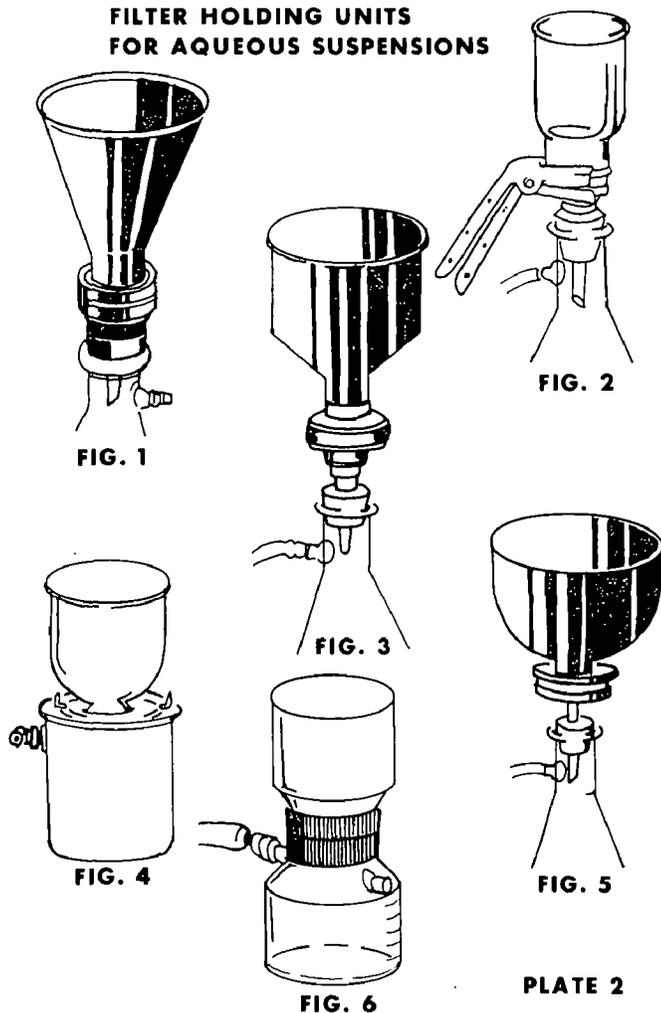
f Millipore "Sterifil" filter unit (Plate 2, Figure 6)

A funnel and flask unit of polycarbonate with filter base and support of polypropylene. Manufacturers tables should be referred to regarding chemicals which may be present in the sample and their effect on the holder and flask elements. This unit can be safely sterilized under steam pressure.

4 Care and maintenance of filter holding units

- a Filter holding units should be kept clean and free of accumulated foreign deposits.
- b Metal filter holding units should be protected from scratches or other physical damage which could result in formation of surface irregularities. The surfaces in contact with membrane filters should receive particular care to avoid formation of shreds of metal or other irregularities which could cause physical damage to the extremely delicate filters.
- c Some filter holding units have rubber components. The rubber parts may in time become worn, hardened, or cracked, necessitating replacement of the rubber part involved.
- d The locking rings used in some kinds of filter holders have two or more small wheels or rollers which engage on parts of the filter holding assembly. Occasional adjustment or cleaning is necessary to insure that the wheels turn freely and function properly. On some units, the wheels are plastic, and are not intended to turn. When worn flat, they should be loosened, turned a partial turn, and tightened again.

FILTER HOLDING UNITS FOR AQUEOUS SUSPENSIONS



B Membrane Filters and Absorbent Pads

- 1 The desired properties of membrane filters have been discussed elsewhere. Typical examples, commercially available include:
 - a Millipore Filters, Type HA, white, grid-marked, 47 mm in diameter
 - b S & S Type B-9, white, black-grid mark, 47 mm diameter

- c Oxid cellulose acetate membrane filters, 4.7 cm, grid-marked
- 2 An absorbent pad for nutrient is a paper filter disk, usually the same diameter as the membrane filter. Absorbent pads must be free of soluble chemical substances which could interfere with bacterial growth. They should be of such thickness that they will retain 1.8 - 2.2 ml of liquid culture medium. During incubation of cultures on membrane filters an absorbent pad saturated with liquid culture medium is the substrate for each filter. Absorbent pads are supplied with the purchase of membrane filters. Additional absorbent pads may be purchased separately. Sterilization in an autoclave is recommended for absorbent pads.

Almost any form of culture container is acceptable if it is made of impervious bacteriologically inert material. The culture container should, of course, be large enough to permit the membrane filters to lie perfectly flat. The following are widely used:

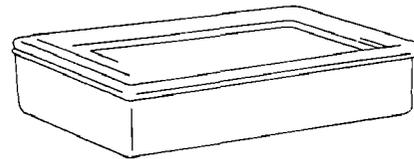
1 Glass petri dishes

Conventional borosilicate glass culture dishes are widely used in laboratory applications of membrane filters. For routine work, 60 mm X 15 mm petri dishes are recommended. The common 100 mm X 15 mm petri dishes are acceptable, but are subject to difficulties.

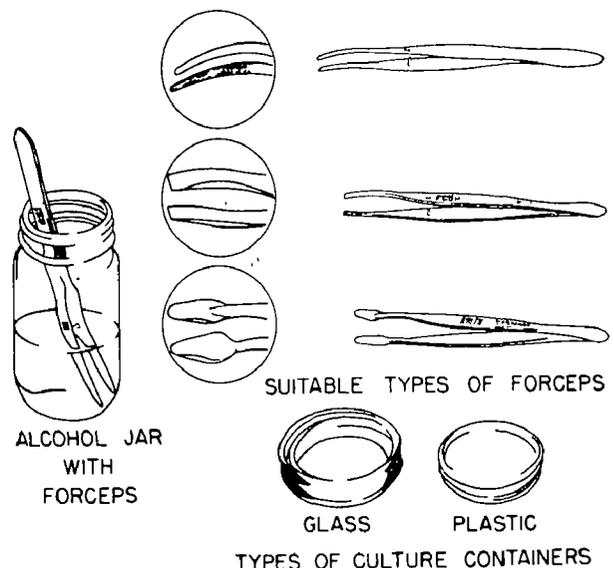
C Vacuum

Water can be filtered through a membrane filter by gravity alone, but the filtration rate would be too slow to be practical. For routine laboratory practice, two convenient methods are available for obtaining vacuum to hasten sample filtration.

- 1 An electric vacuum pump may be used connected to a filtration apparatus mounted in a suction flask. The pump need not be a high-efficiency type. For protection of the pump, a water trap should be included in the system, between the filtration apparatus and the vacuum pump.
- 2 A water pump, the so-called "aspirator" gives a satisfactory vacuum, provided there is reasonably high water pressure.
- 3 In emergency, a rubber suction bulb, a hand pump, or a syringe, may be used for vacuum. It will be necessary to include some form of valve system to prevent return flow of air.



HYDRATOR



ALCOHOL JAR WITH FORCEPS

SUITABLE TYPES OF FORCEPS

GLASS PLASTIC
TYPES OF CULTURE CONTAINERS

D Culture Containers (Plate 3)

Most membrane filter cultures are incubated in individual containers.

PLATE 3

2 Plastic petri dishes

Plastic containers have been developed for use with membrane filter cultures. Their cost is fairly low, and single-service use feasible. They cannot be heat-sterilized, but are supplied sterile. They must be free from soluble toxic substances. They can either be loose-fitting or of a tight lid to base friction fit.

E Other Equipment and Supplies Associated with Sample Filtration

1 Suction flask (Plate 4)

- a Most types of filter holding apparatus are fitted in a conventional suction flask for sample filtration. While other sizes may be used, the 1-liter size is most satisfactory.
- b The suction flask can be connected to the vacuum facility with thick-walled rubber tubing. Latex rubber tubing, 3/16" inside diameter, with wall thickness 3/32", is suggested. This tubing does not collapse under vacuum, yet it is readily closed with a pinch clamp.
- c A pinch clamp on the rubber tubing is a convenient means of cutting off the vacuum from the suction flask during intervals when samples are not actually being filtered. It is most convenient to have the vacuum facility in continuous operation during sample filtration work.
- d In laboratories conducting a high volume of filtration work, the suction flask may be dispensed. Filter-holding manifolds are available to receive up to three filtration units. The filtrate water is collected in a trap (in series with the vacuum source) which is periodically emptied.
- e Another arrangement can be made for dispensing with the suction flask. In this case, the receptacle element of the filtration unit is mounted in

the bench top. Instead of using a suction flask, the lower element of the filter holding unit has a dual connection with the vacuum source and with the laboratory drain. A solenoid-operated valve is used to determine whether the vacuum system or the drain line is in series with the filtration unit.

2 Ring stand with split ring (Optional) (See Plate 4)

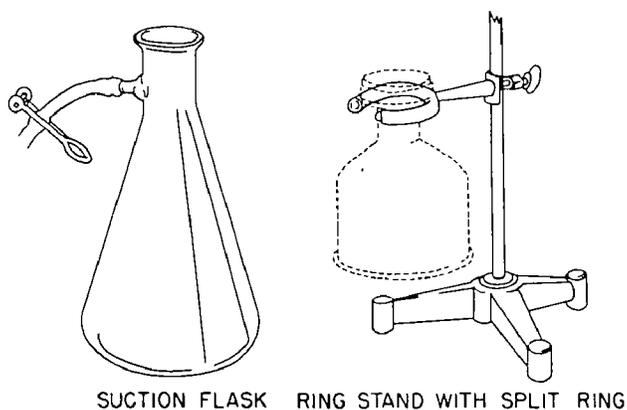


PLATE 4

When the filter holding unit is disassembled after sample filtration, the worker's hands must be free to manipulate the membrane filter. Upon disassembly of the filter holding unit, many workers place the funnel element, inverted, on the laboratory bench. Some workers, to prevent bacterial contamination, prefer a rack or a support to keep the funnel element from any possible source of contamination. A split ring on a ring stand is a convenient rack for this purpose.

3 Graduated cylinders

In laboratory practice, 100 ml graduated borosilicate glass cylinders are satisfactory for measurement of samples greater than 20 ml.

4 Pipettes and cans

- a Graduated Mohr pipettes are needed for many procedures, such as measurement of small samples, and for preparing and dispensing culture media. Pipettes should be available in 1 ml and 10 ml sizes.
- b Holding cans may be round or square but must not be made of copper. Aluminum or stainless steel are acceptable.

5 Alcohol jar with forceps (Plate 3)

- a All manipulation of membrane filters is with sterile forceps. For sterilization, forceps are kept with their tips immersed in ethanol or methanol. When forceps are to be used, they are removed from the container and the alcohol is burned off.
- b Forceps may be straight or curved. They should be designed to permit easy handling of filters without damage. Some forceps have corrugations on their gripping tips. It is recommended that such corrugations be filed off for membrane filter work.

6 A gas burner or alcohol burner is needed to ignite the alcohol prior to use of forceps.

7 Dilution water

The buffered distilled water described in "Standard Methods for the Examination of Water and Wastewater" for bacteriological examination of water is used in membrane filter methods. Dilution water is conveniently used in 99 ± 2 ml amounts stored in standard dilution bottles. Some workers prefer to use 9.0 ± 0.2 ml dilution blanks.

8 Culture medium

Bacteriological culture media used with membrane filter techniques are discussed at length in another part of this manual.

F Incubation Facilities

1 Requirements

a Temperature

For cultivation of a given kind of bacteria, the same temperature requirements apply with membrane filter methods as with any other method for cultivating the bacteria in question. For example, incubation temperature for coliform tests on membrane filters should be $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

b Humidity

Membrane filter cultures must be incubated in an atmosphere maintained at or very near to 100% relative humidity. Failure to maintain high humidity during incubation results in growth failure, or at best, in small or poorly differentiated colonies.

2 The temperature and humidity requirements can be satisfied in any of several types of equipment.

- a A conventional incubator may be used. With large walk-in incubators, it is extremely difficult to maintain satisfactory humidity. With most conventional incubators, membrane filter cultures can be incubated in tightly closed containers, such as plastic petri dishes. In such containers, required humidity conditions are established with evaporation of some of the culture medium. Because the volume of air in a tightly closed container is small, this results in negligible change in the culture medium. If glass petri dishes or other loosely fitting containers are used, the containers should be placed in a tightly closed container, with wet paper or cloth inside to obtain the required humidity conditions. A vegetable crisper, such as used in most home refrigerators,

is useful for the purpose. (See Plate 3)

- b A covered water bath maintaining $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ is necessary for the fecal coliform test and this will necessitate the use of a water-bath having forced circulation of water.

III STERILIZATION OF MEMBRANE FILTER EQUIPMENT AND SUPPLIES

A Filter Holding Unit

1 When is sterilization necessary?

- a The filter holding unit should be sterile at the beginning of each filtration series. A filtration series is considered to be interrupted if there is an interval of 30 minutes or longer between sample filtrations. After such interruption any further sample filtration is treated as a new filtration series and requires a sterile filter holding unit.
- b It is not necessary to sterilize the filter holding unit between successive filtrations, or between successive samples, of a filtration series.

After each filtration the funnel walls are flushed with sterile water to free them of bacterial contamination. If properly done, the flushing procedure will remove bacteria remaining on the funnel walls and prevent contamination of later samples.

2 Methods for sterilization of filter holding unit

- a Sterilization in the autoclave is preferred. Wrap the funnel and receptacle separately in Kraft paper and sterilize in the autoclave 15 minutes at 121°C . At the end of the 15 minutes holding period in the

autoclave, release the steam pressure rapidly, to encourage drying of the filter holding unit.

- b The unit may be sterilized by holding it 30 minutes in a flowing steam sterilizer.
- c The unit may be immersed 2 to 10 minutes in boiling water. This method is recommended for emergency or field use.
- d Some units (Millipore Stainless Unit) are available with accessories permitting anhydrous sterilization with formaldehyde. The method consists of introduction of methanol into a wick or porous plate in the sterilization accessory, assembly of the filter holding unit for formaldehyde sterilization, ignition of the methanol, and closure of the unit. The methanol is incompletely oxidized in the closed container, resulting in the generation of formaldehyde, which is bactericidal. The filter holding unit is kept closed for at least 15 minutes before use.
- e Ultraviolet lamp sterilizers are convenient to use. A device now commercially available for ultraviolet sterilization of membrane filter funnel units.

B Sterilization of Membrane Filters and Absorbent Pads

1 Membrane filters

- a Membranes are supplied in units of 10 in kraft envelopes, or in packages of 100 membranes. They may be sterilized conveniently in the packets of 10, but should be repackaged if supplied in units of 100. Large packages of filters can be distributed in standard 100 mm \times 15 mm petri dishes, or they can be wrapped in kraft paper packets for sterilization.

b Sterilization in the autoclave is preferred. Ten minutes at 121°C or, preferably, at 116°C is recommended. After sterilization the steam pressure is released as rapidly as possible, and the filters are removed from the autoclave and dried at room temperature. Avoid excessive exposure to steam.

c In emergency, membrane filters may be sterilized by immersion in boiling distilled water for 10 minutes. The filters should first be separated from absorbent pads and paper separators which usually are included in the package. The boiling water method is not recommended for general practice, as the membranes tend to adhere to each other and must be separated from one another with forceps.

2 Absorbent pads for nutrient

a Unsterile absorbent pads can be wrapped in kraft paper or stacked loosely in petri dishes, and autoclaved with membrane filters (ten minutes or longer at 121°C or 116°C).

b After sterilization absorbent pads for nutrient should be dried before use.

C Glassware

1 Sterilization at 170°C for not less than 1 hour is preferred for most glassware (pipettes, graduated cylinders, glass petri dishes). Pipettes can be sterilized in aluminum or stainless steel cans, or they may be wrapped individually in paper. The opening of graduated cylinders should be covered with paper or metal foil prior to sterilization. Glassware with rubber fittings must not be sterilized at 170°C, as the rubber will be damaged.

2 Sterilization in the autoclave, 15 minutes at 121°C, is satisfactory, and preferred by many workers. When sterilizing pipettes it is important to

exhaust the steam pressure rapidly and vent the containers momentarily. This allows the vapor to leave the can and prevents wet pipettes.

D Culture Containers

1 Glass petri dishes

a Petri dishes may be sterilized in aluminum or stainless steel cans, or wrapped in kraft paper or metal foil. They can be wrapped individually or, more conveniently, in rolls of up to 10 dishes.

b Preferably, sterilize glass petri dishes at 170°C for at least 1 hour.

c Alternately, they may be sterilized in the autoclave, 15 minutes at 121°C. After sterilization steam pressure should be released rapidly to facilitate drying of the dishes. Other suggested methods for sterilization of plastic dishes include exposure to ethylene oxide vapor (0.5 ml ethylene oxide per liter of container volume), or exposure to ultraviolet light. Ethylene oxide is a dangerous chemical being both toxic and explosive, and it should be used only when more convenient and safer methods are not available.

2 Plastic culture containers

a Because of the thermo-labile characteristics of the plastic, these containers cannot be heat sterilized. Manufacturers supply these in a sterile condition.

b For practical purposes, plastic dishes may be sterilized by immersion in a 70% solution of ethanol in water, for at least 30 minutes. Dishes must be allowed to drain and dry before use, as ethanol will influence the performance of culture media.

This outline was prepared by H. L. Jeter, Director, National Training Center, MDS, WPO, EPA, Cincinnati, OH 45268.

MEMBRANE FILTER EQUIPMENT FOR FIELD USE

I INTRODUCTION

One of the most troublesome problems in bacterial water analysis is the occurrence of changes in the bacterial flora of water samples between the time of sample collection and the time the actual bacterial analysis is started. Numerous studies have been made on this problem. From these have come such recommendations (Standard Methods, 10th ed) as holding the sample at 0-10°C and starting laboratory tests as soon as possible after collection of the sample. Recommendations of Standard Methods, 12th Edition, was to hold the sample as close as possible to the temperature of the source and to start the laboratory tests preferably within 1 hour and always within a maximum of 30 hours after collection. Changes in the 13th edition of Standard Methods (1971) will again call for the icing of samples, and further, that samples of environmental waters be held for not more than 8 hours total elapsed time before samples are plated or used for microbiological testing. The 30 hour maximum elapsed sample holding time will still be retained for potable water samples.

- A They would be useful in certain routine water quality control operations. Examples include such places as on board ships; some airlines, particularly in overseas operations; and some national parks. In each example, it is seen that there is an obvious difficulty in getting water samples to the examining laboratory in time for early examination.
- B In addition, such units would be invaluable in emergencies when existing laboratories are overburdened or inoperative. Portable kits already have proven extremely helpful in testing many small water supplies in a short period of time. Further there is a predictable need for such equipment in the event of a wartime civil defense disaster. Experience of the Germans in the vicinity of Hamburg during World War II lends support to this concept.

The purpose of this discussion is to introduce some of the portable equipment which has been developed and to point out noteworthy features of each. Actual practice and experience with these units reveal strong point and weaknesses in each type.

The membrane filter method has been accepted by the Federal Government for the bacteriological examination of water under its jurisdiction. This acceptance was based on methods developed and procedures applied in fixed laboratories. While the use of field kits is not excluded, no special concession has been made regarding the standards of performance of membrane filter field kits. Thus, in planning to use a membrane filter field kit for the bacteriological examination of water, it is the responsibility of the individual laboratory to establish beyond reasonable doubt, by comparison with Standard Methods fermentation tube tests or established laboratory membrane filter methods, the value of use of the membrane filter field kit in determining the sanitary quality of water supplies examined.

II TYPES OF COMMERCIALY AVAILABLE MEMBRANE FILTER EQUIPMENT FOR FIELD USE

A Sabro Water Laboratory

This unit represents a fixed membrane filter laboratory in miniature, with adaptations for special situations to be encountered in the field. Notable features:

- 1 The funnel unit supplied on older units is glass. A newer model has been released with an all-metal funnel unit.
- 2 The vacuum source is a hand pump (modified bicycle pump) or, optionally, an all-metal syringe.
- 3 The manufacturer sells prepared ampouled medium, in a liquid state. The medium should be kept at a cool

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temperature, out of the light. Its useful shelf life is uncertain, but limited tests by this agency indicate that the medium performs acceptably with storage up to one year.

- 4 Incubation of the cultures is in an incubator drawer having a capacity of 18 1-ounce culture containers, or 36 plastic containers, and operates electrically at 110V, and with suitable converters, at 6V, 12V. A battery also can be used with this unit.
- 5 Sterilization of the funnel unit is carried out by a "light flaming technique" or, optionally, by immersion of the funnel unit in hot or boiling water.
- 6 Useful accessories provided include thermometer, alcohol lamp, measuring cup, and forceps.

B Millipore Field Monitor Units

These units differ radically from any other field equipment that has appeared. Significant reductions in bulk of equipment have been brought about through major changes in function and design of the usual equipment. Notable features:

- 1 The funnel unit has been eliminated in its usual form. This has been done by development of a carefully fitted, single-use combination filtration unit and culture container. This feature eliminates most of the handling and use of accessory equipment.
- 2 The vacuum source is an all metal syringe with a fitting providing for direct connection to the culture container.
- 3 The culture medium provided by the manufacturer includes M-Endo Broth, MF, ready-to-use, in glass ampoules. These ampoules are so designed as to permit easy introduction of the culture medium into the culture container. Alternately, the manufacturer makes available a delayed-incubation medium in the ampoules. Other culture media can be used at the discretion of the user, but some difficulty can be anticipated in introducing the medium without special equipment.

- 4 Incubation of the cultures is provided in the field through use of an associated portable incubator and equipment carrying kit. This incubator has room for about 25 cultures. It is electrically operated, and through selection of available switching positions, operates at 6V, 12V, 110V, or at 220V.

- 5 Sterilization of components in the field is unnecessary. The culture containers and plastic tubes are single-use units supplied in a sterile condition. Samples do not come in contact with the syringe until after they have passed through the filter.

C Millipore Field Unit for Military Use

- 1 A modified Millipore field unit based on the case and incubator described in B, 4 above, has been adopted by the U.S. Department of Defense. This unit includes a miniaturized stainless metal funnel unit instead of the Monitors.
- 2 The vacuum source is an all-metal syringe.
- 3 Sterilization of funnel unit is by formaldehyde generated through incomplete combustion of methyl alcohol.

III COMMON DIFFICULTIES ENCOUNTERED IN COMMERCIALY AVAILABLE FIELD EQUIPMENT

- A The most conspicuous problem arising with field use of most units is their ultimate reliance on a fixed laboratory for essential supplies.
- B These portable laboratories will permit simultaneous incubation of up to 30 membrane filters.
- C For protracted field work, a fairly large amount of reserve supplies and equipment will be necessary. Such a reserve would include culture media, membrane filters, culture containers, fuel, and other expendable supplies required in the field, organized in a supplementary carrying case.
- D No currently available field unit provides illumination or optical assistance for interpretation of results.

E Some of the sterilization methods recommended by manufacturers are unacceptable. If field sterilization in boiling water is needed, then there must be a heat source and a metal can or beaker. Such equipment could be carried in the case suggested in C above.

D Sterilization of funnel units, graduated cylinders, media, etc., would be through immersion in boiling water for 2 minutes or longer, as indicated for the material being sterilized. Provision for boiling water is easy through use of a small camp stove or other simple burner.

IV IMPROVISED FIELD EQUIPMENT

The initial cost of most of the commercially manufactured units has met some objection. This factor, coupled with need for additional accessory supplies and equipment, has aroused interest in improvised units. Such a unit could consist largely of equipment normally used in a fixed laboratory, packaged in one or two fiberboard cases.

Improvised equipment, such as discussed above, would have great usefulness in emergencies, where commercially available membrane filter field units are not on hand.

A The funnel unit could be one of the familiar stainless steel units used in many laboratories; or it could be specially designed, smaller than ordinarily used, permitting use of up to a dozen or more upper filter holding elements in the field.

V In a separate outline are detailed descriptions of procedures for use of commercially available membrane filter field equipment. In some cases the suggested methods are different from those recommended by the manufacturers. In each case such departures are based on a series of experimental studies made by this agency, which suggested need for modification of existing recommendations.

B The vacuum source could be the modified bicycle pump (leathers reversed), and provided with a by-pass valve. The suction flask could be the standard side-arm glass flask, or a metal unit could be devised.

C M-Endo Broth or LES Endo agar are suitable media. Both are available as dehydrated medium which must be reconstituted and boiled in the field. M-Endo Broth MF is now available in liquid form, sterile, in sealed ampules. A shelf life of approximately one year is stated when stored under moderate temperatures in the dark.

REFERENCE

- 1 Laubusch, E. J. What You Should Know About the Membrane Filter, Public Works, 89: 106-13, 162-68, 1958.

LES MF Holding Medium - Coliform requires merely dissolving in distilled water. No heating is necessary. Such medium would be an advantage where applicable.

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PRINCIPLES OF CULTURE MEDIA FOR USE WITH MEMBRANE FILTERS

I INTRODUCTION

A Many kinds of membrane filter media have been described for use in bacteriological tests on water. This is noteworthy in view of the relatively few years the filters have been widely available in this country. This discussion is to consider several of these media in terms of their purposes, composition, and the ways in which they are used.

B Basic Considerations

- 1 Filtration of water sample through a membrane filter results in deposition of bacteria and particles of suspended matter on the filter surface. The bacteria can be cultivated in place if suitable culture medium is made available for their growth.
- 2 The bacteria are cultivated by placing the membrane on a pad of absorbent paper saturated with liquid culture medium, or on an agar medium. The culture medium diffuses through the pores of the filter, and is available to the bacteria on the opposite surface. Proper time, temperature, and humidity of incubation results in development of bacterial colonies. In principle, each bacterial cell multiplies to become a single bacterial colony.
- 3 Some culture media, satisfactory for tube cultures or agar plate cultures, do not perform well when used with membrane filters due to a selective adsorptive property of the filter itself. In the process of diffusion through the pores some components of the culture medium may be removed completely, or reduced in concentration. Thus, the composition of a given culture medium at the filter surface where it is available for bacterial growth may be different from its composition beneath the membrane filter.

There is evidence that improved cultural results sometimes are obtained with increased concentration of certain nutritive constituents of membrane filter culture media.

- 4 Pure cultures may be recovered from membrane filters and subjected to supplementary biochemical, cultural, and serological procedures for identification studies or for verification of interpretations based on direct observation of membrane filter cultures.

The same use can be made of agar plating media; however the membrane filter offers advantages due to the ability to concentrate organisms from a large volume of sample in which the organisms are present in low density.

C Applications of Membrane Filter Culture Media

The composition of bacteriological culture media designed for tube or plate cultures should be subjected to critical study before they are applied to membrane filter procedures. Media based on well-known bacteriological media have been modified for use with membrane filters for the following purposes in testing water.

- 1 Bacterial plate counts
- 2 Media for bacterial indicators of pollution
 - a Coliform organisms
 - b Fecal streptococcus group
 - c Clostridium perfringens
- 3 Salmonella and other enteric bacterial pathogens

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D Constituents of Membrane Filter Culture Media

Membrane filter media for the differentiation and counting of special groups of bacteria are based on the same principles used in differential agar plate media. Thus, the components of a differential medium for membrane filter cultures include:

- 1 Substances favoring growth of the organisms for which the medium is designed. Inclusion of special peptones, fermentable carbohydrates, yeast or meat extracts, water and chemicals to adjust pH to a desired level are common methods of favoring growth of desired organisms.
- 2 Differential indicator system. The purpose of the indicator system is to produce characteristic colonies of the desired bacterial groups for easy recognition when present in a mixture with extraneous types of colonies. This is done through inclusion of (a), a component which is chemically changed by the organisms to be differentiated, and (b), indicator substances, which give visible evidence of an intermediate or end product resulting from a chemical change of substance (a).
- 3 Selective inhibitors. Some bacterial groups to be tested may be overwhelmingly outnumbered by extraneous types of bacteria. In such cases, it is necessary that substances be included in the medium which (a), prevent growth of a maximum number of kinds of extraneous bacteria, and (b), have minimum adverse affect on growth of the kind of bacteria for which the medium is designed.

E Variety of Methods of Using Media Available with Membrane Filter Methods

1 Single-stage tests

After sample filtration, the membrane filter is placed on a designated culture medium, and left there throughout the incubation period. The culture results are examined and interpreted directly.

2 Multi-stage tests

A membrane filter can be transferred from one culture medium to another without disturbance of bacteria or colonies on the filter. This is unique with membrane filter methods, and lends itself to a variety of cultural and testing procedures.

- a The membrane filter, after sample filtration, can be incubated for a specified time on one medium, then transferred to a second medium. The method permits initiation of growth on enrichment medium, after which the membrane filter can be transferred to a less productive medium. With growth already begun, some differential culture media give better quantitative production than would be the case without preliminary incubation.
- b After incubation on one or more media, colonies on the membrane filter can be subjected to biochemical tests with reagents too toxic to include in the culture medium. Such reagents may be flooded over the growth on the filter, or the filter may be placed on an absorbent pad saturated with the reagent, in order to make such tests.
- c A third type of multi-stage test is one in which the membrane filter, after sample filtration, is placed temporarily on a medium containing a bacteriostatic agent. In the presence of such a substance, bacterial growth is inhibited or slowed greatly, but the organisms are not killed. During a limited period, the membrane filters may be transported or stored at ambient temperatures. The filter can be transferred later to a suitable medium and incubated for development of colonies.

II CULTURE MEDIA FOR TOTAL BACTERIAL COUNTS ON MEMBRANE FILTERS

A Concepts

- 1 Strictly, a "total" bacterial count medium is nonexistent. No single medium and incubation procedure can provide simultaneously the full range of oxygen requirements, needs for special growth substances, needs for pH requirements, etc., of all the kinds of bacteria found in water.
- 2 Actually, "total" bacterial counts are counts of the bacteria developing visible colonies on a defined culture medium at a known pH after incubation for a set time and temperature under aerobic conditions.
- 3 Within the foregoing limitations, the following criteria offer a useful basis for selection of membrane filter media to be used for estimates of the bacterial density in water.
 - a The medium and its method of use should produce a maximum number of colonies from all types of water. The colony yield should compare favorably with the bacterial counts determined as described in "Standard Methods for the Examination of Water, Sewage and Industrial Wastes," 13th Ed. (1971).
 - b The colonies should develop rapidly to a sufficient size to be counted after a minimum incubation period. At present, best results with membrane filter methods are obtained after about 18 hours incubation.
 - c The medium should be one which is reproducible and routinely available in laboratories.

B Composition of Total Count Media for Membrane Filters

Almost any rich, general growth promoting culture medium is acceptable for total bacterial counts on membrane filters. Several

such media have been suggested especially for membrane filter methods. These differ only in minor aspects, and can be discussed as a group. For details of composition and specific applications of each, see the media formulations elsewhere in this manual.

- 1 Growth promoting substances: All the substances included in these media are included to encourage growth of a maximum number of kinds of bacteria. Most workers agree that the peptone should be used in twice the concentration usually found in conventional tube or agar plating media.
- 2 Indicator substances are unnecessary with total count media.
- 3 Substances for the selective inhibition of certain bacterial groups are not included in total count media.

C Problems Encountered with Total Count Media

Bacterial colonial growth habits on membrane filters are similar to their surface growth habits on similar agar plate media.

- 1 As with agar plate media, some species of bacteria grow continuously, spreading over the surface of a membrane filter, tending to obscure nonspreading colonies which otherwise could be counted.
- 2 Some samples contain an appreciable amount of particulate matter. In sample filtration, this is deposited on the surface of the membrane filter with the bacteria. When the culture medium diffuses through the filter, a capillary film of liquid culture medium accumulates around the particles of extraneous matter. Bacteria not ordinarily considered "spreaders" sometimes develop confluent colonies due to the film of liquid medium accumulating around such particles.

- D What is the best total count medium for use with membrane filters?

Because of the relative ease of preparation, most workers prefer the commercially prepared dehydrated media. Difco M-Enrichment Broth (B 408) or Baltimore Biological Laboratories' M-Enrichment Broth (No. 331) are used interchangeably. Total colony productivity of these media is equivalent to that of media prepared from the individual components.

III CULTURE MEDIA FOR TOTAL COLIFORM TESTS ON MEMBRANE FILTERS

A Concepts

The nature of membrane filter culture methods imposes a different definition of coliform bacteria than the Standard Methods definition.

- 1 Standard Methods fermentation tube method. "The coliform group includes all of the aerobic and facultative anaerobic Gram-negative nonsporeforming rod-shaped bacteria which ferment lactose with gas formation within 48 hours at 35°C."
- 2 Membrane Filter Methods: "In the membrane filter procedure, all organisms that produce a colony with a metallic sheen in 22-24 hours are considered members of the coliform group. The sheen may appear as a small central focus or cover the entire colony." The guiding principle is that any amount of sheen is considered positive.
- 3 The Standard Methods definition of coliforms requires demonstration of the ability of organisms to produce gas through the fermentation of lactose. The membrane filter method does not lend itself to the demonstration of gas production. It relies instead on the development of a particular type of colony on an Endo type of culture medium. The culture medium is one in which lactose, basic fuchsin, and sodium sulfite comprise an indicator system to cause differentiation of

coliform colonies. While the bacterial groups measured by membrane filter methods are not identical with the group measured by Standard Methods procedures, they are believed to be essentially the same, and to have equal sanitary significance.

B Composition of Coliform Media for Membrane Filters

Several different media have been suggested for coliform tests on membrane filters. The components of these media can be classed into three convenient groups for general considerations.

- 1 Growth-promoting substances. Growth of bacteria on all the media is favored by the inclusion of such components as peptones (as Neopeptone, Thiotope Casitone, Trypticase, and other proprietary peptones), yeast extract, dipotassium phosphate (for adjustment of reaction of the medium), and distilled water. Lactose is included in all these media. It serves doubly, to favor growth of coliform bacteria, and as an essential component of the systems for differentiating coliform colonies.
- 2 Two kinds of differential indicator systems are available for demonstration of lactose fermentation on membrane filters.
 - a Lactose-basic fuchsin-sodium sulfite system (Endo type media).
 - 1) Media using this system include lactose and a suitable concentration of basic fuchsin which has been partially decolorized with sodium sulfite.
 - 2) The basic fuchsin-sodium sulfite complex requires very careful standardization. An excess of either component results in an unsatisfactory culture medium.
 - 3) The indicator system demonstrates lactose fermentation as follows:

- a) The coliform bacteria produce aldehyde as an intermediate product of the fermentation of lactose.
 - b) The aldehyde is "complexed" by the sodium sulfite-basic fuchsin indicator. In this process a reaction occurs in which red color is restored to the basic fuchsin. Colonies of bacteria fermenting lactose assume the color of the restored fuchsin. As the restored dye accumulates, it apparently precipitates on the colony, giving the colony a characteristic green-gold surface sheen. The reaction occurs best in an alkaline medium. The culture medium is adjusted to pH 7.5.
- 4) Endo type media require very careful standardization for successful use in the laboratory. Most workers prefer to use a commercially prepared and standardized medium. M-Endo Broth MF is the recommended coliform medium for use with membrane filters.
- b pH indicator system
- 1) Media using this system rely on detection of pH change due to the accumulation of organic acids, end products of lactose fermentation.
 - 2) Bromocresol purple, for example, is a pH indicator, approaching yellow at more acid pH. Colonies fermenting lactose and accumulating organic acids therefore turn yellow.
 - 3) Studies in England with membrane filters for coliform tests have been based on a modification of MacConkey's Medium, using this principle of colony differentiation in coliform tests.
- 3 Inhibitory substances in membrane filter coliform media
- a Confusing and erroneous results in coliform detection can be caused by
 - 1) the overgrowth of the membrane filter by extraneous nonlactose fermenting bacteria, preventing coliform colonies from developing the characteristic color and sheen; and
 - 2) the development of sheen colonies of lactose fermenting bacteria which produce acid but not gas in the fermentation of lactose.
 - b These difficulties can be reduced through incorporating of substances harmless to coliform bacteria but which have inhibitory effect on growth of extraneous forms. Attention must be given to the concentration of such substances, as excessive amounts also will reduce the productivity of the medium for coliform colonies. The following components of various culture media have proven useful in suppressing growth of noncoliform bacteria on membrane filters.
 - 1) Basic fuchsin-sodium sulfite. Although these compounds are included in Endo-type media for their role in differentiating coliforms from other types of colonies, they are effective in preventing the growth of many of the noncoliform bacteria occurring in water samples.
 - 2) Ethanol (95%... NOT denatured) is included in M-Endo Broth MF. In the concentration used, ethanol suppresses growth of some kinds of noncoliform bacteria, and tends to limit the colony size of others. In addition, the ethanol seems to increase the solubility of some of the other components of the media.

- 3) Sodium desoxycholate or bile salts are used in such media as M-Endo Broth MF, and in the modified MacConkey's Medium for membrane filters used in British studies. They are included primarily for their inhibitory effect against Gram-positive cocci and spore formers.

C Methods Available for Using Coliform Media with Membrane Filters

1 Single-stage coliform tests

- a After sample filtration, the membrane filter is incubated for the desired time on a selective coliform differentiating medium.
- b The coliform colonies are counted without further tests.
- c M-Endo Broth MF and LES Endo Agar Media are alternate standard single-stage coliform media.

2 Two-stage coliform tests

a Immediate coliform test

- 1) After sample filtration, the membrane filter is incubated $1\frac{1}{2}$ - 2 hours on the enrichment medium of lauryl tryptose broth.
- 2) The membrane is then transferred to a new absorbent pad saturated with the standard differential medium for coliform bacteria, and incubated for 20-22 hours at 35 ± 0.5 C.
- 3) The coliform colonies are counted without further tests.
- 4) This test procedure, based on EHC Endo Medium, was described in the 10th edition of Standard Methods. With the 12th edition, an official two-stage coliform test has been adopted, based on LES Endo Agar Medium.

b Delayed incubation coliform test

- 1) After sample filtration, the membrane filter is placed on an absorbent pad saturated with benzoated Endo Medium or with LES Holding Medium. The filter may be preserved up to 72 hours at ambient temperatures. During this time it can be transported or stored. Growth is stopped or greatly reduced.
- 2) The membrane filter can be transferred to a fresh absorbent pad, saturated with such a medium as M-Endo Broth MF, or to LES Endo Agar and incubated up to 24 hours.
- 3) The differentiated coliform colonies are counted as with other membrane filter coliform media.
- 4) This test procedure makes it possible to filter samples in the field, place the filters on preservative medium, then mail or transport them to the laboratory for completion of the bacteriological examination. The procedure is designed to eliminate the need for maintaining sample temperature in the interval between sample collection and initiation of the bacteriological examination. In addition, the method should produce results more nearly reflecting the quality of the source water than is available with other methods of collecting and testing samples.

3 Verified membrane filter coliform test

- a This is used to verify the interpretation of differentiated colonies on any type of membrane filter coliform medium. The test is suggested for: self-training of laboratory workers, for evaluation of new or experimental

media, and in any water examination in which the interpretation of results is in doubt or likely to be involved in legal controversy.

- b The test consists of obtaining pure cultures from differentiated coliform-like colonies on membrane filters, and subjected them to further cultural and biochemical tests to establish their identity as Gram-negative non-sporeforming bacilli which ferment lactose with gas production. The technical procedures are described elsewhere in this manual.

IV MEDIUM FOR THE FECAL COLIFORM TEST

A Concepts

The selective effect of elevated temperature has been the most important development in fecal coliform tests since 1904. In that year, Eijkman discovered that coliform bacteria from the gut of warm-blooded animals produced gas from glucose at 46°C, while the majority of coliform bacteria from other sources did not. Media variations were of only secondary importance.

Much medium variation has resulted from attempts to select for Escherichia coli, only, as the fecal coliform. While E. coli is usually the predominant coliform in human (and animal) feces, other types are present, including the alleged soil and plant coliform, Aerobacter aerogenes in very large numbers. All coliforms demonstrated by isolation to have arisen in feces are called here fecal coliforms and are measured empirically by the fecal coliform tube test. Membrane filter tests reflect divergence of attitude on indicators of fecal origin. Delaney et al. (1962) have published: Measurement of E. coli Type I by the Membrane Filter. Geldreich et al. (1965) have presented: Fecal-Coliform-Organism Medium for the Membrane Filter Technique.

Temperatures are the same but media are different. Because the fecal coliform test appears more convenient, it will be emphasized.

B Composition of Fecal Coliform Medium MFC

- 1 MFC medium is a rich growth medium containing lactose, proteose peptone no. 3, tryptone and yeast extract. A level of .3% sodium chloride produces favorable osmotic balance. Vigorous growth results. A practical result is shortening of test time to 24 hours.

The growth constituents are similar to those of the tube test for fecal coliform. Both have 0.15% bile salts to select for coliforms but elevated temperature is the more important selective factor.

- 2 The indicator system of aniline blue results in blue fecal coliform colonies. Nonfecal coliform colonies, generally few, are gray to cream-colored.

C Special Problems with MFC Broth Medium

- 1 Temperature control must be accurate. Current recommendations call for $44.5 \pm 0.2^\circ\text{C}$ and the temperature to be maintained in a water incubator of forced circulation.
- 2 Temperature equilibration must be rapid. Nonfecal coliforms may initiate growth at lower temperatures and subsequently give false positive blue colonies when incubated at 44.5°C. No more than 20 minutes lapse of time is recommended from filtration to incubation. Submergence in waterproof plastic bags reduces actual temperature equilibration to 10 - 12 minutes.
- 3 Rosolic acid presents some problems in preparation. It is practically insoluble in water and of limited stability in alkaline solution. A 1% solution in 0.2 N NaOH should be prepared and this added to the medium as recommended by the manufacturer

V MEDIA FOR FECAL STREPTOCOCCUS TESTS

A Introduction

- 1 The development of membrane filter culture media for the fecal streptococci reflects the continuing interest in this group of bacterial indicators of pollution. The productivity of enterococcus media recently has been greatly increased
- 2 Standards of performance of a good fecal streptococcus medium correspond with those of a good coliform medium on membrane filters. Thus, the requirements of productivity, specificity, ease of use, and reproducibility of the medium, are equally applicable to medium for the detection and enumeration of the fecal streptococci.

B M-Enterococcus Agar

- 1 In 1955, Slanetz, Bent, and Bartley described a modification of the Chapman mitis-salivarius medium, and reported satisfactory results on membrane filters for detection of enterococci from water.

The following year, Slanetz, Bartley, and Ray described a modification in which 1% agar was included in the medium. With this medium, the membrane filter is placed on the agar surface for incubation. This contrasts with the usual practice of using fluid media to saturate absorbent pads as the substrate for incubating membrane filter cultures. The authors reported generally larger enterococcus colonies and improved colony production with this modification of the medium.

2 Composition of the medium

- a The medium is a tryptone, glucose, general growth media buffered with dipotassium hydrogen phosphate. Yeast extract is included to provide vitamins and other growth factors. Many bacteria would thrive if inhibitor were not included.

- b The inhibitor sodium azide is a powerful suppressor of all except fecal streptococci. It may act in this manner by interfering with aerobic respiration.

False positives, that is red colonies which are not fecal streptococci, are known to occur. A verification procedure is included in the 13th edition of Standard Methods for fecal streptococcus detection.

c Safety note:

Sodium azide is poisonous. In handling dehydrated culture medium containing sodium azide, care should be taken to avoid any action which might create an airborne dust of the medium. If any powdered medium does get blown into the air, it is advisable to get away from the immediate vicinity of the dust until it settles.

- d The indicator is 0.1% 2, 3, 5, triphenyl tetrazolium chloride in the medium. The indicator is colorless in the medium (oxidized state). The metabolizing bacterial colonies reduce the 2, 3, 5 triphenyl tetrazolium chloride to the insoluble and red colored formazan (reduced state). Colonies appear flat, light pink to smooth raised dark red with pink margins after 48 hours incubation. Colonies tend small, 0.5 - 2 mm.

C KF Agar

- 1 This streptococcus medium was developed at SEC by Kenner, et al. and designated KF agar.

While KF medium is productive for detection and enumeration of the Fecal Streptococcus Group, its use

is hampered by nonspecificity. Studies have demonstrated that the medium supports growth of *S. bovis*, and other forms common in animals, but not numerous in the fecal excreta of humans.

2 Composition of KF Agar

- a Nutritive requirements of the fecal streptococci are supplied by peptone, yeast extract, sodium glycerophosphate, maltose, lactose, and distilled water.
- b The indicator system is phenol red and 2, 3, 5 triphenyl tetrazolium chloride. On KF Agar used with membrane filters, the fecal streptococcus colonies develop as small colonies, up to 2 mm in diameter, colored various shades from pale pink to a dark wine color.
- c The selective component of KF Agar is sodium azide, used in 0.04% concentration.

3 Miscellaneous applications of M-Enterococcus Agar and KF Agar

- a Both media can be used in a single stage test for fecal streptococci. Colonies are counted and reported after 48 hours incubation at $35 \pm 0.5^{\circ}\text{C}$.
- b Alternately, they can be used as a holding medium in a delayed incubation test for the fecal streptococcus group.
- c Both mediums can be used as pour plates.

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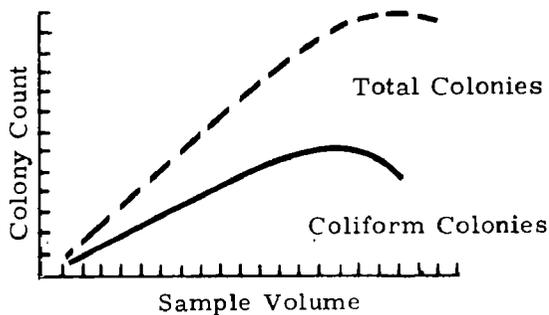
SELECTION OF SAMPLE FILTRATION VOLUMES FOR MEMBRANE FILTER METHODS

I INTRODUCTION

A Wide Range of Filtration Volumes

- 1 The membrane filter permits testing a wide range of sample volumes, from several hundred milliliters to as little as 0.0001 ml, or even less. Suitable dilution of sample volumes smaller than 1.0 ml may be required for accuracy of sample measurement.

- 2 While the method lends itself to a wide range of sample volumes, the filter has limitations in the number of isolated (or countable) differentiated colonies which can develop on the available surface area. Figure 1 illustrates a common pattern of colony counts over a wide range of sample filtration volumes.



- a The graph is based on coliform determinations using M Endo Broth MF. The line designated "Total Colonies" includes both coliform and noncoliform colonies. The "Coliform Colonies" line refers only to differentiated colonies having the typical color and sheen of coliform colonies on the medium.

- b For both the total colonies and the coliform colonies there is a proportional relationship between colony count and sample volume over much of the range of sample volumes. With increasing colony counts, there are some levels above which the proportional relationship fails, both for total and for coliform colonies.

- c In the straight lines in Figure 1, where there is proportionality between colony counts and filtration volumes, it is possible to compute density of bacteria in the sample, based on the equation:

$$\text{No. organisms per 100 ml} = 100 \times \frac{\text{No. colonies counted}}{\text{No. ml of sample filtered}}$$

The equation is not quantitatively reliable in the curved portions of the lines.

B Scope of this Presentation

- 1 To explain limitations on quantitatively reliable colony counts on membrane filters.

- 2 To present numbers of colonies acceptable for quantitative tests with available media.

- 3 To demonstrate the different quantitative bacterial density ranges determined by single-volume filtrations, with available media.

- 4 To demonstrate quantitative ranges covered by a series of filtration volumes with currently-used differential media.

- 5 To provide guidance for selection of sample filtration volumes under the following practical conditions:
 - a When there is need to determine compliance with established bacteriological water quality standards.
 - b When there is need to determine density of a specified bacterial group.
 - 1) In the absence of prior bacteriological data, and
 - 2) When prior bacteriological data are available.

- b Selectivity of medium. Highly selective media permit growth of relatively few colonies of extraneous, unwanted, bacteria. The available area of the filter is occupied primarily by colonies of the group tested. Thus, with a highly selective medium such as that used for fecal streptococci, it is reasonable to expect good quantitative results with relatively high colony counts. Conversely, media having limited selectivity (such as Endo-type media for coliforms) supports growth of considerable numbers of extraneous bacterial colonies, and it is necessary to place arbitrary limitations on the number of colonies per membrane in quantitative studies.

II LIMITATIONS ON COLONY COUNTS USED FOR QUANTITATIVE WORK

A Bacterial Density

- 1 The minimum sample volume should result in production of at least 20 colonies of the bacteria being counted. Sample volumes yielding lesser numbers of colonies are subject to unacceptably large random variations in the computed bacterial density, determined as above (I, A, 2).
- 2 The maximum acceptable colony density, for quantitative determinations, is variable with the bacterial group tested and the medium used. Factors influencing maximum acceptable colony density include:
 - a Size of colonies. In principle, each colony should represent one bacterial cell deposited on the filter, or, conversely, each bacterial cell deposited on the filter should result in production of a recognizable colony. Media producing relatively large colonies (as in the fecal coliform test) will support smaller numbers of colonies on the filter than media producing smaller colonies (such as fecal streptococci). If the colony size is large and the number of bacteria deposited on the filter is great, some colonies will represent two or more cells initially deposited on the filter, and the quantitative reliability of the test is impaired.

- c Biochemical interference between neighboring colonies. Associated with the physical crowding effects noted in (b) above, sheen production of coliform colonies may be inhibited by overcrowding of colonies. This reinforces the need for restriction of colony density on the filter.

B Suspended Matter

- 1 Particulate matter in the sample can be a limitation in application of membrane filters, especially when the amount of suspended matter is relatively great and the bacterial density is low.
- 2 Difficulties from suspended matter in the sample may be apparent in several ways.
 - a The pores of the filter may be occluded, limiting the volume of sample that can be filtered. This problem has been noted in waters rich in clays and in waters containing large populations of certain diatoms or other algae.
 - b Fibrous matter can be troublesome, due to the tendency for a capillary film of liquid culture medium to form around the fibers. Colonies in contact with such fibers tend to grow along

the path of the fibers, assuming highly irregular forms. Sometimes these colonies cover abnormally large areas of the filter surface.

- c A more or less continuous mat of particles may be collected from some samples, with each particle soon surrounded by a film of liquid culture medium. On such filters, distinct colonies usually fail to develop as discrete entities, but grow in a more or less continuous film over the entire surface of the filter.

- 3 Problems due to particulate matter often can be reduced by filtration of the selected volume of water in two or more smaller increments, through separate filters. In effect, this is a means of enlarging the available surface area of the filter.

Prefiltration of the sample through a coarse filter for preliminary removal of extraneous particulate matter is not recommended in quantitative work. Prefiltration invariably results in removal of unpredictably large numbers of bacterial cells.

In some cases the problem of particulates cannot be solved, and it must then be conceded that the membrane filter method is not acceptable for such samples. It then becomes necessary to resort to other procedures, such as the dilution tube method or agar plating methods.

III LIMITS ON NUMBER OF COLONIES ON FILTERS WITH VARIOUS MEDIA

Referring to Figure 1, a specific number of colonies is not shown for acceptable proportionality between colony number and filtration volume. Fixed limits cannot be stated for all test situations, for these limits are somewhat variable from one culture medium to another and from one sample source to another:

The recommended limits shown in Table 1 are empirical values based on research experience. It is believed that quantitative determinations of acceptable statistical reliability can be obtained if the determinations are based on colony counts within the limitations shown.

IV RANGE OF BACTERIAL DENSITIES COVERED BY SINGLE-VOLUME FILTRATIONS

- A The equation used in Section I of this outline can be used with any sample filtration volume to determine the bacterial density range over which acceptable counts can be made.

For example, assume that a sample of 10 ml is used for a quantitative determination of total coliforms. Based on Table 1, quantitative determinations should be based on a filtration volume yielding 20 - 80 coliform colonies. Compute the coliforms per 100 ml based on 20 colonies and on 80 colonies per filter. This will be the bacterial density range covered by a 10 ml filtration volume, thus:

for 20 colonies:

$$\begin{aligned} \text{No. coliforms per 100 ml} &= 100 \times \frac{20}{10} \\ &= 200 \end{aligned}$$

and for 80 colonies,

$$\begin{aligned} \text{No. coliforms per 100 ml} &= 100 \times \frac{80}{10} \\ &= 800 \end{aligned}$$

Thus, a 10 ml sample portion is appropriate for determination of total coliforms in the range 200 - 800 per 100 ml.

- B Table 2 illustrates the ranges covered for several filtration volumes, with colony counts in the ranges 20 - 60, 20 - 80, and 20 - 100 per filtration volume.

Table 1. RECOMMENDED COLONY COUNT RANGES FOR QUANTITATIVE DETERMINATIONS WITH MEMBRANE FILTER TESTS

Test	No. colonies		Medium	Remarks
	Minimum	Maximum		
Total Coliform	20	80	M Endo Broth MF, LES Endo Medium	Not more than 200 colonies of all types
Fecal Coliform	20	60	M FC Broth	
Fecal Streptococci	20	100	M Enterococcus Agar, KF Agar	
Total Counts	20	200	M Enrichment Broth	Spreaders may require adjustment

Table 2. RANGES COVERED BY REPRESENTATIVE FILTRATION VOLUMES

Ml sample filtered	Bacterial count per 100 ml based on			
	20 colonies	60 colonies	80 colonies	100 colonies
100	20	60	80	100
10	200	600	800	1000
1	2000	6000	8000	10,000
0.1	20,000	60,000	80,000	100,000
0.01	200,000	600,000	800,000	1,000,000

C Application of a Series of Filtration Volumes

- 1 Examination of Table 2 shows that for quantitative work on membrane filters, to extend the range of any test, it is necessary to filter two or more different sample volumes. The worker uses the one sample volume yielding a quantitatively acceptable number of colonies to compute the bacterial count per 100 ml.
- 2 Further, it can be seen that varying the filtration volumes by decimal increments will be inappropriate; there are values within the total range covered in which the colony number would fall outside the critical counting range for the test being made.
- 3 In order to give maximum assurance that a series of varying filtration volumes will yield at least one membrane

with an acceptable number of colonies, the range of filtration volumes should be along these lines:

- a Total coliform counts should be based on filtration volumes varying by a factor of 4, or less.
- b Fecal coliform counts should be based on filtration volumes varying by a factor of 3, or less.
- c Fecal streptococcus counts should be based on filtration volumes varying by a factor of 5, or less.

V SELECTING FILTRATION VOLUMES FOR MEMBRANE FILTER TESTS

A Total Coliform Counts

- 1 Determination of compliance with existing bacterial quality standards.

- a For all tests to determine whether water meets PHS Drinking Water quality standards, minimum sample sizes are prescribed as 50 ml, with 100 ml sample volumes suggested.
- b With tests in which it is assumed that coliforms are present in some numbers, and the test is to determine whether some limiting standard (as 1000 per 100 ml in natural bathing waters, prescribed by some agencies), another approach is suggested. Here, select the sample filtration volume which would be quantitatively most acceptable to count coliforms at the limiting value. For example, with a limiting value of 1000 per 100 ml:

$$\text{No. organisms per 100 ml} = 100 \times \frac{\text{No. colonies counted}}{\text{No. ml of sample filtered}}$$

This previously given equation can be rearranged to:

$$\text{Sample filtration volume in ml} = 100 \times \frac{\text{No. colonies counted}}{\text{No. organisms/100 ml}}$$

and from this:

$$\begin{aligned} \text{Sample filtration volume in ml} &= 100 \times \frac{50}{1000} \\ &= 5 \end{aligned}$$

(The value 50 is the midrange number of colonies for an acceptable colony count of 20 - 80 for computing coliforms per 100 ml)

- 2 In quantitative work, to determine number of coliforms per 100 ml the worker may or may not have prior information or standards to use as guidance in selecting filtration volumes.
 - a In absence of prior bacteriological data
 - 1) Unpolluted raw surface water, 1, 4, 15, and 60 ml samples will cover a count range of 33 - 8000 per 100 ml.

- 2) Polluted raw surface water, 0.02, 0.08, 0.15, and 0.5 ml samples will cover a count range of 4000 to 400,000 per 100 ml.
- 3) Sewage and dilute sewage, with filtration volumes of 0.0003, 0.001, 0.003, and 0.01, will cover a count range of 200,000 to 27,000,000 per 100 ml.

- b If prior coliform data are available

Use the equation:

$$\text{Basic filtration volume in ml} = 100 \times \frac{50}{\text{Average coliform count}}$$

Example: Assume that prior data indicate average coliform count of 35,000 per 100 ml. Using the equation:

$$\begin{aligned} \text{Basic filtration volume in ml} &= 100 \times \frac{50}{35,000} \\ &= 0.143 \text{ ml} \end{aligned}$$

Round off the filtration volume to 0.15 ml.

To assure a reasonable count-range, filter increments of 0.04 and 0.60 ml in addition. This will provide for acceptable coliform counts in the range of 3300 to 200,000 per 100 ml.

B Fecal Coliform Counts

- 1 Currently, no drinking water standards are based on fecal coliform organisms.

Many states have environmental water quality standards which are based on fecal coliform organisms.

- 2 Determination of fecal coliforms in the absence of prior data.

- a Unpolluted raw surface water:
 - Filter 1, 3, 10, and 30 ml sample portions. These volumes will cover a fecal coliform range of 67 - 6000 per 100 ml.

b Polluted raw surface water: Filter portions of 0.1, 0.3, 1.0, and 3.0 ml. This will cover a fecal coliform count range of 670 to 60,000 per 100 ml.

c Sewage and dilute sewage: Filter sample portions of 0.0003, 0.001 and 0.003 ml. This will provide for counts of 670,000 to 20,000,000 per 100 ml.

3 Determination of fecal coliforms in presence of prior data

a When previous fecal coliform counts are available:

$$\text{Filtration volume in ml} = 100 \times \frac{40}{\text{Av. fecal coliform count per 100 ml}}$$

Example: Prior data show 8000 fecal coliforms per 100 ml.

$$\text{Basic filtration volume in ml} = 100 \times \frac{40}{8000} = 0.5$$

Filter volumes of 0.15, 0.5 and 1.5 ml. This will be suitable for fecal coliform counts over the range 1300 to 40,000.

b When previous total coliform data are available but no fecal coliform data are available, use the total coliform value as above, but filter 3x and 9x the computed basic volume.

Example (from above): Computed basic value = 0.5 ml

Filter volumes of 0.5, 1.5 and 5.0 ml.

C Fecal Streptococcus Determinations

1 In absence of prior data

a Unpolluted raw surface water: Filter sample portions of 1, 5, 25 and 100 ml. This will provide for fecal streptococcus counts in the range 20 to 10,000 per 100 ml.

b Polluted surface water: Filter sample portions of 0.1, 0.5, and 2.0 ml. This will provide for fecal streptococcus counts in the range 1000 to 100,000 per 100 ml. Provision for rather high counts of fecal streptococci is made because of possible situations in which pollution of the water originates from domestic or wild animals. In the event that such pollution is highly improbable, a filtration series of 0.2, 1.0, and 5.0 ml (covering a count range of 400 to 50,000 per 100 ml) would be more appropriate.

2 When prior data are available

a If coliform, but not fecal streptococcus data are available, compute a basic filtration volume as in A, 2 above, but use the average coliform count as a point of reference. If significant pollution from domestic or wild animals is believed present, filter 0.2X, 1X and 5X the basic filtration volumes. If the pollution levels are believed due primarily to human sources, use 1X, 5X and 25X the basic filtration volumes.

b If prior streptococcus data are available, use the equation

$$\text{Basic filtration volume in ml} = 100 \times \frac{60}{\text{Av. Streptococcus count per 100 ml}}$$

and filter 0.2X, 1X, and 5X the basic filtration volume for streptococci.

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DETAILED MEMBRANE FILTER METHODS

I BASIC PROCEDURES

A Introduction

Successful application of membrane filter methods requires development of good routine operational practices. The detailed basic procedures described in this Section are applicable to all membrane filter methods in water bacteriology for filtration, incubation, colony counting, and reporting of results. In addition, equipment and supplies used in membrane filter procedures described here are not repeated elsewhere in this text in such detail.

Workers using membrane filter methods for the first time are urged to become thoroughly familiar with these basic procedures and precautions.

B General Supplies and Equipment List

Table 1 is a check list of materials.

C "Sterilizing" Media

Set tubes of freshly prepared medium in a boiling waterbath for 10 minutes. This method suffices for medium in tubes up to 25 x 150 mm. Frequent agitation is needed with media containing agar.

Alternately, coliform media can be directly heated on a hotplate to the first bubble of boiling. Stir the medium frequently if direct heat is used, to avoid charring the medium.

Do not sterilize in the autoclave.

*When an agar medium is used, absorbent pads are not used. The amount of medium should be sufficient to make a layer approximately 1/8" deep in the culture container. In the 50 mm plastic culture containers this corresponds to approximately 6-8 ml of culture medium.

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the Office Of Water Programs, Environmental Protection Agency.

D General Laboratory Procedures with Membrane Filters

1 Prepare data sheet

Minimum data required are: sample identification, test performed including media and methods, sample filtration volumes, and the bench numbers assigned to individual membrane filters.

2 Disinfect the laboratory bench surface.

Use a suitable disinfectant solution and allow the surface to dry before proceeding.

3 Set out sterile culture containers in an orderly arrangement.

4 Label the culture containers.

Numbers correspond with the filter numbers shown on the data sheet.

5 Place one sterile absorbent pad* in each culture container, unless an agar medium is being used.

Use sterile forceps for all manipulations of absorbent pads and membrane filters. Forceps sterility is maintained by storing the working tips in about 1 inch of methanol or ethanol. Because the alcohol deteriorates the filter, dissipate it by burning before using the forceps. Avoid heating the forceps in the burner as hot metal chars the filter.

Detailed Membrane Filter Methods

Table 1. EQUIPMENT, SUPPLIES AND MEDIA

Item	Total Coliforms			Fecal Coliform	Fecal Streptococcus	Verified Tests
	M-Endo Broth	L. E. S. Coliform	Delayed Coliform			
Funnel unit assemblies	X	X	X	X	X	
Ring stand, with about a 3" split ring, to support the filtration funnel	X	X	X	X	X	
Forceps, smooth tips, type for MF work	X	X	X	X	X	
Methanol, in small wide-mouthed bottles, about 20 ml for sterilizing forceps	X	X	X	X	X	
Suction flasks, glass, 1 liter, mouth to fit No. 8 stopper	X	X	X	X	X	
Rubber tubing, 2-3 feet, to connect suction flask to vacuum services, latex rubber 3/16" I.D. by 3/32" wall	X	X	X	X	X	
Pinch clamps strong enough for tight compression of rubber tubing above	X	X	X	X	X	
Pipettes, 10 ml, graduated, Mohr type, sterile, dispense 10 per can per working space per day. (Resterilize daily to meet need).	X	X	X	X	X	
Pipettes, 1 ml, graduated, Mohr type, sterile, dispense 24 per can per working space per day. (Resterilize daily to meet need).	X	X	X	X	X	
Pipette boxes, sterile, for 1 ml and 10 ml pipettes (sterilize above pipettes in these boxes).	X	X	X	X	X	
Cylinders, 100 ml graduated, sterile, (resterilize daily to meet need).	X	X	X	X	X	
Jars, to receive used pipettes	X	X	X	X	X	X
Gas burner, Bunsen or similar laboratory type	X	X	X	X	X	
Wax pencils, red, suitable for writing on glass	X	X	X	X	X	
Sponge in dilute iodine, to disinfect the desk tops	X	X	X	X	X	
Membrane filters (white, grid marked, sterile, and suitable pore size for microbiological analysis of water)	X	X	X	X	X	
Absorbent pads for nutrient, (47 mm in diameter), sterile, in units of 10 pads per package. Not required if medium contains agar.	X	X	X	X	X	
Petri dishes, disposable, plastic, 50 x 12 mm, sterile	X	X	X	X	X	
Waterbath incubator 44.5 ± 0.2°C				X		
Vegetable crispers, or cake boxes, plastic, with tight fitting covers, for membrane filter incubations	X	X	X		X	
Fluorescent lamp, with extension cord.	X	X	X	X	X	X
Ring stand, with clamps, utility type	X	X	X	X	X	

Table 1. EQUIPMENT, SUPPLIES AND MEDIA (Cont'd)

Item	Total Coliforms					Verified Tests
	M-Endo Broth	L. E. S. Coliform	Delayed Coliform	Fecal Coliform	Fecal Streptococcus	
Half-round glass paper weights for colony counting, with lower half of a 2-oz metal ointment box	X	X	X	X	X	
Hand tally, single unit acceptable, hand or desk type	X	X	X	X	X	
Stereoscopic (dissection) microscope, magnification of 10X or 15X, preferable binocular wide field type	X	X	X	X	X	X
Bacteriological inoculating needle						X
Wire racks for culture tubes, 10 openings by five openings preferred, dimensions overall approximately 6" x 12"						X
Phenol Red Lactose Broth in 16 X 150 mm fermentation tubes with metal caps, 10 ml per tube						X
Eosin Methylene Blue Agar (Levine) in petri plates, prepared ready for use						X
Nutrient agar slants, in screw capped tubes, 16 x 126 mm						X
Gram stain solutions, 4 solutions per complete set						X
Microscope, compound, binocular, with oil immersion lens, microscope lamp and immersion oil						X
Microscope slides, new, clean, 1" x 3" size						X
Water proof plastic bags for fecal coliform culture dish incubation				X		
M-Endo medium, MF dehydrated medium in 25 x 95 mm flat bottomed screw-capped glass vials, 1.44 g per tube, sufficient for 30 ml of medium	X		X			
Ethanol, 95% in small bottles or screw-capped tubes, about 20 ml per tube	X	X	X			
Sodium benzoate solution, 12% aqueous, in 25 x 150 mm screw-capped tubes, about 10 ml per tube			X			
L. E. S. Endo Agar MF, dehydrated M-Endo medium, 0.36 g per 25 x 95 mm flat bottomed screw-capped glass vial, plus 0.45 g agar, for 30 ml		X				
Lactose Lauryl Sulfate Tryptose Broth in 25 x 150 mm test tube without included gas tube, about 25 ml, for enrichment in L. E. S. method		X				

Table 1. EQUIPMENT, SUPPLIES AND MEDIA (Cont'd)

Item	Total Coliforms			Fecal Coliform	Fecal Streptococcus	Verified Test
	M-Endo Broth	L. E. S. Coliform	Delayed Coliform			
M-FC Broth for fecal coliform, dehydrated medium in 25 × 95 mm flat bottomed screw-capped glass vials, 1.11 g per tube, sufficient for 30 ml of culture medium				X		
Rosolic acid, 1% solution, in 0.2N NaOH, in 25 × 150 mm flat bottomed screw-capped tubes, about 5 ml per tube, freshly prepared				X		
M-Enterococcus Agar, dehydrated medium in 25 × 150 mm screw-capped tubes, sufficient for 30 ml, 1.26 g per tube					X	
Dilution bottles, 6-oz, preferable boro-silicate glass, with screw-cap (or rubber stopper protected by paper), each containing 99 ml of sterile phosphate buffered distilled water	X	X	X	X	X	
Electric hot plate surface	X	X	X	X	X	
Beakers, 400 - 600 ml (for waterbath in preparation of membrane filter culture media)	X	X	X	X	X	
Crucible tongs, to be used at electric hot plates, for removal of hot tubes of culture media for boiling waterbath	X	X	X	X	X	

- 6 Deliver enough culture medium to saturate each absorbent pad, using a sterile pipette.

Exact quantities cannot be stated because pads and culture containers vary. Sufficient medium should be applied so that when the culture container is tipped, a good-sized drop of culture medium freely drains out of the absorbent pad.

- 7 Organize supplies and equipment for convenient sample filtration. In training courses, laboratory instructors will suggest useful arrangements; eventually the individual will select a system of bench-top organization most suited to his own needs. The important point in any arrangement is to have all needed equipment and supplies conveniently at hand, in such a pattern as to minimize lost time in useless motions.

- 8 Lay a sterile membrane filter on the filter holder, grid-side up, centered over the porous part of the filter support plate.

Membrane filters are extremely delicate and easily damaged. For manipulation, the sterile forceps should always grasp the outer part of the filter disk, outside the part of the filter through which the sample passes.

- 9 Attach the funnel element to the base of the filtration unit.

To avoid damage to the membrane filter, locking forces should only be applied at the locking arrangement. The funnel element never should be turned or twisted while being seated and locked to the lower element of the filter holding unit. Filter holding units featuring a bayonet joint and locking ring to join the upper element to the lower element require special care on the part of the operator. The locking ring should be turned sufficiently to give a snug fit, but should not be tightened excessively.

- 10 Shake the sample thoroughly.

- 11 Measure sample into the funnel with vacuum turned off.

The primary objectives here are: 1) accurate measurement of sample; and 2) optimum distribution of colonies on the filter after incubation. To meet these objectives, methods of measurement and dispensation to the filtration assembly are varied with different sample filtration volumes.

- a With samples greater than 20 ml, measure the sample with a sterile graduated cylinder and pour it into the funnel. It is important to rinse this graduate with sterile buffered distilled water to preclude the loss of excessive sample volume. This should be poured into the funnel.
- b With samples of 10 ml to 20 ml, measure the sample with a sterile 10 ml or 20 ml pipette, and pipette on a dry membrane in the filtration assembly.
- c With samples of 2 ml to 10 ml, pour about 20 ml of sterile dilution water into the filtration assembly, then measure the sample into the sterile buffered dilution water with a 10 ml sterile pipette.
- d With samples of 0.5 to 2 ml, pour about 20 ml of sterile dilution water into the funnel assembly, then measure the sample into the sterile dilution water in the funnel with a 1 ml or a 2 ml pipette.
- e If a sample of less than 0.5 ml is to be filtered, prepare appropriate dilutions in sterile dilution water, and proceed as applicable in item c or d above.

When dilutions of samples are needed, always make the filtrations as soon as possible after dilution of the sample; this never should exceed

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the Office of Water Programs, Environmental Protection Agency.

30 minutes. Always shake sample dilutions thoroughly before delivering measured volumes.

12 Turn on the vacuum.

Open the appropriate spring clamp or valve, and filter the sample.

After sample filtration a few droplets of sample usually remain adhered to the funnel walls. Unless these droplets are removed, the bacteria contained in them will be a source of contamination of later samples. (In laboratory practice the funnel unit is not routinely sterilized between successive filtrations of a series). The purpose of the funnel rinse is to flush all droplets of a sample from the funnel walls to the membrane filter. Extensive tests have shown that with proper rinsing technique, bacterial retention on the funnel walls is negligible.

13 Rinse the sample through the filter.

After all the sample has passed through the membrane filter, rinse down the sides of the funnel walls with at least 20 ml of sterile dilution water. Repeat the rinse twice after all the first rinse has passed through the filter. Cut off suction on the filtration assembly.

14 Remove the funnel element of the filter holding unit.

If a ring stand with split ring is used, hang the funnel element on the ring; otherwise, place the inverted funnel element on the inner surface of the wrapping material. This requires care in opening the sterilized package, but it is effective as a protection of the funnel ring from contamination.

15 Take the membrane filter from the filter holder and carefully place it, grid-side up on the medium.

Check that no air bubbles have been trapped between the membrane filter and the underlying absorbent pad or agar. Relay the membrane if necessary.

16 Place in incubator after finishing filtration series.

Invert the containers. The immediate atmosphere of the incubating membrane filter must be at or very near 100% relative humidity.

17 Count colonies which have appeared after incubating for the prescribed time.

A stereoscopic microscope magnifying 10-15 times and careful illumination give best counts.

For reporting results, the computation is:

$$\text{bacteria/100 ml} =$$

$$\frac{\text{No. colonies counted} \times 100}{\text{Sample volume filtered in ml}}$$

Example:

A total of 36 colonies grew after filtering a 10 ml sample. The number reported is:

$$\frac{36 \text{ colonies}}{10 \text{ ml}} \times 100 = 360 \text{ per 100 ml}$$

Report results to two significant figures.

Example:

A total of 40 colonies grew after filtering a 3 ml sample.

This calculation gives:

$$\frac{40 \text{ colonies}}{3 \text{ ml}} \times 100 = 1333.33 \text{ per 100 ml}$$

But the number reported should be 1300 per 100 ml.

II MF LABORATORY TESTS FOR COLIFORM GROUP

A Standard Coliform Test (Based on M-Endo Broth MF)

1 Culture medium

- a M-Endo Broth MF Difco 0749-02 or the equivalent BBL M-Coliform Broth 01-494

Preparation of Culture Medium (M-Endo Broth) for Standard MF Coliform Test

Yeast extract	1.5	g
Casitone or equivalent	5.0	g
Thiopeptone or equivalent	5.0	g
Tryptose	10.0	g
Lactose	12.5	g
Sodium desoxycholate	0.1	g
Dipotassium phosphate	4.375	g
Monopotassium phosphate	1.375	g
Sodium chloride	5.0	g
Sodium lauryl sulfate	0.05	g
Basic fuchsin (bacteriological)	1.05	g
Sodium sulfite	2.1	g
Distilled water (containing 20.0 ml ethanol)	1000	ml

This medium is available in dehydrated form and it is recommended that the commercially available medium be used in preference to compounding the medium of its individual constituents.

To prepare the medium for use, suspend the dehydrated medium at the rate of 48 grams per liter of water containing ethyl alcohol at the rate of 20 ml per liter.

As a time-saving convenience, it is recommended that the laboratory worker preweigh the dehydrated medium in closed tubes for several days, or even weeks, at one operation.

With this system, a large number of increments of dehydrated medium (e.g., 1.44 grams), sufficient for some convenient (e.g., 30 ml) volume of finished culture medium are weighed and dispensed into screw-capped culture tubes, and stored until needed. Storage should preferably be in a darkened desiccator.

A supply of distilled water containing 20 ml stock ethanol per liter is maintained.

When the medium is to be used, it is reconstituted by adding 30 ml of the distilled water-ethanol mixture per tube of pre-weighed dehydrated culture medium.

- b Medium is "sterilized" as directed in I, C.

- c Finished medium can be retained up to 96 hours if kept in a cool, dark place. Many workers prefer to reconstitute fresh medium daily.

- 2 Filtration and incubation procedures are as given in I, D.

Special instructions:

- a For counting, use the wide field binocular dissecting microscope, or simple lens. For illumination, use a light source perpendicular to the plane of the membrane filter. A small fluorescent lamp is ideal for the purpose.
- b Coliform colonies have a "metallic" surface sheen under reflected light which may cover the entire colony, or it may appear only in the center. Non-coliform colonies range from colorless to pink, but do not have the characteristic sheen.
- c Record the colony counts on the data sheet, and compute the coliform count per 100 ml of sample.

B Standard Coliform Tests (Based on L. E. S. Endo Agar)

The distinction of the L. E. S. count is a two hour enrichment incubation on LST broth. M-Endo L. E. S. medium is used as agar rather than the broth.

1 Preparation of culture medium (L. E. S. Endo Agar) for L. E. S. coliform test

a Formula from McCarthy, Delaney, and Grasso (2)

Bacto-Yeast Extract	1.2 g
Bacto-Casitone	3.7 g
Bacto-Thiopeptone	3.7 g
Bacto-Tryptose	7.5 g
Bacto-Lactose	9.4 g
Dipotassium phosphate	3.3 g
Monopotassium phosphate	1.0 g
Sodium chloride	3.7 g
Sodium desoxycholate	0.1 g
Sodium lauryl sulfate	0.05 g
Sodium sulfite	1.6 g
Bacto-Basic fuchsin	0.8 g
Agar	15 g
Distilled water (containing 20 ml ethyl alcohol)	1000 ml

- b To rehydrate the medium, suspend 51 grams in the water-ethyl alcohol solution.
- c Medium is "sterilized" as directed in I, C.
- d Pour 4-6 ml of freshly prepared Agar into the smaller half of the container. Allow the medium to cool and solidify.

2 Procedures for filtration and incubation

- a Lay out the culture dishes in a row or series of rows as usual. Place these with the upper (lid) or top side down.
- b Place one sterile absorbent pad in the larger half of each container (lid). Use sterile forceps for all

manipulations of the pads. (Agar occupies smaller half or bottom).

- c Using a sterile pipette, deliver enough single strength lauryl sulfate tryptose broth to saturate the pad only. Avoid excess medium.
- d Follow general procedures for filtering in I, D. Place filters on pad with lauryl sulfate tryptose broth.
- e Upon completion of the filtrations, invert the culture containers and incubate at 35°C for 1 1/2 to 2 hours.

3 2-hour procedures

- a Transfer the membrane filter from the enrichment pad in the upper half to the agar medium in the lower half of the container. Carefully roll the membrane onto the agar surface to avoid trapping air bubbles beneath the membrane.
- b Removal of the used absorbent pad is optional.
- c The container is inverted and incubated 22 hours \pm 2 hours \pm 0.5°C.

4 Counting procedures are as in I, D.

- 5 L. E. S. Endo Agar may be used as a single-stage medium (no enrichment step) in the same manner as M-Endo Broth, MF.

C Delayed Incubation Coliform Test

This technique is applicable in situations where there is an excessive delay between sample collection and plating. The procedure is unnecessary when the interval between sample collection and plating is within acceptable limits.

1 Preparation of culture media for delayed incubation coliform test

- a Preservative media M-Endo Broth base

To 30 ml of M-Endo Broth MF prepared in accordance with directions in II, A, 1 of this outline, add 1.0 ml of a sterile 12% aqueous solution of sodium benzoate.

L. E. S. MF Holding Medium-Coliform: Dissolve 12.7 grams in 1 liter of distilled water. No heating is necessary. Final pH 7.1 ± 0.1 . This medium contains sodium benzoate.

b Growth media

M-Endo Broth MF is used, prepared as described in II, A, 1 earlier in this outline. Alternately, L. E. S. Endo Medium may be used.

2 General filtration followed is in I, D.

Special procedures are:

- a Transfer the membrane filter from the filtration apparatus to a pad saturated with benzoated M-Endo Broth.
- b Close the culture dishes and hold in a container at ambient temperature. This may be mailed or transported to a central laboratory. The mailing or transporting tube should contain accurate transmittal data sheets which correspond to properly labeled dishes.

Transportation time, in the case of mailed containers, should not exceed three days to the time of reception by the testing laboratory.

- c On receipt in the central laboratory, unpack mailing carton, and lay out the culture containers on the laboratory bench.
- d Remove the tops from the culture containers. Using sterile forceps, remove each membrane and its absorbent pad to the other half of the culture container.

- e With a sterile pipette or sterile absorbent pad, remove preservative medium from the culture container.
- f Place a sterile absorbent pad in each culture container, and deliver enough freshly prepared M-Endo Broth to saturate each pad.
- g Using sterile forceps, transfer the membrane to the new absorbent pad containing M-Endo Broth. Place the membrane carefully to avoid entrapment of air between the membrane and the underlying absorbent pad. Discard the absorbent pad containing preservative medium.
- h After incubation of 20 ± 2 hours at 35°C , count colonies as in the above section A, 2.
- i If L. E. S. Endo Agar is used, the steps beginning with (e) above are omitted; and the membrane filter is removed from the preservative medium and transferred to a fresh culture container with L. E. S. Endo Agar, incubated, and colonies counted in the usual way.

D Verified Membrane Filter Coliform Test

This procedure applies to identification of colonies growing on Endo-type media used for determination of total coliform counts. Isolates from these colonies are studied for gas production from lactose and typical coliform morphology. In effect, the procedure corresponds with the Completed Test stage of the multiple fermentation tube test for coliforms.

Procedure:

- 1 Select a membrane filter bearing several well-isolated coliform-type colonies.
- 2 Using sterile technique, pick all colonies in a selected area with the inoculation needle, making transfers into tubes of phenol red lactose broth (or lauryl sulfate tryptose lactose

broth). Using an appropriate data sheet record the interpretation of each colony, using, for instance, "C" for colonies having the typical color and sheen of coliforms; "NC" for colonies not conforming to coliform colony appearance on Endotype media.

3 Incubate the broth tubes at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

4 At 24 hours:

- a Read and record the results from the lactose broth fermentation tubes. The following code is suggested:

Code

- O No indication of acid or gas production, either with or without evidence of growth.
- A Evidence of acid but not gas (applies only when a pH indicator is included in the broth medium)
- G Growth with production of gas. If pH indicator is used, use symbol AG to show evidence of acid. Gas in any quantity is a positive test.

- b Tubes not showing gas production are returned to the 35°C incubator.

- c Gas-positive tubes are transferred as follows:
- 1) Prepare a streak inoculation on EMB agar for colony isolation, and using the same culture.

2) Inoculate a nutrient agar slant.

3) Incubate the EMB agar plates and slants at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

5 At 48 hours:

- a Read and record results of lactose broth tubes which were negative at 24 hours and were returned for further incubation.

- b Gas-positive cultures are subjected to further transfers as in 4c. Gas-negative cultures are discarded without further study; they are coliform-negative.

- c Examine the cultures transferred to EMB agar plates and to nutrient agar slants, as follows:

1) Examine the EMB agar plate for evidence of purity of culture; if the culture represents more than one colony type, discard the nutrient agar culture and reisolate each of the representative colonial types on the EMB plate and resume as with 4c for each isolation. If purity of culture appears evident, continue with c (2) below.

2) Prepare a smear and Gram stain from each nutrient agar slant culture. The Gram stain should be made on a culture not more than 24 hours old. Examine under oil immersion for typical coliform morphology, and record results.

6 At 72 hours:

Perform procedures described in 5c above, and record results.

- 7 Coliform colonies are considered verified if the procedures demonstrate a pure culture of bacteria which are gram negative nonspore-forming rods and produce gas from lactose at 35°C within 48 hours.

E Fecal Coliform Count (Based on M-FC Broth Base)

The count depends upon growth on a special medium at $44.5 \pm 0.2^{\circ}\text{C}$.

- 1 Preparation of Culture Medium (M-FC Broth Base) for Fecal Coliform Count

a Composition

Tryptose	10.0 g
Proteose Peptone No. 3	5.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose	12.5 g
Bile salts No. 3	1.5 g
Rosolic acid* (Allied Chemical)	10.0 ml
Aniline blue (Allied Chemical)	0.1 g
Distilled water	1000 ml

- b To prepare the medium dissolve 37.1 grams in a liter of distilled water which contains 10 ml of 1% rosolic acid (prepared in 0.2 N NaOH).

Fresh solutions of rosolic acid give best results. Discard solutions which have changed from dark red to orange.

- c To sterilize, heat to boiling as directed in I, C.
- d Prepared medium may be retained up to 4 days in the dark at 2-8°C.
- 2 Special supplies
- Small water proof plastic sacks capable of being sealed against water with capacity of 3 to 6 culture containers.
- 3 Filtration procedures are as given in I, D.
- 4 Elevated temperature incubation
- a Place fecal coliform count membranes at $44.5 \pm 0.2^{\circ}\text{C}$ as rapidly as possible.

Filter membranes for fecal coliform counts consecutively and immediately place them in their culture containers. Insert as many as six culture containers all oriented in the same way (i.e., all grid sides facing the same direction) into the sacks and seal. Tear off the perforated top, grasp the side wires, and twirl the sack to roll the open end inside the folds of sack. Then submerge the sacks with culture containers inverted beneath the surface of a $44.5 \pm 0.2^{\circ}\text{C}$ waterbath.

- b Incubate for 22 ± 2 hours.

5 Counting procedures

Examine and count colonies as follows:

- a Use a wide field binocular dissecting microscope with 5 - 10X magnification.
- b Low angle lighting from the side is advantageous.
- c Fecal coliform colonies are blue, generally 1-3 mm in diameter.
- d Record the colony counts on the data sheet, and report the fecal coliform count per 100 ml of sample. (I, D, 17 illustrates method)

III. TESTS FOR FECAL STREPTOCOCCAL GROUP-MEMBRANE FILTER METHOD

A 48 hour incubation period on a choice of two different media, giving high selectivity for fecal streptococci, are the distinctive features of the tests.

*Prepare 1% solution of rosolic acid in 0.2 N NaOH. This dye is practically insoluble in water.

A Test for Members of Fecal Streptococcal Group (Tentative, Standard Methods) M-Enterococcus Agar Medium

1 Preparation of the culture medium

a Formula (The Difco formula is shown, but equivalent constituents from other sources are equally acceptable).

Bacto tryptose	20.0 g
Bacto yeast extract	5.0 g
Bacto dextrose	2.0 g
Dipotassium phosphate	4.0 g
Sodium Azide	0.4 g
Bacto agar	10.0 g
2, 3, 5, Triphenyl tetrazolium chloride	0.1 g

b The medium is prepared by rehydration at the rate of 42 grams per 1000 ml of distilled water. It is recommended that the medium in dehydrated form be preweighed and dispensed into culture containers (about 25 x 150 mm) in quantities sufficient for preparation of 30 ml of culture medium (1.26 g per tube).

c Follow I, C, for "sterilizing" medium and dispense while hot into culture containers. Allow plates to harden before use.

2 List of apparatus, materials, as given in Table I.

3 Procedure, in general, as given in I.

Special instructions

a Incubate for 48 hours, inverted, with 100% relative humidity, after filtrations are completed. If the entire incubator does not have saturated humidity, acceptable conditions can be secured by placing the cultures in a tightly closed container with wet paper, towels, or other moist material.

b After incubation, remove the cultures from the incubator, and count all colonies under wide field binocular dissecting microscope with magnification set at 10X or 20X. Fecal streptococcus colonies are 0.5 - 2 mm in diameter, and flat to raised smooth, and vary from pale pink to dark red in color.

c Report count per 100 ml of sample. This is conveniently computed:

$$\text{No. fecal streptococci per 100 ml} =$$

$$\frac{\text{No. fecal streptococcus colonies counted}}{\text{Sample filtration volume in ml}} \times 100$$

B Test for Members of Fecal Streptococcal Group based on KF-Agar

1 Preparation of the culture medium

a Formula: (The dehydrated formula of Bacto 0496 is shown, but equivalent constituents from other sources are acceptable). Formula is in grams per liter of reconstituted medium.

Bacto proteose peptone #3	10.0 g
Bacto yeast extract	10.0 g
Sodium chloride (reagent grade)	5.0 g
Sodium glycerphosphate	10.0 g
Maltose (CP)	20.0 g
Lactose (CP)	1.0 g
Sodium azide (Eastman)	0.4 g
Sodium carbonate (Na ₂ CO ₃ reagent grade)	0.636 g
Brom cresol purple (water soluble)	0.015 g
Bacto agar	20.0 g

b Reagent

2, 3, 5-Triphenyl tetrazolium chloride reagent (TPTC)

This reagent is prepared by making a 1% aqueous solution of the above chemical passing it through a Seitz filter or membrane filter. It can

- be kept in the refrigerator in a screw-capped tube until used.
- c The dehydrated medium described above is prepared for laboratory use as follows:
- Suspend 7.64 grams of the dehydrated medium in 100 ml of distilled water in a flask with an aluminum foil cover.
- Place the flask in a boiling water-bath, melt the dehydrated medium, and leave in the boiling waterbath an additional 5 minutes.

Cool the medium to 50°-60° C, add 1.0 ml of the TPTC reagent, and mix.

For membrane filter studies, pour 5-8 ml in each 50 mm glass or plastic culture dish or enough to make a layer approximately 1/8" thick. Be sure to pour plates before agar cools and solidifies.

For plate counts, pour as for standard agar plate counts.

NOTE: Plastic dishes containing media may be stored in a dark, cool place up to 30 days without change in productivity of the medium, provided that no dehydration occurs. Plastic dishes may be incubated in an ordinary air incubator. Glass dishes must be incubated in an atmosphere with saturated humidity.

- 2 Apparatus, and materials as given in Table 1.
- 3 General procedure is as given in I.

Special instructions

- a Incubate 48 hours, inverted with 100% relative humidity after filtration.

- b After incubation, remove the cultures from the incubator, and count colonies under wide field binocular dissecting microscope, with magnification set at 10X or 20X. Fecal streptococcus colonies are pale pink to dark wine-color. In size they range from barely visible to approximately 2mm in diameter. Colorless colonies are not counted.
- c Report fecal streptococcus count per 100 ml of sample. This is computed as follows:

No. fecal streptococci per 100 ml =

$$\frac{\text{No. fecal streptococcus colonies}}{\text{Sample filtration volume in ml}} \times 100$$

C Verification of Streptococcus Colonies

- 1 Verification of colony identification may be required in waters containing large numbers of Micrococcus organisms. This has been noted particularly with bathing waters, but the problem is by no means limited to such waters.
- 2 A verification procedure is described in "Standard Methods for the Examination of Water and Wastewater," 13th ed. (1971). The worker should use this reference for the step-by-step procedure.

IV PROCEDURES FOR USE OF MEMBRANE FILTER FIELD UNITS

A Culture Media

- 1 The standard coliform media used with laboratory tests are used.
- 2 To simplify field operations, it is suggested that the medium be sent to the field, preweighed, in vials or capped culture tubes. The medium then requires only the addition of a suitable volume of distilled water-ethanol prior to sterilization.

- 3 Sterilization procedures in the field are the same as for laboratory methods.
- 4 Laboratory preparation of the media, ready for use, would be permissible provided that the required limitations on time and conditions of storage are met.

B Operation of Millipore Water Testing Kit, Bacteriological

- 1 Supporting supplies and equipment are the same as for the laboratory procedures.
- 2 Set the incubator voltage selector switch to the voltage of the available supply, turn on the unit and adjust as necessary to establish operating incubator temperature at $35 \pm 0.5^{\circ}\text{C}$.
- 3 Sterilize the funnel unit assembly by exposure to formaldehyde or by immersion in boiling water. If a laboratory autoclave is available, this is preferred.

Formaldehyde is produced by soaking an asbestos ring (in the funnel base) with methanol, igniting, and after a few seconds of burning, closing the unit by placing the stainless steel flask over the funnel and base. This results in incomplete combustion of the methanol, whereby formaldehyde is produced. Leave the unit closed for 15 minutes to allow adequate exposure to formaldehyde.

- 4 Filtration and incubation procedures correspond with laboratory methods.
- 5 The unit is supplied with a booklet containing detailed step-by-step operational procedures. The worker using the equipment should become completely versed in its contents and application.

- C Other commercially available field kits should be used according to manufacturer's instructions. It is emphasized that the standards of performance are required for field devices as for laboratory equipment.

D Counting of Colonies on Membrane Filters

1 Equipment and materials

Membrane filter cultures to be examined

Illumination source

Simple lens, 2X to 6X magnification

Hand tally (optional)

2 Procedure

- a Remove the cultures from the incubator and arrange them in numerical sequence.
- b Set up illumination source as that light will originate from an area perpendicular to the plane of membrane filters being examined. A small fluorescent lamp is ideal for the purpose. It is highly desirable that a simple lens be attached to the light source.
- c Examine results. Count all coliform and noncoliform colonies. Coliform and noncoliform colonies. Coliform colonies have a "metallic" surface sheen under reflected light, which may cover the entire colony or may appear only on the center.

Noncoliform colonies range from colorless to pink or red, but do not have the characteristic "metallic" sheen.

d Enter the colony counts in the data sheets.

e Enter the coliform count per 100 ml of sample for each membrane having a countable number of coliform colonies. Computation is as follows:

No. coliform per 100 ml =

$$\frac{\text{No. coliform colonies on MF}}{\text{No. milliliters sample filtered}} \times 100$$

- 2 McCarthy, J. A., Delaney, J. E. and Grasso, R. J. Measuring Coliforms in Water. Water and Sewage Works. 1961: R-426-31. 1961.

This outline was prepared by H. L. Jeter, Director, National Training Center, EPA, WPO, Cincinnati, OH 45268.

Descriptors: Biological Membranes, Coliforms, Fecal Coliforms, Fecal Streptococci, Filters, Indicator Bacteria, Laboratory Equipment, Laboratory Tests, Membranes, Microbiology, Water Analysis

REFERENCES

- 1 Standard Methods for the Examination of Water and Wastewater. APHA, AWWA, WPCF. 12th Edition. 1965.

COLONY COUNTING ON MEMBRANE FILTERS

I INTRODUCTION

On removal of membrane filter cultures from the incubator, the worker has several tasks to perform, leading to the reporting of results of the bacteriological examination. These steps, together with the selection and use of associated equipment, are considered in this discussion. The following topics are included:

- A Precautions on removal of membrane filter cultures from the incubator.
- B Selection of the best membrane filter for colony counting (when more than one membrane filter per sample was prepared, representing a graded series of sample increments.)
- C Use of grid systems on filter surfaces as counting aids.
- D Recognition and counting of desired colonies, including selection and use of optical equipment.
- E Calculations for reporting number of test organisms per 100 ml of sample.

II REMOVAL OF CULTURE FROM INCUBATOR

- A Incubation time and temperature recommendations should be closely adhered to. This applies particularly to total coliform counts. Some of our earlier training manuals have suggested counting of colonies after as few as 16 hours of incubation at 35°C. Currently, 22 ± 2 hours is preferred.
- B All membrane filter cultures should be incubated in the inverted position, with measures to avoid loss of culture medium through leakage or evaporation. Sometimes an excessive amount of culture medium is applied initially, or additional

moisture finds its way into the culture container during incubation. In such cases, when the culture is removed from the incubator, it should be turned "right side up" in such a way as to avoid flooding the filter with excess liquid. If excessive liquid is present, open the culture container cautiously, and pour off the excess.

C Drying Filters Before Colony Counts

- 1 Some workers advise opening all cultures (especially total coliform tests when Endo-type media are used) for a short time (15 minutes to 1 hour) for partial drying of coliform colonies before counting. Advocates of this step report that the typical surface sheen characteristic of coliform colonies is improved by this step.
- 2 Use of preliminary drying procedures is a matter of personal preference. In the opinion of the writer, the benefit of preliminary drying is at best debatable, and at worst, may interfere with subsequent study of the bacterial colonies. Correct use of acceptable lighting and optical equipment is a far more important factor in ease and accuracy of recognition of differentiated colonies.

III SELECTION OF ACCEPTABLE MEMBRANE FILTER CULTURE FOR EXAMINATION

A Non-Quantitative Tests

In bacteriologic examination of treated waters, where waters meeting requirements result in development of very few or no coliform colonies, the typical filtration volume is 100 ml, and but one filtration is made per sample. In this case, there is no problem: the one membrane

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filter preparation is the basis of bacteriologic evaluation of the sample.

B Quantitative Tests

1. When the bacteriological water quality standard is for some fixed limiting value, such as 70 per 100 ml for shellfish waters, again only a single sample filtration volume may be used. In such a case, the filtration of a single portion of 50 ml will show directly whether the water meets bacteriologic standards, or if the limiting standard is being exceeded.

2. On the other hand, if the objective of the test was to show how many coliforms were present per 100 ml of sample, then it is necessary to filter a series of sample increments from each sample, each increment being placed on a separate membrane filter. At the end of the incubation period, the series of membrane filters representing each sample must be inspected, with selection of the membrane filter bearing the number of colonies most suitable for reporting quantitative results. This is summarized in Table 1, below:

The lower limit of 20 is set arbitrarily, as a number below which statistically valid results become increasingly questionable with smaller numbers of colonies. The upper limits represent numbers above which interference from colony crowding, deposition of extraneous material, and other factors appear to result in increasingly questionable results. It is emphasized that these limiting values are empirical, based on laboratory observations alone, and do not represent results of theoretical calculations. It follows that it is quite possible, with some sample sources, to obtain acceptable quantitative results with colony counts higher than the recommendations, but the minimum limit of 20 colonies appears to apply to the majority of sample sources."

3. If no membrane filter bears a number of colonies within the recommended limits for the test, the worker has a choice between - a) collecting a new sample and repeating the test; and b) using whatever results actually were obtained, reporting an "educated guess" as to the number of organisms per 100 ml. In the latter case, it is most

Table 1. NUMBERS OF COLONIES ACCEPTABLE FOR QUANTITATIVE DETERMINATIONS

<u>Test</u>	<u>Colony Counting Range</u>		<u>Remarks</u>
	<u>Minimum</u>	<u>Maximum</u>	
Total coliform	20	80	200 limit overall
Fecal coliform	20	60	
Fecal streptococcus	20	100	

strongly urged that each result of this type be specifically identified with a qualifying statement, such as "Estimated count, based on non-ideal colony density on filter."

- 4 Sometimes two or more filters, of a series of filtration volumes from a sample, produce colony counts within the recommended counting range. Colony counts should be made on all such filters. See Section VI of this outline for calculations based on such results. These problems may arise from the selection of a too-close range of sample filtration volumes, from colony differentiation failures related to overcrowding on the filters, or from physico-chemical interference with colony development related to material in the sample deposited in or on the filters.

IV USE OF GRID SYSTEMS IN COLONY COUNTS

- A Most manufacturers provide grid-imprinted membrane filters for bacteriologic use. The ink used in such filters must be biochemically inert to the test organisms, and, of course, must be applied in such a manner as not to degrade the quality of the filter. **Examples of such gridding have appeared from various manufacturers as follows:**

- 1 ... effective filtering area subdivided into squares equal to 1/100 the effective filtering area (when a filtering unit with funnel-diameter of 35 mm is used).
- 2 ... grid markings which subdivide the effective filtering area into squares equal to 1/100 the effective filtering area (9.6 cm² for 47 mm diameter filters).
- 3 ... filters subdivided so that each square of the grid represents 1/60 of the effective filtering area.

- B Some special studies may require use of membrane filters without grid markings. For example, the ink in some filters prevents growth of *Brucella melitensis*. In such cases it may be necessary to improvise a viewing grid which can be placed over the culture after incubation and colony development.

C Applications of Grids

- 1 The grid dimensions are of no particular significance in colony counting, provided that their size permits easy and continuous orientation in counting of colonies. To be sure, a rough estimate of the total number of differentiated colonies on a filter is possible by counting a representative number of squares and multiplying colony count by the appropriate factor. For example, with many filters, colonies in ten squares can be counted, multiplied by 10, and the product is a rough estimate of the total number on the entire filter. It is emphasized that such procedure is for rough estimates only, and should not be condoned in quantitative work with membrane filters.
- 2 The primary usefulness of the grid system is for orientation during the counting procedure. Some colonies will touch lines on a grid system, and a uniform practice must be established to avoid missing some colonies or counting others twice. The procedure used by the writer is as follows:
 - a Counts are made in an orderly back-and-forth sweep, from top to bottom of the filter. See Figure 1.
 - b Inevitably, some colonies will be in contact with grid lines. A suggested routine procedure for counting colonies in contact with lines is indicated in Figure 2. Colonies are counted in the squares indicated by the arrows, and no effort is made to decide whether "most of the colony" is in one or the other square.

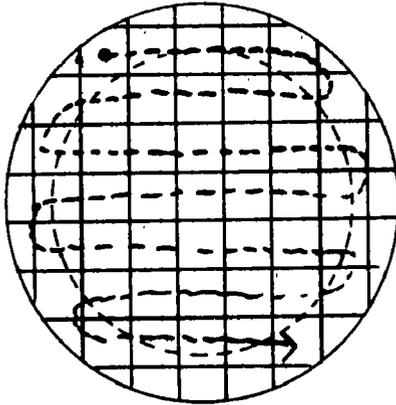


Figure 1. The dashed circle indicates the effective filtering area. The dashed back-and-forth line indicates the colony counting pathway.

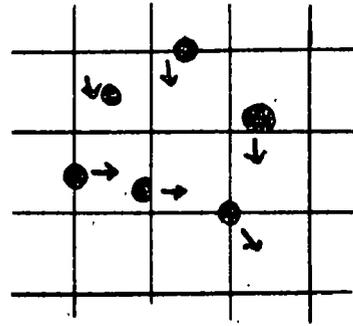


Figure 2. Enlarged portion of grid-marked square of filter, with various ways colonies can be in contact with grid-lines. Colonies are counted in squares indicated by the arrows.

V COUNTING OF COLONIES

A Equipment

- 1 A hand-tally is a useful device while counts are being made.
- 2 Optical assistance in colony counts is strongly recommended. Dependence on naked-eye counts often results in too-low results.
 - a Preferably, use a wide-field binocular dissecting microscope with magnification of 10X or 15X.
 - b Optionally, but less desirably, a simple lens with magnification at least 5X can be used, provided that acceptable illumination also is present.
- 3 Lighting equipment
 - a For coliform counting, a large light source is mandatory. Fluorescent lamps in housings permitting placement close to and as directly as possible over the membrane filter is the best lighting arrangement known to the writer. Incandescent lamps, whether simple light bulbs in a table

lamp or in elaborate microscope lamp housings, are not satisfactory for coliform colony counting on membrane filters with Endo-type media.

- b For fecal coliforms or fecal streptococci, the lighting requirements are not so severe; in this case almost any sufficiently bright light source, which can be placed above the filter (either at a high or at a low angle) will suffice.

4 Lighting arrangement and counting

- a As above, for coliform counting, the fluorescent lamp should be at a high angle (as nearly as possible directly over the membrane filter) so placed that an image of the light source is reflected off the colony surfaces into the microscope lens system. Properly placed, the light will demonstrate the "golden metallic" surface luster of coliform colonies, which may cover the entire colony, or may appear only in an area in the center of the colony. The worker must learn to recognize the difference between the typical golden sheen of coliform colonies and the merely shiny surface of non-coliform colonies.

- b Other types of colonies (fecal coliforms, fecal streptococci, etc.) do not require such rigid control of the light source. Low-angle lighting can be helpful, to give a relief of the colony profile from the colony surface. This is valuable with small colonies, such as frequently encountered in streptococcal studies. In such cases, almost any light source is acceptable, provided that it is bright enough and that it is applied from somewhere above the membrane filter.
- c The typical appearance of various types of colonies is related to the culture media applied; therefore, this is not discussed in detail at this point. See the outlines on culture media and on laboratory procedures for specified indicator organisms for such information.
- d In colony counting, count all colonies individually, even if they are in contact with each other (this is contrary to usual practice in colony counting in agar cultures in Petri dishes). Such colonies are recognized quite easily when a microscope is used for colony counting as recommended. Colonies which have grown into contact almost invariably show a very fine line of contact. The worker must learn to recognize the difference between two or more colonies which have grown into contact with each other, and single, irregularly shaped, colonies which sometimes develop on membrane filters. Such colonies almost invariably are associated with a fiber or particulate material deposited on the filter, and tend to develop along a path conforming to the shape and size of the fiber or particulates.

VI CALCULATIONS

A Counting Units

- 1 In reporting densities of indicator organisms (coliforms, fecal coliforms,

fecal streptococci), bacterial counts always are reported in numbers per 100 ml. In standard practice, results are expressed to two significant figures. For example, if the calculation indicates 75,400, or even 75,444 organisms per 100 ml, the results would be reported as 75,000 per 100 ml in each case. (The digits 7 and 5 are the significant figures; the three zeros only locate the decimal point.)

- 2 When "total" bacterial counts are reported, common practice is to report in number per ml, not the number per 100 ml.
- 3 Quantitative work on enteric pathogens is, at this time, limited to reporting of occurrence of designated enteric pathogens, correlated with measured density of pollution indicating bacterial groups. At such time as the numerical determination of enteric pathogens becomes feasible, it is anticipated that reports will be in terms of count per 100 ml, or even larger volume units.

B Typical Calculations

- 1 Select the membrane filter bearing the acceptable number of colonies for reporting, and calculate indicators per 100 ml according to the general formula:

$$\frac{\text{No. colonies of indicator organism}}{\text{No. ml of sample filtered}} \times 100 = \text{No. indicator organisms per 100 ml}$$

- 2 Example:

- a Assume that for a total coliform count, volumes of 50, 15, 5, 1.5, and 0.5 ml produced coliform colony counts of 200, 110, 40, 10, and 5, respectively.
- b First, the worker actually would not have counted coliform colonies on all these filters. He would have selected, by inspection, the membrane filter(s) most likely to have 20-80 coliform colonies, limiting actual counting to such colonies (this does take some practice and skill in making quick estimates, but comes with experience).

c Having selected the membrane filter probably most useful for reporting purposes, coliform colonies are counted according to accepted procedures, and the general formula is applied:

$$\text{Coliforms per 100 ml} = \frac{40}{5} \times 100$$

$$\text{Coliforms per 100 ml} = 800$$

C Special Situations in Calculating Densities of Indicator Organisms

1 Assume a coliform count in which the volumes of 1, 0.3, 0.1, 0.03, and 0.01 ml, respectively, produced coliform colony counts of TNTC, TNTC, 75, 30, and 8, respectively.

a Here, two sample volumes resulted in production of coliform colonies in the acceptable counting range.

b Suggestion: Compile the filtration volumes and colonies from both acceptable filters, as follows:

<u>Volume, ml</u>	<u>Count</u>
0.1	75
<u>0.03</u>	<u>30</u>
0.13	105

Calculate coliforms per 100 ml from the composite result:

$$\text{Coliforms per 100 ml} = \frac{105}{0.13} \times 100$$

$$\text{Coliforms per 100 ml} = 81,000$$

2 Assume a coliform count in which sample volumes of 1, 0.3, and .01 ml produced colony counts of 14, 3, and 0, respectively.

a Here, no colony count falls within recommended limits.

b Suggestion: Calculate on the basis of the most nearly acceptable value,

and report with qualifying remark, thus:

Use 14 colonies from 1 ml of sample:

$$\frac{14}{1.0} \times 100 = 1400$$

Report: "Estimated Count, 1400 per 100 ml, based on non-ideal colony count"

3 Assume a coliform count in which the volumes 1, 0.3, and 0.01 ml produced coliform colony counts of 0, 0, and 0, respectively.

a Here, no actual calculation is possible, even for "estimate" reports.

b Suggestion: Calculate the number of estimated coliforms per 100 ml that would have been reported if there had been 1 coliform colony on the filter representing the largest filtration volume, thus:

$$\text{Use 1 colony, and 1 ml: } \frac{1}{1} \times 100 = 100$$

Report: "Less than 100 coliforms per 100 ml".

4 Assume a coliform count in which the volumes of 1, 0.3, and 0.01 ml produced coliform colony counts of TNC, 150, and 110 colonies.

a Here, all colony counts are above the recommended limits.

b Suggestion: Use Example 2, above, and report an estimated count based on non-ideal colony counts:

$$\frac{110}{0.01} \times 100 = 1,100,000$$

Report: "Coliform count estimated at 1,100,000 per 100 ml, based on non-ideal colony count".

- 5 Assume that, in Example 4, the volumes of 1.0, 0.3, and 0.01 ml, all produced too many coliform colonies to show separated colonies, and that the laboratory bench record showed TNTC (Too Numerous to Count).

Suggestion: Use 80 colonies as the basis of calculation with the smallest filtration volume, thus:

$$\frac{80}{0.01} \times 100 = 800,000$$

Report: "> 800,000 coliforms per 100 ml sample. Filters too crowded."

VII CONCLUSION

The foregoing discussion has presented a number of factors which determine the quantitative reliability of membrane filter results. It cannot be too strongly emphasized that the correct use of acceptable colony counting equipment is one of the most important single factors in successful application of membrane filter methods. Here, there is perhaps a greater exercise of personal skill and judgment than in any other aspect of membrane filter methodology. There is no substitute for practice and experience, supported by liberal use of supporting colony verification studies, to produce a skilled worker in colony counts on membrane filters.

This outline was prepared by Harold L. Jeter, Director, National Training Center, EPA, Cincinnati, OH 45268.

VERIFIED MEMBRANE FILTER TESTS

I INTRODUCTION

- A The purpose of a verified membrane filter test procedure is to establish the validity of colony differentiation and interpretation in the test being applied. Specifically, a verified membrane filter test may prove useful 1) as a self-training device for new workers, 2) as a research tool in evaluation of new membrane filter media and procedures, or 3) to provide supporting evidence of colony interpretation in cases where the analytical results may be subject to professional or official challenge.
- B Reduced to essentials, a verified membrane filter test consists of 1) interpretation of the colonies appearing on a selective, differential medium, 2) recovery of purified bacterial cultures from differentiated colonies, and 3) application

of supplemental test procedures to determine the validity of the original interpretation of the membrane filter colonies.

- C In this discussion, primary attention is given to a verified membrane filter coliform test. In addition, verification procedures are presented for members of the fecal coliform group and for fecal streptococci.

II VERIFIED TEST FOR MEMBERS OF THE COLIFORM GROUP

- A An abbreviated procedure corresponds to the Confirmed Test of Standard Methods through use of lactose broth (or lactose lauryl tryptose broth) followed by confirmation in brilliant green lactose bile broth. The procedure is shown diagrammatically as follows:

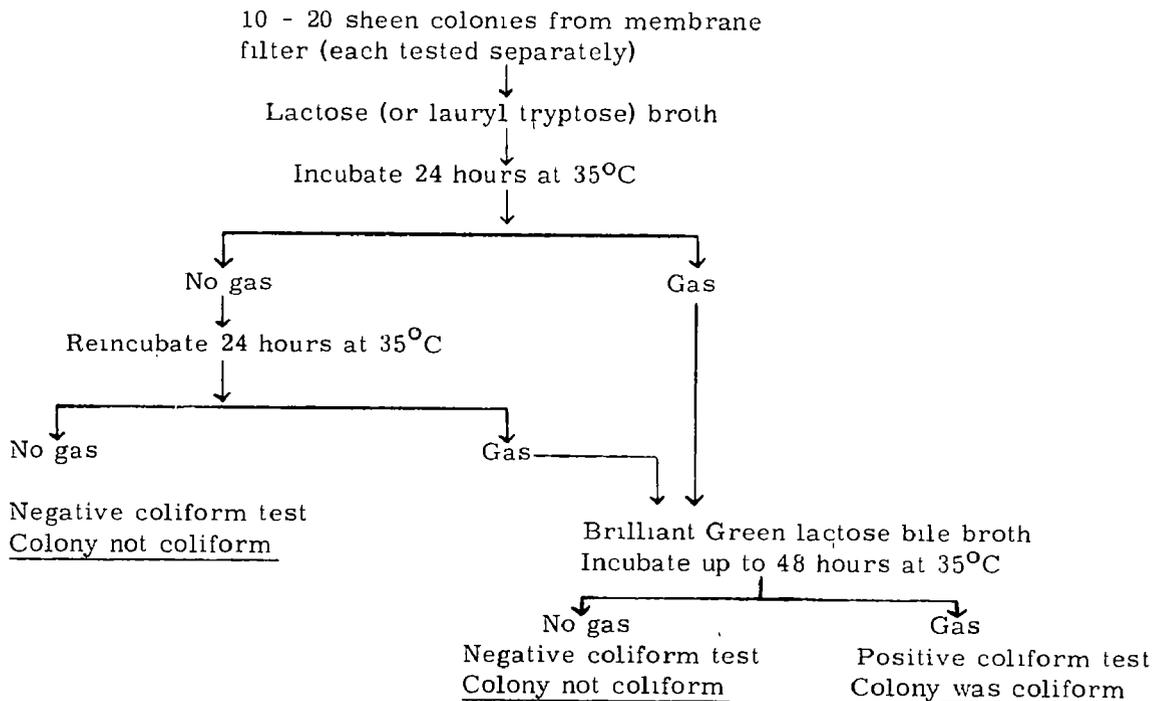


Diagram 1. ABBREVIATED COLIFORM VERIFICATION PROCEDURE

B A more elaborate verification of membrane filter test for coliforms resembles the Completed Test of Standard Methods. The test is started in exactly the same way as the abbreviated test, and may be represented diagrammatically as a continuation from the lactose broth stage of Diagram 1. See Diagram 2.

C While the diagrams (1 and 2) are presented in terms of sheen colonies (interpreted as coliforms), the careful worker also should subject a similarly representative number of non-sheen colonies (judged to be noncoliforms) to the same test procedure. This will reveal whether the medium being studied fails to differentiate appreciable numbers of colonies which in reality are coliforms, even

though they did not demonstrate the desired differential characteristic.

III VERIFICATION OF FECAL COLIFORM TESTS ON MEMBRANE FILTERS

A The procedure described here is based on the principle that, with use of m-FC Broth and incubation in a water bath at 44.5°C for 24 hours, fecal coliform colonies on membrane filters develop a blue color, (sometimes a greenish-blue). Extraneous bacteria are believed to fail to develop colonies, or else consist of such colonies develop some color other than the blue color of fecal coliforms (colorless, buff- or brownish-color, or even red colonies may develop on the medium).

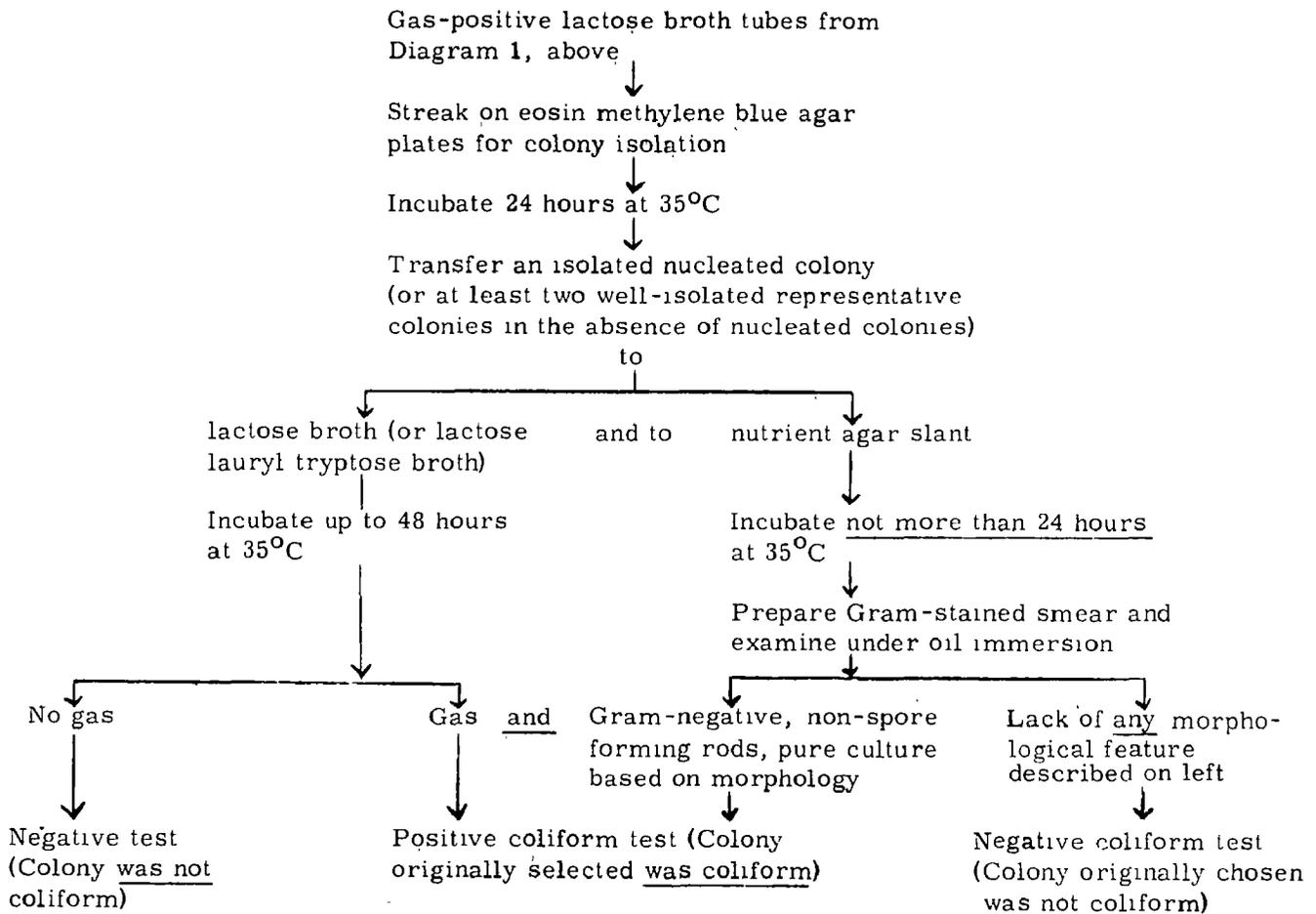


Diagram 2. EXTENDED COLIFORM VERIFICATION PROCEDURE

B The verified test for fecal coliforms is indicated in Diagram 3, below:

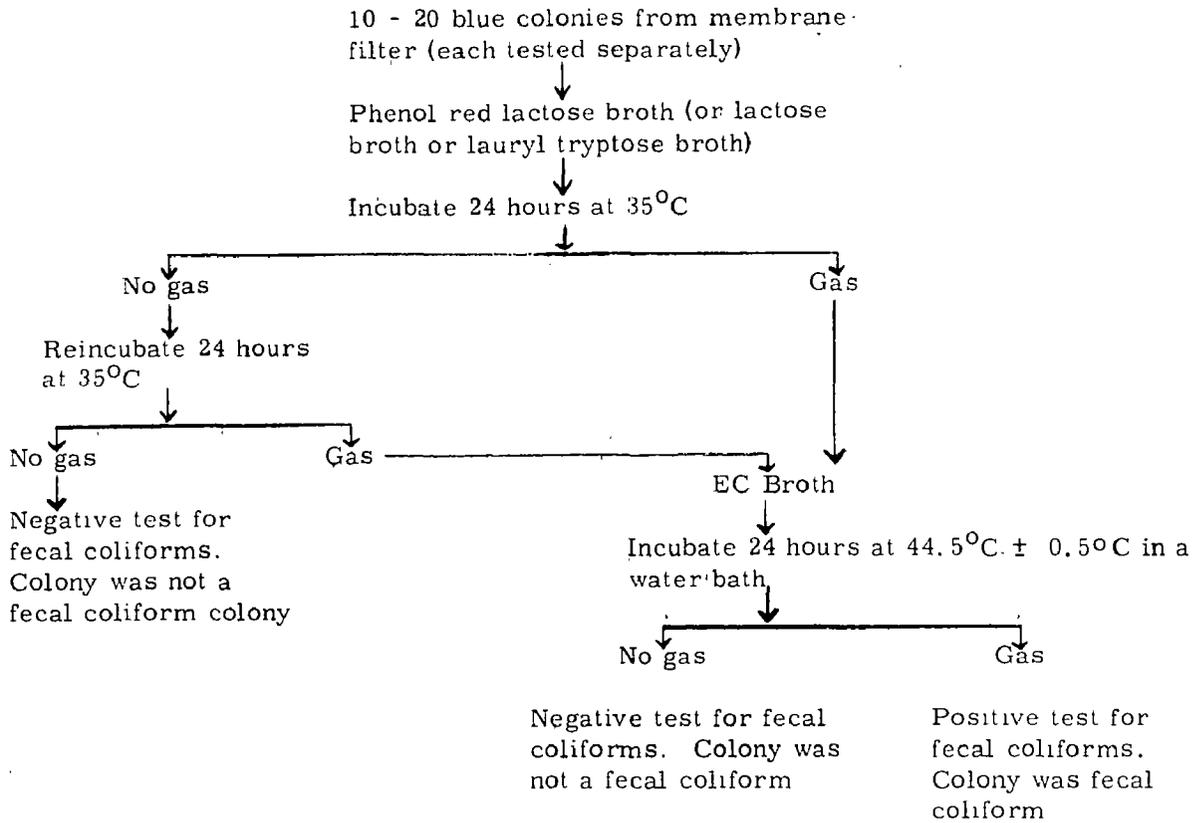


Diagram 3. A VERIFICATION PROCEDURE FOR FECAL COLIFORMS ON MEMBRANE FILTERS

IV VERIFICATION OF FECAL STREPTOCOCCUS COLONIES ON MEMBRANE FILTERS

A The procedure is used in the evaluation of results from a medium similar to the m-Enterococcus Agar (Slanetz) described in the current edition of Standard Methods. The membrane filter procedure utilizes 48 hour incubation at 35°C, and colonies which are pink to red, either in their entirety or only in their centers, are regarded as fecal streptococci. Most such colonies are 1-2 mm in diameter, and some may be larger. Occasionally, some samples may be encountered in

which numerous extremely small colonies, approximately 0.1 mm in diameter, are present in great numbers. Almost invariably, these are not fecal streptococci. See diagram 4 for a representation of a verification test.

V CALCULATIONS BASED ON VERIFICATION STUDIES

A A percent verification can be determined for any colony-validation test:

. Percent verification =

$$\frac{\text{No. of colonies meeting verification test}}{\text{No. of colonies subjected to verification}} \times 100$$

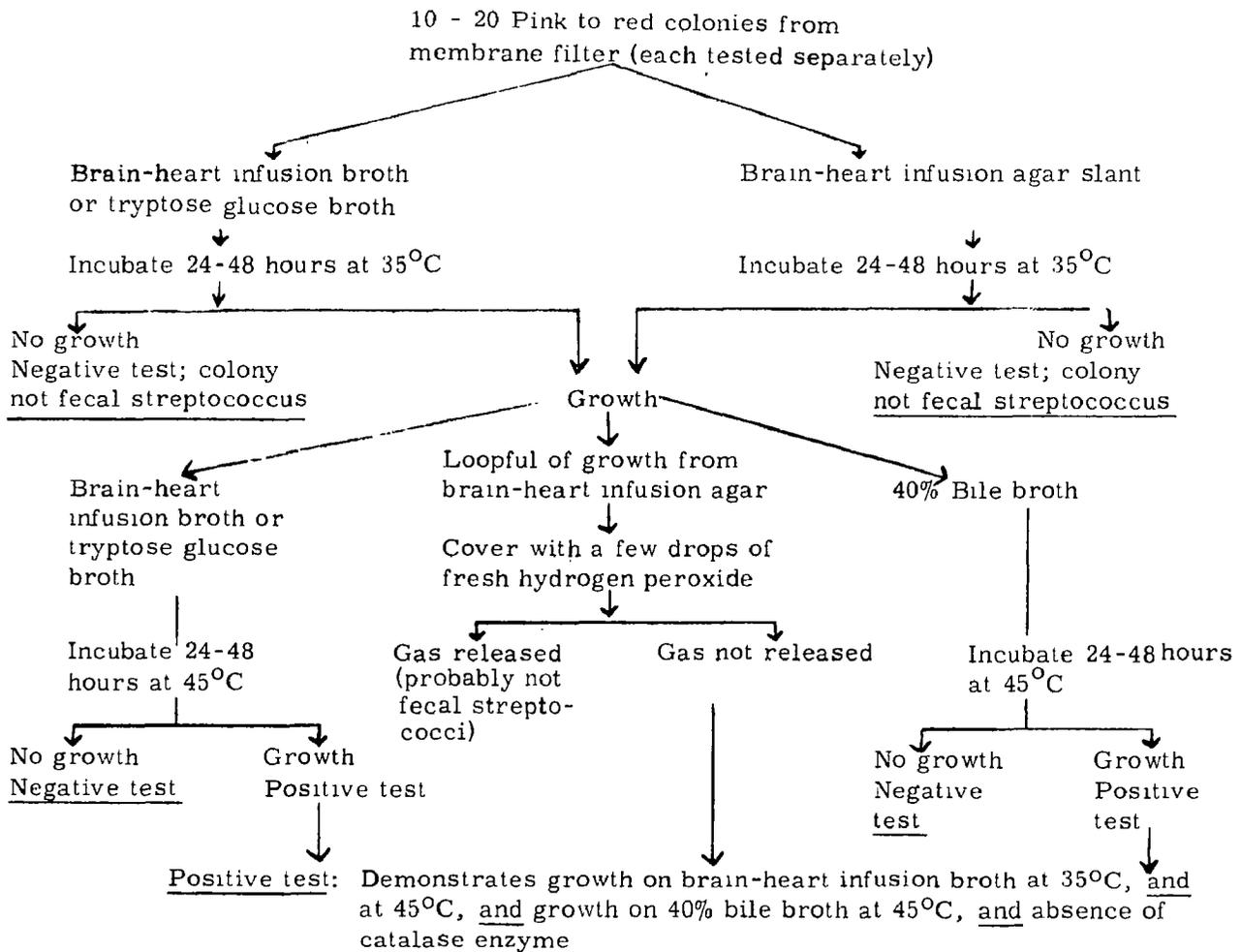


Diagram 4. FLOW SHEET AND SUGGESTED SEQUENCE OF TESTS TO PERFORM VERIFICATION STUDIES ON COLONIES BELIEVED TO BE FECAL STREPTOCOCCI

Example: Twenty-five sheen colonies on Endo-type membrane filter medium were subjected to verification studies shown in Diagram 1. Twenty-two of these colonies proved to be coliforms according to provisions of the test:

$$\text{Percent verification} = \frac{22}{25} \times 100 = 88$$

B A percent verification figure can be applied to a direct membrane filter count per 100 ml to determine the verified membrane filter count per 100 ml of the test organism.

Verified count per 100 ml of the test organism

$$\frac{\text{Percent verification}}{100} \times \text{count per 100 ml of test organism}$$

Example: For a given sample, by a direct membrane filter test, the fecal coliform count was found to be 42,000 per 100 ml. Supplemental studies on selected colonies showed 92% verification.

$$\begin{aligned} \text{Verified fecal coliform count} &= \frac{92}{100} \times 42,000 \\ &= 0.92 \times 42,000 \\ &= 38,640 \end{aligned}$$

$$\text{Rounding off:} = 39,000 \text{ per 100 ml}$$

- C A percentage of false-negative tests also can be determined (See II, C)

Percent false negative =

$$\frac{\text{No. "negative" colonies found positive}}{\text{Total No. "negative" colonies tested}} \times 100$$

Example: On a total coliform test, 25 nonsheer (coliform negative) colony types were subjected to the coliform verification procedure shown in Diagram 1. Two of these colonies proved to be coliform colonies.

$$\begin{aligned} \text{Percent false negatives} &= \frac{2}{25} \times 100 \\ &= 8 \end{aligned}$$

VI SOME APPLICATIONS OF PERCENT VERIFICATION CALCULATIONS

- A In comparisons between two or more different membrane filter media, the medium which has the highest percentage of verification, and the lowest percentage of false negatives (based on a broad range of sample types and sources) is the better medium.
- B In productivity comparisons between two or more different membrane filter media, the medium which produces the highest verified membrane filter counts per 100 ml (based on a broad range of sample types and sources) is the better medium.
- C The worker is cautioned NOT to apply percentage of verification determined from one sample, to other samples. For example, do not determine a percentage verification on m-Endo broth for a sample taken from the Ohio River on September 6, and then seek to apply that percentage verification to another coliform determination from the Little Miami River, on the same date. Even the application of the verification percentage to another Ohio River sample, either on the same date from a different station, or on another date from the same station, should be undertaken with great caution. Such

application of verification percentages from one sample to another should be taken only after sufficient studies have been made demonstrate the suitability of such a procedure.

VII USE OF VERIFICATION STUDIES IN MF-MPN COMPARISONS

- A Comparisons of data obtained from MF versus MPN methods have been the source of great concern to microbiologists. For the current basis of comparisons, see Standard Methods (either 11th or 12th edition) "-- with a proviso that it should be used for determining the potability of drinking water only after parallel testing had shown that it afforded information equivalent to that given by the standard multiple-tube test."
- B Some workers have sought to apply this requirement on the basis of statistical calculations, based on comparisons of numerical values from membrane filter tests with numerical values obtained from multiple-tube tests. Further study of this problem, and methods different workers have applied to the problem, can be made on the basis of the appended reference list.
- C Numerical comparisons between raw or verified membrane filter results on split samples, compared with multiple-tube results, also should take into account the question of the reliability of the multiple-tube test. The numerical results of the Completed Test for coliforms, for example, can be compared with the results of the Confirmed Test, to determine a percentage of verification for the multiple-tube test:

$$\begin{aligned} \text{Percent verification} &= \\ &= \frac{\text{Completed Test Coliforms per 100 ml}}{\text{Confirmed Test Coliforms per 100 ml}} \times 100 \end{aligned}$$

Example: On a given sample, the test was carried to the Completed Test stage. Afterward, both a Confirmed Test and a Completed Test coliform result were obtained, consisting of

Table 1. VALIDITY OF MF AND MPN "CONFIRMED TEST"*

Source	Number of supplies	MF Coliform Test			MPN Confirmed Test		
		Minimum	Maximum	Percent verified	Minimum	Maximum	Percent verified
Wells - Springs	16	1.0	7,600	96.6	7.0	11,000	64.6
Lakes - Lagoons	23	1.0	420,000	79.6	79	490,000	70.9
Creeks	19	32	260,000	75.8	120	460,000	66.4
Rivers	22	320	890,000	69.7	700	350,000	75.7
Sewage	11	1,400,000	28,000,000	68.6	460,000	49,000,000	73.8
Totals	91			78.1			70.3

*All coliform values are per 100 ml of sample

49,000 per 100 ml for the Confirmed Test and 33,000 per 100 ml for the Completed Test.

$$\text{Percent verification} = \frac{33,000}{49,000} \times 100 = 67$$

See Table 1 for some studies of MF verification studies, and parallel multiple-tube verification studies (Confirmed Test carried to Completed Test). These studies have been conducted in research laboratories of this Center, and demonstrate the difficulty and problems associated comparative evaluation of membrane filter versus multiple-tube methods. The student is invited to study this table at leisure.

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DETERMINING ACCEPTABILITY OF MEMBRANE FILTER METHODS
IN WATER QUALITY TESTS

I INTRODUCTION

A Historical

The membrane filter technique was presented in the 10th Edition of "Standard Methods for the Examination of Water and Waste Water" in 1955, as a tentative method. Five years later, the method became an accepted standard procedure, with the 11th Edition. However, the approval was limited by a proviso that the membrane filter method should be used for determining the potability of drinking waters only after parallel testing had shown that it afforded information equivalent to that given by the standard multiple-tube test. Presumably it was intended that similar tests should be extended in application of membrane filter methods to bacterial tests on non-potable waters.

B Factors in Acceptance of Membrane Filter Methods

- 1 The decision to accept membrane filter methods for determining bacterial quality of water may be based on one or a combination of the following:
 - a Applicability to the water samples tested
 - b Availability and quality of special laboratory equipment and supplies
 - c Technical skill of laboratory workers
 - d Convenience, i. e., speed of obtaining results
 - e Economic factors

f Administrative attitudes

- 2 The demonstration of equivalent interpretation of bacterial quality of water, stipulated as a qualifying proviso of acceptance in Standard Methods, includes 1, a-c. On the other hand, rejection of membrane filter methods on the basis of noncompliance with the limiting proviso of Standard Methods may be due to any one of these three basic causes with two of the three representing rather easily corrected deficiencies in the laboratory. Carried to a logical conclusion, much of the determination of "acceptability" of membrane filter methods is, in fact, an evaluation of the "fitness" of the laboratory to perform the testing procedure. This may be quite a different matter from the inherent applicability of the method. Workers seeking to evaluate the suitability of membrane filter methods for their own water testing programs should undertake evaluation studies with great care, to assure that the testing program actually defines the factors tested.

C Scope of This Discussion

- 1 On the basis of the above comments, it is worthwhile to consider some significant factors which may influence decisions relating to use of membrane filter methods.
- 2 In addition, the discussion includes program currently used by the State of Ohio in certifying water treatment quality control laboratories to use membrane filter methods.

II MEMBRANE FILTER ACCEPTANCE FACTORS

A Applicability to the Water Samples Tested

- 1 This can be determined only after items 2, and 3, (following) have been established at acceptable standards.
- 2 The validity of the multiple tube method used in comparisons should be established.
 - a There is a tendency to apply the Confirmed Test as the standard of comparison with results obtained from membrane filter tests, without demonstrating that the Confirmed Test results are, in fact, valid.
 - b See Table 1. (Reference no. 4). The table demonstrates discrepancies between results obtained from Confirmed Test vs the Completed Test in a range of sample types studied in the Cincinnati laboratories. When study of the multiple tube test shows appreciable disagreement between Confirmed Test and Completed Test results on the samples tested, results from the Completed Test should be used as the basis of comparison with results from membrane filter tests.

- c When individual multiple tube results are compared with the split-sample membrane filter test results, the results may be considered in agreement if the membrane filter result falls within the 95% confidence limits of the bacterial count obtained by the parallel-test multiple tube. There is a tendency for numerical results from membrane filter methods to be somewhat lower than corresponding multiple-tube results. This should not be surprising or disturbing; mathematicians have drawn attention to an inherent bias in the computations of the Tables of Most Probable Numbers, estimated to range from 15 - 25% high.
- d A sufficient number of split-sample comparisons should be made to establish a valid comparison of results. Many workers assume that 100 split samples is an acceptable basis of comparison, provided that they represent samples from all seasons of the year.

Table 1. VALIDITY OF MEMBRANE FILTER RESULTS AND OF MULTIPLE TUBE CONFIRMED TEST IN A SPLIT SAMPLE SERIES

Source	Number of Samples	MF Coliform Test*		Average % Verified**	MPN Confirmed Coliform Test*		Average % Verified
		Minimum	Maximum		Minimum	Maximum	
Wells-Springs	16	1	7,600	96.6	7	11,000	64.6
Lakes-Lagoons	23	1	420,000	79.6	79	490,000	70.0
Creeks	19	32	260,000	75.8	120	460,000	66.4
Rivers	22	320	890,000	69.7	700	350,000	75.7
Sewage	11	1,400,000	28,000,000	68.6	460,000	49,000,000	73.8

* All coliform results in count per 100 ml
 ** Through performance of completed test

3 Numerical agreement between membrane filter vs multiple dilution tube results demonstrates acceptability of the membrane filter method for the water samples tested. Lack of such agreement, when traced to the waters tested, may be from any of several causes, such as high turbidity and low coliform counts, unacceptably large numbers of non-coliform colonies and small numbers of coliform colonies growing on the membrane filters, or presence of deleterious chemicals which are deposited in or on the filters, with development of unreasonably low numbers of colonies.

4 A series of verified coliform tests, still another useful test in establishing the validity of membrane filter methods for the samples tested, is described elsewhere in this course manual. Table 1 includes data obtained as a result of including verified membrane filter coliform tests in a series of comparisons between membrane filters vs multiple tube tests. The results of this study suggest that results obtained by membrane filter methods are somewhat more reliable than the results from the Confirmed Test in the sample series described.

B Availability and Quality of Special Laboratory Equipment and Supplies

1 Descriptions of acceptable membrane filter equipment, supplies, and media are found elsewhere in this course manual. Rigid adherence to specifications is important in all bacteriological work, and is vital in comparative studies of this type.

2 Almost any single item of supply or equipment, if misused or badly selected, may bring about unsatisfactory results. The following are among the most flagrant areas of deviation from recommended equipment and supplies:

a Use of membrane filters not meeting recommended specifications.

- b Improperly selected or prepared culture medium.
- c Lack of suitable equipment to maintain proper levels of moisture and recommended temperature ranges of culture during incubation.
- d Lack of recommended optical assistance for counting colonies, or misuse of such equipment.
- e Lack of recommended illuminating equipment for colony counts, or misuse of recommended equipment.

C Technical Skill of Laboratory Workers

- 1 Membrane filter methods require application of laboratory skills and judgment of at least the same order as that required for multiple-tube tests.
- 2 Training is a recommended starting point in acquisition of the required skills. At this Training Center, 4-1/2 days of basic training includes lectures, demonstrations, and intensive, repetitive, laboratory work under supervision of qualified instructors. It is recommended that this amount of formal training should not be appreciably reduced.
- 3 Extended individual practice in membrane filter methods is recommended following a training course. Only after such practice, with liberal use of verified membrane filter coliform tests to establish reliability of colony interpretation, should the individual undertake comparisons between membrane filter vs multiple tube tests to comply with the limited-acceptance proviso of Standard Methods.

D Convenience

- 1 The time for obtaining coliform results is one day for membrane filter methods, versus 2-4 days for the Confirmed Test and up to a week for the Completed Test.

- 2 Space requirements for media preparation and storage, glassware preparation and storage, incubator space, all are smaller with membrane filter methods than with multiple-tube methods.

E Economic Factors

- 1 Much of the convenience of membrane filter methods, described in D above, also covers economic implications.
- 2 Cost comparisons between membrane filter methods and multiple tube methods include many debatable considerations. Personnel at this Center have tended to regard the two methods as being roughly equal in cost per test.
- 3 In a special study, workers of the Illinois Department of Health (McCaffrey, unpublished) reported a saving of \$0.08 per sample with use of membrane filter methods. The comparison was based on more than 30,000 samples for each method.

F Administrative Attitudes

Decisions regarding use, or even consideration of introduction of membrane filter methods in a given laboratory may be determined by laboratory administrative management, without reference to any findings within the laboratory, or even without consultation with laboratory personnel. Several different aspects of this problem may develop:

- 1 Prejudgment without experimental data is sometimes encountered. In candor, it must be recognized that this is a very real problem, and has been a significant factor in slowness of acceptance, or even consideration, of membrane filter methods in some laboratories.
- 2 Acceptability of evidence based on membrane filter methods in legal procedures has concerned some administrators. Membrane filter methods have been used to an increasing extent in numerous water pollution control activities of this organization.

- 3 Some workers have been reluctant to convert to membrane filter methods in water quality control investigations, due to an obvious contrast between the membrane filter method and methods used in previous investigations on the same, or similar, body of water based on multiple tube methods. Often, these workers fail to recognize that several features of multiple tube methods today are not identical with those in use 10 or 20 years ago. The overall acceptance of the membrane filter method may be just as seriously handicapped by too-ready an administrative acceptance of the method without adequate equipping or training of the laboratory and its personnel to apply the method. In this case, the method can acquire a poor reputation, not due to the method itself, but to its routine use before the necessary skills and material resources are available.

III State of Ohio's Procedures for Establishing Acceptability of Use of Membrane Filter for Bacteriological Examination of Drinking Water

A Background

- 1 The Ohio Department of Health does accept the use of membrane filter for bacteriological examination of potable water.
- 2 In other States, the individual should check with the regulatory agency of the State as to acceptability; other States may have a different position or conditions surrounding acceptance.

B Conditions of Acceptance

Ohio Department of Health's acceptance of membrane filter procedure is subject to three conditions:

- 1 Formal training of laboratory personnel responsible for bacteriological examinations
- 2 Laboratory survey and approval of both equipment and procedures

- 3 Parallel testing of raw and finished water supplies
- C Formal Training
- 1 This requirement is fulfilled by successful completion of training courses at the National Training Center, Cincinnati, Ohio.
 - 2 The training personnel of the Robert A. Taft Laboratories have worked closely with the Ohio Health Department, and similar agencies in other states, in providing training to meet special requirements.
- D Laboratory Survey
- 1 The survey is made by Ohio's main laboratory bacteriologists.
 - 2 The survey is made only after a period of parallel tests of split samples using both membrane filter and multiple tube methods.
 - 3 The request for survey by state personnel must originate at the local level.
 - 4 The survey covers both personnel and equipment in the laboratory.
- E Parallel Testing
- 1 The Ohio Department of Health requires parallel testing using the multiple tube technique and the membrane filter technique.
 - 2 The test should include at least a period of 3 months with a minimum of 100 samples of raw water and 100 samples of finished water included in the tests.
 - a Practice in identifying coliform colonies is required. Preparation of synthetic raw water samples is required if the raw water is free of coliform bacteria.
 - b Practice in all aspects of laboratory techniques in membrane filter tests is required.
 - c The limitations of the membrane filter must be recognized, for easy recognition.
 - 3 Results of parallel testing procedure are reported on special forms designed by the Ohio Department of Health.
 - 4 Several precautions are presented for those running parallel tests:
 - a Use 100 ml samples for finished waters and samples collected from taps in the distribution system.
 - b Use synthetic raw water if the natural raw water lacks coliforms.
 - c Clear any questions or problems with the Central Laboratory or Central Office personnel before the three-month parallel testing period is completed.

ACKNOWLEDGMENT:

This outline includes certain materials made available to the National Training Center by Robert S. McEwen, Engineer, Water Supply Unit, Ohio Department of Health, Columbus, Ohio.

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COLLECTION AND HANDLING OF SAMPLES FOR BACTERIOLOGICAL EXAMINATION

I INTRODUCTION

The first step in the examination of a water supply for bacteriological examination is careful collection and handling of samples. Information from bacteriological tests is useful in evaluating water purification, bacteriological potability, waste disposal, and industrial supply. Topics covered include: representative site selection, frequency, number, size of samples, satisfactory sample bottles, techniques of sampling, labeling, and transport.

II SELECTION OF SAMPLING LOCATIONS

The basis for locating sampling points is collection of representative samples.

- A Take samples for potability testing from the distribution system through taps. Choose representative points covering the entire system. The tap itself should be clean and connected directly into the system. Avoid leaky faucets because of the danger of washing in extraneous bacteria. Wells with pumps may be considered similar to distribution systems
- B Grab samples from streams are frequently collected for control data or application of regulatory requirements. A grab sample can be taken in the stream near the surface.
- C For intensive stream studies on source and extent of pollution, representative samples are taken by considering site, method and time of sampling. The sampling sites may be a compromise between physical limitations of the laboratory, detection of pollution peaks, and frequency of sample collection in certain types of surveys. First, decide how many samples are needed to be processed in a day. Second, decide whether to measure cycles of immediate pollution or more average pollution. Sites for measuring cyclic pollution are immediately below the pollution source. Sampling is frequent, for example, every three hours.

A site designed to measure more average conditions is far enough downstream for a complete mixing of pollution and water.

Keep in mind that averaging does not remove all variation but only minimizes sharp fluctuations. Downstream sites sampling may not need to be so frequent

Samples may be collected 1/4, 1/2 and 3/4 of the stream width at each site or other distances, depending on survey objectives. Often only one sample in the channel of the stream is collected. Samples are usually taken near the surface

- D Samples from lakes or reservoirs are frequently collected at the drawoff and usually about the same depth and may be collected over this entire surface
- E Collect samples of bathing beach water at locations and times where the most bathers swim.

III NUMBER, FREQUENCY AND SIZE OF SAMPLES

- A For determining sampling frequency for drinking water, consult the USPHS Standards.
 - 1 The total number, frequency, and site are established by agreement with either state or PHS authorities.
 - 2 The minimum number depends upon the number of users. Figure 1 indicates that the smaller populations call for relatively more samples than larger ones. The numbers on the left of the graph refer to actual users and not the population shown by census
 - 3 In the event that coliform limits of the standard are exceeded, daily samples must be taken at the same site. Examinations should continue until two consecutive samples show coliform level is satisfactory. Such samples are to be considered as special samples and shall not be included in the total number of samples examined.
 - 4 Sampling programs described above represent a minimum number which may be increased by reviewing authority.

- B For stream investigations the type of study governs frequency of sampling.
- C Collect swimming pool samples when use is heavy. The high chlorine level rapidly reduces the count when the pool is not in

use. Residual chlorine tests are necessary to check neutralization of chlorine in the sample.

- D Lake beaches may be sampled as required depending on the water uses

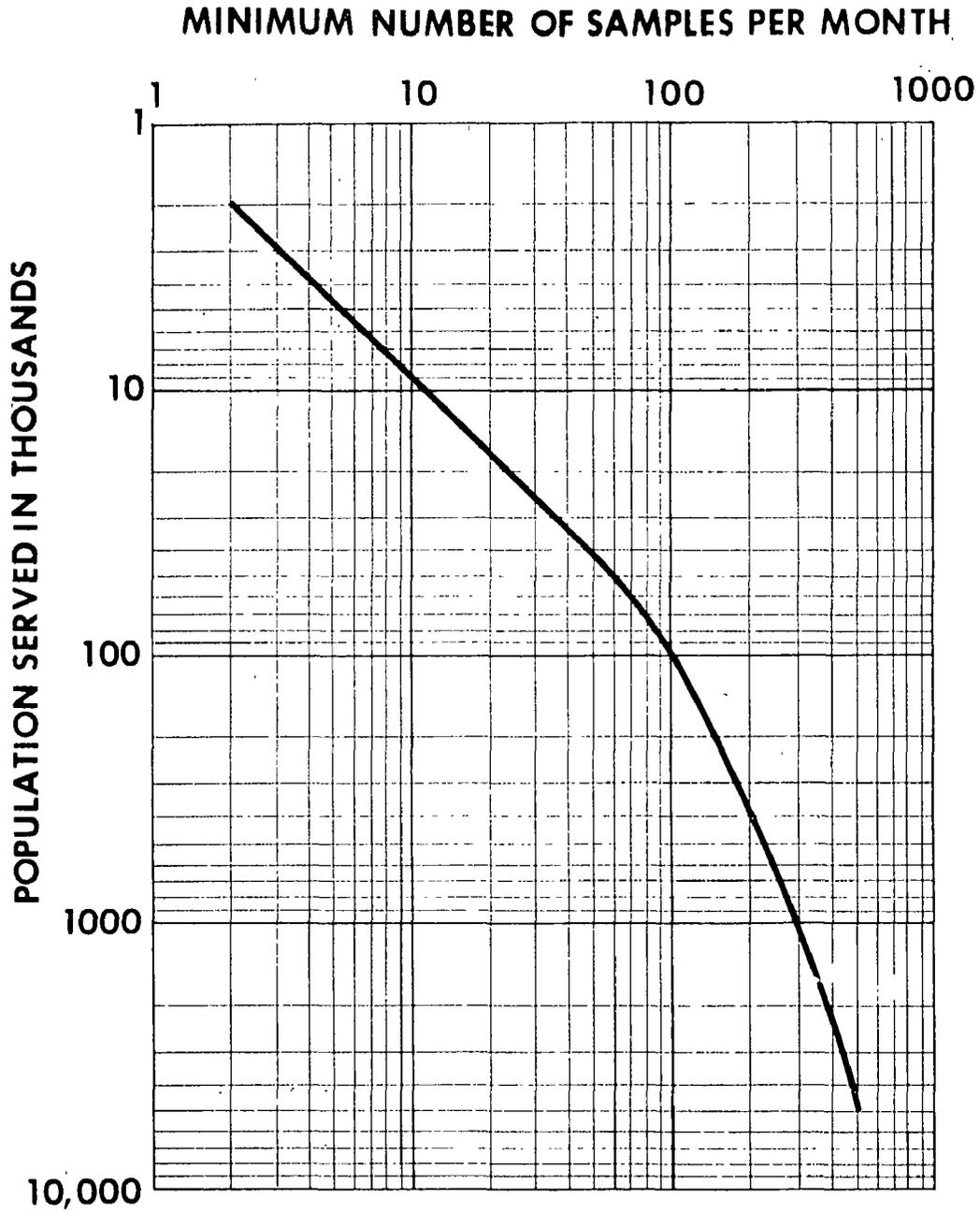


FIGURE 1

- E Salt water or estuarine beaches are sampled as needed with frequency depending on use.
- F Size of samples depends upon examination anticipated. Generally 100 ml is the minimum size.

IV BOTTLES FOR WATER SAMPLES

- A The sample bottles should have capacity for at least 100 ml of sample, plus an air space. The bottle and cap must be of bacteriological inert materials. Resistant glass or heat resistant plastic are acceptable. At the National Training Center, wide mouth ground-glass stoppered bottles (Figure 2) are used.

All bottles must be properly washed and sterilized. Protect the top of the bottles and cap from contamination by paper or metal foil hoods. Both glass and heat

resistant plastic bottles may be sterilized in an autoclave. Hold plastic at 121°C for at least 10 minutes. Hot air sterilization, 1 hour at 170°C, may be used for glass bottles.

- B Add sodium thiosulfate to bottles intended for halogenated water samples. A quantity of 0.1 ml of a 10% solution provides 100 mg per liter concentration in a 100 ml sample. This level shows no effect upon viability or growth.
- C Supply catalogs list wide mouth ground glass stoppered bottles of borosilicate resistance glass, specially for water samples.

V TECHNIQUE OF SAMPLE COLLECTION

Follow aseptic technique as nearly as possible. Nothing but sample water must touch the inside of the bottle or cap. To avoid loss of sodium thiosulfate, fill the bottle directly and do not rinse. Always remember to leave an air space.

- A In sampling from a distribution system, first run the faucet wide open until the service line is cleared. A time of 3-5 minutes generally is sufficient. Reduce the flow and fill the sample bottle without splashing. Some authorities stress flaming the tap before collection. A chlorine determination is often made on the site.

- B The bottle may be dipped into some waters by hand. Avoid introduction of bacteria from the human hand and from surface debris. Some suggestions follow: Hold the bottle near the base with one hand and with the other remove the hood and cap. Push the bottle rapidly into the water mouth down and tilt up towards the current to fill. A depth of about 6 inches is satisfactory. When there is no current move the bottle through the water horizontally and away from the hand. Lift the bottle from the water, spill a small amount of sample to provide an air space, and return the uncontaminated cap.

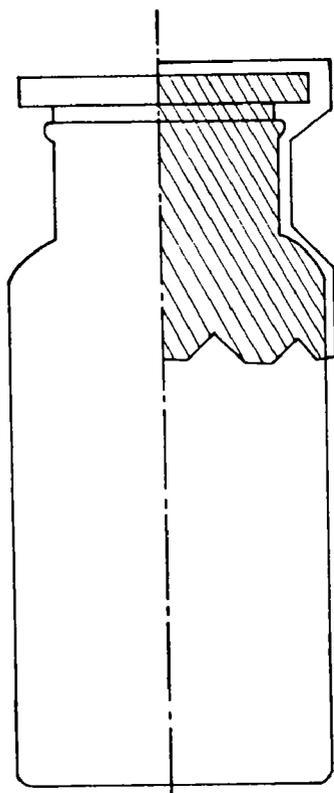


FIGURE 2

- C Samples may be dipped from swimming pools. Determine residual chlorine on the pool water at the site. Test the sample at the laboratory to check chlorine neutralization by the thiosulfate.
- D Sample bathing beach water by wading out to the two foot depth and dipping the sample up from about 6 inches below the surface. Use the procedure described in V. B.
- E Wells with pumps are similar to distribution systems. With a hand pumped well, waste water for about five minutes before taking the sample. Sample a well without a pump by lowering a sterile bottle attached to a weight. A device which opens the bottle underneath the water will avoid contamination by surface debris.
- F Various types of sampling devices are available where the sample point is inaccessible or depth samples are desired. The general problem is to put a sample bottle in place, open it, close it, and return it to the surface. No bacteria but those in the sample must enter the bottle.
- 1 The J - Z sampler described by Zobell in 1941, was designed for deep sea sampling but is useful elsewhere (Figure 3). It has a metal frame, breaking device for a glass tube, and sample bottle. The heavy metal messenger strikes the lever arm which breaks the glass tubing at a file mark. A bent rubber tube straightens and the water is drawn in several inches from the apparatus. Either glass or collapsible rubber bottles are sample containers.

Commercial adaptations are available.

- 2 Note the vane and lever mechanism on the New York State Conservation Department's sampler in Figure 4. When the apparatus is at proper depth the suspending line is given a sharp pull. Water inertia against the vane raises the stopper and water pours into the bottle. Sufficient sample is collected prior to the detachment of the stopper from the vane arm allowing a closure of the sample bottle.

The New York State Conservation Department's sampler is useful for shallow depths and requires nothing besides glass stoppered sample bottles.

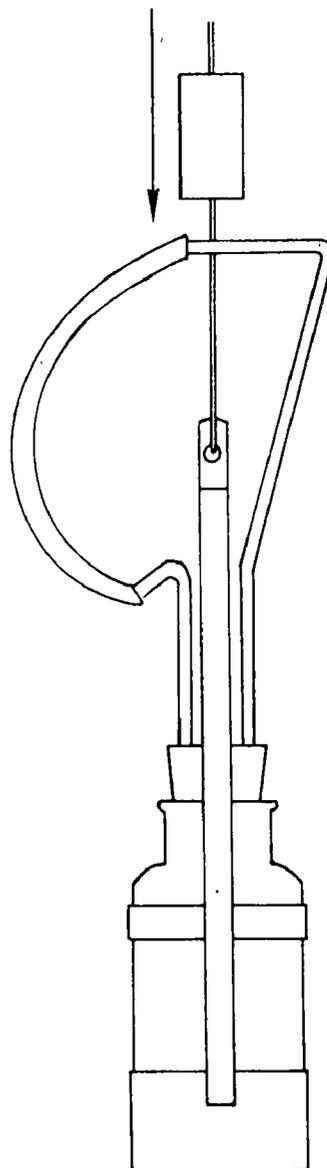


FIGURE 3

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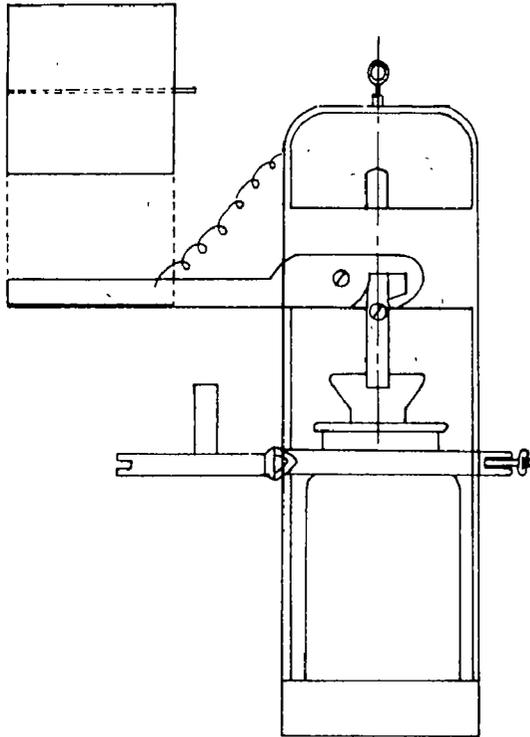


FIGURE 4

- 3 A commercial sampler is available which is an evacuated sealed tube with a capillary tip. When a lever on the support rack breaks the tip, the tube fills. Other samplers exist with a lever for pulling the stopper, while another uses an electromagnet.

VI DATA RECORDING

- A Information generally includes: date, time of collection, temperature of water, location of sampling point, and name of the sample collector. Codes are often used. The location description must be exact enough to guide another person to the site. Reference to bridges, roads, distance to the nearest town may help. Use of the surveyors' description and maps are recommended. Mark identification on the bottles or on securely fastened tags. Gummed tags may soak off and are inadvisable.

- B While a sanitary survey is an indispensable part of the evaluation of a water supply, its discussion is not within the scope of this lecture. The sample collector could supply much information if desired

VII SHIPPING CONDITIONS

- A The examination should commence as soon as possible, preferably within one hour. A maximum elapsed time between collection and examination is 30 hours for potable water samples and 8 hours for other water samples (collection 6 hours and laboratory procedures 2 hours). Standard Methods (13th Edition) recommends icing of samples between collection and testing.

VIII PHOTOGRAPHS

- A photograph is a sample in that it is evidence representing water quality. Sample collectors and field engineers may carry cameras to record what they see. Pictures help the general public and legal courts to better understand laboratory data.

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Descriptors: Bacteria, Sample, Sampling,
Water Sampling, Handling, Preservation,
Samplers, Surface Waters, Distribution
Systems

RECOVERY AND IDENTIFICATION OF SALMONELLA AND SHIGELLA
FROM ENVIRONMENTAL WATERS

I INTRODUCTION

A Significant Factors Relating to Enteric Diseases

- 1 Salmonellosis (species of Salmonella other than S. typhi). An illness characterized by the usual inception of gastroenteritis which can further manifest itself in an enteric fever and/or septicemia. The usual incubation period is from 6 to 48 hours and more commonly symptoms appear in about 12 hours. A period of communicability can occur within the intervals of 3 days to 3 weeks but a permanent carrier condition can occur in a small percentage of cases.

- 2 Typhoid Fever (Salmonella typhi)

This representative of the Salmonella genus can have a 10% mortality rate unless immediate treatment is instituted wherein a 2-3% rate is usual. The organism is found in the blood during the first two weeks and in the feces and urine after the initial two weeks of malady. About 50 types have been identified by means of the Vi phage. The usual incubation period varies from 1 to 3 weeks. The permanent carrier rate is from 2-5% and as much as 10% of the patients excrete the bacilli for a period of three months.

- 3 Shigellosis (various species of genus Shigella)

An acute infection of the intestine characterized by stools of blood, mucous, and pus in the severe cases. The usual incubation period is from 1 to 7 days but the usual case occurring in 4 days. A few individuals become carriers for a year or two but rarely longer.

B Relative Incidence

- 1 Almost exclusive transmittance of these

enteric diseases is by the Water-Food-Milk route as shown in figure 1.

- 2 Increased numbers of outbreaks have been attributed to Salmonellosis within recent years but an apparent disparity exists between reported disease and population of reporting areas. This data is shown in figures 2 and 3.
- 3 Reporting of etiological agents responsible for outbreaks has been undetermined in a significant number of cases. Figure 4 illustrates this for the reporting period 1952 to 1959.

C Increased Interest in Pathogen Isolations

- 1 Increased desire to obtain more complete knowledge of state of water sample.
- 2 Development and augmentation of media for pathogenic analysis.
- 3 Analysis in special areas where coliform isolations yield low counts.
- 4 Areas where pathogen analysis and recoveries can relate pollution sources from specific areas.
- 5 Passage and implementation of Public Law 660 one section of which calls for the abatement of pollution of interstate or navigable waters which endangers the health or welfare of any persons.

D Development of Isolation Technique

- 1 Early interest in the detection of Salmonella typhi and Shigella Species due to explosive outbreaks mainly by the water route.
- 2 Development of serological techniques which have aided in the detection and identification of pathogens as well as providing an impetus in taxonomic developments.

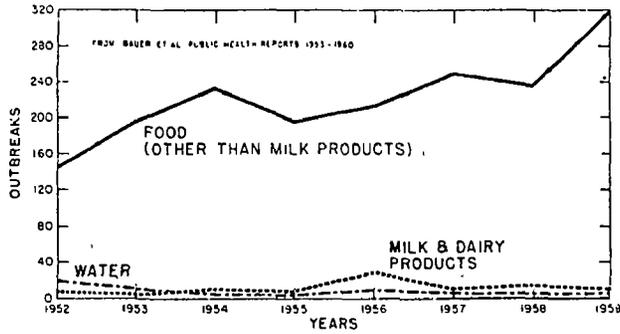


FIGURE 1

FOOD, MILK, AND WATERBORNE DISEASE
OUTBREAKS REPORTED IN U.S.A. 1952-59

REPORTED INCIDENCE OF HUMAN SALMONELLOSIS*
UNITED STATES, 1942-1963

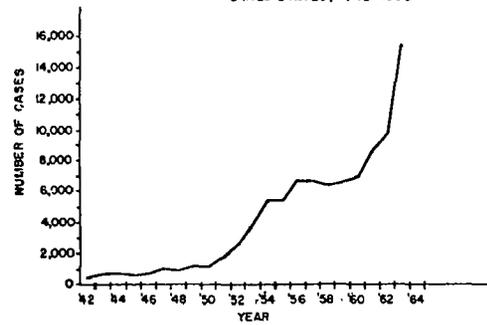


FIGURE 2

* Other than typhoid fever
Source: MMWR Annual Supplements, 1951, 1951, & 1963

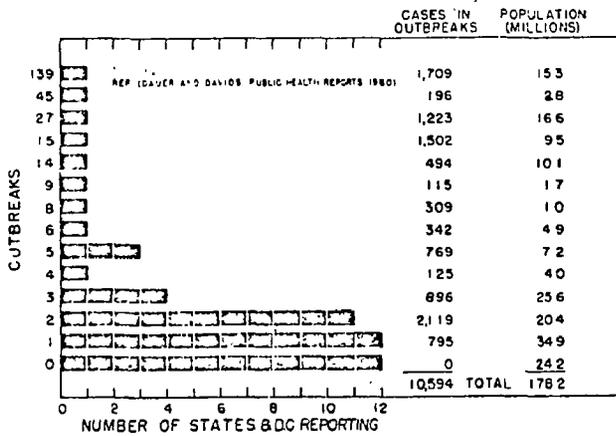
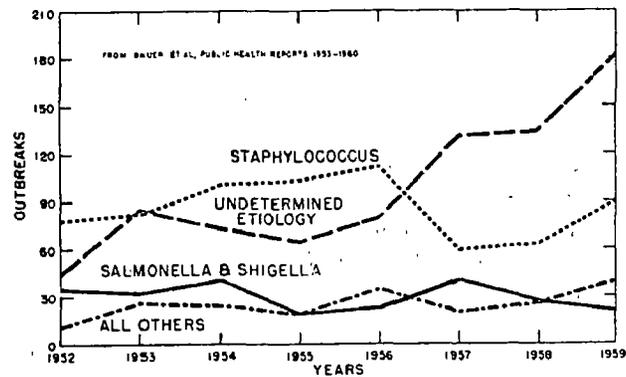


FIGURE 3

DISPARITY BETWEEN
REPORTED FOODBORNE DISEASE
AND
POPULATION OF REPORTING AREAS - 1959

FIGURE 4

TYPES OF FOODBORNE DISEASE OUTBREAKS
REPORTED IN U.S.A. 1952-59



- 3 Increased knowledge of the physiology and nature of enteric pathogens in the following areas:
 - a Preservation and transport of fecal and contaminated samples
 - b Enrichment procedures
 - c Selective procedures providing enhanced recoveries
 - d Methodology of sample concentration of microbial flora

II RECOVERY AND IDENTIFICATION

A Procedural Sequence - Description

1 Concentration

In most cases it is necessary to provide a relatively large volume of sample in order to isolate pathogenic organisms from the water sample. This is due to the relatively small numbers of pathogens in relation to the indicator organisms of pollution since contribution of these pathogens is made only by frank cases, subclinical infections, and carriers and even by these contributors the emissions may be sporadic and always in smaller numbers than the coliform and fecal streptococcal organisms.

2 Enrichment

In order to provide for an optimal opportunity of isolation it is necessary, in most cases, to incorporate an enrichment stage within the procedural sequence. This is generally done by the use of broths which are intended to provide optimal growth conditions for the enteric pathogens and, to some extent, suppress the coliform and non-pathogenic organisms.

3 Selection

Selectivity procedures separates the pathogens from the surviving non-pathogens, usually upon selective agar

plates, and from their visual growth characteristics can provide a culture which is a presumptive pathogen to be further scrutinized by biochemical procedures.

4 Biochemical

Cultures isolated during the selective procedures are further subjected to biochemical tests to provide cultures which can be identified as specific pathogens. Many non-pathogenic enteric organisms can resemble enteric pathogens upon selective plates and thus it is usually necessary to provide both selection and biochemical testing in order to significantly reduce the numbers of organisms which are carried to the identification procedures which are the serological procedures.

5 Identification

- a This is a serological procedure which identifies the more than 1,000 serotypes of Salmonella and the more than 30 serotypes of Shigella. Serological identification depends upon the antigenic pattern exhibited by the somatic and flagellar (if motile) components of the cell. Identification procedures can also include bacteriophage typing technique.

B Procedural Sequence - Methodology

1 Concentration

a Moore Swab Technique

This technique involves the immersion of gauze pads for varying periods whereby passage of water in the sample area acts to concentrate particulate matter and microbial flora. In most cases this technique is used to obtain indications of pathogens without regard to a quantitative estimate of numbers of species present in a given volume. Recently, however, a methodology has been developed wherein a counter

registers flow-through of environmental waters and thereby gives quantitative estimation. This technique has found increased use in estuarine and tidal waters where organism concentration can occur over several tidal cycles.

b Diatomaceous Earth Technique

This is a procedure whereby the high filtration capability of diatomaceous earth is used to concentrate a relatively large volume of microbial flora. This method has the advantage of allowing a filtration of a known volume of water and the quantitation of species isolated to volume filtered can be documented.

c Membrane Filtration

Membrane filtration is a technique of limited use since large volumes of sample may mechanically clog pore surfaces and prevent filtration. This method is useful for waters of very low organic and particulate matter content. Again a value can be found for species isolated to volume filtered if the membrane is totally immersed in the enrichment broth.

2 Enrichment

a Selenite Broth

This is an enrichment broth which has the advantage of inhibiting the nonpathogenic colon bacilli for the first 8-12 hours of incubation while allowing the salmonellae, particularly S. typhi, to multiply fairly rapidly.

b Tetrathionate Broth

This is an enrichment broth which, again, restricts the coliform organisms and allows the salmonellae to flourish.

While the two broths listed above are the most generally used enrichment broths, it must be mentioned that a gram negative broth (GN Broth) can be utilized as both a carrying medium as well as an enrichment broth combination. Some investigators have noted good results from the use of a non-inhibitory broth, such as lauryl tryptose broth, followed by polyvalent "O" antiserum addition after incubation, centrifugation of the broth, and streaking the sediment on the primary plating media. A multitude of modifications have been made to the above enrichment broths such as the addition of various antibiotics, bile, brilliant green, various amino acids, etc., and use of these additives should be made only after investigation of the literature to ascertain the reasons and advantages.

c Traditionally, the temperature of incubation for the enteric pathogens has been 37°C - that temperature which has been considered optimal for growth, and therefore, recovery. Within recent years, however, the utilization of higher temperatures for enrichment and selection have given indications of increased recoveries due to the suppression of the nonpathogens and a decrease in the lag phase of the pathogens upon inoculation.

3 Selection

a Brilliant Green Agar

Typical salmonellae on Brilliant Green agar form a pinkish-white colony with a red background when isolation has occurred. Nonpathogenic and lactose fermenters usually form greenish colonial forms or may, at times, produce other colorations. This medium should be incubated a full 48 hours, after a 24 hour observation and picking of typical colonial forms, to allow late or partially inhibited forms to attain macroscopic size. If no typical forms are observed on the plate is

crowded it would be well to pick a few colonies from the plate and process them through the biochemical schema since it is possible that typical colorations can be masked by other more numerous bordering colonies.

b SS Agar

On this medium typical colonies of Salmonella and Shigella are colorless to opaque and, in the case of H₂S producers, may show a blackening within the "eye" of the colony. Typical lactose fermenters show various colorations which can vary from off-pinks to deep reds. Other non-pathogenic colonial forms may show yellow hues. This selective plate is, in general, more productive in numbers of non-pathogens compared to Brilliant Green agar and Bismuth Sulfite agar but is a good plate to run in conjunction with the Brilliant Green agar plate since it allows S. typhi and shigellae to grow which may not be the case with Brilliant Green agar which is definitely toxic to S. typhi.

c Bismuth Sulfite Agar

This medium is reputed to be the best available medium for the isolation of S. typhi. Luxuriant growth of the salmonellae can be expected upon this medium and typical forms usually have a blackening and/or metallic sheen of which the blackening may extend beyond the colony to give a "halo" effect. A few of the salmonellae may produce green colorations and thus it would be wise to pick some of these representatives from each plate in addition to the typical forms as mentioned above.

d EMB Agar

This medium is almost completely uninhibitory to the pathogenic forms as well as to the non-pathogens and thus it can be seen that, in the plate streaking process, less inoculum

should be applied to the plates or more loop flaming intervals practiced to obtain desired isolation. Usually a 24 hour incubation limit is the best that can be expected due to over-growth with continued incubation. Upon this medium, the Proteus organisms are likely to produce their characteristic "swarming" phenomenon and thus further reduce the isolation capability. Typical pathogens are colorless and can produce a "dark eye" usually blue or purple. Typical non-pathogens and lactose fermenters can produce varicolored forms and some of the coliforms can produce a metallic sheen. Many investigators recommend the utilization of this plate, despite its apparent disadvantages, due to the fact that it can allow some of the more fastidious species of the Salmonella and Shigella to multiply.

In addition to the above more commonly used media, it has been the practice of some investigators to utilize the following media for primary isolation:

MacConkey Agar
ENDO Agar
Desoxycholate Citrate Agar
BCP-D Agar (Brom Cresol
Purple Desoxycholate Agar)

Again, as in the case of the enrichment broths, various additives such as antibiotics, dyes, etc., have been added to the standard formulation of the various media and a literature review must again be necessary to ascertain the advantages and situations where applied before utilizing these altered formulations.

4 Biochemical Tests

At this point the pure cultures obtained from the selective plates may number from several to hundreds depending upon the number of samples being processed and the numbers of characteristic colonial forms exhibited by the primary isolation media. If the numbers

of cultures are relatively small and the need for prompt confirmations are necessary the whole battery of biochemical tests can be applied at once and those showing characteristic patterns for the Salmonella-Shigella groups can be immediately subjected to the serological tests and, if necessary, prompt mailing of cultures to the Diagnostic Centers can immediately follow. Usually the numbers of cultures obtained from the primary isolation media will be numerous and the need of rapid results is not the case and for this situation it is recommended that a sequential pattern of biochemical testing be followed which will result in a great saving of media and valuable bench time for the laboratory personnel. If the latter case is the prevailing situation the following pattern of biochemical tests are recommended:

a 1st Biochemical

Urea Agar or Urea Broth

Urea positive cultures should be immediately discarded as indicative of the Proteus group or other non-pathogenic forms and the urea negative cultures subjected to the 2nd biochemical series. Although the Urea broth or agar should be incubated for the recommended time periods it will be generally found that the 24 hour period will be sufficient to indicate the majority of the positive cultures and, at this point, the cultures which are negative should be further processed, but should be further incubated as the second series is being processed.

b 2nd Biochemical

<u>Media</u>	<u>Purpose of Test</u>
Decarboxylase Media	Presence or absence of enzyme system
Citrate	Utilization of Citrate as carbon source
TSI	Fermentation pattern; H ₂ S production

<u>Media</u>	<u>Purpose of Test</u>
Lactose Broth	Fermentation Capability
Saccharose Broth	Fermentation Capability
Salicin Broth	Fermentation Capability
KCN Broth	Growth Capability in presence of CN ⁻ group
SIM	Production of Indol, Motility, H ₂ S Production
Raffinose Broth	Fermentation Capability

A adherence to the biochemical patterns established for the Salmonella - Shigella groups will decide if the cultures are to be further processed to the 3rd biochemical series. The point should be mentioned that at times aberrant cultures will be encountered and as such they will not satisfy all of the classical reactions attributed to occur to each of the pathogenic groups. In all cases, therefore, it will be necessary to review all of the reactions as a whole and not to discard cultures on the basis of a small number of apparent discontinuities.

c 3rd Biochemical

Dextrose Broth, Mannitol Broth, Maltose Broth, Dulcitol Broth, Xylose Broth, Rhamnose Broth, Inositol Broth

This series of biochemical tests is to establish further patterns of fermentation capabilities of the isolates. This series of tests is indicated only to reduce the possible number of positive cultures that are sent for confirmation. For example, if one isolates 15 positive cultures of Salmonella group C₁ and in completing the 3rd Biochemical series it is found that, on the basis of comparing fermentative reactions, the cultures can be roughly separated into 3 differing patterns, it is more likely than not that in sending these three "differing" cultures for confirmation

they will confirm as three different species within the C₁ grouping. If the testing laboratory is equipped for flagellar analysis the elimination of the 3rd biochemical series can be eliminated as complete confirmation procedures can be completed for all of the isolates.

5 Identification

a Somatic Antigen Identification

In the serological examination of presumptive Salmonella cultures the somatic or "O" antigens are identified first and this is accomplished by using a dense suspension of a fresh culture in physiological saline and performing a slide agglutination test first with the polyvalent "O" antiserum and then with each representative of the groups. Interferences may be present due to the presence of the Vi antigen which is found only in a limited number of salmonellae (including S. typhi) and in a number of the non-pathogenic organisms. If a positive is indicated with the Vi antiserum, it can be eliminated from the salmonellae representatives by a boiling process and the somatic grouping procedure resumed for group identification. Serological identification of the shigellae follows the same pattern as that of the salmonellae somatic analysis and it is not necessary to do a flagellar grouping since all species of the Shigella are non-motile.

b Flagellar Antigen Identification

Although the slide test is used for this analysis by some laboratories, the tube test is recommended to eliminate cross-reactivity. Fresh motile cultures (there is a small number of non-motile salmonellae) are subjected

to the tube test with specific antigens which occur within the positive "O" groupings previously identified and thus a representative within these groups can be identified as to a specific species. Since the salmonellae usually contain two distinct phases of flagellar antigens it may be necessary to isolate the other phase than that identified in order to obtain complete identification. This is accomplished by the use of semisolid medium wherein one phase is held back by the appropriate antiserum while the yet unidentified phase is allowed to "ray out" from the line of inoculation to be isolated and identified.

III FUTURE DEVELOPMENTS

Increased emphasis upon the recoveries of pathogenic microorganisms from environmental waters will provide an impetus in the areas of improved utilization of current techniques and the development of more rapid methodologies. Some of the areas in which these developments are expected to occur are:

- A Increased understanding of the physiology of these pathogens and from this such developments as the recent indications that elevated temperature techniques (Spino, 1966) can improve recoveries.
- B Continued development of serological procedures and the possible use of such techniques as the fluorescent antibody methodology to provide a rapid appraisal of environmental waters.
- C Increased capability of typing by the use of bacteriophages for species other than S. typhi.

GROUP DIFFERENTIATION OF ENTEROBACTERIACEAE BY BIOCHEMICAL TESTS

1st BIOCHEMICAL

POSITIVES OBTAINED ONLY WITH
PROTEUS-PROVIDENCE GROUP

UREA AGAR

2nd BIOCHEMICAL

Shigella-Escherichia		Salmonella-Arizona-Citrobacter			
Shigella Group	Escherichia Group	Salmonella Group	Arizona Group	Citrobacter Group ⁽¹⁾	
-	d	+	+	-	LYSINE DECARBOXYLASE
- or (+)	d	(+)	(+)	(+)	ARGININE DIHYDROLASE
-*	d	+	+	d	ORNITHINE DECARBOXYLASE
d	+	-	-	-	INDOL
-	d	+	+	+	MOTILITY
-	-	+	+	+	H ₂ S (TSI AGAR)
-	-	-	-	+	KCN
-	-	+	+	+	SIMMONS' CITRATE
-*	+ or x	-	+ or x	+ or x	LACTOSE
-*	d	-	-	d	SUCROSE
-	d	-	-	d	SALICIN
d	d	-	-	d	RAFFINOSE

OTHER INFORMATION

	Shigella-Escherichia		Salmonella-Arizona-Citrobacter		
	Shigella Group	Escherichia Group	Salmonella Group	Arizona Group	Citrobacter Group ⁽¹⁾
GELATIN (22°C.)	-	-	-	(+)	-
Organic Acids					
D-tartrate					
L-tartrate					
I-tartrate					
Citrate					
Mucate					

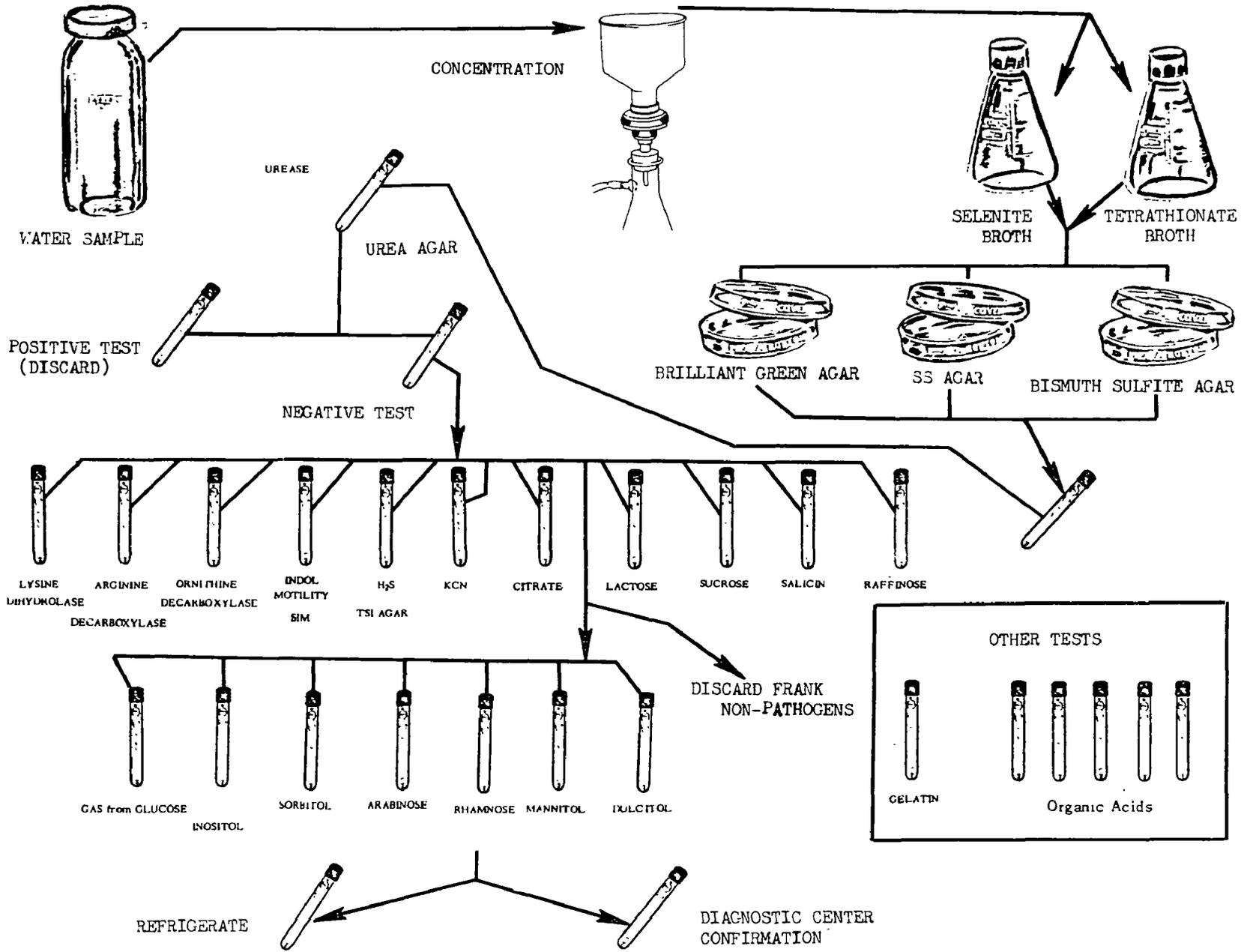
3rd BIOCHEMICAL

Shigella-Escherichia		Salmonella-Arizona-Citrobacter			
Shigella Group	Escherichia Group	Salmonella Group	Arizona Group	Citrobacter Group ⁽¹⁾	
-*	+	+	+	+	GAS from GLUCOSE
-	-	d	-	- or x	INOSITOL
d	d	+	+	+	SORBITOL
d	+	+(2)	+	+	ARABINOSE
d	d	+	+	+	RHAMNOSE
d	+	+	+	+	MANNITOL
d	d	+(2)	-	d	DULCITOL

d DIFFERING BIOCHEMICAL TYPES
 x LATE OR IRREGULARLY POSITIVE
 (+) DELAYED POSITIVE
 + POSITIVE - NEGATIVE

(1) FORMERLY ESCHERICHIA FREUNDII
 (2) SOME SPECIES DO NOT FERMENT DULCITOL PROMPTLY
 S.CHOLERAE SUIS DOES NOT FERMENT ARABINOSE

* CERTAIN BIOTYPES PRODUCE GAS
 S. SONNEI FERMENTS LACTOSE & SUCROSE & DECARBOXYLATES ORNITHINE



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TESTING THE SUITABILITY OF DISTILLED WATER
FOR THE BACTERIOLOGY LABORATORY

I INTRODUCTION

A Standard Methods for the Examination of Water and Wastewater (12th Edition) states;

"Only distilled water or demineralized water which has been tested and found free from traces of dissolved metals and bactericidal and inhibitory compounds may be used for the preparation of culture media and reagents. Bactericidal compounds may be measured by a biologic test procedure" This outline describes a suitable procedure.

B A need for such a test has been shown in the lack of reproducibility of plate counts and a possible cause of inconsistent results in split sample examinations.

II THEORY OF THE TEST PROCEDURE

A Growth of Aerobacter aerogenes in a chemically defined minimal growth medium. The addition of a toxic agent or a growth promoting substance will alter the 24 hr population by an increase or decrease of 20% or more, when compared to a control.

III APPARATUS AND MATERIALS

A Glassware - rinse all glassware in freshly redistilled water from a glass still. The sensitivity of the test depends upon the cleanliness of the sample containers, flasks, tubes, and pipettes. Use only borosilicate glassware.

B Culture - any strain of coliform IMViC type --++ (A. aerogenes). This can be easily obtained from any polluted river or sewage sample.

IV REAGENTS

A Use reagents of the highest purity. Some brands of potassium dihydrogen phosphate (KH_2PO_4) have large amounts of impurities. The sensitivity of the test is controlled in part by the purity of the reagents employed.

1 Carbon source - Sodium citrate, reagent, crystals ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) 0.29 g dissolved in 500 ml of redistilled water.

2 Nitrogen source - Dissolve 0.60 g of ammonium sulfate, reagent, crystals, (NH_4)₂SO₄) in 500 ml of redistilled water.

3 Salt mixture solution - Dissolve the following compounds in 500 ml of redistilled water.

Magnesium sulfate, reagent, crystals ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) 0.26 g.

Calcium chloride, reagent, crystals ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) 0.17 g.

Ferrous sulfate, reagent, crystals ($\text{FeSO}_4 \cdot \text{H}_2\text{O}$) 0.23 g.

Sodium chloride, reagent, crystals (NaCl) 2.50 g.

4 Phosphate buffer solution - Use a 1 to 25 dilution of a stock phosphate solution prepared by dissolving 34.0 gm of potassium dihydrogen phosphate (KH_2PO_4) in 500 ml of distilled water, adjusting to pH 7.2 with 1 N NaOH and diluting to 1 liter with distilled water.

5 Toxic control - dissolve 0.40 grams $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 100 ml of redistilled water. Dilute 1:1000 for 1 mg per liter Cu before use.

Testing the Suitability of Distilled Water

B Sterilization of Reagents

Unknown distilled water sample - either boil for one minute or sterilize by membrane filtration.

Prepare reagents with redistilled water heated to boiling for 1 to 2 minutes. Phosphate buffer solution may be sterilized by MF filtration or boiling.

C Solutions are useful up to two weeks when stored at 5°C in sterilized glass stoppered bottles. The salts solution must be stored in the dark because sunlight results in copious ferric ion precipitation. A slight turbidity arising in the first 3 - 5 days does not detract from the usefulness of the reagents.

V PROCEDURE

A Collect 150 - 200 ml of water sample in a sterile borosilicate glass flask and sterilize. Label 3 flasks or tubes: A, B, and F. Add water Samples and redistilled water to each flask as indicated at the bottom of the page.

B Add a suspension of *Aerobacter aerogenes* (IMViC type --++) of such density that each flask will contain 25 - 75 cells per ml. Make an initial bacterial count by plating a 1 ml sample in plate count agar. Incubate tests A-F at 32° or 35°C for 20 - 24 hr. Make plate counts using dilutions of 1, 0.1, 0.01, 0.001 and 0.0001 ml.

VI PREPARATION OF A BACTERIAL SUSPENSION

A Bacterial Growth

On the day prior to performing the distilled water suitability test, inoculate a strain of *Aerobacter aerogenes* onto a nutrient agar slant with a slope of approximately 2 - 1/2 inches in length contained in a 125 mm X 16 mm screw cap tube. Streak the entire agar surface to develop a continuous growth film and incubate 18 - 24 hrs at 35°C.

B Harvesting Viable Cells

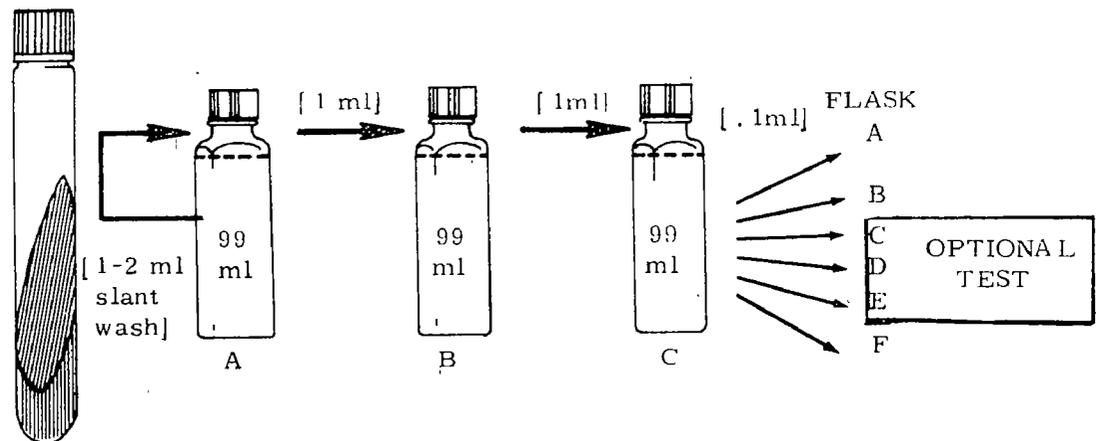
Pipette 1 - 2 ml of sterile dilution water from a 99 ml water blank onto the 18 - 24 hr culture. Emulsify the growth on the

STANDARD TEST

	Control A	Unknown Dist. Water B	Toxic Control F
Media Reagents			
Citrate	2.5	2.5	2.5
Ammonium sulfate	2.5	2.5	2.5
Salt mixture	2.5	2.5	2.5
Phosphate buffer (7.3 + .1)	1.5	1.5	1.5
Water, 1 mg per liter Cu	X	X	21.0
Unknown water	X	21.0	X
Redistilled water	21.0	X	X
TOTAL VOLUME	30.0	30.0	30.0

OPTIONAL TEST

	Food Available C	Nitrogen Source D	Carbon Source E
OPTIONAL TEST			
X	2.5	X	X
X	X	2.5	2.5
2.5	2.5	2.5	2.5
1.5	1.5	1.5	1.5
X	X	X	X
21.0	21.0	21.0	21.0
5.0	2.5	2.5	2.5
OPTIONAL TEST	30.0	30.0	30.0
OPTIONAL TEST			



slant by gently rubbing the bacterial film with the pipette, being careful not to tear the agar, and pour the contents back into the original 99 ml water blank.

C Dilution of Bacterial Suspension

Make a 1 - 100 dilution of the original bottle into a second water blank, and a further 1 - 100 dilution of the second bottle into a third water blank, shaking vigorously after each transfer. Then pipette 0.1 ml of the third dilution (1:1, 000, 000) into each of the flasks A, B, and F (see Standard Methods for Examination of Dairy Products, 12th ed.). This procedure should result in a final dilution of the organisms to a range of 25-75 viable cells for each ml of test solution.

D Verification of Bacterial Density

Variations among strains of the same organism, different organisms, media, and surface area of agar slopes will possibly necessitate adjustment of the dilution procedure to arrive at a specific density range between 25 - 75 viable cells. To establish the growth range numerically for a specific organism and medium, make a series of plate counts from the third dilution to determine the bacterial density. Then choose the proper volume from this third dilution which when diluted by the 30 ml in the flasks A, B, and F will

contain 25 - 75 viable cells per ml. If the procedures are standardized as to surface area of the slant and laboratory technique, it is possible to reproduce results on repeated experiments with the same strain of microorganisms.

E Procedural Difficulties:

- 1 Chlorine or chloramine distilling over into receiver. Distilled water should be checked by a suitable quantitative procedure like the starch-iodide titration. If chlorine is found, sufficient sodium thio-sulfate or sodium sulfite must be added.
- 2 Unknown water sample stored in soft glass containers or glass containers without liners for metal caps.
- 3 Contamination of reagents of distilled water with a bacterial background.
- 4 Incorrect dilution of A. aerogenes to get 25 - 75 cells per ml.
- 5 Gross contamination of the sample determined by the initial colony count before incubation.

F Calculation:

- 1 For growth inhibiting substances:

$$\frac{\text{colony count per ml Flask B}}{\text{colony count per ml Flask A}}$$

- a Ratio 0.8 to 1.2 (inclusive) shows no toxic substances.

b Ratio less than 0.8 shows growth inhibiting substances in water sample.

2 For toxic control

$$\frac{\text{colony count per ml Flask F}}{\text{colony count per ml Flask A}} = \text{Ratio}$$

OPTIONAL TEST

3 *For nitrogen and carbon sources that promote growth**

$$\frac{\text{colony count per ml Flask C}}{\text{colony count per ml Flask A}} = \text{Ratio}$$

4 *For nitrogen sources that promote growth**

$$\frac{\text{colony count per ml Flask D}}{\text{colony count per ml Flask A}} = \text{Ratio}$$

5 *For carbon sources that promote bacterial growth**

$$\frac{\text{colony count per ml Flask E}}{\text{colony count per ml Flask A}} = \text{Ratio}$$

2 When the ratio exceeds 1.2, it may be assumed that growth stimulating substances are present. However, this procedure is an extremely sensitive test and ratios up to 3.0 would have little significance in actual practice. Therefore, Test C, D, and E do not appear necessary except in special circumstances, when the ratio is between 1.2 and 3.0.

3 Usually Flask C will be very low and flasks D and E will have a ratio of less than 1.2 when the ratio of Flask B/Flask A is between 0.8 and 1.2. The limiting factors of growth in Flask A are the nitrogen and organic carbon present. An extremely large amount of ammonia nitrogen with no organic carbon could increase the ratio in Flask D above 1.2 or the absence of nitrogen with high carbon concentration could give ratios above 1.2 in Flask E with an A/B ratio between 0.8 and 1.2.

4 A ratio below 0.8 indicates the water contains toxic substances and this ratio includes all allowable tolerances. As indicated in item 2 (above), the 1.2 ratio could go as high as 3.0 without any undesirable results.

C Interpretation of Results:

1 The colony count from Flask A after 20 - 24 hours, at 35°C will depend on the number of organisms initially planted in Flask A and on the strain of A. aerogenes used in the test procedures. This is the reason the control Flask A must be run for each individual series of tests. However, for a given strain of A. aerogenes under identical environmental conditions, the terminal count should be reasonably constant when the initial plant is the same.

5 We are unable to recommend corrective measures in specific cases of defective distillation apparatus. However, a careful inspection of the distillation equipment and a review of production and handling of the distilled water should enable the local laboratory personnel to correct the cause of the difficulty.

Thus, it is essential that the initial colony count on Flask A and Flask B should be approximately equal to secure accurate data.

*Do not attempt to calculate ratios, 3, 4, or 5 when ratio 1 indicates a toxic reaction.
 **Ratio in excess of 1.2 indicates available source for bacterial growth.

CASE EXAMPLES

Test results for various distilled water samples

<u>SOURCE</u>	<u>TEST COUNT</u>	<u>CONTROL COUNT</u>	<u>RATIO</u>	<u>INTERPRETATION</u>
1	< 100	120,000	-----	Toxic Substance
2	74,000	170,000	0.4	Toxic Substance
3	18,000	14,000	1.3	Excellent water
4	21,000	14,000	1.5	Excellent water
5	310,000	60,000	5.2	Growth Substance
6	850,000	37,000	22.9	Growth Substance

REFERENCES

1 Standard Methods for Examination of Water and Wastewater. 12th Edition. 1965. p 578.

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IDENTIFICATION OF THE FECAL STREPTOCOCCI

I DEFINITION

The term fecal streptococci means any strain of streptococci commonly found in significant numbers in the feces of humans and other warm-blooded animals.

II THE GENERAL PROCEDURE FOR ISOLATION OF STRAINS IS:

- A Select a natural source where streptococci exist.
- B Inoculate a portion of natural sample into a selective streptococci growth medium. After incubation, a nearly pure mixture of streptococcal strains usually results because the selective medium is designed to inhibit the growth of other bacteria.
- C Pick a single cell to obtain a pure strain. The most common way is cultivating a single cell on solid medium, followed by picking the resulting colony.

III HISTORY

Selective media have been most successful with the fecal streptococci. Sodium azide, potassium tellurite, or thallium acetate function to inhibit nonstreptococcal growth. Sodium azide is the most common selective agent in current use.

- A As early as 1940 a broth containing .02 percent sodium azide was suggested for estimating sewage streptococci. Three years later, a medium was proposed which the authors considered almost complete evidence for Streptococcus fecalis when growth and acid occurred at 45.5°C. The medium contained 0.05 percent sodium azide and brom cresol purple which was to indicate pH change. In 1951, a modification appeared, described as a buffered glycerol glucose medium. This medium has been found lacking in productivity in comparative studies carried out at this Center.

IV SELECTIVE AGENTS AND CURRENT METHODS

- A The presumptive portion of the multiple tube test contains 0.02 percent sodium azide. The confirmatory portion contains 0.04 percent sodium azide and ethyl violet dye. Since the confirmatory ethyl violet-azide broth is very inhibitory, the polluted sample is inoculated first into the presumptive medium and then, after growth, transferred (triple 3mm loop) into the confirmatory medium. Growth there may be checked for the presence of Gram-positive cocci in pairs and chains, by the Gram-stain method. Either broth may be streaked on brain-heart infusion agar or other streptococcal media.
- B The membrane filter method utilizes a general growth medium, with yeast extract and 0.04 percent sodium azide. The 12th edition of Standard Methods indicates that confirmation of colonies appearing may be required. In some problem waters, selectivity is not complete. Verified identification as listed in Standard Methods requires four steps.

- 1 Isolation of a pure strain by picking inoculum from a single colony into broth and incubation at 35°C.
- 2 Testing an actively growing transfer culture for catalase enzyme activity.
- 3 Testing for growth in 40 percent bile broth.
- 4 Testing for growth at 45°C in broth.

Confirmed fecal streptococci should have a negative catalase reaction and should grow both in 40 percent bile and at 45°C.

m-Enterococcus agar is currently the Standard Methods 1965 medium but still has limitations where animal pollution is evident. First, bathing pool waters may

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the Federal Water Pollution Control Administration and the U. S. Department of the Interior

contribute micrococci which are distinguished by the presence of catalase enzyme. Second, work by Rose and Litsky (1965) shows 0.04 percent sodium azide inhibits some fecal streptococci. They report preliminary enrichment increases subsequent counts two-fold.

C KF liquid medium and agar have had some use. KF is a very rich growth medium containing 0.04 percent sodium azide. Also, the agar may be used for membrane filter determinations similarly to m-Enterococcus agar.

D Either the membrane filter technique or the agar pour plate method offers the most convenient method for strain isolation. If samples are turbid or contain debris, the pour plate is superior. Besides, clogging the membrane filter, debris causes colonies to grow together. A pour plate of either KF agar or m-Enterococcus agar aids discrete colony formation. Total colonies appearing are the same, whether on membrane filter or in pour plates. After colonies are picked into broth and purification is certain, further biochemical characteristics can be studied.

V ISOLATED STRAINS ARE IDENTIFIED BY MORPHOLOGICAL AND BIOCHEMICAL TESTS

A The fecal streptococci are cocci occurring commonly in pairs or short chains. They are nonmotile, staining purple (positive) with the Gram stain. They average about 0.5 to 1 micron in diameter and may exhibit pleomorphism.

B Streptococcal colonies typically grow slowly and colonies are small, (0.5-2mm). For these reasons, 40 - 48 hour incubation times are used. Colored pigments are not commonly produced.

C In a natural environment the biochemical activities vary greatly. For classification, certain activities are selected and measured. Careful techniques and selectivity of biochemical procedures must be exercised in the identification of various fecal streptococci.

D Useful differential tests are:

1 The 45°C Growth Test - Incubate streptococci in brain heart infusion

broth at 45°C for two days and examine for growth.

2 The 10°C Growth Test - Identical to the 45°C test except for temperature, and five to seven day incubation.

3 Tolerance Test in 40 Percent Bile- Add 40 ml of sterile 10 percent Oxgall solution to 60 ml of sterile brain heart infusion broth. The 10 percent Oxgall solution is equivalent in concentration to fresh bile originally used in this test. Positive cultures show growth within one to three days.

4 Sodium Chloride (6.5 percent) Tests- Inoculate brain heart infusion broth containing 6.5 percent sodium chloride and observe for growth of streptococci within three to seven day period.

5 Growth at pH 9.6 - Add sterile 38 percent sodium phosphate solution ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$) to sterile brain heart infusion broth to give a pH 9.6 reaction. This usually requires approximately 5 ml of phosphate solution per 100 ml of medium. Observe for growth of streptococci at one, two, three and 7 days.

6 Hydrolysis of Starch - Add sufficient 10% soluble starch to brain heart infusion agar to give a 1% starch content. Pour into petri dishes, allow to harden, and inoculate unknown strains by streaking. After two days, flood surface with gram's iodine. Note clear zones indicating starch hydrolysis.

7 Potassium Tellurite Reduction - Add sufficient sterile warmed (50°C) 4% K_2TeO_3 (1ml/100 med) to brain heart infusion agar to have a final concentration of 1:2500 potassium tellurite. Cool, pour and harden agar in a petri dish. Streak unknown cultures and observe 1-7 days. Black colonies indicate if reaction is positive.

8 Methylene Blue Test - Add sufficient 1 percent sterile solution of methylene blue to sterile skim milk to have a final concentration of 0.1 percent methylene blue. Observe reactions at one, two, three and seven days. (Note blue milk becoming white for positive reduction).

9 Litmus Milk Reaction - Sterilize the

litmus milk at 115.2°C (10 lb for 10 minutes). Read reactions of streptococci at one, two, three and seven days. (Observe pink of acid formation, white of reduction, coagulation, proteolysis).

10 The Azide Dextrose and Ethyl Violet Azide Growth Tests - Standard Methods 12th edition, page 620 describes preparation and performance of these tests.

11 The Catalase Test - Transfer growth from a brain heart infusion agar slant or agar plate to a glass slide. After dropping some 3% hydrogen peroxide on the cells, watch for bubbles. Catalase enzyme, if present, cleaves hydrogen peroxide to water and visible oxygen gas.

12 Serology

Serology depends upon the presence of specific chemical-complexes in capsules surrounding the bacteria, in cell wall or inside the bacteria. An individual bacterium has a unique chemical composition. It is more or less a carbon copy of all other members of the same strain which share identical chemical-complexes. But the less closely related a strain is, the fewer chemical-complexes are shared. Bacterial strains are grouped together by their common chemical-complexes. Specific chemical-complexes are called antigens. The science of their manipulation and interpretation is serology. Much theoretical work has been done on serology of the fecal streptococci. Apparently, Streptococcus fecalis, S. durans and some S. bovis, S. equinus share a common group antigen, called group D. Serology has not been useful in pollution studies.

VI By use of standard tests, the streptococci can be separated into groups. For sanitary interpretation, the groups should clearly differentiate between strains of fecal and nonfecal origin. Species groups are commonly used since species are correlated to their origin. Figure 1 lists the biochemical characteristics of the different groups. The enterococcus group includes Streptococcus fecalis, its varieties, and S. durans (faecium).

A Since a fecal streptococci is defined here

as one found in significant numbers in feces, the fecal environment determines which group predominates. Human feces contains chiefly the enterococcus group and occasionally Streptococcus salivarius may occur in a small number of individual human fecal samples. The S. equinus and S. bovis groups are predominant streptococci in cows, pigs and sheep, although some enterococcal group is present, Kenner, et al. (1967) (Figure 2) report S. bovis composes 48.4% of the total streptococcal flora of cows, 41.5% of sheep, and 8.8% of pigs. S. salivarius has been observed only two times in domestic animal feces. S. equinus occurs in appreciable numbers in feces of cows and pigs, although early investigators named it as the predominant streptococci in horse manure.

B While referring strains to species groups is useful in some studies, the procedure is involved and lengthy. For sanitary meaning, a simple test differentiating fecal from nonfecal strains would be useful. Geldreich et al. (1964) proposed a simple temperature-growth test. Presence or absence of growth at 10 or 45°C. divides the streptococci into four different groups indicative of different health hazards.

1 Growth at both 10 and 45°C

a Present evidence indicates the common fecal streptococci are generally distributed in the feces of warm-blooded animals. These organisms probably mean fecal pollution when present in water.

b The term common fecal streptococci includes the original enterococcus group, which has at present, a confused meaning. The common fecal streptococci grow in 6.5 percent NaCl and at pH 9.6. Specifically they would be classified as S. fecalis, S. fecalis v. liquefaciens, S. fecalis v. zymogenes and S. durans, depending on additional biochemical tests.

2 Growth at 45°C only. The high temperature fecal group includes the S. equinus strains, S. bovis strains, and most strains of S. salivarius. Their presence probably signified fecal pollution in water.

Figure 1. BIOCHEMICAL REACTIONS OF 4,633 CULTURES ISOLATED FROM HUMANS AND OTHER WARM BLOODED ANIMALS*¹

No. of strains	Growth at		Growth in		Reduction of		Final pH in 1% glucose	Hydrolysis of starch	Arginine hydrolysis	Litmus Milk				Fermentation 1% Sorbitol	Species
	10°C	45°C	6.5% NaCl	Broth pH 9.6	Methylene blue (0.1%)	K ₂ TeO ₃ 1:2500				Acid	Coag	Reduct	Pepton		
2,706	+	+	+	+	+	+	3.9-4.6	0	+	+	+	(+)	±	+	<i>S. fecalis</i> var.
43	+	+	+	+	+	0	4.0-4.5	0	+	+	+	+	0	0	<i>S. durans</i> (faecium)
*** 823	(+)	+	+	+	(0)	±	3.9	0	(+)	(+)	(+)	(+)	(0)	(+)	<i>S. fecalis</i> biotype I
	(+)	+	+	0	±	±	6.5	0	(+)	(+)	(+)	(+)	(0)	(+)	<i>S. fecalis</i> biotype II
	(+)	+	0	+	±	±		0	(+)	(+)	(+)	(+)	(0)	(+)	<i>S. fecalis</i> biotype III
	+	+	0	0	0	±	0	0	(+)	(+)	(+)	(+)	(+)	(+)	<i>S. fecalis</i> biotype IV
	698	0	+	0	0	0	0	4.0-4.5	+	0	+	+	(+)	0	not used
93	0	+	0	0	0	(0)	4.0-4.5	+	0	0	0	0	0	not used	<i>S. equinus</i>
78	0	(+)	0	0	0	+	4.0-4.5	0	±	+	+	+	0	not used	<i>S. salivarius</i>
192	(+)	+	(+)	(+)	(+)	+	4.0-4.5	+	+	+	+	(+)	±	not used	Atypical ** <i>S. fecalis</i> var.

*All strains were catalase-negative; all grew in 40% bile broth, except *S. salivarius*.

**Atypical strains usually isolated from fermenting vegetation, isolated only from cat feces (10 strains), and dog feces (182 strains).

(+) = usually positive, (0) = usually negative; ± = variable.

***Biotypes arbitrarily based on growth in 6.5% NaCl broth and pH 9.6 broth, allowing four types.

¹B. A. Kenner and P. C. Haley

Figure 2. DISTRIBUTION OF FECAL STREPTOCOCCAL GROUPS IN FECES OF HUMANS AND OTHER WARM-BLOODED ANIMALS SOURCE ON THE FECAL SAMPLES¹

		Human	Cow	Sheep	Pigs	Chickens	Turkeys	Ducks	Cats	Dogs	A Rodents	Totals
No. of fecal samples		22	12	10	10	10	9	8	10	24	22	137
Total no. of strains		1,065	438	371	360	368	349	318	268	557	606	4,700
S. fecalis var.	No.	940	97	142	194	223	249	130	215	160	356	2,706
	%	88.26%	22.15%	38.27%	53.89%	60.6%	71.35%	40.88%	80.22%	28.73%	58.75%	57.57%
S. durans (faecium)	No.	13	13	6	2	1	0	0	0	8	0	43
	%	1.22%	2.97%	1.62%	0.56%	0.27%	0%	0%	0%	1.44%	0%	0.91%
S. fecalis biotypes	No.	34	74	54	106	143	94	52	43	88	135	823
	%	3.19%	16.90%	14.56%	29.44%	38.86%	26.93%	16.35%	16.04%	15.8%	22.28%	17.51%
S. bovis	No.	0	212	154	32	0	5	126	0	94	75	698
	%	0%	48.4%	41.51%	8.89%	0%	1.43%	39.62%	0%	16.88%	12.38%	14.85%
S. equinus	No.	0	41	14	26	1	1	10	0	0	0	93
	%	0%	9.36%	3.77%	7.22%	0.27%	0.29%	3.14%	0%	0%	0%	1.98%
S. salivarius	No.	76	1	1	0	0	0	0	0	0	0	78
	%	7.14%	0.23%	0.27%	0%	0%	0%	0%	0%	0%	0%	1.66%
Atypical S. fecalis types*	No.	0	0	0	0	0	0	0	10	182	0	192
	%	0%	0%	0%	0%	0%	0%	0%	3.73%	32.68%	0%	4.09%
Streptococci X ¹	No.	0	0	0	0	0	0	0	0	25	40	65
	%	0%	0%	0%	0%	0%	0%	0%	0%	4.49%	6.6%	1.38%
S. anginosum	No.	2	0	0	0	0	0	0	0	0	0	2
	%	0.19%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0.04%

*See Figure 1

X - Unidentified strains

A - Rabbits (10 samples), Rat (7), Chipmunk (2), Guinea Pig (2), and Raccoon (1).

¹B. A. Kenner and P. C. Haley

Streptococcus bovis characteristically hydrolyses starch, as does S. equinus, when the 1% starch is incorporated into brain heart infusion agar which contains a small amount of dextrose. S. equinus does not ferment lactose. S. salivarius does not hydrolyse starch, but does ferment lactose.

- 3 Growth at 10°C only. The low temperature group includes the lactic acid streptococci found in soil and plants. They have never been found in animals or their feces. They neither indicate fecal pollution or cause disease themselves and do not grow in KF streptococcus medium.
- 4 Growth at neither 10 or 45°C. These are a group of no sanitary significance and they are rare in water, but do include the pyogenic or pathogens of the genera.

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INTRODUCTION TO STATISTICS

Part I

Summarization of Data

I INTRODUCTION

The body of statistical methods comprises two groups generally called descriptive statistics and statistical inference. Descriptive statistics encompasses primarily graphical techniques for the presentation of data while statistical inference is basically a mathematical approach to the problem of inferring from a part to the whole.

II BASIC GRAPHICAL TECHNIQUES

In this section we consider the non-mathematical techniques of summarizing a set of data in order that the meaningful information can be extracted from it.

Consider the data in Table 1 which represent aqueous fluoride ion concentrations determined by a colorimetric procedure.

TABLE 1

F ⁻ , mg/liter		
0.68	0.77	0.85
0.71	0.79	0.85
0.72	0.80	0.87
0.74	0.81	0.90
0.75	0.82	0.91
0.77	0.83	0.95

A Frequency Table

As the first step in summarizing a set of data we form a frequency table. (See Table 2). To construct the table the data are divided into a number of intervals of equal length called class intervals and the number of results falling into each interval is recorded in the "frequency" column.

The number of class intervals chosen is arbitrary. However, it is a rule of thumb to choose the length of the class interval so that 7 to 15 intervals will include all the data under consideration.

TABLE 2

Class Interval	Frequency
.65 au* .70	1
.70 au .75	3
.75 au .80	4
.80 au .85	4
.85 au .90	3
.90 au .95	2
.95 au 1.00	1

* and under

B Frequency Polygon

As a further step we can graph the frequencies recorded in the frequency table. One way of doing this is to plot the frequency along the ordinate (vertical axis) and the midpoint of the class interval along the abscissa (horizontal axis). The line connecting the plotted points in Figure 1 form a frequency polygon.

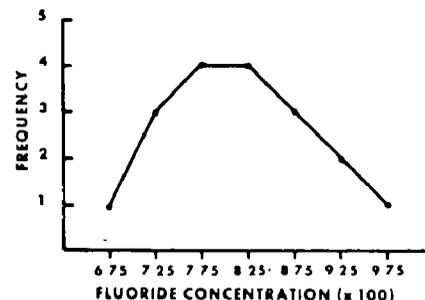


FIGURE 1

C Histogram

Another method of graphing the frequency information is to construct a histogram as shown in Figure 2. The histogram is a two dimensional graph in which the length of the class interval is taken into consideration. Each class interval becomes the base of a rectangular bar whose height is equal to the corresponding frequency.

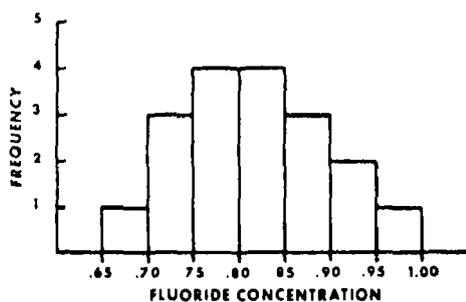


FIGURE 2

D Histogram of Relative Frequency

The histogram can be a very useful tool in statistics especially if we convert the given frequency scale to a relative scale such that the sum of all the ordinates equals one. Thus, each ordinate value is derived by dividing the original frequency by the number of observations in the sample. For the data in Table 1 we would divide each of the frequency values in Table 2 by 18.

The advantage of constructing a relative frequency histogram like the one in Figure 3 is that we can interpret areas under the histogram as probabilities provided we assume a scale on the abscissa such that each class interval is of unit length. Then the probability that a given value will fall in any one interval is the area under the histogram in that interval.

For example, the probability that a value will fall between .70 and .75 is equal to the area under the histogram in that interval which is $\frac{3}{18} \times 1 = \frac{1}{6}$.

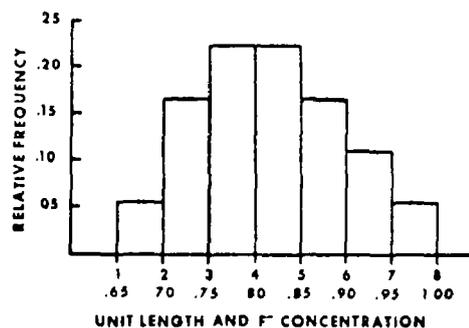


FIGURE 3

E Cumulative Frequency Distribution

From a frequency table (See Table 2) we can construct a cumulative frequency table as shown in Table 3. The cumulative frequency table gives the number of observations less than a given value. The cumulative frequency values in Table 3 are found by summing the frequencies in Table 2.

TABLE 3

F ⁻ Conc.	Cum. Frequency
under .70	1
" .75	4
" .80	8
" .85	12
" .90	15
" .95	17
" 1.00	18

If we convert the cumulative frequency values to relative frequencies as we did to construct the relative frequency histogram, we can plot the relative values to form a cumulative frequency curve as shown in Figure 4. The probability that a result will fall below a given value can be read from the cumulative frequency curve. For example, to find the probability that a result will be less than

0.85 mg/liter we read up to the curve at the point $x = .85$ and across to the value .67 on the probability axis.

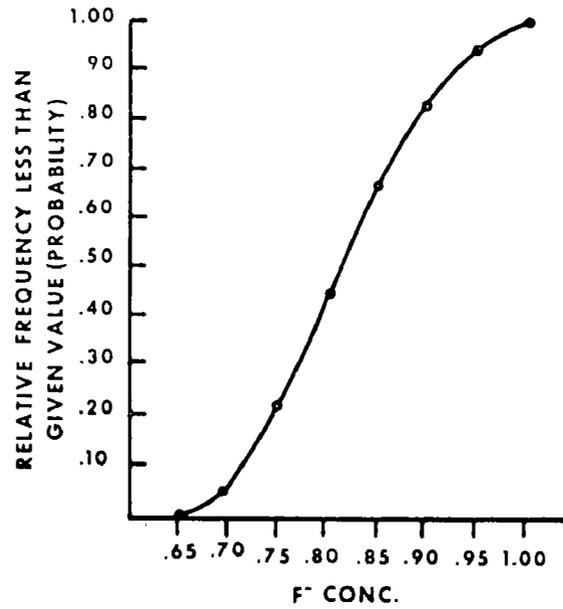


FIGURE 4

INTRODUCTION TO STATISTICS

Part II

Measures of Central Tendency and Dispersion

I INTRODUCTION

From a practical point of view, statistical inference is the application of mathematical tools to analyze and interpret the results of investigations in the physical and social sciences with respect to the hypotheses under investigation. The need for a statistical approach rests on the well-founded assumption that any such result contains an inherent component of error or random variation which introduces uncertainty into any conclusions which may be drawn from the observed results.

A Mathematical Distributions

It is further assumed that the error components can be described by a mathematical function called a probability distribution. Knowledge of the relevant type of theoretical distribution provides us with the means of assessing the uncertainty (risk of being incorrect) in conclusions drawn from observed results.

B Population Parameters

The concept of distribution pertains to the totality of possible results which would be observed in an infinite number of repetitions of an experiment or investigation. This theoretically infinite number of results is called a population. Any measurable characteristic of a population distribution is called a parameter. For each type of distribution there exists a characteristic set of parameters. Specific values for the parameters of a given distributional form define a particular distribution of that type. In almost every case the entire population can never be observed and therefore the values for the parameters are unknown. Population parameters are denoted by lower case Greek letters.

C Sample Statistics

The observed results from a particular investigation are called a sample of the relevant population. In almost every case the sample is a subset of the population. A statistic is a function of the sample points (i. e., observed results) which usually estimates a population parameter. Sample statistics are denoted by English letters.

D Classification of Statistical Measures

It is implied above that the type of population distribution determines the particular function of the observed results (i. e., sample statistic) which will provide the "best" estimate of the corresponding population parameter. For this reason it is advantageous to assume that any given sample came from a population with a specified form of distribution. The use of graphical techniques to indicate the form of distribution should now be apparent.

In most situations the distributions or frequency functions of interest have a single peak and characteristic dispersion of area about this peak. Parameters which locate the distribution (by the peak, center of gravity, midpoint, etc.) are called location parameters and the corresponding sample statistics are classified as measures of central tendency. Parameters which describe the dispersion of area are called scale parameters and the corresponding sample statistics are classified as measures of dispersion. Ideally, one would like to encounter only distributions for which one measure of central tendency and one measure of dispersion summarize all of the relevant information in the sample about the population.

II MEASURES OF CENTRAL TENDENCY

In this section and the next we define and lay out the computational format for the more commonly used measures in statistics. We let x_i denote a typical observed result so that $[x_1, x_2, \dots, x_n]$ represents a sample of n observations.

A The Mean

The most commonly used measure of central tendency is the mean or arithmetic average. We denote the sample mean by \bar{x} and the population mean, of which \bar{x} is an estimate, by μ . The computational formula is:

$$\bar{x} = \frac{\sum x_i}{n}$$

For the fluoride data in Table 1, we calculate the sample mean as follows:

$$\bar{x} = \frac{\sum x_i}{n} = \frac{14.52}{18} = 0.807$$

TABLE 1

F ⁻ , mg/liter		
0.68	0.77	0.85
0.71	0.79	0.85
0.72	0.80	0.87
0.74	0.81	0.90
0.75	0.82	0.91
0.77	0.83	0.95

B The Median

Another common measure of central tendency is the median. The median is the midpoint of an array of numbers (ordered according to value). To find the sample median we need only arrange the data in ascending or descending order and pick the middle value. When there is an even number of observations take the

average of the two middle values. For the data in Table 1 the median is 0.805.

C The Mode

The mode is another measure of central tendency, although it is of little practical importance. The mode is the most frequently occurring value. Therefore, the population mode is the value corresponding to the peak of the frequency distribution curve. Frequency distributions with more than one peak are called multimodal. In a symmetrical frequency curve the mean, median, and mode are all equal.

III MEASURES OF DISPERSION

A The Standard Deviation

As with measures of central tendency, there are several measures of dispersion, the most common of which is the standard deviation. We denote the sample by s and the population value of which s is an estimate by σ . The computational formula is:

$$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n - 1}}$$

However, computation using this formula is tedious and it is relatively simple to show the following relationship:

$$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n - 1}} = \sqrt{\frac{n \sum x_i^2 - (\sum x_i)^2}{n(n - 1)}}$$

The derived formula is preferable because of its adaptability to the desk calculator. We calculate the sample standard deviation of the fluoride data in Table 1, as follows:

$$\begin{aligned} s &= \sqrt{\frac{(18)(11.8048) - (14.52)^2}{18(17)}} \\ &= \sqrt{\frac{212.4864 - 210.8304}{306}} \\ &= \sqrt{\frac{1.656}{306}} = \sqrt{0.0054} = 0.073 \end{aligned}$$

B The Variance

The sample value s^2 is referred to as the sample variance and is merely the square of the standard deviation. Its formula is:

$$s^2 = \frac{\Sigma(x_i - \bar{x})^2}{n - 1}$$

The population variance is represented by σ^2 . Its formula is:

$$\sigma^2 = \frac{\Sigma(x_i - \mu)^2}{n}$$

This is the same as the formula for s^2 except we use the true population mean μ , rather than its estimate \bar{x} and divide by n instead $n - 1$.

Obviously in calculating the sample variance the true mean is not known and we, therefore, use the estimate of the mean from the data. In calculating the sample mean and then using it to calculate the variance of the same data we lose what is called a degree of freedom. It can be shown that the estimate of the variance must be based upon the sum of independent squared terms. We average our values over $n - 1$ because this is the number of independent squared terms that we will have when using a mean that has been estimated from the sample. In line with above, we should draw the distinction between the variance of the sample and the sample variance. The variance of the sample, we would calculate for its own sake and divide by n . However, we calculate the sample variance as an estimate of the population variance and divide by $n - 1$. There is no practical use of the variance of the sample, therefore, we always calculate the sample variance dividing by $n - 1$ which we shall call the number of degrees of freedom in our sample. As a rule, in any calculation, for every parameter that must be estimated, one degree of freedom (d.f) is lost.

C The Range

The range is also used as a measure of dispersion. It is the difference between the highest and lowest values in a set of data.

$$R = \max(x_i) - \min(x_i)$$

For the data in Table 1 the range is then:

$$R = 0.95 - 0.68 = 0.27$$

A rough estimate of s can be made by dividing the range of the sample by the square root of n , the number of observations, when n is small.

$$s \approx \frac{0.27}{\sqrt{18}} \approx \frac{0.27}{4} \approx 0.067$$

The use of the range is limited to instances where the labor of computing the standard deviation is impractical.

IV THE NORMAL DISTRIBUTION

A The most important theoretical distribution in statistics is the familiar bell-shaped normal distribution which is symmetric about its peak (See Figure 1) The following assumptions give rise to this distributional form:

- 1 Values above or below the mean are equally likely to occur.
- 2 Small deviations from the mean are extremely likely.
- 3 Large deviations from the mean are extremely unlikely.

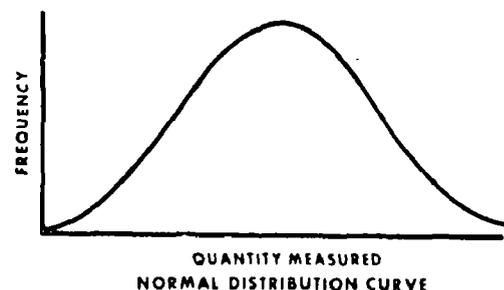


FIGURE 1

The normal distribution is completely defined by its mean, μ , and its standard deviation σ in the following manner:

- 1 The area under the normal curve between μ minus σ and μ plus σ is 68 percent of the total area, to the nearest 1 percent.
- 2 The area under the normal curve between μ minus 2σ and μ plus 2σ is 95 percent.
- 3 The area under the normal curve between μ minus 3σ and μ plus 3σ is 99.7 percent of the total area, to the nearest 0.1 percent.

If a frequency curve is a good approximation to the normal curve, these characteristics of the normal curve can be used to find information about the frequency distribution.

V. TRANSFORMATION OF DATA

A Skewed Distribution

In some areas of investigation one often encounters distributions which are not symmetric. For example, distributions of bacterial counts are often characterized by many more extremely high counts relative to the median than extremely low counts. The frequency curves of these distributions have a long right tail as shown in Figure 2. Distributions of this type display positive skewness.

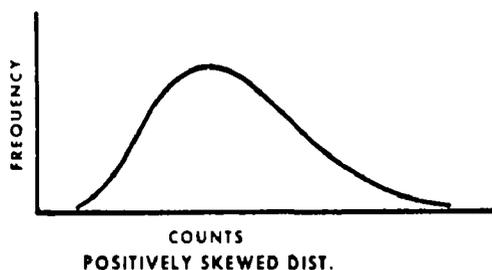


FIGURE 2

B Logarithmic Transformation

For many reasons, both practical and theoretical, we prefer to work with symmetric distributions like the normal curve. Therefore, it is desirable to transform skewed data in such a way that a symmetric distribution results, resembling the normal. One way of deriving an approximately normal distribution from a positively skewed distribution is by expressing the original data in terms of logarithms. An artificial sample of coliform counts and their logarithms are shown in Table 2. Comparison of the frequency tables for the original data and the logs (Table 3 and Table 4, respectively) clearly shows that the logarithms more closely approximate a symmetric distribution.

TABLE 2

Coliform MPN	MPN/100 ml log MPN
11	1.041
27	1.431
36	1.556
48	1.681
80	1.903
85	1.929
120	2.080
130	2.114
136	2.134
161	2.207
317	2.501
601	2.779
760	2.881
1020	3.009
3100	3.491

TABLE 3

Class Interval	Frequency (MPN)
0 au 400	11
400 au 800	2
800 au 1200	1
1200 au 1600	0
1600 au 2000	0
2000 au 2400	0
2400 au 2800	0
2800 au 3200	1

TABLE 4

Class Interval	Frequency (log MPN)
1.000 au 1.300	1
1.300 au 1.600	2
1.600 au 1.900	1
1.900 au 2.200	5
2.200 au 2.500	1
2.500 au 2.800	2
2.800 au 3.100	2
3.100 au 3.400	0
3.400 au 3.700	1

C Measures of Central Tendency of Skewed Distributions

If the logarithms of data from a positively skewed distribution are approximately normally distributed, we say that the original data have a log-normal distribution. The best estimate of central tendency of log-normal data is the geometric mean defined as:

$$\bar{x}_g = \sqrt[n]{(x_1)(x_2)\dots(x_n)}$$

Notice that

$$\log \bar{x}_g = \frac{\Sigma(\log x_i)}{n}$$

so that the geometric mean of the original data is equal to the antilog of the arithmetic mean of the logarithms. It is of interest that the population geometric mean is equal to the population median. Thus, in the case of data from a skewed distribution, the sample median is a better estimate of central tendency than the arithmetic mean of the original data. For the coliform data in Table 2 we calculate:

$$\log \bar{x}_g = \frac{\Sigma(\log x_i)}{n} = \frac{32.737}{15} = 2.1825$$

$$\bar{x}_g = \text{antilog}(2.1825) \approx 152,$$

$$\text{median} = 130,$$

$$\bar{x} = \frac{\Sigma x_i}{n} = \frac{6632}{15} = 442.$$

This outline was prepared by John H. Parker, Former Statistician, Analytical Reference Service, Training Program.

Descriptors: Frequency Analysis, Histograms, Statistical Methods, Statistical Models, Statistics, Variability

ECOLOGY AND SANITATION OF BIVALVES

Part 1

Some important facts on anatomy, physiology and ecology of the oyster, quahog, soft clam and sea mussel in relation to sanitation thereof.

I THE PROBLEM: ITS MAJOR PHASES

A The Water Pumping and Feeding Mechanism

- 1 The first three bivalves (pelecypods, lamellibranchs) are estuarine, not marine, living in shallow, brackish water, coastal areas subject to high turbidity and to pollution from adjoining lands. The mussel while chiefly marine does invade coastal waters.
- 2 The oyster and "little neck" quahogs are the only animals eaten while still alive by civilized races.
- 3 All materials used in building tissue, storage of glycogen, and shell formation are absorbed, or strained, from surrounding waters.
- 4 Pumpage of water through bivalves is effected through coordinated beating of myriads of vibrating hair-like cilia which cover all external body surfaces, while lining the digestive and reproductive tracts, and interior as well as exterior of the gills. Some cilia are under nervous control.
- 5 At frequent intervals among the cilia are unicellular mucus glands which respond directly to a wide variety of tactile and chemical stimuli.
- 6 Mucus discharged from these glands in response to particles striking them entangles the suspended matter, strained from the water by one group of gill cilia, into "food" strings which are transported by a second and a third group of gill cilia toward the mouth.
- 7 Secretion of mucus from the mucous glands is to a high degree controlled quantitatively by nature of the particles in suspension: sand grains, dirt particles, spiny diatoms, evoke copious discharge of mucus resulting in large masses which are mostly rejected before reaching the mouth. Bacteria, unicellular algae including nonspiny diatoms, protozoa and other microscopic animals evoke relatively less mucus hence form thinner food strings which are largely accepted and enter the stomach. Separation of sand, mud particles, and other nonfood materials from food is thus effected to a large degree, but it is not perfect; some sand and useless materials enter the stomach while considerable amounts of food are rejected.
- 8 The entangling of bacteria in mucus, and elimination through ciliary action (processes which occur on the walls of our own respiratory tract) make possible cleansing of grossly polluted sea mussels, Mytilus edulis, in 48 hours entirely through conjoint action of mucous glands and cilia. Even small traces of free chlorine interfere seriously with this biological process since mussels refuse to function. Turbidity speeds bacterial elimination through stimulating mucus secretion. (References: Dodgson, R.W.) Mussel not considered further in this lecture.

SUMMARY: Topics 1-8. Mucus secretion and ciliary activity make possible the life of bivalves in turbid coastal waters. (Incidentally also permit humans to exist in dusty or smoke-filled atmospheres). They are the very key to purification of polluted shellfish.

B Some Basic Anatomy of Bivalves

1 Gross Anatomy

We shall consider the following:

a Mantle. The mantle lines the shells or valves throughout, while the mantle border secretes the shell. In the oyster Crassostrea virginica the mantle halves are separated except around the anterior (front) end, and at one point near the posterior (hind) end where they unite with the gills. Water passes in along the entire ventral (under) side of the bivalve, leaving it along the dorsal (upper) side. Of chief interest to sanitarians: the mantle collects rejected materials concentrating them for expulsion. In the quahog and the clam the mantle halves are attached at two additional points to form two tubes, the siphons, which in the quahog, mercenaria mercenaria, (Venus) are approximately an inch long when extended. They are completely withdrawn when the bivalve closes its shells. In the soft clam, long necked clam, Mya arenaria, the siphons of a large animal may be extended a foot or more. They can not be withdrawn into the shell cavity but remain mostly outside, enclosed in a tough, leathery sheath of periostracum which likewise covers the shell in most bivalves. Water enters through the more ventral of the two siphons, the incurrent, and leaves through the more dorsal, excurrent or anal siphon. Mya cannot close its shells completely, the mantle borders being exposed.

The mantle border in the oyster reveals three reduplications: the outermost secretes the shell; the middle one bears many tentacles hence known as the tentacular border; the innermost, the pallial curtain, can be raised to block entrance or egress of water or to direct expulsion of water over a restricted area to blow away encroaching mud.

In Crassostrea are two excurrent chambers: the cloacal chamber behind the adductor muscle, and the promyal chamber in front of the muscle on the right side. Water leaves over two broad areas on the dorsal side.

b The Gills and Branchial or Gill Chambers. There are two complete gills or four half gills (demibranchs) in each of the three bivalves here considered. Each demibranch consists of two rows of gill filaments enclosing chimney-like excurrent compartments, the water tubes, between them. The gill filaments are comparable to pickets in a fence with openings of variable aperture, the ostia, through which water is driven into the water tubes of the interior. In the oyster a hormone, designated dianthlin, relaxes the tissue surrounding each ostium thus enlarging it to permit passage of eggs. The upper ends of the filaments are attached to body of bivalve, while at the lower ends filaments are attached to body of marginal food collecting furrow in which strong cilia transport mucus strings of food and dirt toward the mouth. Between the outermost demibranch and the mantle on each side is a basal food collecting furrow with three more such furrows between the bases of the inner demibranches, or five basal furrows and four marginal furrows in all.

The space between the mantle halves in which the gills lie is designated the infrabranchial, or incurrent, gill chamber. Water passes from here through the ostia of the gills into the water tubes. The water tubes in turn discharge into the epibranchial, or excurrent, gill chamber. In the two siphonate clams, mercenaria and Mya, incurrent siphon passes water to infrabranchial chamber whence it passes through ostia into water tubes, thence to epibranchial chamber and out through excurrent siphon.

c The palps, or lips, four in number, surround mouth and extend backward to embrace front ends of four demibranchs. Mucus strings carried to palps in nine food-collecting furrows of gills are accepted and passed on to mouth if small in volume, and if

of acceptable nature. Much rejection occurs here, material in oysters being passed over mantle edge at any ciliary current carries particles to this ejection point. In the two clams powerful ciliary tracts on the mantle carry all rejected material to a sinus or depression at base of the incurrent siphon whence it is ejected by quick contraction of adductor muscles which drives a strong stream of water out through the siphon. These rejected masses, commonly called pseudo faeces, will be designated as "rejecta" as contrasted with the "dejecta" or true faeces which have been through the digestive system. Accumulation of rejecta is important to sanitarians since a clam harvested just before "blowing off" may contain up to half a teaspoonfull of sediment hence in moderately polluted waters would be expected to contain a much higher bacterial load than one just recently blown. Mya should show greater differences than mercenaria owing to larger masses accumulated prior to "blowing off".

2 Microscopic anatomy

The gill filaments in the oyster are folded into folds or plicae each containing twelve or less filaments thus greatly increasing the number of filaments possible in a given length of gill axis. This folding of the walls of the gill greatly increases the pumping power of the oyster while aiding in separating food from dirt on the gill surface. In transverse section a plica shows each filament to be hollow containing a blood vessel. Four groups of cilia occur on each filament, to wit:

Latera cilia: the "water pumpers", adjacent to the ostia.

Latero-frontal cilia: the "strainers", interlock with those of adjoining filaments.

Frontal cilia: the "food pushers" move mucus strings across face of the filament and into food-collecting furrows.

Abfrontal cilia: "accessory pumpers" inside the gill aid in pushing excurrent water toward exist.

- 3 SUMMARY: a quick review of water passage through bivalves will be given.

C Ecology

- 1 Accumulation of rejecta; oyster may bury itself in highly turbid water. Mudding, lethal effects of H_2S .
- 2 Concentration of bacteria in mud on shells. Oyster shell difficult to clean; ridges, perforations of boring sponge, Cliona; retention by barnacles.
- 3 Effects of salinity changes: outside limits 5 to 30 parts per mille. Desirable range 12 to 28; adjustment. Tidal influences on feeding in the oyster. Day and night pumping in water with low buffering potential, in swamp water.
- 4 Temperature effects; oyster: reduction of pumpage below $10^{\circ}C$, cessation in most between $5 - 6^{\circ}$, but occasional oyster may be slightly active down to temperatures approaching 0° . Above 30° activation in northern oysters, 35° in Gulf oysters. Hibernation a relative, not absolute term, round.
- 5 Water pumpage by shellfish. Deficiencies in turbidity clearing methods. Accurate measurement in oyster using Nelson rubber apron method, 1935, yielded 26 liters per hour in oyster 11.5×9 cm. Loosanoff at Milford, Conn. obtains average of 33 l.p.h. with maximum of 48 l.p.h.
- 6 Feeding: leucocytes engulf bacteria. Dr. Stauber has traced bacterial spores over much of body of oyster, not digested by the leucocytes. Much food present in stomach of feeding oyster, very little in mercenaria. Passage of food into digestive gland and its digestion there; a possible explanation of fall in bacterial score of mercenaria during storage.

7 Keeping quality of shellfish. Dugal's findings on anaerobiosis; its application to oysters; "closed respiratory system" in *mercenaria* vs. "open system" in *Mya*. Possible effects of oxygen and lower pH in *Mya* on bacterial score. Anaesthetic effect of CO₂ on ciliary activity.

8 Elimination of mud by quahogs after removal from water. An as yet unsolved problem in shellfish sanitation is explanation of consistently lower bacterial scores in quahogs as compared with those of clams and oysters removed simultaneously from the same waters. A chance observation during late autumn 1957 offers a possible explanation. Several dozen quahogs were raked from exposed tidal flats of Cape May County in front of the New Jersey Oyster Research Laboratory. A thin layer of finely divided mud of consistency of thick cream covered the flats at the time. After washing thoroughly in clean bay water the bivalves were transported by car six miles to my home. Several hours later on picking up several to eat raw the ventral margins of the shells were found to be covered with a copious discharge of thick mucus containing much mud. Since the shells of the quahogs had been thoroughly washed and drained after harvesting this mucus and mud must have been extruded subsequently between the apposed ventral mantle borders by ciliary action. Slight relaxation of the adductor muscles to permit escape of the material between the valves must have occurred.

It is emphasized that this extrusion took place out into the air and not into water as is usual. Also, presence of the overlying mud on the flats is significant. Dodgson at Conway, North Wales, found more rapid elimination of bacteria from turbid water than from relatively clear water correlated with heavier mucus secretion stimulated by particles of dirt striking the gills and mantle. Confirmation of the above experience is seen in the fact that only rarely have I found any retained mud in quahogs when

opened, whereas clams which have not been floated in clean water invariably have revealed small to very large accumulations of sand and mud in the cloacal sinus. Oysters may or may not contain appreciable amounts of silt when opened, hence may show wide variations in bacterial scores.

9 Burial of oysters in their own rejecta. In an important series of three papers published in the Journal of the Institute of Marine Science, No. 2, Vol. 4, for July 1957, Dr. E. J. Lund presents interesting data on rate of deposition of rejecta from oysters as compared with settling of silt due to gravity. In one series of laboratory experiments in relatively low turbidities, with light transmission from 70 to 95 per cent, a single layer of oysters covering half the bottom area deposited sufficient silt to cover themselves completely in 36 days. In a second series the volume of sediment thrown down by oysters was eight times the amount deposited by gravity alone during the same period. In a third series of observations this deposit of oyster rejecta ranged from six to twelve times the amount accumulating through the force of gravity.

In open waters with good circulation on the bottom these deposits are largely swept away by currents. In relatively quiet waters, however, the silt accumulates, cutting off oxygen with resultant large production of hydrogen sulfide. Death of oysters may follow where reducing conditions of bottom muds develop to sufficiently high levels to result in strong hydrogen sulfide production.

Significance of these observations to shellfish sanitation presents two aspects. First, at even low levels of coliforms in the water their numbers in rejecta on and around the oyster's shells will be enormously greater than in overlying waters. Second, on removal of polluted shellfish to clean waters it may be expected that turbidity will hasten the

cleansing process as already demonstrated thirty years ago by Dodgson for the mussel, Mytilus edulis. Where purification is carried out in taken it is suggested that small amounts of laundry starch be added to the water to stimulate mucus secretion. Some by the starch will be eaten by the shellfish with

possibility of increasing glycogen reserves of value in anaerobic respiration.

This outline was prepared by the late Thurlow C. Nelson, formerly "The Julius Nelson Professor of Zoology", Rutgers University, New Brunswick, New Jersey.

DESCRIPTION OF ACCOMPANYING PLATE

Figure 8

O. virginica from the right side, right pallium dissected away exposing promyal chamber and the right epibranchial chamber posteriorly to its minimum size beneath the adductor muscle at point X. The region A to B with arrows directed inward is the incurrent area; from B to C with arrows pointing outward from the cloacal and promyal chambers is the excurrent area. Natural size.

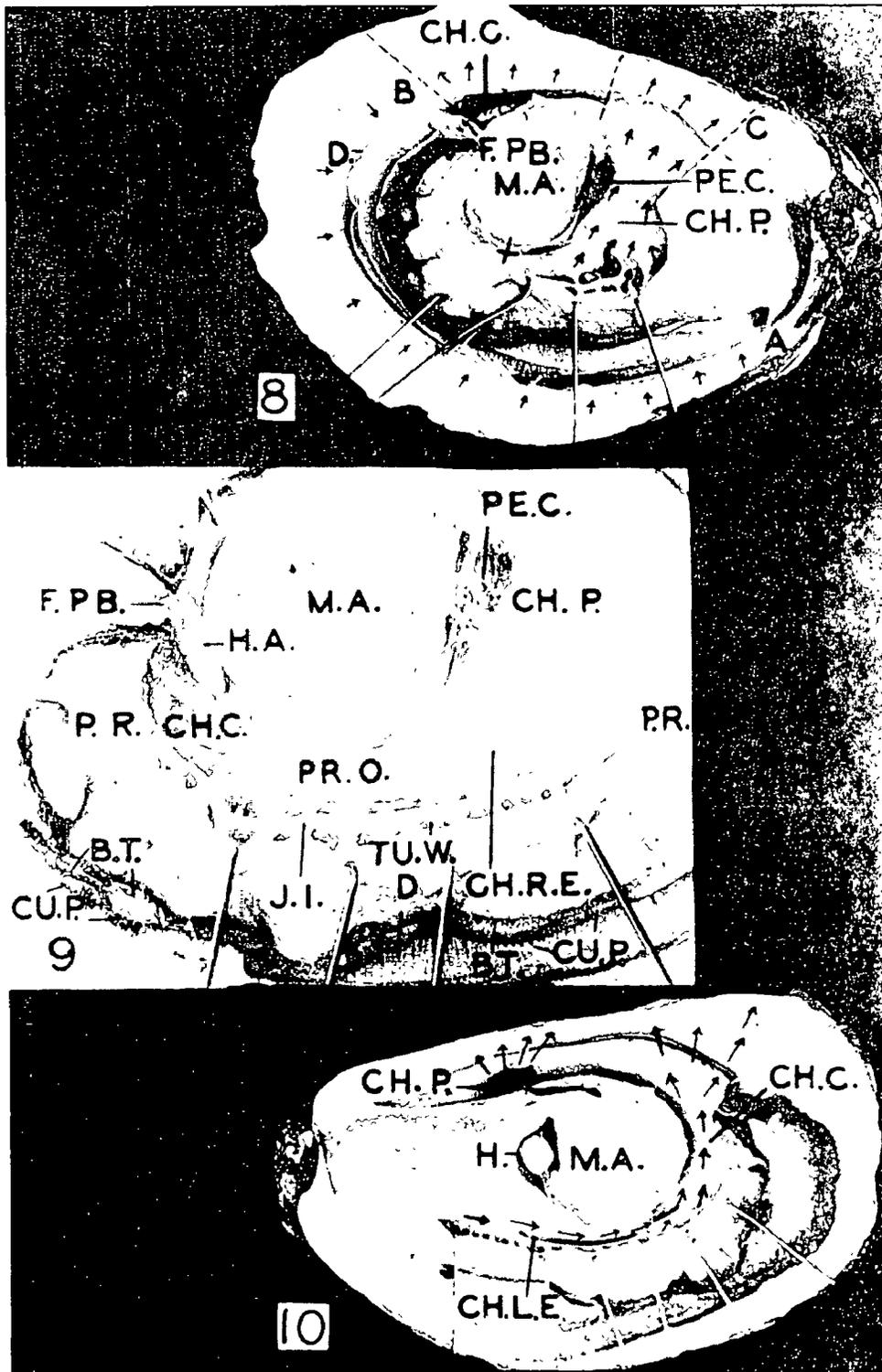
Figure 9

O. virginica dissected from right side, X2, showing relation of the water tubes of the right demibranchs to the right epibranchial, cloacal and promyal chambers, and the fixed position of the oral process. B.T., tentacular border; CH.C., cloacal chamber;

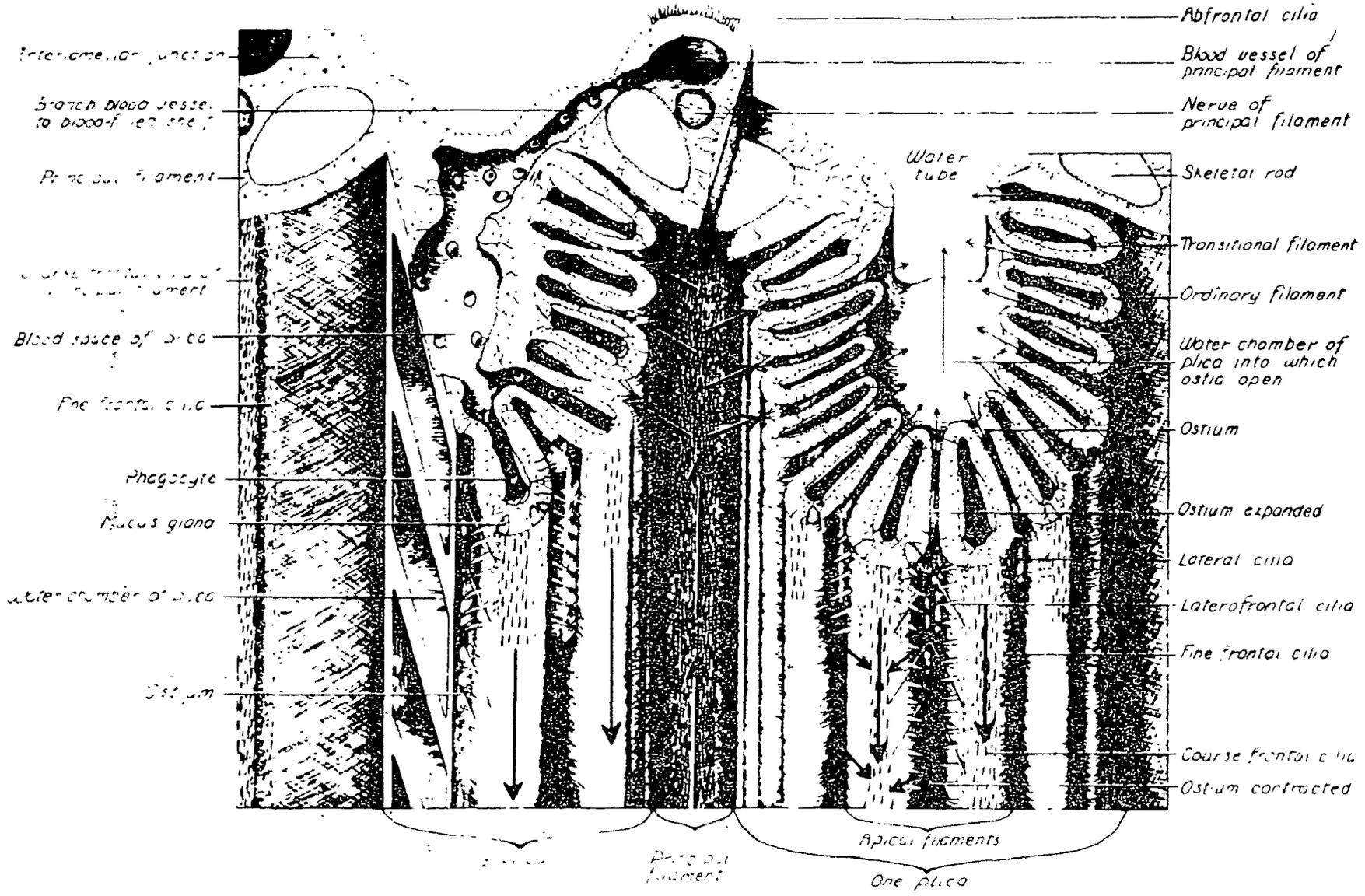
CH.P., promyal chamber; CH.R.E., right epibranchial chamber; CU.P., pallial curtain; D., demibranch; F.PB., pallio-branchial fusion; H.A., accessory heart of Hopkins; J.I., interlamellar junction; M.A., adductor muscle; P.R., right pallium; PE.C., pericardial cavity; PR.O., oral process; TU.W., water tube.

Figure 10

O. virginica from the left side to show route of water discharged from the left demibranchs and from posterior portion of the right demibranchs. A roll of black paper is inserted into the promyal chamber. CH.L.E., left epibranchial chamber; H., heart; other abbreviations as in Figure 9. Natural size.



Ref: T. C. Nelson, J. Morphology 63, 33 (1938)



(For Administrative Use Only)

Ref: Personal Communication T. C. Nelson (1957)

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CONTAMINANTS OF SHELLFISH

Part 2

I INTRODUCTION

- A This outline deals with the effects of contaminants in the marine environment on shellfish, and their uses as human food.
- B Contaminants enter shellfish from the marine environment primarily via two routes;
- 1 The body surface - gaseous exchange, and elements in the ionic state as well as small molecules may enter directly through the soft body surface.
 - 2 The digestive tract - carried in by feeding and absorbed either by the mucus, as part of the food, or as separate entities.
- C Mollusks are particularly vulnerable to salinity and other environmental chemical changes.
- 1 Marine species are in osmotic equilibrium with their environment.
 - 2 Some resist the temporary tidal changes in salinity by closure of the shell valves.

II ESTUARINE - SHELLFISH MANAGEMENT

According to Tarzwell's (1963) concept: contamination is the occurrence of any material in or any change in character of shellfish that interferes with, lessens, or destroys their use as a human food item.

- A The need for recognition of estuaries as a national resource, particularly the shellfish which abound in these estuaries, has been highlighted recently.
- B The following principles will aid in estuarine and shellfish management.

- 1 Shellfish are a renewable, manageable natural resource of significant economic value to many coastal communities, and should be treated as are other natural resources such as forest, water and agricultural lands.
- 2 Shellfish culture and harvesting represents a beneficial use of water in the estuaries. This should be recognized by State and Federal Agencies in planning and carrying out pollution prevention and abatement programs and in comprehensive planning for the use of these areas.
- 3 The goals of the National Shellfish Sanitation Program are:
 - a The continued safe use of this natural resource and
 - b Active encouragement of water quality programs which will preserve and restore all possible coastal areas for this beneficial use.

III CONTAMINANTS OF SHELLFISH

- A Marine Biotoxins - Among the several types of biologically produced marine toxins, paralytic shellfish poison (PSP), found in shellfish of northern waters, is the most frequent and most studied.
- 1 PSP is also called saxitoxin or mussel poison.
 - 2 Another marine biotoxin found in shellfish and associated with "red tides" is produced by the dinoflagellate Gymnodinium breve. It is believed that this poison is identical to the fish poison known as Ciguatera.
- B Bacterial Accumulation by Shellfish

- 1 Many bacterial diseases, such as typhoid fever, are transmitted to humans from eating contaminated shellfish.
 - a Bacterial accumulation is correlated to pumping activity and temperature.
 - b Oysters may filter only 30% of known numbers of E. coli out of the water.
- 2 The influence of shucking, packing and storage upon bacterial growth in the shucked product is of prime consideration.
 - a Time and temperature are the two prime factors; fortunately they can be controlled to certain practical limits.
 - b It is important that the shellfish and the shucked products be processed as soon as possible and stored at low temperatures.

C Effects of Pollution from Waterfowl

- 1 In Great South Bay, Long Island, over-fertilization of the waters from duck farm wastes led to great blooms of undesirable plankton which interfered with the feeding of oysters.
- 2 High counts of fecal streptococci in certain oyster-producing tidal streams of Delaware were attributed to the large numbers of waterfowl nesting in the adjacent marshes.

D Transmission of Human Viruses

- 1 Evidence has been established for the transmission of infectious hepatitis by raw shellfish.
- 2 The common mussel (Mytilus edulis) can become contaminated by the polio-myelitis virus. Some oysters can take up the polio virus in two hours, but self-purification did not occur in experiments of 6-day's duration.

E Suspended-Silt

Water-borne sediments, as pollutants of the shellfish environment, are important because they interfere with the process of water-pumping and feeding.

- 1 Concentrations of 0.25 grams/l of silt reduced pumping of oysters by 57%; 0.5 g by 68%, 1 g by 81% and 3-4 g/l by 94%.
- 2 In open areas, tidal and other water currents sweep away silt; in sheltered areas deposits accumulate, to cover shellfish.

F Pesticides

- 1 DDT in sufficient quantities kills oysters, but of more importance, lesser amounts of DDT seriously disrupt the normal activity of the oyster.
 - a DDT levels may be so low as to be barely detectable in the seawater with no apparent affect on the oysters, but the DDT may make the oyster unpalatable.
 - b Oysters may concentrate DDT in their tissues 20,000 times greater than that in the seawater within 7 days exposure.
- 2 Even at concentrations well below 1.0 ppm most chlorinated hydrocarbon insecticides - including aldrin, DDT, dieldrin, andrin, and lindane - markedly inhibit shell growth.
- 3 There is a great difference in the toxicity of insecticides to bivalve larvae.
 - a At a concentration of 0.05 ppm, DDT caused over 90% mortality of oyster larvae and almost entirely prevented growth.
 - b In 5.0 ppm lindane the growth of clam larvae was somewhat faster than in control cultures.

G Reaction of Oysters to Chlorinated Seawater

- 1 Oysters respond to an initial contact with chlorine (concentrations between 0.01 and 0.2 ppm) by cessation of feeding current and shell closure.
- 2 Apparently tolerance is developed to repeated exposure and the valves may remain open and pumping of water may continue at concentrations greater than those which caused the initial response.

H Metals, Particularly Radionuclides

Shellfish have a propensity for the uptake, accumulation, and storage for several months of heavy metals, including their radionuclides.

- 1 Certain elements, particularly trace metals such as copper and zinc, are essential for maintenance of living systems, but when they exceed normal concentrations in the environment, as from industrial wastes, they can be exceedingly toxic.
- 2 Although mollusks may contain relatively high levels of metallic radionuclides, they do not constitute a health hazard.
- 3 An outbreak of a severe neurologic disorder of humans in Minamata, Japan, during the 1950's was related to the ingestion of seafood contaminated by mercuric compounds.

I Effects of Hydrocarbons

- 1 In Louisiana, accidental oil spillage did not kill any oysters on commercial beds, but it did render them unpalatable for periods from several days to several weeks.
- 2 Rates of feeding are affected by hydrocarbons.

J Pulp Mill Wastes

- 1 Pulp-mill waste was determined to be the principal cause of a steady decline since 1916 in the productivity of oyster beds in the Upper York River, Virginia. The pulp-mill effluent reduced the total time the oyster shells were open and also depressed the rate of water-pumping.
- 2 Kraft (sulfate) pulp-mill wastes contain more toxic constituents than sulfite pulp-mill waste liquors per unit volume.
- 3 Three ways in which pulp-mill wastes are harmful to marine life:
 - a Direct toxicity
 - b Indirect effect through reduction of oxygen in the water
 - c Long-term effects on the bottom and water adjacent to it.

K Other Contaminants

- 1 pH - at low pH levels (4.25), oysters show abnormal shell movement and reduced pumping.
- 2 Rhodamine B dye - hard clams showed normal siphoning only at concentrations of 4.7 mg/l or less.

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This outline was prepared by Carl N. Shuster, Jr., Director, Northeast Research Center, Shellfish Sanitation Branch, Division of Environmental Engineering and Food Protection, BSS, PHS, DHEW, Narragansett, Rhode Island.

THE DEVELOPMENT OF BACTERIOLOGICAL STANDARDS FOR SHELLFISH GROWING AREAS

I INTRODUCTION

A Responsibilities

The National Shellfish Sanitation Program is a cooperative program between the shellfish industry and both state and federal agencies responsible for the sanitary control of shellfish shipped in interstate commerce. This program has been in existence since 1925.

B Early Considerations

The possible association between the consumption of raw shellfish and the incidence of Typhoid Fever was suspected for many years. As early as 1902, following an outbreak of Typhoid Fever, Dr. Gurion stated in Public Health Bulletin No. 86: "there is a zone of pollution established by the mere fact of the existence of a populated city upon the banks of a stream or tidal estuary which makes the laying down of oysters and clams in these waters a pernicious matter." The difficulty, in these early days, of isolating pathogens and the lack of knowledge pertaining to the indicator/pathogen relationship resulted in the collection of masses of data aimed at establishing a safe level of indicator organisms or determining the population levels that would reduce to an insignificant degree the health hazard potential associated with the consumption of raw shellfish.

II HISTORICAL

A Development of Test Procedures

The early studies of pollution indicating bacteria resulted in the development of the multiple tube fermentation test for Bacillus coli (formerly referred to as the "coli-aerogenes" group and now known

as the coliform group of bacteria). Historically, the development of this test procedure can be summarized as follows:

- 1 With the publication of "Elements of Water Bacteriology" (Prescott and Winslow¹, 1904) two basic concepts were stated which laid the groundwork for the development of bacteriological standards for shellfish growing areas:
 - a The detection of B. coli in a large proportion of 1 cc volumes or dilutions less than 1 cc is an indication of recent pollution..
 - b The finding of B. coli in large samples of water or in an occasional small sample does not have any special significance. No attempt was made to define the term "large proportion" however, the concept of using the presence of the bacterial indicator in 1 ml volumes to imply the existence of a possible health hazard is well established.
- 2 Eijkman² proposed a test for pollution indicator organisms in 1904 which recommended glucose as the energy source and an incubation temperature of 46° C. It was his contention that "coli bacteria" associated with warm blooded animals would grow under these conditions but that related organisms isolated from soil, plants, and so called "safe" waters would not survive at that temperature. Thus the elevated temperature test became the Eijkman test, the forerunner of what is known today in sanitary microbiology as the "fecal coliform" test.

- 3 Prior to the development of the MPN tables, a "score" system was devised to denote sanitary quality. This number system was based on the number of positive tubes and the dilution factors but it appears, at this time, that the primary interest in standards within the control agencies was oriented toward shellfish meats and not the growing areas.
- 4 The table below comparing "Coliform Organism Scores" with MPN Values, was taken from the 1946 Manual of Recommended Practice for Sanitary Control of the Shellfish Industry (Public Health Bulletin 295). Mr. Furfari³ states: "I suspect this (the score system) might have contributed to a delay in the ultimate derivation of a standard for water."

COMPARISON OF COLIFORM ORGANISM SCORES WITH MOST PROBABLE NUMBER OF COLIFORM ORGANISMS PER 100 MILLILITER.			
Score	MPN	Score	MPN
1.....	20	41.....	1,300
2.....	45	50.....	2,400
3.....	78	140.....	3,500
4.....	130	230.....	5,400
5.....	230	320.....	9,200
14.....	330	410.....	16,000
23.....	490	500.....	24,000
32.....	790	1,400.....	35,000

5 MPN tables for evaluating coli-aerogenes tests were published by Hoskins⁴ in 1934.

B Development of Standards

- 1 Following the 1924 outbreak of Typhoid Fever a Committee on the Sanitary Control of Shellfish in the United States was formed and the Frost Report⁵ given in 1925, stated:

"The committee is not prepared to recommend any precise bacterial standards for waters from which the taking of shellfish is permitted until additional data which are now being collected have been assembled and considered. In the light of present knowledge it would probably be unfair and unnecessary to apply to such waters the rigid standards which are applied to the drinking water supplied in interstate commerce. It is considered, however, that the waters should ordinarily not show the presence of B. coli in 1cc amounts, test for B. coli being made in 10 cc, 1 cc, and 0.1 cc amounts, according to the Standard Methods of the American Public Health Association."

Had the word "ordinarily" been left out of this statement and the recommendations, as proposed, been accepted the maximum allowable count for "open" growing areas would have been equivalent to an MPN of 23.

- 2 In October 1926 a Subcommittee on Bacteriological Examination was formed and requested to review recommendations relating to the techniques, significance, and uses of bacteriological examinations. A tentative report of this Subcommittee⁶ is quoted as follows:

"Areas which may be approved for the taking of shellfish without serious question. This class includes the areas which are so protected against human fecal contamination by distance from sources of such pollution, by

dilution and by the time afforded for natural purification, that there is no discoverable likelihood of dangerous contamination. On bacteriological examination such waters may be expected to show the presence of organisms of the coliform aerogenes group more or less frequently in 10 cc portions but usually not in 1 cc or smaller amounts* (original italics). The footnote reads: "The specification is given above as it was formulated in conference. It has since been proposed that it be amended to read: but not in the majority of 1 cc portions. Members of the sub-committee are requested to express their preference as between the original and the amended forms."

Furfari³, at this point, states that:

"the thinking on the proportion of 1 cc sample has progressed from not ordinarily show to usually not (more or less frequently) to but not in the majority.

- 3 In 1933 Carl Green⁷ reported that some states were specifying maximum "scores" of 2.3 and 3.6 for water standards. Equivalent MPN values for these "scores" would be 45 and 130/100 ml sample. He also reported that other states were recommending a maximum "score" of 2.5, an MPN equivalent of approximately 62/100 ml, or not more than 50% of the 1 ml portions. Green also urged that the USPHS establish a nation wide standard. At this time the concept of 50% of 1 ml portions as a possible working standard for shellfish growing areas was rapidly becoming a real possibility.
- 4 Miller⁸ stated in a publication in 1935, "The Public Health Service... has arrived at the conclusion that generally not more than 50% of the 1 cc tubes in an area should show the presence of B. coli if that area is to be used for the taking of shellfish for market. The word "generally" was not defined.

The precedent, therefore, had been set for "weasel words" in future standards.

- 5 Dr. C. A. Perry⁹, referee of the A.P.H.A. committee on bacteriological methods made the following proposals for changes in the standard procedure for the examination of shellfish in 1936:
 - a The new procedure should include such edible mollusks as oysters, clams and mussels.
 - b Escherichia coli rather than the colon group should be the index of pollution for both shellfish and shellfish waters.
 - c A new procedure should include methods for the examination of shellfish waters as well as shellfish.
 - d The whole oyster rather than just the shell liquid should be examined.
 - e Escherichia coli results should be expressed as most probable numbers rather than as a score.
 - f Certain recommendations should be made in regard to amount of pollution which should ordinarily be tolerated.
- 6 In separate publications in 1937 Miller¹⁰ and Shea¹¹ referred to the "unwritten standard" of not more than 50% of 1 cc positive tubes. Sheas' report stated that:

"The United States Public Health Service has arrived at the conclusion that waters used in the production of shellfish for the market should in general not show the presence of coli-aerogenes organisms in 1 cc portions of samples more than fifty percent of the time."

It is interesting to note that the Minimum Requirements for Endorsement of State Shellfish Control Measures and Certifications for Shippers in Interstate Commerce¹², published in 1937, did not give a bacteriological standard for shellfish growing areas.

7 In 1941 a study was completed and the results published in "A Report on the Public Health Aspects of Clamming in Raritan Bay."¹³ By comparing epidemiological data fo known cases of typhoid presumed to be of shellfish origin with coliform populations of shellfish and shellfish waters, it was concluded that "70 coliforms/100 ml, on the basis of average results from waters overlying the shellfish beds, is recommended as the limiting allowable standard for the taking of hard clams to be eaten raw."

8 The 1946 publication of the Manual of Recommended Practice for Sanitary Control of the Shellfish Industry¹⁴ defined the bacteriological criteria of growing areas as follows: "The median bacteriological content of samples of water. . . shall not show the presence of organisms of the coliform group in excess of 70/100 ml of water."

9 The present bacteriological standard for approved and conditionally approved shellfish growing areas, as stipulated in the 1965 Revision of the National Shellfish Sanitation Program Manual of Operations, Part I¹⁵, is as follows:

a The coliform median MPN of the water does not exceed 70/100 ml.

b Not more than 10% of the samples ordinarily exceed an MPN of 230/100 ml for a 5 tube decimal dilution test (or 330/100 ml where the 3 tube decimal dilution test is used) in those portions of the area most probably exposed to fecal contamination during the most unfavorable hydrographic and pollution conditions.

c These foregoing limits need not be applied if it can be shown by detailed study that the coliforms are not of fecal origin and do not indicate a public health hazard.

10 A supplementary standard for an approved growing area was recommended by the 5th National Shellfish Sanitation Workshop¹⁶ in 1964. It states, "In an approved shellfish growing area, a median fecal coliform MPN of 7.8 shall not be exceeded and not more than 10% of all samples tested shall exceed an MPN/100 ml in excess of 33 (46/100 ml where the 3 tube decimal dilution test is used). This recommendation is still under study.

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This outline was prepared by D. A. Hunt, Chief, Standards Development Section, Shellfish Sanitation Branch, Bureau of Compliance, FDA.

SHELLFISH GROWING AREA SURVEYS

I INTRODUCTION

A Important Public Health Concern

The safety of a raw food resource depends in large measure on the water quality criteria used and its application to determine approved shellfish growing areas. (Entire shellfish, excluding shell, of course, is often consumed in the uncooked state.)

B Reliability a Part of Criteria

Since the safety of the raw food product has to be consistently met, the water criteria has to be one that is also consistently met and not just one that is satisfied on the average, as is the case in many other design or survey situations. The question of variability is covered in the phrase, "most unfavorable hydrographic and pollution condition," which have to be considered in the criteria and which, unfortunately, are most often overlooked.

C Four Types of Contamination

As indicated in previous lecture the types of contamination which have to be considered in area studies may be considered in four categories:

- 1 Bacteriological - Including virus (infectious hepatitis), parasites (E. histolytica), etc.
- 2 Biological - Paralytic shellfish poison or other marine biotoxins
- 3 Chemical - Metals, pesticides, etc.
- 4 Radiological

D Four Groups of Area Classification

Reference should be made to Part I of the manual on "Sanitation of Shellfish Growing Areas," 1965 Revision of PHS Publication No. 33, for details of the following classes

and to Figure 1 in the publication and Figure 8 attached which shows schematically some possible interrelationships.

- 1 Approved areas - Survey indicates shellfish from area may be used as raw food product.
- 2 Conditionally approved areas - Survey indicates that shellfish from area may be used as a raw food product provided certain specified and controlled conditions are met, i. e., proper control of treatment plant effluents.
- 3 Restricted areas - Survey indicates that shellfish from the area may be used as a raw food product only when some further practice is employed to improve the product before marketing. Product may be relayed into an approved area for a specified time, or it may be processed in a depuration plant.
- 4 Prohibited areas - Survey indicates that shellfish from the area should not be used, except that salvage operations may be permitted under extensive and detailed supervision and control.

E Frequency of Survey - A Criteria

The proper area classification also depends upon how often an area is surveyed and what type of survey is made.

- 1 Unsurveyed - Automatically prohibited. "All actual or potential growing areas which have not been subjected to sanitary surveys shall be automatically classified as prohibited." (Part I, Section C, Item 2d, of manual.) (An area is considered "guilty" until proved otherwise. The purpose of the survey is to determine what portion of the area may not be guilty of contributing to a public health hazard.)

- 2 Ten-year intervals - Complete resurvey required every ten years for area in an approved category (approved, conditionally approved, or restricted).
- 3 Two-year intervals - A reappraisal is made of each such approved category area every two years to determine if there has been any change in the factors influencing the sanitary quality of the area. If any change is determined, then a complete survey is needed to support area classification.
- 4 Wet storage area - Yearly - "The temporary storage of shellfish from approved sources, intended for marketing, in tanks containing sea water or in natural bodies of water, including storage in floats."

- b Background of standard and meaning - Although the level of bacteria in shellfish is related to the level in the water, it varies for species and seasons (see Figures 1, 2, and 3). The standard is based upon the more easily tested overlying waters and is considered as indicating the relative amounts of pollution and possibility of the presence of pathogenic organisms.

- 2 Direct source - For small sources discharging directly to a growing area, such as from boats or shoreline homes, the bacteriological indicator is no longer useful, numerically, in area classification or in giving an indication of the safety of the area. In the direct source situation it becomes a matter of evaluating the chance of the problem existing in a given situation and its relationships to the probable amount of product that might be affected. For example, more distance should be required as protection around a direct pollution source where wet storage or even relaying was to be practiced as compared with a similar use of the area as a natural bed.

II SURVEY BASED ON BACTERIOLOGICAL CRITERIA

A Two General Types of Pollution Sources

- 1 Remote pollution source from a community - "The coliform median MPN of the water does not exceed 70 per 100 ml, and not more than 10 percent of the samples ordinarily exceed an MPN of 230 per 100 ml for a 5-tube decimal dilution test (or 330 per 100 ml where the 3-tube decimal dilution test is used) in those portions of the area most probably exposed to fecal contamination during the most unfavorable hydrographic and pollution conditions." (See Figure 6)

- a Margin of safety - The present standard has passed the test of "it works" although the epidemiological investigations have not established a direct numerical correlation between the bacteriological quality of the water and the degree of hazard to health.

B Source of Contamination - Starting Point of Problem and Survey

- 1 Area study - Determine location and type of existing and potential pollution sources. This includes sources of untreated wastes, treated wastes, pumping stations, industrial waste sources, sludge dumps, small direct waste sources from shore, marinas, boat anchorages, etc.
- 2 Determine most unfavorable pollution condition - This has to be evaluated for each pollution source, both existing and potential. In some cases, as is the case for resort areas, the seasonal pattern has to be determined. For sewage treatment an analysis has to be made of the various units and a determination made of the level of operation that might be consistently relied upon.

(Any operation below this level is cause for closure of a portion of the approved area).

C Hydrography

- 1 Tidal currents - Study tidal pattern for extreme conditions and study velocity and direction of current relationship to tidal phase.
- 2 Wind-driven currents - Determine the effect of wind direction and magnitude on the tidal current pattern.
- 3 Estuarine circulation patterns - Determine by salinity cross-section studies the type of estuarine circulation pattern in the growing area.
- 4 Determine fresh water run-off - Study fresh water run-off levels for extreme conditions.
- 5 Determine most unfavorable hydrographic condition - With knowledge of the area determine what hydrographic situation or situations would create the most unfavorable pollution level condition in the shellfish growing area.

D Water Quality Study

- 1 Special standard methods - "Recommended Procedures for the Bacteriological Examination of Sea Water and Shellfish," 3rd Edition, 1962, of the American Public Health Association. (See Figures 6 and 7.)
- 2 Location - Need to establish stations for sampling which may be re-occupied for serial sampling.
- 3 Determine efficiency of receiving water as treatment process - Determine the fate of a conservative (not time variable) pollutant (Figure 4) and non-conservative (time variable) pollutant (Figure 5) in the body of receiving water.

a Bacteriological survey - If possible conduct study during period when most unfavorable conditions occur; otherwise, extrapolate data from survey conditions to most unfavorable situation.

b Time in transit studies - Determine transit time from pollution source to various parts of shellfish area.

E Area Classification (See Figure 8)

- 1 Analyze data and determine pollution levels under most unfavorable situations (Figures 9 and 10).
- 2 Conditionally approved areas - The conditions have to be built into the criteria. Some example of conditions which must be met in order for areas to remain in an acceptable classification for direct harvesting are:
 - a Operation of sewage plant - At level of operation always in excess of specified level for area classification.
 - b Alarms on pumping stations to provide notice of changed conditions.
 - c Seasonal considerations such as increase in population and boat usage.
- 3 Restricted areas - "The coliform median MPN of the water does not exceed 700 per 100 ml and not more..."

III BIOLOGICAL CRITERIA

A Paralytic Shellfish Poison

Epidemiological investigations have indicated that some 200 to 600 micrograms of poison will produce symptoms in susceptible persons, and a death has been attributed to the ingestion of a probable 480 micrograms of the poison. This is a naturally occurring poison.

B Establish Conditions

Areas should be surveyed to establish conditions of occurrence. When determined, control stations should be monitored in accordance with prevailing pattern. (Figures 11 and 12)

C Closure Level

"A quarantine is imposed against the taking of shellfish when the toxicity reaches 80 micrograms per 100 grams of the edible portion of raw shellfish."

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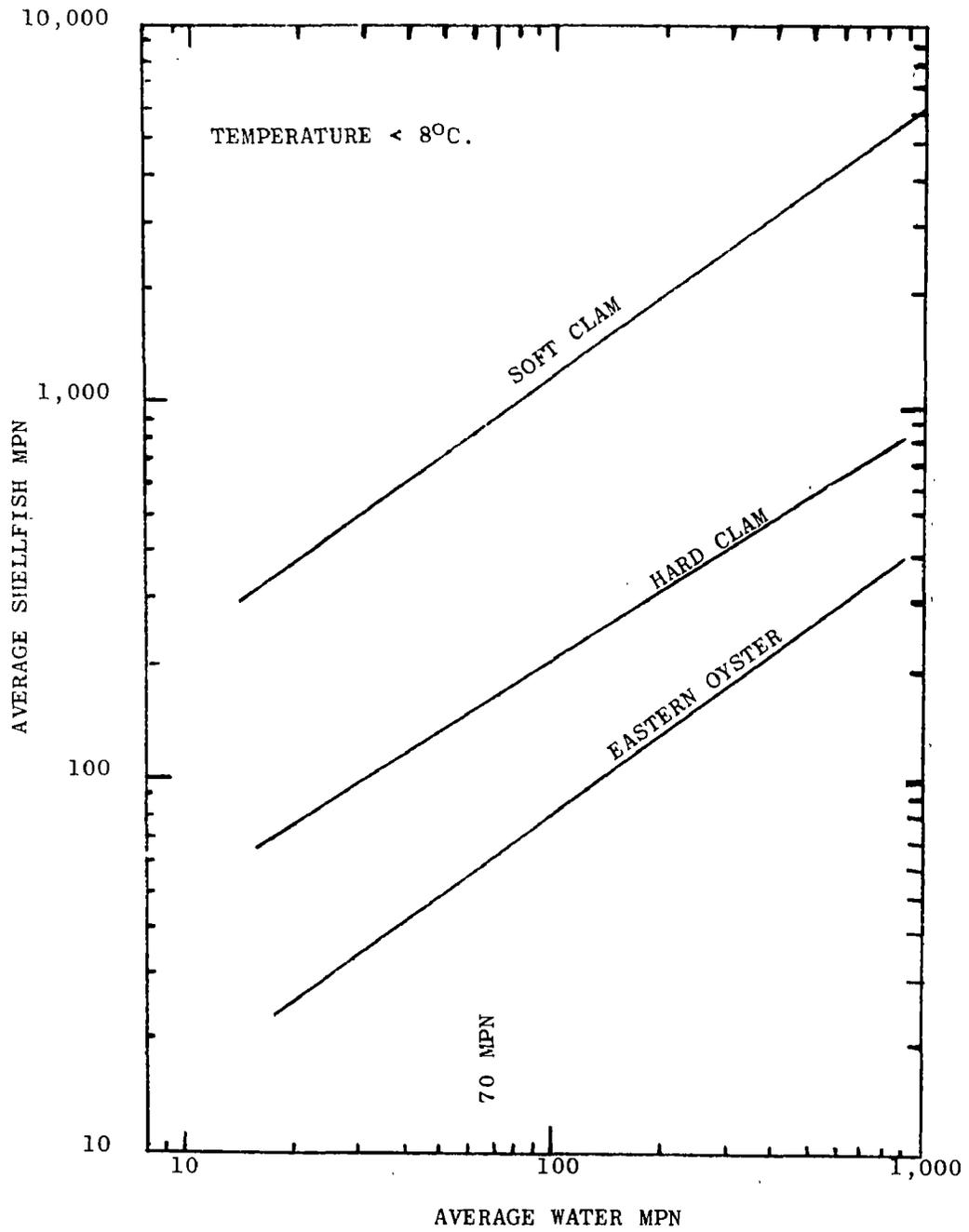


FIGURE I

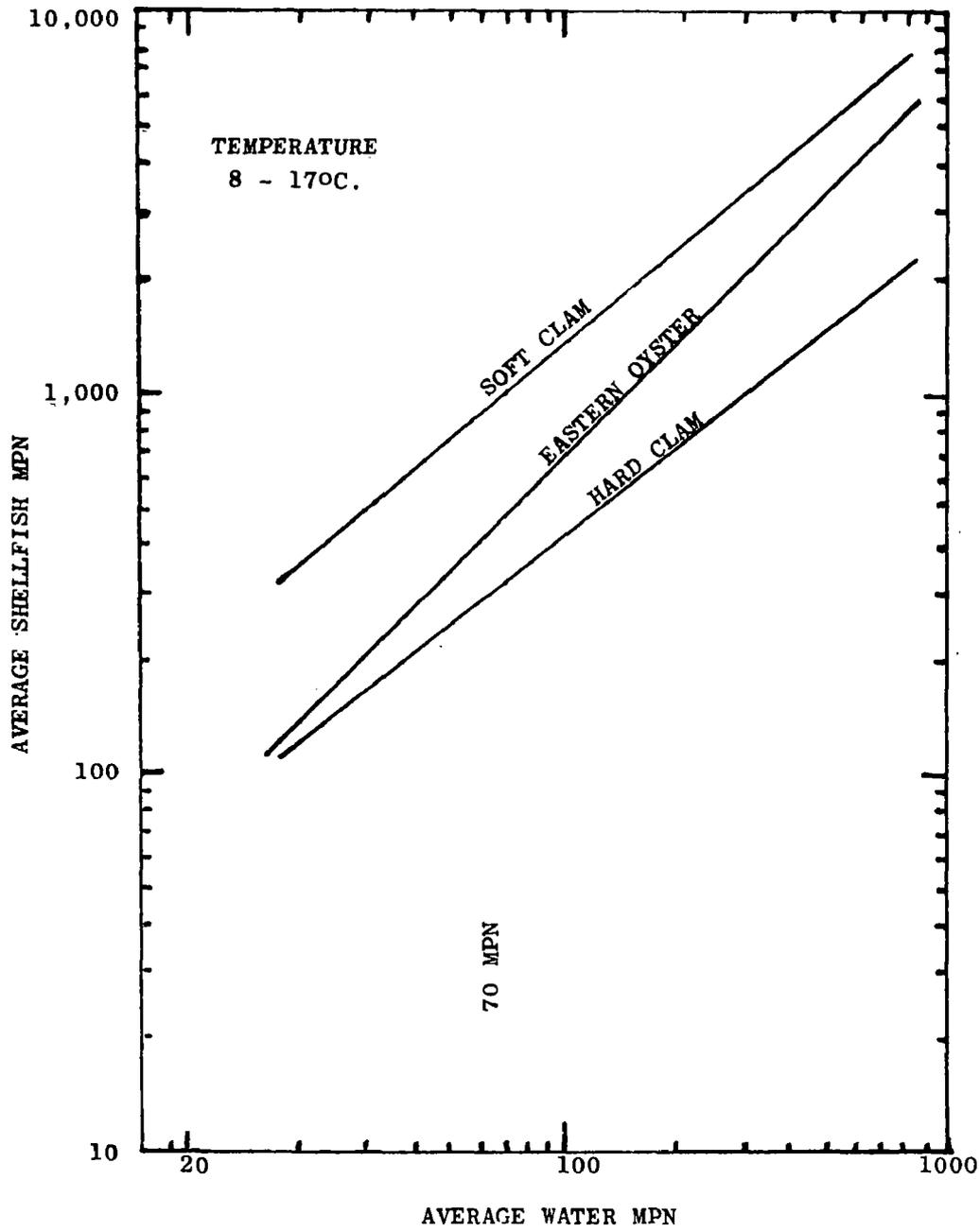


FIGURE II

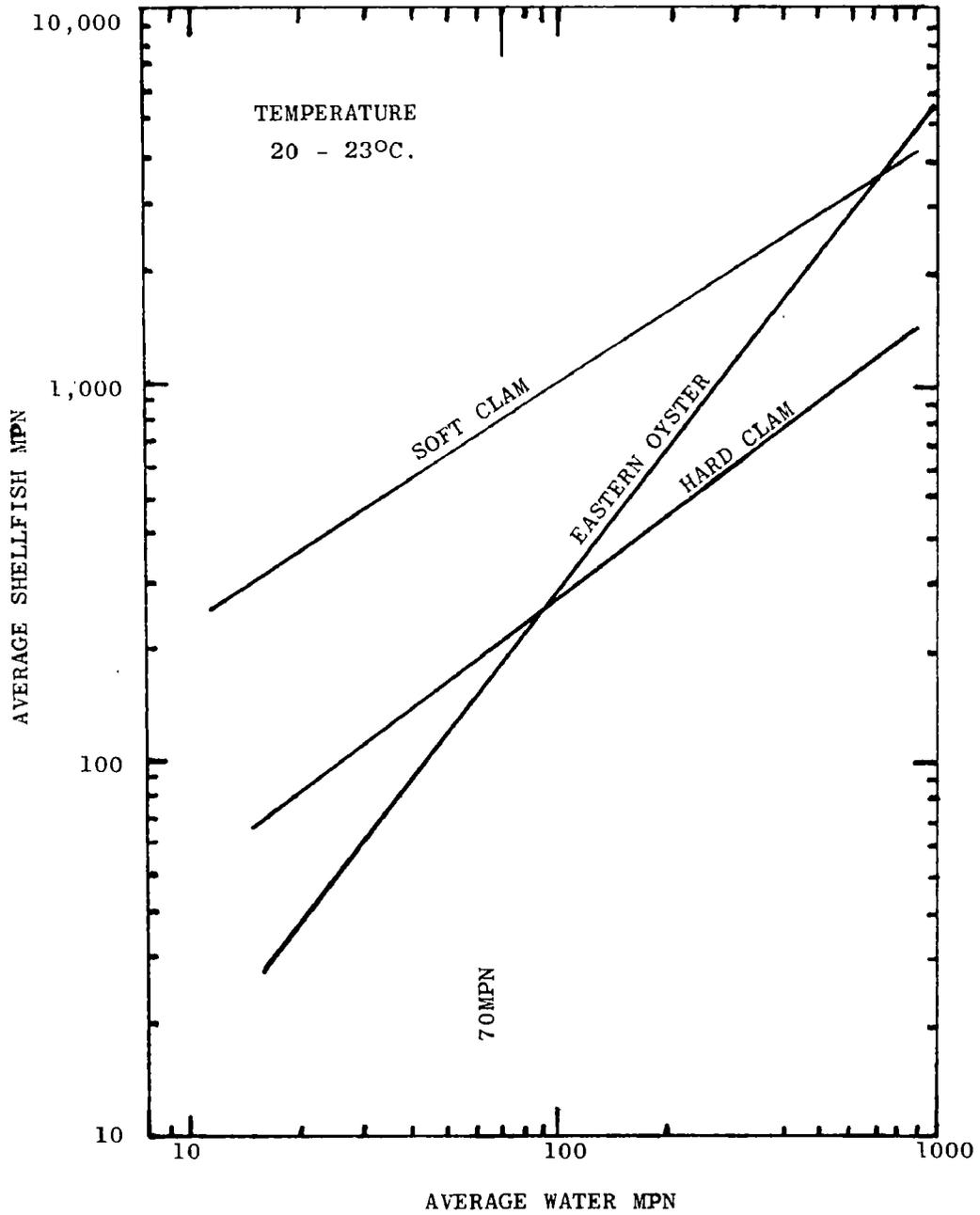


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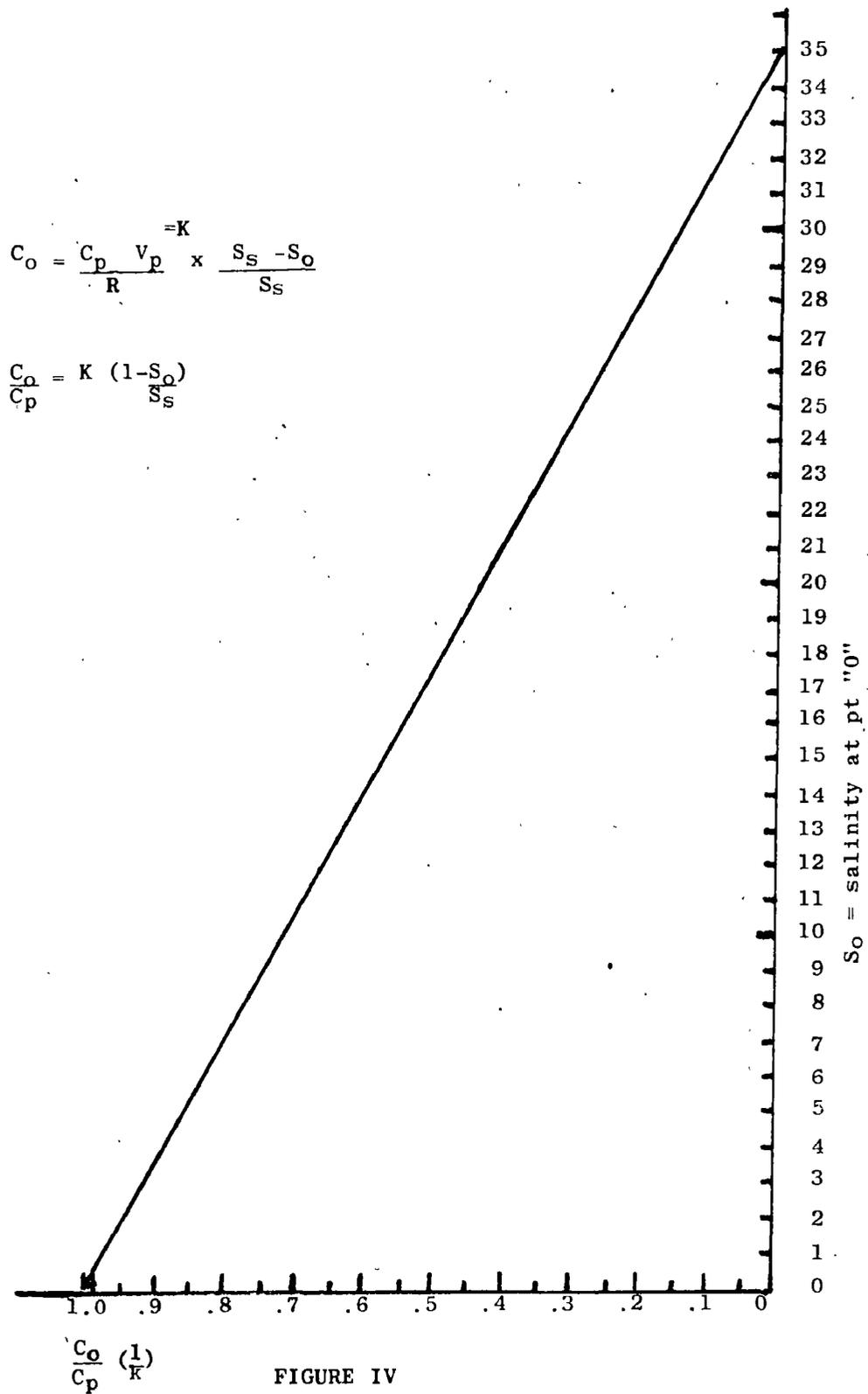


FIGURE IV

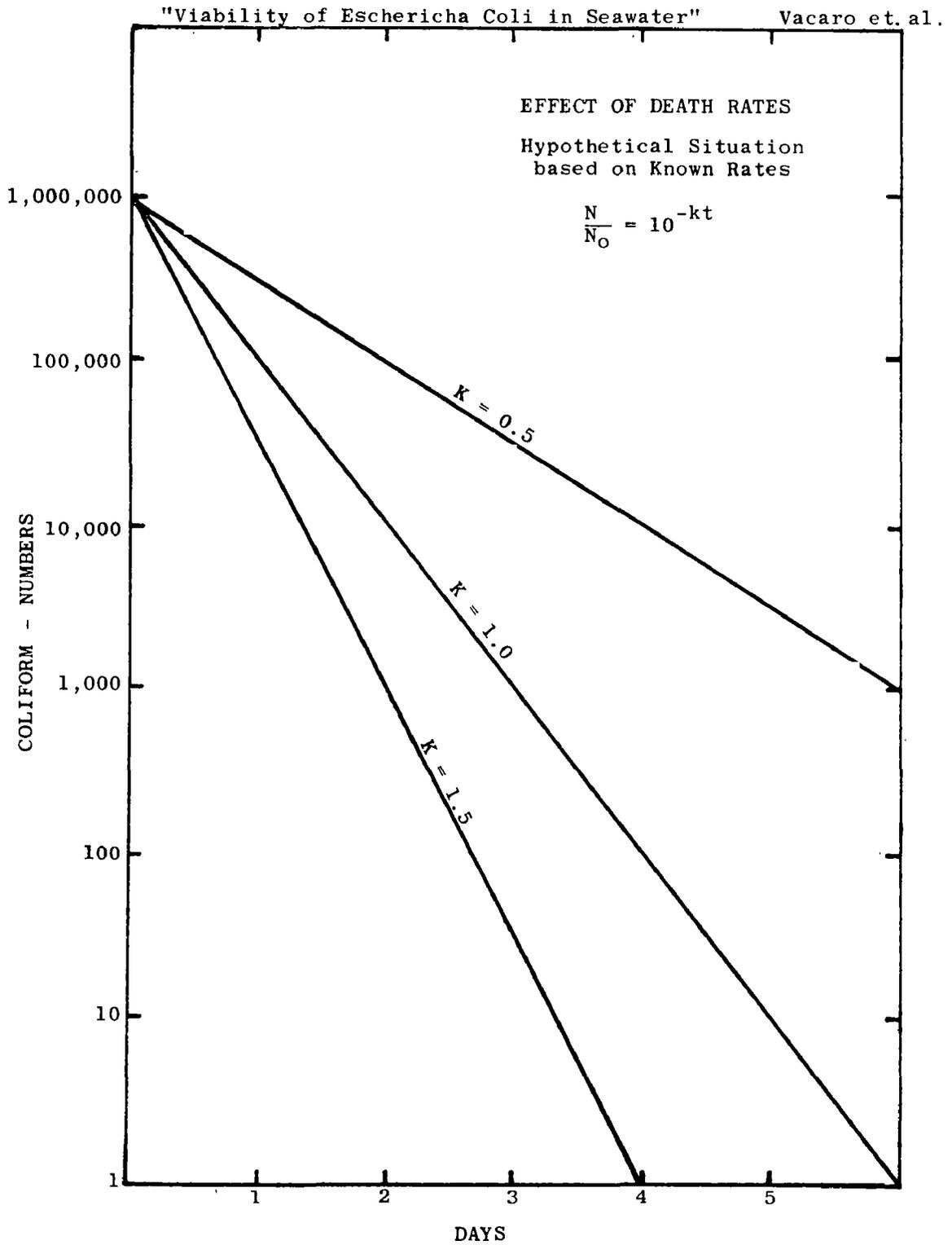


FIGURE V

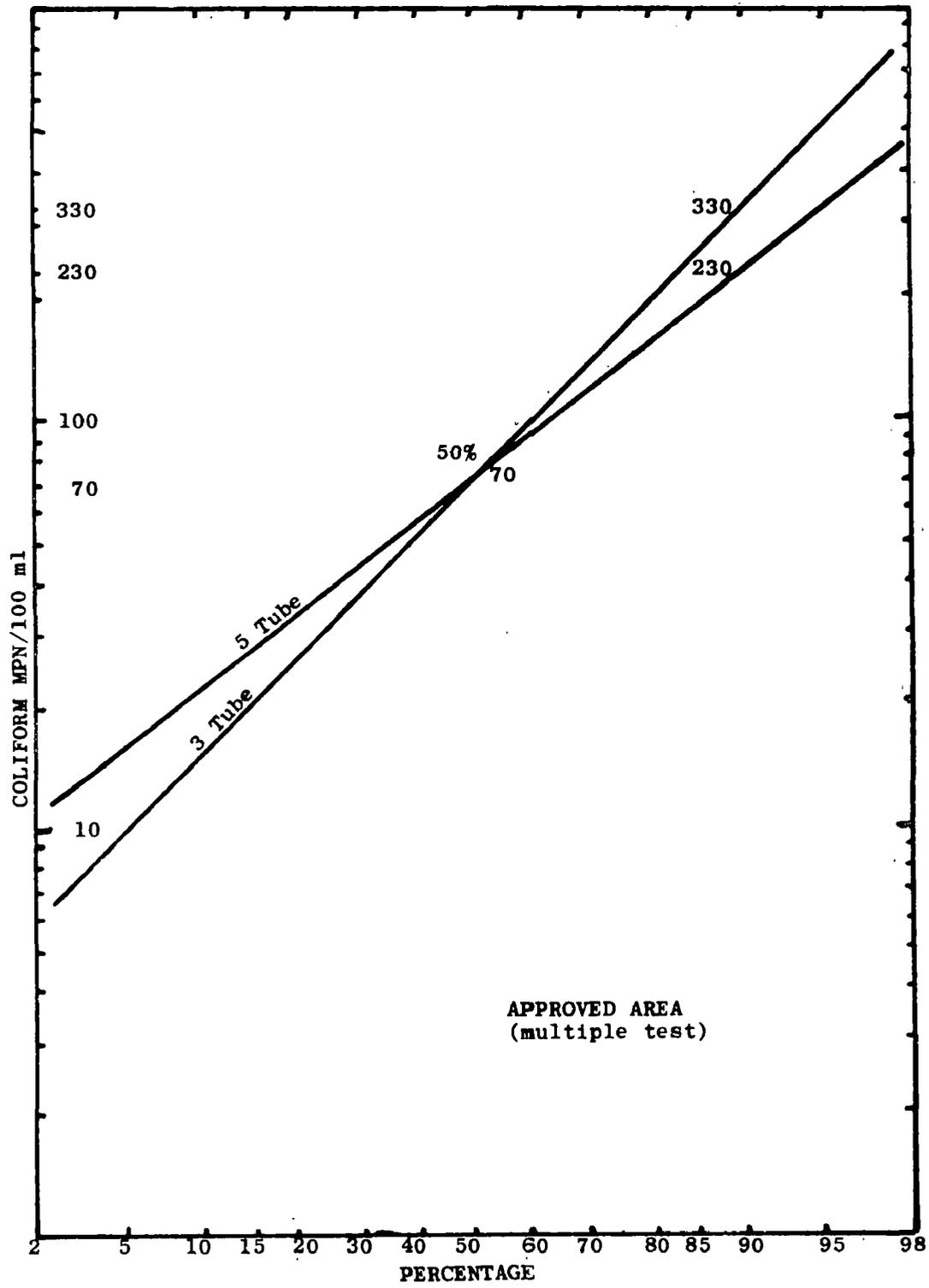


FIGURE VI

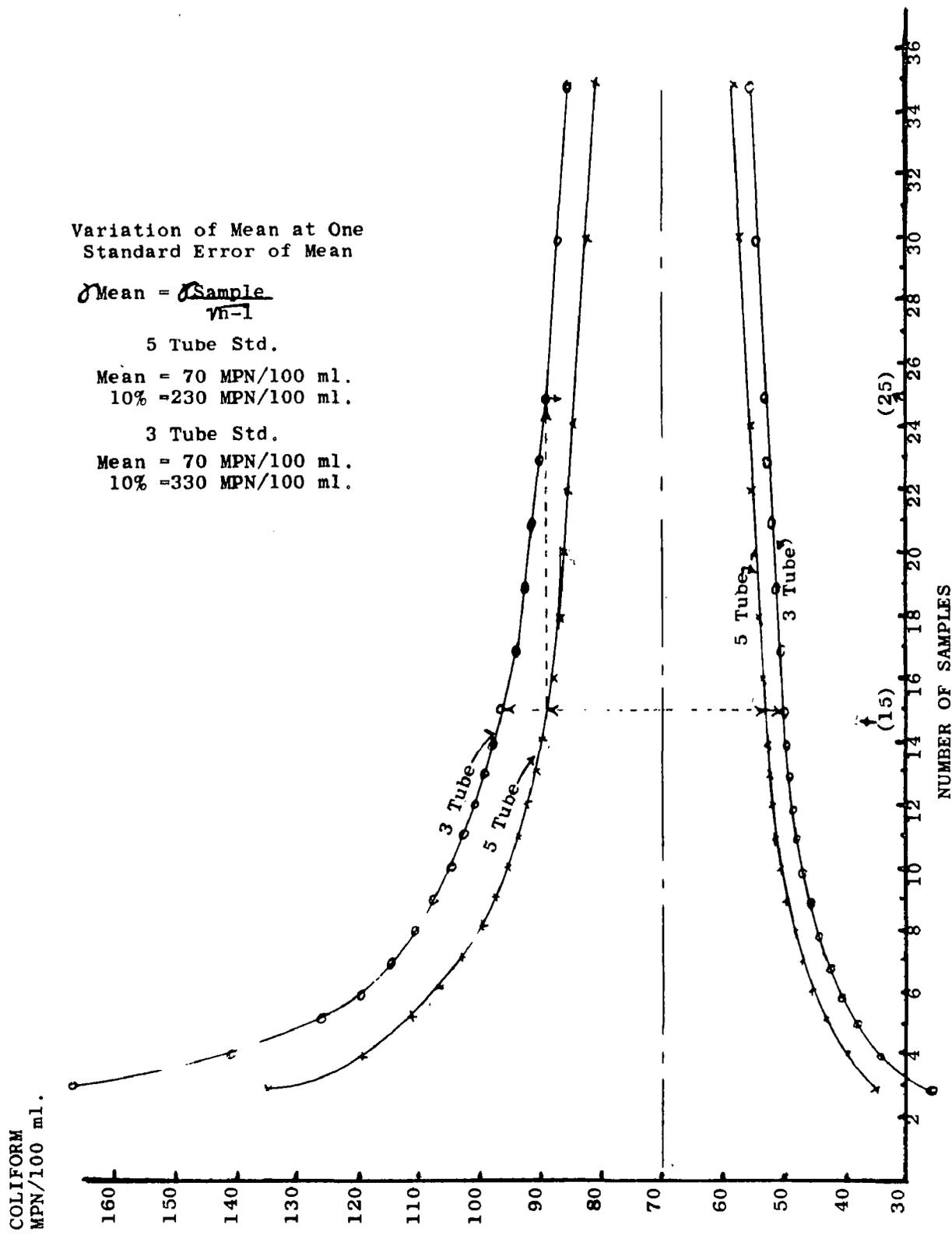
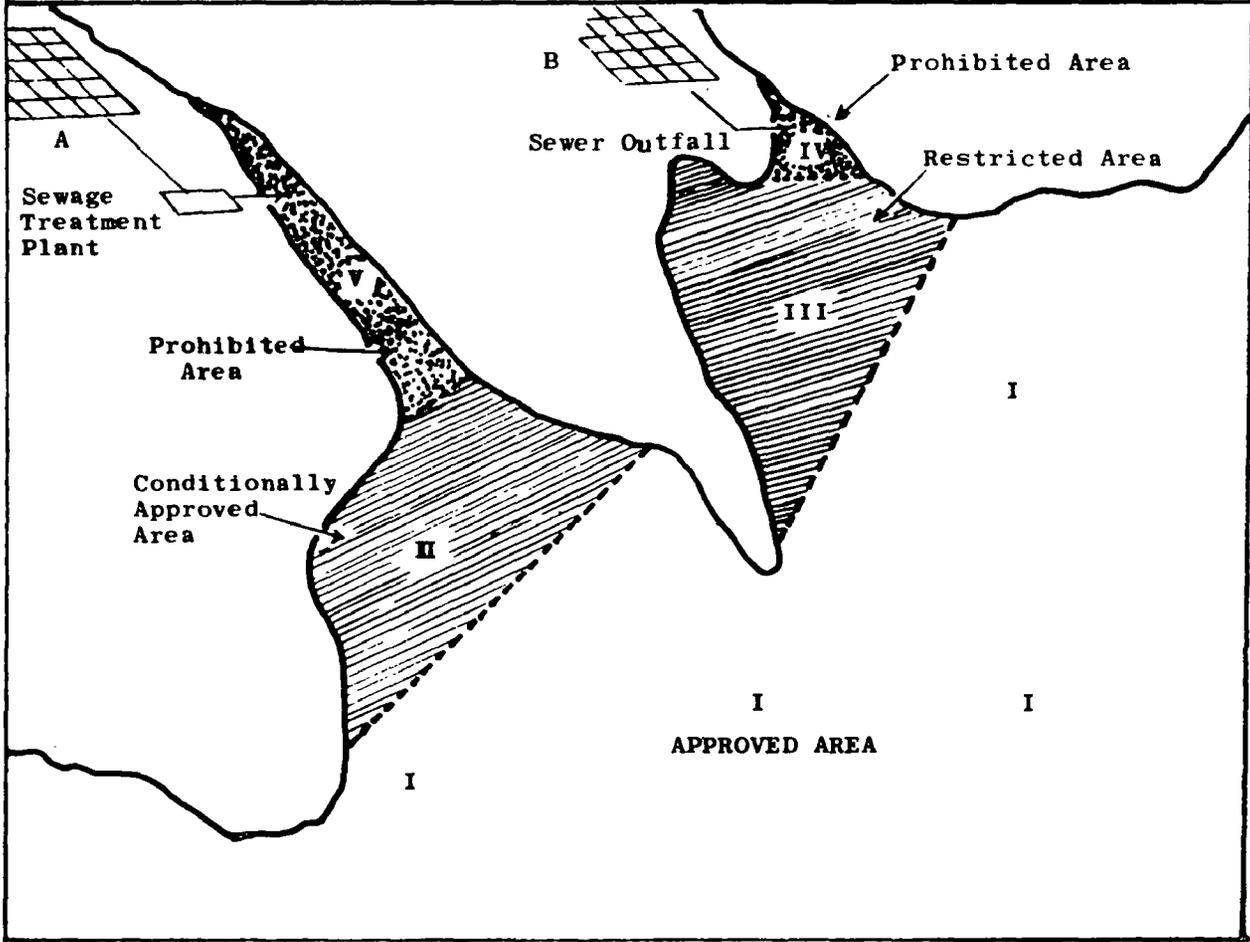


FIGURE VII

FIGURE VIII



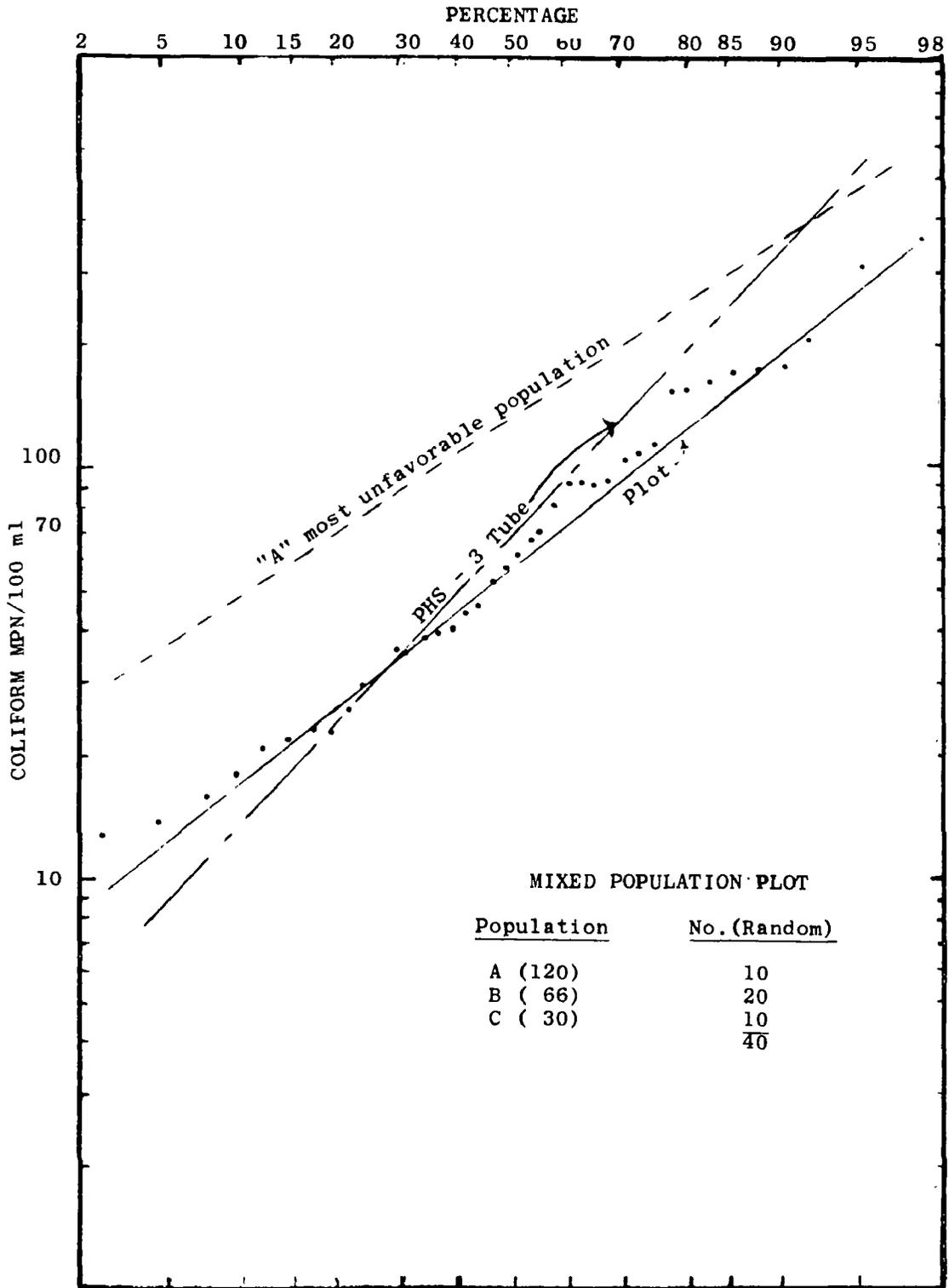


FIGURE IX

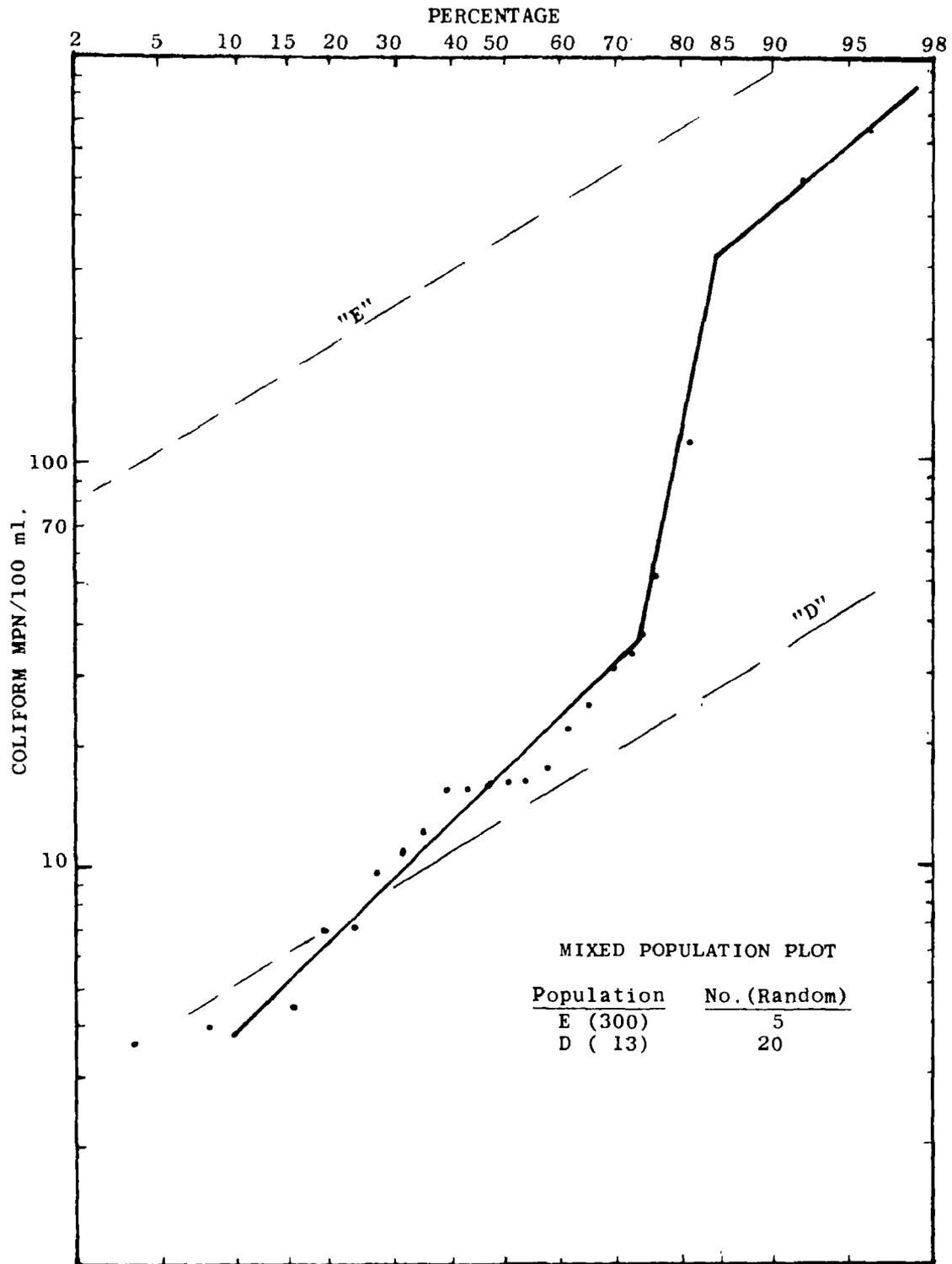
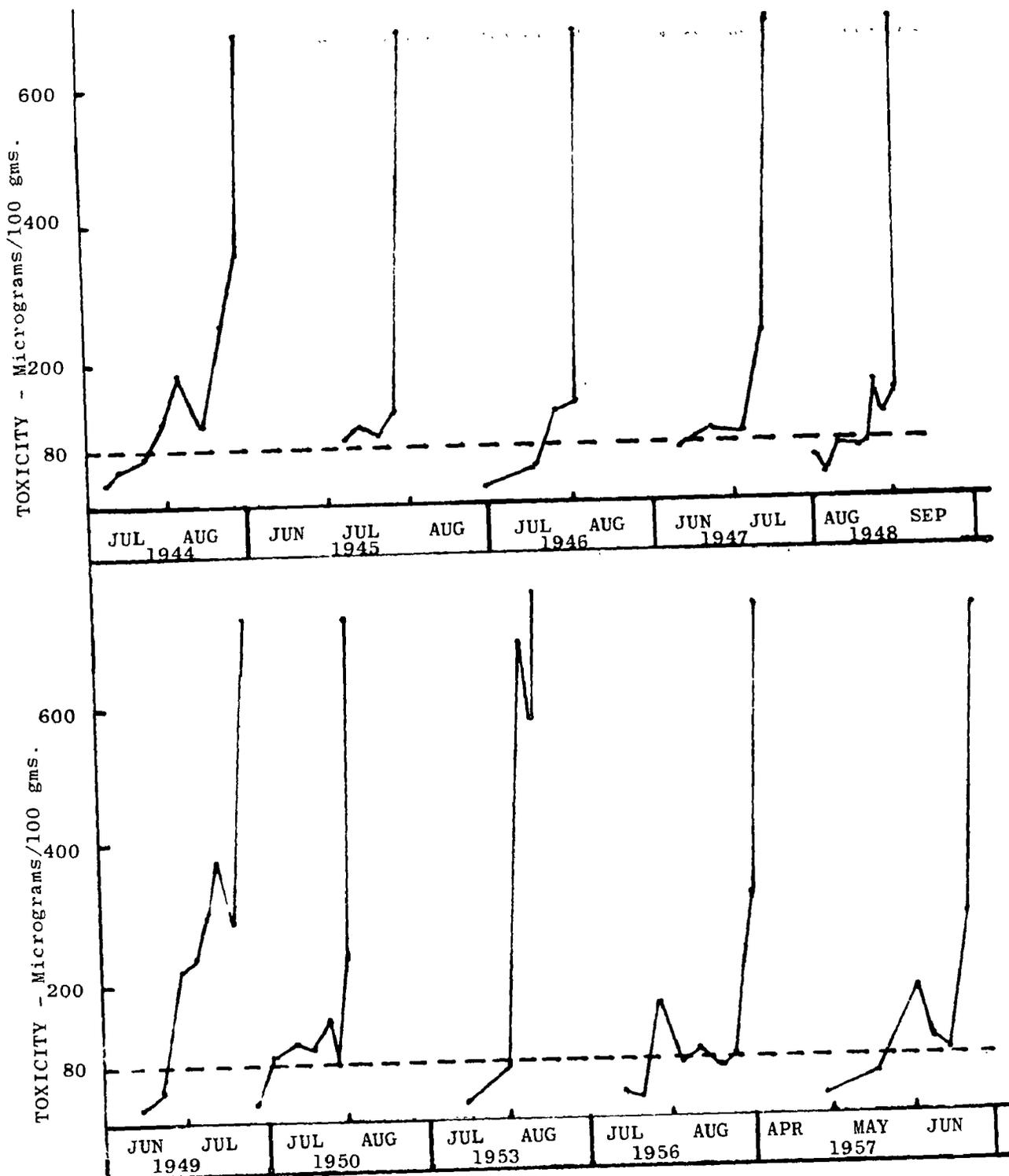


FIGURE X



Initial Toxicity Rise -
Key Station, Head Harbour, N.B.

FIGURE XI

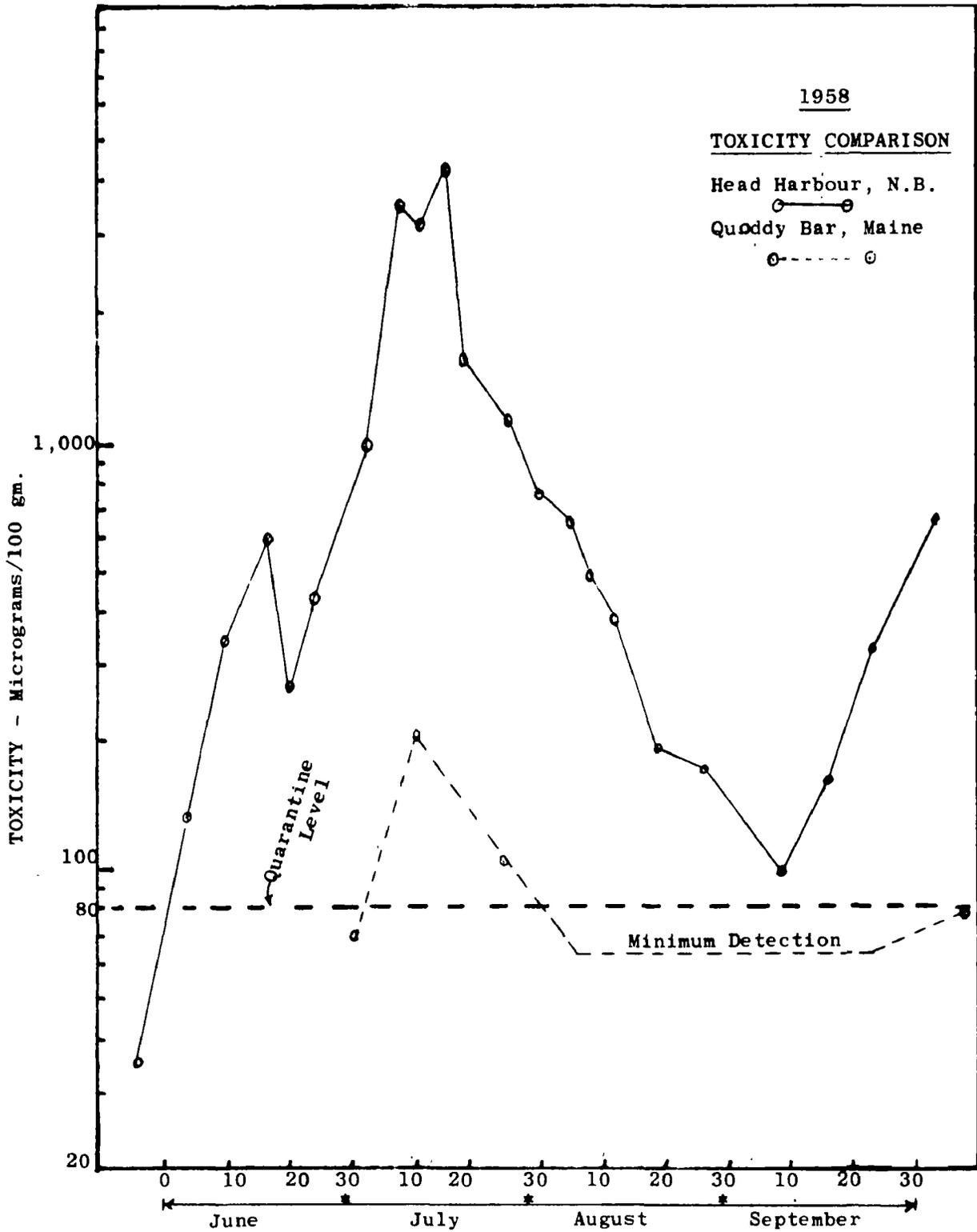


FIGURE XII

BIOLOGICAL ASPECTS OF NATURAL SELF PURIFICATION

I INTRODUCTION

- A The results of natural self purification processes are readily observed. Did they not exist, sewage (and other organic wastes) would forever remain, and the world as we know it would long ago have become uninhabitable. Physical, chemical, and biological factors are involved. The microscopic and macroscopic animals and plants in a body of water receiving organic wastes are not only exposed to all of the various (ecological) conditions in that water, but they themselves create and profoundly modify certain of those conditions.
- B Since toxic chemicals kill some of or all of the aquatic organisms, their presence disrupts the natural self purification processes, and hence, will not be considered here. The following discussion is based solely on the effects of organic pollution such as sewage or other readily oxidizable organic wastes.
- C This description is based on the concept of a "stream" since under the circumstances of stream or river flow, the events and conditions occur in a linear succession. The same fundamental processes occur in lakes, estuaries, and oceans, except that the sequence of events may become telescoped or confused due to the reduction or variability of water movements.
- D The particular biota (plants and animals, or flora and fauna) employed as illustrations below are typical of central United States. Similar or equivalent forms occur in similar circumstances in other parts of the world.
- E This presentation is based on an unpublished chart produced by Dr. C.M. Tarzwell and his co-workers in 1951. Examples from this chart are employed in the presentation.

II THE STARTING POINT

- A A normal unpolluted stream is assumed as a starting point. (Figure 1)
- B The cycle of life is in reasonably stable balance.
- C A great variety of life is present, but no one species or type predominates.
- D The organisms present are adjusted to the normal ranges of physical and chemical factors characteristic of the region, such as the following:
 - 1 The latitude, turbidity, typical cloud cover, etc. affect the amount of light penetration and hence photosynthesis.
 - 2 The slope, cross sectional area, and nature of the bottom affect the rate of flow, and hence the type of organisms present deposition of sludge, etc.
 - 3 The temperature affects both certain physical characteristics of the water, and the rate of biological activity (metabolism).
 - 4 Dissolved substances naturally present in the water greatly affect living organisms (hard water vs. soft water fauna and flora).
- E Clean water zones can usually be characterized as follows:
 - 1 General features:
 - a Dissolved oxygen high
 - b BOD low
 - c Turbidity low
 - d Organic content low

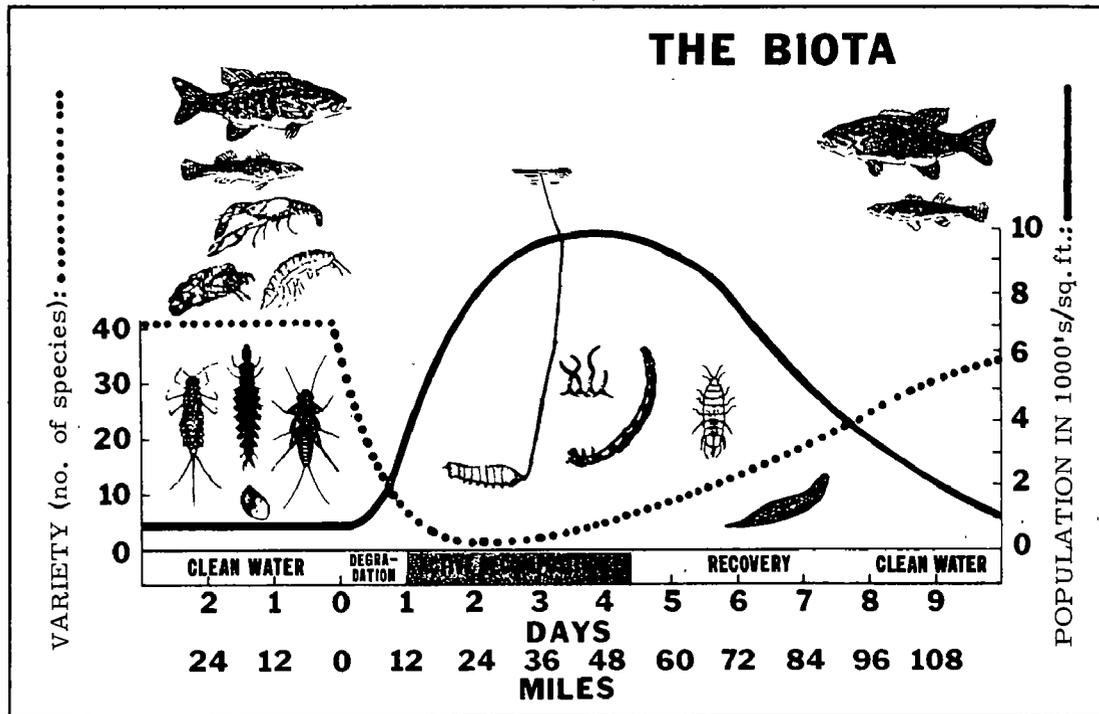


Figure 1: Relations between variety and abundance (production) of aquatic life, as organic pollution (discharged at mile 0) is carried down a stream. Time and distance scales are only relative and will be found to differ in nearly every case. After Bartsch and Ingram.¹

- e Bacterial count low
 - f Numbers of species high
 - g Numbers of organisms of each species moderate or low
 - h Bottom free of sludge deposits
- 2 Characteristic biota includes a wide variety of forms such as:
- a A variety of algae and native higher (vascular, or rooted) plants
 - b Caddis fly larvae (Trichoptera)
 - c Mayfly larvae (Ephemeroptera)
 - d Stonefly larvae (Plecoptera)
 - e Damselfly larvae (Zygoptera)
 - f Beetles (Coleoptera)
 - g Clams (Pelecypoda)
 - h Fish such as:
 - Minnows (Notropid types)
 - Darters (Etheostomatidae)
 - Millers thumb (Cottidae)
 - Sunfishes and basses (Centrarchidae)
 - Sauger, yellow perch, etc. (Percidae)
 - Others
- 3 Organisms characteristic of clean lakes, estuaries, or oceanic shores might be substituted for the above, and likewise in the following sections. However, it should be recognized that no single habitat is as thoroughly understood in this regard as the freshwater stream.
- well organized events are initiated. Important items to observe in interpreting the pollutional significance of stream organisms are the following:
 - B Numbers of species present, they tend to decrease with pollution.
 - C Numbers of individuals of each species tends to increase with pollution.
 - D Ratios between types of organisms are disturbed by pollution.
 - 1 Clean water species intolerant of organic pollution tend to become scarce and unhealthy.
 - 2 Animals with air breathing devices or habits tend to increase in numbers.
 - 3 Scavengers become dominant
 - 4 Predators disappear
 - 5 Higher plants, green algae, and most diatoms tend to disappear.
 - 6 Blue green algae often become conspicuous
 - E The importance of observations on any single species is very slight.

III POLLUTION

- A With the introduction of organic pollution (Figure 1, day 0), a succession of fairly

IV THE ZONE OF RECENT POLLUTION

- A The zone of recent pollution begins with the act of pollution, the introduction of excessive organic matter: food for microorganisms (Figure 1, day 0)
- B There follows a period of physical mixing.
- C Many animals and plants are smothered or shaded out by the suspended material.
- D With this enormous new supply of food material, bacteria and other saprophytic microorganisms begin to increase rapidly.

E The elimination of intolerant predatory animals allows the larger scavengers to take full advantage of the situation.

F This explosive growth of organisms, particularly fungi and bacteria, draws heavily on the free dissolved oxygen for respiration, and may eventually eliminate it.

G The number of types of organisms diminishes but numbers of individuals of tolerant types may increase.

H Zone of degeneration, or recent pollution, can usually be characterized as follows:

1 General features:

- a DO variable, 2 ppm to saturation
- b BOD high
- c Turbidity high
- d Organic content high
- e Bacterial count variable to high
- f Number of species declines from clean water zone
- g Number of organisms per species tends to increase
- h Other: Slime may appear on bottom

2 Characteristic biota:

- a Fewer higher plants, but rank heavy growth of those which persist
- b Increase in tolerant green, and blue green algae
- c Midge larvae (Chironomidae) may become extremely abundant
- d Back swimmers (Corixidae) and water boatmen (Notonectidae) often present
- e Sludge worms (Tubificidae) common to abundant.

f Dragonflies (Anisoptera) often present have unique tail breathing strainer

g Fish types, eg:

- Fathead minnows (Pimephales promelas)
- White sucker (Catostomus commersonni)
- Bowfin (Amia calva)
- Carp (Cyprinus carpio)

V THE SEPTIC ZONE

A The exact location of the beginning of the septic zone, if one occurs, varies with season and other circumstances. (Figure 1, day 1)

B Lack of free DO kills many microorganisms and nearly all larger plants and animals, again replenishing the mass of dead organic material.

C Varieties of both macro and micro-organisms and adjustable types (facultative) that can live in the absence of free oxygen (anaerobic) take over.

D These organisms continue to feed on their bonanza of food (pollution) until it is depleted.

E The numbers of types of organisms is now at a minimum, numbers of individuals may or may not be at a maximum.

F The septic zone, or zone of putrefaction can usually be characterized as follows:

1 General features:

- a Little or no DO during warm weather
- b BOD high but decreasing
- c Turbidity high, dark; odoriferous
- d Organic content high but decreasing

- e Bacterial count high
- f Number of species very low
- g Number of organisms may be extremely high
- h Other: Slime blanket and sludge deposits usually present, oily appearance on surface, rising gas bubbles

2 Characteristic biota:

- a Blue green algae
- b Mosquito larvae
- c Rat-tailed maggots
- d Sludge worms (Tubificidae and similar forms). Small, red, segmented (annelid) worms seem to be characteristic of this zone in both fresh and salt waters, the world around.
- e Air breathing snails (Physa for example)
- f Fish types: None

3 Note: Fortunately, all polluted waters do not always degenerate to "septic" conditions.

VI THE RECOVERY ZONE

- A The septic zone gradually merges into the recovery zone. (Figure 1, day 4)
- B As the excessive food reserves diminish so do the numbers of anaerobic organisms and other pollution tolerant forms.
- C As the excessive demand for oxygen diminishes, free DO begins to appear and likewise oxygen requiring (aerobic) organisms.
- D As the suspended material is reduced and available mineral materials increase due to microbial action, algae begin to increase often in great abundance.

E Photosynthesis by the algae releases more oxygen, thus hastening recovery.

F Since algae require oxygen at all times for respiration (like animals), heavy concentrations of algae will deplete free DO during the night when it is not being replenished by photosynthesis.

G Consequently this zone is characterized by extreme diurnal fluctuations in DO.

H With oxygen for respiration and algae, etc. for food, general animal growth is resumed.

I The stream may now enter a period of excessive productivity which lasts until the accumulated energy (food) reserves have been dissipated.

J Zone of recovery may usually be characterized as follows:

1 General features:

- a DO 2 ppm to saturation
- b BOD dropping
- c Turbidity dropping, less color and odor
- d Organic content dropping
- e Bacterial count dropping
- f Numbers of species increasing

g Numbers of organisms per species decreasing, (with the increase in competition)

h Other: Less slime and sludge

2 Characteristic biota

- a Blue green algae
- b Tolerant green flagellates and other algae
- c Rooted higher plants in lower reaches
- d Midge larve (Chironomids)

- e Black fly larvae (Simulium)
- f Giant water bugs (Belostoma spp.)
- g Clams (Megalonais)
- h Fish types:
 - Green sunfish (Lepomis cyanellus)
 - Common sucker (Catostomus commersonni)
 - Flathead catfish (Pylodictis olivaris)
 - Stoneroller minnow (Campostoma anomalum)
 - Buffalo (Ictiobus cyprinellus)

3 Excessive production and extreme variability often characterize middle and lower recovery zones.

4 Unfortunately, many waters once polluted never completely "recover". Repollution is the rule in many areas so that after the initial pollution, clear out delineation of zones is not possible. Characterization of these waters may involve such parameters as productivity, BOD, some "index" figure, or other value not included here.

VII CLEAN WATER ZONE

A Clean water conditions again obtain when productivity has returned to a normal, relatively poor level, and a well balanced varied flora and fauna are present. (Figure 1, day "10") Conditions may usually be characterized as follows:

B General features: similar to upstream clean water except that it is now a larger stream.

C Characteristic biota: similar to upstream clean water fauna and flora except that species include those indigenous to a larger stream.

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BIOTA OF WASTEWATER TREATMENT PLANTS
(MICROSCOPIC INVERTEBRATES)

I GENERAL CONSIDERATIONS

- A Community rather than individual as a unit for study of the process; quantitative relationship among different populations - "population dynamics".
- B Sequential transformation of organic matter through the microbial life - a transference of materials and energy between microbial populations led to the development of functional synecology or productive ecology.
- C Microbes considered here include bacteria, protozoa, and microscopic metazoa; algae and fungi are important groups included elsewhere in more detail.
- D All microbial groups originate from a) the waste itself, b) washing waters, c) soil, d) dust from air, and e) incidental sources; only those members that can survive and establish themselves in the community are important; some are transient.
- E Some variations in composition of the microbial community in domestic sewage treatment due to climatic and other ecological factors; industrial wastes with specific waste matter may call for development of more restricted microbial community for degradation.
- F Most active microbial groups are: True bacteria, filamentous bacteria, fungi, protozoa; nematodes, rotifers, oligochaetes, and water-mites.

II BACTERIA

- A No ideal method for studying distribution and ecology of bacteria in waste-treatment. Total bacterial counts made on nutrient agar or gelatine reflect only a portion of the bacterial flora present.

- B Pseudomonads are probably the most versatile in their ability to attack a great variety of organic compounds, including petroleum products, phenolics, cyanides. Others, such as Achromobacter, Alcaligenes, Chromobacterium, Flavobacterium, Aerobacter, and Micrococcus, are also important genera. Actinomyces are prominent in wastes rich in cellulose and Bacillus organisms are starch attackers. Sulfur and iron bacteria are predominant in wastes rich in respective compounds.
- C Actinomyces, Bacillus spp., Aerobacter spp., and nitrogen-fixation bacteria are primarily soil dwellers and are almost always present in any type of wastes in small numbers.
- D Parasitic and pathogenic bacteria, if present, are transient.
- E In extended aeration process with high dissolved oxygen, predominant species are limited to pseudomonads, Zoogloea ramigera, and Sphaerotilus.

III PROTOZOA

A Classification

- 1 Single-cell animals in the phylum Protozoa in the animal kingdom.

or
- 2 A separate kingdom, Protista, to include protozoa, algae, fungi, and bacteria.

- a Mastigophora (flagellates) - only the subclass Zoomastigina (non-pigmented) included; four orders:

- 1) Rhizomastigina - amoeba-flagellates with 1 or more flagella; examples: Mastigamoeba, Actinomonas, Rhizomastix
 - 2) Protomonadina - with 1 or 2 flagella; comprising most of the free-living forms; examples: Peranema, Bodo, Monas, Pleuromonas
 - 3) Polymastigina - with 3-8 flagella; mostly parasitic in gut of man and animals
 - 4) Hypermastigina - with numerous flagella; all parasitic in insect intestine
- b Ciliophora or Infusoria (ciliates) - largest class of protozoa; no pigmented members; most important group of protozoa in waste treatment; 2 subclasses:
- 1) Ciliata - cilia present during the entire trophic life; comprising most of the common ciliates; examples: Paramecium, Colpidium, Colpoda, Euplotes, Stylonychia, Vorticella, Opercularia, Epistylis, Carchesium
 - 2) Suctorina - cilia present while young and tentacles during trophic life
- c Sarcodina (amoebae) - pseudopodia (false feet) for locomotion and food-capturing; cell without cell-wall; some with test or shell; 2 subclasses:
- 1) Rhizopoda - pseudopodia without axial filaments; 5 orders:
 - a) Proteomyxa - with radiating pseudopodia; no test or shell
 - b) Mycetozoa (slime-molds) forming plasmodium; resembling fungi in sporangium formation.
 - 2) Actinopoda - with spinous pseudopodia; 2 orders:
 - a) Heliozoa - without central capsule; usually spherical in form with many radiating axopodia; examples: Actinosphaerium, Actinophrys
 - b) Radiolaria - pelagic in various oceans
 - 3) Sporozoa - no organ of locomotion; all parasitic (Plasmodium, Coccidia)
- d) Testacea - amoeba with single test or shell; examples: Arcella, Diffugia
- e) Foraminifera - large amoeba with calcareous shell; all marine forms
- c) Amoebina - true amoeba; pseudopodia in the form of lobopodia, no test or shell, cyst formation frequent; a few capable of flagellate transformation; examples: Naegleria, Amoeba, Hartmannella, Endamoeba

B General Morphology

1 Zoomastigina:

With the exception of Rhizomastigina which is amoeboid, the body has definite shape (oval, leaf-like, pear-like, etc.); most free-living forms with 1-2 flagella, some with 3 or more flagella, few forming colonies, cytostome present in many for feeding on bacteria; relatively small size (15-40 μ)

2 Ciliophora:

Most highly developed protozoa, with few exceptions, a macro- and a micro-nucleus; adoral zone, mouth, oral groove, usually present in swimming and crawling forms; stalked form with

conspicuous ciliation of a disc-like anterior region and little or no body cilia; cyst formed in most species

3 Sarcodina:

Cytoplasmic membrane but not cell-wall; cytoplasm with distinct ectoplasm and endoplasm in many common spp.; nucleus with large nucleolus in most of the free-living forms; some with the body enclosed in a test or shell and moving by protruding pseudopodia outside of the enclosure through an opening; few capable of temporary transformation into flagellate; fresh-water actinopods usually spherical with many radiating axopodia; some Testacea spp. containing symbiotic algae - mistaken for pigmented amoebae; cysts with single or double wall and 1-2 nuclei, parasitic amoebae forming cysts with 4 or more nuclei

C General Physiology

1 Zoomastigina

Free-living forms normally holozoic; food supply mostly bacteria; relatively aerobic, therefore, among the first to disappear in anaerobic conditions; reproduction by simple fission and occasionally by budding.

2 Ciliophora:

Holozoic; true ciliates concentrating food particles, i.e., bacteria, by ciliary movement around the mouth-part; suctoria sucking through tentacles, bacteria, small algae and protozoa constituting main food under normal conditions; not as aerobic as flagellates - a few surviving under highly anaerobic conditions, such as Metopus; reproducing by simple fission, conjugation, or encystation.

3 Sarcodina:

Mostly holozoic; feeding through engulfing by pseudopodia, food supply of small

amoebae mostly bacteria; large amoebae engulfing larger organisms; shelled amoebae, i.e., Arcella, feeding on a variety of organisms or saprozoic, reproduction by simple fission and encystation.

IV NEMATODA

A Classification

- 1 All in the phylum Nemata (nonsegmented round worms); 2 subphyla:

Secernentea (phasmids) 6 orders: Tylenchida (spear in mouth), Rhabditida (rhabditoid eosophagus), Strongylida (parasitic), Ascaridida (parasitic), Spirurida (parasitic), and Camallanida (parasitic), with the exception of tylenchids, all with papillae on male tail

Adenophora (aphasmids) 5 orders: Dorylaimida (spear in mouth), Chromodorida, Monhysterida, Enoplida, and Diocytophymatida; no papillae on male tail; no excretory canal

- 2 Nematodes encountered in polluted water and in sewage treatment mostly belonging to order Rhabditida and few in orders Dorylaimida and Tylenchida; those in Rhabditida being bacteria-feeders and those in the latter two, feeding on algae and other zoomicrobes; examples of rhabditids: Rhabditis, Diplogaster, Diplogasteroides, Monochoides, Cephalobus, Cylindrocorpus, Turbatrix, examples of the other two: Dorylaimus, Aphelenchoides

B General Morphology

Round, slender, nonsegmented (some with markings on outside); most of the free-living forms microscopic in size although dorylaimids up to several mm in length, sex separated but some parthenogenetic complete alimentary tract with elaborate mouth parts with or without spear (or stylet); no circulatory or respiratory system

C General Physiology

Most sewage treatment plant dwellers feeding on bacteria; others preying on protozoa, small nematodes, rotifers, etc.; clean water species vegetarians, DO diffused through cuticle; rhabditids tolerating lower DO than clean water spp; reproduction - eggs - larvae 4 molts) - adults

V ROTIFERS

A Classification:

- 1 Classified either as a class of the phylum Aschelminthes (various forms of worms) or as a separate phylum (Rotifera); commonly called wheel animalcules, on account of circular appearing movement of cilia around head (corona); corona contracted when crawling or swimming and expanded when attached to catch food.
- 2 Of the 3 classes, 2 (Seisonidea and Bdelloidea) grouped by some authors under Digononta (2 ovaries) and the other being Monogononta (1 ovary); Seisonidea containing mostly marine forms.
- 3 Class Bdelloidea containing 1 order (Bdelloida) with 4 families, Philodineidae being the most important.
- 4 Class Monogononta comprising 3 orders: Ploima with 14 families, Flosculariaceae with 4 families, and Collothecaceae with 1 family; most important genera included in the order Ploima (i. e., Brachionus, Keratella, Monostyla, Trichocerca, Asplanchna, Polyarthra, Synchaeta, Microcodon), common genera under the order Flosculariaceae: Floscularia, Limnias, Conochilus, and Atrochus
- 5 Unfortunately orders and families of rotifers based on character of corona and trophi (chewing organ), which are difficult to study, esp the latter; the foot and cuticle much easier to study.

B General Morphology and Physiology

- 1 Body weakly differentiated into head, neck, trunk, and foot, separated by folds; in some, these regions are merely gradual changes in diameter of body and without a separate neck, segmentation external only.
- 2 Head with corona, dorsal antenna, and ventral mouth; mastax, a chewing organ, located in head and neck, connected to mouth anteriorly by a ciliated gullet and posteriorly to a large stomach occupying much of the trunk.
- 3 Common rotifers reproducing parthenogenetically by diploid eggs; eggs laid in water, cemented to plants, or carried on femals until hatching.
- 4 Foot, a prolongation of body, usually with 2 toes; some with one toe; some with one toe and an extra toe-like structure (dorsal spur).
- 5 Some, like Philodina, concentrating bacteria and other microbes and minute particulate organic matter by corona; larger microbes chewed by mastax; some such as Monostyla feeding on clumped matter, such as bacterial growth, fungal masses, etc. at bottom; virus generally not ingested - apparently undetected by cilia.
- 6 DO requirement somewhat similar to protozoa, some disappearing under reduced DO, others, like Philodina, surviving at as little as 2 ppm DO.

VI SANITARY SIGNIFICANCE

- A Pollution tolerant and pollution nontolerant species - hard to differentiate - requiring specialist training in protozoa, nematodes, and rotifers.
- B Significant quantitative difference in clean and polluted waters - clean waters containing large variety of genera and species but quite low in densities.

C Aerobic sewage treatment processes (trickling filters and activated sludge processes, even primary settling) ideal breeding grounds for those that feed on bacteria, fungi, and minute protozoa and present in very large numbers; effluents from such processes carrying large numbers of these zoomicrobes; natural waters receiving such effluents showing significant increase in all 3 categories.

D Possible Pathogen Carriers

- 1 Amoebae and nematodes grown on pathogenic enteric bacteria in lab; none alive in amoebic cysts; very few alive in nematodes after 2 days after ingestion; virus demonstrated in nematodes only when very high virus concentrations present; some free-living amoebae parasitizing humans.
- 2 Swimming ciliates and some rotifers (concentrating food by corona) ingesting large numbers of pathogenic enteric bacteria, but digestion rapid; no evidence of concentrating virus; crawling ciliates and flagellates feeding on clumped organisms.
- 3 Nematodes concentrated from sewage effluent in Cincinnati area showing live *E. coli* and streptococci, but no human enteric pathogens.

VII EXAMINATION OF SEWAGE TREATMENT EFFLUENT, AND SLUDGE FOR MICROBES

A Bacteria - Not Included

B Zoomicrobes -

The 12th edition of the Standard Methods (1965) has a part on Biologic Examination of Water, Wastewater, Sludge, and Bottom Materials, in which the sludge of sewage treatment is discussed, but very briefly. Much of the materials are concerned with sediment at bottom of natural bodies of water. Chang described a method for examination of water for nematodes, but

the method is not applicable to sewage treatment, sludge, or effluent.

- 1 Waste treatment - the method bound to be qualitative; material scraped from stones in trickling filters or the floc masses in activated sludge examined in slide-coverslip preparations for poor, moderate, or rich zoobiota; material relatively rich in zoobiota indicating satisfactory treatment process; protozoa, rotifers, and nematodes predominant, especially protozoa; bristle worms and watermites in smaller numbers; springtails and insect larvae present as grazing fauna on top of trickling filters.
- 2 Sludge - representative samples suspended in known quantities of dilution water and thoroughly shaken; filtered through bolting cloth or metal screen of comparable pore size to remove extraneous dead clumped matter; filtrate examined in Sedgewick Rafter (SR) counting cell for various zoomicrobes; fresh sludge desired or samples refrigerated.
- 3 Sewage effluent - samples "fixed" with formalin, merthiolate, or similar chemical not desirable for examination for zoomicrobes; 50-200 ml filtered through a 7- or 14-micron membrane and strained material washed with a few mls of dilution and examined in an SR cell for zoo-microbes quantitatively or qualitatively.

VIII USE OF ZOOMICROBES AS POLLUTION INDEX

A Idea not new, protozoa suggested long ago; many considered impractical because of the need of identifying pollution-intolerant and pollution-tolerant species - proto-zoologist required.

B Can use them on a quantitative basis - nematodes, rotifers, and nonpigmented protozoa present in small numbers in clean water. Numbers greatly increased

when polluted with effluent from aerobic treatment plant or recovering from sewage pollution; no significant error introduced when clean-water members included in the enumeration if a suitable method of computing the pollution index developed.

- C Most practical method involves the equation: $(A + B)/A = Z.P.I.$, where
 A = number of pigmented protozoa,
 B = other zoomicrobes, in a unit volume of sample, and Z. P. I. = zoological pollution index. For relatively clean water, the value of Z. P. I. close to 1; the larger the value above 1, the greater the pollution by aerobic effluent, or sewage during recovery. This is based on the fact that pigmented protozoa are members of clean water micro-fauna (stabilization pond excluded).

IX CONTROL

- A Chlorination of Effluent and Settling
- B Prolongation of Detention Time of Effluent
- C Modification of Waste Treatment
- D Elimination of Slow Sand Filters in Nematode Control

X LIST OF COMMON ZOOLOGICAL ORGANISMS FOUND IN SEWAGE TREATMENT PROCESS - TRICKLING FILTERS AND ACTIVATED SLUDGE PROCESS

PROTOZOA

Sarcodina - Amoebae

- Amoeba proteus; A. radiosa
- Hartmanella spp.
- Arcella vulgaris
- Naegleria gruberi
- Actinophrys sol

FLAGELLATA

- Bodo caudatus
- Pleuromonas jaculans
- Oikomonas termo
- Cercomonas longicauda
- Peranema trichophorum

Swimming type

Ciliophora:

- Colpidium colpoda
- Colpoda cucullus
- Glaucoma
- Paramecium caudatum; P. bursaria

Stalked type

- Opercularia spp. (short stalk dichotomous)
- Vorticella spp. (stalk single and contractile)
- Epistylis plicatilis (like Opercularia more colonial)
- Carchesium spp. (like Vorticella but colonial, both have spiral coiled stalk when contracted)

Crawling type

- Euplotes spp.
- Stylonychia mytilus
- Urostyla spp.
- Oxytricha spp.

NEMATODA

- Diplogaster spp.
- Monochoides spp.
- Diplogasteroides spp.
- Rhabditis spp.
- Pelodera spp.
- Aphelenchoides sp.
- Dorylaimus sp.

Cylindrocorpus sp.
Cephalobus sp.
Rhabdolaimus sp.
Monhystera sp.
Trilobus sp.

ROTATORIA

Diglena
Monstyla
Polyarthra
Philodina
Keratella
Brachionus

OLIGOCHAETA (bristle worms)

Aelosoma hemprichi (Aelosomatidae)
Aulophorus vagus (Naididae)
Tubifex tubifex (Tubificidae)
Pachydrilus lineatus (Enchytraeidae)

INSECT LARVAE

Metriocnemus spp. (midge)
Orthocladus spp. (midge)
Psychoda spp. (filter fly)

OTHER ARTHROPODA

Hydrochna sp. (Acarina, mite)
Platysieus tenuipes (Acarina, mite)
Hypogastrura (= Achorutes sub-viatica)
viaticus (Collembola, Springtail)
Folsomia sp. (Collembola, Springtail)
Tomocerus sp. (Collembola, Springtail)

MOLLUSCA

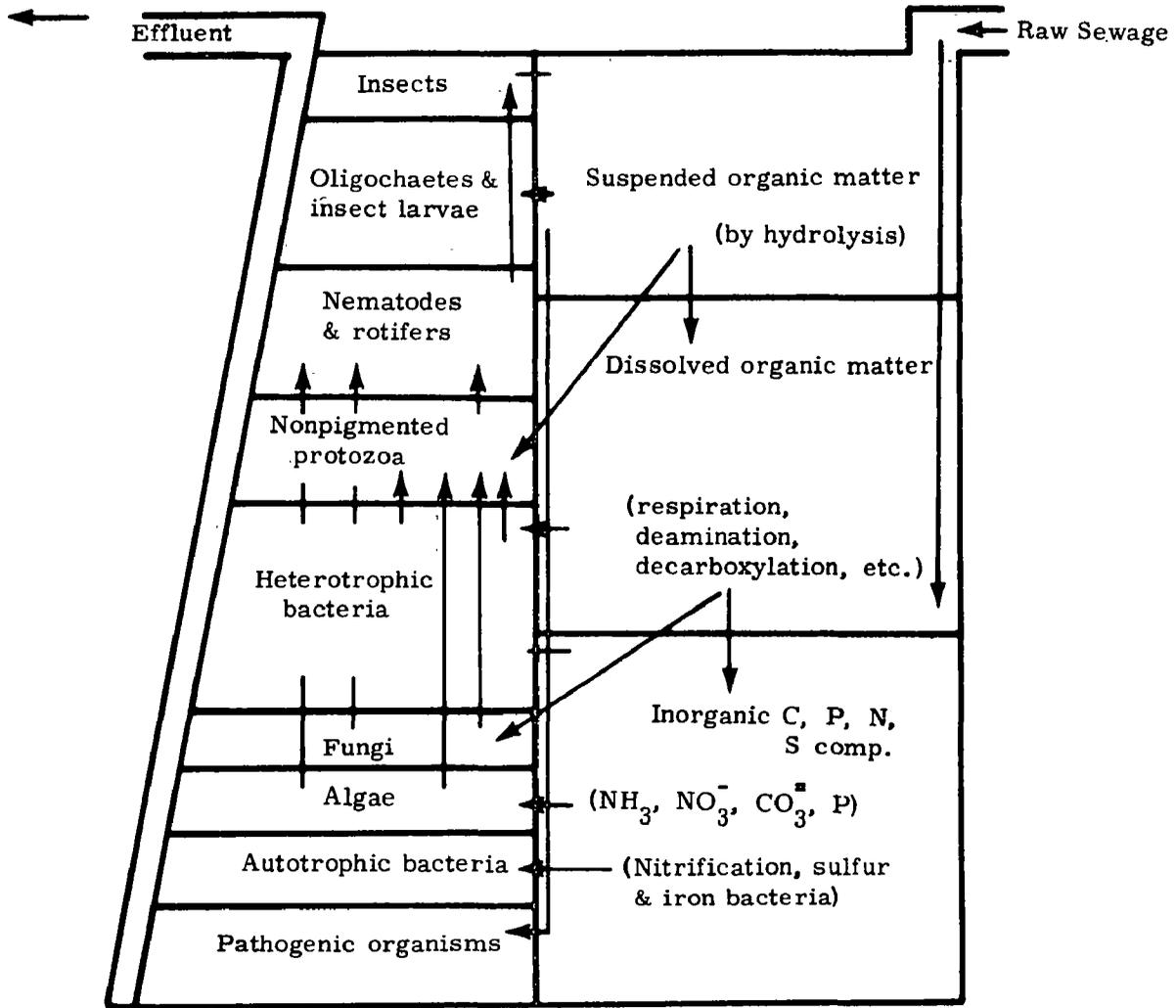
Lymnaea spp. (pulmonate snail)
Physa sp. (pulmonate snail)

XI POPULATION DYNAMICS AND THE FOOD CHAIN IN AEROBIC SEWAGE TREATMENT PROCESSES (Figure 1 and 2)

A Aerobic bio-oxidation of waste materials comparable to a food chain through which the dead organic matter is converted to inorganic matter during the stabilization process, e.g., waste organic matter → bacterial phase → zoological phase → inorganic matter.

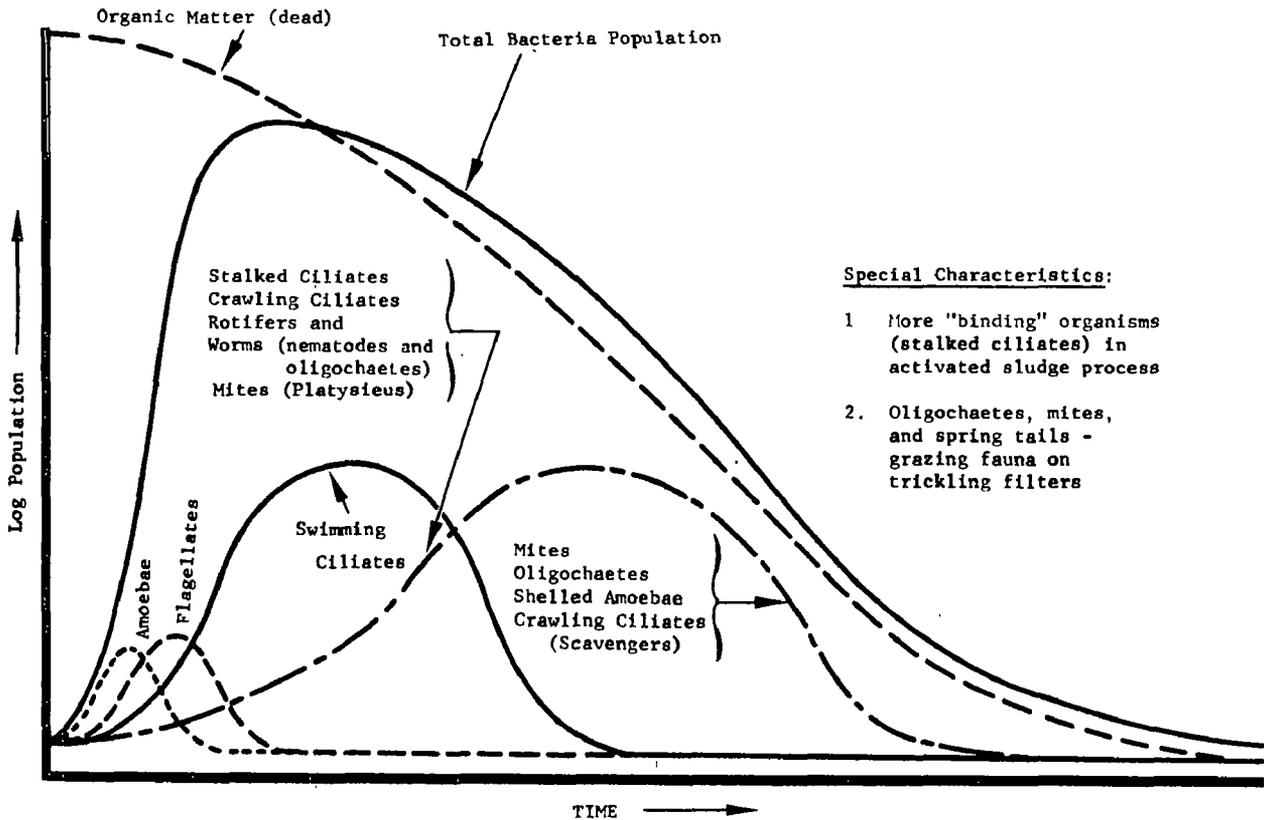
B Systematics, physiology and biochemistry involved in explaining the "chain reaction"; knowledge inadequate and fragmental, ecological study limited to principles governing the relationship of different groups of flora and fauna with each other and with the environment

C With adequate DO supply, bacterial population increases rapidly in the presence of rich organic food; flagellates and amoebae, which feed on bacteria and other small particulate matter in clumped material (such as growth film and floc masses), first show increase in population size, as suspended bacterial population increases to a high level, swimming ciliates, which feed actively on the suspended bacteria, also increase; increased consumption of bacteria and reduced supply of dead organic matter results in decline in bacterial population, which, in turn, results in a decline in the swimming ciliate population; the presence of large populations of small protozoa (ciliates, flagellates, and amoebae) results in an increase in populations of rotifers, nematodes, stalked ciliates, and crawling ciliates, which feed on the small protozoa and bacteria that are lodged in clumped masses; eventually, scavengers, such as mites, shelled amoebae, certain nematodes, and bristle worms become predominant, and bacteria and small protozoa populations drop to the pre-cycle level; rotifers that can concentrate bacteria in suspension, (such as Rotifer and Philodina, and nematodes), which have long surviving time, may remain for a long time; these zoomicrobes appear in the effluent in proportion to their respective population during treatment - nematodes, rotifers, ciliates predominant with small numbers of flagellates and amoebae; bristle worms unpredictable; mites few.



Food Chain in Aerobic Sewage Treatment Processes

Figure 1.



Population dynamics in Aerobic Sewage Treatment Process

Figure 2.

D Since sewage effluent from aerobic treatment processes are rich in nonpigmented zoomicrobes, discharge of effluent into natural causes great increase in their members; unpolluted waters usually have a much higher algae-to-nonpigmented-zoomicrobes ratio. The great increase

in the latter in water resulted from effluent pollution is likely to change this ratio, thus giving the basis for the Z.P.I. This analysis is not applicable to stabilization ponds due to the large algal population present in their effluents.

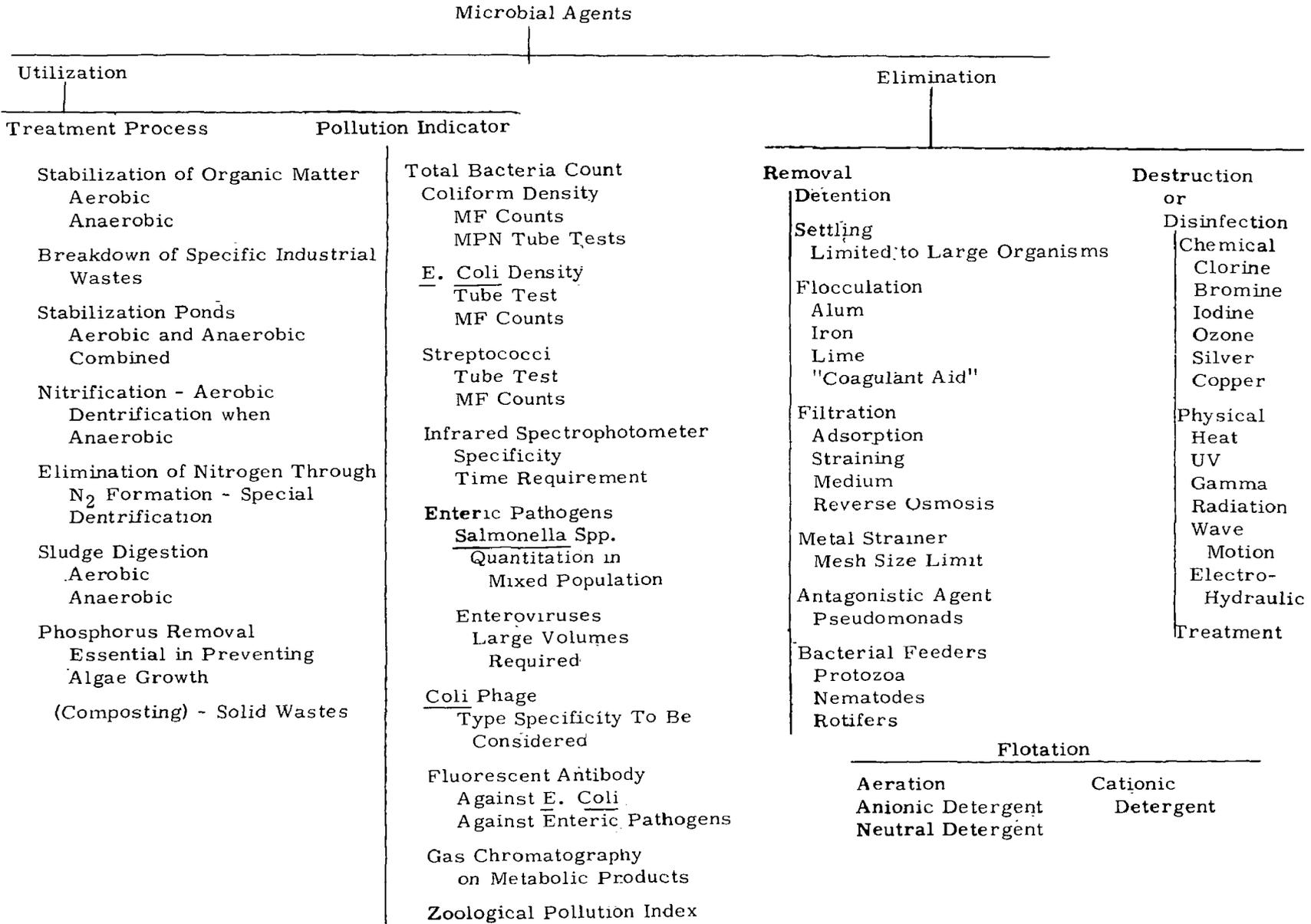
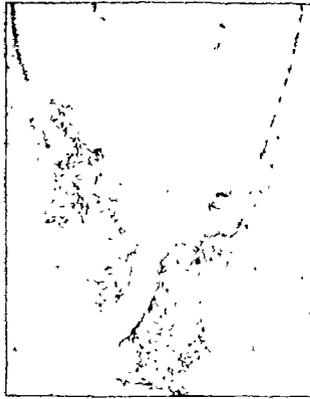


Figure 3

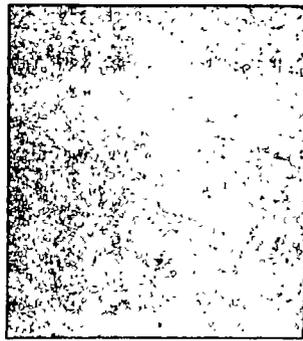
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a. Peranema trichoporum, 25u



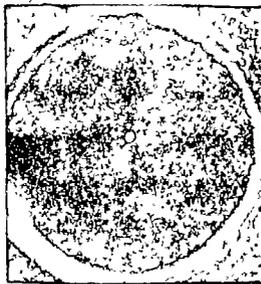
b. Naegleria gruberi amoeba stage, 18u



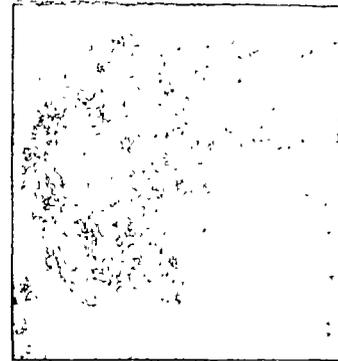
c. N. gruberi flagellate stage



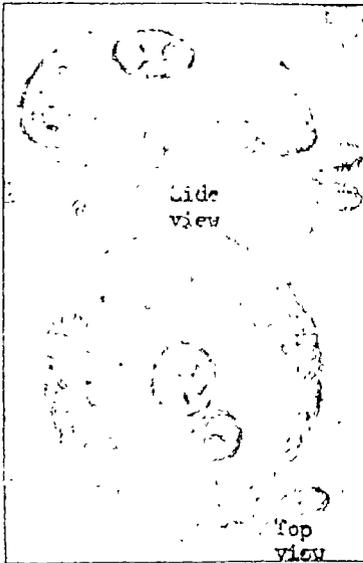
d. N. gruberi, cyst (2 nuclei) 12u



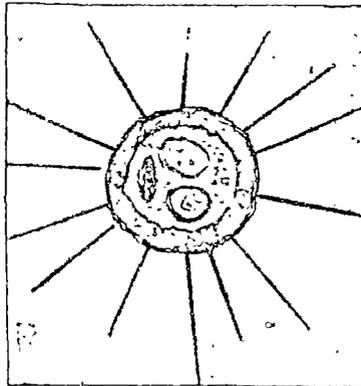
e. N. gruberi, cyst (1 nucleus)



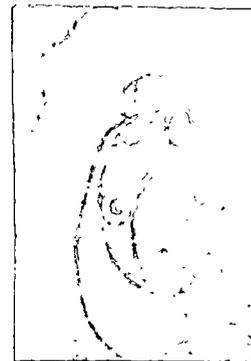
f. Endamoeba histolytica cyst (4 nuclei) 16u



g. Arcella vulgaris 40u



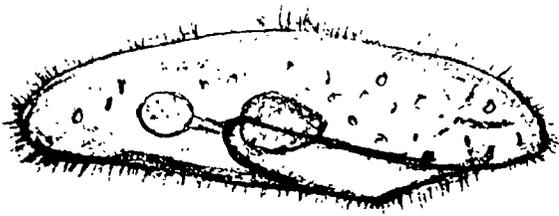
h. Actinosphaerium sp. 200-300u (with a navicula inside)



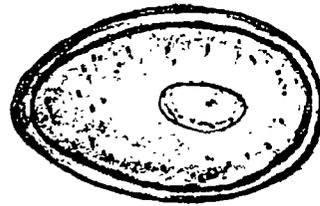
i. Bodo caudatus 10-20u

Fig. 1

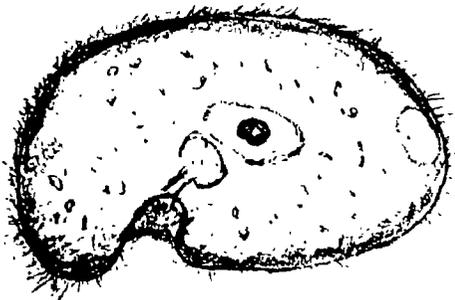
S. L. Chang, 1963



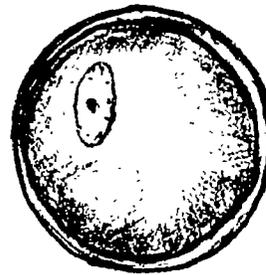
a. Paramecium caudatum
200 - 260u



b. P. caudatum
cyst



c. Colpoda sp. 20-120u



d. Colpoda cyst



Side view



Top view

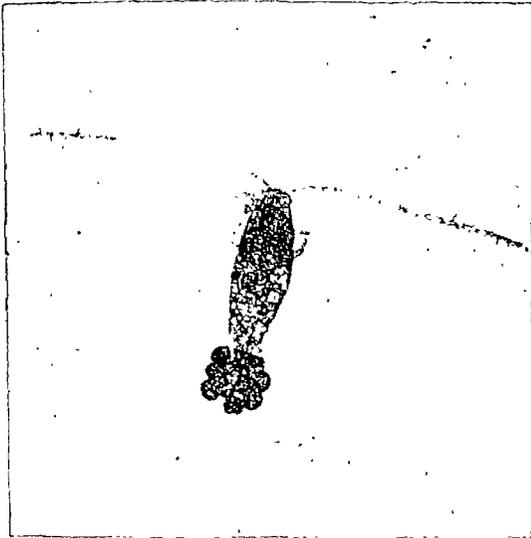
e. Euplotes carcinatus
70u



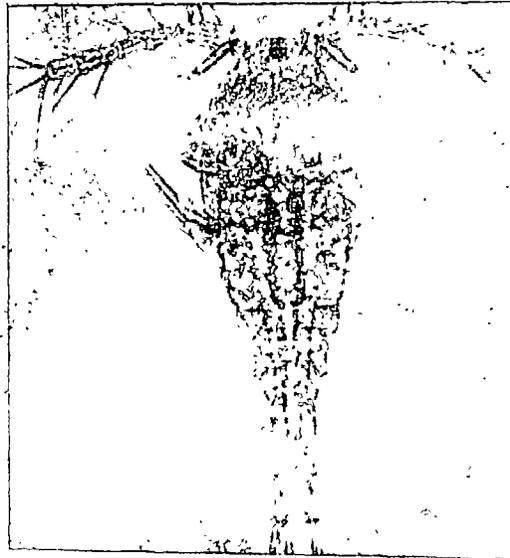
f. Vorticella 35-157u

Fig. 2

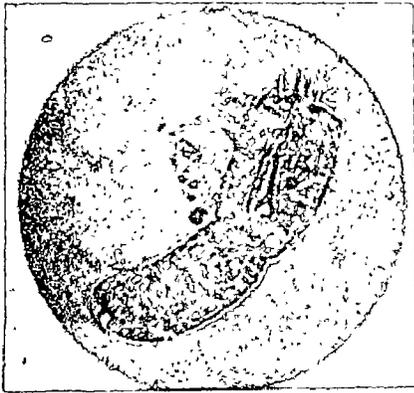
S. L. Chang, 1963



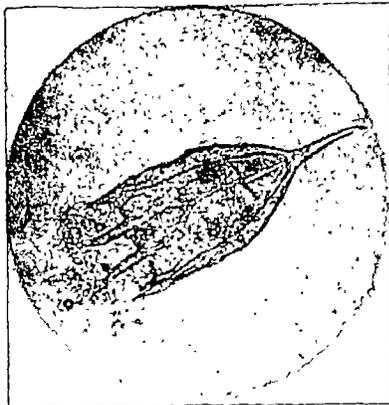
a. Diaptomus sp. 2 mm.
(2 egg sacs)



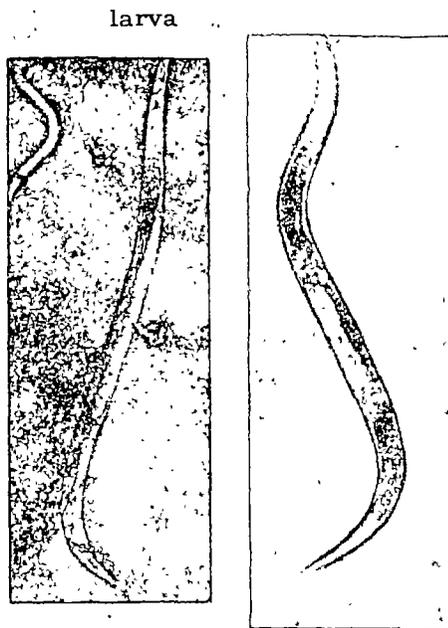
b. Cyclops sp. 2 mm.



c. Philodina sp. 45u



d. Anurea cochlearis 125u



larva
male female
e. Diplogaster nudicapitatus
about 1 mm.

Fig. 3

S. L. Chang, 1963

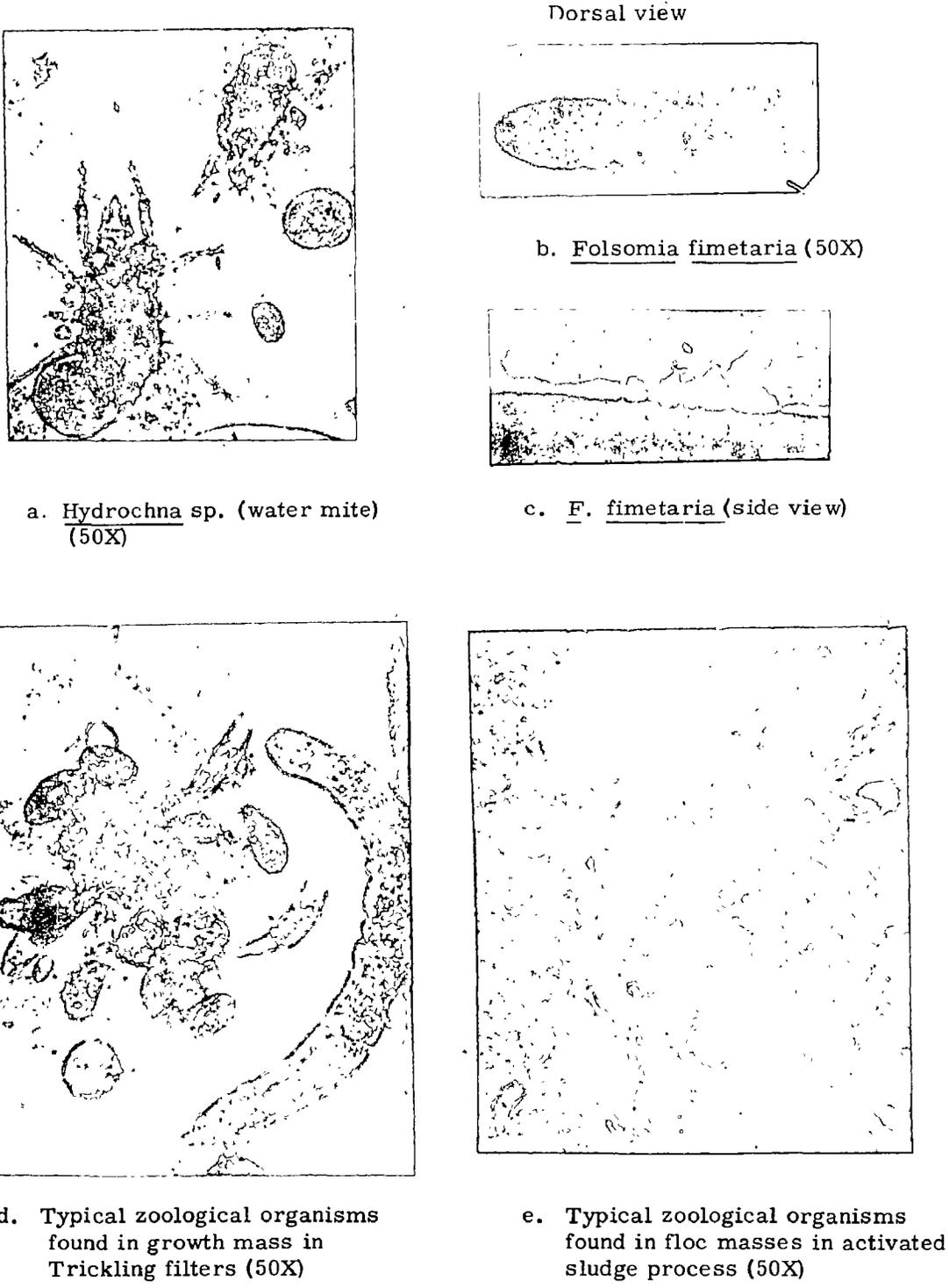
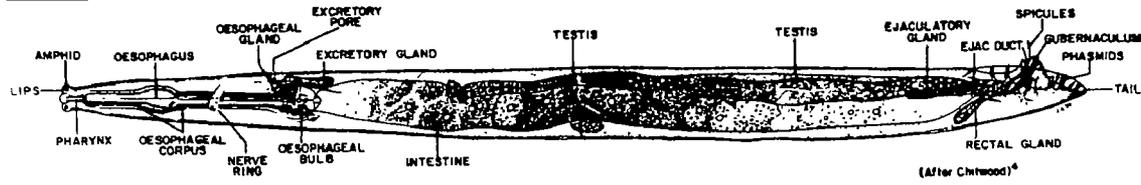


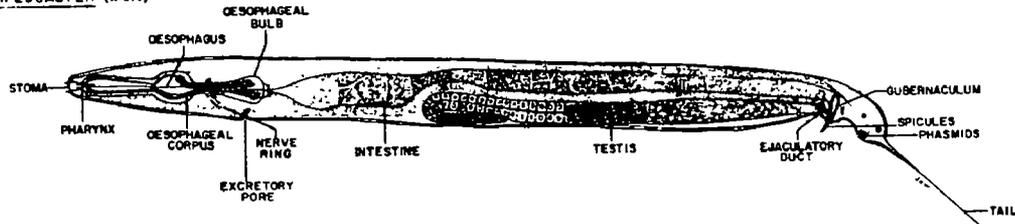
Fig. 4

S. L. Chang, 1963

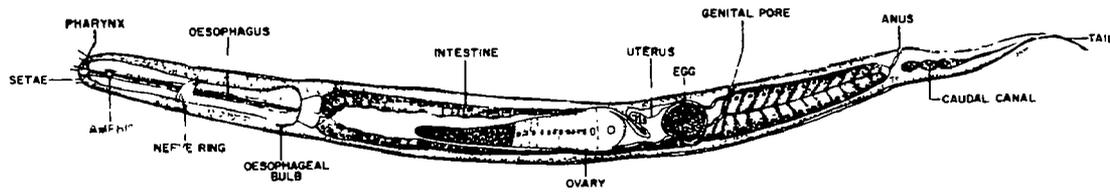
RHABDITIS (Male)



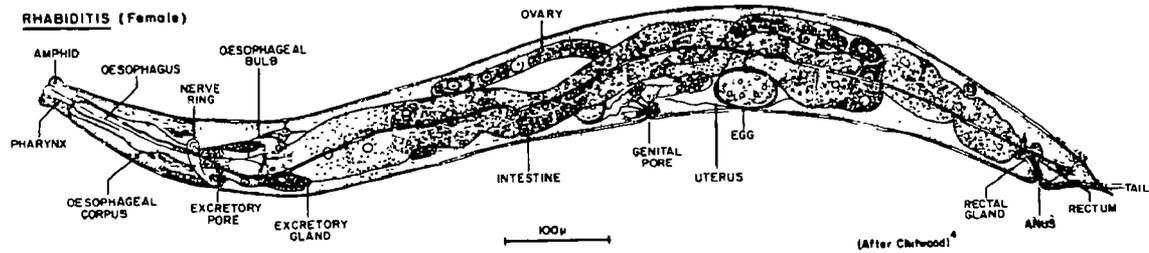
PLAGASTER (Male)



MONHYSTERA (Female)



RHABDITIS (Female)



Free Living Nematodes
(Nemathelminthes)

WASTEWATER TREATMENT - THE RESULT OF NATURAL PHENOMENA

Part 1

I INTRODUCTION

All sewage treatment is accomplished by application of biological, physical, chemical processes. These processes are natural phenomena which have been in operation since primeval time. Man has not always understood these processes and in fact we may not have a complete understanding of them at this time; nevertheless, it is by means of these phenomena that sewage treatment is possible.

II PHYSICAL PROCESSES

A Specific Density

The density of waste solids, coupled with the law of gravity, provides a physical phenomena resulting in removal of wastes. Sedimentation has been observed by man for thousands of years and a study of geologic formations reveal that sedimentation has been continuing for millions of years. In nature, the pools in streams, lakes, and estuaries provide the necessary conditions of quiescence to allow gravity separation of settleable solids.

In using the physical laws relating to gravity and specific density, man has used two processes:

- 1 Sedimentation in tanks built to provide quiescence, and
- 2 Centrifuge separation

B Particle Size Distribution

Screening sewage flows to remove large particles is merely an application of size selection. Screens abound in nature as settled rock deposits which prevent movement of twigs, sticks, leaves and other solids. The earth itself acts as a fine screen and filter, removing all water-borne material except those that are dissolved. In treatment plants, bar racks and sand filters are applications of these natural conditions.

C Reaeration

Few people have failed to take the time to see a waterfall or to enjoy the scenic beauty of a fast-flowing and turbulent mountain stream. These are nature's examples of reaeration facilities. In addition to these dramatic aerators, there is a constant exchange of molecules of oxygen and other atmospheric gases across the liquid-gas interface of rivers, lakes, ponds, and oceans. The wind provides mixing energy to carry the dissolved gases to portions of the water mass below the surface. Utilizing these principles as treatment processes, man injects air into the waste flow by use of air under pressure; the making of waterfalls by pumping the liquid into the air fountain-like; or, by creating an infinitely large surface area with depth being merely a thin film as the liquid trickles downward over beds of rock.

III BIOLOGICAL PROCESSES

In the real world the aquatic community is very complex, consisting of organisms of every size from the virus to the fishes. Each has a definite role in the community and in a natural environment--one unaffected by wastes from man's activities--there is a very great variety of different kinds and species. All are present in such numbers as will maintain a balance with the food supply available.

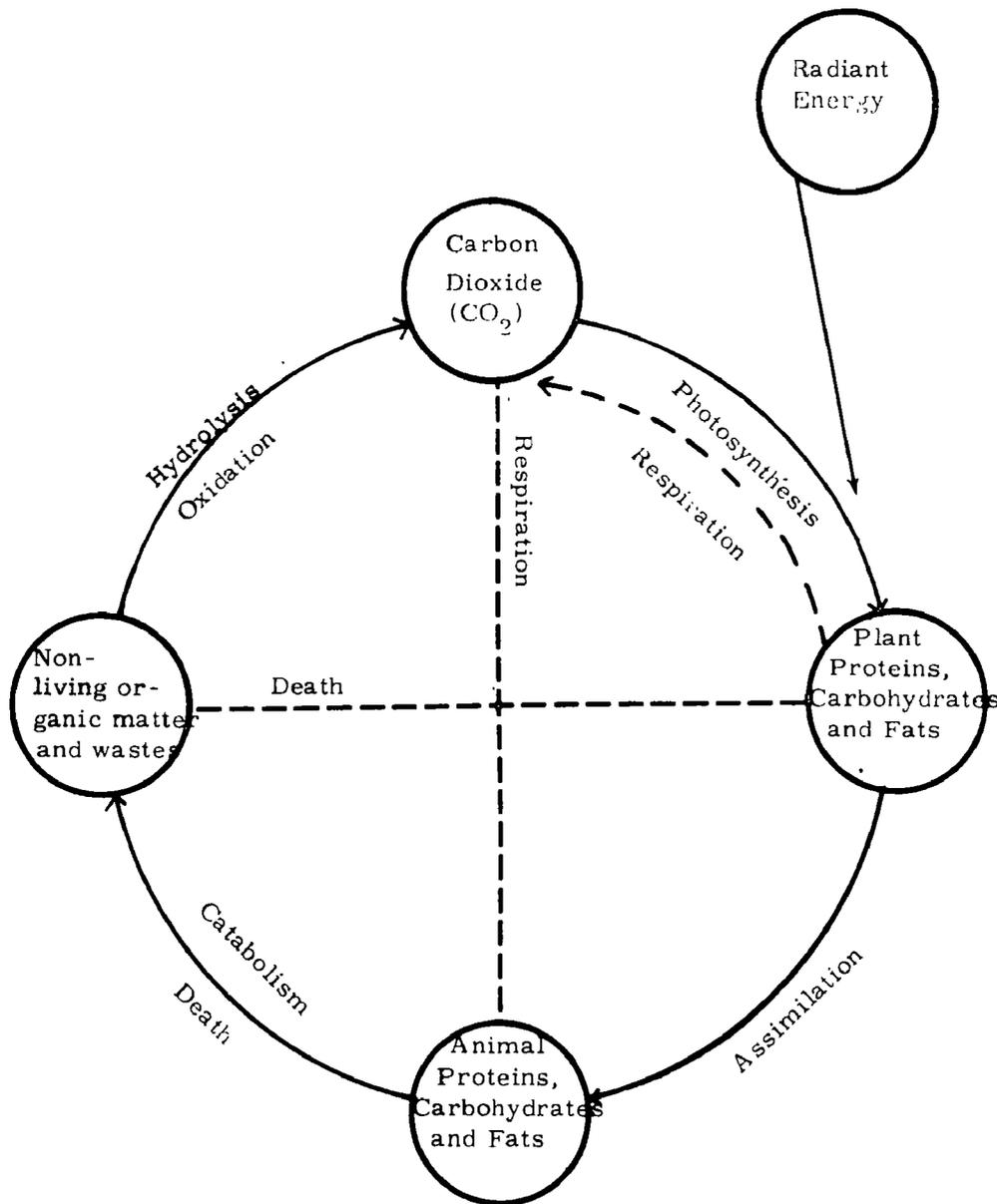
Action by bacteria in this community breaks down complex organic matter to simpler molecular forms. These become the basic building blocks for new growth by other microorganisms. These in turn are a food source for yet other, more complex, organisms. This activity of decomposition and growth is a continuous one--such that the process is cyclic.

All the elemental components of organic material--carbon, nitrogen, sulfur, etc.--are cyclic. The carbon cycle (Figure 1) is an example. It can be seen that once elements making up organic matter, from any source, enter the aquatic environment they will continue in the cycle indefinitely unless they are removed as a "Harvest" as fish or other product.

IV CHEMICAL PROCESSES

Chemical processes in the aquatic environment are intimately connected with biological activity and proceed simultaneously with photosynthesis, assimilation and decomposition.

In addition, the chemistry of water is a function of the solubility and presence of inorganic salts in the environment.



Carbon Cycle
Figure 1

The salt content of the oceans, the Dead Sea, the Salton Sea, and Great Lake are examples which indicate that once inorganic salts enter the aquatic environment they remain indefinitely as an integral part. It is only by means of evaporation that water high in inorganic salts is returned, in the form of rain and snow, to the fresh water state.

V LIMITATIONS OF NATURAL TREATMENT

- A Although wastes discharged into the aquatic environment enter the cycle previously described, time is required to reach a new balance during which time water quality may be seriously impaired. In addition, the new balance may not be a desirable one as excessive nutrients may bring about blooms of organisms causing nuisance conditions and/or foul odors and tastes.
- B It is axiomatic that elements of wastes removed prior to discharge into the aquatic environment do not enter these cycles and therefore cannot cause adverse effects.

C A stream has capacity to accept organic wastes and through natural processes to self-purify; however, its capacity to assimilate wastes without seriously affecting water quality for other uses is limited by such factors as stream flow, reaeration rate, temperature, etc.

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AEROBIC BACTERIAL SYSTEMS FOR INDUSTRIAL WASTES

Part 2

I INTRODUCTION

Natural processes carried out by common soil organisms under controlled conditions. To completely understand the process one must have some basic understanding of the microorganisms involved.

II BACTERIA

A General Structure - See Figure 1

B Size and Shape

Cocci, about 1 micron in diameter

Rods, from 0.5 to 2 microns by 0.75 to 10 microns

Spirals, about 1 micron by 10 microns

C Composition - $C_5H_7O_2N$ (average formula)

D Growth Curve - See Figure 2

III BACTERIAL REACTIONS

A Basic Concept

Bacteria are trying to make more bacteria from the substrate, but this requires energy. To obtain energy the bacteria takes some of the substrate (a minimum amount as the bacteria wants to use as much of the substrate as possible for new cells) and breaks it down to end products, thereby releasing energy, and this energy is used to create a new cell.

B General Reaction

Organic matter + bacteria + trace inorganics + N and P + H₂
Acceptor \longrightarrow end products + energy + more bacteria (See Figure 3).

C Enzymes

Organic catalysts which promote biological reactions. True catalysts since rate of reaction is proportional to the amount of enzyme present, also the enzyme is always regenerated. Most common make-up of enzyme:

Protein + metallic activator + coenzyme

Some common classifications of enzymes:

Extracellular - hydrolytic only.

Intracellular - all reactions; hydrolysis, energy, and synthesis.

Constitutive - always present in cell, whether being used or not.

Adaptive - must be continuously stimulated by substrate.

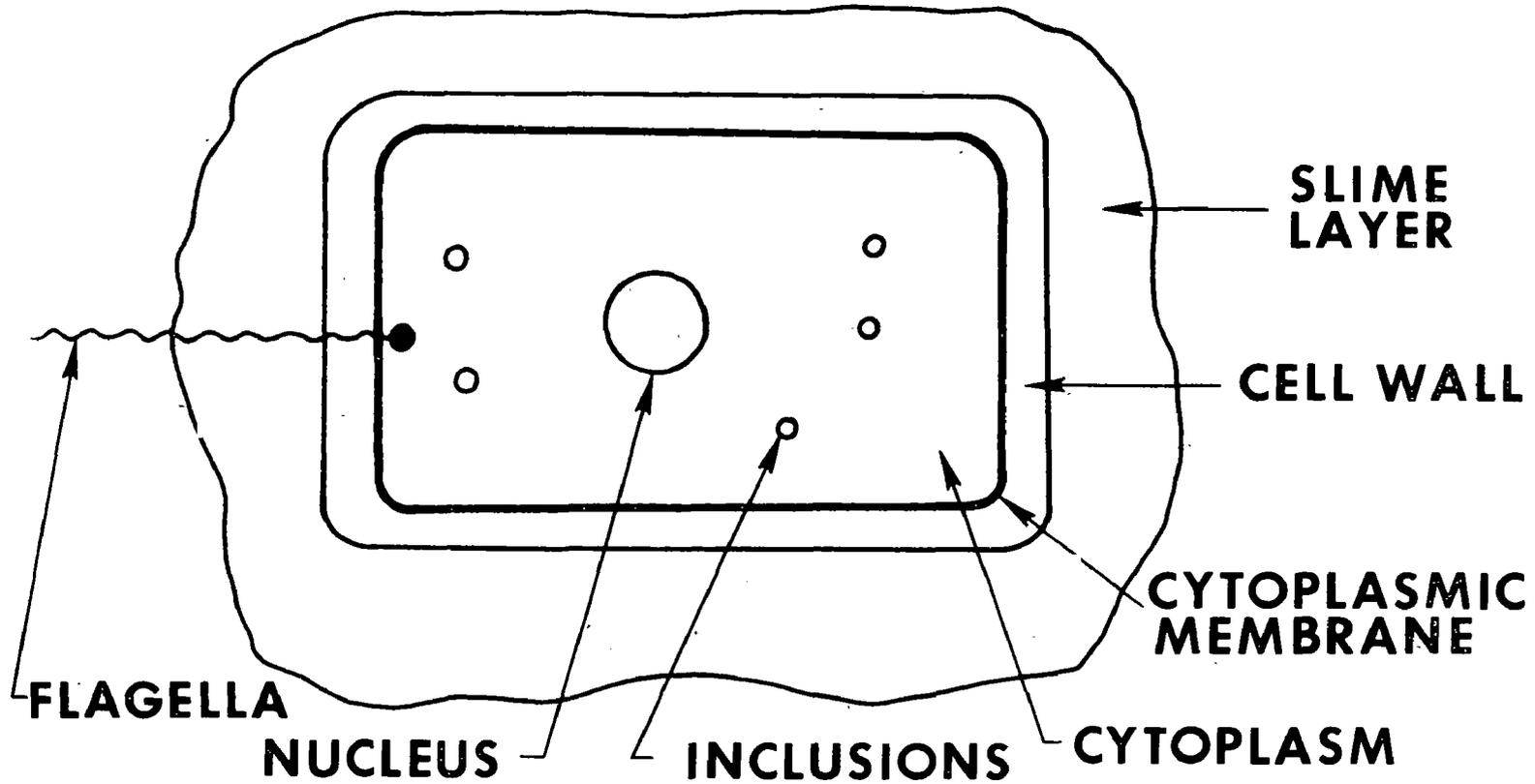
D Oxidative Phosphorylation (See Figure 4)

This is the mechanism by which the bacteria generate energy. The first step is the removal of hydrogen from the organic molecule by the coenzyme DPN⁺. These hydrogens are then passed from enzyme to enzyme until they are finally linked to oxygen (the hydrogen acceptor in this case) to form water. This process releases 30-50 KCal/cycle.

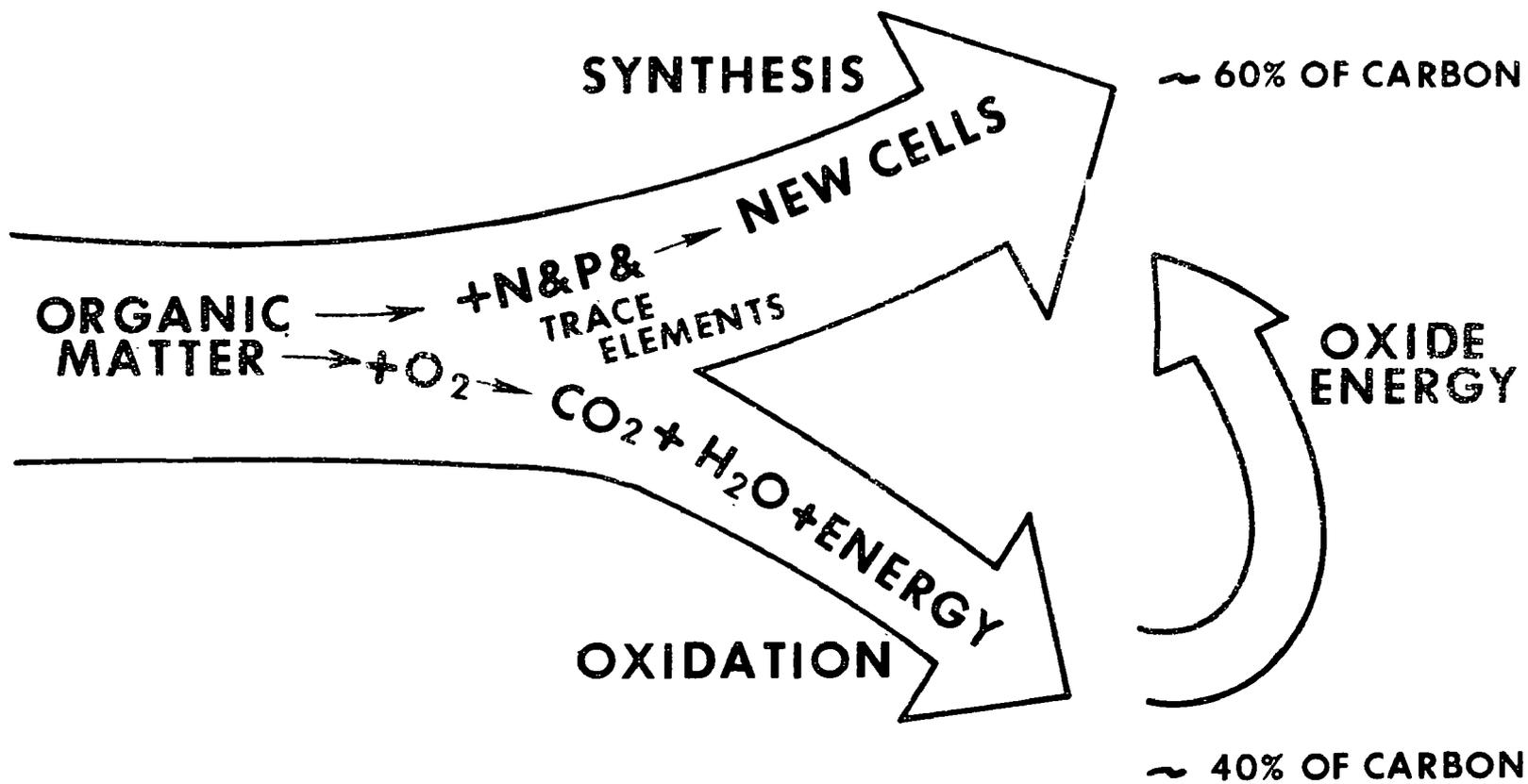
E Energy Transfer

The question is, what does the bacteria do with all this energy, how does it store it and then reuse it? The coenzyme adenosine triphosphate (ATP) contains three high energy phosphate bonds which store the energy. Three ATP are formed per cycle of oxidative phosphorylation. When a cell needs energy for a synthesis (cell building) reaction, some ATP is converted to ADP with the resulting release of energy.

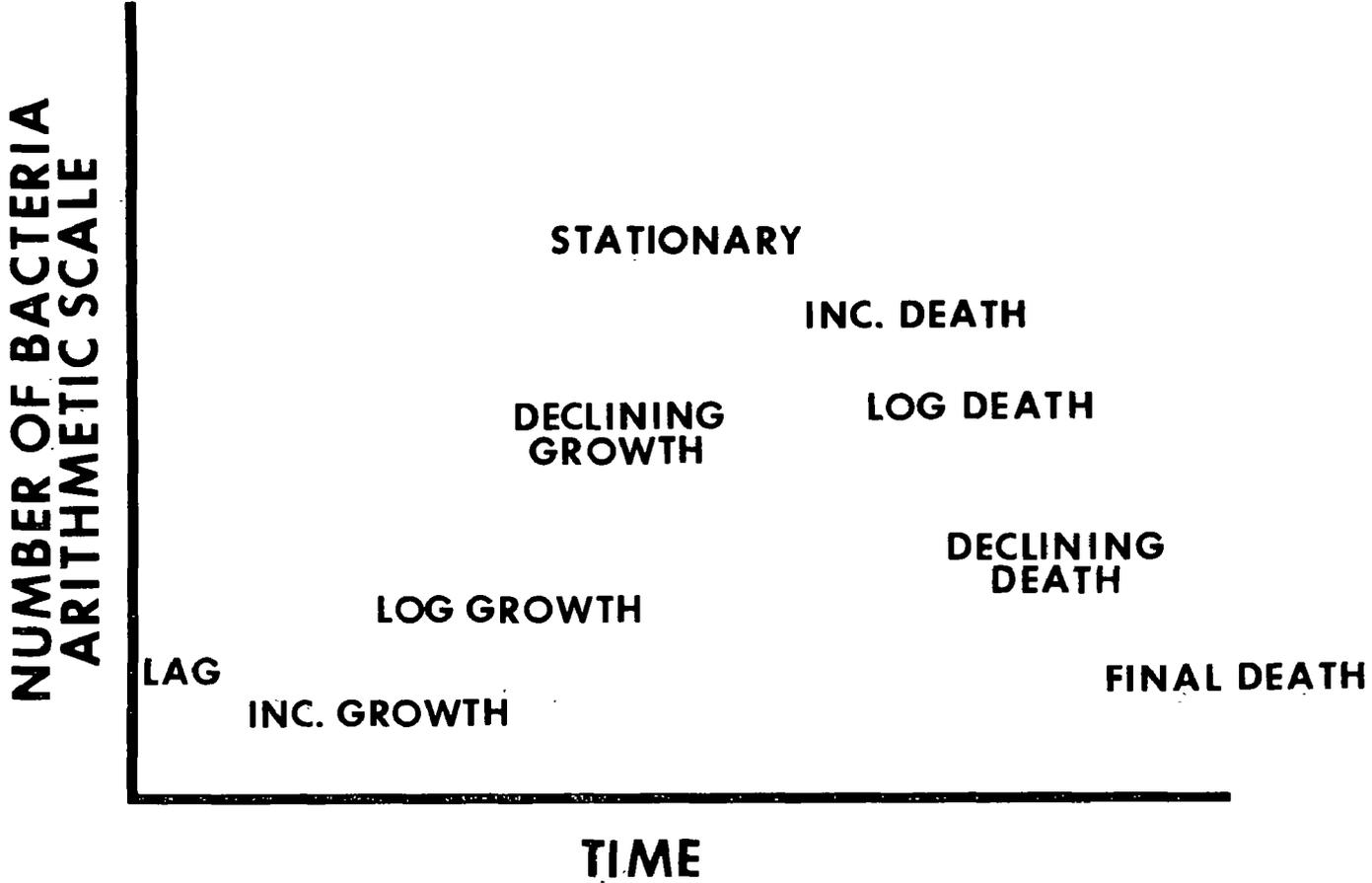
GENERAL STRUCTURE OF BACTERIA



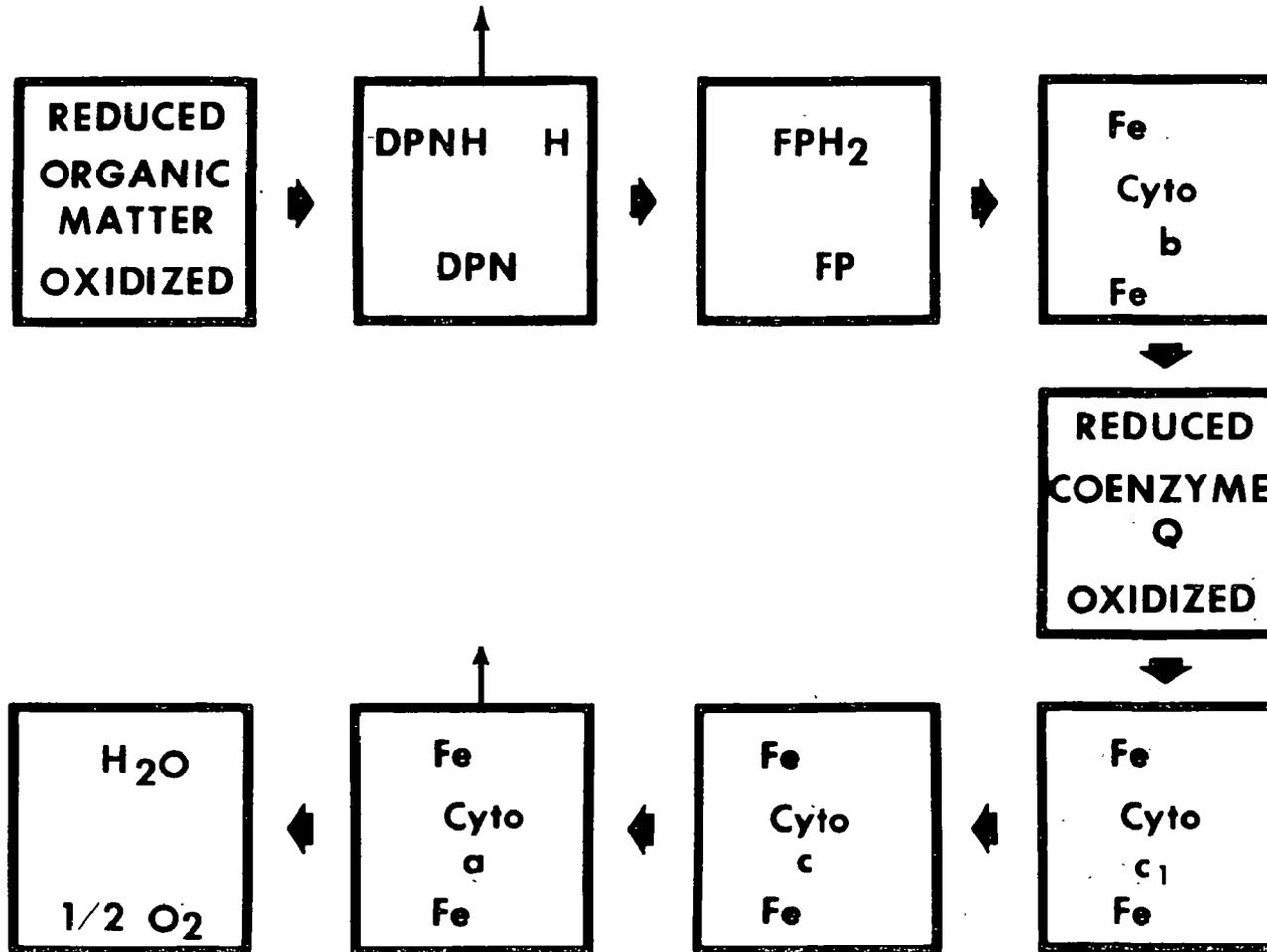
GENERAL METABOLIC REACTION



TYPICAL BACTERIAL GROWTH CURVE



OXIDATIVE PHOSPHORYLATION



F Oxidation

Complete oxidation is a rather complex process involving many hydrogen removal steps as well as the addition of water and splitting of organic molecules. In general, a given organic substrate must be biologically converted to pyruvic acid ($C_3H_4O_3$) before entering the Krebs or energy cycle. This is a nine-step cycle during which there is the loss of 2 CO_2 and the generation of 15 ATP. Note that pyruvic acid loses 1 CO_2 getting into the Krebs cycle so all three carbons in the pyruvic acid are oxidized to CO_2 .

G Synthesis

Synthesis pathways are not the reverse of oxidation pathways, but a complete synthesis scheme has been worked out so an entire cell, with all of its complex components, may be created from a simple substrate.

IV FLOCCULATION

The mechanism by which the newly created cells are coagulated into large enough masses to settle out in the settling tank. Mechanism is not well understood.

V OTHER ORGANISMS OF IMPORTANCE IN AEROBIC TREATMENT SYSTEMS

A Autotrophic Bacteria

Nitrifying bacteria which convert ammonia nitrogen to nitrites and nitrates. Note, denitrifying bacteria are not autotrophic.

B Fungi

Oxidize organics well, but usually do not settle well.

C Protozoa and Rotifers

Feed on dispersed bacterial cells and thereby allow treatment systems to produce a very clear effluent.

VI PRACTICAL APPLICATION

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UNIT OPERATIONS IN WASTE TREATMENT

I INTRODUCTION

A Definitions

- 1 Unit operation⁽¹⁾ a particular kind of a physical change that is repeatedly and frequently used as a step in the process for industrial chemicals and related materials. Examples include filtration, evaporation, distillation, heat transfer, fluid transfer, sedimentation and mixing.
- 2 Unit process⁽¹⁾ a particular kind of chemical reaction and equipment to which the same basic designs and operation may be applied. Oxidation, coagulation, disinfection, hydrolysis and chemical absorption are common examples.
- 3 Process - a series of actions or operations conducting to an end. A continuing operation or treatment consisting of a combination of unit operations. For example, the activated sludge process includes mixing, fluid and gas transfer and clarification among unit operations; oxidation, hydrolysis and coagulation either biological or chemical among unit processes.
- 4 Wastewater treatment⁽²⁾ any process to which wastewater is subjected to remove or alter its objectionable components.
 - a Wastewater treatment may also be defined as a series of unit operations designed to produce a product "clean water" from a raw material "waste water."
 - b Treatment is a means to renovate used water to meet a specific beneficial reuse requirement.
 - c Conventional treatment is commonly classified by stage or degree of treatment such as preliminary or pretreatment, primary, secondary, or advanced treatment. Processes such as activated sludge, trickling filtration or oxidation pond treatment are commonly used. Each of these can be more precisely described and better understood in terms of the unit operations involved

d Unit operations for purposes of this outline include both "unit operations" (A 1) and "unit processes" (A 2) to distinguish unit process from the more generally applied term "process" which may include many unit operations or unit processes.

B Increasing stress on environmental quality means that wastewater treatment must be upgraded. Upgrading treatment means: removal of a larger fraction of conventionally removed components and removal of additional items presently not significantly removed by conventional treatment. This also means treatment of a larger fraction of collectable wastewaters for a greater variety of used water types and components for 24 hours per day, 365 days per year

- 1 The unit operation concept tends to focus attention upon the specific components to be removed and upon fundamental units most suitable for that function. The unit operation approach offers a wider selection for design purposes than that available in empirical plant design. The treatment therefore may be more specific, better tailored to the situation and show a better cost/benefit ratio
- 2 Implementation of treatment operations requires motivated and trained manpower. Personnel training along the unit operations route shortens the time and promotes better comprehension by focusing upon the unit operations or tasks most commonly used. Rotation among assignments is a smoother and progression more likely because the individual trained in unit operations tends to recognize familiar unit operations in the new assignment; his learning requirements consist of the differences, such as a different sequence of familiar tasks, a smaller number of new unit operations, and different handling techniques because of material or situation. Learning is split into fundamental units. Personal progress, job satisfaction, and competence increase with the recognition of proficiency of the smaller "bits."

C This outline considers selected unit operations of sanitary engineering (3, 4) and processes based upon them. Tables presented later summarize interrelations and the means whereby these are combined into processes or stages of treatment.

- 1 Unit operations are the fundamental "building blocks" of treatment.
- 2 Unit operations are the alternate routes to a given objective. Solids-liquid separations may be achieved by many different operations; some are favored in one situation, others limited by that situation.

D The following sections consider individual unit operations and their characteristics as guidelines for selection or design. These notes are general in nature and subject to the influence of waste characteristics, local conditions, practice, economics and water quality requirements of the situation. Each unit operation is characterized in terms of:

- 1 Favorable application factors
- 2 Limiting application factors that may encourage selection of an alternate operation for a particular situation.

II PHYSICAL UNIT OPERATIONS - SOLID-LIQUID SEPARATIONS

The separation of solids from liquid, or the reverse, is of primary importance to wastewater treatment. Various unit operations or adaptations of them to achieve this objective may be used to remove objectionable components, to protect process equipment, to simplify subsequent operations, to increase stability of process water, to make the water more amenable for treatment or to complete the process. Separations may be a part of pretreatment, an integral process step, or a means of upgrading process effluents. No single operation appears more frequently, in more numerous adaptations, at more stages in processing, and is more critical in product water upgrading than solids-liquid separation.

A Gravity Sedimentation

- 1 Favorable aspects: This unit operation is by far the least expensive and feasible route for a large variety of separations. May be adapted for separation of a variety of materials having a specific gravity sufficiently different from that of water and immiscible in it such as: High density sand, gravel or scale, moderate density organic suspended materials, low density floatable materials.

Requires simple and generally available equipment. Operating variables are known and generally controllable to favor reliable treatment.

- 2 Limitations: Adversely affected by variations in wastewater characteristics and flow. Requires a moderately large capital, equipment and area investment. Sludge detention conducive to solids liquefaction and feedback. Affected by short circuiting, turbulence, distribution, temperature or density changes. Relatively slow operation in most situations.

B Surface Filtration

1 Coarse or fine screens

- a Favorable: Inexpensive simple operation and equipment. Reliable removal of discrete solids larger than the screen openings. Equipment available and operating practice known. Simplifies subsequent operations. Low area requirement.
- b Limitations: Susceptible to plugging, large quantities of wet, difficult-to-handle solids. Variable loading may result in operating and performance problems associated with higher loads.

2 Microscreens

- a Favorable: Produces an effluent of low suspended solids (<10 mg/l) and low turbidity (2JTU) at low capital, operating time and area cost at rated loading. Simple operating requirements. Equipment availability good.
- b Limitations: Poor tolerance for high suspended solids feeds (>50 mg/l). Tends to plug filter surface. Affected by changes in waste characteristics. Solids breakthrough at excessive loading.

3 Diatomaceous earth filtration

- a Favorable: Produces a high quality effluent low in suspended solids and turbidity (0.1 to 1.0 JTU). Low area requirement. Pressure buildup rather than solids breakthrough warning of overloads.

- b Limitations: High pressure drop through the filter; rapidly increases with solids loading. Tends to plug. Low output/sq. ft. / unit time with high suspended solids feed.

4 Vacuum filtration

- a Favorable: Suitable for treatment of a variety of solids-liquid concentrates. Large choice of filter media including string, coil, cloth (natural or synthetic) screens. Versatile in adaptability for varying conditions and loading. Low area requirement. High capacity per unit area.
- b Limitations: Complex operation, high maintenance cost. Usually requires chemical coagulation or coagulant aids. High capital and operating cost. Requires frequent attention to maintain capacity during varying load and sludge characteristics. High cake moisture content produces a poor quality filtrate.

C Bed Filtration

Many options are available such as type of media (sand, coal, gravel, synthetics, etc.) size of media (from fine sand to rock or manufactured media) and flow direction (up flow or down flow, compressed or expanded bed). Fine media and downflow operation may resemble operational characteristics of surface filtration. Coarse media, multi media, expanded beds represent filtration in depth. In some situations such as trickling filtration, the process is largely a biological phenomena rather than intrinsically filtration.

- 1 Sand or single media filtration. Characterized by a high rate of head loss development with high solids loading.
 - a Favorable: High quality effluents produced. Increased solids, oxygen demand, and organism removals specially with low application rates. Beneficial for upgrading reasonably good quality treated effluents. Dependable polishing step. Simple operational control.
 - b Limitations: Large area requirement. Usually requires pretreatment for removal of most of the solids. High head loss development specially for

high rate application. Usually an intermittent operation. Low capacity per unit time. Media replacement based upon incidence of "balling," backwash losses, deposition on the grains, and nature of feed stock contamination. Possible odor development. Backwash water may be voluminous and generally requires retreatment.

2 Soil percolation

- a Favorable: Generally a dependable method of effluent disposal where land is available. Returns both water and wastewater nutrients to the food chain. Useful for land reclamation purposes. Requires simple operation and low operational cost. Versatile for use with a wide variety of wastewater types
- b Limitations: Commonly limited with respect to application rates. Requires a large land area for intermittent operation. Capital cost primarily related to land area requirements. Disinfection commonly required. Good agricultural practice needed to support good engineering. A cover crop, tilling and drainage control generally required. Subject to seasonal, soil, and topographical factors. Odors and health hazards tend to produce a poor public image. Ground or surface water hazard potential.

3 Multi media filtration

The use of two or more filter media in which both size and density are variable makes it possible to distribute trapped particulates in a wide zone with respect to filter depth. Usually a larger sized lower density media are placed over a fine high density media. Larger particulates are trapped in the upper zone while the fine media upgrade effluent clarity. Head loss development occurs more gradually to permit longer runs of high product quality as compared with single media filtration.

- a Favorable: Head loss distributed throughout the bed, builds up more slowly to permit higher rate and volume application. Dependable high quality effluent production. Generally high capacity characteristics.

Capable of being tailored to fit a particular feed and effluent quality requirement.

- b Limitations: Design generally requires more careful evaluation of feed and product quality requirements. Solids load and nature are critical. Requires more careful control during backwashing to make it a more complex operation than sand filtration. Intermittent operation. Media and equipment more expensive. Usually requires pretreatment. Backwash water requires retreatment--may require more backwash water or more time than for single media filtration. Media replacement may be higher.

D Pressure Flootation

- 1 The operation consists of aeration of part or all of the liquid flow in a covered tank to trap exhaust air and provide a controlled pressure rise. Under pressure more gas will dissolve than can be retained at normal pressures. Discharge of the pressurized liquid to the clarifying compartment permits release of excess dissolved gas. The released gas tends to associate with oil, scum and particulates to favor separation from water as a floatable concentrate. Variables include time pressure, turbulence, air-liquid-solid interface area and nature, and association tendencies in both pressure and clarifying compartments.

a Favorable

The floatation process is highly versatile for separation of oils emulsions or particulates. It may be used for thickening or clarification with or without conditioning chemicals such as surface active materials, coagulants or other separation aids. It is possible to employ higher loading and higher overflow rates than for sedimentation. A higher solids concentration factor may be achieved. More complete clarification of hard-to-separate materials is possible. Usually requires less area per unit of capacity.

- 1) Activated sludge concentration by floatation is becoming

increasingly popular because the hydrated solids are amenable to the floatation process to a greater extent than for sedimentation

- 2) Oil and surface active agents tend to be more completely separated by floatation to produce a better clarified product water.

- b Limitations: Usually requires very careful design and operation for a specific situation. The complex operation is sensitive to feed stock variations. Generally more complex equipment requiring closer control. More amenable to moderately concentrated feeds. Thickening operations may require duplicate solids handling for removal of floatable and settleable fractions. The supernatant zone commonly has a high solids concentration requiring retreatment. Clarification commonly is improved by increasing feed stock concentration. More expensive in capital and operating cost than sedimentation

E Centrifugation

The centrifuge has a long history for dependable separation of liquid-solid suspensions according to specific gravity differences. Solid bowl, basket, or disc type machines are available. Horizontal solid bowl units appear to have the greatest potential in sanitary engineering. Organic sludge from water and grit from organic sludge separations are attractive. Variables include feed rate, solids-liquid characteristics, feed concentration, temperature, chemical additives; machine variables include bowl design, rotational speed, contained volume, input, distribution and takeout mechanisms.

- 1 Favorable: Highly versatile; may be designed for high sludge concentration or high separation on a variety of feeds. High capacity-low area requirements. Low capital cost per unit of capacity. Capable of solids concentrations up to 30 to 35 percent with favorable loads and operation; continuous performance
- 2 Limitations: High power requirements (~0.5 HP/gpm). Usually necessary to make a choice between high solids concentration and high solids recovery--unlikely to have both. Reduced feed

concentration tends to reduce both concentration and recovery of centrifuged solids. Centrate generally requires retreatment. Requires suitable design for a particular function, material and flow and performs best at rated loading.

III CHEMICAL UNIT OPERATIONS

For purposes of this outline a chemical unit operation refers to a particular kind of chemical transformation of feed stock (I. c.). The transformations may be inseparably associated with both physical and biological changes, for example, hydrolysis, oxidation and disinfection are closely related to biological changes, while coagulation, flocculation are closely related to physical operations. These transformations may be natural in origin or induced by chemical additions. In a multicomponent wastewater the control of treatment largely is a compromise situation among operations of biological-chemical-physical natures, some favoring, some interfering, with the intended function.

A Neutralization

The combination of excess acids and alkaline materials to form a salt and water is a recurrent natural process called Neutralization. Among other components, organisms release both carbon dioxide (acidic) and ammonia (alkaline) which neutralize each other to form ammonium bicarbonate and water. Under certain conditions nature tends to inhibit itself where an excess of acid or alkaline materials are favored such as in low pH deep waters or high pH surface waters. Growth may become limited because of pressure retention of excess CO_2 or rapid assimilation of CO_2 respectively. This unbalance is unlikely to be as serious as that due to local discharge of acid or alkali released from manmade sources. Neutralization is a common requirement.

- 1 Favorable: Neutralization enhances the probability for biological and certain chemical transformations. Commonly reduces corrosivity of acid waters.

Increases acceptability of acid or alkaline waters for beneficial reuse in water supply, recreation, wildlife, agricultural industry and esthetics.

- 2 Limitations: Generally high operating and control cost. May produce gross quantities of solids for disposal or increase the dissolved solids in the water. Requires close control to prevent excessive additions.

B Oxidation

The oxidation of organic waste components in water is a primary consideration in wastewater stabilization. This process is intimately linked with solids-liquid separation. For example, organic soluble compounds may be oxidized biochemically to form settleable agglomerates of cell mass, to removable gaseous CO_2 and to less reactable water. Nitrogen compounds may be converted to the oxidized state and reduced to less reactable and removable nitrogen gas.

- 1 The use of oxygen (aeration or surface oxygenation) from air is by far the most used unit operation for supplying essential oxygen for intermediate and terminal stabilization.
 - a Favorable: Generally available, low cost. Necessary pumping, cleaning and transfer equipment available at reasonable cost. Moderate power cost. Transfer capability reasonably good. Dependable supply.
 - b Limitations: Limited solubility of oxygen in contact with air. Large capital investment in tankage and space. Air solubility and transfer limitations generally mean a low to moderate rate process.
- 2 The use of commercial oxygen instead of air permits a five-fold increase in oxygen partial pressures.
 - a Favorable: Higher oxygen partial pressures permit higher solubility of oxygen in water and greater

- oxygen transfer rate in high demand situations. More likely to maintain a higher residual DO in high rate situations, or through the clarifier stage. More complete stabilization possible in less tankage and/or time. High oxygen tension favors sludge oxidation--lowers solids accumulation.
- b Limitations: Better design requirements necessary to maintain high oxygen use efficiency. More complex system, more costly. Covered tanks and oxygen production facilities nearby usually required to favor cost/benefit ratios. Requires better operational control.
- 3 Ozone is another form of oxygen used primarily for special purposes.
- a Favorable: Ozone is used primarily for odor control because of its high oxidizing energy and high activity in water. Capable of reacting with components that may not react with oxygen under similar conditions.
 - b Limitations: Ozone (O_3) is a highly unstable compound. Generally cannot be stored or made in high concentrations. Usually requires formation on site and use in pre-treated water. High unit cost. Complex control.
- 4 Chlorine is commonly considered for disinfection; disinfecting properties are inherently associated with oxidizing energy.
- a Favorable: Commercially available chemical, control equipment available, operating controls generally known. High energy material. Relatively simple operation. Versatile material capable of use in a variety of situations. Cost higher than that for oxygen but has a greater reactivity for many beneficial operations. Rapid reaction in most situations.
 - b Limitations: Hazardous-nonspecific toxicity in air or water. Chlorine reaction produces HCl during reaction. Usually requires neutralization. Highly corrosive in water solution or wet gas. Certain components such as ammonia preferentially react with chlorine to cause high chlorine demands. Requires close control. Generally requires pretreatment to avoid excessive chlorine dosage.
- 5 Peroxy-acid oxidizing agents
- Permanganate and dichromate are the most common peroxy-acids used in sanitary engineering. Permanganate is relatively pH independent, dichromate is an effective oxidant only under acid conditions. Both have high oxidizing energy for special purposes.
- a Favorable: High oxidizing energy. Capable of being separated from product water. Adaptable for special requirements such as destruction of most organic materials or color. Permanganate may occur naturally in water and in excess its color is its own indicator.
 - b Relatively high cost per unit of oxidizing energy. Excess reagent contributes to poor quality water. Close control required. Commonly does not oxidize ammonia nitrogen. May require catalysis to reduce delayed reactions.
- C Hydrolysis
- The addition of water to split large molecules into two or more simpler substances is an inevitable part of biodegradation. Cell mass may be hydrolyzed to form smaller component parts that are partially oxidized to yield energy for building another crop of cells. The process will continue as long as oxidation energy is sufficient for growth. Treatment tends to produce a low energy

discharged effluent in which hydrolysis and oxidation become lower rate operations - (stabilized).

- 1 Favorable: Items favoring hydrolytic cleavage (liquefaction) include high or low pH, hydrolase enzymes or other catalysts, high temperatures, low or negative oxidation reduction potential (low oxygen tension) or anything favoring introduction of water into a complex molecule.
- 2 Limitations: Any situation favoring resynthesis of hydrolyzed components into larger molecules reduces the net effect of hydrolysis. Algal photosynthesis, bacterial or plant growth, absence of toxic components, and favorable conditions for growth usually are associated with high rate hydrolysis in a high energy situation but growth may be the predominant reaction. Any situation favoring dehydration limits hydrolysis.

IV PROCESSES USED FOR THE REMOVAL AND DISPOSAL OF ORGANIC MATERIAL

Isolation and stabilization are the key factors in wastewater treatment. Unstable intermediates must be stabilized to be acceptable as gaseous or solid residues. Isolation refers to separation of gaseous and solids residues from the recombining media--water. It is not possible to isolate or to stabilize to "end" products--somewhere in time recycle will occur. Each treatment operation is intended to hasten recycle for beneficial use and delay other types of recycle.

A Table 1 summarizes the functions of various stages of treatment. Certain unit operations are repeated at each stage in a different manner.

TABLE 1

WASTEWATER TREATMENT STAGES

Preliminary or Pretreatment:

1. Removal of roots, rocks, rags
2. Removal of sand, grit, gravel
3. To "freshen" the wastewater by short term aeration, chlorination, grinding or otherwise protect and promote subsequent treatment

Primary Treatment

Removal of readily settleable or floatable components

Secondary Treatment

Conversion of soluble or colloidal components to removable form with partial stabilization in process (commonly biodegradation).

Advanced Treatment of Wastewaters

Biological chemical or physical treatment of used water to meet specific reuse quality requirements. May consist of general or specific item clean-up.

Solids Disposal

Nonpollutional takeout favoring conversion to stable residues and separation of gas, liquid, and solid phases.

B Tables 2 and 3 list selected unit operations and the stages or processes in which they may be used. Note that many of these may occur repeatedly and that there is the possibility of including one or more options in any given treatment process

depending upon performance requirements and nature of the problem. These lists of physical (2) and chemical (3) operations are not complete. It is not likely that all of those listed may be included in any modification of a treatment facility. The problem is to select a series of operations suitable to meet the performance requirements of the situation at a favorable cost/benefit ratio.

C Several processes are possible for secondary treatment. Each of these have several modifications. The most common are based upon biological-physical unit operations. Chemical-physical operations may be used to upgrade the overall removal, or to remove specific components commonly not sufficiently removed by treatment. The same processes may be used for advanced treatment providing the degree of removal is upgraded on all components of interest to meet specific reuse requirements.

TABLE 2
UNIT OPERATIONS BY TREATMENT STAGE OR
PROCESS - PHYSICAL TRANSFORMATIONS

Unit Operation	Stage or Process*									
	Pre	Pri	Sec	Adv	AS	TF	OP	SP	D	
Fluid Transfer										
liquid pumping	x	x	x	x	x	x	x	x	x	x
mixing	x	x	x	x	x	x	x	x	x	x
sludge pumping		x	x	x	x	x		x	x	
process residues-scum	x	x	x	x	x	x				
Gas Transfer										
into process-oxygenation	x		x	x	x			x		
stripping	x		x	x	x			x		
mixing	x		x	x	x			x		
Solids Transfer										
applied chemicals		x	x	x	x			x		
process residues	x	x	x	x	x	x		x		x
Heat Transfer				x				x		x
Solids-Liquid Separation										
coarse screening	x									
microscreening			x	x	x	x				
gravity sedimentation	x	x	x	x	x	x	x	x		x
filtration			x	x	x	x	x	x		
evaporation-drying				x				x		x
distillation				x						
floatation	x	x	x	x	x	x		x		
thickening		x	x	x	x	x		x		
centrifugation		x	x	x	x	x		x		
adsorption			x	x	x	x	x	x		
elutriation	x	x	x	x	x	x	x	x		x
flocculation		x	x	x	x	x	x	x		x

TABLE 3
UNIT OPERATIONS BY TREATMENT STAGE OR PROCESS -
CHEMICAL TRANSFORMATIONS

Unit Operation	Stage or Process*								
	Pre	Pri	Sec	Adv	AS	TF	OP	SP	D
Oxidation Reduction									
wet combustion (biol-chem)	x	x	x	x		x	x	x	x
dry combustion				x				x	x
corrosion	x	x	x	x	x	x	x	x	x
bleaching (color removal)			x	x	x	x	x		
Disinfection	x	x	x	x	x	x	x	x	
Hydrolysis (liquefaction)		x	x	x	x	x	x	x	x
Solids-Liquid Separation									
coagulation		x	x	x	x	x	x	x	
precipitation		x	x	x	x	x	x	x	
ion exchange				x					
electrodialysis (phy-chem)				x					
complexation		x	x	x	x	x	x	x	x
assimilation (biol-chem)			x	x	x	x	x	x	
absorption (phy-chem)			x	x	x	x	x	x	
Neutralization		x	x	x	x	x	x	x	x

*Coding Tables 1 and 2

Pre - Preliminary or pretreatment stage

Pri - Primary clarification stage

Sec - Secondary treatment stage

Adv - Advanced treatment stage

AS - Activated sludge treatment process

TF - Trickling filtration treatment process

OP - Oxidation pond treatment process

SP - Sludge processing stage

D - Disposal stage (solids)

1 Activated sludge

This process is based upon a mixed fluid suspension of solids concentrates from previous operations and raw wastewater in the presence of excess oxygen to rapidly stabilize the incoming pollutants by biological growth, transfer to the solids phase, agglomeration and solids liquid separation.

- a Favorable: Versatile process capable of being adapted to high performance on most types of organic contaminants. Generally capable of high efficiency in stabilization and clarification. Lower tankage and area requirements than for most biological processes. May be modified to achieve high removal of nitrogen phosphorus and solids. Adaptable to a wide range in removal efficiency.
- b Limitations: Requires close control of load ratios and operating conditions. High oxygen requirements. Subject to upset by qualitative and quantitative shock loads. Unmodified process commonly shows poor removal of nitrogen and phosphorus. Subject to the variations of characteristic of biological treatment. High operating cost.

2 Trickling filtration

Employs an attached media of sewage slimes on the support surface for transfer and stabilization of organic pollutants in the influent.

- a Favorable: Versatile process capable of being adapted for intermediate performance on most types of organic waste. Low operating cost. Adaptable for a fairly wide range of removal on substances showing good solids transfer efficiency.
- b Limitations: High capital cost for land and tankage (rock or slag). High pumping cost on manufactured media. Generally not amenable for

coagulation and clarification. Fewer operating controls possible. Subject to the variations characteristic of biological treatment even though it may not be as noticeable due to generally turbid effluents.

3 Oxidation ponds

Employs natural purification phenomena of sedimentation, aerobic and anaerobic degradation, algal photosynthesis usually in a sacrificial pond or series of ponds.

- a Favorable: Capable of high treatment efficiencies with low operational cost. Adaptable to low or high removal efficiencies depending on land and capacity or time availability. Useful on a wide variety of wastewaters. Land is available for upgrading treatment and other uses as needed.
- b Limitations: Generally limited to application where land costs are low. Subject to poor performance during ice cover, overloads, spring warmup and unexpected boil-up. May be an odor nuisance at times. Generally a low rate process with poor solids recovery characteristics. Appears to have a tendency to poorer performance after several years of operation.

4 Physical chemical treatment operations

Physical chemical treatment by lime precipitation and activated carbon adsorption is becoming recognized to an increasing degree.

- a Favorable: Is a versatile process capable of being adapted to very high degrees of treatment on a variety of wastewaters. Recovery of added lime and regeneration of spent carbon by controlled incineration permits chemical reuse and reduces solids disposal. Relatively low capital costs and space requirements. Capable of application over

wide flow variations with dosage and regeneration time control. Freedom from toxic effects.

- b Limitations: Generally higher operating cost. More complex process requires precise operational control. May require pretreatment. Chemical reuse almost mandatory to limit solids disposal requirements. Operating history for wastewater applications scant.

D Sludge Processing or Disposal Routes

1 Wet combustion by aerobic biological processes

Activated sludge, trickling filtration and oxidation ponds involve a certain amount of processing and disposal of solids to a degree limited by the amount of carbon dioxide and water formed in process. Aerobic sludge digestion accentuates solids disposal.

- a Favorable: Generally a conventional bio-chemical process using established procedures. Time, oxygen supply and favorable conditions are basic requirements. Versatile for a variety of wastes. Generally capable of a high degree of stabilization.
- b Limitations: Process limited to a residue solids level containing about 40% volatile content (10 to 20% of the feed volatile solids). High liquid recycle of N, P, and solids content. Generally a long term operation. Process interference serious for low temperatures, toxic agents, or unfavorable pH. Generally produces a low concentration sludge (~ 2%).

2 Wet combustion--elevated temperature and pressure

Wet combustion of organic wastes at various pressures in enclosed vessels with liquid temperatures of 350° F or above have been effective for separation of a highly stable mineralized ash.

- a Favorable: Capable of producing an easily separated high-mineral ash. Rapid process low area requirements, low solids disposal volume.
- b Limitations: Requires complex equipment and high heat requirements. Good control essential. Residual liquid has a high color and contaminant level.

3 Anaerobic digestion

This process is both a sludge conditioning and a solids separation process. Sludge residuals after digestion are more concentrated in solids content and decreased in volume and mass due to escape of methane and carbon dioxide gas or elutriate.

- a Favorable: Versatile and dependable process of organic stabilization for suitable loading, mixing and temperatures. Low cost operation. Produces a usable product gas. Residual solids relatively stable, improved in concentration, and drainability.
- b Limitations: Capital costs relatively high for space and tankage. Susceptible to shock loading, temperature changes, poor mixing or toxicity. Once upset--it requires appreciable time and effort to restore good performance. Recycle liquids are high in dissolved and suspended solids; are difficult to treat.

4 Drying and incineration

Many modes of drying such as drying beds, land spreading, flash drying are possible with wet sludge. Incineration in a fluidized bed, rotary kiln or multiple hearth are used. The multiple hearth is one example of drying and incineration.

- a Favorable: Dependable, versatile operation for thick sludges where

heat release is close to heat requirements for water evaporation and temperature rise. Can be controlled to produce clean stack gases and stable mineral solids residue. Rapid process. Low area requirements. Control techniques well established.

- b Limitations: Solids feed of low heat and high water content may require excessive auxiliary fuel cost. Generally requires stack gas reburning and solids recovery to meet air quality requirements. Generally costs about \$50 plus per ton of dry solids for operation. Requires close control of feed, burner temperature and other operating variables.

5 Wet disposal of sludge

Application of wet sludge to spoil areas, stripped terrain, farm or marginal land has been common practice for a long time. Piping instead of truck hauling is receiving increased consideration to extend the disposal area.

- a Favorable: Costs of disposal may be reduced by avoiding costly drying operations. Pumping of wet sludge is more economical than hauling. Possibilities for use of the organic and water for reclamation of waste land is attractive as a means of recycling the wastes into the food chain. Isolation possibilities are improved by remote application from population centers.

- b Limitations: Good engineering and farming practice are required. The local residents do not appreciate receiving waste materials from elsewhere unless practice and public relations are top rate. Possible hazards from surface ground water and air pollution dim the good neighbor policy.

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