



ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2007) - Part 7 – Methods for the enumeration of heterotrophic bacteria

Methods for the Examination of Waters and Associated Materials

The Microbiology of Drinking Water (2007) - Part 7 - Methods for the enumeration of heterotrophic bacteria

Methods for the Examination of Waters and Associated Materials

This booklet contains two methods for the enumeration of heterotrophic bacteria and replaces the earlier booklet published in 2002.

Within this series there are separate booklets, each 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 - The isolation and enumeration of *enterococci* by membrane filtration

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 11 - Taste, odour and related aesthetic problems

Part 12 - Methods for micro-organisms associated with taste, odour and related aesthetic problems.

The Microbiology of Drinking Water (2006)

Part 5 - The isolation and enumeration of *enterococci* by membrane filtration

Part 9 - The isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube-most probable number techniques

The Microbiology of Drinking Water (2007)

Part 13 - The isolation and enumeration of aerobic spore-forming bacteria by membrane filtration

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products but serves only as an illustrative example of the type of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used and all should be confirmed by validation of the method.

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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, soils (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 these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the Agency's web-page (www.environment-agency.gov.uk/nls) or by post.

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
July 2006

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 heterotrophic bacteria by pour and spread plate techniques

A1 Introduction

The colony count of heterotrophic bacteria may be enumerated by pour or spread plate techniques using yeast extract agar⁽¹⁾ or R2A agar⁽²⁾. Petri dishes are typically incubated at 22 °C and 37 °C. In some cases, incubation at 30 °C can be undertaken. The most useful application of the estimation of heterotrophic bacteria populations is the detection of significant changes in trends in the bacterial content of waters. However, meaningful comparisons, between results for a particular sample or location, can only be made if the same method is used. Details of the method should be stated in the report. Heterotrophic bacteria counts at 37 °C are useful to assess the quality of relatively unpolluted groundwaters and can provide an early indication of more serious pollution. The significance of the heterotrophic bacterial populations in water treatment and supply are described elsewhere⁽³⁾ in this series.

A2 Scope

These methods are suitable for the testing of drinking waters, including samples from all stages of treatment and distribution, and source waters.

Users wishing to employ these methods should verify their performance under their own laboratory conditions⁽⁴⁾.

A3 Definitions

In the context of these methods, heterotrophic bacterial colony forming units comprise bacteria grown on an appropriate non-selective medium under the conditions specified. Some yeasts and moulds are capable of growing on the media outlined in this method. Whilst moulds are readily distinguished, certain yeasts may produce bacterial-like colonies, and as such, will be included in any colony count obtained.

A4 Principle

Pour plates are prepared by mixing test volumes of the water sample with molten yeast extract agar or R2A agar in Petri dishes. Following incubation under the conditions specified, the number of the colonies that develop is counted.

Spread plates are prepared by spreading test volumes of the water sample onto the surface of pre-dried yeast extract agar or R2A agar in Petri dishes. Following incubation under the conditions specified, the number of the colonies that develop is counted.

A5 Limitations

In the UK, the pour plate technique employing yeast extract agar is used for drinking water compliance monitoring. Yeast extract agar is a nutrient-rich medium and is known to support the growth of only a small percentage of heterotrophic bacteria present in water. For special or unusual investigations, such as consumer complaints relating to taste or odour, it may be advantageous to use R2A agar. The R2A medium is a low nutrient formulation that enhances the recovery of disinfectant-damaged organisms, and those organisms with a low nutrient requirement that are inhibited by the higher concentrations of nutrients present in yeast extract agar medium.

The spread plate technique may give higher recoveries than the pour plate technique, as organisms in the spread plate method are not heat-shocked by the addition of molten agar, as may occur in the pour plate method.

If estimations of total bacterial populations are needed these may be obtained by direct counting techniques using microscopic methods employing acridine orange⁽⁵⁾ or 4', 6-diamidino-2-phenylindole⁽⁶⁾. Respiring populations may be estimated using formazan-based substrates such as 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride⁽⁷⁾ or 5-cyano-2, 3-ditolyl-tetrazolium chloride⁽⁸⁾.

A6 Health and safety

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations⁽⁹⁾ and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere⁽⁴⁾ in this series.

A7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere⁽⁴⁾ in this series. Principally, incubators (fan assisted, static temperature) 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 a 1.8 % m/v solution of Na₂S₂O₃.5H₂O per 100 ml of sample, or equivalent).

A7.2 Incubators capable of maintaining temperatures of 22.0 ± 1.0 °C, and 30.0 ± 1.0 °C or 37.0 ± 1.0 °C.

A7.3 Sterile 1 ml pipettes (preferably plugged), or pipettor with sterile 1 ml pipette tips (preferably with filter protection).

A7.4 Colony counter, magnifying lens or image analysis colony counting system.

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 the method⁽⁴⁾. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality. Unless otherwise stated chemical constituents should be added as anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

A8.1 Yeast extract agar

Yeast extract	3 g
Peptone	5 g
Agar	12 g
Water	1 litre

Suspend the ingredients in the water and heat to dissolve. Dispense the medium (typically, 15 - 20 ml) into tubes capable of being capped, or in larger volumes (for example, 400 ml) into screw-capped 500 ml bottles. Sterilise the medium by autoclaving at 121 °C for 15 minutes. The final pH value of the medium after sterilisation should be 7.2 ± 0.2 . The medium may be stored at room temperature but should be used within 1 month. Alternatively, Petri dishes containing medium to be used in the spread plate technique may be stored at a temperature of 5 ± 3 °C for up to 1 month, if protected against dehydration. Deterioration of the medium may occur during storage and it is advisable that the performance of medium stored for 2 - 4 weeks should be verified.

A8.2 R2A agar

Yeast extract	500 mg
Proteose peptone No. 3 or polypeptone	500 mg
Casamino acids	500 mg
Glucose	500 mg
Soluble starch	500 mg
Dipotassium hydrogen phosphate	300 mg
Magnesium sulphate heptahydrate	50 mg
Sodium pyruvate	300 mg
Agar	12 g
Water	1 litre

Dissolve all the ingredients, except the agar, in the water. Adjust the pH value to 7.2 with solid dipotassium hydrogen phosphate or potassium dihydrogen phosphate. Add the agar and heat to dissolve. Dispense the medium (typically, 15 - 20 ml) into tubes capable of being capped, or in larger volumes (for example, 400 ml) into screw-capped 500 ml bottles. Sterilise by autoclaving at 121 °C for 15 minutes. The final pH value should be 7.2 ± 0.2 . The medium may be stored at room temperature but should be used within 1 month. Alternatively, Petri dishes containing medium to be used in the spread plate technique may be stored at a temperature of 5 ± 3 °C for up to 1 month, if protected against dehydration. Deterioration of the medium may occur during storage and it is advisable that the performance of medium stored for 2 - 4 weeks should be verified.

A8.3 Other media

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

A9 Analytical procedure

A9.1 Sample preparation

The volumes, and dilutions, of samples should be chosen (and typically, 1 ml of sample or sample dilution is used) so that the number of colonies to be counted on the plate lies, ideally, between 30 and 300. With some waters, it may be advantageous to plate out a series of dilutions of sample so that the number of colonies in any one of the Petri dishes from each incubation temperature is likely to fall within this range. For treated waters, inoculate 1 ml of the sample; for polluted waters prepare dilutions of the sample, using an appropriate diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) before plating out. The pipetting of very small volumes should be avoided due to increased volume errors associated with dispensing very small volumes, and the increased difficulty in ensuring even mixing in, or spreading on, the medium.

When testing samples of drinking water originally supplied in sealed bottles or other containers⁽¹⁰⁾, only one set of Petri dishes as described in sections A9.2 and A9.3 need be prepared.

A9.2 Pour plate sample processing

Heat the tube or bottle of medium and melt the medium. Once molten, the medium should be kept, but for no more than 4 hours, at 46 ± 2 °C until required. Any un-used medium should be discarded. The whole volume of agar should be at this temperature before use. For each incubation temperature, pipette 1 ml of undiluted sample into empty sterile Petri dishes. If dilutions have been prepared, pipette 1 ml of each dilution for each incubation temperature, starting with the highest dilution. If a water bath is used to keep the medium at 46 ± 2 °C remove the water from the outside of the container, so as to avoid contamination, prior to pouring the medium into the Petri dishes. Pour 15 - 20 ml of molten agar medium into each Petri dish within 20 minutes of dispensing the 1 ml sample volumes and cover the dishes with lids. Mix the sample and medium by rapid, but gentle, clockwise and anti-clockwise circular movements for approximately 10 seconds. Lift the lids slightly during mixing so that the agar does not adhere to the lids, and keep the Petri dishes flat on the bench. Allow the agar to solidify, invert the Petri dishes and incubate them in this position.

A positive control sample containing target bacteria and a blank control should be prepared for each incubation temperature with each batch of samples.

A9.3 Spread plate sample processing

If Petri dishes are to be prepared from agar medium contained in tubes or bottles, heat the tube or bottle of medium and melt the medium. Once molten, the medium should be kept, but for no more than 4 hours, at 46 ± 2 °C until required. Any un-used medium should be discarded. The whole volume of agar should be at this temperature before use. If a water bath is used to keep the medium at 46 ± 2 °C remove the water from the outside of the container, so as to avoid contamination, prior to pouring the medium into the Petri dishes. Pour 15 - 20 ml of molten agar medium into each Petri dish and allow the medium to solidify. If pre-prepared Petri dishes are used, allow the dish to equilibrate to room temperature. The dish should be dried of excess moisture before use. For each incubation temperature, pipette 0.1 ml of the sample, or diluted sample, onto the surface of pre-dried agar medium contained in each of the Petri dishes. 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 the inverted position.

A positive control sample containing target bacteria and a blank control should be prepared for each incubation temperature with each batch of samples.

A9.4 Incubation of plates

Generally, for yeast extract agar, incubate one of the sets of Petri dishes at 22 °C for 68 ± 4 hours, and the other set at 37 °C for 44 ± 4 hours. Petri dishes incubated at 37 °C may be examined after 21 ± 3 hours if necessary, for instance if an early indication of growth is required. When testing samples of drinking water originally supplied in bottles or other containers⁽¹⁰⁾ incubate only one set of Petri dishes at 37 °C for 44 ± 4 hours.

For R2A agar, incubate one set of Petri dishes at 22 °C for 5 - 7 days and the other set at 30 °C for 3 days.

A9.5 Reading of results

For each temperature, count the colonies on the Petri dish inoculated with the undiluted sample. A colony counter with magnifying glass will facilitate counting. Typically, drinking water will often exhibit counts of less than 30 from the undiluted sample, whilst untreated water samples may have counts that are considerably greater. If the count is greater than 300, and dilutions have been prepared, then count the colonies from a Petri dish showing between 30 and 300 colonies. If all Petri dishes show more than 300 colonies, record the result as greater than 300 at the highest dilution. If the number of colonies exceeds 300, and an attempt is made to count all colonies present, the report of the count should be accompanied with a statement that the count is only an estimate, and as such, may not reflect the true count.

Spreading colonies can hinder the count, and the following guidance should be used as a basis for counting. If situations such as

- (i) a chain of colonies that appears to be caused by the disintegration of a single clump of organisms; or
- (ii) a spreading growth that develops as a film at the bottom of the Petri dish; or
- (iii) a colony that forms in a film of water at the edge of, or over the surface of the agar

occur, then these should be counted as a single colony forming unit.

If the colonies cannot be counted immediately after being removed from the incubator, then the Petri dishes should be kept at 5 ± 3 °C for no longer than 24 hours.

A10 Calculations

Calculate the number of colonies per millilitre of sample. Divide the count by the actual volume (in millilitres) of sample pipetted into, or spread onto, the medium, taking into account any dilution carried out.

A11 Expression of results

The count is quoted in terms of the number of colony forming units per millilitre. Counts greater than 300 per Petri dish are recorded as greater than 300 colony forming units per millilitre. Counts are increased *pro rata* if dilutions are prepared and used. If the number of colonies exceeds 300, and an attempt is made to count all colonies present, the report of the count should be accompanied with a statement that the count is only an estimate, and as such, may not reflect the true count. The term “too numerous to count” should not be used without further qualification.

A12 Quality assurance

The performance of new batches of the culture media (YEA or R2A) should be checked with regard to the recovery of target organisms and the physical properties (for example pH and sterility). Quantitative procedures for evaluation of recovery are described elsewhere⁽¹¹⁾. Laboratories should use reference strains of micro-organisms obtained directly from a recognised national or international collection, where these exist, for establishing acceptable performance of the media. Petri dishes should be incubated for 24 hours at 22 °C, and

30 °C or 37 °C as appropriate. Alternatively, aliquots of a raw water, of known microbial quality, may be used. Further details are given elsewhere⁽⁴⁾ in this series.

A13 References

1. The Examination of Waters and Water Supplies, 7th Edition. Churchill Ltd., London. E Windle Taylor, 1958.
2. A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology*, D J Reasoner and E E Geldreich, 1985, **49**, pp1-7.
3. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
4. 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.
5. Use of Nucleopore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology*, J E Hobbie, R J Daley and S Jasper, 1977, **33**, pp1225-1228.
6. The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography*, K G Porter and Y S Feig, 1980, **25**, pp943-948.
7. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. *Applied and Environmental Microbiology*, R Zimmermann, R Iturriaga and J Becker-Birck, 1978, **36**, pp926-935.
8. Use of a fluorescent redox probe for direct visualisation of actively respiring bacteria. *Applied and Environmental Microbiology*, G G Rodriguez, D Phipps, K Ishiguro and H F Ridgway, 1992, **58**, pp1801-1808.
9. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.
10. European Union (1998) Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. *Official Journal of the European Communities*, 5.12.98, L 330/32-L 330/53.
11. DD CEN ISO/TS 11133-2:2003, Microbiology of food and animal feeding stuffs – Guidelines on preparation and production of culture media - Part 2: Practical guidelines on performance testing of culture media, British Standards Institution.

B The enumeration of heterotrophic bacteria by a multiple substrate most probable number technique

Details of this method are included for information purposes only as an illustrative example of the type of products available. Information on the routine use of this method would be welcomed to assess its full capabilities. It has not been subjected to widespread use or verification of its performance. Data, from a multi-laboratory comparison study organised under the auspices of the Standing Committee of Analysts compared the results generated using this method with those obtained using the YEA pour plate method, and are given in Appendix 1. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance.

B1 Introduction

The count of heterotrophic bacteria may be enumerated by a multiple substrate most probable number (MPN) procedure, typically incubated at 22 °C and 37 °C. In some cases, incubation at 30 °C can be undertaken. The most useful application of the estimation of heterotrophic bacterial populations is the detection of significant changes in trends in the bacterial content of waters. However, comparisons, between results for a particular sample, can only be made if the same method is used. Details of the method should be stated in the report. Heterotrophic bacteria counts at 37 °C are useful to assess the quality of relatively unpolluted groundwaters and can provide an early indication of more serious pollution. The significance of the heterotrophic bacterial populations in water treatment and supply are described elsewhere⁽¹⁾ in this series.

B2 Scope

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

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

B3 Definitions

In the context of this method, heterotrophic bacteria comprise bacteria that express one or more of a suite of selected enzymes (for example, including glycosidases and phosphatases) under the conditions specified. Some yeasts and moulds may be capable of expressing the target enzymes, and as such, would be included in any MPN count.

B4 Principle

Organisms are grown in a defined fluorogenic multi-enzyme substrate medium in, for example, a 50-well reaction module. A fixed aliquot of sample (for example, 4 ml) is added to the module, of which a defined volume (for example, 0.5 ml) is equally dispersed via capillary channels into 50 reagent-coated reaction wells. When the module lid is fitted the excess sample remaining is then absorbed (by absorbent material attached to the lid). After incubation at the selected temperature, the reaction chamber is examined under UV light and the number of wells showing blue-white fluorescence counted. From this count, an MPN is derived (see Table B1).

B5 Limitations

The method is suitable for most types of water. Waters with high turbidities, however, may impede capillary action, which is an essential feature of this specific product necessary for the distribution of the water sample into the reaction wells.

B6 Health and safety

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations⁽³⁾ and appropriate risk assessments should be made before adopting these methods. 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 performance criteria outlined elsewhere⁽²⁾ in this series. Principally, incubators (fan assisted, static temperature) 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 Na₂S₂O₃.5H₂O per 100 ml of sample, or equivalent).

B7.2 Incubators capable of maintaining temperatures of 22.0 ± 1.0 °C, and 30.0 ± 1.0 °C or 37.0 ± 1.0 °C.

B7.3 Sterile pipettes (preferably plugged) capable of delivering 4 ml, or pipettor with appropriate sized sterile pipette tips (preferably with filter protection).

B7.4 MPN reaction modules as supplied by the manufacturer (for example, a 50-well system).

B7.5 Ultra-violet (365-366 nm) lamp and viewer.

B8 Media and reagents

Alternative formulations of the test system may become available, and may possess variations to their formulation. The performance of all media and reagents should be verified prior to their use in the method. Variations in the preparation and storage should also be verified. Water, where applicable, should be distilled, deionised or of similar quality. Unless otherwise stated chemical constituents, where applicable, should be added as anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

B8.1 *Quanti-Disc*^{TM(4)}

This is an example of a commercially available product with 50 substrate-coated reaction wells. The reaction wells contain a selection of minimal nutrients and substrates selected for the detection of a range of enzymes widely expressed by water associated heterotrophic bacteria.

B8.2 *Other media*

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

B9 Analytical procedure

B9.1 *Sample preparation*

For waters where an MPN above 100 is expected then dilutions may need to be prepared. The dilutions inoculated should be chosen so that the number of wells showing blue-white fluorescence to be counted in the module lies, if possible, between 5 and 45 (equivalent to a MPN of 11 and 230 respectively, see Table B1). With some waters, it may be advantageous to prepare dilutions of sample for inoculating a series of modules so that the number of wells from any one of the modules from each incubation temperature is likely to fall within this range. For treated waters, inoculate 4 ml of the sample (or 4 ml of a diluted sample). For polluted waters, dilute the sample with an appropriate diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) before inoculating modules.

When testing samples of drinking water originally supplied in sealed bottles or other containers⁽⁵⁾, only one set of modules as described in section B9.2 need be prepared.

B9.2 *Sample processing*

For each incubation temperature, starting with the highest dilution, pipette 4 ml of each of the diluted samples, if prepared, and 4 ml of the original sample, into separate MPN reaction modules. Remove the lid of the module and pipette the aliquot of sample into the centre of the reaction module, so as to cover the central disc area completely. This can be achieved by dispensing in a circular motion onto the central area. Hold the pipette or pipettor vertically during delivery of the sample. After dispensing the sample, leave the module on a level surface for at least three seconds before replacing the lid. This allows the sample to be drawn into the reaction wells prior to the excess sample being taken up by an absorbent block fitted in the module lid.

A positive control sample containing target bacteria and a blank control should be prepared for each incubation temperature with each batch of samples.

B9.3 *Incubation of plates*

Incubate one set of reaction modules at 22 °C for 68 ± 4 hours and the other set at 37 °C for 44 ± 4 hours. Do not invert the reaction modules during incubation. Modules incubated at 37 °C may be examined after 21 ± 3 hours if necessary, for instance if an early indication of bacterial numbers is required. When testing samples of drinking water supplied in bottles or other containers⁽⁵⁾ incubate only one set of reaction module at 37 °C for 44 ± 4 hours.

B9.4 *Reading of results*

Examine each module with an ultra violet lamp. The lamp should be placed approximately 150 - 200 mm from the module. Count the number of wells showing blue-white fluorescence (see Figure 1) in the module containing the undiluted sample. If all the wells show blue-white fluorescence then the number of wells showing blue-white fluorescence in the module containing a diluted sample should be counted and this should be between 5 and 45.

If all modules show blue-white fluorescing wells, record the result as being greater than 50 at the highest dilution.

B10 Calculations

The MPN of heterotrophic bacteria (colony count bacteria) is determined by reference to appropriate tables, see for example Table B1. The MPN is derived from the number of wells showing blue-white fluorescence. For example, if there are 35 wells showing blue-white fluorescence in the reaction module (as in Figure 1), then from Table B1, the MPN of heterotrophic (colony count) bacteria is 120 per millilitre of sample, or diluted sample, examined. Any dilutions need to be taken into account in calculating the final result.

Figure 1 Example of a 50-well reaction module for the enumeration of heterotrophic plate count bacteria with 35 wells showing blue-white fluorescence



B11 Expression of results

The result is quoted in terms of the MPN per millilitre. An MPN of greater than 391 per reaction module is recorded as greater than 391 MPN per millilitre. Numbers are increased *pro rata* if dilutions are prepared and used.

B12 Quality assurance

The performance of new batches of reaction modules should be checked with regard to the recovery of target organisms. Laboratories should use reference strains of micro-organisms obtained directly from a recognised national or international collection, where these exist, for establishing acceptable performance of the media. Reaction modules should be incubated for 24 hours at 22 °C, and 30 °C or 37 °C as appropriate. Alternatively, aliquots of a raw water, of known microbial quality, may be used. Further details are given elsewhere⁽²⁾ in this series.

B13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *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.
4. IDEXX Laboratories, Newmarket Business Park, Studlands Park Avenue, Newmarket, Suffolk, CB8 7ER, UK.
5. European Union (1998) Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. *Official Journal of the European Communities*, 5.12.98, L 330/32-L 330/53.

Table B1 MPN (and 95 % confidence intervals) per millilitre for a 50-well multiple substrate reaction module

Wells showing blue-white fluorescence	MPN per ml	95 % Confidence Limits		Wells showing blue-white fluorescence	MPN per ml	95 % Confidence Limits	
		Lower	Upper			Lower	Upper
0	< 2	0	< 14	26	73	50	110
1	2	0	14	27	78	53	110
2	4	1	16	28	82	56	120
3	6	2	19	29	87	60	130
4	8	3	22	30	92	63	130
5	11	4	25	31	97	67	140
6	13	6	28	32	102	71	150
7	15	7	32	33	108	75	150
8	17	9	35	34	114	80	160
9	20	10	38	35	120	85	170
10	22	12	42	36	127	90	180
11	25	14	45	37	135	95	190
12	27	16	48	38	143	100	200
13	30	17	52	39	151	110	210
14	33	19	56	40	161	110	230
15	36	21	59	41	172	120	240
16	39	24	63	42	183	130	260
17	42	26	67	43	197	140	280
18	45	28	71	44	212	150	300
19	48	30	75	45	230	160	330
20	51	33	80	46	253	170	370
21	55	35	84	47	281	190	420
22	58	38	89	48	322	210	490
23	62	41	93	49	391	240	640
24	65	44	98	50	> 391	> 240	> 640
25	70	46	100				

Appendix 1 Comparative evaluation of a 50-well multi-substrate reaction module method (Quanti-Disc™) with the yeast extract agar pour plate method for the enumeration of heterotrophic bacteria

1 Introduction

The Quanti-Disc™ method may be used for the detection and enumeration of heterotrophic bacteria in water. The medium, which is pre-deposited into a 50-well reaction module, contains multiple fluorogenic enzyme substrates that detect viable bacteria by testing enzyme activities expressed by these organisms. The substrates fluoresce when metabolised by the target bacteria. The sample, or diluted sample, is added to a Quanti-Disc module, incubated and all wells examined for those that show blue-white fluorescence. From appropriate tables, the number of wells showing blue-white fluorescence corresponds to a Most Probable Number (MPN) of total bacteria in the original sample. The Quanti-Disc method has been shown to yield similar results at 37 °C and higher results at 22 °C to those obtained using a yeast extract agar pour plate procedure⁽¹⁾.

This appendix summarises a comparative evaluation between the Quanti-Disc method and the yeast extract agar pour plate method⁽¹⁾ for the quantification of heterotrophic bacterial populations in drinking water and similar water samples. Data analyses were performed according to the principles outlined in MoDW (2002) - Part 3⁽²⁾ and ISO 17994:2004⁽³⁾. Reference MoDW (2002) - Part 3⁽²⁾ was used as stage 1 analysis of data from each participating laboratory. Reference ISO 17994:2004⁽³⁾ was used to assess the comparability between the trial method (i.e. the Quanti-Disc method) and the reference method (i.e. MoDW (2002) - Part 7- Methods for the enumeration of heterotrophic bacteria⁽¹⁾).

2 Definitions

The two methods for the enumeration of heterotrophic plate count organisms are based on different detection systems. In this context, plate count organisms enumerated by the yeast extract agar pour plate method are defined as those micro-organisms that grow in yeast extract agar at the selected temperatures and times to become colonies of sufficient size to be counted. The test, therefore, relies on the ability of viable target organisms to grow in the specified medium. In contrast, plate count organisms enumerated by the Quanti-Disc method are defined as those that express one or more specified target enzymes at the selected temperatures and times. This test, therefore, relies on the expression of enzymes by viable target organisms.

3 Methodology

Seven geographically diverse laboratories, comprising five water utility laboratories and two public health laboratories, participated in this trial. A training session using the trial protocol was conducted at each laboratory and was overseen by recognised international experts. A number of water samples (totalling 1953) typical of those normally tested by each laboratory was analysed. These waters comprised natural samples predominately from chlorinated supplies, including samples taken for regulatory and operational monitoring of mains drinking water, potable water tanks, drinking water fountains, tap water in commercial buildings and food manufacturers, and chlorinated drinking water from ships. A small number of samples (less than 10 %) originated from unchlorinated sources such as bore-holes and wells, raw water from surface water sources, mineral and other bottled water, and unchlorinated water from hospital facilities (for example dialysis and endoscopy units).

Aliquots of each water sample were tested (in duplicate) by the Quanti-Disc method (i.e. the trial method) and the yeast extract agar pour plate method (i.e. the reference method). For each method, one aliquot was incubated at 37 °C for 44 ± 4 hours and the other aliquot was incubated at 22 °C for 68 ± 4 hours⁽¹⁾.

The trial method was carried out according to the manufacturer's instructions, and the corresponding MPN obtained from an MPN Table. The reference yeast extract agar method was carried out according to MoDW (2002) - Part 7 - Methods for the enumeration of heterotrophic bacteria⁽¹⁾. The yeast extract agar pour plate results were obtained by counting the colonies on each Petri dish using a Quebec dark field colony counter, or similar apparatus. Positive controls with known strains of bacteria and negative controls with sterile water were tested using both methods throughout the trial in accordance with recognised procedures^(1, 2).

When both of the methods yielded counts of zero, (i.e. in the reference method no colony growth was observed, and in the trial method no wells exhibited blue-white fluorescence) the results from both methods were excluded from the following statistical analyses. In addition, when the results for one or both methods exceeded the counting range for that method, results from both methods were excluded from the statistical analyses. The counting range for the Quanti-Disc method was deemed to have been exceeded when a MPN of greater than 391 was recorded. The counting range for the yeast extract agar method was deemed to have been exceeded when a count was recorded as "too numerous to count". However, for the yeast extract agar method, samples yielding counts of greater than 300 cfu were estimated⁽¹⁾. These estimated results were rounded to the nearest ten and, although exceeding the ideal counting range for the yeast extract agar pour plate method (i.e. 300 cfu/ml), these estimated counts were not excluded in the following statistical data analyses. However, these situations only arose in 3 out of the 1953 results. MPN counts from Quanti-Discs were reported as whole integers.

4 Results

A combined total of 1953 water samples was analysed, of which 633 results were collated at 37 °C and 758 results were collated at 22 °C. The remaining samples (i.e. 562) gave zero counts by both methods. The analysable results are summarised (by laboratory) in Table 1.

Table 1 Numbers of samples with suitable data for analysis

Laboratory	37 °C			22 °C		
	Chlorinated	Unchlorinated	All	Chlorinated	Unchlorinated	All
1	84	0	84	135	0	135
2	167	7	174	136	5	141
3	103	24	127	108	21	129
4	110	0	110	215	0	215
5	22	32	54	16	18	34
6	59	0	59	66	0	66
7	25	0	25	38	0	38
	570	63	633	714	44	758

4.1 Statistical evaluation – preliminary statistical analyses

4.1.1 Test for normality

The analysable results from all the laboratories are presented in Figure 1 as scatter plots for the combined \log_{10} -transformed data. The data were tested for normality using the Kolmogorov-Smirnov test on both untransformed and \log_{10} -transformed data. Neither sets of data for either the 37 °C nor the 22 °C comparisons were normally distributed (in all cases $p < 0.01$), although the \log_{10} -transformed data appears more closely related to a normal distribution than the untransformed data (Figures 2 and 3). This is to be expected as occasional observations display variations much greater than random (Poisson) variation^(2, 4). Consequently, non-parametric statistics were applied for the preliminary analysis of the data⁽²⁾.

Figure 1 Scatter plots of \log_{10} -transformed paired difference counts at 37 °C and 22 °C from all water samples

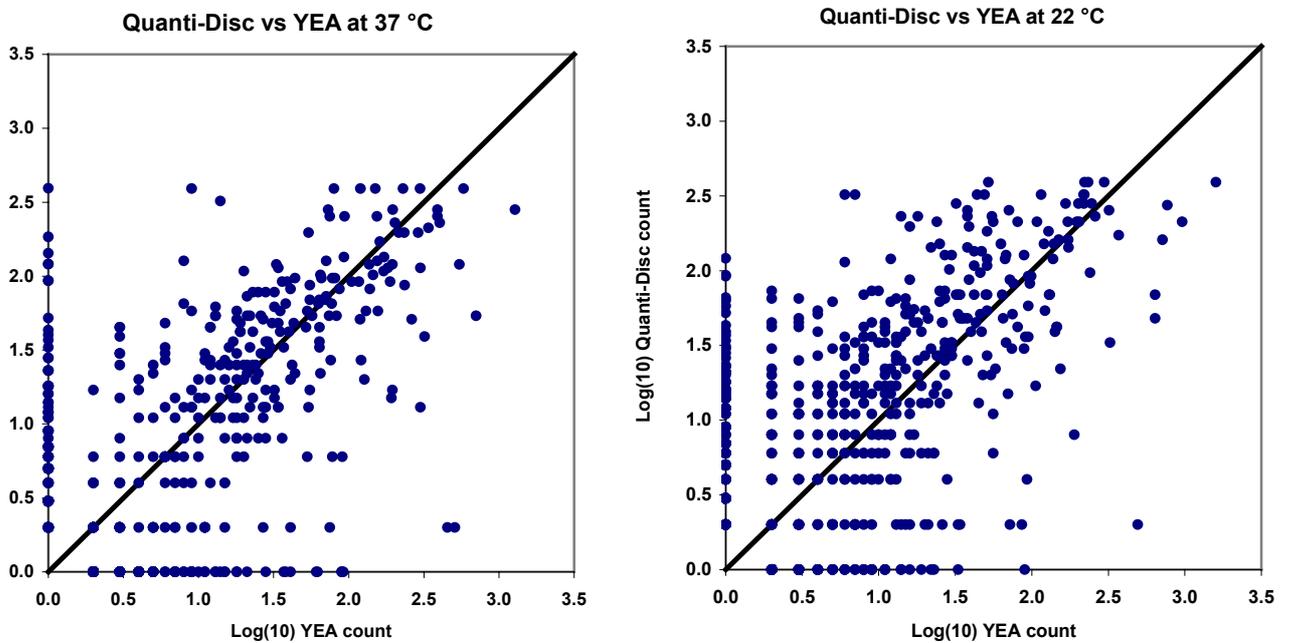


Figure 2 Kolmogorov-Smirnov normality test of untransformed and \log_{10} -transformed paired difference counts at 37 °C from all water samples

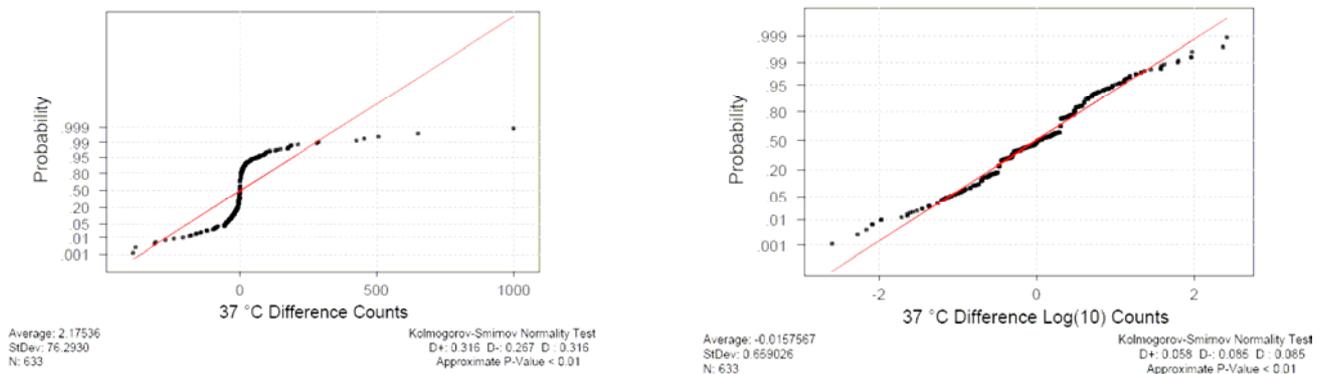
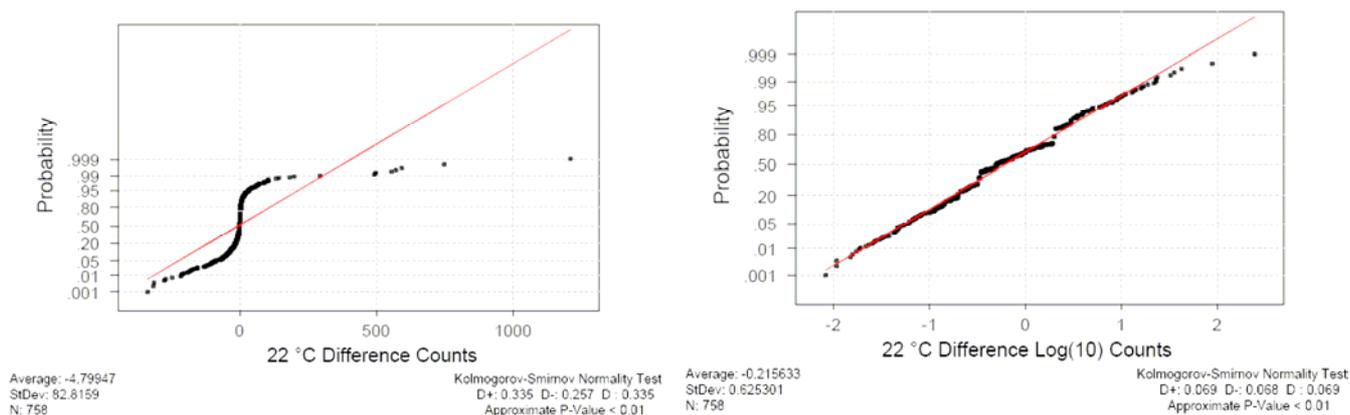


Figure 3 Kolmogorov-Smirnov normality test of untransformed and \log_{10} -transformed paired difference counts at 22 °C from all water samples



The paired differences were derived for each temperature. For the 37 °C data the number of pairs in which the Quanti-Disc MPN value was higher (i.e. 297) was similar to the number of pairs in which the yeast extract agar count was higher (i.e. 316), with a median difference of zero and a range of -999 to +382. For the 22 °C data, however, the number of pairs in which the Quanti-Disc MPN value was higher (i.e. 473) was markedly greater than the number of pairs in which the yeast extract agar count was higher (i.e. 268), with a median difference of +2.0 and a range of -1209 to +339.

4.1.2 Non-parametric analysis of data from each laboratory

The non-parametric statistics for both untransformed and \log_{10} -transformed data for each laboratory are presented in Tables 2 and 3. The analysis of the 37 °C data (Table 2) shows that for three of the laboratories (laboratories 2, 4 and 6) there is no significant difference between the two methods. Also two laboratories (laboratories 1 and 5) record higher counts with Quanti-Disc significantly more often, whilst the remaining two laboratories (laboratories 3 and 7) recorded higher counts with yeast extract agar significantly more often. The differences between the laboratories are not sufficient to merit exclusion of any of the data. The null hypothesis, that the median difference for all the data is zero, is accepted by the Wilcoxon signed rank test. The median difference (Quanti-Disc – yeast extract agar) for the untransformed data is +0.50 with 95 % confidence interval of -0.50 to +1.00 and for the \log_{10} -transformed data the median difference is zero with 95 % confidence interval of -0.06 to +0.04.

Table 2 Non-parametric statistics of untransformed and log₁₀-transformed paired sample results where the trial method gave lower, equal or higher plate counts at 37 °C than the reference method for the seven participating laboratories

Lab	Quanti-Disc			n	p*	Median difference Untransformed (95 % confidence interval)	Median difference Log ₁₀ -transformed (95 % confidence interval)
	lower	equal	higher				
1	20	2	62	84	< 0.001	2.00 (1.57 to 2.00)	0.48 (0.30 to 0.48)
2	84	4	86	174	0.939	0.00 (-1.00 to 1.39)	0.00 (-0.15 to 0.15)
3	84	7	36	127	< 0.001	-2.00 (-2.04 to -1.00)	-0.30 (-0.30 to -0.18)
4	57	2	51	110	0.630	-1.00 (-1.00 to 2.00)	-0.06 (-0.30 to 0.12)
5	18	2	34	54	0.038	7.50 (0.36 to 17.86)	0.14 (0.01 to 0.25)
6	34	0	25	59	0.298	-1.00 (-2.00 to 2.92)	-0.30 (-0.30 to 0.22)
7	19	3	3	25	0.001	-1.00 (-2.60 to -1.00)	-0.30 (-0.30 to -0.30)
	316	20	297	633			

* binomial probability, parameter p = 0.05

The analysis of the 22 °C data (Table 3) shows that for four of the laboratories (laboratories 2, 3, 5 and 7) there is no significant difference between the two methods. However, for two of these laboratories (laboratories 2 and 3) there is a tendency for higher counts to be recorded more frequently by Quanti-Disc. The other three laboratories (laboratories 1, 4 and 6) record higher counts with Quanti-Disc significantly more often. The differences between the laboratories are not sufficient to merit exclusion of any of the data. The null hypothesis, that the median difference for all the data is zero, is not accepted by the Wilcoxon signed rank test. The median difference for the untransformed data is +3.50 with 95 % confidence interval of +2.50 to +4.50 and for the log₁₀-transformed data, the median difference is +0.22 with 95 % confidence interval of +0.17 to +0.27.

Table 3 Non-parametric statistics of untransformed and log₁₀-transformed paired sample results where the trial method gave lower, equal or higher plate counts at 22 °C than the reference method for the seven participating laboratories

Lab	Quanti-Disc			n	p*	Median difference Untransformed (95 % confidence interval)	Median difference Log ₁₀ -transformed (95 % confidence interval)
	lower	equal	higher				
1	29	2	104	135	< 0.001	4.00 (2.00 to 4.00)	0.48 (0.48 to 0.56)
2	59	5	77	141	0.145	2.00 (-0.60 to 4.60)	0.13 (-0.01 to 0.28)
3	57	1	71	129	0.251	2.00 (-1.00 to 2.00)	0.04 (-0.04 to 0.20)
4	66	4	145	215	< 0.001	2.00 (2.00 to 2.00)	0.32 (0.26 to 0.48)
5	18	0	16	34	0.864	-1.00 (-3.00 to 5.37)	-0.04 (-0.28 to 0.15)
6	18	3	45	66	0.001	12.0 (3.20 to 22.00)	0.64 (0.30 to 0.85)
7	21	2	15	38	0.404	1.00 (-1.42 to 1.00)	-0.10 (-0.30 to 0.18)
All	268	17	473	758			

* binomial probability, parameter p = 0.05

4.1.3 Non-parametric analysis of data from chlorinated and unchlorinated water samples

Non-parametric statistics were applied to both untransformed and log₁₀-transformed data for combined samples (from all laboratories) that were from chlorinated and unchlorinated

sources (Table 4). The data set for the unchlorinated samples, however, is very small. The analysis of the 37 °C data shows that for both types of water there is no significant difference between the two methods. For the 22 °C data higher counts were recorded with Quanti-Disc significantly more often from chlorinated water samples, but there was no significant difference between the two methods for the unchlorinated water samples. The differences between the water types are not sufficient to merit exclusion of the data from unchlorinated samples.

Table 4 Non-parametric statistics of untransformed and log₁₀-transformed paired sample results where the trial method gave lower, equal or higher plate counts than the reference method for chlorinated and unchlorinated water samples at 37 °C and 22 °C

Type	Quanti-Disc			n	p*	Median difference untransformed (95 % confidence interval)	Median difference log ₁₀ -transformed (95 % confidence interval)
	lower	equal	higher				
37 °C							
Chl [†]	282	17	271	570	0.671	0.00 (-1.00 to 1.00)	0.00 (-0.14 to 0.04)
Unchl	34	3	26	63	0.366	-1.00 (-4.00 to 1.00)	-0.04 (-0.22 to 0.05)
All	316	20	297	633			
22 °C							
Chl	247	16	451	714	< 0.001	2.00 (2.00 to 2.00)	0.30 (0.27 to 0.37)
Unchl	21	1	22	44	1.000	0.50 (-2.00 to 3.00)	0.01 (-0.18 to 0.13)
All	268	17	473	758			

[†] Chl = chlorinated water samples, Unchl = unchlorinated water samples

* binomial probability, parameter p = 0.05

4.2 Statistical evaluation – ISO 17994:2004 analysis

The combined paired results data from all of the participating laboratories for each incubation condition were compared using the mean relative difference procedure of ISO 17994:2004⁽³⁾. Since the objective of the study was to compare a trial method with an established reference method in terms of being “at least as reliable”, it is considered that the “one-sided” comparison according to ISO 17994:2004 is appropriate. In a “one-sided” comparison, the lower D value only is set^(2, 3) and this value was -10. The results of the mean relative difference analyses are summarised in Table 5.

Table 5 Mean relative difference analysis (trial method – reference method) of paired sample results from the trial method (Quanti-Disc) and the reference method (yeast extract agar pour plate) for water samples analysed at 37 °C and 22 °C

	37 °C for 44 ± 4 hours (n = 633)	22 °C for 68 ± 4 hours (n = 758)
Mean relative difference	3.8	49.6
Standard deviation	151.8	144.0
x_L lower confidence interval of the expanded uncertainty around the mean	- 8.3	39.1
x_H higher confidence interval of the expanded uncertainty around the mean	15.8	60.0
Outcome	Not different	Different – higher recovery by trial method

For the 37 °C data set, the trial method (Quanti-Disc) and the reference method (yeast extract agar pour plate) are “not different” with the lower and higher confidence intervals of the expanded uncertainty around the mean (x_L and x_H) being -8.3 and +15.8, respectively. In contrast, for the 22 °C data set, the expanded uncertainty intervals, x_L and x_H , were both greater than zero (39.1 and 60.0, respectively), with the trial method (Quanti-Disc) giving significantly higher results.

This analysis is based upon data sets of which 90 % or more of the samples were derived from water sources that were subject to chlorination. The data demonstrate that the Quanti-Disc method gave similar results to the yeast extract agar pour plate method for the analysis of plate count bacteria from chlorinated waters at 37 °C. In addition, the data also demonstrate that the Quanti-Disc method yielded higher results than the yeast extract agar pour plate method for the analysis at 22 °C. Although the non-parametric analysis indicates similar performances at both temperatures for unchlorinated waters, the data set is too small to merit separate analysis by mean relative difference statistics.

5 Conclusions

This inter-laboratory study compared the Quanti-Disc™ method against the yeast extract agar pour plate method⁽¹⁾ for the enumeration of heterotrophic plate count bacteria from waters. Seven laboratories in the United Kingdom participated in the study, analysing a range of waters, predominantly samples from water sources that had been subjected to chlorination.

Non-parametric statistical analyses of the trial results showed that between the laboratories there were no major differences in the performance of the Quanti-Disc method compared to the yeast extract agar pour plate method. This is despite the fact that the two methods are based on different principles for the enumeration of heterotrophic bacteria in water samples. The same was also found when the data were split between samples sourced from chlorinated waters and those from unchlorinated sources, although the data set for the unchlorinated samples is small.

For this inter-laboratory study, the mean relative difference analyses using statistical procedures described in ISO 17994:2004 indicate that when the Quanti-Disc method for the

analysis of heterotrophic bacteria plate count at 37 °C is compared to the yeast extract agar pour plate reference method, similar results are produced. For heterotrophic bacteria plate counts at 22 °C, the Quanti-Disc method yielded higher results significantly more often when compared to the yeast extract agar pour plate reference method.

6 References

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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. ISO 17994:2004 - Water Quality - Criteria for establishing equivalence between microbiological methods. Geneva: International Organization for Standardization.
4. ISO/TR 13843:2000(E) - Water Quality - Guidance on validation of microbiological methods. Geneva: International Organization for Standardization.

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Severn Trent Laboratories (Coventry)
Severn Trent Laboratories (Runcorn)

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