Method for the Selective Enumeration of Blue-Green Bacteria in Water

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A membrane filter method for the selective enumeration of blue-green bacteria has been developed which, on the basis of studies with laboratory 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.

There appear to be no very satisfactory methods available for the enumeration of blue-green bacteria in water. Allen and Stanier (3) employed a dilution to extinction method for the enumeration of blue-green bacteria by using high temperature and a nitrogen-deficient medium as selective factors. In the same year, Jurgensen and Davey (6) reported the use of pour plates and a nitrogen-deficient agar medium to determine the number of blue-green bacteria in soils. Both methods have obvious limitations since only a relatively small segment of the total viable blue-green population is measured: those that fix atmospheric nitrogen and grow at higher temperatures.

In view of the wide application of the membrane filter technique for enumeration of microorganisms (8, 14), it is surprising that it has not until now been used for the estimation of blue-green bacteria. It is the purpose of this report to describe a membrane filter technique for estimating blue-green populations in water.

MATERIALS AND METHODS

Cultures. The following axenic blue-green bacterial cultures received from R. Y. Stanier were used during this investigation: Anacystis nidulans, strain 6301 and Berkeley isolate, strain 6312 (Stanier typological group IA) (13); Berkeley isolate, strain 6903 (Stanier typological group IB); Gloeocapsa alpicola, strain 6306 (Stanier typological group IIA); Berkeley isolate, strain 6601 and Gloeocapsa sp. 6909 (Stanier typological group IIIB); Chlorella frischi (Stanier typological group III); Anabaena sp. and Plectonema sp.

Eukaryotic axenic algal cultures of Chlorella vulgaris, Chlamydomonas reinhardtii, and Scenedesmus quadricauda were supplied by D. G. Wallen. Two isolates of the division Chlorophyta from this laboratory were also used during preliminary studies. In all except one experiment (Table 5) logarithmic cultures were used for experimental counts.

Media and conditions of cultivation. The basic medium (BG11 broth) used for the growth and maintenance of cultures and from which the final membrane filter medium (BGY agar) was developed, was that of Hughes et al. (6) as modified by Allen and Stanier (3). BGY agar was solidified with 1% Difco agar as described by Allen (2) and contained 0.01% yeast extract and 20 μg of cycloheximide per ml (Sigma Chemicals). Each component of the medium was sterilized separately and the complete medium was adjusted to pH 8.0 with N NaOH.

All incubations were at 30°C with illumination supplied by cool white fluorescent light (40 CW). For routine purposes a light intensity of 100 foot candles was employed as measured with a Tri-Lux foot candle meter (Grosen GMBH, Erlangen, West Germany).

Samples. Surface samples from various bodies of water in Essex County, Ontario, were collected in sterile 100-ml screw-capped bottles, returned to the laboratory, and if possible processed the same day. All samples were agitated for 30 min on a wrist-action shaker and when necessary diluted in BG11 broth.

Membrane filter technique. Membrane filters of cellulose acetate (Millipore Corp.) or mixed esters of cellulose (R-B filters, Ltd.) with an average pore diameter of 0.45 μm were used. Appropriate volumes of 10 to 100 ml of diluted or undiluted sample were passed through the membranes by using a house vacuum after which the filtration apparatus was rinsed thoroughly with BG11 broth. Membranes were then placed on the growth medium in 50-mm sealed petri dishes, incubated as described, and examined periodically for the appearance of colonies. Counts were made after 2 weeks by using an Olympus dissecting microscope.

Other methods. For surface platings 0.1 ml of the water sample was spread over the surface of a BGY agar plate with a sterile bent glass rod. The plates were inverted, incubated as described, and examined periodically for blue-green bacterial colonies.

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In the tube dilution technique 1-ml quantities from each of a series of 10-fold dilutions of water samples were inoculated into five, 10-ml volumes of BG11 broth. The tubes were incubated for 4 weeks and examined for the presence of blue-green bacteria. Most probable numbers (MPN's) were computed by using the tables in Standard Methods for the Examination of Water and Wastewater (4).

Direct microscopic counts were made with a Brightline Hemocytometer.

Counts were analyzed statistically by means of the $t$ test (11, 12).

**RESULTS**

As shown in Table 1, membrane filters on BG11 agar yielded consistently higher counts from logarithmic cultures than were obtained on pads saturated with BG11 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 counts obtained by the two methods (Table 2) except in the instance of Chlorogleaa fritschii and Plectonema sp. where the counts were significantly different. Nevertheless, even in the latter cases recovery on the membrane filter was approximately 80%.

Morris (7), and Palmer and Maloney (9) 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 10 types of blue-green bacteria. In this investigation, 20 $\mu$g/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 $\mu$g/ml. In tests of field samples (Table 4), the addition of 20 $\mu$g of cycloheximide per ml to the solid medium eliminated virtually all eukaryotic contaminants and indeed, for some samples, allowed somewhat increased colony counts of blue-green bacteria.

BG11 medium plus cycloheximide gave reasonably good results with most samples but, in some instances, counts obtained were not consistent in the various dilutions. Since some blue-greens are stimulated by organic growth

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**Table 1. Comparison of membrane filter counts on BG11 agar versus pads saturated with BG11 broth**

| Culture                  | Counts per ml | $t$   |
|--------------------------|--------------|------|
|                          | Agar | Pads |      |
| *Gloeocapsa alpicola* 6308 | 830  | 570  | 3.464* |
| Berkeley isolate 6312    | 1,120| 610  | 7.107* |
| *Gloeocapsa* sp. 6909    | 880  | 410  | 8.975* |
| Berkeley isolate 6903    | 250  | 210  | 0.797  |
| *Anabaena* sp.           | 420  | 280  | 2.878* |
| *Chlorogleaa fritschii*  | 2,770| 2,220| 3.780* |
| *Anacystis nidulans* 6301| 2,710| 1,640| 3.341* |
| *Plectonema* sp.         | 640  | 430  | 2.778* |
| Berkeley isolate 6501    | 1,210| 730  | 2.007  |

* Average of four counts.

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**Table 2. Comparison of direct microscope and membrane filter counts**

| Culture                          | Counts per ml | Direct counts | Membrane filter counts | $t$   |
|----------------------------------|---------------|---------------|------------------------|------|
| *Gloeocapsa alpicola* 6308       |               | 2,770         | 2,500                  | 0.0469 |
| Berkeley isolate 6312            |               | 2,630         | 2,680                  | 0.797  |
| *Gloeocapsa* sp. 6909            |               | 4,870         | 4,330                  | 1.543  |
| Berkeley isolate 6903            |               | 2,110         | 1,850                  | 1.032  |
| *Anabaena* sp.                   |               | 3,870         | 3,710                  | 0.598  |
| *Chlorogleaa fritschii*          |               | 5,960         | 4,750                  | 4.703* |
| *Anacystis nidulans* 6301        |               | 1,420         | 1,660                  | 1.208  |
| *Plectonema* sp.                 |               | 4,760         | 3,860                  | 4.989* |
| Berkeley isolate 6501            |               | 3,240         | 3,030                  | 1.441  |

* Average of four counts.

* Indicates significant difference, critical value of $t$ for $n=4$ is 2.447.

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**Table 3. Effect of cycloheximide (actidione)**

| Eukaryotic algae | Cycloheximide (µg per ml) * | 5  | 10  | 20  | 30  | 40  | 50  |
|------------------|-----------------------------|----|-----|-----|-----|-----|-----|
| *Scenedesmus quadricauda* | + - | - | - | - | - | - |
| *Chlorella vulgaris* | + | + | - | - | - | - |
| *Chlamydomonas reinhardtii* | + | - | - | - | - | - |
| Isolate 34       | + | - | - | - | - | - |
| Isolate 12       | - | - | - | - | - | - |
| Blue-green bacteria | + | + | + | + | + | + |
| Filamentous types | + | + | + | + | + | + |
| Unicellular types (5 strains) | + | + | + | + | + | + |

* (+) Indicates presence of colonies for eukaryotic algae and counts equivalent to controls for blue-green bacteria.

* (-) Indicates absence of colonies.
factors (1, 10), 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, the medium enriched with 0.01% yeast extract yielded significantly higher counts from stationary phase cultures of several of the test strains. Stationary cultures were used since, in being to some degree nutrient depleted and stressed, they were thought most likely to exhibit stimulation. Counts obtained from field samples incubated on the enriched yeast extract medium were in most cases significantly higher than controls (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

| Sample site     | Counts per ml* | Yeast extract | No yeast extract | t*  |
|-----------------|----------------|---------------|------------------|-----|
| Dump Pond (1)   | 220            | 132           | 7.038*           |     |
| Sales Pond      | 1,600          | 1,040         | 4.850*           |     |
| L. Erie, W. Beach | 5,060         | 3,360         | 7.646*           |     |
| Dump Pond (2)   | 86             | 50            | 3.242*           |     |
| Detroit River   | 372            | 206           | 8.989*           |     |
| Marsh (Pt. Pelee) | 18             | 13            | 1.651            |     |

* Average of four counts.

Superscript b indicates significant difference, critical value of \( t_{0.05}\) = 2.447.

Table 7. Effect of light intensity on colony counts (counts per ml*)

| Sample site        | Light intensity (foot candles) | 50 | 100 | 150 |
|--------------------|--------------------------------|----|-----|-----|
| Sales Pond         | 980 x                         | 2,010 | 740 x  |
| Detroit River      | 210 x                         | 360 x | 170 x  |
| Dump Pond          | 220 x                         | 220 x | 150 y  |
| L. Erie, E. Beach  | 8,800 x                       | 11,400 x | 5,950 x |
| River Canard       | 1,510 x                       | 1,630 x | 890 y  |
| Cedar Creek        | 1,760 x                       | 2,170 y | 1,160 z |
| Pt. Pelee Marsh    | 170 x                         | 170 x  | 180 x   |
| L. Erie, W. Beach  | 4,610 x                       | 4,760 x | 2,590 y |

* Means of four counts. Means followed by a given letter are statistically different from means not followed by that letter.

Table 8. Comparison of membrane filter and MPN counts

| Sample site         | Counts per ml* | Membrane filter | MPN | t*  |
|---------------------|----------------|-----------------|-----|-----|
| Sales Pond          | 2,010          | 1,600           | 1,797 |     |
| Detroit River       | 360            | 220             | 2.286 |     |
| Dump Pond           | 220            | 220             | 0    |     |
| L. Erie, E. Beach   | 11,400         | 2,400           | 6.011* |     |
| River Canard        | 1,630          | 540             | 3.046 |     |
| Cedar Creek         | 2,170          | 920             | 5.495* |     |
| Pt. Pelee Marsh     | 168            | 110             | 2.251 |     |
| L. Erie, W. Beach   | 4,760          | 2,400           | 9.849* |     |

* Average of four counts.

Superscript b indicates significant difference, critical value of \( t_{0.05}\) = 3.182.
three light intensities tested, 100 foot candles in general yielded the highest colony counts. Consequently, 100 foot candles were chosen for routine use.

As shown in Table 8, colony counts using the membrane filter technique were generally higher, and frequently significantly so, than those obtained by the MPN method.

The attempt to compare the membrane filter technique with surface plating was made difficult by the tendency of colonies on the agar surface to spread (Fig. 1A). By contrast, colonies on the filters generally remained discrete and easily counted (Fig. 1B). For the few samples for which counts were available by both methods, the results obtained were comparable (Table 9).

### DISCUSSION

The results indicate that the membrane filter technique using BGY agar 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 of somewhat limited precision and less convenient in many respects. Surface plating, although rapid and easily carried out, is limited by the small sample size that may be applied and by the tendency of colonies to spread. On the filters, however, spreading (gliding?) appears to be restricted with the result that colonies are more easily counted.

The method described here should be of considerable practical utility to those interested in monitoring, for example, blue-green populations in domestic water supplies and purification plants as well as to those with more general ecological concerns such as studies of distribution, productivity, and interactions with other organisms of blue-green bacteria in freshwater and marine systems. Finally, as will be noted in a subsequent report, the membrane filter technique has also been found to facilitate the isolation of axenic cultures of a variety of blue-green bacteria.

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