Designation: D932 – 20

Standard Practice for Filamentous Iron Bacteria in Water and Water-Formed Deposits[1]

This standard is issued under the fixed designation D932; 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 practice covers the determination of filamentous iron bacteria (FIB) by microscopic examination. This practice 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 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

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

1.4 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D887 Practices for Sampling Water-Formed Deposits
D1129 Terminology Relating to Water
D1193 Specification for Reagent Water
D3370 Practices for Sampling Water from Flowing Process Streams
D5465 Practices for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this standard, refer to Terminology D1129.

4. Summary of Practice

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**).[3] However, Starkey (**3**) reports another type which is classified among the true bacteria. Detection and identification is accomplished by microscopic examination of sediment from the sample.

4.2 This practice provides a qualitative indication of the density of the filamentous iron bacteria and the severity of the clogging problem in pipes caused by these bacteria.

5. Significance and Use

5.1 Filamentous 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 slimy 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 250 mL conical bottles.

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

6.3 *Filter Paper or Blotter.*

6.3.1 For 8.3.2.1 – Grade 5 (nominal 2.5 µm particle-size retention).

6.3.2 For 9.3 – any absorbent paper medium will suffice.

6.4 *Containers,* sterile 1 L glass or plastic (can be autoclavable).

[1] This practice is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The boldface numbers in parentheses refer to a list of references at the end of this standard.

6.5 *Membrane Filter,* 0.45 µ nominal pore size, with appropriate filter-holding and vacuum assembly (see 9.2).

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

6.7 *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 or equivalent disposable plastic pipettes.

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

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

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.[4] 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 D1193, Type II.

7.3 *Hucker's modification of the Gram stain* (**4**).

7.3.1 *Crystal Violet Solution*—Dissolve 2.0 g of crystal violet (90 % dye content) in 20 mL of ethyl alcohol (95 % v/v).

7.3.2 *Ammonium Oxylate Solution*—Dissolve 0.8 g of ammonium oxalate monohydrate $(NH_4)_2C_2O_4 \cdot H_2O$) in 80 mL of water.

7.3.3 *Ammonium Oxalate-Crystal Violet Solution*—Combine crystal violet (7.3.1) and ammonium oxylate (7.3.2) solutions and mix well to ensure that the salts are dissolved completely.

7.4 *3N 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.

8. Sampling

8.1 Collect the samples in accordance with either Practices D887 or D3370, whichever is applicable.

8.2 Obtain a 500-mL (1-pt) sample of water, using a sterile 1-L (1-qt) bottle.

NOTE 1—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 Sample concentration by following either 8.3.2 or 8.3.3.

8.3.1 If the population is not sufficiently dense to be visible to the naked eye, samples should be concentrated before staining and microscopic examination.

8.3.2 *Filtration*—Use a small side stream filter to collect the sample to be examined.

8.3.2.1 Filter the water suspected of containing iron bacteria through a Grade 5 (nominal 2.5 µm particle-size retention) filter paper (6.3.1 or some other comparable media) for 24 h.

8.3.2.2 Adjust the side-stream filter flow rate to match the maximum filtration capacity of the filter medium used.

8.3.3 *Centrifugation:*

8.3.3.1 Divide the 500 mL sample (8.2) equally, by weight, among four 250 mL centrifuge bottles (6.1).

8.3.3.2 Centrifuge the subsamples at 9000 to 12 000 × g for 10 min.

8.3.3.3 Decant the supernate from each 250-mL bottle.

8.3.3.4 Resuspend the pellet from one centrifuge bottle into 20 mL of phosphate buffer or physiological saline (Practices D5465).

8.3.3.5 Transfer the suspension (8.3.3.4) to a second, pellet-containing centrifuge bottle and repeat 8.3.3.4.

8.3.3.6 Repeat 8.3.3.4 and 8.3.3.5 until all pellets and been consolidated into a single 20-mL suspension.

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

8.5 Collect mud samples from the mud-water interface in order to obtain maximum bacterial populations.

8.6 Transfer the deposit or mud samples to wide-mouth bottles and add sterile phosphate buffer or physiological saline (Practices D5465) 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 (6.8) and apply a cover glass (6.2).

9.1.1 Use a spatula (6.9) or wide-mouth pipet to transfer the sample to the slide.

9.1.2 When flocs of material are encountered, use a pipet; as the flocs settle to the tip when the pipet is held in a vertical position, and concentrate in the first drop.

9.1.3 In the case of very dilute solids or a water sample, concentrate the organisms by centrifuging (8.3.3), pour off the supernatant liquid, and repeat if necessary.

9.1.4 Alternatively, filter a suitable volume (10 to 500 mL; based on estimated population density) through a 0.45-µm membrane filter in an appropriate membrane filtration assembly (6.5: holder, tubing, trap, flasks and vacuum pump).

NOTE 2—For this test, it is not necessary to sterilize the filter assembly for each sample, but the assembly should be thoroughly cleaned between tests.

9.2 Examine the slide under the microscope to determine if encrusted or colorless sheaths are present.

[4] *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

9.2.1 Observe at least 20 microscope fields.

9.2.2 Record the presence of the twisted stalks of *Gallionella* at this point, since treatment with acid in accordance with 9.3 will dissolve the delicate stalks.

9.3 Place a drop of HCl solution (7.4) 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 (6.3.2).

9.4 Continue this procedure until no more yellow ferric chloride is evident in the solution.

NOTE 3—In order to prevent the sample from being drawn to the absorbent material, control the flow of the liquid.

NOTE 4—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 (7.5) under the cover glass until the color of the liquid becomes yellow or the filter paper becomes colored.

NOTE 5—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.

9.6.1 Observe at least 20 fields.

9.7 *Detection of Siderocapsa:*

9.7.1 Prepare a new slide by placing a drop of the sample on a clean slide and allowing it to air-dry.

9.7.2 Stain the slide for 1 min with ammonium oxalate-crystal violet solution (7.3.3), wash it with water, and allow it to dry. Examine the slide under an oil-immersion lens for the presence of *Siderocapsa*, which will appear violet colored.

9.7.2.1 Observe at least 20 fields.

9.8 Table 1 and Figs. 1-10 (**3**) may be used to differentiate the various types of filamentous iron bacteria. This practice provides an indication of the density of the iron bacteria and the severity of the clogging problem in pipes caused by these bacteria.

10. Report

10.1 Compute concentration factor of observed microscope field.

10.1.1 Calibrate the surface area of the microscope field.

10.1.2 Compute concentration factor for volume placed onto microscope slide.

10.1.3 Compute fraction of 10.1.2 observed per microscope field.

10.1.4 From 10.1.2 and 10.1.3, compute lower limit of detection (LLD) in filaments/mL, filaments/g, or filaments/cm^2 of original sample.

10.2 Computer either average percentage of coverage or average number of filaments of each type of filamentous iron bacterium per field.

10.3 Report *Present* or *Absent* and LLD.

10.3.1 If filaments are present, report relative abundance of the organisms present.

10.3.1.1 Report average percentage of coverage per field observed, or

10.3.1.2 Report average number of filaments counted per field.

NOTE 6—When mixed population have been observed, preferably,

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

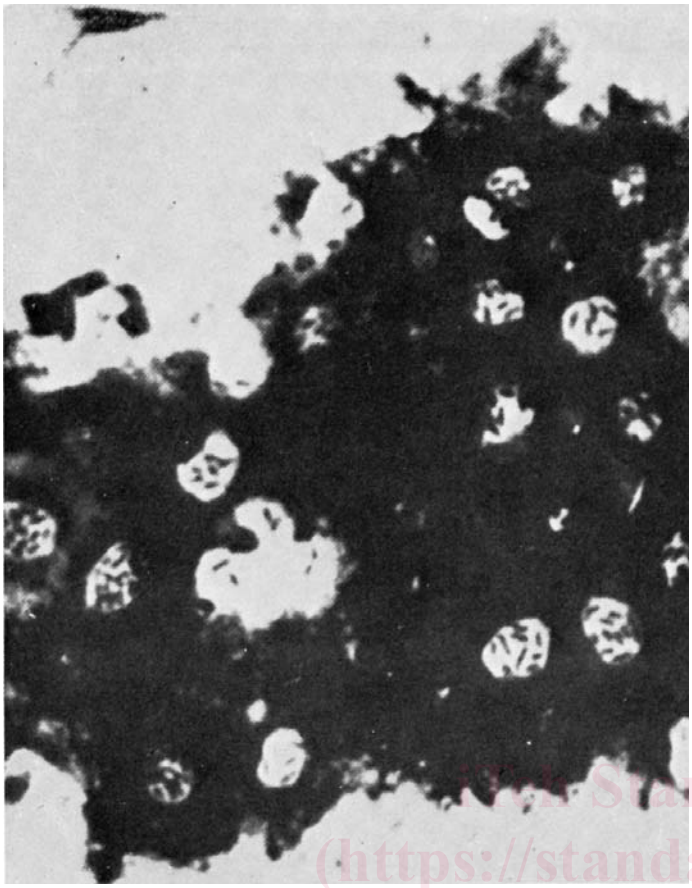
Genus: *Leptothrix* (Figs. 7 and 8)

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

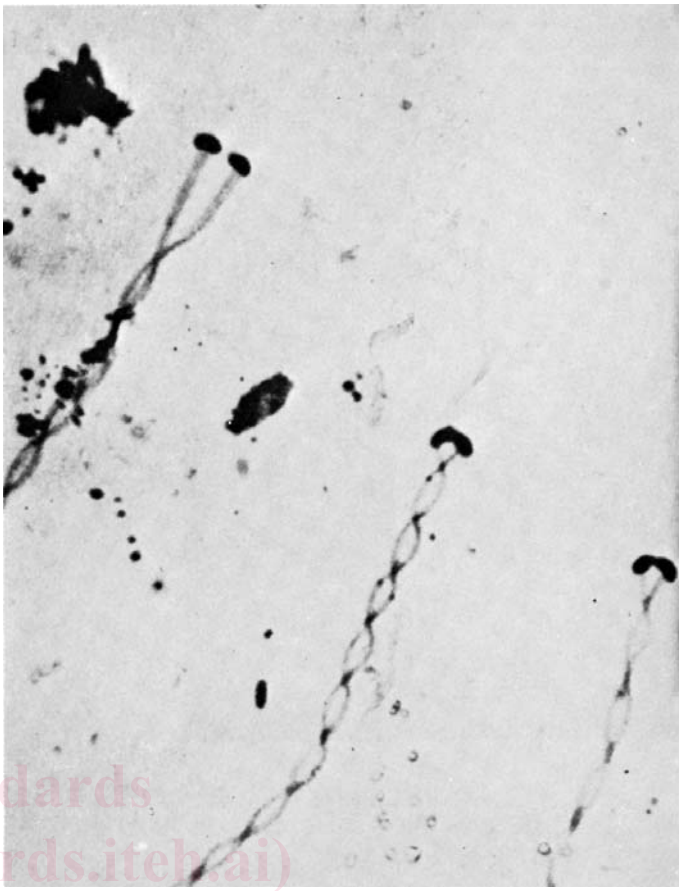
Genus: *Clonothrix* (Fig. 9)

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.



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

FIG. 1 *Siderocapsa treubii*



NOTE 1—Cells at the ends of excretion bands undergoing division. Magnification about 1180 × . Fig. 3 of Ref (**6**).

FIG. 2 *Gallionella major*

report by taxon (for example, 10 % *Crenothrix polyspora*; 30 % *Leptothrix ochracea*, etc.).

10.3.2 Report *Absent* only after examination of several slides.

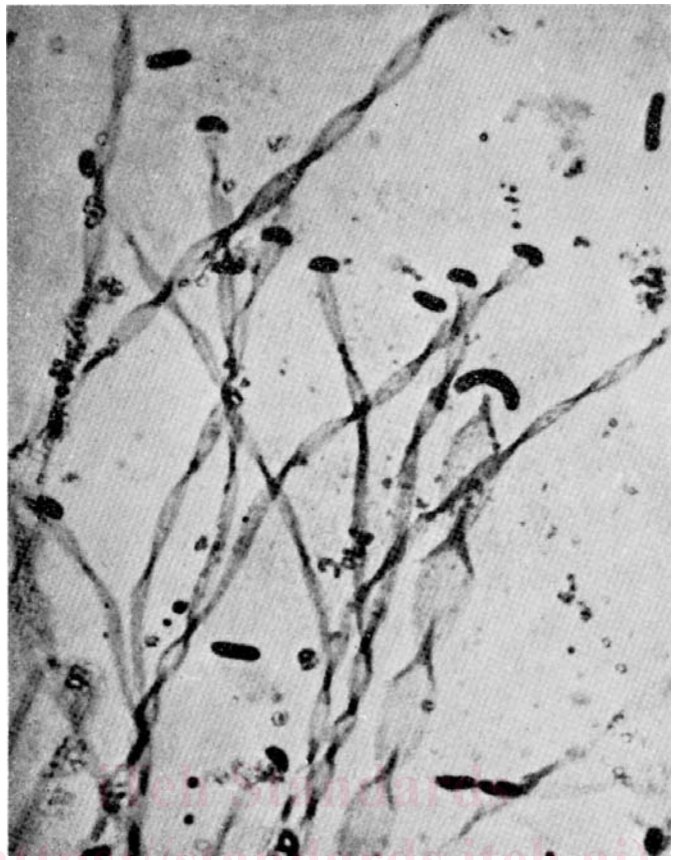
10.3.3 In accordance with 10.1.4, include LDL in *Absent* report: for example, <1 filament/50 mL.

11. Precision and Bias

11.1 This standard is a qualitative type test. Consequently precision and bias statements cannot be provided.

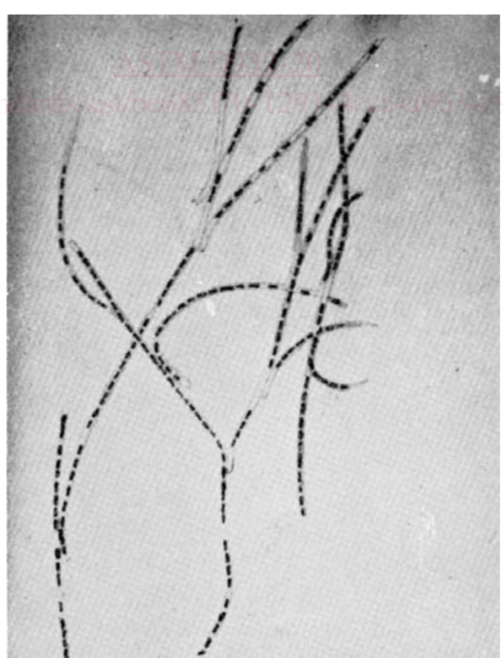
12. Keywords

12.1 biofouling; *Clonothrix*; *Crenothrix*; *Dioymohelix*; filamentous bacteria; *Gallionella*; iron bacteria; iron deposits; IRB; *Leptothrix*; microbiologically influenced corrosion; MIC; *Siderocapsa*; *Sphaerotilus*



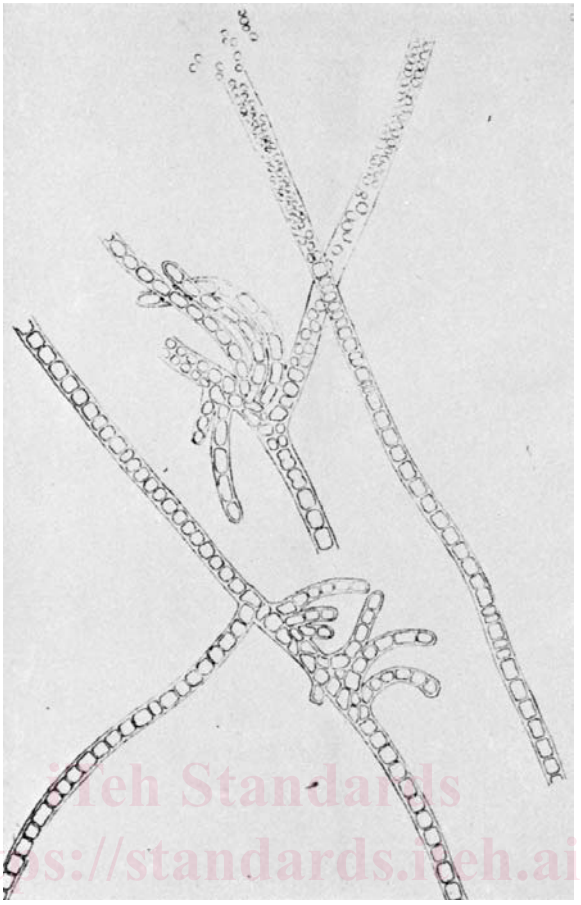
NOTE 1—Curved cells at the ends of excretion bands. Magnification about 1120 × . Fig. 6 of Ref (6).

FIG. 3 *Gallionella major*



NOTE 1—Sketch showing false branching. Magnification about 230 × . Fig. 3*b* of Ref (7).

FIG. 4 *Sphaerotilus dichotoma*



NOTE 1—Sketch showing details of false branching of cells within sheath. Magnification about 380 × . Plate 1, Fig. A of Ref (7).

FIG. 5 *Crenothrix polyspora*

NOTE 1—Cells enclosed within a sheath of ferric hydrate and showing false branching. Magnification about 390 × .

FIG. 6 *Crenothrix polyspora*