

HPA STANDARD METHOD

# AEROBIC COLONY COUNT BY THE POUR PLATE METHOD

W 4

Issued by Standards Unit, Evaluations and Standards Laboratory  
Centre for Infections

## AEROBIC COLONY COUNT BY THE POUR PLATE METHOD

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## **Suggested citation for this document:**

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# AMENDMENT PROCEDURE

<b>Controlled document reference</b>	<b>W 4</b>
<b>Controlled document title</b>	<b>Aerobic Colony count by the pour plate method</b>

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from [standards@hpa.org.uk](mailto:standards@hpa.org.uk).

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
7/ 08.08.08	4.1	4.2	1	<b>Title</b>	Changed to Aerobic colony count by the pour plate method
				<b>Background</b>	Updated to include information on ACC pre and post disinfection. Paragraph added to cover aircraft and purified waters
				<b>Definitions</b>	Note updated
				<b>Safety considerations</b>	Use of protective gloves added
				<b>Equipment</b>	Temperature range for waterbath amended. Incubator at 21°C and information note added
				<b>Culture media and reagent</b>	TGEA added
				<b>Sample processing</b>	Inoculation and Incubation split into two sections and updated with incubation ranges clearly set out for all water samples.  Use of hand lens added.
				<b>Reporting</b>	Updated
				<b>Quality Control</b>	Updated
				<b>Reference Facilities</b>	Heading added
				<b>Appendix</b>	Updated
				<b>References</b>	Updated

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# AEROBIC COLONY COUNT BY THE POUR PLATE METHOD

## SCOPE OF DOCUMENT

The method is applicable to the enumeration of aerobic viable micro-organisms in all types of water. The target count is in the range of 0 to 300 organisms per mL of water when 1 mL volumes are analysed. Counts in excess of 300 per mL can be determined by using suitable dilutions.

## INTRODUCTION

### Background<sup>2</sup>

Waters of all kinds invariably contain a variety of micro-organisms derived from various sources such as soil and vegetation and estimation of the overall numbers provide useful information for the assessment and surveillance of water quality. Most bacteria capable of growth in potable water and natural surface waters in temperate climates will grow better in culture media at 22°C than at higher temperatures. Organisms that grow best at 37°C usually grow less readily in potable water and are likely to have gained access from external sources particularly of human or animal origin. These two groups of organisms are counted separately and used to assess the general quality of potable water. A World Health Organisation (WHO) meeting in 2002 identified that the aerobic colony count (ACC) is not a good indicator of risk to health. ACC are useful for assessing the integrity of ground water sources and the efficiency of water treatment processes such as coagulation, filtration and disinfection and provide an indication of the cleanliness and integrity of the distribution system<sup>3</sup>. However it should be noted that ACC are generally only useful when carried out regularly and when carried out pre and post disinfection.

### Pool waters

In pool waters, the ACC at 37°C is used as these organisms are most likely to have been derived from bathers and are a measure of the adequacy of disinfection of the pool water. Microbiological guidelines and standards are available for potable and pool waters<sup>4-14</sup>.

The main value of ACC lies in the detection of changes from those expected, based on frequent, long-term monitoring. Any sudden increase in the count can be an early warning of serious pollution and calls for immediate investigation. It is therefore important that the same technique and media should always be used to examine a given water sample. The method of choice for potable and pool waters in the UK is the pour-plate method using yeast extract agar (YEA). This method is based on EN ISO 6222:1999 (BS 6068-4.5:1999)<sup>15</sup> as specified in the EC Drinking Water Directive and described in the Microbiology of Drinking Water 2007 document<sup>11</sup> and EN ISO 7218:2007<sup>16</sup>.

### Water from cooling systems

In water from cooling systems with cooling towers or evaporative condensers, the ACC is assayed to determine if the water treatment is controlling microbial growth. Since the water in cooling systems is usually between 25°C and 35°C and the majority of the organisms growing in such systems grow at 30°C this temperature is recommended for the incubation of ACC on cooling systems. The target counts in the Health and Safety Executive (HSE) guidelines are based on incubation at 30°C for at least 48 hours<sup>17</sup>.

### Private water supplies

Where the samples are only collected at infrequent intervals there is little value in performing the ACC. Thus the regulations only require the routine determination of ACC for private water supplies of Classes A, B, 1 or 2 and not for others<sup>18</sup>. However when investigating problems the ACC may be useful to assist with the interpretation of the results.

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### **Tank Disinfection**

The determination of the ACC can also be a useful hygiene check following disinfection and flushing of a system for example after construction and leak testing of a new domestic water system, cleaning and disinfecting a tank or repairs on a water system. In this case, if the cleaning and disinfection have been adequate one would normally expect the ACC to be comparable to and no more than 10 times greater than that of the incoming water.

### **Ships and aircraft<sup>19,20</sup>**

As for systems on land, there should be no significant increase over “expected levels” of ACC in water of satisfactory quality on ships. The “expected levels” may be difficult to assess on board a vessel, and for this reason ACC is not recommended for routine monitoring. During a sanitation inspection of a ship it is recommended that Port Health Officers sample the potable water and the laboratories test for *E. coli*, enterococci, and coliform bacteria. ACC counts should only be requested following a system disinfection after a hygiene problem has been identified. High ACC do not in themselves represent a health risk but should not be present in ship’s water systems as they may indicate inadequacies in disinfection and / or maintenance of tanks and distribution systems in ships.

Similarly on aircrafts the ACC results are useful in assessing the effectiveness of disinfection and can also be used to monitor changes in water quality over time. Where microbiological testing is necessary water should be tested for coliform bacteria, *E. coli*, enterococci, ACC and *P. aeruginosa*. Essentially the quality standards should be the same as those for public water supplies with the addition of *P. aeruginosa* and ACC as these are indicators of the efficacy of disinfection. *E. coli* and enterococci are used as faecal indicators, coliform bacteria are useful as indicators of poor hygiene and the ACC and *P. aeruginosa* counts as checks on disinfection and re-growth within distribution.

### **Purified waters (Post reverse osmosis) and dialysis fluids**

The UK Renal Association recommends a more sensitive pour plate method for dialysis fluids/waters<sup>21</sup> and recommends a longer incubation time and a low nutrient medium suitable for bacteria found in purified water. Hence the same pour plate method procedures described in this method are used with a Tryptone Glucose Extract Agar (TGEA) test medium and 21°C ± 1°C for 7 days incubation.

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# 1 DEFINITIONS

For the purpose of this method the following definition applies:

*Aerobic Colony Count (culturable micro-organisms)*

All aerobic bacteria, yeasts and moulds capable of forming colonies in or on the medium specified, under the test conditions described.

**Note:** Various synonyms are frequently used instead of “Aerobic colony count”. These include heterotrophic colony count, viable count, plate count and culturable micro-organisms etc. This document uses aerobic colony count which is synonymous with the term colony count used in the Drinking Water Regulations<sup>22</sup>.

# 2 PRINCIPLE

Measured volumes of the sample or dilutions of the sample are mixed with molten YEA / TGEA as appropriate in sterile Petri dishes, and incubated under the conditions specified. The number of colony-forming units (cfu) per millilitre (mL) of the sample is calculated from the number of colonies.

# 3 SAFETY CONSIDERATIONS<sup>23-30</sup>

Normal microbiology laboratory precautions apply

## 3.1 SAMPLE TRANSPORT AND STORAGE

Compliance with current postal and transportation regulations is essential.

## 3.2 SAMPLE PROCESSING

Care must be taken when using a boiling waterbath for melting agar. Use heat and water resistant protective gloves and do not put the face or hands over the bath when opening to remove objects.

***The above guidance should be supplemented with local COSHH and risk assessments***

# 4 EQUIPMENT

Usual laboratory equipment and in addition:

- Waterbath: 44°C - 47°C
- Boiling waterbath
- Incubator: 22°C ± 1°C
- Incubator: 21°C ± 1°C (If processing purified waters)
- Incubator: 30°C ± 1°C (If processing water from cooling systems)
- Incubator: 37°C ± 1°C
- Petri dishes: 90 mm diameter (sterile)
- Automatic pipettors and associated sterile pipette tips capable of delivering up to 10 mL and 1 mL volumes (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)

**Information note:** Bottles of agar should be allowed to cool before putting into the waterbath at 44°C – 47°C.

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## 5 CULTURE MEDIA AND REAGENTS

Equivalent commercial dehydrated media may be used; follow the manufacturer's instructions.

### *Peptone saline diluent (Maximum recovery diluent)*

Peptone	1.0 g
Sodium chloride	8.5 g
Water	1 L
pH	7.0 ± 0.2 at 25°C

### *Yeast extract agar (YEA)*

Yeast extract	3.0 g
Peptone	5.0 g
Agar	12.0 g
Water	1 L
pH	7.2 ± 0.2 at 25°C

### *Tryptone Glucose Extract Agar (TGEA)*

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	9.0 g
pH	7.0 ± 0.2

## 6 SAMPLE PROCESSING

### 6.1 SAMPLE PREPARATION AND DILUTIONS

Water samples should be received and handled as described in [W 1 - General technique for the detection and enumeration of bacteria by negative pressure membrane filtration](#). In brief, the nature of the request and condition of the sample is to be noted on arrival, and the sample stored at 2°C - 8°C until processed. Samples should be examined as soon as is practicable on the day of collection. In exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours before the commencement of analysis.

Following the procedures laid down in [W 1 - General technique for the detection and enumeration of bacteria by negative pressure membrane filtration](#), select suitable volumes for analysis and prepare tenfold dilutions if counts are expected to be in excess of 300 cfu per mL.

### 6.2 INOCULATION

Invert the sample bottle rapidly several times in order to mix the sample thoroughly. If the bottle is full, remove the stopper or cap and retain in the hand avoiding contamination. Pour off some of the contents, replace the stopper or cap and again shake the bottle in order to distribute any organisms uniformly through the water. Aseptically measure a 1 mL volume of sample or dilution into a Petri dish using a 1 mL graduated pipette or automatic pipettor.

Aseptically pour 15 - 20 mL of molten agar YEA / TGEA as appropriate, which has been cooled to ≤ 45°C, into each Petri dish. Avoid pouring the molten agar directly on to the inoculum. The molten agar should be poured within 20 minutes of dispensing the 1 mL sample volumes and used preferably within 4 hours. Any unused medium should be discarded after 8 hours.

Immediately mix the sample and agar carefully to obtain a homogenous distribution of the micro-organisms within the medium. It is essential to keep the Petri dish flat on the bench throughout the procedure.

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## 6.3 INCUBATION

Allow the agar to set, invert the Petri dishes and incubate as follows:

***Potable waters including abattoirs, natural mineral waters, mains water, drinking waters in containers, private water supplies, ice (melted) and vending machines***

Inoculate two Petri dishes and incubate one at 22°C ± 1°C for 68 hours ± 4 hours and the other at 37°C ± 1°C for 44 hours ± 4 hours. Petri dishes incubated at 37°C ± 1°C may be examined after 21 ± 3 hours if an early indicator of growth is required and then re-incubated if the ACC does not exceed 300 cfu.

**Information note:** Incubating as close as possible to the upper time limit can significantly increase the ability to count micro colonies.

***Pool waters***

Incubate at 37°C ± 1°C for 24 hours ± 1 hour.

***Cooling waters***

Incubate at 30°C ± 1°C for 49 hours ± 1 hour.

***Purified waters***

Incubate at 21°C ± 1°C for 7 days

## 6.4 COUNTING/READING OF COLONIES

Examine the plates as soon as they are removed from the incubators. If this is not possible, then store at 2°C - 8°C for no longer than 24 hours.

Count the colonies present in or on plates containing less than 300 colonies. Counts may be enumerated using automated colony counters. If necessary, use a hand lens or low power binocular microscope.

Spreading colonies can interfere with counts. A chain of colonies that appears to be formed by the disintegration of a clump of organisms, a spreading growth developing as a growth on the bottom of the Petri dish or a colony that forms in a film of water at the edge or on the surface of the Petri dish, should be regarded as single colonies. Depending on the source of water it may be advisable to identify further if there is a predominant colony type eg pseudomonads.

## 7 CALCULATION OF RESULTS

Calculate the ACC as follows:

ACC per mL of water =

$$\frac{\text{Number of colonies}}{\text{Volume Tested}} \times \text{Dilution Factor}$$

For samples in which 1 mL of undiluted sample was plated then the count per mL will be equal to the number of colonies counted.

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## 8 REPORTING

Express the result as the number of colony forming units (cfu) per mL of the sample for each temperature of incubation.

If there are no cfu in or on the plates inoculated with the undiluted sample, report as:

**None detected in 1 mL**

Results may be reported as 0 / specified volume with an explanatory note stating that 0 = Not Detected.

If cfu are detected report as:

**a per mL**

where **a** is the number of cfu counted in 1 mL of sample.

If there are more than 300 colonies on the plate and dilution is not performed report as:

**'Greater than 300 cfu per mL'**

Do not use the term 'too numerous to count'.

## 9 QUALITY CONTROL

The following procedures should be performed once daily when the ACC method is used.

The quantitative internal quality controls are to be carried out using a suspension of the positive control organism known to contain sufficient organisms to give 20-100 cfu per mL.

The internal quality controls are to be carried out as follows:

**Potable waters including abattoirs, natural mineral waters, mains water, drinking waters in containers, private water supplies, ice (melted) and vending machines**

*Positive control*

*Klebsiella aerogenes* NCTC 9528

Inoculate 1 mL of suspension into each of 2 Petri dishes. Add about 15 - 20 mL of cooled molten agar, YEA / TGEA as appropriate to the plates at the same time as the pour plate method is carried out on test samples. Incubate one control plate with the test plates at 37°C ± 1°C and the other plate with the test plates at 22°C ± 1°C for the appropriate times.

*Negative control (blank)*

Sterility checks should be performed at the end of each session. Aseptically pour about 20 mL of molten agar, YEA / TGEA as appropriate, cooled to ≤ 45°C, into 2 Petri dishes. This should be done at the same time as the test samples are inoculated. Incubate the control plates with the test plates at 37°C ± 1°C and 22°C ± 1°C for the appropriate times.

**Pool waters**

For pool waters only one positive and one negative control plate is required and they are incubated at 37°C ± 1°C.

### AEROBIC COLONY COUNT BY THE POUR PLATE METHOD

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### **Cooling waters**

For cooling waters only one positive and one negative control plate is required and they are incubated at 30°C ± 1°C for 49 hours ± 1 hour.

### **Purified waters**

For purified waters only one positive and one negative control plate is required and they are incubated at 21°C ± 1°C for 7 days.

## **10 REFERENCE FACILITIES**

N/A

## **11 ACKNOWLEDGEMENTS AND CONTACTS**

This National Standard Method has been developed, reviewed and revised by the Water Working Group for Standard Methods ([http://www.hpa-standardmethods.org.uk/wg\\_water.asp](http://www.hpa-standardmethods.org.uk/wg_water.asp)). The contributions of many individuals in Food, Water and Environmental laboratories, reference laboratories and specialist organisations who have provided information and comment during the development of this document are acknowledged.

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# APPENDIX: FLOWCHART SHOWING ENUMERATION OF AEROBIC COLONY COUNT BY THE POUR PLATE METHOD

Transport to laboratory at 2°C – 8°C out of direct sunlight  
in suitable container



Store at 2°C – 8°C in the dark and examine on the day of collection if possible  
otherwise within 24 hours of collection



Mix sample well and make any necessary dilutions in peptone saline diluent



Dispense a 1 mL amount of each dilution into an empty Petri dish



Pour 15 - 20 mL of appropriate molten agar (tempered to 44°C - 47°C) into each  
Petri dish and mix carefully with the inoculum



Allow agar to set, invert plate and incubate at 22°C ± 1°C for 68 hours, 37°C ± 1°C for 21 or 44 hours or  
21°C ± 1°C for 7 days

(Waters from cooling systems incubate at 30°C ± 1°C for 49 hours)



Count colonies



Calculate ACC

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