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AMERICAN SOCIETY FOR TESTING AND MATERIALS
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Standard Test Method for Iron Bacteria in Water and Water-Formed Deposits[1]

This standard is issued under the fixed designation D 932; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers the determination of iron bacteria by examination under the microscope. The method provides for the identification of the following genera of bacteria found in water and water-formed deposits: *Siderocapsa*, *Gallionella (Dioymohelix)*, *Sphaerotilus*, *Crenothrix*, *Leptothrix*, and *Clonothrix*.

1.2 *This standard does not purport to address the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

D 887 Practices for Sampling Water-Formed Deposits[2]

D 1129 Terminology Relating to Water[3]

D 1193 Specification for Reagent Water[3]

D 3370 Practices for Sampling Water from Closed Conduits[3]

3. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminology D 1129.

4. Summary of Test Method

4.1 The iron bacteria are generally filamentous, typically found in fresh water, and frequently surrounded by a sheath which is usually encrusted with iron or manganese, or both (**1, 2**).[4] However, Starkey (**3**) reports another type which is classified among the true bacteria. Detection and identification is accomplished by microscopical examination of sediment from the sample. Table 1 and Figs. 1-10 (**3**) may be used to differentiate the various types. This test method provides an indication of the density of the iron bacteria and the severity of the clogging problem in pipes caused by these bacteria.

5. Significance and Use

5.1 Iron bacteria is a general classification for microorganisms that utilize ferrous iron as a source of energy and are characterized by the deposition of ferric hydroxide in their mucilaginous sheaths. The process is continuous with these growths, and over a period of time large accumulations of slimey brown deposits can occur. Iron bacteria may clog water lines, reduce heat transfer, and cause staining; objectionable odors may arise following death of the bacteria. The organic matter in the water is consequently increased, and this in turn favors the multiplication of other bacteria.

6. Apparatus

6.1 *Centrifuge*, complete with conical tubes.

6.2 *Microscope* that provides a magnification of 400 to 1000× and is complete with a suitable light source. A dark-field condenser is desirable.

6.3 *Pipets*, Mohr-type, 10-mL, with an opening 3 to 4 mm in diameter, for thick samples, and 1-mL Mohr-type pipets for thin samples.

6.4 *Spatula*, small and narrow, for handling thick samples.

6.5 *Membrane Filter*, with appropriate filter-holding assembly (see 9.2).

7. Reagents

7.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type II.

7.3 *Ammonium Oxalate-Crystal Violet Solution*—Prepare Hucker's modification of the Gram stain (**4**) by mixing a solution of 2.0 g of crystal violet (90 % dye content) in 20 mL of ethyl alcohol (95 % with a solution of 0.8 g of ammonium

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[1] This test method is under the jurisdiction of ASTM Committee D-19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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[2] *Annual Book of ASTM Standards*, Vol 11.02.

[3] *Annual Book of ASTM Standards*, Vol 11.01.

[4] The boldface numbers in parentheses refer to the list of references at the end of this test method.

[5] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."

TABLE 1 Key for Identification of Bacteria

I. TRUE BACTERIA:
Capsulated coccoid or short rods

Genus: *Siderocapsa* (Fig. 1)

The organisms are coccoid or short rods, occurring in groups of 1 to 30 but generally less than 10, surrounded by a mucoid capsule. The deposit surrounding the capsule is rust-brown due to the presence of hydrous ferric oxide.

II. STALKED BACTERIA:
Twisted or straight bands resembling a ribbon or a row of beads. Bacteria are rod-shaped and borne at the top of the stalk.

Genus: *Gallionella (Didymohelix)* (Figs. 2 and 3)

The stalks are slender (1 to 3 μm), dichotomously branched, composed of colloidal hydrous ferric oxide. The bacteria are frequently overlooked and the stalk considered as the bacterium.

III. FILAMENTOUS BACTERIA:
A. Not encrusted with iron:

Genus: *Sphaerotilus* (Fig. 4)

The filaments are attached, colorless, may show false branching. The cells are rod-shaped or oval, 1.5 to 4 μm in diameter, surrounded by a firm sheath which is entirely organic and not impregnated with iron.

B. Encrusted with iron:
(1) Not branched:

Genus: *Crenothrix* (Figs. 5, 6, and 10)

The filaments are usually attached to a firm substrate, and are differentiated into a base and a tip. The sheath is plainly visible and is thin and colorless at the tip, becoming thick and encrusted with iron oxide at the base. The cells vary from cylindrical to spherical, the diameter being between 2 and 9 μm. Spherical, nonmotile reproductive bodies are formed. False branching may occur due to germination of spores within the sheath.

(2) May be branched:

(a) Cells from 0.5 to 1 μm in diameter

Genus: *Leptothrix* (Figs. 7 and 8)

The filaments contain colorless, cylindrical cells which first have a thin colorless sheath that later becomes encrusted with iron oxide.

(b) Cells 2 μm or more in diameter

Genus: *Clonothrix* (Fig. 9)

Filaments attached, show false branching. The sheaths are organic and encrusted with iron hydroxide or manganese, are broader at the base, and taper to the tip, varying from 7 to 2 μm. The cells are colorless, cylindrical, 2 by 10 μm. The filaments are colorless when young, becoming dark, yellowish-brown with age. Forms spherical reproductive cells on the short branches of the younger portions of the filaments.

oxalate monohydrate $(NH_4)_2C_2O_4 \cdot H_2O$) in 80 mL of water.

7.4 *Hydrochloric Acid* (1 + 4)—Mix 1 volume of hydrochloric acid (HCl, sp gr 1.19) with 4 volumes of water.

7.5 *Iodine Solution*—Prepare Gram's modification of Lugol's solution (**4**) by dissolving 1 g of iodine in a solute containing 2 g of potassium iodide (KI) in 10 mL of water and diluting the resulting solution to 300 mL with water.

7.6 *Filter Paper or Blotter.*

7.7 *Slides*, standard type, 25 by 76-mm (1 by 3 in.) with either plain or frosted end.

7.8 *Cover Glasses*, round or square type, 19 mm (¾ in.) in diameter.

8. Sampling

8.1 Collect the samples in accordance with either Practices D 887 or D 3370, whichever is applicable.

8.2 Obtain a 500-mL (1-pt) sample of water, using a sterile 1-L (1-qt) bottle. The bottle should not be more than half-filled because of the oxygen demand of suspended matter; filling the bottle may cause the sample to become anaerobic.

8.3 If the number of iron bacteria are very low or that they are just becoming established in the system, use a small side stream filter to collect the sample to be examined. The water suspected of containing iron bacteria should be filtered through a highly retentive filter paper (or some other comparable media) for 24 h. Centrifugation or membrane filtration is satisfactory also. The flow rate of the water should be at the maximum filtering capacity of the material employed.

8.4 Regardless of the method used to concentrate the solids in the water, keep them moist until examined.

8.5 Mud samples should be collected from the mud-water interface for maximum bacterial populations.

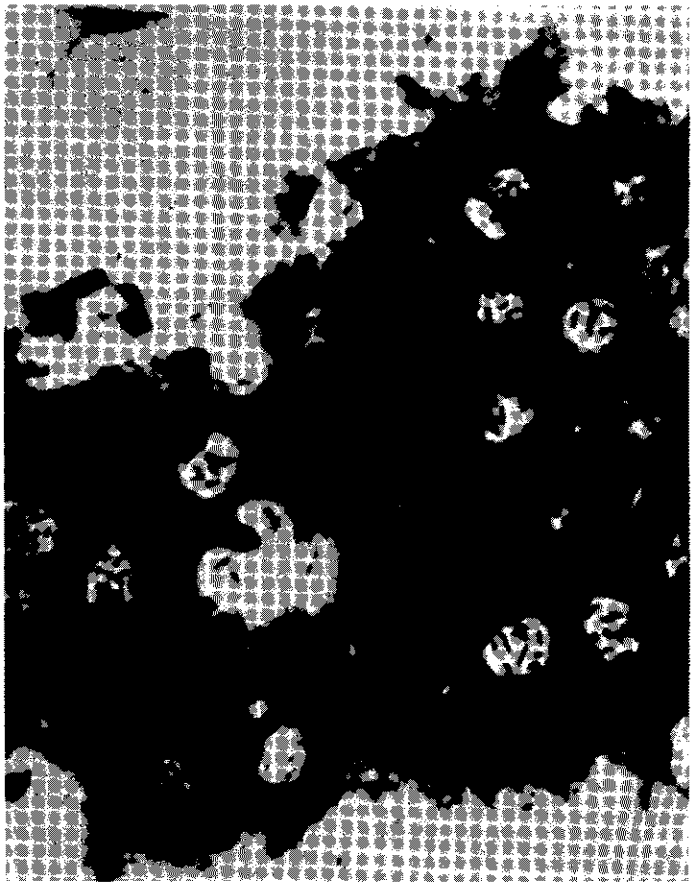

FIG. 1 *Siderocapsa treubii.* **Multiple colonies surrounded by ferric hydrate. Magnification about 500 ×. Fig. 4 of Ref (5)**

FIG. 2 *Gallionella major.* **Cells at the ends of excretion bands undergoing division. Magnification about 1180 × . Fig. 3 of Ref (6)**

FIG. 3 *Gallionella major.* **Curved cells at the ends of excretion bands. Magnification about 1120 × . Fig. 6 of Ref (6)**

8.6 Transfer the deposit or mud samples to wide-mouth bottles and add clean, chlorine-free water to cover the deposits and maintain moisture until examined. Protect the samples from sunlight and hold at 4°C during transportation and storage.

8.7 As soon as possible after collection of the solids, microscopically examine them for the presence of iron bacteria.

9. Procedure

9.1 Place a portion of the sample on the slide and apply a cover glass. A spatula or wide-mouth pipet can be used to transfer the sample to the slide. Use a pipet when flocs of material are encountered, as the flocs settle to the tip when the pipet is held in a vertical position, and concentrate in the first drop. In the case of very dilute solids or a water sample, concentrate the organisms by centrifuging, pour off the supernatant liquid, and repeat if necessary.

9.2 An alternative procedure is to filter a suitable volume of the dilute solids or the entire water sample through a 0.45-μm membrane filter in an appropriate membrane filtration assembly (holder, tubing, trap, flasks and vacuum pump). For this test it is not necessary to sterilize the filter assembly for each sample, but the assembly should be thoroughly cleaned between tests.

9.3 Examine the slide under the microscope to determine if

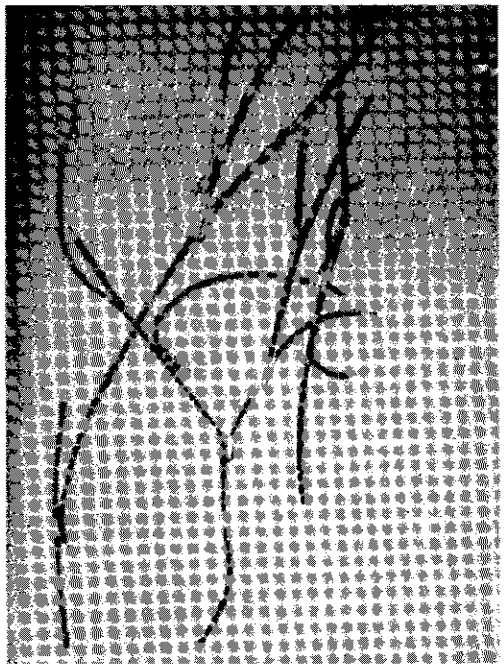

FIG. 4 *Sphaerotilus dichotoma.* **Sketch showing false branching. Magnification about 230 × . Fig. 3*b* of Ref (7)**

encrusted or colorless sheaths are present. Note the presence of the twisted stalks of *Gallionella* at this point, since treatment with acid in accordance with 9.4 will dissolve the delicate

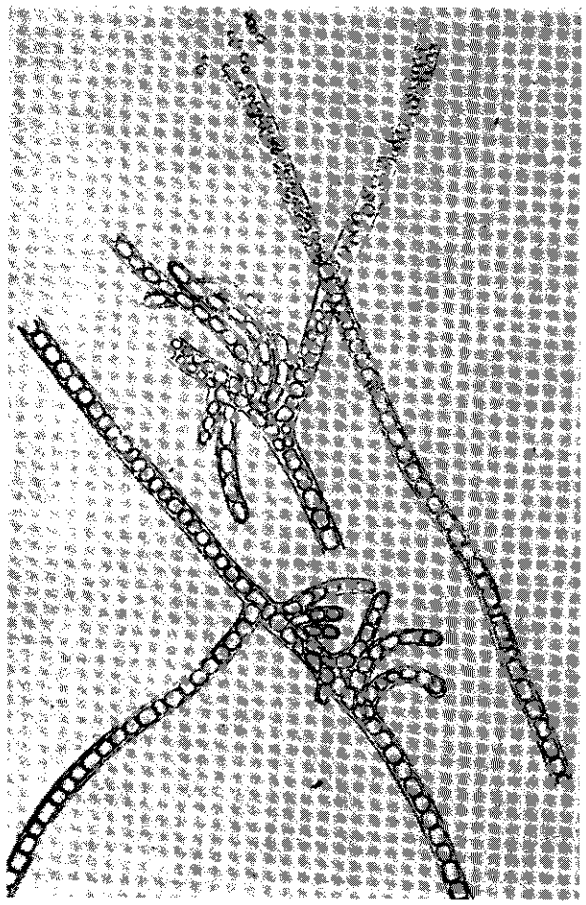

FIG. 5 *Crenothrix polyspora.* **Sketch showing details of false branching of cells within sheath. Magnification about 380 ×. Plate 1, Fig. A of Ref (8)**

stalks.

9.4 Place the HCl (1 + 4) at one side of the cover glass and draw it underneath by absorbing the liquid at the opposite side by means of a filter paper or blotter. Continue this procedure until no more yellow ferric chloride is evident in the solution. Take care that the flow of the liquid is not fast, or the sample may be drawn to the absorbent material. This treatment removes the iron deposited in the sheaths of the bacteria and allows the cells to be seen.

9.5 In a similar manner, rinse the iodine solution under the cover glass until the color of the liquid becomes yellow or the filter paper becomes colored. The iodine stains the bacterial cells brown and makes them more easily visible.

9.6 Examine the slide under a microscope, using a high-power, dry objective, for the presence of *Sphaerotilus*, *Crenothrix*, *Leptothrix*, and *Clonothrix*. If used carefully, an oil-immersion lens may be helpful.

9.7 Prepare a new slide by placing a drop of the sample on a clean slide and allowing it to air-dry. Then stain it for 1 min with ammonium oxalate-crystal violet solution, wash it with water, and allow it to dry. Examine the slide under anoil-immersion lens for the presence of *Siderocapsa*, which will appear violet colored.

10. Report

10.1 The report shall state "Present" or" Not found, probably absent." Make a statement as to the relative abundance of the organisms present. Make a negative report only after examination of several slides.

11. Precision and Bias

11.1 Since this standard is a qualitative type test, precision and bias statements cannot be provided.

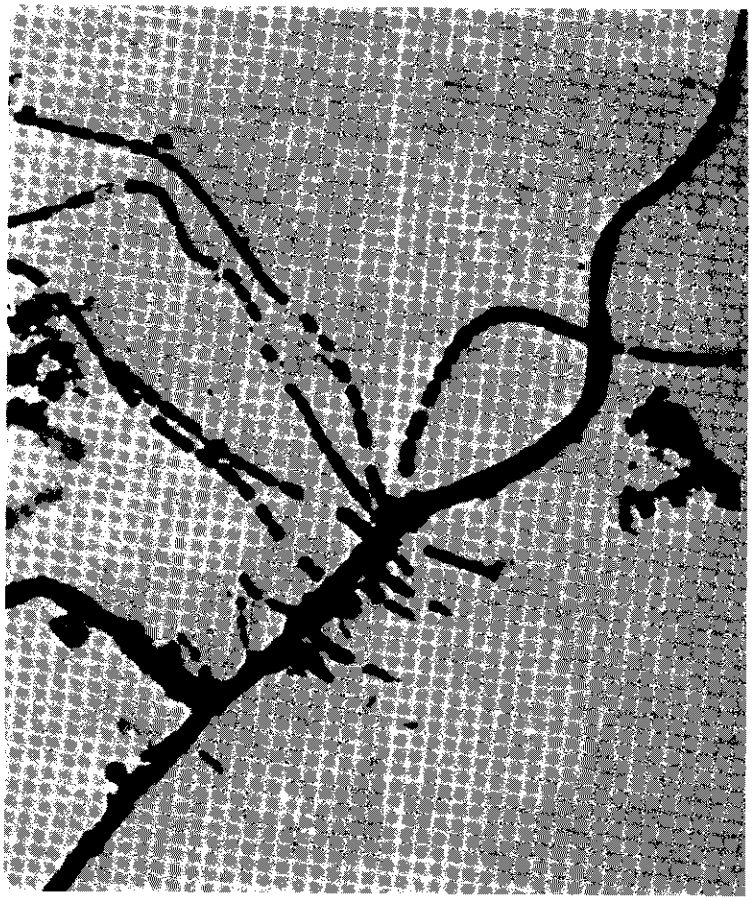

FIG. 6 *Crenothrix polyspora.* **Cells enclosed within a sheath of ferric hydrate and showing false branching. Magnification about 390 ×. Plate 3, Fig. B of Ref (8)**

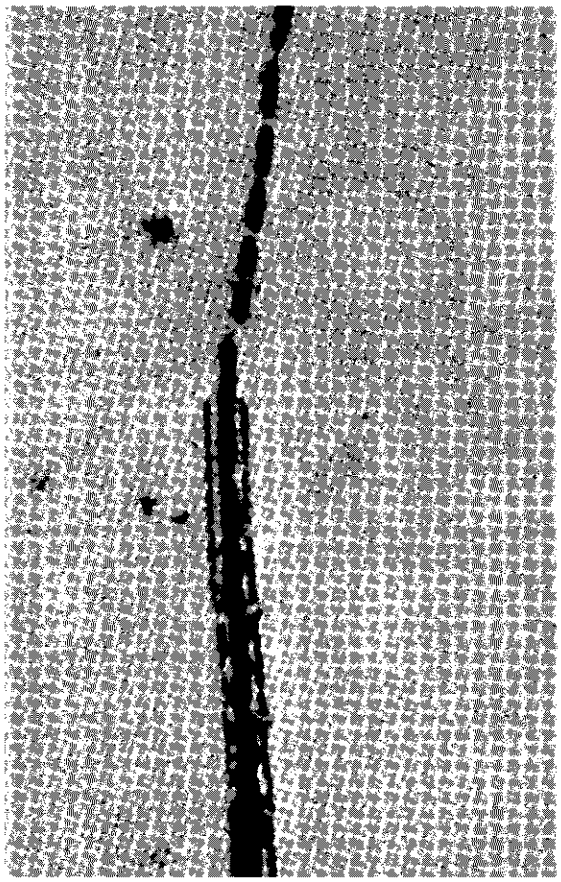

FIG. 7 *Leptothrix ochracea.* **Cells coming out of their sheath. Magnification about 2200 × . Plate 4, Fig. 20 of Ref (9)**

FIG. 8 *Leptothrix ochracea.* **Sheaths from an accumulation of precipitated ferric hydrate in iron bearing water. Magnification about 390 × . Fig. 5 of Ref (7)**

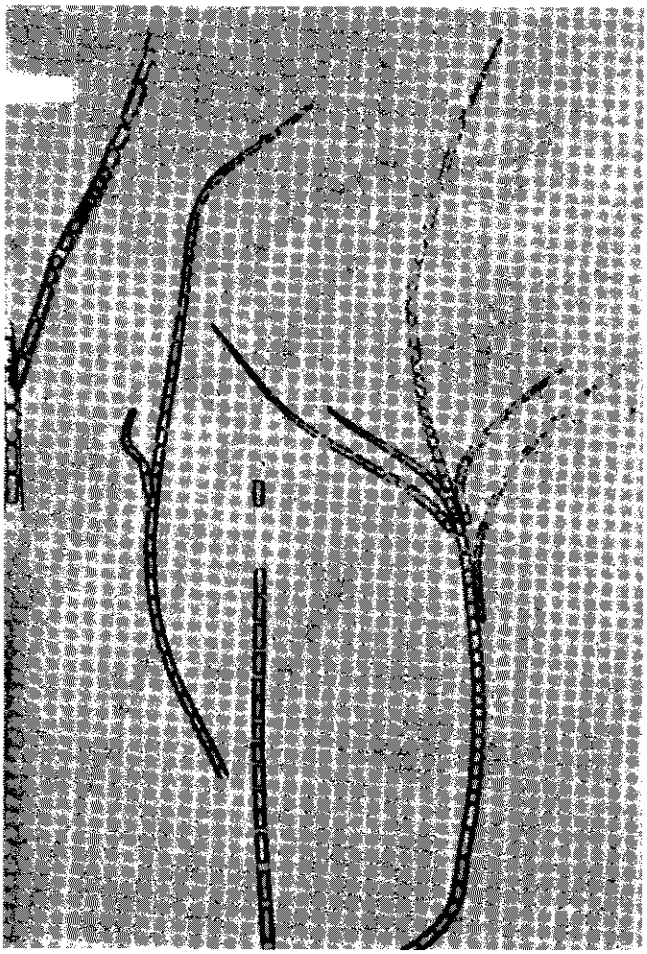

FIG. 9 *Clonothrix ferruginea.* **Sketch showing cells enclosed within sheath and false branching. Magnification about 430 ×. Fig. 4 of Ref (7)**

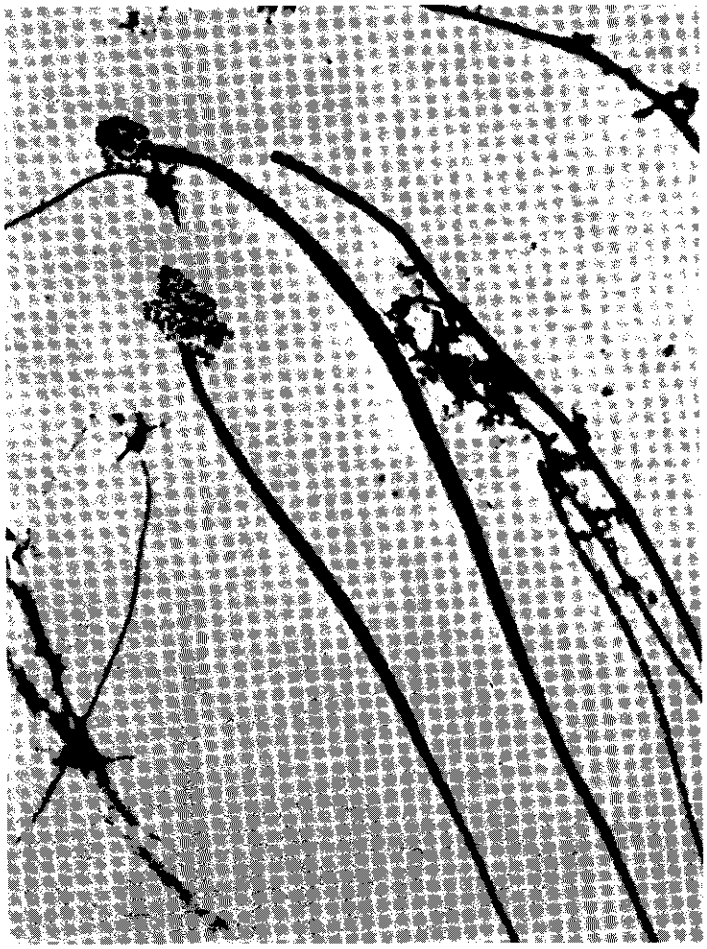

FIG. 10 *Crenothrix polyspora.* **Conidia can be seen inside and coming out at ends of filaments. Magnification about 345 × . Fig. 5 of Ref (9)**

REFERENCES

(1) Bergey, D. H., *Manual of Determinative Bacteriology*, 8th Edition, Williams & Wilkins Co., Baltimore, MD, 1974.

(2) Salle, A. J., *Fundamental Principles of Bacteriology*, McGraw-Hill Book Co., Inc., New York, NY, 1943, pp. 516–519.

(3) Starkey, R. L.," Transformation of Iron by Bacteria in Water," *Journal of the American Water Works Association*, Vol. 37, 1945, pp. 963–984.

(4) *Manual of Methods for Pure Culture Study of Bacteria,* Biotech Publications, Geneva, NY, 1946, Chapter IV, pp. 46–48.

(5) Hardman, Yvette, and Henrici, A. T., "Studies of Fresh Water Bacteria V. Distribution of *Siderocapsa treubii* in Some Lakes and Streams," *Journal of Bacteriology*, Vol 37, 1939, p. 97.

(6) Mitchell, R., *Water Pollution Microbiology Vol. 1*, Wiley-Interscience, New York, NY, 1972.

(7) *Standard Methods for the Examination of Water and Waste Water.* American Public Health Assoc., 19th Edition, 1976, p. 1000.