A Study of the Distribution of Heterotrophic Bacteria in the Great Lakes.

I. The Heterotrophs in Lake Water.

by

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INTRODUCTION

The study of heterotrophic bacteria in fresh water lakes has been traditionally associated with those bacteria of intestinal origin. As indicators of domestic wastes, the intestinal group (coliforms and fecal streptococci) have proved invaluable. With increasing use of fresh water as industrialization and agriculture expand, the problem of water pollution becomes, aside from the public health aspects, one of eutrophication of the environment and its associated nuisances. The effect of this nutrient enrichment on the native bacterial flora of lakes has received little attention; conversely the effect of the resident bacterial population on nutrients has received little more than tacit recognition of the chemical conversions the various groups of bacteria effect. One of the primary reasons has been lack of knowledge on the composition of the bacterial plankton, bacterial periphyton, and benthic bacteria and their specific biochemical roles.

This study was undertaken to obtain information on the types of heterotrophic bacteria in various areas of the Great Lakes and to relate the distribution of these types to 2 different levels of pollution. Along with knowledge of the nutrition and physiology of these organisms, some of the bacterial types could hopefully be used as indicators of the biochemical condition of the environment.

Previous Work:

There are few published works on the types of bacteria in lakes. Henrici (1933), Henrici and McCoy (1938), Taylor (1940), and Potter and Baker (1961), were primarily concerned with broad morphological and biochemical groupings without attempting to classify the isolated organisms in any taxonomic scheme. Scarce (1965) identified 500 cultures from Lake Michigan waters and found that members of the genera *Achromobacter* and *Flavobacterium* were most common, comprising approximately 95% of the total colonies isolated from 20°C and 35°C plate counts. No attempts were made to relate the types of organisms recovered to any features of the environment.

Materials and Methods:

Water samples collected during regular monitor surveys of Lakes Ontario and Superior in 1967, and from two stations in Lake Ontario in 1968, served as the source of bacterial isolates. Collection areas in Lake Ontario are shown in Fig. 1. Lake Superior samples were collected on one monitor survey from Marathon to Nipigon Bay.

Plate count analyses were performed on appropriate dilutions of refrigerated samples less than twelve hours old, using the membrane filtration technique. Black Millipore filters with grids (HABG 047 SO) were employed. The medium was Difco M-plate count broth, buffered with 0.1% K_2HPO_4. Incubation was at 20°C in a humid atmosphere for 60 and later 72 hours. All bacteria-like colonies appearing on the membrane were counted with the aid of a stereomicroscope at 15 x magnifications.

Coliform analyses were performed on the same samples, using the MF technique and M-Endo Broth. The "*in situ*" water temperature was recorded for each sample.

Isolation and Purification of Cultures:

From membrane filters containing between 20 and 200 colonies, ten colonies were isolated from one sector of a plate. The isolates were streaked on Nutrient and later on Trypticase-Soy Agar, which proved a superior growth medium, particularly for yellow-pigmented bacteria. Plates were incubated at 20°C for five to seven days or until well-isolated colonies of a few millimeters diameter were obtained.

If necessary, a second streaking was performed. After incubation, the colonial morphology was described from plates bearing like well-isolated colonies. Slants of Nutrient Agar or Trypticase-Soy Agar from colonies were prepared and incubated at 20°C for 24-48 hours, or until growth was visible. Gram stains (Kopeloff-Beerman modification, Manual of Microbiological Methods, p. 16) were done to test for purity. If the culture was pure, the Gram-reaction, shape and size of the cells was recorded.

Diagnostic Tests:

Unless stated otherwise, incubation was at 20°C. All cultures were tested for the presence of the enzymes catalase and oxidase (Kovacs, 1956), Motility (hanging drop method with two passages through Nutrient or Trypticase-Soy Broth), acid and gas production in Phenol-red Dextrose Broth, reaction in the glucose Oxidation-Fermentation Medium of Hugh and Leifson (1953), and growth on MacConkey Agar.

Those cultures identified as members of the family Enterobacteriaceae were further tested for reduction of nitrate, fermentation of lactose in Brom-cresol Purple Broth, production of indole, the methyl-red and Voges-Proskauer reactions, citrate utilization, gelatin liquifaction, presence of lysine and ornithine decarboxylases, and the arginine dihydrolase, and for the enzymes urease, and phenyl alanine deaminase.


LAKE ONTARIO
Trent R.
Kingston
Cobourg
Black R.
Oshawa
Toronto
Humber R.
Credit R.
Hamilton Bay
Oswego
Rochester
Niagara Falls
L. Erie
LEGEND: x'S indicate areas from which bacterial cultures were obtained.

Incubation was at 35°C. Members of the *Aeromonas* - *Vibrio* group were tested for gas production from glucose Brom-cresol Purple Broth, gelatin liquifaction, and for arginine dihydrolase, and lysine and ornithine decarboxylases. Spore stains (Method-Shaeffer and Fulton's-Method in Cowan and Steel, 1965, P. 143) were performed on Gram-positive bacteria and Gram-negative bacteria which failed to grow on MacConkey Agar.

Non-routine tests included penicillin sensitivity using Sensi-discs of two I.U. concentrations, swarming on 0.1% Yeast-extract agar, H_2S production in JSI or Kligler's Iron Agar, and reactions in various carbohydrate broths, for those few cultures which failed to fit into the regular scheme.

Detailed descriptions of the reagents, media, and test procedures are presented in Appendix 1. Approximately 600 cultures were tested in duplicate as a check upon methods.

Fig. 2a. Determinative Scheme for the Identification of Gram-negative Rods. See also Fig. 2b.

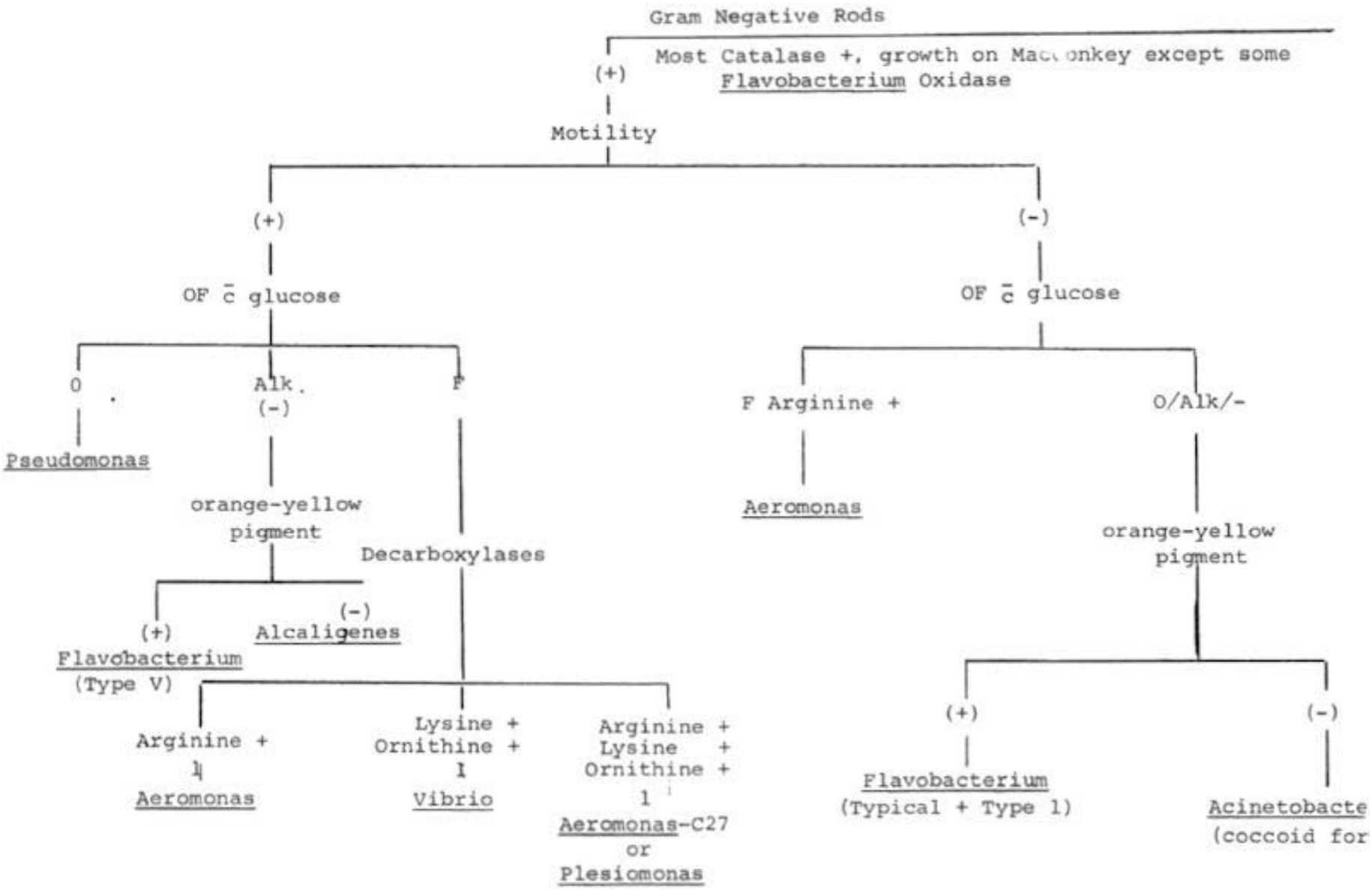

Fig. 2b. Determinative Scheme for the Identification of Gram-negative Rods. See also Fig. 2a.

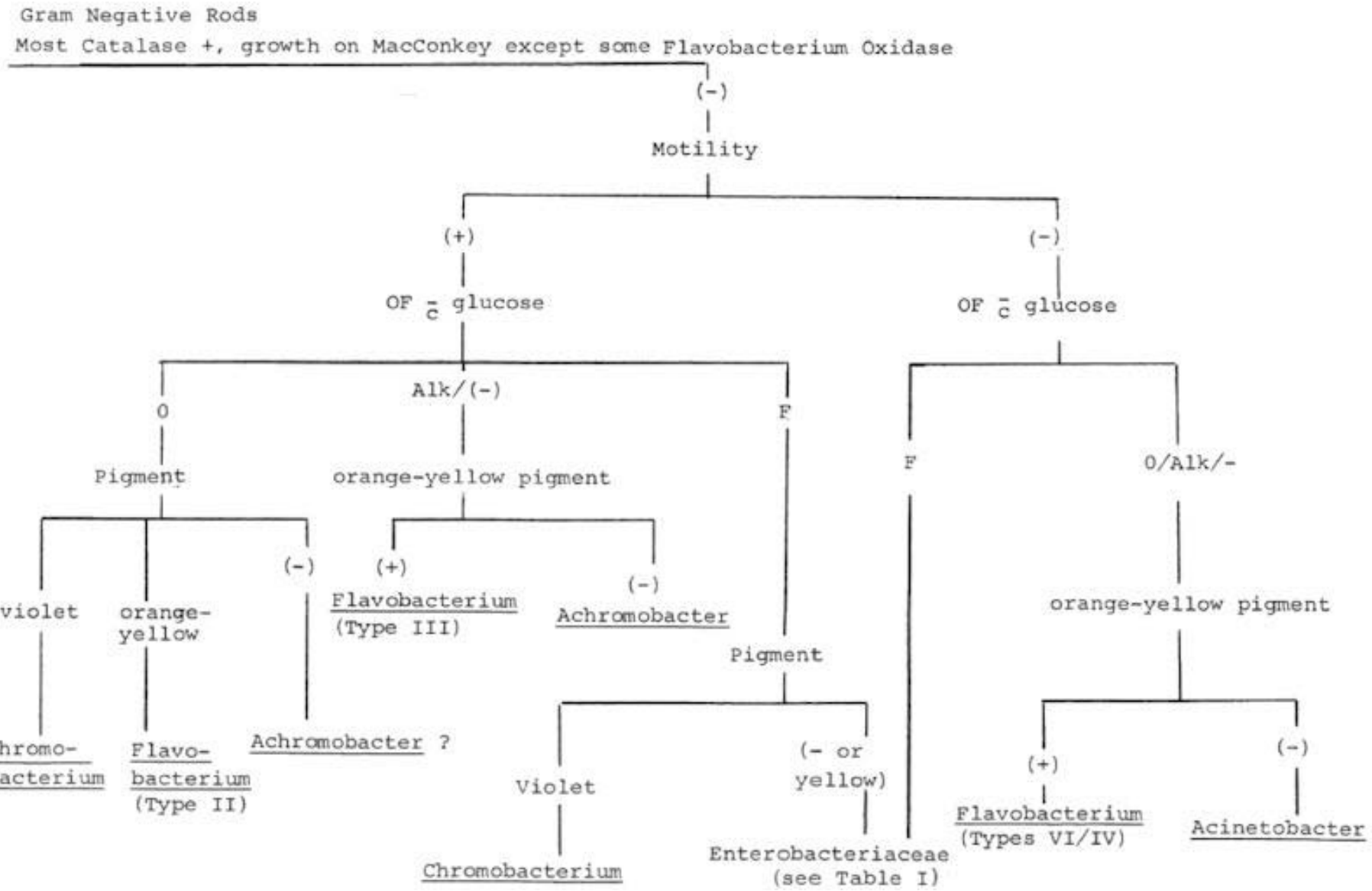

Fig. 3. Determinative Scheme for the Identification of Gram-Positive Bacteria.

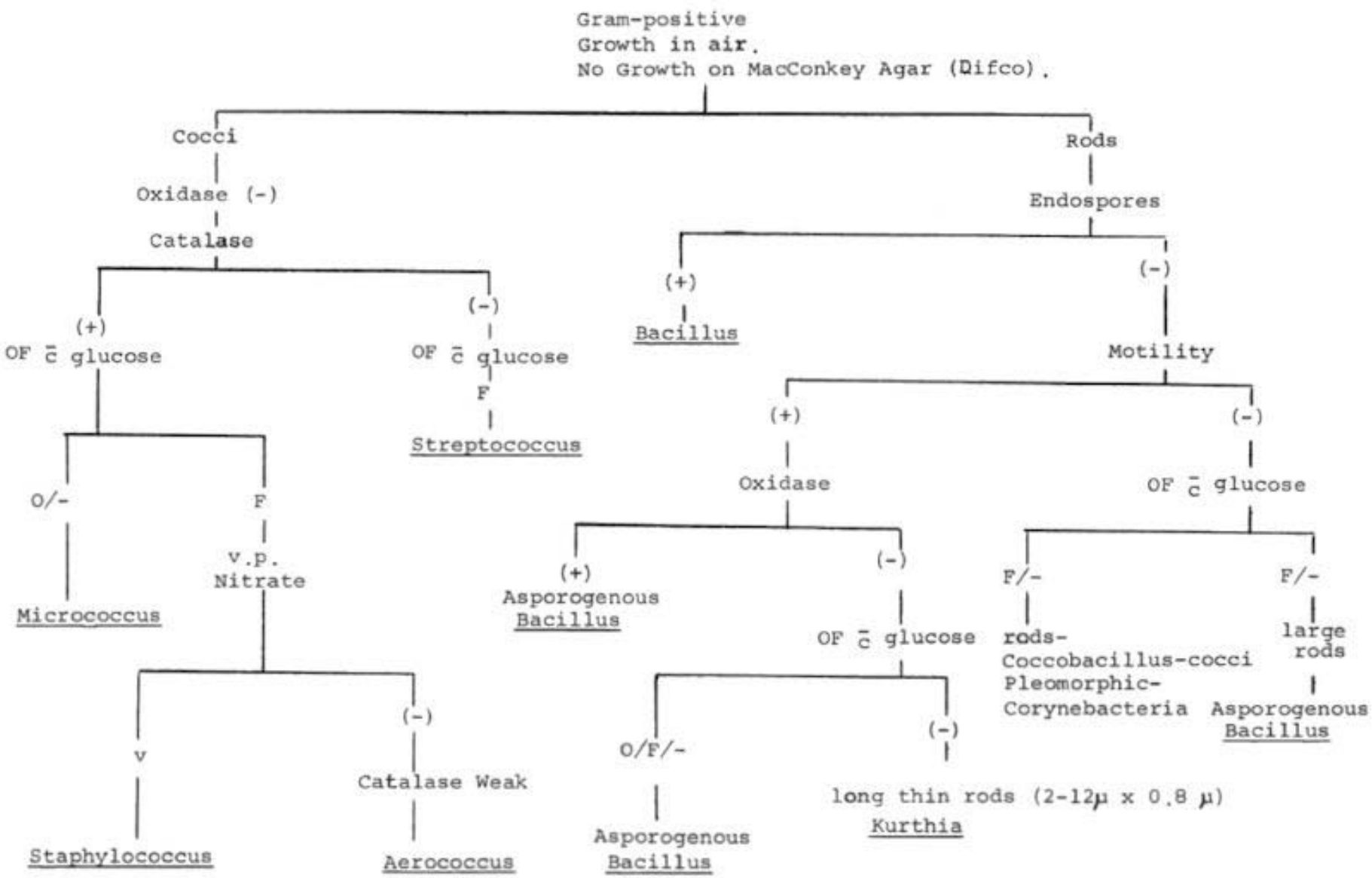

Table I: Biochemical Tests employed in the Differentiation of Genera within the Family Enterobacteriaceae, frequently for isolates encountered on Total Plate Counts.

Biochemical Tests	Motile Rods							Non-motile Rods	
Nitrate	+	+	+	+	+	+	v	+	+
Lactose (Acid)	+	+	v	-	v	-	v	+	v
Indole	+ (-)	- (+)	- (+)	-	-	v	v	-	v
Methyl Red	+ (-)	+	-	-	-	v	v	v	+
Voges-Proskauer	-	-	+ (-)	+ (-)	+	v	v	v	-
Citrate	-	+	+	+	+	v	+	+	-
Gelatin	- (+)	- (+)	- (+)	-	+	v	+	-	-
Lysine	v	-	v	+	+	-	-	+	v
Arginine	v	+	v	-	-	-	--	-	v
Ornithine	v	v	+	+	+	v	-	-	v
Urease	-	v	v	-	-	+	-	+	-
Phenylalanine	-	-	-	-	-	+	v	-	-
Pigment	-	-	-	-	Red (-)	-	Yellow	-	-
	Escherichia	*Citrobacter*	*Enterobacter*	*Hafnia*	*Serratia*	*Proteus*	*Erwinia*	*Klebsiella*	A - D

LEGEND: + positive reaction:
- negative reaction
v varies with different species
( ) - reaction of some biotypes

RESULTS AND DISCUSSION

Identification Schemes:

The determinative schemes developed for the identification of the Lakes Ontario and Superior isolates are presented in Figs. 2 and Table I for the Gram-negative bacteria and in Fig. 3 for the Gram-positive bacteria. Approximately 1700 cultures from waters of Lake Ontario and 230 from Lake Superior were identified to genus employing these schemes: the percentage occurrence of each genus among the total isolates for the two lakes is presented in Table II.

Types of Heterotrophs from Lakes Ontario and Superior:

The predominance of Gram-negative bacilli in the aquatic environment (both marine and fresh water) has been well-established. Among the 1688 isolates from Lake Ontario and the 230 from Lake Superior, approximately 90% were Gram-negative rods, 8% Gram-positive rods, and 2% Gram-positive cocci. The main genera represented among Lake Ontario isolates were *Acinetobacter* (22%), *Flavobacterium* (21%) and *Pseudomonas* (14%). Among the Lake Superior isolates, *Pseudomonas* (41%) was the most frequently isolated with lesser numbers of *Acinetobacter* (21%). In Lake Michigan, Scarce (1965) found very few of his plate count isolates were *Pseudomonas* (3%): *Achromobacter* (including *Acinetobacter* of this report) and *Flavobacterium* comprised 95% of the isolates, and no Enterobacteriaceae were found.

Members of the family Enterobacteriaceae made up 5% of the Lake Superior isolates and 18% of the Lake Ontario isolates. Scarce employed Tryptone Glucose Extract Agar as growth medium, while buffered m-Plate Count Broth was used in this study, Whether or not the differences in the proportions of various genera isolated is a reflection of differences in the microbial flora of the three lakes, or of methodological differences, can only be determined by more extensive investigation.

Relationship of Lake Ontario Isolates to Level of Pollution and Water Temperature:

The plate count isolates within each genus from Lake Ontario were grouped according to two criteria - coliform density and "*in situ*" water temperature of the samples from which they were obtained, The resulting frequency distribution for each genus were tested by the method of Chi-square to determine if there was any association between the percentage frequency of isolation of each genus and coliform level or water temperature. In Table III is presented the grouping according to coliform level, with class intervals of one log cycle: in Table IV, according to water temperature, with class intervals of 5°C. The calculated χ^2 values are included. The Lake Superior isolates were not similarly grouped because none of the coliform densities in the samples from which these cultures were isolated exceeded 10 per 100 ml. and the water temperatures were less than 12°C.

Miscellaneous:

Results from grouping the isolates according to coliform and temperature levels revealed that *Alcaligenes, Micrococcus* and *Bacillus* species remained a relatively constant segment of the heterotrophic bacterial flora regardless of the level of pollution or temperature of the water. Staphylococci and coryneforms were isolated infrequently and *Chromobacterium*, only regularly and in small numbers in late fall and winter when water temperatures were approximately 2 - 4°C. Many of the *Chromobacterium* cultures had to be grown in natural light to promote formation of their characteristic violet pigment.

Table II: Generic Distribution of Bacterial Isolates from Lakes Ontario and Superior.

	Percentage of Total Number of Isolates within each genus.	
GENUS	LAKE ONTARIO	LAKE SUPERIOR
Alcaligenes	6.0	5.0
Micrococcus	2.0	1.0
Staphylococcus	0.2	-
Bacillus	8.0	12.0
Aeromonas	8.0	4.0
Pseudomonas	14.0	41.0
Flavobacterium	21.0	8.0
Acinetobacter	21.0	21.0
Arthrobacter - Cornebacterium	1.0	1.0
Chromobacterium	0.8	-
Enterobacteriaceae	18.4	5.0
Enterobacter	8.7	2.0
Proteus	6.2	-
Citrobacter	0.6	1.0
Klebsiella	1.8	2.0
Escherichia	0.5	-
Hafnia	0.5	-
Erwinia	0,2	-
Yeast	0.1	2.0
Total Number of Isolates	1688	230

Aeromonas:

The proportion of *Aeromonas* isolates did not vary significantly with coliform level (χ^2 = 0.291) but appeared to be influenced by water temperature. (χ^2 = 5.75 > 3.84 = χ^2, 0.05 level). The greater proportion of *Aeromonas* isolates was collected when the water temperature was above 10°C, the class interval of 10-15°C, containing the highest percentage (13.2%). *Aeromonas* species have a wide range of habitats, including water, diseased fish and amphibia, and man. Eddy (1960) isolated strains which he grouped into three classes, depending upon their temperature requirements - those with an optimum temperature below 20°C, those with an optimum temperature between 25°C and 30°C, and those with an optimum temperature near 37°C. One strain which grew well at 37°C, also grew fairly rapidly at 1.5°C.

The *Aeromonas* isolates from Lake Ontario were placed in four groups - Group I resembling *Aeromonas liquefaciens*; Group II, A. *formicans*; Group III, *A. shigelloides* of Cowan and Steel (1965) and variously "Paracolon C-27" (Ferguson and Henderson, 1947) or *Plesiomonas* (Habe and Schubert, 1962); and Group IV, *A. salmonicide*. The characteristics and percentage of the total *Aeromonas* isolates within each group are presented in Table V. Subgroup IV was the most frequently isolated, comprising 45% of the total *Aeromonas* isolates. It resembles *A. salmonicida* - a true psychropile and the only non-motile species in the genus, whose habitat is freshwater lakes, streams, rivers and ponds, where it may cause furunculosis in fish. The remaining Aeromonas subgroups, with the possible exception of III, may be considered typical water organisms (Eddy, 1960). Bonde (1966) found that *Aeromonas* was the dominating group of bacteria in certain areas of a polluted marine sound, and that it was especially numerous in the sediments.

Table III: Generic Distribution of Bacterial Isolates from Lake Ontario, grouped according to coliform density of the sample source.

NUS	LEVELS OF COLIFORMS/100 ml										X^2
	0-10		10-100		100-1000		1> 1,000		Total		
	No.	% †	No.	%	No.	%	No.	%	No.	%	
Acaligenes	23	4.6	27	6.9	27	7.1	20	4.8	97	5.75	0.883
Micrococcus	9	1.8	7	1.8	8	2.1	4	1.0	28	1.7	0.430
Staphylococcus	-	-	1	0.3	-	-	2	0.5	3	0.2	NC
Bacillus	53	10.6	39	9.9	25	6.5	13	3.1	130	7.7	4.74
Aeromonas	41	8.2	25	6.4	29	7.6	34	8.2	129	7.6	0.291
Pseudomonas	68	13.6	49	12.5	63	16.5	60	14.4	240	14.2	0.603
Flavobacterium	146	29.3	72	18.4	69	18.1	63	15.1	350	20.7	5.77
Acinetobacter	30	6.0	72	18.4	107	28.0	157	37.7	366	21.7	24.5***
Arthrobacter Corynebacterium	5	1.0	4	1.0	4	1.0	7	1.7	20	1.2	0.273
Chromobacterium	1	0.2	2	0.5	6	1. 6	4	1.0	13	0.8	1.31
Enterobacteriaceae	121	24.3	94	24.0	44	11.5	52	12 5	311	18 .4	8.17*
Enterobacter	56	11.2	44	11.2	27	7.1	19	4.6	146	8.7	3.8
Proteus	55	11.0	41	10.4	3	0.8	6	1.4	105	6.2	15.7**
Citrobacter	2	0.4	3	0.8	3	0.8	2	0.5	10	0.6	0.195
Klebsiella	2	0.4	4	1.0	8	2.1	16	3.8	30	1.8	3.72
Escherichia	1	0.2	1	0.3	1	0.3	6	1.4	9	0.5	2.00
Hafnia	4	0.8	1	0.3	2	0.5	-	-	7	0.5	0.897
Erwinia	1	0.2	-	-	-	-	2	0.5	3	0.2	NC
Yeast	1	0.2	-	-	-	-	-	-	1	0.1	NC
Total No. Isolates	498		392		[3]82		416		1688		-

NC - not calculated
† - percentage of total number of isolates in class belonging to each genus
* - significant at the 5% level
** - significant at the 1% level
*** - significant at the 0.1% level.

Table IV: Generic Distribution of Bacterial Isolates from Lake Ontario, grouped according to *"in situ"* water temperature of the source sample.

GENUS	TEMPERATURE LEVEL						χ^2
	<5-10°C		10-15°C		>15°C		
	No.	% †	No.	%	No.	%	
Alcaligenes	54	7.4	23	5.0	20	4.1	1.20
Micrococcus	19	2.6	3	0.6	6	1.2	1.95
Staphylococcus	1	0.1	-	-	2	0.4	NC
Bacillus	63	8.6	31	6.7	36	7.3	0.34
Aeromonas	24	3.4	61	13.2	44	8.9	5.75*
Pseudomonas	126	17.1	84	18.2	30	6.1	6.54*
Flavobacterium	183	24.9	69	15.0	98	19.9	2.29
Acinetobacter	186	25.3	94	20.4	86	17.4	1.55
Arthrobacter-Corynebacterium	8	1.1	9	2.0	3	0.6	1.38
Chromobacterium	13	1.8	-	-	-	-	3.22
Enterobacteriaceae	57	7 8	86	18.7	168	34.1	17.30**
Enterobacter	35	4.8	52	11.3	59	12.0	2.47
Proteus	8	1.1	13	2.8	84	17.0	21.98**
Citrobacter	-	-	3	0.6	7	1.4	2.59
Klebsiella	12	1.6	8	1.7	10	2.0	0.52
Escherichia	1	0.1	3	0.6	5	1.0	1.93
Hafnia	1	0.1	4	0.9	2	0.4	1.03
Erwinia	-	-	2	0.4	1	0.2	NC
Yeast	-	-	-	-	1	-	-
Total No. Isolates	734		461		493		1688

† - Percentage of total number of isolates in class belonging to each genus
* - significant at 5% level of probability
** - significant at 1% level.
NC - not calculated.

He leaves it open as to whether these organisms are of pollutional origin or a segment of the native population characteristic for that special environment. The data from the lake water isolates suggest that these organisms are, in fact, a part of the natural bacterial population, little affected by level of pollution, since their numbers were relatively constant at all coliform levels.

Pseudomonas:

As can be seen in Table III, the relative proportion of *Pseudomonas* isolates was similar in all coliform classes ($\chi^2 = 0.603$). A significant drop in the proportion of *Pseudomonas* cultures isolated was noted at water temperatures above 15°C ($\chi^2 = 6.54 > 3.84 = \chi^2$, 0.05 level), suggesting that the major portion of *Pseudomonas* isolates were natural water forms (many of which are psychrophilic), rather than the potential pathogen *Ps. aeruginosa*. The latter has been suggested by Hoadley and McCoy (1966) as an indicator of sewage pollution of approximately half the *Pseudomonas* isolates tested for fluorescein production in Drake's medium (1966), 40% were positive and probably *Ps. fluorescens* a common soil and water inhabitant. *Pseudomonas* species are noted for their versatility in substrate utilization and probably represent a very active component of the natural population of lake waters. *Pseudomonas* types comprised 50% of the cultures isolated from Port Hope Harbour in Lake Ontario and at least 41% of those from Lake Superior.

Table V: Characteristics of *Aeromonas* subgroups and percentage of total *Aeromonas* isolates within each group.

	Subgroups			
Characteristic	I	II	III	IV
Motility	+	+	+	-
Gas from glucose	+	-	-	+
Lysine Decarboxylase	-	-	+	-
Arginine Dihydrolase	+	+	+	v
Ornithine Decarboxylase	-	-	v	-
Percentage of Total Isolates	19.0	14.3	21.4	45.3
Subgroup resembles	*A. liquefaciens*	*A. formicans*	*A. Shigelloides* (C-27 or *Plesiomonas*)	*A. Salmonicide*

v = different reaction with different strains.

Flavobacterium:

Although the incidence of *Flavobacterium* isolated decreased sharply in classes above 0-10 coliforms per 100 ml,, the overall difference among classes was not significant (χ^2 = 5.77 < 5.99 = χ^2, 0.05 level). Neither did the incidence of *Flavobacterium* relate to water temperature. Members of the genus *Flavobacterium* are considered true water organisms. They are found in the marine as well as the freshwater environment, and are often incriminated, along with *Pseudomonas, Alcaligenes* and *Acinetobacter* in the spoilage of fresh fish under refrigeration temperatures. The genus is, at the present time, only poorly characterized. Virtually any Gram-negative rod possessing a yellow-orange pigment can be assigned to the group. Another genus with which it is often confused is *Cytophaga*. Hendrie *et al* (1968) consider two subgroups:

a) Non-motile rods, not identifiable as *Cytophaga*, Gram-negative, oxidative, or not attacking sugars.

b) Gram-negative rods, motile by usually few peritrichous flagella, and oxidative in sugars.

In this study, isolates were placed in the genus *Flavobacterium* if they possessed the following characteristics:

> Yellow-orange, pigmented, Gram-negative bacilli, forming filaments in young cultures (24-48 hours), and exhibiting little or no growth on MacConkey Agar (Kazanas. 1967). Not fermentative in Hugh and Leifson's medium with glucose.

Based on variable motility, oxidase, and O-F with glucose, seven subgroups were formed; the characteristics and proportion of *Flavobacterium* isolates within each subgroups are presented in Table VI. Sixty-three per cent of the cultures were oxidase negative, 79% non-motile, and 90% yielded a negative or alkaline reaction in Hugh

and Leifson's medium with glucose. Subgroup IV was most frequently isolated comprising 47.3% of the isolates. Next was subgroup I, its oxidase positive counterpart, Based on Hendrie *et al* (1968), our "typical" subgroup could possibly be better ascribed to the genus *Cytophaga*, comprising 4.3% of the Gram-negative orange-yellow pigmented isolates; subgroups I, IV and VI to Hendrie's group (a) which would encompass 75% of the isolates, and subgroup II (1.7%) to Hendrie's group (b).

Nineteen per cent of the yellow-pigmented Gram-negative rods isolated in this study are not accounted for by the definition of Hendrie *et al* (1968). This is one area of bacterial taxonomy where much research is required. More widespread use of biochemical methods for e.g., DNA base composition, cell-wall analyses, and immuno-fluorescence techniques should help in clarifying the relationships among this heterogeneous group of bacteria, lumped into the genus *Flavobacterium.*

Acinetobacter:

Though not apparently associated with temperature (Table IV, $\chi^2 = 1.55 < 3.84 = \chi^2$, 0.05 level) , the percentage incidence of *Acinetobacter* among the plate count isolates was directly related to increasing level of coliforms (Table III). Only six per cent of the plate count isolates within the 0-10 coliform class could be assigned to the genus *Acinetobacter*, 18.4% within the class 10-100, 28.0% in the class interval 100-1000 and 37.7% of the isolates in the class >1000 coliforms per 100 ml.

Four subgroups within the genus *Acinetobacter* were observed, a large segment in each yielding pink or red colonies on MacConkey Agar. The characteristics and relative proportions of the total *Acinetobacter* isolates within each group are given in Table VII. The most important quantitatively was subgroup I, comprising 82% of the *Acinetobacter* isolates. This subgroup, along with II and together accounting for 93%

of the total *Acinetobacter* isolates, is the typical soil and water form of Baumann (1968). It is able to grow on mineral media with single straight chain hydrocarbons or organic acids, and nitrate or ammonia as the sole source of nitrogen. In well-aerated mineral-acetate media, generation times of 40 to 55 minutes have been observed. Lowering the pH below neutrality selectively favours the predominance of *Acinetobacter*; this fact cannot account for the large proportion of *Acinetobacter* isolates in Lake Ontario since the pH seldom is below 7.9. Under certain conditions, *Acinetobacter* may surpass the pseudomonads in growth and in decomposition of simple carbon compounds.

Subgroup III (3.1% of the isolates) is regarded by Baumann *et al* (1968) as a typical member of the genus *Moraxella*, ecologically distinct from the oxidase negative forms, and not reported to have been isolated from soil and water. They do not possess the capacity for assimilating the large variety of organic compounds that subgroups I and II possess.

The relationship of *Acinetobacter* to increasing level of pollution, as measured by coliform density, is substantiated by the Lake Superior isolates and a limited number from the St. Mary's River. Of the 21% of the *Acinetobacter* isolates from Lake Superior (coliform density less than 10 per 100 ml.), only 5.7% belonged to subgroups I and II compared to 93% for the Lake Ontario isolates. Of a small number of isolates from the St. Mary's River (26), 50% were *Acinetobacter*, and all of these belonged to subgroups I and II. None of the coliform densities for the St. Mary's River samples from which the isolates were obtained was less than 1000 per 100 ml. From the results of this study on the distribution of *Acinetobacter*, it would appear that these organisms are indeed a natural inhabitant of the aqueous environment.

Table VI: Characteristics of *Flavobacterium* Subgroups, and percentage of total *Flavobacterium* isolates within each group.

	Subgroup						
Characteristic	Typical	I	II	III	IV	V	VI
Motility	-	-	+	+	-	+	-
Catalase	+	+	+	+	+	+	+
Oxidase	+	+	-	-	-	+	-
Glucose	A	A/-	A	A	A/-	-	A
O-F	0	-/ALK	0	-	-	-	0
Percentage of Total Isolates	4.3	23.6	1.7	10.5	47.3	8.8	4.0

Table VII: Characteristics of *Acinetobacter* subgroups and percentage of total *Acinetobacter* isolates within each group.

	Subgroup			
Characteristic	I	II	III	IV
Motility	-	-	-	-
Catalase	+	+	+	+
Oxidase	-	-	+	+
O-F	-/ALK	0	-	0
Percentage of Total Isolates	81.9	11.3	3.1	3.7
Percentage of LF* within each subgroup	53.1	62.5	45.5	23.1

* LF - red or pink colonies on MacConkey Agar.

Their increased frequency of isolation as level of coliform increased, suggests that pollution favours their proliferation. These organisms perhaps could serve as indicators of nutrient enrichment of the environment, much as certain of the fecal bacteria serve as indicators of the disease potential of the environment. The prime difference here is that one group of bacteria is being added via the pollutant, while the second represents organisms already present, responding to the pollutant.

Baumann (1968) describes a medium consisting of 0.2% sodium acetate (trihydrate) , 0.2% KNO_3, and 0.02% $MgSO_4$.$7H_2O$ in 0.04 m KH_2PO_4 - Na_2HPO_4 buffer (pH 6.0) containing 20 ml per litre of Hutner's mineral base (Cohen *et al*, 1957) for enrichment of *Acinetobacter*. It might be profitable to employ this medium in an MPN or MF procedure in conjunction with the medium of Mandel *et al* (1964) in comparing the types of organisms recovered, and to relate their numbers to other pollution parameters useful in evaluation of the water quality of lakes.

Enterobacteriaceae:

The classification of the Enterobacteriaceae isolates according to coliform level and water temperature yielded surprising results. Rather than increasing with increasing coliform level, the percentage of Enterobacteriaceae decreased significantly (χ^2 = 8.17 > 5.99 = χ^2 , 0.05 level). The incidence of the genus *Proteus* accounted primarily for this decrease. When classed according to temperature levels, the percentage of Enterobacteriaceae increased significantly with increasing temperature level (χ^2 = 21.98 > 3.84 = χ^2 , 0.05 level), again attributable to the isolation pattern of the genus *Proteus*. The results of the Chi-square tests prompted a re-grouping of the *Proteus* isolates in two-way classification, according to both water temperature and coliform level. A Chi-square test for independence of proportions was performed. The results are presented in Table VIII. As can be seen in the table, the bacterium rarely

Table VIII: Percentage of *Proteus* among the total isolates grouped according to coliform level and "*in situ*" water temperature of the sample from which they were obtained.

Water Temperature (°C)	Coliforms per 100 ml			
	0-10	10-100	100-1000	>1000
< 5-10	1.1	2.7	0.5	-
10-15	6.2	1.5	2.4	0.8
> 15	25.9	24.6	-	4.9

$\chi^2 = 15.19^*$, d.f. = 6.

* significant at the 5% level of probability.

occurred on plate counts at the two highest coliform levels, but was isolated frequently at the lower levels when water temperatures were above 15°C, and particularly along the Niagara Peninsula from the Niagara River to Stoney Creek. The observation that Enterobacteriaceae multiply in Bay of Quinte waters as temperatures increase was made by Dutka (personal communication), working with pure and mixed cultures. The incidence of other genera within the family Enterobacteriaceae on total plate counts was not significantly associated with either coliform level or water temperature. *Enterobacter* was the most frequently isolated; *Escherichia* was recovered on rare occasions from 20°C plates.

SUMMARY AND CONCLUSIONS:

I. A determinative scheme is presented for the identification of water bacteria isolated from total plate counts. As with any scheme, there are certain limitations. Better methods for identifying the yellow pigmented Gram-negative bacteria, and the Gram-positive bacteria occurring in lakes are required.

II. Gram-positive bacteria formed only a small proportion (10%) of the total number of heterotrophic bacteria isolated from Great Lakes water. Preliminary investigations with different media to be reported in a later paper, suggest that this may be in part due to the isolation medium.

III. The occurrences of *Pseudomonas, Aeromonas* and *Proteus* were related to water temperature. The proportion of *Pseudomonas* decreased when the water temperature was above 15°C, while that of *Proteus* increased. The percentage of *Aeromonas* was greatest at temperatures between 10 and 15°C. Prevalent among the *Aeromonas* isolates was the potential fish pathogen *A. salmonicida*.

IV. The incidence of the genus *Acinetobacter* was significantly associated with the level of pollution, as measured by coliform density per 100 ml. Since 93% of the Lake Ontario isolates within the genus *Acinetobacter* were recognized as belonging to a group of natural soil and water forms, it appears that these organisms are responding to enrichment of the environment, rather than being of pollutional origin themselves. As such they may serve as a useful indicator of eutrophication in the fresh water lakes. Two media are suggested for use in their detection.

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APPENDIX I

METHODS EMPLOYED IN THE ISOLATION AND IDENTIFICATION OF BACTERIA FROM 20°C PLATE COUNTS

by

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1969

IDENTIFICATION TESTS FROM GREAT LAKES' PLATE COUNTS

APPENDIX I

Colony Isolation and Purification:

Isolates were taken from plates containing 20-200 colonies. Each plate was divided into sectors and ten colonies were isolated from the same sector and streaked onto ten separate T. Soy Agar plates. Each isolate was numbered as follows: prefix - P.C., followed by the lab number and an alpha notation, A-J. The plates were incubated at 20°C for 5 to 7 days and examined daily for well isolated colonies.

One colony was picked from each plate and re-streaked onto another T. Soy Agar plate. If two or more distinct colonies were observed on any one plate, one colony of each type was picked and streaked onto a separate T. Soy Agar plate. Each was numbered with the lab number and the alpha notation (upper case) of the original plate and a separate small alpha notation (lower case). These were incubated at 20°C for five days.

The plates were observed for purity on the basis of colonial morphology. Two T. Soy Agar slants were then prepared from the same colony, one being the working slant, the other for stock culture. They were both incubated at 20°C for 48 hours.

Both slants were observed for purity on the basis of the Gram stain and if both were pure, the stock was placed in the refrigerator and a group of primary and secondary tests were done from the working slant to establish the identity of the culture. Mixed cultures were re-streaked onto T. Soy Agar plates.

DIAGNOSTIC TESTS:

Primary Tests

Gram Stain: (Kopeloff - Beerman Modification)

Solution A		Solution B	
Crystal Violet	- 1 gm	$NaHCO_{3,}$	- 1 gm
Distilled Water	- 100 ml	Distilled Water	- 20 ml

Burke's Iodine		Burke's Counterstain	
Iodine	- 1 gm	Safranin O (85% dye content)	- 2 gm
Potassium Iodide	- 2 gm		
Distilled Water	- 100 ml	Distilled Water	- 100 ml

Decolorizing Agent

Ethyl Alcohol - 95% - 7 Parts

Acetone - 3 Parts

1) Heat-fixed smears were flooded with Solution A: a few drops of Solution B were added, and the mixture allowed to stand for 3 minutes.

2) The slides were rinsed with Burke's iodine, covered with fresh iodine solution and allowed to stand for 2 - 3 minutes.

3) The slides were rinsed with tap water, and then with acetone-alcohol solution. The slides were flooded with the decolorizer for 8 - 10 seconds, and then rinsed with tap water.

4) The slides were counterstained with Burke's safranin for 5 - 10 seconds.

5) The slides were rinsed in tap water, dried, and examined.

Results: Gram-positive organisms, blue, Gram-negative organisms, red.

Catalase Activity:

A portion of the growth was placed on a slide. Three drops of 3% H_2O_2 were added and the slide was examined immediately and after two minutes. The ebullition of gas was indicative of a positive reaction.

Oxidase Activity:

Equal amounts of oxidase reagents (A: - 1% naphthol in 95% ethanol, B: - dimethyl-p-phenylene diamine dihydro-chloride preserved by the addition of 0.1% aqueous ascorbic acid) were added to a filter paper placed in a petri dish. A portion of the growth was removed with a platinum loop and smeared on the reagent impregnated paper. The appearance of a blue colour within 30 seconds constituted a positive reaction.

Motility:

Two successive transfers were made through T. Soy broth, incubating the first tube for 48 hours, the second for 24 hours at 20°C. A hanging drop slide was prepared from the 24 hours broth culture and observed for motility. If no growth appeared in the 24 hour tube, a hanging drop slide was prepared from the 48 hours broth culture. If

negative for motility, a third tube was incubated and read after the appearance of visible growth.

Growth on MacConkey:

MacConkey agar plates were streaked, one half plate per isolate and incubated for 48 hours at 20°C. The plates were examined and the observations were recorded as follows:

LF - red or pink colonies
NLF - colorless or yellowish colonies
NG - no apparent growth.

Acid from Carbohydrates:

Tubes of phenol red dextrose broth were inoculated, incubated at 20°C and examined daily for seven days for acid production (indicator change from orange to yellow), acid and gas production or alkalinity. An uninoculated tube served as a control.

Oxidation or Fermentation of Glucose (Hugh & Leifson, 1953):

Tubes of O-F medium with 1% glucose were stab-inoculated, incubated at 20°C, and examined daily for 7 days. An uninoculated tube was incubated as a control. If no reaction was observed after seven days, the test was repeated using the O-F medium enriched with 0.1% yeast extract. A yellow zone appearing in the surface layer of agar in the tube was indicative of an oxidative attack on glucose: yellow throughout the tube, of a fermentative attack: blue, and alkaline reaction; green, no attack on glucose.

Spore Stain:

Spore stains were done on all Gram-positive organisms and certain Gram-negative organisms which failed to grow on MacConkey agar. Prepared slides were flooded with 5% aqueous malachite green and steamed for 6 minutes. They were then rinsed with water, blotted and counterstained for 3 minutes with 0.5% aqueous safranin (85% dye content).

Spores stained green; vegetative cells red.

Secondary Tests:

Secondary tests were done on all cultures of the family Enterobacteriaceae and of the genera *Aeromonas* and *Pseudomonas* and on all orange pigmented bacteria. Incubation was at 35°C for the Enterobacteriaceae or at 20°C, if no growth was evident. Biochemical tests for all other groups were performed with 20°C incubation.

Enterobacteriaceae

Citrate Utilization:

Slants of Simmons' citrate were inoculated by streaking the surface of the slant with a light inoculum. Tubes were incubated and examined daily up to seven days. A colour change from green to blue, and appearance of growth was indicative of citrate utilization.

Indole Production:

Tubes of tryptone broth were inoculated and incubated for 48 hours. Three drops of Kovac's reagent were added to the broth cultures and the tubes were shaken and allowed to stand for a few minutes. The production of a deep red colour in the reagent layer was indicative of the presence of indole.

Methyl Red Test:

Tubes of MR-VP broth were inoculated and incubated for 5 days. Two to three drops of methyl red solution were added to the broth cultures. The tubes were shaken and examined. The production of a red colour constituted a positive reaction; yellow, a negative reaction.

Voges-Proskauer Test for acetylmethylcarbinol:

Tubes of MR-VP broth were inoculated and incubated for 48 hours. To each tube 0.6 ml. of a 5% alpha-naphthol solution (in absolute ethyl alcohol) and 0.2 ml. of 40% potassium hydroxide solution were added. The tubes were well shaken, allowed to stand for 1-2 hours and examined at regular intervals. The production of a red colour was indicative of a positive reaction.

Lactose Fermentation:

Tubes of Brom-cresol purple broth with 1% lactose were inoculated and incubated for up to seven days. An indicator change from purple to yellow with or without gas production was indicative of lactose fermentation.

Gelatin Hydrolysis:

Nutrient gelatin plates were inoculated by heavily streaking a portion of the plate, incubated at 20°C for 48 hours and then left at room temperature for an additional 24 hours. The surfaces of the plates were flooded with 5-10 ml. of acid mercuric chloride solution (Frazier) and examined for zones of clearing.

Urease Activity:

Slants of Christensen's urea medium were inoculated by streaking the surface of the slant with a light inoculum. The tubes were incubated and examined daily for 5 days. The production of a red colour was indicative of a positive reaction.

Phenylalanine Deamination:

Slants of phenylalanine agar were inoculated by streaking the surface of the slant with a heavy inoculum. The tubes were incubated for 48 hours. 0.2 ml. 10% $FeCl_3$ aqueous solution was allowed to run down over the growth on the slants. The development of a green colour on the slant and in the fluid at the base of the slant was indicative of a positive reaction.

Nitrate Reduction:

Tubes of nitrate broth were inoculated and incubated for 48 hours. To each tube, 5 drops of nitrite reagent A (0.8% sulphanilic acid in 5 N - acetic acid) was added, followed by 5 drops nitrite reagent B (0.5% alpha- naphthylamine in 5 N - acetic acid). The production of a red colour was indicative of the presence of nitrite.

A small amount of powdered zinc was added to the tubes not showing a red colour. The presence of unreduced nitrate in the medium was revealed by the development of a red colour.

Enterobacteriaceae and Aeromonas

Decarboxylase Tests (Moller Base):

Tubes of four media (1% arginine, 1% lysine, 1% ornithine in Moller Base and control - Moller Base alone) were inoculated and several drops of sterile liquid paraffin were added to the surface of each of the media. The tubes were incubated at 20°C and examined daily up to seven days for colour change. The production of a violet colour subsequent to a yellow colour in the amino-acid containing tubes was indicative of decarboxylation. Control tubes remained yellow.

Pseudomonas:

Fluorescein production tubes of Drake's basal medium were inoculated, incubated at 20°C and examined daily up to 5 days. Growth and fluorescence when examined under a U.V. light (wave length - Long Wave UVL-21) indicated the presence of the pigment fluorescein.

Orange Pigmented Cultures Test for Swimming:

A tube of T. Soy broth was inoculated with the test culture and incubated for 48 hours. To the centre of a well dried yeast extract (0.1%) agar plate, 0.1 ml of the broth cultures was added. The plates were incubated and examined daily up to 5 days for the appearance of a thin film of growth radiating from the central inoculum over the surface of the agar.

Penicillin Sensitivity:

A Sensi-disc, containing 2 I.U. penicillin was placed in the centre of a T. Soy Agar plate, previously spread with a 0.1 ml. aliquot of the test culture (24-48 hour T. Soy broth suspension). Plates were incubated and examined daily for five days, for evidence of penicillin sensitivity (clear zone surrounding the Sensi-disc).

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