ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2012) - Part 7 – Methods for the enumeration of heterotrophic bacteria

Methods for the Examination of Waters and Associated Materials

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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 2007.

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 that are 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.

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

The Microbiology of Drinking Water (2002)
Part 1 - Water quality and public health
Part 3 - Practices and procedures for laboratories
Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment.

The Microbiology of Drinking Water (2004)
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 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

The Microbiology of Drinking Water (2009)
Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)

The Microbiology of Drinking Water (2010)
Part 2 - Practices and procedures for sampling
Part 5 - The isolation and enumeration of enterococci by membrane filtration
Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

Contents

Methods for the enumeration of heterotrophic bacteria

A   The enumeration of heterotrophic bacteria by pour and spread plate techniques   7

B   The enumeration of heterotrophic bacteria by a multiple substrate most probable number technique   15

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. Users should ensure they are aware of the most recent version they seek.

Mark Gale
Secretary
March 2012

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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 is usually enumerated by pour or spread plate techniques using yeast extract agar[1]. Petri dishes are typically incubated at 22 °C and 37 °C. A low nutrient medium, for example R2A agar[2], may be more suitable for certain types of investigation and 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.

If required estimations of total bacterial populations by direct counting techniques may be obtained using microscopic methods employing acridine orange[4] or 4', 6-diamidino-2-phenylindole[5]. Respiring populations may be estimated using formazan-based substrates such as 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride[6] or 5-cyano-2, 3-ditolyl-tetrazolium chloride[7]. These approaches are not considered in this document.

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[8].

A3 Definitions

In the context of these methods, heterotrophic bacterial colony forming units comprise bacteria grown on the chosen medium under the conditions specified. Some yeasts and moulds are capable of growing on media used for heterotrophic plate counts. Whilst moulds are readily distinguished, and should not be included in the count, certain yeasts may produce bacteria-like colonies, and as such, would be included in the colony count obtained.

A4 Principle

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

There is some evidence to suggest that in the pour plate technique the addition of molten agar to the sample may cause heat stress to organisms[9], due to the temperature of the molten agar, and thereby affect the count subsequently obtained. Since this is one of several factors that may influence the growth of heterotrophic colony bacteria, this should be taken into account when counts obtained by the spread plate and pour plate techniques are compared.

A6   Health and safety

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations[10] and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[8] in this series.

A7   Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[8] 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 a sufficient quantity of a suitable de-chlorinating agent, for example a solution of sodium thiosulphate pentahydrate. This can be used 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).

A7.2   Incubators capable of maintaining temperatures of 22.0 ± 1.0 °C and 37.0 ± 1.0 °C. For some applications 30.0 ± 1.0 °C may be preferred.

A7.3   Sterile 1 ml pipettes, or pipettor with sterile 1 ml pipette tips.

A7.4   Waterbath or incubator capable of maintaining a temperature of 45.0 ± 1.0 °C for holding tubes or bottles of agar in a molten state ready for use. Alternatively, a media preparator or agarclave may be used.

A7.5   Automatic agar plate pouring equipment, including those with an integrated dish mixer, may be used. These should be validated to demonstrate control of cross contamination, volume dispensed and mixing and be regularly calibrated and checked. Equipment having an internal UV lamp is not recommended as this can adversely affect samples dispensed into the Petri dishes. UV lamps should be switched off or removed for this application.

A7.6   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[8]. 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 large volumes (for example, 400 ml) into screw-capped 500 ml bottles. Sterilise the medium by autoclaving at 121 ± 3°C for 15 minutes. The final pH value of the medium after sterilisation should be 7.2 ± 0.2. The medium may be used freshly prepared after equilibrating the temperature to 45.0 ± 1.0 °C, or stored at room temperature and used within 1 month. Alternatively, Petri dishes containing medium to be used in the spread plate technique should preferably be used as fresh as possible but may be stored at a temperature of 5 ± 3 °C for up to 1 month, if protected against dehydration.

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 ± 3 °C for 15 minutes. The final pH value should be 7.2 ± 0.2. The medium may be used freshly prepared after equilibrating the temperature to 45.0 ± 1.0 °C or stored at room temperature and used within 1 month. Alternatively, Petri dishes containing medium to be used in the spread plate technique should preferably be used as fresh as possible but may be stored at a temperature of 5 ± 3 °C for up to 1 month, if protected against dehydration.

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*

Samples should be adequately mixed before testing. 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, if possible, between 10 and 300[11]. With some waters, it may be advisable to use a series of dilutions of sample so that the number of colonies on any one of the plates from each incubation temperature is likely to fall within this range. For treated waters, inoculate 1 ml of the sample: for polluted waters prepare a series of dilutions of the sample with an appropriate sterile diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) before plating out. Alternatively, when using spread plates, it may be advantageous to use a smaller volume, usually 0.1 ml, of sample or diluted sample.

The precision and accuracy of dispensing the sample or dilution of sample can be markedly affected by the pipetting technique used. Pipettes and pipettors should be suitable for the volumes to be dispensed. Attention should be paid to detail, such as the angle of the pipette and its depth in the sample, as well as to avoiding contamination of pipettors and other samples.

In the UK two Petri-dishes as described in sections A9.2 and A9.3 are required for all drinking water samples including those taken to monitor water supplied in bottles by a water undertaker as an alternative supply[12]. This differs from the requirement of the European Directive for which two plates are only a requirement for water in sealed bottles or other containers[13] intended for sale and tested at the point of filling, otherwise only a plate at 22°C is required.

A9.2 *Pour plate sample processing*

The method can be performed using freshly prepared molten medium or by heating a tube or bottle of previously sterilised medium until it is molten throughout. Once molten, the medium should be equilibrated to 45 ± 1 °C and may then be kept until required, preferably for no more than 4 hours[11] from the time the agar reaches 45 ± 1 °C. Molten agar stored for more than four hours at 45 ± 1 °C may still enable satisfactory growth to occur, and hence suitable counts to be made, and may be appropriate for the examination of samples received outside of normal working hours. The routine use of molten agar stored for more than 4 hours should be validated in the laboratory.

In duplicate, starting with the most dilute sample solution, pipette 1 ml of each of the diluted samples, if prepared, and 1 ml of the original sample, into separate, empty sterile Petri dishes. If a water bath is used to keep the medium at 45 ± 1 °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. This may be done manually, by 'hand plate pouring', or using plate pouring equipment which may include an

integrated Petri dish mixer. Typically this equipment uses a peristaltic pump in conjunction with sterilised tubing. Dispensed volume checks should be made for each tubing-set as tubing may vary.

For hand poured plates 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 lid, and keep the Petri dishes flat on the bench. Allow the agar to solidify and incubate in an inverted position.

Alternatively, if using plate pouring equipment with an integrated mixer, mix for the time determined during validation to obtain evenly distributed colonies. The plate pouring equipment may include a 'cold plate' to aid agar solidification.

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 may be kept at 45 ± 1 °C (until required) preferably for no more than 4 hours[11]. If a water bath is used to keep the medium at 45 ± 1 °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 prepared Petri dishes are used, allow the dish to equilibrate to room temperature. The dish should be dried to remove excess moisture before use. In duplicate, pipette 0.1 ml of the sample, or diluted sample, onto the surface of pre-dried agar medium contained in a 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, and incubate in the inverted position.

A9.4   *Incubation of plates*

For yeast extract agar, incubate one of the Petri-dishes at 22 °C for 68 ± 4 hours, and the other Petri-dish 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.

It should be noted that significant changes in count can occur within these tolerances and that for trending purposes incubation times should be kept as consistent as possible.

The requirement to incubate plates at both 22 °C and 37 °C applies to all drinking water in the UK including bottled water supplied as an alternative to mains water as a contingency arrangement[12] and water put into bottles or other containers intended for sale[13].

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

A9.5   *Reading of results*

Colonies may be of various shapes and sizes, count all the colonies in or on the medium containing the original sample. A plate counter or magnifying lens should be used to ensure that very small colonies are counted. Count colonies up to 300 per plate. If the count is greater than 300 then count the colonies from a Petri-dish containing diluted sample, which shows a count between 10 and 300 colonies. If all Petri-dishes show more than 300 colonies, record the result as greater than 300 at the highest dilution.

When very small colonies are present that are indistinguishable from artefacts such as dust etc, it may be appropriate, provided the Petri-dish has not already been incubated for the maximum incubation time, to consider incubating the Petri-dish for an additional period up to the maximum allowed by the method, to encourage further growth.

In some circumstances, where the number of colonies exceeds 300 and the colonies are well defined and discrete, it may be deemed more useful to obtain an estimate of the count. In such circumstances it may be advantageous to use a counting grid, for example a Quebec grid[14]. The number of colonies in a suitable number, usually a minimum of 5, of randomly selected large grid squares should be counted. After adding together the grid square counts the sum obtained should be multiplied by the appropriate factor (the number of grid squares making up the total area of the plate divided by the number of grid squares counted) to estimate the total number of colonies on the plate rounded to the nearest whole number. Where an attempt is made to count all colonies present, even though this may be greater than 300, the circumstances should be documented and the count recognised as only an estimate since it may not reflect the true count.

The presence of spreading colonies can hinder the count and for guidance the following should be regarded as constituting a single colony forming unit:

(i)   A chain of colonies that appears to be caused by the disintegration of a single clump of organisms.

(ii)   A spreading growth that develops as a film at the bottom of the Petri-dish.

(iii)   A colony that forms in a film of water at the edge of, or over the surface of, the agar.

If the colonies cannot be counted immediately after being removed from the incubator, then the Petri-dishes may be kept at $5 \pm 3\,°C$ for no longer than 24 hours. However, they should be brought back to room temperature before reading since condensation may hinder counting.

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. If no dilutions were performed counts greater than 300 per Petri-dish are recorded as >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

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *Micrococcus luteus*). Petri-dishes should be incubated at 22

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°C, and 30 °C or 37 °C for a time appropriate to the intended application. Alternatively, aliquots of raw water, known to contain suitable numbers of target bacteria, may be used. Sterility should also be checked by the inclusion of negative, un-inoculated, controls. Further details are given elsewhere[8] 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.   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.

5.   The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography*, K G Porter and Y S Feig, 1980, **25**, pp943-948.

6.   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.

7.   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.

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

9.   Stress: a factor to be considered in heterotrophic micro-organism enumeration from aquatic environments. *Applied Microbiology*, D A Klein and S Wu, 1974, **27**, pp429-431.

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

11.   BS EN ISO 8199:2007 Water Quality – General guidance on the enumeration of micro-organisms by culture.

12.   The Water Supply (Water Quality) Regulations 2000 (Amendment) Regulations 2007 Statutory Instrument 2007 No. 2734

13.   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.

14   An improved dark-field Quebec colony counter. *Journal of Milk Technology*, O W Richards and P C Heijn, 1945, **8**, p253

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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 example to illustrate the type of products available. Information on the routine use of methods of this type would be welcomed to fully assess their capabilities. This methodology 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. However, it should be noted that the exact product formulation used in this comparison *Quanti-Disc™*[4] may no longer be available. Users of this methodology 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 enzymes (for example, glycosidases, phosphatases, etc) 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 multiple 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 prevent or impede capillary action, which is an essential feature, necessary for the distribution of the water sample into the reaction wells.
In the example method described the limit of detection would be 2 MPN per millilitre.

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 $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

B7.2 Incubators capable of maintaining temperatures to the tolerances specified by the product manufacturer, for example for *Quanti-Disc™* 22.0 ± 2.0 °C, and 30.0 ± 2.0 °C or 37.0 ± 2.0 °C.

B7.3 Sterile pipettes capable of delivering 4 ml, or pipettor with appropriate sized sterile pipette tips.

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

Different commercial formulations of these media and reagents may be available. Commercial formulations should be used and stored according to manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the method[2].

B8.1 *Quanti-Disc™*[4]

This is an example of a commercial product with 50 substrate-coated reaction wells. Inclusion here does not constitute an endorsement of this product but serves to illustrate the type of products that are available. 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.

In the UK two MPN modules as described in sections B9.2 and B9.3 are required for all drinking water samples including those taken to monitor water supplied in bottles by a water undertaker as an alternative supply[5]. This differs from the requirement of the European Directive for which two MPN modules are only a requirement for water in sealed bottles or other containers[6] intended for sale and tested at the point of filling, otherwise only a plate at 22°C is required.

B9.2 S*ample processing*

In duplicate, starting with the most dilute sample solution, 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.

B9.3 *Incubation of plates*

Incubate one of the duplicate reaction modules at 22 °C for 68 ± 4 hours and the other duplicate reaction module 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. The requirement to incubate MPN modules at both 22 °C and 37 °C applies to all drinking water in the UK including bottled water supplied as an alternative to mains water as a contingency arrangement[5] and water put into bottles or other containers intended for sale[6] .
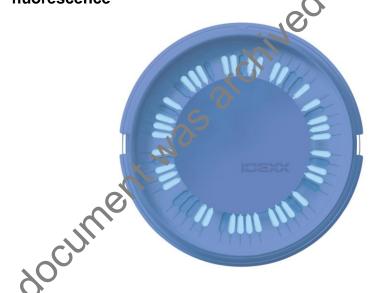
B9.4 *Reading of results*

Examine the 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 original 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 the MPN for 50 positive wells at the highest dilution tested.

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 > 391 MPN per millilitre. Numbers are increased *pro rata* if dilutions are prepared and used.

B12 Quality assurance

New batches of media and reaction modules should be tested with appropriate reference strains of target bacteria (for example, *Micrococcus luteus*). 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) (currently undergoing revision) - 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   The Water Supply (Water Quality) Regulations 2000 (Amendment) Regulations 2007 SI 2007 No. 2734

6.   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 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 (e.g. 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 > 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 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

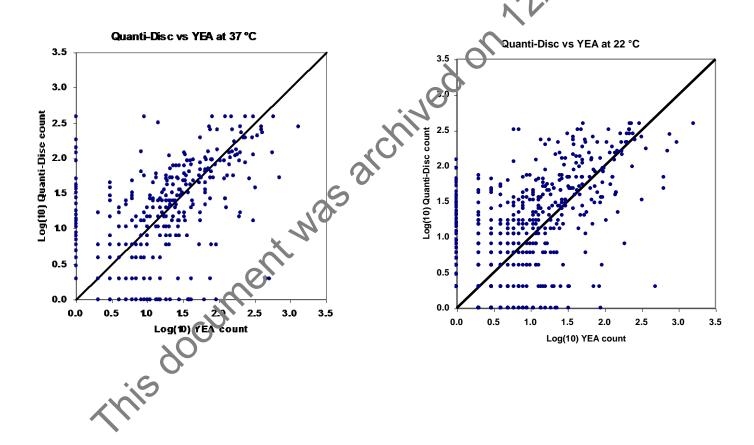**Figure 2   Kolmogorov-Smirnov normality test of untransformed and log$_{10}$-transformed paired difference counts at 37 °C from all water samples**
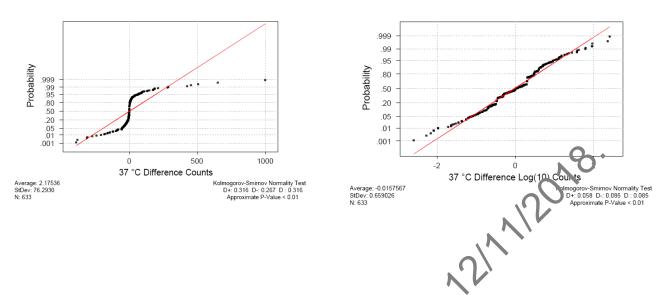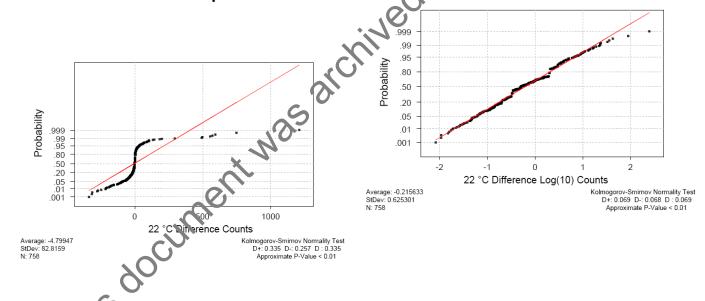

Figure 3   Kolmogorov-Smirnov normality test of untransformed and log$_{10}$- transformed paired difference counts at 22 °C from all water samples

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 (297) was similar to the number of pairs in which the yeast extract agar count was higher (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 (473) was markedly greater than the number of pairs in which the yeast extract agar count was higher (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

24

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.

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. Although for three of these laboratories 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 $\log_{10}$-transformed data, the median difference is + 0.22 with 95 % confidence interval of + 0.17 to + 0.27.

Table 2 **Non-parametric statistics of untransformed and $\log_{10}$-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**

	Quanti-Disc					Median difference Untransformed (95 % confidence interval)	Median difference $\log_{10}$-transformed (95 % confidence interval)
Lab	lower	equal	higher	n	p		
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

Table 3 **Non-parametric statistics of untransformed and log$_{10}$-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 lower	equal	higher	n	p*	Median difference Untransformed (95 % confidence interval)	Median difference Log$_{10}$-transformed (95 % confidence interval)
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 log$_{10}$-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 log$_{10}$-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 lower	equal	higher	n	p*	Median difference Untransformed (95 % confidence interval)	Median difference Log$_{10}$-transformed (95 % confidence interval)
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
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.

The mean relative difference analyses using procedures described in ISO 17994:2004 indicate that the Quanti-Disc method can be considered equivalent (i.e. gives similar results) for the analysis of heterotrophic bacteria plate count at 37 °C when compared to the yeast extract agar pour plate reference method. 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

1.   Environment Agency (Standing Committee of Analysts). The Microbiology of Drinking Water (2002) – Part 7 – Methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques. Methods for the Examination of Waters and Associated Materials. Nottingham: Environment Agency.

2.   Environment Agency (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. Nottingham: Environment Agency.

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4.   ISO/TR 13843:2000(E) Water Quality – Guidance on validation of microbiological methods. Geneva: International Organization for Standardization.

7   Acknowledgements

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. In addition, if users wish to receive advance notice of forthcoming publications, please contact the Secretary.

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Without the good will and support given by these individuals and their respective organisations SCA would not be able to continue and produce the highly valued and respected blue book methods.

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