standing committee of analysts

The Microbiology of Drinking Water (2020) – Part 7

Methods for the enumeration of heterotrophic bacteria

Methods for the Examination of Waters and Associated Materials

The Microbiology of Drinking Water (2012) - 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 2012.

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

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

The Microbiology of Drinking Water
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

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 sludge's, 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 (established 1972 by the Department of the Environment). At present, there are seven 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, Inorganic and physical methods, Metals and metalloids
4 Solid substances
5 Organic impurities
6 Biological, biodegradability and inhibition methods
7 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 SCA's web-page:- http://www.standingcommitteeofanalysts.co.uk/Contact.html

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.

Rob Carter
Secretary
Feb 2020

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 resources are; HSE website HSE: Information about health and safety at work ; RSC website http://www.rsc.org/learn-chemistry/collections/health-and-safety
"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 "Biological Agents: Managing the Risks in Laboratories and Healthcare Premises", 2005 and "The Approved List of Biological Agents" 2013, produced by the Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE).

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 larger 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.

† - The 120 minute time period is only validated for Yeast Extract Agar (YEA) only. (Appendix 1)

Therefore 20 minutes applies for all other agar unless a statistically valid determination of an acceptable time limit between pipetting and agar pouring has been undertaken.

In duplicate, starting with the most dilute sample, 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, to avoid contamination, prior to pouring the medium into the Petri dishes.

Pour 15 - 20 ml of molten agar medium into each Petri dish as soon as possible, and within no more than 120 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 ± 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 °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

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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

Appendix 1

Evaluation of the time between pipetting and pouring heterotrophic plate counts using Yeast Extract Agar (YEA)

Introduction

Yeast Extract Agar is routinely used for the enumeration of heterotrophic bacteria from drinking water. The Microbiology of Drinking Water (2012) - Part 7 – Methods for the enumeration of heterotrophic bacteria [1] states 'Pour 15 - 20 ml of molten agar medium into each Petri dish within 20 minutes of dispensing the 1 ml sample volumes.'

The requirement for a time limit between inoculating plates and pouring agar was first specified in Report 71, The Bacteriological Examination of Water Supplies, published in 1934. It stated that 'The time that elapses between preparation of the dilutions and the pouring of plates should in no case exceed 15 minutes.' This then continued to be detailed in the 1939, 1949, 1956 and 1969 editions. The time limit was then set at 20 minutes in the publication of The Microbiology of Water 1994 - Part 1 - Drinking Water[2]

There is no rationale for the time limit detailed in Report 71 or The Microbiology of Water 1994 - Part 1. It has been documented by Edwin E. Geldreich that it is important to limit the time between pipetting and pouring in order to reduce any impact that light and increased temperature can have on the viability of bacterial vegetative cells.

The SCA Working Group 2 has conducted a multi-laboratory study on the impact of extending the 20 minute time limit between pipetting and pouring on the recovery of heterotrophic bacteria from water.

Study Design

The aim of the multi-laboratory study was to investigate the significance of extending the time between pipetting and pouring on the recovery of heterotrophic bacteria from water.

The participating laboratories were asked to prepare spiked samples which were then analysed as per The Microbiology of Drinking Water (2012) - Part 7. One set of the pipetted spiked samples were then poured at each of the following time points; 20, 30, 40, 50, 60, 80, 100 and 120 minutes.

After appropriate incubation colonies were counted and the results recorded on a supplied pro-forma Excel spreadsheet.
The study covered a range of spiked surface and ground drinking water samples, raw water, reference material e.g. Lenticule AQC samples and the data included both samples analysed by hand pouring and using an automated pourer stacker.

Data Analysis

Data was received from six laboratories and analysed according to ISO 17994[3] by assigning counts from times greater than 20 minutes as the Trial Method and corresponding counts from 20 minutes as the Reference Method. The data was censored by the removal of pairs of data where both counts were zero or where at least one count of a paired count was recorded as either >300 or TNTC (too numerous to count). Results from one laboratory had to be removed for HPC 37 results due to Bacillus like colonies impacting the ability of the samples to be enumerated accurately.

The paired count data was transferred to an Excel 2013 spreadsheet and analysed according to the mean relative difference approach of ISO 17994. Briefly, the relative difference (x) of each pair of counts was calculated using the equation x = 100(ln(a) - ln(b)), where ln(a) is the natural logarithm of the count by the trial method (times >20 minutes), and ln(b) is the natural logarithm of the count by the reference method (20 minutes). Data with a zero count by one method had plus one (i.e. count +1) added to each pair of the counts prior to log-transformation.

As the objective of the study was to show there was no difference between the trial method and the established reference method, it was considered that the 'two-sided' comparison according to ISO 17994 was appropriate. The percentage value of the upper and lower limits was set at ± 10% as suggested by ISO 17994.

HPC 30ºC Comparison

For this study the term HPC 30ºC refers to heterotrophic plate count analysis incubated for 68 ± 4 hours at 30ºC. There were 29 paired counts for each time point. The results of the ISO 17994 analysis demonstrated that for all time points the outcome of the analysis is inconclusive (i.e. more samples are needed). Using the number of samples formula in ISO 17994 the total number of paired counts needed to achieve a conclusive outcome is 47 (i.e. 18 more).

There was a low number of data points within the 30ºC dataset due to only one laboratory submitting results for this analysis.

HPC 37ºC Comparison

For this study the term HPC 37ºC refers to heterotrophic plate count analysis incubated for 44±4 hours at 37ºC. There were a minimum of 308 paired counts for each time point and the results of the ISO 17994 analysis of the data is presented in Table 1.

The outcome of the analysis demonstrates that there was not a significant difference in heterotrophic plate count results at 37ºC between pouring at 20 minutes and time intervals greater than 20 minutes.

Time points 80 and 100 minutes results were indifferent when compared to results at 20 minutes. This indicates that the difference between the results are of statistical significance but the difference is too small to be of microbiological practical significance as detailed in ISO 17994:2014.

Table 1 Outcome of mean relative difference analysis of the paired counts of HPC 37ºC at all time points 308 samples per time point according to ISO 17994.

Time Point (minutes)	Number of paired counts	Mean Relative Difference	Standard Deviation	W *	XL **	XU ***	Outcome
30	309	3.25	32.11	3.65	-0.40	6.91	Not different
40	309	2.75	28.89	3.29	-0.53	6.04	Not different
50	308	2.90	32.40	3.69	-0.79	6.59	Not different
60	308	3.35	32.45	3.70	-0.35	7.05	Not different
80	308	5.14	39.52	4.50	0.64	9.65	Indifferent
100	309	4.76	34.48	3.98	0.84	8.68	Indifferent
120	309	3.87	41.39	4.71	-0.84	8.58	Not different

* Half width of the 'confidence interval' around the mean relative difference.
** Value of the relative difference at the lower 'confidence limit.'
*** Value of the relative difference at the upper 'confidence limit.'

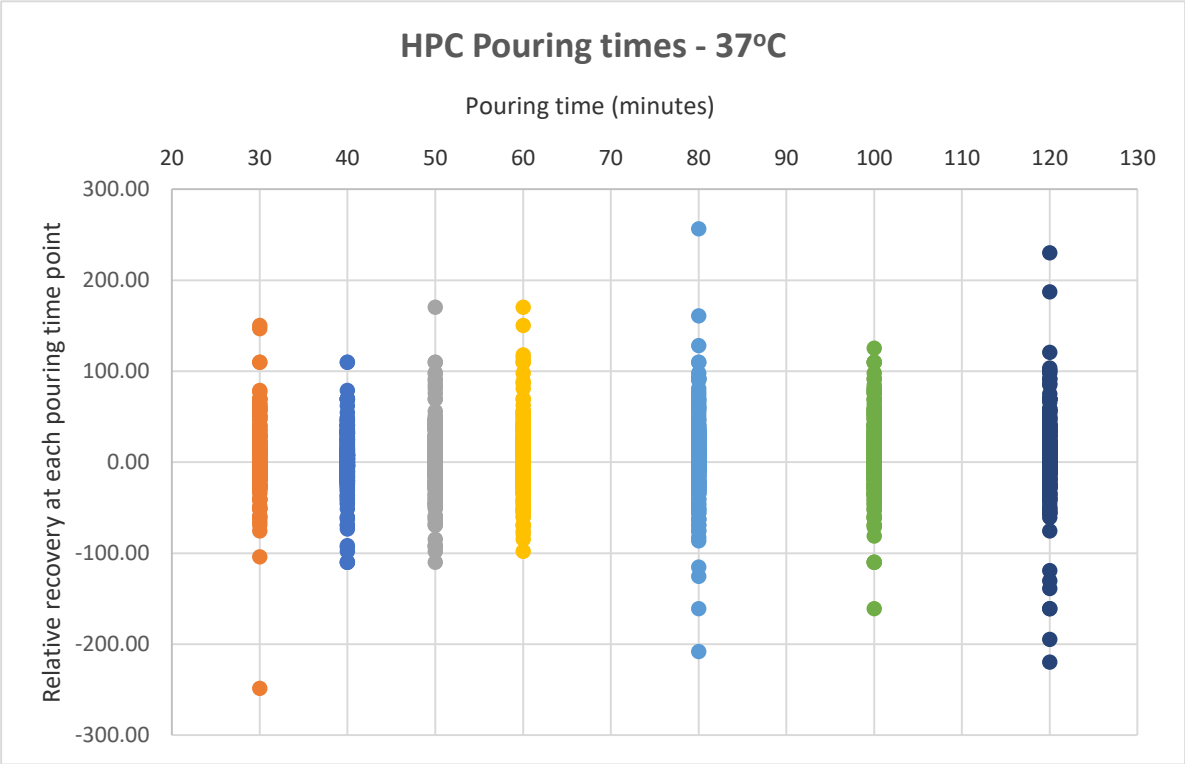

Figure 1 - Plot of the difference in counts of HPCs incubated at 37 °C at all time points for pouring agar.

HPC 22ºC Comparison

For this study the term HPC 22 ºC refers to heterotrophic plate count analysis incubated for 68 ± 4 hours at 22 ºC. There was a minimum of 387 paired counts for each time point and the results of the ISO 17994 analysis of the data is presented in Table 2. The outcome of the analysis demonstrates that there was not a significant difference in heterotrophic plate count results at 22 ºC between pouring at 20 minutes and time intervals greater than 20 minutes.

At the time point of 120 minutes the results were indifferent when compared to results at 20 minutes. This indicates that the difference between the results are of statistical significance, but the difference is too small to be of microbiological practical significance as detailed in ISO 17994:2014.

Table 2 - Outcome of mean relative difference analysis of the paired counts of HPC 22ºC at all time points from 387 samples per time point according to ISO 17994.

Time Point (minutes)	Number of paired counts	Mean Relative Difference	Standard Deviation	W *	XL **	XU ***	Outcome
30	388	0.31	33.27	3.38	-3.06	3.69	Not different
40	387	-2.56	30.42	3.09	-5.65	0.53	Not different
50	388	0.13	32.55	3.31	-3.18	3.43	Not different
60	388	-2.78	34.90	3.54	-6.32	0.76	Not different
80	387	-2.68	33.17	3.37	-6.05	0.69	Not different
100	388	-2.16	38.17	3.88	-6.03	1.72	Not different
120	387	-4.28	31.96	3.25	-7.53	-1.03	Indifferent

* Half width of the 'confidence interval' around the mean relative difference.
** Value of the relative difference at the lower 'confidence limit.'
*** Value of the relative difference at the upper 'confidence limit.'

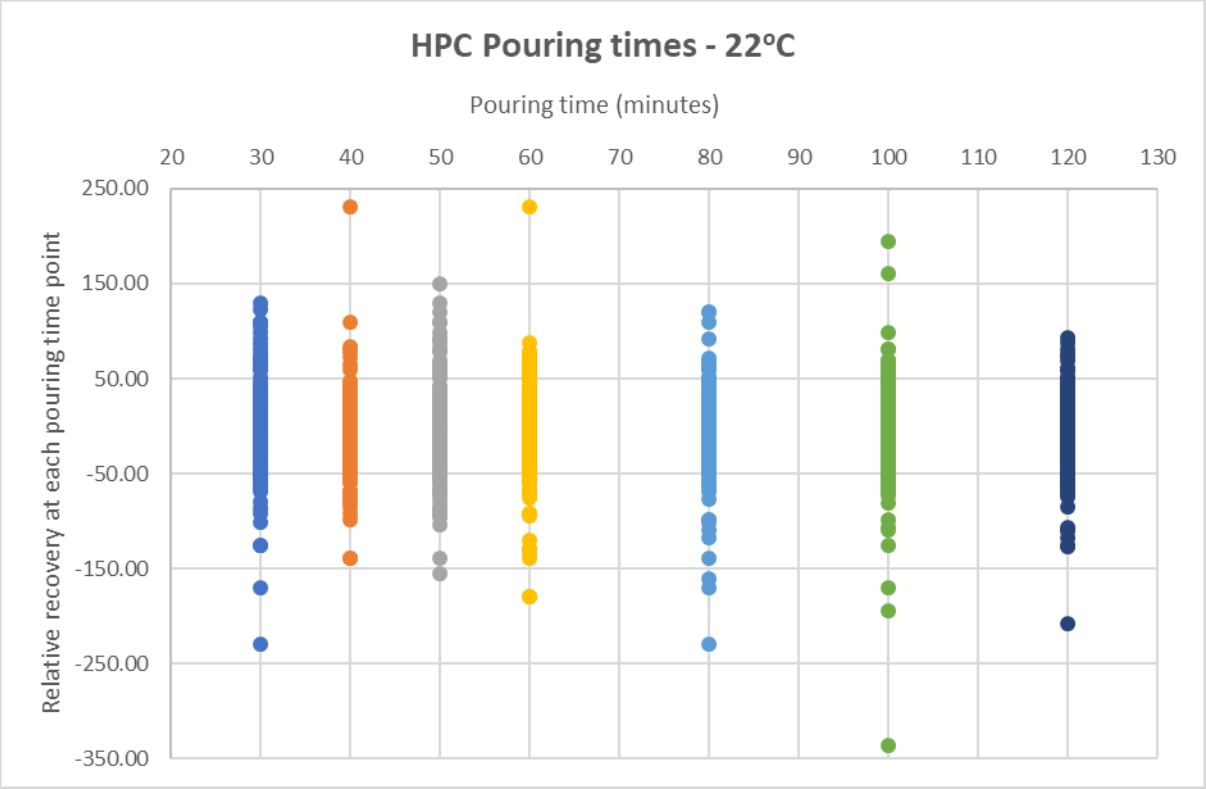

Figure 2 - Plot of the difference in counts of HPCs incubated at 22°C at all time points for pouring agar.

Conclusions and Recommendations

This study assessed the recovery of heterotrophic bacteria using Yeast Extract Agar pour plate method comparing samples poured at 20 minutes and time intervals of 30, 40, 50, 60, 80, 100 and 120 minutes. The outcomes of ISO 17994 analysis of the data indicate that plates poured up 120 minutes after pipetting provide equivalent recovery of heterotrophic bacteria using the Yeast Extract Agar pour plate method.

Thus, it is recommended that The Microbiology of Drinking Water (2012) - Part 7 is amended to allow the time limit between dispensing a 1ml sample volume and pouring molten yeast extract agar medium into each Petri dish to 120 minutes. The following wording is suggested to replace the second paragraph of A 9.2:

"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 as soon as possible, but within no more than 120 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.

This trial has been carried out using Yeast Extract Agar (YEA) only, if laboratories are using alternative agar then this exercise must be repeated using the specific agar to determine an acceptable time limit between pipetting and pouring of the agar.

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The investigation was organised and the data analysed by Matthew Jones, Dŵr Cymru

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[1] The Microbiology of Drinking Water (2012) - Part 7 – Methods for the enumeration of heterotrophic bacteria Methods for the Examination of Waters and Associated Materials.
Standing Committee of Analysts, Environment Agency, Nottingham, UK.

[2] The Microbiology of Water (1994) - Part 1 - Drinking Water. Standing Committee of Analysts, Environment Agency, Nottingham, UK.

[3] ISO 17994:2014, Water quality – Requirements for the comparison of the relative recovery of microorganisms by two quantitative methods. International Organization for Standardization, Geneva, Switzerland.

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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Members assisting with this method

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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