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The selective enumeration and isolation of blue-green bacteria from water

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THE SELECTIVE ENUMERATION AND ISOLATION OF BLUE-GREEN BACTERIA FROM WATER

by

WALTER F. HODGSON

A thesis
Submitted to the Faculty of Graduate Studies through the
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ABSTRACT

A membrane filter method for the selective enumeration of blue-green bacteria has been developed which on the basis of studies with axenic stock cultures and field tests has proved to be both practical and reproducible. The filters are incubated under specified conditions of temperature and illumination on a mineral salts agar medium supplemented with yeast extract and containing cycloheximide to eliminate eukaryotic contaminants. Axenic cultures of many blue-green bacteria have been obtained with relative ease either directly or after preliminary enrichment from membrane filter colonies by means of ommimixer-glass bead dispersion followed by dilution and surface plating on mineral salts agar.

ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to Dr. H.D. McCurdy, Jr., Department of Biology, University of Windsor, for his advice and encouragement during the course of this study and for his guidance in the preparation of this thesis.

The author is grateful also to Dr. D.G. Wallen, Department of Biology and Dr. J.K. Bewtra, Department of Civil Engineering, both of the University of Windsor, for their aid in the reviewing of this thesis.

A special thanks is extended to Messrs. F.M. Lukacs and T.D. Edwards for their assistance with research materials.

This investigation was supported by grant A1022 from the National Research Council of Canada.

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INTRODUCTION

There appear to be no very satisfactory methods reported in the literature for the enumeration of blue-green bacteria from natural waters on a routine basis.

Allen and Stanier (1968) suggested the dilution to extinction method for the enumeration of blue-green bacteria using high temperature and a nitrogen-deficient medium as selective factors. In the same year, Jurgensen and Davey (1968) reported the use of the agar pour plate method and a nitrogen-deficient medium to determine the number of blue-green bacteria in soils. Both methods however have obvious limitations since only a relatively small segment of the total viable blue-green population is measured, those that fix atmospheric nitrogen and are able to grow at higher temperatures.

Various modifications of the membrane filter technique have gained wide acceptance in sanitary, clinical and industrial microbiology as reviewed by Mulvany (1969). The method has been especially useful when applied to liquid samples for the purpose of determining viable counts (Jannasch and Jones, 1959) and direct microscopic counts of microorganisms entrapped on the filters (Erhlich, 1955; Ecker and Lockhart, 1959; Jannasch, 1958; Jannasch and Jones, 1959). The membrane filter technique is now the method of choice for the estimation of fecal coliforms and streptococci from some water samples.

Recently, Simard and Blackwood (1970) used membrane filters to estimate viable yeast populations from natural waters. Although almost exclusively used for enumeration of chemoorganotrophs, Swoager

and Lindstrom (1971) reported the application of an anaerobic modification of the method for the determination of viable counts of Rhodospirillaceae. The only other application of the membrane filter technique to estimate numbers of photosynthetic organisms is that reported by McNabb (1960) who used direct microscopic counts for total phytoplankton populations in freshwater.

In view of the wide application of the technique, it is surprising that the membrane filter method has not been used for the estimation of blue-green bacteria. It is therefore the purpose of this report to describe the successful application of the membrane filter technique to the selective enumeration of viable blue-green populations from natural waters and additionally to describe how the method may be adapted to the isolation of blue-green bacteria in pure culture.

The difficulty in obtaining axenic cultures of blue-green bacteria may be attributed largely to the presence of a copious slime or sheath in which most blue-greens are embedded and in which other prokaryotes are frequently entrapped. Numerous techniques have been tried to obtain pure cultures with varying degrees of success including standard surface plating methods (Gerloff et al., 1950; Allen, 1952; Stanier et al., 1971), ultraviolet irradiation (Gerloff et al., 1950), antibiotics (Tchan and Gould, 1961), cloning of hormogonia (Bunt, 1961), detergents and phenol (McDaniel et al., 1962), atomizer treatment (Wiedeman et al., 1964), heat treatment (Wieringa, 1969), and phototactic migration (Stanier et al., 1971). These techniques, however, are either too

laborious or relatively inefficient for use on a routine basis.

In this report it is demonstrated that the discrete colonies formed on the membrane filters, coupled with glass bead dispersion and surface plating, provide a convenient and rapid means for the isolation of many blue green bacteria in pure culture.

MATERIALS AND METHODS

Cultures

The following axenic blue-green bacterial cultures received from Dr. R.Y. Stanier were used during this study: *Anacystis nidulans*, strain 6301 and Berkeley isolate, strain 6312 (Stanier typological group IA, Stanier *et al.*, 1971); Berkeley isolate, strain 6903 (Stanier typological group IB); *Gleocapsa alpicola*, strain 6308 (Stanier typological group IIA); Berkeley isolate, strain 6501 and *Gleocapsa* sp. 6909 (Stanier typological group IIB); *Chloroglea fritschii* (Stanier typological group III); *Anabaena* sp. and *Plectonema* sp.

Eukaryotic algal cultures of *Chlorella vulgaris*, *Chlamydomonas reinhardtii* and *Scenedesmus quadricauda* were supplied by Dr. D.G. Wallen. Two isolates of the division Chlorophyta from this laboratory were also used during preliminary studies.

Media and Conditions of Cultivation

The basic mineral medium used for the maintenance of cultures and for all experimental work was that of Hughes *et al.* (1958) as modified by Allen and Stanier (1968) and designated BG11 (see Appendix). Each component of the medium was prepared and autoclaved separately and the complete basic medium was adjusted to pH 8.0 with N NaOH. As required, the medium was solidified with 1% Difco Bacto agar as described by Allen (1968). All incubations were at 30 C under cool fluorescent lights and intensity was measured using a Tri-Lux footcandle meter (Grossen GMBH, Erlangen, West Germany).

Membrane Filter Technique

Membrane filters of cellulose acetate (Millipore Corporation) or mixed esters of cellulose (R-B filters, Ltd.) with an average pore diameter of 0.45 μ were used. Surface samples from various bodies of water in Essex County, Ontario were collected in 100 ml sterile screw-capped bottles, returned to the laboratory and if possible processed the same day. All samples were agitated for 30 minutes on a wrist action shaker and when necessary, diluted in BG11 broth prior to filtration. Appropriate volumes of 10 to 100 ml of diluted or undiluted sample were passed through the membrane using low house vacuum after which the filtration apparatus was rinsed thoroughly with BG11 broth. Membranes were then placed on the growth medium (see below), incubated as described and examined twice weekly for the appearance of colonies. Counts were made using an Olympus dissecting microscope.

Comparison of Agar versus Saturated Pads Underlay or Substrate

Nine logarithmically growing blue-green bacterial cultures (see above) were filtered through membranes which were placed on either BG11 agar or on absorbent cellulose pads saturated with BG11 broth and incubated as described. Colony counts obtained on the two media from the same samples were then compared. On the basis of the results, all subsequent work was carried out using BG11 agar medium.

Comparison of Direct Microscopic and Membrane Filter Counts

Logarithmically growing cultures of nine blue-green bacteria were counted both by the membrane filter technique and direct micro-

scopic examination using a Brightline Hemocytometer and the counts compared.

Incorporation of Cycloheximide (Actidione)

During preliminary work with field samples, it was observed that the predominance of eukaryotic algal colonies on the filters often made counting of blue-green bacterial colonies exceedingly difficult. Therefore, filter sterilized cycloheximide (Sigma Chemicals) was added to the BG11 agar in concentrations of from 5 to 50 ug/ml in order to determine the optimum concentration permitting growth of the nine test strains of blue-green bacteria while inhibiting eukaryotic algae. In subsequent work 20 µg/ml of the antibiotic was incorporated into the solid medium on a routine basis. (see Appendix).

Effect of Yeast Extract on Colony Counts

Difco yeast extract (0.01%) was added to the BG11 agar in an attempt to increase colony counts and to obtain consistency among the various dilutions used. Initially, counts using nine test strains of non-logarithmic blue-green bacterial cultures, incubated with and without yeast extract, were compared. Field samples were then tested using the enriched medium. In subsequent work with water samples, filters were incubated on BG11 agar containing yeast extract. (see Appendix).

Effect of Light Intensity

Field samples were processed as described and the membranes incubated under 50, 100 and 150 foot candles light intensity in order to determine light conditions giving maximum colony counts.

Most Probable Number (MPN) Method

Aliquots from each of a series of tenfold dilutions of water samples were inoculated into five 10 ml volumes of BG11 broth. The tubes were incubated as described for 4 weeks and then examined microscopically for the presence of blue-green bacteria. MPN's were computed using tables in Standard Methods for the Examination of Water and Wastewater (1971). The MPN results obtained were compared to those from identical water samples using the membrane filter technique.

Comparison of Surface Plating and Membrane Filter Counts

BG11 agar containing cycloheximide and yeast extract was dispensed in 30 ml volumes in petri plates. One ml and 0.1 ml volumes of water samples were spread over the surface of agar plates with a sterile bent glass rod. Plates were incubated inverted, with illumination from below, and examined frequently for the presence of blue-green bacterial colonies. Counts obtained were compared with those from the membrane filter method.

Isolation

A modification of the technique of McCurdy (1963) used for the isolation of Myxobacteria was adapted for use during this study.

Initially, blue-green bacterial colonies were picked from the filters with a fine needle using a dissecting microscope and transferred to BG11 broth for enrichment. When growth was evident, 0.5 ml of a culture was placed in a sterile Omnimixer vessel (Ivan Sorvall, Norwalk, Conn.) containing glass beads (-100 +120, Flexolite Ltd., St. Thomas, Ont.) and 2.0 ml of BG11 broth. Cells were dispersed for from

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60 to 90 seconds at a rheostat setting of 80 while the vessel was immersed in ice water. After dispersion the liquid was examined microscopically to ensure the presence of intact cells and then diluted using 10-fold dilutions. One-tenth ml of each dilution was spread over the surface of a predried (20 minutes at 37 C) BG11 agar plate with a sterile bent glass rod. Plates were incubated in the inverted position, illuminated from below and examined daily under a dissecting microscope for the appearance of isolated blue-green bacterial colonies. Such colonies were picked and transferred to BG11 broth and agar.

In later work it was found that Omnimixer treatment of colonies picked directly from the filters followed by dilution and plating gave more rapid and consistent results than the initial broth enrichment procedure.

Cultures were tested for purity by inoculation into tubes of Difco nutrient broth, Oxoid trypticase-soy broth, and a dilute medium containing 0.05% peptone and 0.01% yeast extract in tap water. Tubes were incubated at 30 and 37 C in the dark for several weeks and examined for the growth of bacterial contaminants. Blue-green bacterial cultures were also examined by phase microscopy for the presence of contaminants. Cultures considered pure were those failing to exhibit growth in the bacterial test media and which, after repeated microscopic observation, showed no evidence of contamination.

For identification purposes wet mounts of the isolates were

made using both BG11 broth and India ink. The references used for identification were Stanier et al., (1971), Kenyon et al., (1972), Prescott (1970), and Desikachary (1959).

Photomicrographs were taken with a Carl Zeiss (Jena) Nfpk microscope and a Carl Zeiss (Jena) automatic exposure device using high contrast ASA 64 black and white film (Eastman Kodak, Rochester, N.Y.).

EXPERIMENTAL RESULTS

As shown in Table 1, membrane filters on BG11 agar yielded consistently higher counts from laboratory cultures, in the logarithmic phase, than were obtained on pads saturated with broth. Incubation on the solid medium also resulted in increased colony size and minimized problems due to evaporation.

A comparison of direct microscopic and membrane filter counts, when applied to logarithmic cultures, showed substantial agreement between the two methods (Table 2), indicating good recovery on the filters and justification for continuing with the development of the method.

Morris (1966). and Palmer and Maloney (1955). showed that cycloheximide inhibits the growth of eukaryotic algae while not affecting the growth of blue-green bacteria. Therefore, since it was necessary to eliminate eukaryotes from the membrane filters, cycloheximide was added to the solid medium. Table 3 shows the effect of the antibiotic upon the growth of several eukaryotic algae and ten types of blue-green bacteria. In this study, 20 ug/ml was the minimum concentration inhibiting eukaryotes while having no effect upon the growth of blue-green bacteria. Growth of the latter was unaffected even at a concentration of 50 ug/ml. In tests of field samples (Table 4), the addition of 20 ug/ml of cycloheximide to the solid medium virtually eliminated eukaryotic contaminants and allowed somewhat increased colony counts of blue-green bacteria.

The medium, as described thus far, gave reasonably good results with most samples but in some instances, counts obtained at the high dilutions were not consistent with those of low dilutions. Some blue-greens are known to be stimulated by organic growth factors (Pringshiem, 1949; Allen, 1952; and Provasoli, 1958). It seemed possible that non-photosynthetic bacteria present at the lower dilutions were providing growth factors which being absent at the higher dilutions resulted in lower counts. Therefore the effect of the addition of yeast extract to the medium was examined. As shown in Table 5, consistently higher counts were obtained using nine test strains of blue-green bacteria. The rather striking increase in colony counts may be attributed to the fact that no effort was made to ensure the use of logarithmic cultures and most were probably in the stationary phase. Counts obtained from field samples incubated on the enriched yeast extract medium were substantially higher (Table 6), and counts obtained at the various dilutions were consistent.

Preliminary studies involved a range of light intensities. It was necessary, therefore, to establish light conditions yielding maximum counts on the filters and which would avoid the bleaching of colonies often observed at the higher intensities. As can be seen in Table 7, of the three light intensities tested, 100 foot candles yielded the highest colony counts. Consequently, 100 foot candles were chosen as one of the routine conditions of incubation.

As given in Table 8, colony counts using the membrane filter technique were consistently higher than those obtained by the MPN method.

Table 9 shows that comparable counts were obtained between the membrane filter technique and surface plating. However, considerable difficulty in counting was experienced with the latter method due to spreading of the colonies over the agar surface (Figure 1b), which explains why so few comparisons between the two methods are available. On the filters, colonies generally remained discrete and easily countable (Figure 1a).

During the course of this study 24 blue-green bacteria of both unicellular and filamentous types were isolated in pure culture from water sources in Essex County, Ontario. Of two alternative procedures attempted, that involving direct Omnimixer treatment of colonies picked from filters, followed by plating, gave the more favourable results. During a single run, 8 axenic cultures of blue-green bacteria were obtained in less than 3 weeks by direct transfer. The isolates were keyed out according to the literature available and Table 10 lists the organisms and the sources of isolation. Photomicrographs of the isolates are shown in Figures 2 to 4.

DISCUSSION

The results indicate that the membrane filter technique using BG11 agar containing cycloheximide and yeast extract is both a practical and reproducible method for the selective enumeration of blue-green bacteria in water. By comparison, the MPN method, although commonly used in the enumeration of other bacteria, is statistically of somewhat limited precision and less convenient in many respects. Surface plating, although rapid and easily carried out, is limited in its usefulness by the small sample size that may be applied and by the tendency of colonies to spread over the agar surface. On the filters however, spreading appears to be restricted with the result that colonies are more easily counted.

The membrane filter technique may be expected to be of considerable practical utility. It may be applied to monitoring nuisance populations in domestic water supplies and purification plants, to studies of blue-green bacteria associated with the production of toxic factors, to studies of the relationship between cyanophage and fluctuations in blue-green bacterial populations and to other studies in which blue-green bacteria may affect the quality and character of water supplies. The method may also be used in general ecological studies involving determinations of biomass, distribution and productivity of blue-green bacteria in freshwater and marine systems. With minor modifications it may be possible to adapt the method to the study of

certain soils.

An important consequence of the use of membrane filters is the ease with which axenic cultures may be obtained from the colonies produced. By direct transfer of the colonies to an Omnimixer, followed by dispersion and plating, a large number of cultures can be procured in a relatively short period of time. As evidenced by the number and variety of morphological types obtained, the method is both efficient and rapid, a significant improvement over previous techniques which are both laborious and too frequently unsuccessful. The availability of an easy-to-use pure culture method may serve to stimulate further studies leading to the elucidation of problems of taxonomy, gliding motility, nutrition and metabolism in blue-green bacteria.

Figure 1a and b. Comparison of Colonies on Agar and the Membrane Filter.

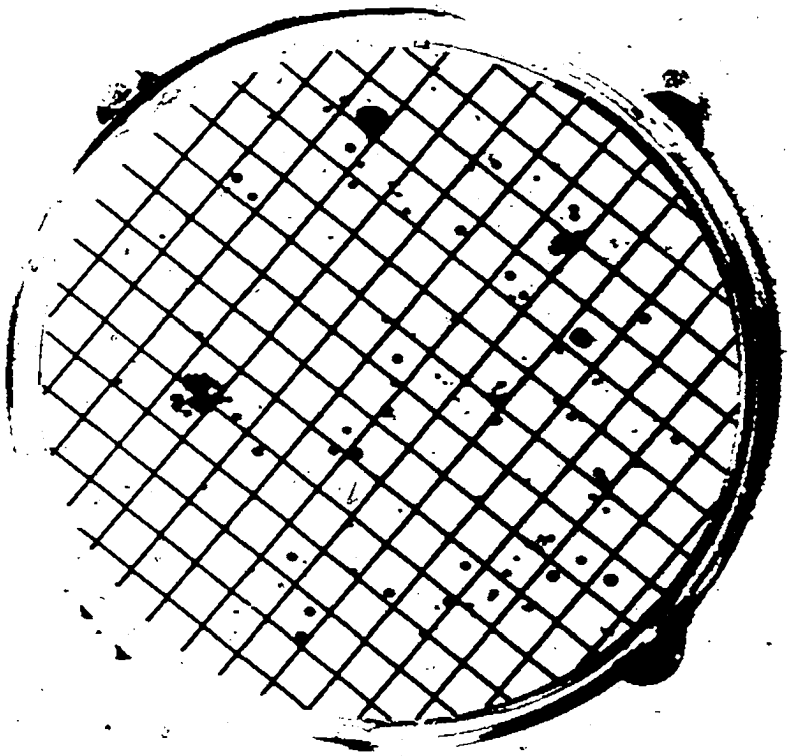


Figure 2. Phase Micrographs of Axenic Blue-Green Bacterial Isolates. Isolates 1 to 8. x 1,350. Isolate numbers correspond to the listing in Table 10.

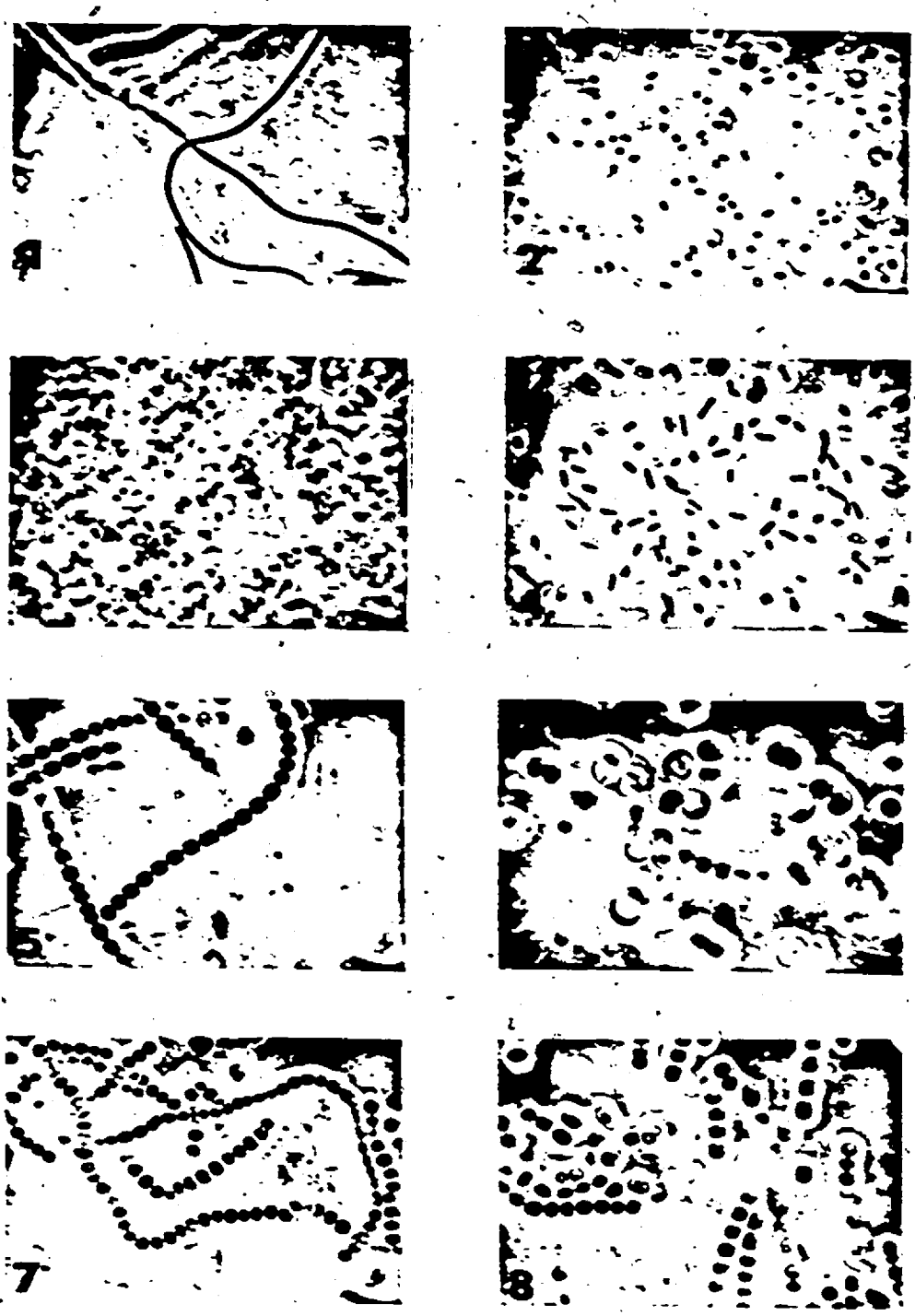
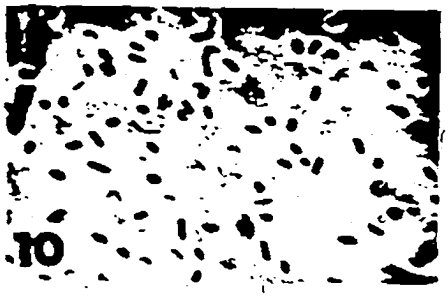
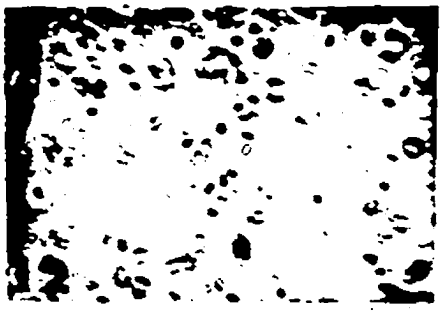
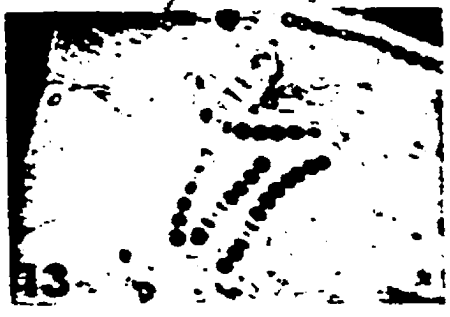


Figure 3. Phase Micrographs of Axenic Blue-Green Bacterial Isolates. Isolates 9 to 16. x 1,350. Isolate numbers correspond to the listing in Table 10.







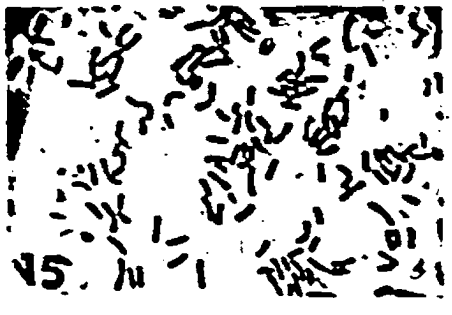
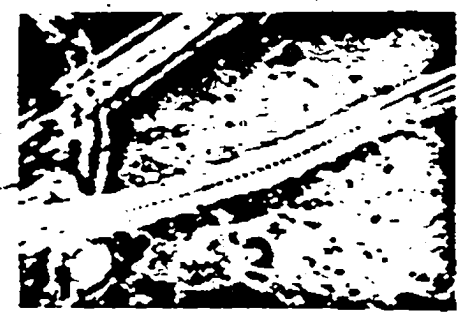
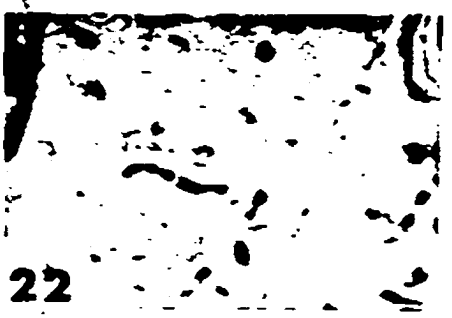


Figure 4. Phase Micrographs of Axenic Blue-Green Bacterial Isolates. Isolates 17 to 24. x 1,350. Isolate numbers correspond to the listing in Table 10.





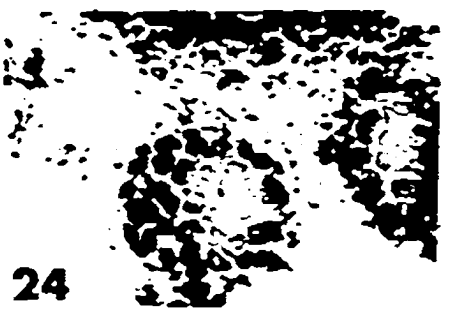


Table 1. Comparison of Membrane Filter Counts on Agar versus Pads Saturated with Broth.

Culture	Counts ml^{-1}*	
	Agar	Pads
Gleocapsa alpicola 6308	830	570
Berkeley isolate 6312	1120	610
Gleocapsa sp. 6909	880	410
Berkeley isolate 6903	250	210
Anabaena sp.	420	280
Chloroglea fritschii	2770	2220
Anacystis nidulans 6301	2710	1640
Plectonema sp.	640	430
Berkeley isolate 6501	1210	730

* Average of four counts

Table 2. Comparison of Direct Microscopic and Membrane Filter (M.F.) Counts.

Culture	Counts ml^{-1}*	
	Direct counts	M.F. Counts
Gleocapsa alpicola 6308	2770	2500
Berkeley isolate 6312	2630	2680
Gleocapsa sp. 6909	4870	4330
Berkeley isolate 6903	2110	1850
Anabaena sp.	3870	3710
Chloroglea fritschii	5960	4750
Anacystis nidulans 6301	1420	1660
Plectonema sp.	4760	3860
Berkeley isolate 6501	3240	3030

* Average of four counts.

Table 3. Effect of Cyclohexamide (Actidione).

Eukaryotic algae	Cycloheximide, ug ml^{-1}*					
	5	10	20	30	40	50
Scenedesmus quadricauda	+	-	-	-	-	-
Chlorella vulgaris	+	+	-	-	-	-
Chlamydomonas reinhardtii	+	+	-	-	-	-
Isolate 34	+	-	-	-	-	-
Isolate 12	-	-	-	-	-	-
Blue-green bacteria						
(a) Filamentous types (5 strains)	+	+	+	+	+	+
(b) Unicellular types (5 strains)	+	+	+	+	+	+

* (+) = growth

(-) = absence of growth

Table 4. Effect of Cycloheximide on Counts from the Field (counts ml^{-1} *)

Source	Control Plates No cycloheximide		Test Plates Cycloheximide 20 ug/ml	
	Eukaryotes	Blue-Green Bacteria	Eukaryotes	Blue-Green Bacteria
Cedar Creek	17,500	1,760	0	2,170
River Canard	21,300	1,000	2	1,630
L. Erie. E. beach	17,200	10,500	0	11,400
Detroit River	9,340	300	0	360
Sales Pond	1,490	2,200	0	2,010

* Average of four counts

Table 5. Effect of 0.01% Yeast Extract on Colony Counts

Culture	Counts ml^{-1}*	
	No Yeast Extract	Yeast Extract
Gleocapsa alpicola 6308	1120	1490
Berkeley isolate 6312	1890	2410
Berkeley isolate 6903	510	680
Gleocapsa sp. 6909	830	1150
Anabaena sp.	880	830
Chloroglea fritschii	2770	2910
Anacystis nidulans 6301	2380	3010
Plectonema sp.	1220	1730
Berkeley isolate 6501	750	1180

* Average of four counts

Table 6. Effect of 0.01% Yeast Extract on Colony Counts from Water Samples.

Sample Site	Counts ml^{-1}*	
	Yeast Extract	No Yeast Extract
Dump Pond (1)	220	132
Sales Pond	1600	1040
L. Erie W. Beach	5060	3350
Dump Pond (2)	86	50
Detroit River	372	206
Marsh (Pt. Pelee)	18	13

* Average of four counts

Table 7. Effect of Light Intensity on Colony Counts (counts ml^{-1}*)

Sample site	Light intensity (foot candles) 50	100	150
Sales Pond	980	2010	740
Detroit River	210	360	170
Dump Pond	220	220	150
L. Erie E. Beach	8800	11400	5950
River Canard	1510	1630	890
Cedar Creek	1760	2170	1160
Pt. Pelee Marsh	170	170	180
L. Erie W. Beach	4610	4760	2590

* Average of four counts

Table 8. Comparison of Membrane Filter (M.F.) and Most Probable Number (M.P.N.) Counts.

Sample site	Counts ml^{-1}*	
	M.F.	M.P.N.
Sales pond	2010	1600
Detroit River	360	220
Dump pond	220	220
L. Erie, E. beach	11400	2400
River Canard	1630	540
Cedar creek	2170	920
Pt. Pelee Marsh	168	110
L. Erie, W. beach	4760	2400

* Average of four counts

Table 9. Comparison of Membrane Filter (M.F.) and Surface Plate Counts (Agar).

RUN #	Counts ml^{-1}*	
	M.F.	AGAR
1.	21	22
2.	16	15
3.	22	20

* Average of four counts

Table 10. List of Isolates: Essex County, Ontario

Isolate		Source
1.	Lyngbya sp.	Detroit River
2.	Chroococcus sp.	Lake St. Clair
3.	Aphanocapsa sp.	Detroit River
4.	Synechococcus sp.	Winogradsky Column
5.	Anabaena sp.	Detroit River
6.	Aphanocapsa sp.	Sales farm pond
7.	Anabaena sp.	Lake St. Clair
8.	Anabaena sp.	Lake St. Clair
9.	Aphanocapsa sp.	Sales farm pond
10.	Synechococcus sp.	Winogradsky Column
11.	Chroococcus sp.	Marsh, Pt. Pelee National Park
12.	Anacystis sp.	Marsh, Pt. Pelee National Park
13.	Anabaena sp.	Lake St. Clair
14.	Oscillatoria sp.	Lake St. Clair
15.	Synechococcus sp.	Winogradsky Column
16.	Aphanocapsa sp.	Detroit River
17.	Chloroglea sp.	Detroit River
18.	Anabaena sp.	Lake St. Clair

(Table 10 - cont.)

19. *Oscillatoria* sp.	Lake St. Clair
20. PP 14	Marsh, Pt. Pelee National Park
21. PP 7	Marsh, Pt. Pelee National Park
22. C - 2	Detroit River
23. C - 3	Detroit River
24. PP - 2	Marsh, Pt. Pelee National Park

APPENDIX

Modified BG11 medium for enumeration procedure

	g/liter		g/liter
$NaNO_3$	1.5	EDTA	0.001
K_2HPO_4	0.039	Citric acid	0.006
$MgSO_4.7H_2O$	0.075	Fe citrate	0.006
Na_2CO_3	0.020	Microelements*	1.0 ml
$CaCl_2$	0.027	Cycloheximide	20 ug/ml
$Na_2SiO_3.9H_2O$	0.058	Yeast extract	0.01

* Composition (g/liter): H_3BO_4, 2.86; $MnCl_2$, 0.181; $ZnSO_4.7H_2O$, 0.222; $Na_2MoO_4.2\ H_2O$, 0.391; Cn $SO_4.5\ H_2O$, 0.079; Co $(NO_3)_2.6H_2O$, 0.0494.

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