ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2004) - Part 12 - Methods for the isolation and enumeration of micro-organisms associated with taste, odour and related aesthetic problems

Methods for the Examination of Waters and Associated Materials

The Microbiology of Drinking Water (2004) - Part 12 – Methods for the isolation and enumeration of micro-organisms associated with taste, odour and related aesthetic problems

Methods for the Examination of Waters and Associated Materials

This booklet contains four methods for the isolation and enumeration of micro-organisms that may be related to microbially-mediated taste, odour and related aesthetic problems in drinking waters.

Within this series there are separate booklets dealing with different topics concerning the microbiology of drinking water. Other booklets include

The Microbiology of Drinking Water (2002)
Part 1 - Water quality and public health
Part 2 - Practices and procedures for sampling
Part 3 - Practices and procedures for laboratories
Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)
Part 5 - A method for the isolation and enumeration of enterococci by membrane filtration
Part 7 - Methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment

The Microbiology of Drinking Water (2004)
Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube most probable number techniques
Part 11 - Taste, odour and related aesthetic problems

Whilst specific commercial products may be referred to in this document this does not constitute an endorsement of these particular materials. Other similar materials may be suitable and all should be confirmed as such by validation of the method.

Contents

About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soil (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency. At present, there are nine working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical and physical methods
4 Metals and metalloids
5 General non-metallic substances
6 Organic impurities
7 Biological methods
8 Biodegradability and inhibition methods
9 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with this booklet are listed at the back of the booklet.

Publication of new or revised methods will be notified to the technical press. An index of methods and details on how to obtain copies are available from the Agency's internet web-page (www.environment-agency.gov.uk/nls) or from the Secretary.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary

February 2004

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

A The enumeration of actinomycetes by a membrane filtration technique

A1 Introduction

Members of the actinomycetes group occur ubiquitously in the environment, typically in soil, decomposing organic matter and aquatic habitats. Growth of some of these bacteria in source waters and in drinking water distribution systems can result in the release of substances imparting undesirable tastes and/or odours to the water. Several members of this group, particularly species of *Streptomyces*, are capable of producing geosmin and 2-methylisoborneol, which impart characteristic earthy or musty odours to waters. The significance of actinomycetes in water treatment and supply are described elsewhere in this series[(1)].

A2 Scope

This method is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those source waters of moderate turbidity.

Users wishing to employ this method should verify its performance under their own laboratory conditions[(2)].

A3 Definitions

In the context of this method, actinomycetes are bacteria which produce dry, raised and powdery looking colonies which can be chalky white, pink, red, brown, yellow or green in colour. Some slower growing colonies of actinomycetes may exhibit a wrinkled, waxy "star-shaped" appearance.

Actinomycetes are Gram-positive coccobacillus or rod-shaped, aerobic bacteria of the Order Actinomycetales that form a well-developed branched mycelium.

A4 Principle

Organisms are isolated on a membrane filter placed on the surface of an agar medium containing sodium caseinate. After incubation, membrane filters are examined for colony growth typical of actinomycetes.

A5 Limitations

The method is suitable for most types of aqueous samples, except those with high turbidities. The volume of sample that can be filtered in such cases may be limited due to the membrane filter becoming blocked. Accumulated deposit on the membrane filter may mask or inhibit growth of actinomycetes. Species of the *Streptomyces*, the dominant actinomycetes associated with odour problems in water, can be isolated. Some species of *Nocardia*, *Micromonospora* and *Microbispora* may also be recovered. The maximum number of colonies that should be counted from a single membrane filter is approximately 100.

A6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[(3)] and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[(2)] in this series.

A7 Apparatus

Standard laboratory equipment should be used which conforms to the performances criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted) are required. Other items include:

A7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of 1.8 % m/v solution of $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

A7.2 Incubator capable of maintaining a temperature of 22.0 ± 1.0 ºC.

A7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.

A7.4 Sterile, membrane filters, for example, white, 47 mm diameter, cellulose-based 0.45 μm nominal pore size.

A7.5 Smooth-tipped forceps.

A8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

A8.1 *Actinomycete isolation agar*

Sodium caseinate	2 g
Asparagine	0.1 g
Glycerol	5 g
Sodium propionate	4 g
Dipotassium hydrogen phosphate	0.5 g
Magnesium sulphate heptahydrate	0.1 g
Iron(II) sulphate heptahydrate	0.001 g
Agar	15 g
Water	1 litre

Dissolve all the ingredients in the water. Adjust the pH, if necessary, so that after sterilisation the pH is 8.1 ± 0.2. Sterilise by autoclaving at 121 ºC for 15 minutes. Allow the solution to cool to approximately 50 ºC and distribute into sterile Petri dishes. Sterile media can be stored for up to one month at a temperature between 2 - 8 °C, if protected against dehydration.

Several species of micro-fungi are able to grow on this medium and, if abundant, may overgrow the membrane filter. For waters where this is a problem, or for previously uncharacterised waters, this may be obviated by the addition of 0.05 g/l of cycloheximide to the medium prior to autoclaving.

A8.2 *Other media*

Standard and commercial formulations of other media and reagents used in this method include Ringer's solution and maximum recovery diluent.

A9 Analytical procedure

A9.1 *Sample preparation*

The volumes and dilutions of samples should be chosen so that the number of colonies to be counted on the membrane filter lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter a selection of different volumes so that the number of colonies on any single membrane filter is likely to fall within this range. For treated waters, filter 100 ml of the sample. For waters where high numbers of actinomycetes are expected, either filter smaller volumes or dilute the sample with Ringer's solution or maximum recovery diluent before filtration.

A9.2 *Sample processing*

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter, grid-side upwards, onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) to the funnel before addition of the sample. This aids the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the sample slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and transfer the membrane filter carefully to a Petri dish containing actinomycete isolation agar. Ensure that the surface of the agar is not too dry, as restricted growth may occur during incubation. Ensure that no air bubbles are trapped between the membrane filter and the medium. 'Rolling' the membrane filter onto the medium minimises the likelihood of air bubbles becoming trapped. Cover the membrane filter with the lid of the Petri dish.

When the funnel is removed it can be placed in a boiling water bath if it is to be re-used. Alternatively, pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without boiling provided that the smallest volume or highest dilution of sample is filtered first. For different samples, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath, allow the funnel to cool and repeat the filtration process. If funnels are re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling water for at least one minute. During the filtration of a series of samples the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. Polluted and non-polluted samples should, where known or suspected, be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible and no longer than 2 hours.

Incubate the Petri dishes at 22 ºC for seven days. Plates should be examined for growth at regular intervals during the incubation period.

A9.3 *Reading of results*

The final count is made after seven days of incubation. Count all compact colonies which are dry, raised and powdery in appearance. Generally, actinomycete colonies are chalky white in colour, but may be pink, red, brown, yellow or green. Some slower growing actinomycetes may exhibit a wrinkled, waxy "star-shaped" colony within the incubation time.

A9.4 *Confirmation tests*

Actinomycete morphology may be confirmed by conducting a Gram-stain on selected isolates. The isolates that possess well-developed branched mycelia with long chains of spores on aerial mycelia are often species of *Streptomyces*.

A10 Calculations

The number of actinomycetes is generally expressed as the number of colonies per 100 ml of sample. Calculate the count as follows:

$$\text{Count/100 ml} = \frac{\text{Number of colonies counted on membrane filter x 100}}{\text{Volume of sample filtered (ml)}}$$

A11 Expression of results

Actinomycetes are expressed in colony forming units per volume of sample. For drinking water the volume is typically 100 ml.

A12 Quality assurance

New batches of media should be tested with appropriate reference strains of target actinomycetes (for example, *Streptomyces griseus*). Further guidance on quality assurance are given elsewhere[(2)] in this series.

A13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2004), - Part 11 - Taste, odour and related aesthetic problems, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002), - Part 3 - Practices and Procedures for Laboratories, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

B The enumeration of micro-fungi and yeasts by membrane filtration or spread plate techniques

B1 Introduction

Micro-fungi are a major group of micro-organisms and occur wherever organic matter is present. They are found in natural (untreated) waters and arise predominantly from decaying vegetation or are washed into water courses from soil. Micro-fungi and yeasts are known to occur in treated water. Some micro-fungi cause undesirable changes in the organoleptic quality of treated water. Bio-transformation of chlorinated compounds, to produce more potent tastes and/or odours, has been demonstrated in certain species. The significance of micro-fungi and yeasts in water treatment and supply are described elsewhere in this series[1].

B2 Scope

The method is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those source waters of moderate turbidity.

Users wishing to employ this method should verify its performance under their own laboratory conditions[2].

B3 Definitions

Micro-fungi exhibit mycelial growth and reproduce by spores. The nutritional requirements of micro-fungi and yeasts are such that they grow on simple media. Yeasts exhibit unicellular growth and reproduce by budding or fission.

B4 Principle

Organisms are either isolated on a membrane filter placed on the surface of an agar medium containing mycological peptone, rose bengal and chloramphenicol or directly inoculated onto the medium. After incubation the plates are examined for colony growth typical of micro-fungi and yeasts.

B5 Limitations

The method is suitable for most types of aqueous samples, except those with high turbidities. The volume of sample that can be filtered in such cases may be limited due to blockage of the filter. Accumulated deposit on the membrane filter may mask or inhibit growth of micro-fungi and yeasts. Growth of mycelium and spores takes place, which reduces the significance (in terms of a meaningful indication of fungal biomass) of the number of colonies detected. The number of micro-fungi and yeasts may be distorted by excessive handling of cultures during incubation as spores can be released which produce additional colonies on the medium.

It should be recognised that there is no single medium capable of isolating the entire range of micro-fungi and yeasts that may be present in water supplies. The medium employed in this method, rose bengal chloramphenicol agar, has been widely used in the UK to isolate micro-fungi and yeasts from drinking waters, and is available commercially. While rose bengal is incorporated into the medium to suppress the growth of rapidly growing micro-fungi, it may prevent growth of other species present. During initial investigations, more than one medium may be used (for example, Sabourand dextrose agar, half-strength potato-carrot agar and half-strength cornmeal agar)

to ensure that a broad range of species is recovered. Details on the selection of suitable media have previously been reported[3, 4].

B6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[5] and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

B7 Apparatus

Standard laboratory equipment should be used which conforms to the performances criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted) are required. Other items include:

B7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

B7.2 Incubators capable of maintaining temperatures of 12.0 ± 1.0 ºC and 22.0 ± 1.0 ºC.

B7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.

B7.4 Sterile, membrane filters, for example, white, 47 mm diameter, cellulose-based 0.45 μm nominal pore size.

B7.5 Smooth-tipped forceps.

B8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

B8.1 *Rose bengal chloramphenicol agar*[6]

Mycological peptone	5.0 g
Glucose	10.0 g
Potassium dihydrogen phosphate	1.0 g
Magnesium sulphate heptahydrate	0.5 g
Rose bengal	0.05 g
Chloramphenicol (0.1g in 5 ml of methanol)	5 ml
Agar	15.5 g
Water	1 litre

Suspend all the ingredients in one litre of distilled water and boil to dissolve. Sterilise the medium by autoclaving at 121 ºC for 15 minutes. Allow the solution to cool to approximately 50 ºC, and

distribute into sterile Petri dishes. The final pH should be 7.2 ± 0.2. Sterile media can be stored for up to one month at a temperature between 2 - 8 °C, if protected against dehydration. Rose bengal may degrade in light to form toxic derivatives, thus prepared plates should be stored in the dark.

B8.2 *Other media*

Standard and commercial formulations of other media and reagents used in this method include Ringer's solution and maximum recovery diluent.

B9 Analytical procedure

B9.1 *Sample preparation*

The volumes and dilutions of samples should be chosen so that the number of colonies to be counted on the membrane filter lies, if possible, between 10 and 50, or for spread plate between 10 and 80. For some species of fungi (for example *Rhizopus* species) substantial mycelial growth may occur and this may limit the number of colonies that can be counted on a plate. As the concentration of micro-fungi and yeasts in water may not be known, a range of volumes, from 500 ml to 0.001 ml, may need to be prepared, in duplicate, and processed by both membrane filtration (for the larger volumes) and spread plate (for the smaller volumes) techniques. Paired volumes of each sample should be incubated separately at 12 °C and at 22 °C.

B9.2 *Membrane filtration sample processing*

With some waters, it may be advantageous to filter a selection of different volumes so that the number of colonies on any one of the membrane filters is likely to fall within the range desired. For treated waters, filter two aliquots of 100 ml of the sample. For waters where high numbers of micro-fungi and yeasts are expected, either filter smaller volumes or dilute the sample with Ringer's solution or maximum recovery diluent before filtration.

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter, grid-side upwards, onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) to the funnel before addition of the sample. This aids the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the sample slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and carefully transfer the membrane filter to a Petri dish containing rose bengal chloramphenicol agar. Ensure that the surface of the agar is not too dry, as restricted growth may occur during incubation. Ensure that no air bubbles are trapped between the membrane filter and the medium. 'Rolling' the membrane filter onto the medium minimises the likelihood of air bubbles becoming trapped. Cover the membrane filter with the lid of the Petri dish.

When the funnel is removed it can be placed in a boiling water bath if it is to be re-used. Alternatively, pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without boiling provided that the smallest volume or highest dilution of sample is filtered first. For different samples, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath, allow the funnel to cool and

repeat the filtration process. If funnels are re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling water for at least one minute. During the filtration of a series of samples the filter base need not be sterilised unless it has become contaminated or a membrane filter becomes damaged. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible and no longer than 2 hours.

For each pair of Petri dishes, incubate one at 12 ºC and the second at 22 ºC, for seven days. The Petri dishes should be examined daily, as abundant growth of some micro-fungi may obscure the growth of slower growing species. It may be necessary to record and isolate the colonies before the full period of incubation has been completed. Alternatively, it may be possible to remove the rapid growing fungi to enable the slower growing species to emerge. Microscopic examination of the Petri dishes may assist the detection of slower growing species which do not form colonies that are readily visible to the naked eye.

B9.3 *Spread plate sample processing*

If Petri dishes are to be prepared from rose bengal chloramphenicol agar contained in tubes or bottles, heat the tube or bottle of medium and the melt the medium and keep at 45 - 48 °C. If a water bath is used to keep the medium between 45 - 48 °C then prior to pouring the medium into the Petri dish remove the water from the outside of the container. This should remove or minimise any contamination. Pour 15 - 20 ml of molten agar into each Petri dish and allow the medium to solidify. If a prepared Petri dish is used, allow the dish to equilibrate to room temperature. The dish should be dried to remove excess moisture before use, but ensure that the surface of the agar is not too dry, as restricted growth may occur during incubation. In duplicate, pipette 0.1 ml of the sample, or diluted sample, onto the surface of pre-dried agar medium contained in the Petri dish. Distribute the sample over the surface of the medium with a sterile bent glass or plastic rod. Alternatively, distribute the sample by rotating the dish whilst holding the spreader steady. Allow the inoculum to soak into the agar, place a lid on the Petri dish and incubate in an inverted position.

For each pair of Petri dishes, incubate one at 12 ºC and the second at 22 ºC, for seven days. The Petri dishes should be examined daily, as abundant growth of some micro-fungi may obscure the growth of slower growing species. It may be necessary to record and isolate the colonies before the full period of incubation has been completed. Alternatively, it may be possible to remove the rapid growing fungi (for example, by using sterilised forceps to remove abundant aerial hyphae, taking care not to dislodge any spore structures that may have developed) to enable the slower growing species to emerge. Microscopic examination of the Petri dishes may assist the detection of slower growing species which do not form colonies that are readily visible to the naked eye.

B9.4 *Reading of results*

The final count is made after incubation for seven days. Micro-fungi develop as raised colonies, which take on a wool-like, cotton-like or powder-like appearance. In contrast, yeasts develop as red, opaque colonies and are characterised by a smooth, waxy appearance. Occasionally, aerial mycelium will not be present, as growth may occur in the medium. All compact colonies that conform to the characteristics of micro-fungi and yeasts should be counted.

B9.5 *Confirmation tests*

At present, there is no single source of published information for identifying micro-fungi and yeasts isolated from water. However, specialist schemes are available for specific taxonomic groups[7, 8]. However, interpretation requires experience. Also, the use of the above medium may not allow the production of reproductive structures, which are essential for identification. It may, therefore, be necessary to sub-culture isolates on media more appropriate for sporulation.

B10 Calculations

The number of micro-fungi and yeasts is generally expressed as the number of colonies per 100 ml of sample. Calculate the count as follows:

$$\text{Count/100 ml} = \frac{\text{Number of colonies counted on membrane filter} \times 100}{\text{Volume of sample filtered (ml)}}$$

B11 Expression of results

Micro-fungi and yeasts are expressed in colony forming units per volume of sample. For drinking water the volume is typically 100 ml.

B12 Quality assurance

New batches of media should be tested with appropriate reference strains of target micro-fungi and/or yeast (for example, *Penicillium* species and/or *Rhodotorulla* species). Further guidance on quality assurance are given elsewhere[2] in this series.

B13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2004) - Part 11 - Taste, odour and related aesthetic problems, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

3. Drinking Water Inspectorate, *Significance of fungi in water distribution systems*. Report EPG/1/9/69, 1996. Available from Foundation for Water Research, Marlow (www.fwr.org/dwipage.htm).

4. Methods for the determination of filamentous fungi in treated and untreated waters, *Journal of Applied Microbiology Symposium Supplement*, G C Kinsey, R R Paterson and J Kelley, 1999, **85**, pp214S-224S.

5. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

6. Comparison of an improved Rose Bengal-Chlortetracycline Agar with other media for the selective isolation and enumeration of moulds and yeasts in foods. *Journal of Applied Bacteriology*, B Jarvis, 1973, **36**, pp723-727.

7. *Illustrated Genera of Imperfect Fungi*, 4th Edition, H L Barnett & B B Hunter, 1998, APS Press, The American Phytopathological Society, St. Paul Minnesota.

8. *Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and Key to Species*, T Watanabe, 1994, Lewis Publishers, Boca Raton.

C The estimation of sulphate-reducing bacteria from water or deposits by a dilution series technique

C1 Introduction

Sulphate-reducing bacteria are a diverse group of strictly anaerobic micro-organisms distinguished by their ability to reduce sulphate to sulphide. The organisms can be found in many anaerobic environments, in soil, mud, sediment and sewage. However, they occur most commonly where high concentrations of sulphate are present. This group of bacteria is a significant cause of microbially-induced corrosion and taste and/or odour problems in water systems. In addition, major health hazards may arise due to the production of hydrogen sulphide. The significance of sulphate-reducing bacteria in water treatment and supply are described elsewhere in this series[1].

C2 Scope

The method is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those surface or groundwater source waters of moderate turbidity.

Users wishing to employ this method should verify its performance under their own laboratory conditions[2].

C3 Definitions

In the context of this method, sulphate-reducing bacteria are those bacteria which reduce sulphate to sulphide in modified Starkey's medium A or modified API RP-38 medium.

The two most common genera are *Desulfovibrio* and *Desulfotomaculum*. *Desulfovibrio* are Gram-negative, non-sporing vibrio rods (2.5 - 1.0 μm by 0.5 - 1.5 μm) which are motile with polar flagellae. *Desulfotomaculum* are Gram-negative rods (3 - 9 μm by 0.3 - 1.5 μm) which produce terminal or subterminal spores.

C4 Principle

A serial dilution series of the sample should be prepared within media bottles. After incubation the population of sulphate-reducing bacteria is estimated from the greatest dilution producing a positive reaction.

C5 Limitations

There are many formulations of media used for enumerating sulphate-reducing bacteria. All include a carbon source (normally lactic acid) and small amounts of yeast extract, inorganic salts and a reducing agent (to equilibrate the medium at a low potential). It is possible that viable count procedures used for enumerating sulphate-reducing bacteria may underestimate numbers by a factor of around 1000, compared with numbers produced by an *in-situ* sulphate reduction activity measurement[3]. Due to the difficulties in obtaining accurate counts on agar-based plating media, sulphate-reducing bacteria in environmental samples have been enumerated by serial dilution in completely anaerobic liquid media. Variations of the medium used[4] are available commercially. It is important to ensure that a freshwater-based medium is used.

It should be recognised that the analysis of black deposits and those deposits containing sulphide may make interpretation of the test difficult. Bottles containing black deposits that appear to be positive within a few hours of inoculation should be incubated as normal. In general, only the first

bottle in the dilution series may show black colouration. Where the presence of sulphate-reducing bacteria is in doubt after incubation, a sub-sample of medium exhibiting any presumed positive characteristics should be transferred to a fresh bottle of medium for further incubation and confirmation.

C6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[5] and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

C7 Apparatus

Standard laboratory equipment should be used which conforms to the performances criteria outlined elsewhere[2] in this series. Principally, incubators (fan assisted) are required. Other items include:

C7.1 Sterile sample bottles of appropriate volume, made of suitable material.

C7.2 Incubators capable of maintaining a set temperature to ± 1.0 ºC when set at a temperature between 20 ºC and 30 ºC.

C8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

C8.1 *Modified Starkey's medium A*[6]

Dipotassium hydrogen phosphate (anhydrous)	0.5 g
Ammonium chloride	1.0 g
Sodium sulphate	0.5 g
Calcium chloride dihydrate	0.1 g
Magnesium sulphate heptahydrate	2.0 g
Sodium lactate solution (60 - 70 %)	5.0 ml in 100 ml of water
Sodium thioglycollate	0.1 g
Ascorbic acid	0.1 g
Iron(II) sulphate heptahydrate	0.001 g
Water	to 1 litre

Dissolve all the ingredients, except sodium lactate solution, in approximately 800 ml of water. If necessary use gentle heating. The pH of the solution should be adjusted to 7.2 ± 0.2. Add 5 ml of sodium lactate solution (60 - 70 %) to 100 ml of water and boil the solution. This solution is then added to the other media components and the whole volume made up to 1000 ml with water.

Bottles (of 10 ml capacity) are filled with 9 ml of medium and then stoppered and capped using butyl rubber stoppers and metallic caps. Flushing the bottles with nitrogen prior to capping will displace any residual oxygen, which may allow enhanced recovery of some strains of sulphate-

reducing bacteria. The bottles and contents are then autoclaved at 121 ºC for 15 minutes. The medium should be used freshly prepared.

C8.2 *Modified API RP-38 medium*[7]

Sodium sulphate	1.0 g
Sodium lactate solution (60-70%)	4.0 ml in 100 ml of water
Yeast extract	1.0 g
Ascorbic acid	0.1 g
Magnesium sulphate heptahydrate	0.2 g
Dipotassium hydrogen phosphate	0.01 g
Iron(II) ammonium sulphate hexahydrate	0.2 g
Water	to 1 litre

Dissolve all the ingredients, except sodium lactate solution, in approximately 800 ml of water. If necessary use gentle heating. The pH of the solution should be adjusted to 7.3 ± 0.2. Add 5 ml of sodium lactate solution (60 - 70 %) to 100 ml of water and boil the solution. This solution is then added to the other media components and the whole volume made up to 1000 ml with water.

Bottles (of 10 ml capacity) are filled with 9 ml of medium and then stoppered and capped using butyl rubber stoppers and metallic caps. Flushing the bottles with nitrogen prior to capping will displace any residual oxygen, which may allow enhanced recovery of some strains of sulphate-reducing bacteria. The bottles and contents are then autoclaved at 121 ºC for 15 minutes. The medium should be used freshly prepared.

C9 Analytical procedure

C9.1 *Water samples*

For each sample, six bottles of either modified Starkey's medium A or modified API RP-38 medium are required. Using a disposable 1 ml sterile syringe and needle, 1 ml of sample should be added (through the butyl seal) to a bottle of medium. The syringe and needle should then be discarded and the bottle vigorously shaken. Using a fresh syringe and needle, 1 ml of the inoculated liquid from the first bottle is withdrawn and added into the second bottle in the six-bottle dilution series. Mix the contents of the bottle with vigorous shaking. The procedure is repeated until a series of dilution bottles has been prepared. If a duplicate dilution series is required, 1 ml of the original sample should be taken and processed in a similar manner using a further six bottles of media.

Incubate the prepared bottles at a suitable temperature between 20 ºC and 30.0 ºC, for up to 28 days. The bottles should be examined at regular intervals during the incubation period.

C9.2 *Samples of deposits*

Use either modified Starkey's medium A or modified API RP-38 medium. The number of bottles required will depend upon the expected numbers of sulphate-reducing bacteria in the deposit. Remove the cap from the first bottle in the dilution series and add a known amount of the deposit (by weight or volume). Recap the bottle and shake vigorously to disperse the loose deposits. Using a fresh syringe and needle, 1 ml of the inoculated liquid from the first bottle should be withdrawn and added into the second bottle in the dilution series. Mix the contents of the bottle with vigorous shaking. The procedure should be repeated until a series of dilution bottles has been prepared. If a

duplicate dilution series is required, an identical amount of the original sample should be taken and processed in a similar manner.

Incubate the prepared bottles at a suitable temperature between 20 ºC and 30 ºC, for up to 28 days. The bottles should be examined at regular intervals during the incubation period.

C9.3 *Reading of results*

Bottles in which the contents exhibit a black colouration, or contain a black precipitate within 28 days are regarded as positive. In many instances, colouration or precipitation will appear within 14 days.

C10 Calculations

The number of sulphate-reducing bacteria is estimated from the highest dilution giving a positive result. For example, if the first four bottles in the series exhibit a black colouration, or contain a black precipitate then the number of sulphate-reducing bacteria is estimated to be approximately 10^3/ml. If all six bottles exhibit a black colouration, or contain a black precipitate the number of sulphate-reducing bacteria is recorded as $\geq 10^5$/ml. For duplicate dilution series, numbers are reported as a range between the highest dilution that exhibits a black colouration, or contains a black precipitate and the lowest dilution exhibiting no black colouration, or containing no black precipitate.

C11 Expression of results

Sulphate-reducing bacteria are expressed as an estimated count per volume of sample. For drinking water the volume is typically 1 ml.

C12 Quality assurance

New batches of media should be tested with appropriate reference strains of target sulphate-reducing bacteria (for example, *Desulfovibrio* species or *Desulfotomaculum* species). General guidance on quality assurance is given elsewhere[(2)] in this series.

C13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2004) - Part 11 - Taste, odour and related aesthetic problems, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

3. Sulphate-reducing bacteria and anaerobic corrosion. *Annual Review of Microbiology*, W A Hamilton, 1985, **39**, pp195-217.

4. *Review of current practices for monitoring bacterial growth in oilfield systems*. Corrosion Control Engineering Joint Venture, 1987.

5. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

6. Standard test methods for sulfate-reducing bacteria in water and water-formed deposits. ASTM D 4412-84 (Re-approved 1990), American Society for Testing and Materials, Philadelphia.

7. *API Recommended Practice for the Biological Analysis of Subsurface Injection Waters, Second Edition.* American Petroleum Institute, 1965.

D The detection of iron-precipitating bacteria by a defined enrichment technique and microscopy

D1 Introduction

Iron-precipitating bacteria can obtain energy via the oxidation of iron(II) to iron(III). This oxidation results in the precipitation of iron(III) hydroxide, which can impart a red colour to the water. Typically, the source of iron is pipe-work or the water in supply. Substantial growth of iron-precipitating bacteria can also impart unacceptable odours to drinking water. Bacteria, which are commonly associated with iron precipitation problems, include species of *Gallionella*, *Leptothrix*, *Crenothrix* and *Siderocapsa*. Other bacteria that have been shown to be capable of non-oxidative precipitation of iron include species of *Klebsiella*, *Enterobacter*, *Serratia*, *Corynebacterium*, *Caulobacter* and *Bacillus*. The significance of iron-precipitating bacteria in water treatment and supply are described elsewhere in this series[1].

D2 Scope

The method is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those surface and groundwater source waters of moderate turbidity.

Users wishing to employ this method should verify its performance under their own laboratory conditions[2].

D3 Definitions

In the context of this method, iron-precipitating bacteria are those bacteria which grow in modified W-R medium producing a brown scum or pellicle at the surface of the medium, often with a strong yellow or brown colouration of the medium

Iron-precipitating bacteria may be represented by members from a number of bacterial genera, including sheathed bacteria (for example *Leptothrix*, *Crenothrix* and *Clonothrix*), stalked and budding bacteria (for example *Gallionella* and *Pedomicrobium*) and Gram-negative chemolithotrophic bacteria (for example *Siderocapsa*, *Ochrobium* and *Thiobacillus*).

D4 Principle

An aliquot of sample is added to the growth medium, modified W-R medium. Growth and precipitation of iron within the incubation period indicates the presence of a significant population of bacteria associated with iron-precipitation in the original sample.

D5 Limitations

Using modified W-R medium, growth and enrichment of relatively fast growing bacteria associated with oxidative and non-oxidative iron-precipitation is supported. However, with this medium growth of sheathed or stalked bacteria may not be supported, or only limited growth occurs. These bacteria generally require more sophisticated methods for growth[3].

Some non-microbially-induced oxidation of iron may occur during incubation producing a light brown or green colouration.

D6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[4] and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

D7 Apparatus

Standard laboratory equipment should be used which conforms to the performances criteria outlined elsewhere[2] in this series. Principally, incubators (fan assisted) are required. Other items include:

D7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

D7.2 Incubator capable of maintaining a temperature of 22.0 ± 1.0 ºC.

D8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

D8.1 *Modified W-R single strength medium*[5]

Dipotassium hydrogen phosphate	0.5 g
Magnesium sulphate heptahydrate	0.5 g
Ammonium nitrate	0.5 g
Calcium chloride dihydrate	50 mg
Iron(III) ammonium citrate	6.0 g
Water	1 litre

The medium is prepared as a double strength solution. Dissolve all the ingredients in the water. Adjust the pH of the solution, if necessary, to 6.7 ± 0.2. Distribute in 15 ml volumes into screw-capped bottles of 50 ml capacity, or 25 ml volumes into screw-capped bottles of 100 ml capacity. Sterilise by autoclaving at 121 ºC for 15 minutes, allow the solution to cool and tighten the bottle caps. The medium should be used freshly prepared.

Alternatively, prepare a 20-fold strength solution of modified W-R medium. Aliquots of the concentrated medium (typically, 0.75 ml) are added to 25 ml screw-capped bottles and aseptically evaporated to dryness at 65 ºC. The bottles are then capped and may be stored for up to one year at a temperature between 2 - 8 °C.

D9 Analytical procedure

D9.1 *Using freshly prepared medium*

Prepare double strength modified W-R medium in volumes equal to the intended volume of sample to be tested (typically, 15 - 25 ml) in screw-capped bottles (typically, 50 - 100 ml capacity, depending on volume of sample to be tested). The bottle size chosen should be large enough to ensure a headspace above the sample/medium mixture.

Add the appropriate volume of sample to a bottle containing double strength modified W-R medium, cap and mix well. Incubate the bottle at 22 ºC for up to seven days. The bottle contents should be examined daily during the incubation period. Incubation may be extended to 30 days, if necessary.

A control sample of medium, inoculated with sterile water in place of the sample should also be prepared with each batch of samples and used for comparison.

D9.2 *Using dried medium*

An aliquot of sample (typically, 15 ml) is added to a bottle containing the evaporated contents of a 20-fold strength solution of modified W-R medium and mixed well. Ensure the dried medium dissolves in the sample. If larger sample volumes are to be tested, then the volume of medium and the size of the bottle should be adjusted accordingly. For example, 1.0 ml of 20-fold strength solution of modified W-R medium may be required for 20 ml of sample, or 1.25 ml of 20-fold strength solution of modified W-R medium for 25 ml of sample, etc.

Incubate the bottle at 22 ºC for up to seven days. The bottle contents should be examined daily during the incubation period. Incubation may be extended to 30 days, if necessary.

A control sample of medium, inoculated with sterile water in place of the sample should also be prepared with each batch of samples and used for comparison.

D9.3 *Reading of results*

The presence of iron-precipitating bacteria is typically indicated by growth of a brown scum or pellicle at the surface of the medium, often with a strong yellow or brown colouration of the medium. In some cases, a brown, slimy growth may also occur at the bottom of the bottle, occasionally without surface growth. If iron-precipitating bacteria are not present, the medium may slowly auto-oxidise resulting in a clear green colouration. The control sample of medium inoculated with sterile water should, therefore, be examined with each batch of samples and used for comparison.

D9.4 *Examination of deposits*

In many instances, a microscopical examination will be sufficient to identify the organism causing a water quality problem. If the sample is clear and without any obvious turbidity, the sample should be centrifuged or allowed to settle overnight in order to concentrate any cells or filaments prior to examination. If sediment is present, carefully use a Pasteur pipette, or similar, to remove a small amount of deposit from the bottom of the container and place on a microscope slide. Cover with a cover-slip. Examine the slide under 400 - 1000 x magnification, ideally under phase contrast. If a conventional light microscope is used, identification may be easier if the sample on the slide is stained beforehand, for example with India ink or lactophenol blue. If the sample contains very

little particulate matter, the sample can be filtered through a membrane filter (nominally 0.45 µm pore size). The filter should be dried, for example by placing the filter (grid side up-wards) on adsorbent paper, cleared by the addition of immersion oil, and examined at 1000 x magnification.

Heavy deposits of iron in the sample may obscure any iron bacteria that are present making identification more difficult. This problem may be overcome if the iron deposits are dissolved in dilute hydrochloric acid, oxalic or citric acid solution. Citric acid will not cause lysis of bacterial cells, but is less effective at removing iron. Place a few drops of 1 molar hydrochloric acid, or a solution of oxalic or citric acid (0.5 – 1 % m/v) to one side of the cover-slip and draw the solution under the cover-slip by placing absorbent paper to the opposite side. Slimes, flocs or other visible material can also be examined in a similar manner.

D9.5 *Confirmation tests*

To confirm or identify the presence of bacteria involved in iron-precipitation, conduct a microscopical examination of the contents of the bottles exhibiting growth of bacteria. The structures of several iron bacteria are shown in Appendix D1.

D10 Calculations

This use of this method indicates the presence or absence of iron-precipitating bacteria in the volume of sample examined.

D11 Expression of results

Iron-precipitating bacteria are reported as being detected, or not detected, in the volume of sample examined.

D12 Quality assurance

New batches of media should be tested with an appropriate reference strain of target iron-precipitating bacterium (for example, *Gallionella* species or *Siderocapsa* species) and non-target bacterium (for example, *Pseudomonas aeruginosa*). General guidance on quality assurance is given elsewhere[(2)] in this series.

D13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2004) - Part 11 - Taste, odour and related aesthetic problems, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

3. Detection, Cultivation and Maintenance of Gallionella in Laboratory Microcosms. *Letters in Applied Microbiology*, J Verran, J F D Stott, S L Quarmby and M Bedwell, 1995, **20**, pp341-344.

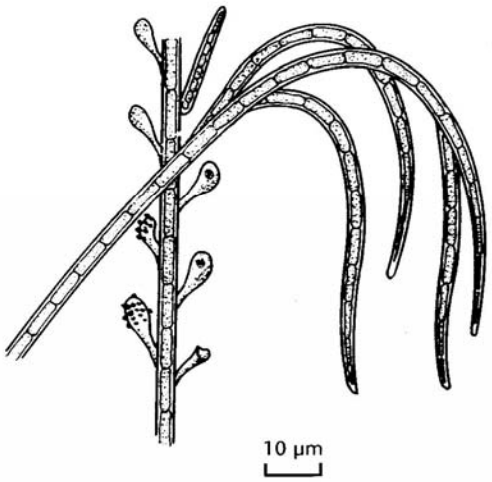
4. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

5. *Methods for Monitoring Iron and Manganese Biofouling in Water Wells*. S A Smith, 1992, American Water Works Association Research Foundation, Denver.

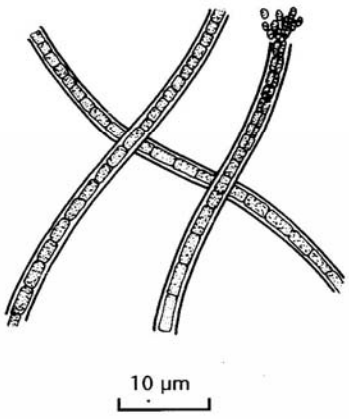
6. The Identification, Cultivation and Control of Iron Bacteria in Ground Water, D R Cullimore and A E McCann in Aquatic Microbiology, (Eds) F A Skinner and J M Shewan, Academic Press, London, 1977, pp219-261.

Appendix D1 Diagrams of various iron bacteria

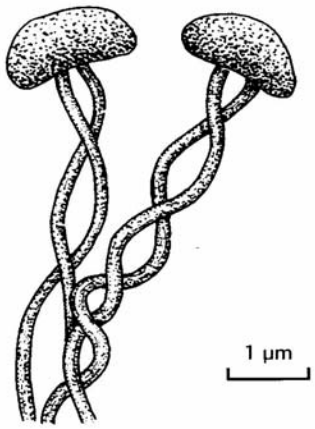
These diagrams are taken from *Iron transformations by freshwater bacteria*, J G Jones, Advances in Microbial Ecology, 1986, 9, pp 149 - 185, (Ed) K C Marshall, Plenum Press, New York.



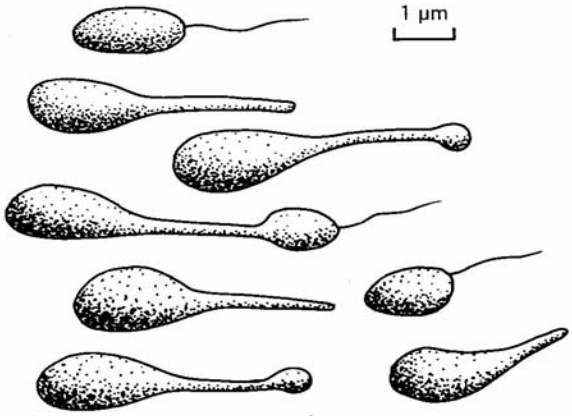
Clonothrix



Crenothrix

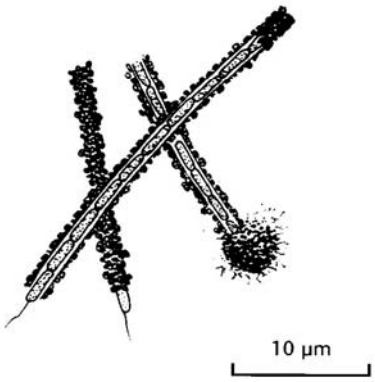


Gallionella

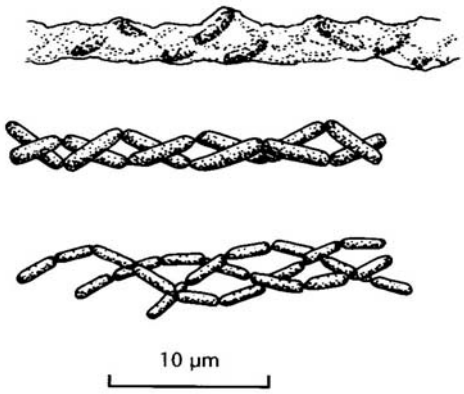


Hyphomicrobium

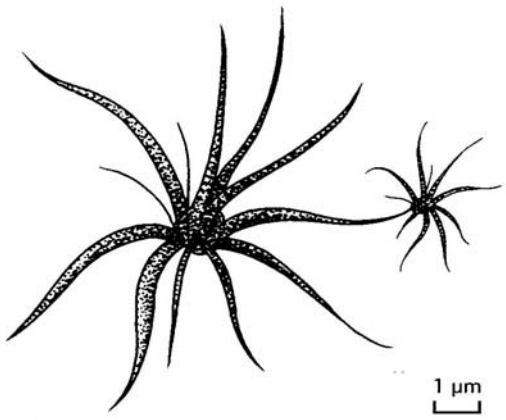
Appendix D1 **Diagrams of various iron bacteria** (continued)



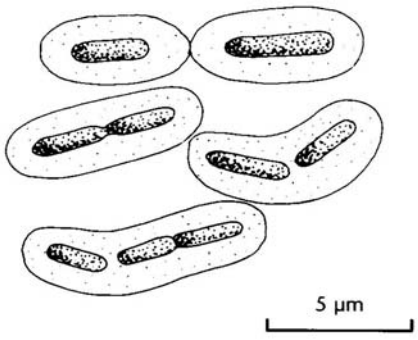
Leptothrix



Lieskeella

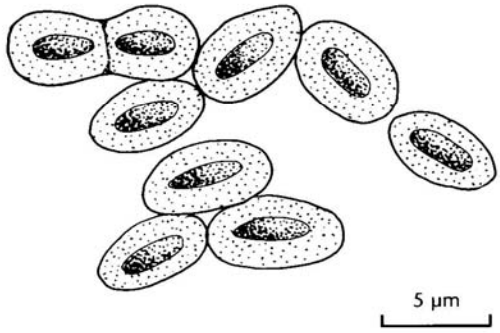


Metallogenium

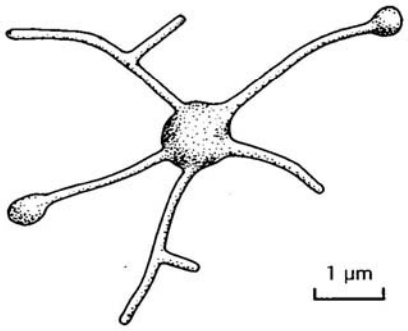


Naumaniella

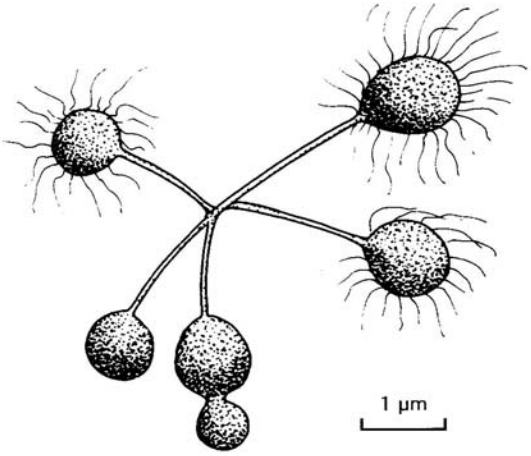
Appendix D1 **Diagrams of various iron bacteria** (continued)



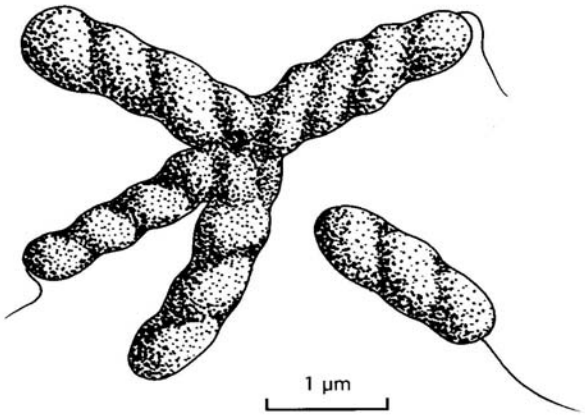
Ochrobium



Pedomicrobium

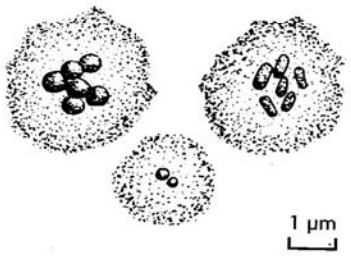


Planctomyces

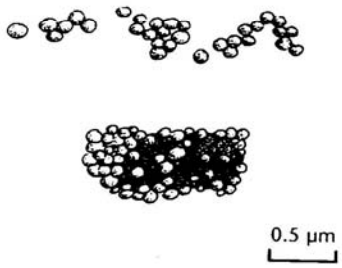


Seliberia

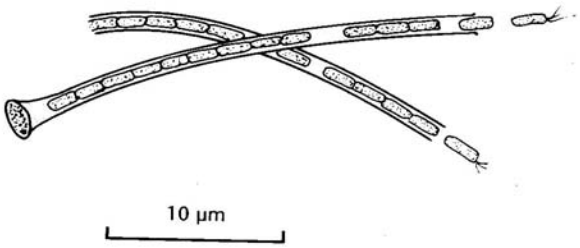
Appendix D1 **Diagrams of various iron bacteria** (continued)



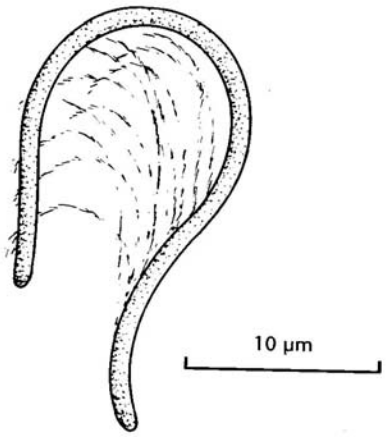
Siderocapsa



Siderococcus



Sphaerotilus



Toxothrix

Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below.

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