Isolation and Counting of *Athiorhodaceae* with Membrane Filters

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The number, type, and distribution of *Athiorhodaceae* in two Central Pennsylvania artificial lakes were investigated with an anaerobic modification of the membrane filter technique.

The classical approach to the ecological study of the photosynthetic bacteria has been to increase the numbers of particular biotypes by enrichment culture (9, 10, 13), isolate by an agar-shake culture procedure (13), and characterize the pure cultures. The process of enrichment has controlled the types and numbers eventually obtained, limiting the ecological validity of this approach.

The membrane filter method for counting coliform bacteria (1) “provides a degree of precision and sensitivity not obtainable with conventional methods—due largely to the ability to concentrate even a few organisms from large volumes and to culture these organisms free from the effects of bacteriostatic agents” (7). A membrane filter culturing technique (2) seems ideal for enumerating and isolating bacteria from sparse populations.

The photosynthetic bacteria can be classified by their primary source of electrons. The *Athiorhodaceae* usually require reduced carbon and are inhibited by reduced inorganic sulfur, whereas the *Chlorobacteriaceae* and *Thiorhodaceae* have a reduced sulfur-based photometabolism. As no one medium could be devised that would permit the growth of all three families on one membrane filter, only the *Athiorhodaceae* were chosen for study. A technique was developed to grow the *Athiorhodaceae* on membrane filters, and this technique was found to be useful in the enumeration and isolation of *Athiorhodaceae* from two central Pennsylvania lakes.

MATERIALS AND METHODS

Test lakes. Two central Pennsylvania artificial lakes were chosen for study. Whipple Dam is part of Whipple Dam State Park, and the area is used mainly for fishing, swimming, and picnicking. The lake covers an area of 18.2 hectares and has a maximum depth of 2.3 m. Stone Valley Dam is under the total control of The Pennsylvania State University, and the area is used mainly for boating, fishing, camping, and picnicking. The lake covers an area of 34.4 hectares and has a maximum depth of 8 m.

Water samples. Samples of water from the spillways of these lakes during spring, summer, and fall of 1969 were obtained in the early afternoon in sterile, 500-ml medicine bottles and were placed on ice and refrigerated in the laboratory until assayed, no more than 24 hr after sampling.

Depth samples were taken at 1-m intervals at the deepest region from Stone Valley Dam on 20 May 1970, 30 June 1970, and 27 July 1970 with a line-operated sampler made from 100-ml dilution bottles fitted with escher stoppers with rubber band returns. A sterile, argon-filled bottle was used for each sample. The steel suspending and operating cables were detachable by means of snap swivels. Samples were taken from the upper layers first to minimize interlayer contamination.

Oxygen determinations were by the Winkler method (8) immediately after sampling.

Membrane filter technique. The procedures in membrane filtration were those outlined in *Techniques for Microbial Analysis* (7). The top of the filter funnel was fitted with a vented rubber stopper to prevent laboratory contamination. Given volumes of the water samples were filtered through 47-mm HA membrane filters (Millipore Corp.) which were then placed on 5 ml of medium in disposable petri dishes (Millipore Corp.). To equalize pressure and insure the creation of anaerobic conditions, the petri dishes were perforated once on the side and three times on the bottom. Controls proved that these holes did not create contamination problems. The filters were then incubated in Brewer jars that were evacuated and filled four times with nitrogen. The Brewer jars were placed in a constant-temperature water bath at either 28 or 18 C and illuminated with 150-w reflector floodlights (approximately 5,000 ft-c at the center of the water bath as measured by a Weston light meter). After 5 days of incubation, the red and brown pigmented colonies

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were first counted and then purified by repeated streakings.

The accuracy of the membrane filter technique for enumerating *Athiorhodaceae* was validated by parallel counts with traditional pour plate counts and most-probable-numbers experiments. Plate counts were made by adding 1 or 10 ml of water sample to 20 ml of basal medium with 2% agar, incubating under the same conditions as the membrane filters, and counting the characteristic pigmented colonies. Most-probable-number experiments were prepared by adding 0.1, 1, and 10 ml of water sample to 30-ml screw top tubes and filling completely with medium. Incubation was at 28°C for 3 weeks and a red-brown color was interpreted as being positive for *Athiorhodaceae*. Numbers were obtained from tables in *Standard Methods for the Examination of Water and Wastewater* (1).

**Media.** The basal medium consisted of 0.3% yeast extract (Difco), 0.2% vitamin-free Casamino Acids, and tap water (pH 6.5). Stock cultures were kept in deep stabs of this medium plus 1.5% agar. Membrane filters were incubated on the basal medium plus 2% agar and 10 μg of atrazine per ml. This basal medium gave highest counts of the several media tested. A yeast extract-based medium as described by Haskins and Kihara (5) was used in the most-probable-number experiments.

Substrate utilization by the isolates was tested with the aseptic addition of 0.2%, neutralized substrate to the medium used by Rollins and Lindstrom (11). Utilization of sulfide was tested by using the medium described by Jones (6) with the addition of the trace element solution used in the defined medium.

**Instrumentation.** Adsorption spectra were prepared by the method of Haskins and Kihara (5) by using a Beckman DK-2A spectrophotometer.

Agitated anaerobic incubation was at 175 rev/min in a New Brunswick Psychrometer incubator at an illumination intensity of 200 to 400 ft-c of incandescent light.

Nitrogen fixation was measured by the acetylene reduction technique (4). The increase in ethylene was measured with a Hewlett-Packard F and M gas chromatograph (model 700) with an H2-flame ionization detector.

**RESULTS**

**Membrane filter technique.** Preliminary experiments with laboratory cultures had indicated that *Athiorhodaceae* could be grown on membrane filters and that this technique could be used for enumeration and isolation. Preliminary experiments with water samples had indicated that algal colonies crowd the filter and their photosynthetic products of O2 would create aerobic conditions. Atrazine (10 μg/ml), an inhibitor of photosystem II, was added to the basal medium to inhibit the algae but not the *Athiorhodaceae*.

Table 1 summarizes the experiment with water samples (taken on 15 September 1969 at the respective spillways) used to validate the membrane filter technique for enumerating *Athiorho-

| Source       | Method | Cell count per 100 ml | Range  |
|--------------|--------|----------------------|--------|
| Whipple Dam  | MPN    | 23                   | 7–70   |
|              | PP     | 77                   | 40–130 |
|              | MF     | 73                   | 10–130 |
| Stone Valley Dam | MPN | 8                    | 1–19   |
|              | PP     | 30                   | 10–50  |
|              | MF     | 27                   | 10–50  |

* a Pour plates.
* b Membrane filtration.

daceae. The most-probable-number counts were often lower, but comparable counts were achieved with the membrane filters and pour plates, suggesting that the membrane filter method yielded reliable counts of *Athiorhodaceae*.

With the membrane filter technique, cell counts of *Athiorhodaceae* were obtained weekly from spillway samples of water during the spring, summer, and fall of 1969. For the water at Stone Valley, the average of six replicate counts varied from a low of 7 cells per 100 ml on 21 September to a high of 290 cells per 100 ml on 21 August. For Whipple Dam water, the membrane filter count (average of six replications) ranged from a low of 4 cells per 100 ml on 13 April to a high of 1,400 cells per 100 ml on 21 June. The highest count obtained was 11,300 cells per 100 ml from the hatch area at Whipple Dam on 20 July. There was unexplained variation in the counts over the season, but counts taken during a day were consistent and the variation within the replications was not great.

Membrane filter counts of *Athiorhodaceae* were made from water samples taken at 1-m depths in the deepest section of Stone Valley Lake on 20 May, 30 June, and 27 August 1970. Oxygen determinations and temperature measurements were made at the time of sampling. The highest count (40 cells per 100 ml) was found at a depth of 3 or 4 m and was always greater than the cell count from the surface water. A thermocline was evident at 30 June (3 to 6 m) and well established (4 to 6 m) by 27 August. The oxygen concentration was below the limit of the Winkler determination at 8 m on 30 June and at 6 m on 27 August. There was no evidence that any population of *Athiorhodaceae* will colonize a specific depth of Stone Valley Lake.

**Preliminary description of isolates.** Pure cultures were isolated from the filters by repeated streaking on plates incubated anaerobically in the light. To detect any contamination, the 40
isolates were each streaked on nutrient agar and the plates were incubated both aerobically and anaerobically. These 40 isolates could not be identified by using the available bacteriochlorophyll-carotenoid absorption spectra and substrate utilization results (Tables 2 and 3). None of the isolates used sulfide or thiosulfate, which is proof that they are indeed *Athiorhodaceae*. The cell-free and methanol-acetone extracts also had absorption spectra characteristic of *Athiorhodaceae*.

The most frequently observed isolates (SV-B and WD-A9) had light brownish-red gummy colonies of 3 to 5 mm in diameter on the filter, were rods of 0.8 by 5 µm, and were considered similar to *Rhodopseudomonas gelatinosa*. Isolates SV-C, SV-D, and SV-G appeared on the filter as gummy red-brown colonies of 3 to 5 mm in diameter, were rods of 1 by 2 µm, and were considered similar to *R. capsulata*. Isolates SV-J and WD-C1 appeared on the filter as purplish-red colonies of 1 mm in diameter, were spirals of 1 by 7 µm with two turns, had a characteristic absorption maximum at 550 or 530 nm, and were considered most closely related to *Rhodospirillum rubrum*. In enrichments from these lakes, *Rhodopseudomonas palustris* was the type usually isolated, but this biotype was seldom isolated with membrane filters. All isolates were motile and all reduced acetylene.

DISCUSSION

The anaerobic membrane filter technique was shown to be a reliable and convenient method for enumerating and isolating *Athiorhodaceae* from aquatic ecosystems. Quantitative data and a wide range of biotypes can be simultaneously obtained with this method. Although anaerobic pour plates are an equally reliable method of enumeration, they do not offer the ease of isolation and the ability to determine the density of sparse populations. Most-probable-number experiments rely on enrichment techniques in which the less well adapted organisms will not be isolated.

Why were the *Athiorhodaceae* the only family

### Table 2. Substrate utilization of representative isolates

| Substrate | WD-C1 | SV-J | SV-B | SV-C | SV-D | SV-G | WD-A9 |
|-----------|-------|------|------|------|------|------|-------|
| Alanine*  | +     | -    | +++  | +    | ++   | +    | ++++  |
| Asparagine| -     | +    | ++   | +    | +    | -    | +     |
| Aspartate | +     | +    | ++   | +    | ++   | +    | +     |
| Citrate   | -     | +    | +    | +    | +    | +    | +     |
| Dextrose  | +     | +    | +++  | +++  | +++  | +    | +     |
| Fructose  | +     | +++  | +++  | +++  | +++  | +    | -     |
| Fumarate  | +++   | +++  | +++  | +++  | +++  | +    | +     |
| Gluconate | +     | +    | +    | +    | +    | +    | -     |
| Glutamate | +     | +    | +    | +    | +    | +    | -     |
| Glutarate | -     | -    | -    | -    | -    | -    | -     |
| Lactate   | +++   | +++  | +++  | +++  | +++  | +    | +     |
| Leucine   | +     | -    | +    | -    | -    | -    | -     |
| Malate    | ++++  | ++++ | +++  | +++  | +++  | +++  | ++++  |
| Mannitol  | -     | -    | -    | -    | -    | -    | -     |
| Mannose   | +     | +    | +    | +    | +    | +    | -     |
| Propionate| +++   | +++  | +++  | +++  | +++  | +++  | +     |
| Sorbitol  | +     | -    | +    | -    | -    | -    | -     |
| Succinate | +++   | +++  | +++  | +++  | +++  | +++  | +++   |
| Tartrate  | -     | +    | +    | +    | +    | +    | -     |
| Ethanol   | +     | +    | +    | +    | +    | +    | -     |
| S2O32-  | -     | +    | +    | +    | +    | +    | -     |
| Sulfide*  | -     | -    | -    | -    | -    | -    | -     |
| Gelatin* | +     | -    | +    | -    | -    | -    | -     |
| N2 fixation | +    | +    | +    | +    | +    | +    | +     |
| Gum production | -   | -    | +    | -    | -    | -    | +     |

*a Modified Hutner's medium plus 0.2% substrate.

*b Symbols: ++++, heavy growth; +++, good growth; +, fair growth; +, slight growth; -, no growth as compared to the control.

c Malate (0.02 M) plus S2O32- with 0.02 M malate as a control.

d As described by Jones (6), with addition of Hutner's trace elements solution.

e In broth medium.
of photosynthetic bacteria able to grow under the conditions described above? As was stated in the introduction, the *Athiorhodaceae* have a carbon-based photometabolism, whereas the *Thiorhodaceae* and *Chlorobacteriaceae* have a sulfur-based photometabolism. The *Athiorhodaceae* are also slightly more tolerant of O₂, and great pains were not taken to exclude traces of O₂ from the Brewer jars. By switching to a sulfur electron donor and providing more anaerobic conditions, the membrane filter technique could probably be adapted to count and isolate the sulfur photosynthetic bacteria.

Rhodospirilla have traditionally been difficult to isolate. Haskins and Kihara (5) speculate that an overgrowth of the rhodopsudomonads prevents the isolation of rhodospirilla in enrichment cultures. In the present study, rhodospirilla were isolated with ease, and the membrane filter technique would seem to eliminate many microbial interactions since the colonies grow in a somewhat isolated manner. In enrichment cultures, the microflora alter the medium. At first, the heterotrophs are dominant, and, as the medium becomes more reduced, the photosynthetic bacteria can grow. It is unlikely that all of the photosynthetic bacteria will be favored by the altered medium, and the more adapted or more numerous species will predominate. However, on a membrane filter, the isolated colonies can alter only their immediate environment and microbial interactions are not such a factor; thus, a greater variety of photosynthetic bacteria can be isolated.

The streak plate with anaerobic incubation is more convenient and rapid than the agar-shake culture method for purifying isolates. The agar-shake culture method is awkward, and contaminated clones are less easily detected.

The maximum cell densities in the spillway samples of 14 and 2.9 cells per ml may not represent the population at the normal niche of *Athiorhodaceae*. The depth studies indicated that no particular layer of Stone Valley Dam was enriched for *Athiorhodaceae* in spring or summer. Are the *Athiorhodaceae* present in the surface waters only by accident? Shallow, anaerobic, reduced areas of the lake, such as the hatch area of Whipple Dam, or anaerobic bottom muds (12) may serve as reservoirs for the *Athiorhodaceae* and the populations found in the other areas of the lakes may have originated from these reservoirs.

The membrane filter technique first used by Biebl and Drews (2) is a useful and reliable technique for enumerating and isolating *Athiorhodaceae*, and this technique should be useful in the further study of the ecology of the photosynthetic bacteria.

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**Table 3. Absorption spectra of representative isolates**

| Isolate | Morphology | Cell free absolute maxima | Methanol-acetone absolute maxima |
|---------|------------|---------------------------|----------------------------------|
| WD-C1   | Spirillum  | 860,795 590,530 505,375 | 770,585 525,370                   |
| SV-J    | Spirillum  | 877,805 585,550 510,480 | 770,585 535,370                   |
| SV-B    | Rod        | 850,799 585,515 485,455 | 770,585 500,460 375              |
| SV-C    | Rod        | 865,800 585,510 470,455 | 770,585 500,460 435,374           |
| SV-D    | Rod        | 855,798 585,510 475,460 | 770,585 500,460 435,375           |
| SV-G    | Rod        | 860,798 585,510 475,375 | 770,585 500,460 435,375           |
| WD-A9   | Rod        | 865,800 585,510 475,375 | 770,585 500,460 435,375           |

*a* Cells were grown for 5 days in the modified Hutner's medium with malate under N₂-fixing conditions, and the absorption spectra (in nanometers) were prepared as described by Haskins and Kihara (5).

*b* Cells were grown for 3 days in the modified Hutner's medium (10) with 0.05 M HN₄Cl, and the absorption spectra were prepared as described by Cohen-Bazire et al. (3).
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