

## **Bacteriological quality of water in ornamental water features**

### **Introduction**

The risk to the public's health from recreational waters has been well documented. Outbreaks of infectious diseases from waterborne pathogens such as *Legionella*, from Protozoa such as *Gardia* and *Cryptosporidium*, viruses such as *adenovirus* and *norovirus*, has led to the necessity for risk assessments, monitoring and management based on advisory guidelines and legislation.

Mandatory guidelines, legislation and microbiological standards has mainly focused upon swimming pools, hydrotherapy, spa pools and interactive water features due to the risk of faecal contamination from humans and animals, with little concern for the potential risk from decorative water features.

Decorative water features are increasing in popularity and such ornaments can be found within buildings, such as offices to create a tranquil atmosphere, as a design feature in hotel receptions or displayed in public gardens as well as exhibited for sale in garden centres.

Jones *et al* (2003) discusses an outbreak of Pontiac Fever, the milder influenza-type symptom of *Legionellasis* which resulted in 25 patrons of a restaurant, becoming ill. The cause of the outbreak was found to be a number of decorative fountains and misting machines positioned within the restaurant. Another outbreak of Pontiac Fever involving 34 guests was associated with a decorative water fountain positioned in a hotel lobby Gozt *et al*(2001).

Nichols(2006) discusses how indoor water features have been instigated in *Legionella* outbreaks and surmises that this is due to increased temperatures, lack of sun light and enclosed spaces which enhance aerosol transmission.

An investigating into the potential risk to the public from decorative water features should not be discounted. Although they are not designed to be bathed in or for interaction, and therefore theoretically reducing the risk of direct contamination there still remains a risk from contact, shredding of faecal organisms as people and children are drawn to the water feature. The water features may also be susceptible to environmental contamination from plants and earth (Havelaar *et al*, 2001). When the water becomes contaminated and in the absence of a cleaning and disinfection regime bacterial biofilms can establish themselves, subsequent water flow is reduced and they are difficult to eradicate with basic cleaning techniques.

The project seeks to identify if pathogenic bacteria are present in the water contained within these features and whether there is a risk to health which needs to be assessed and managed in order to prevent the risk. The aim of the project is to collect samples of water from approximately 50 decorative water features and analyse for the presence of *Escherichia coli* and *Legionella*.

Havelaar *et al* (2001)state that *E.coli* is an ideal organism to indicate faecal contamination of water sources, because it is easily detectable, does not grow in natural water and behaves similarly to waterborne pathogens.

Legionella is commonly found in warm, damp places and in soil and lives and colonises at temperatures between 20-50°C.

Furthermore based on the results and the observations made during the investigation, recommendations on the management of such features will be made.

### **Methodology**

The brief stated that approximately 50 water samples were required for the project. Seven garden centres were identified within the South Wales area as possible sources of decorative Water feature suppliers. A sample programme worksheet was designed to record sample information. For each feature identified the following information was recorded. (Ref Appendix 1. for table of results)

- Sample code
- Total height of feature in cm
- Height of water discharge in cm
- Photograph of the feature
- Evidence of cleaning procedures
- Evidence of risk assessments or the presence of warning signs
- Additional information about the environment.

On arrival at the garden centres permission to take water samples was requested and relevant centre managers or supervisors were informed of the purpose of the study and the information required. Personnel were asked about cleaning procedures and the availability of risk assessments.

The water features were measured for total height and height of water discharge was taken. Temperature of the water circulated by a feature was taken using a digital probe which was sanitised between use using antibacterial wipes.

The surrounding environment was observed for warning signs, safety instructions for the public and also for any possible sources of contamination. All information was documented on the worksheet. A minimum of 1000ml of water was required for laboratory testing which was collected from the water feature at various points depending upon the water's flow. Water samples were collected aseptically into sterile bottles and were identified by an individual code written on the container label along with description of the sample point, date and time of sampling. The samples were transported under refrigeration to an independent laboratory for analysis.

The laboratory used the M016 test method for the enumeration of total *E.coli* in water using MLGA. Following incubation relevant colonies were enumerated and results recorded (see appendix for comprehensive analysis method). Limitations to the method are that only 90% of *E.coli* species are detected and the specific species or sero type were not identified.

The laboratory employed the BS6068-4.12:1998 ISO 117731:1998 analysis method for the detection and enumeration of *Legionella* was based on the membrane filtration technique (see

Appendix 2. for comprehensive analysis method). Following incubation relevant colonies were enumerated and results recorded.

## **Results**

All garden centres visited agreed to allow sampling to take place and personnel were happy to answer questions regarding cleaning procedures and risk assessments.

The water used in all the decorative features was sourced from the mains supply. The features contained pumps which would circulate the water continuously around the system. An exception to this was where decorative displays were built to demonstrate how they could be incorporate into a garden's landscape. These features differed because water from the various fountains and cascades would stagnate in a shallow pool before re-circulating the system.

### Cleaning procedure

The methods and frequency of cleaning was inconsistent across the garden centres. There were no cleaning schedules produced from any of the garden centres. Cleaning was mostly carried out when excess algae growth affected the aesthetics of the decorative feature or blocked nozzle's thus restricting its' function.

Generally the methods of cleaning employed involved features being drained and brushes used to scrub away algae growth or they were washed clean without the use of chemicals, refilled and treated with chlorine crystals. The use of chlorine was not encouraged by the manufacturers as the features were designed for the garden and any chemicals used could affect wild life. The water which supplied one of the landscaped feature was treated with Ultra Violet light before being re-circulated.

### Risk assessment and warning notices.

When asked none of the garden centres personnel were aware of any risk assessments completed for the water features. The Pool Water Advisory Group (PWAG) states that formal risk assessments is required by Health and Safety work Act 1974 to assess the microbiological risks from interactive water features and the PWAG recommends that decorative water features be included in order to protect the health and safety of the employees. This is especially pertinent when there was clear evidence that many of the decorative features created aerosols and caused splashing, creating a slippery surface around the display area which could result in a slip injury as well as the risk contracting an infectious disease.

Two of the seven garden centres visited were aware of the possible dangers and displayed warnings signs around the features with the following statements:

- All children must be accompanied
- .....Unaccompanied children may result in injuries

- Water fountains may contain bacteria....wash hands after immersion

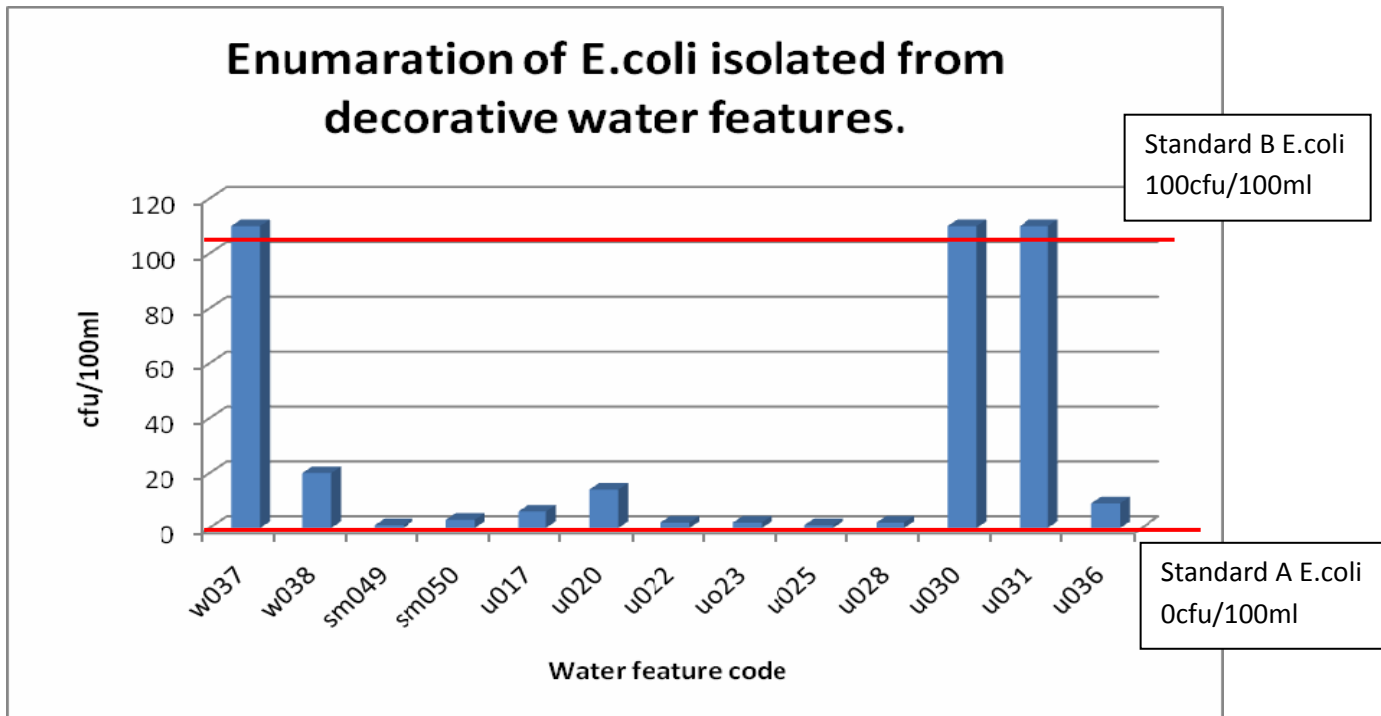


(Pictures of warning signs taken from two of the garden centres)

Microbiological analysis results

The samples were analysed for the presence of *Legionella spp* the results concluded that the organism was not detected in any of the water samples. The risk from the presence of *Legonella* should not be discounted as the majority of features created aerosols which could potentially in the presence of the organism be inhaled and cause respiratory infections.

The samples were also analysed for the presence of *E.coli* . Thirteen of the fifty two samples were found to contain *E.coli* all of which exceeded the microbiological standards recommended for water by Barrell *et al* (2000) in a communicable disease and public health review. Barrell *et al* (2000) recommend guidance levels of 0cfu/100ml *E.coli* for swimming baths, Spa pools and Hydrotherapy pools(see standard A of graph). Three of the samples were >100cfu/100ml which exceeds the recommended value of faecal coliforms in bathing beach water which is 100cfu/100ml(see standard B of graph).



Nine of the thirteen samples taken from the decorative features were found to come from the same garden centre. The water features themselves were in a poor state of cleanliness, and

many of the features were heavily contaminated with algae. One feature was not working correctly because a number of the pipes were clogged.

Two of the samples which exceeded >100cfu/per 100ml were taken from a display which included a collection of decorative features. Water from all the features was pooled before re-circulating also the display was landscaped with plants which could be the cause of the high level of contamination. A further two samples which contained *E.coli* were situated outside which increased the risk of contamination.

### **Discussion/conclusion**

*Legionella* or *E.coli* was not detected in the samples taken from the decorative features from the garden centres which demonstrated an awareness of health and safety by displaying public notices.

The water samples with a detectable level of *E.coli* were taken from features which were landscaped and included plants and soil in the display and also those which were situated outside. The World Health Organisation (WHO)(2001)states that it is difficult to deduce an infective level of any particular pathogen found in water because the risk largely depends on the infectivity and invasiveness of the pathogen as well as the susceptibility and immunity of the individual exposed. In light of this the WHO concludes that any water containing pathogenic organisms cannot be considered safe.

The majority of the decorative water features were prone to splashing and creating water aerosols at heights which children may make contact and inhale the aerosols. Aerosolisation spreads pathogens to the surrounding environment and the WHO(2001) states that it is unclear precisely the infective dose of *Legionella* but are aware that susceptible humans exposed to a low dose for only a few minutes have become ill.

### **Recommendations**

The results identify that there is a potential risk to the public health from these decorative water feature if they are not managed and maintained correctly. Nichols(2006) and Jones *et al* (2006)agree with the recommendations made by the PWAG that the foremost effective way of minimising the risk to the public's health from outbreaks of infectious diseases is through risk assessment and risk management.

Correct management of the features would ensure that cleaning schedules and methods employed for cleaning minimises the risk of contamination. Caution must be taken when treating the water with disinfectants because of splashing.

All decorative water features exhibit areas should display warning signs to ensure that children are kept under supervision and do not make contact with the water contained in the feature or the feature itself and are notified of the importance of wash their hands if they make contact with the water.

Garden centres which design and build landscaped decorative water features need to be aware of the potential source of contamination introduced when encompassing plants and soil into and around the water contact surfaces.

Based on the project results it is recommended that Environmental Health Practitioners request evidence of risk assessments and risk management procedures, in the form of adequate cleaning and disinfection regimes of the water features and surrounding environment, the display of suitable warning signs highlighting the potential risk from contact, along with possibly a microbiological water quality sampling programme.

## References

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[www.pwag.org/technicalrelease2](http://www.pwag.org/technicalrelease2)



## Appendix 2

### FOODLABS LABORATORY TEST METHOD M016 ENUMERATION OF TOTAL COLIFORMS AND ESCHERICHIA COLI IN WATERS USING MLGA

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#### 1.0 INTRODUCTION

Tests for Coliforms and *Escherichia coli* are the most important routine microbiological examinations

carried out on potable water as they provide the most sensitive method for assessing the effectiveness of disinfection and for detecting faecal contamination.

#### 2.0 SCOPE

This method is for the enumeration of Coliforms and *E.coli* present in potable water (in the context of

the method, potable water is all post-treated hot and cold water intended to be safe for human consumption) and is based on the Microbiology of Drinking Water (2002) Part 4B.

Limitations: This method does not apply to non-lactose fermenting bacteria.  $\beta$ -glucuronidase activity is

restricted to only 90% of *E.coli*, therefore 10% of *E.coli* will not be detected using this method.

Oxidase tests should not be performed on colonies taken directly from carbohydrate containing

media, particularly if the organism ferments or oxidises the carbohydrate. The low pH of the test

colony and the surrounding media may result in false negative results.

#### 3.0 APPLICATION

A volume of water is filtered through a sterile membrane. The membrane is placed on the solid

Membrane Lactose Glucuronide agar (MLGA) and incubated at  $30 \pm 1^\circ\text{C}$  for 4 hours to allow for

resuscitation followed by  $37 \pm 1^\circ\text{C}$  for 14 hours. The plates are then examined for typical colonies.

Typical coliform colonies appear yellow. Typical *E. coli* colonies appear green, utilising the chromogenic substrate BCIG to detect  $\beta$ -glucuronidase activity. Typical coliform and *E. coli* colonies

are confirmed.

#### 4.0 APPARATUS

4.1 Cycling Incubator,  $30 \pm 1^\circ\text{C}$  and  $37 \pm 1^\circ\text{C}$

4.2 Incubator,  $37 \pm 1^\circ\text{C}$

4.3 Sterilised Membrane Filtration apparatus

4.4 Sterile gridded cellulose acetate 47mm diameter membranes 0.45 $\mu\text{m}$  nominal pore size

4.5 Smooth tipped tweezers

4.6 90mm sterile Petri dishes

4.7 Incubator,  $44 \pm 1^\circ\text{C}$

#### 5.0 REAGENTS

- 5.1 Membrane Lactose Glucuronide Agar (MLGA) Oxoid CM1031
  - 5.2 Lactose Peptone Water (LPW) See Appendix A
  - 5.3 Maximum Recovery Diluent (MRD) Oxoid CM733, Merck 1.12535
  - 5.4 Nutrient Agar (NA) Oxoid CM3, Merck 1.05450
  - 5.5 Oxidase Test Strips Oxoid MB0266A
  - 5.6 MacConkey Agar (MA) Oxoid CM0007
  - 5.7 Tryptone Water (TW) See Appendix B
  - 5.8 Kovacs' Reagent Merck 1.09293
- For more information on media preparation see SOP 3.

## **FOODLABS LABORATORY TEST METHOD M016 ENUMERATION OF TOTAL COLIFORMS AND ESCHERICHIA COLI IN WATERS USING MLGA**

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### **6.0 PROCEDURE**

For more information on sample processing and aseptic techniques see SOP 2.

For the full procedure of membrane filtration techniques see SOP 7.

For more information on supplementary and confirmation kits see SOP 8.

6.1 For drinking waters, filter 100mls. Where high counts are anticipated, prepare serial decimal

dilutions using 90ml amounts of MRD (5.3).

6.2 Before filtering invert the sample container gently five times, to ensure homogeneous distribution of the micro-organisms.

6.3 Using sterile smooth-tipped tweezers (4.5) place a sterile membrane filter (4.4) grid side up,

onto the sterile filtration apparatus (4.3) and secure the sterile filtration funnel.

6.4 Filter the 100ml of sample and/or dilution. Taking care not to damage the membrane, remove

the filter and using the sterile smooth tipped tweezers (4.5).

6.5 Transfer the membrane to a pre-poured and dried plate containing MLGA (5.1). Place the filter on the agar, grid side up, ensuring that no air bubbles are trapped underneath. Replace the lid of the petri dish.

6.6 Invert the plates and incubate in the appropriately labelled cycling incubator at  $30 \pm 1^\circ\text{C}$  for 4

$\pm 1$  hours. The incubator will then cycle to  $37^\circ\text{C}$  for  $14 \pm 1$  hours.

6.7 After incubation, examine the membranes under a good light for typical colonies. It is important to note the presence of pink colonies (non-target organisms) in numbers that may interfere with the growth of coliform bacteria and *E. coli*. If the growth of pink colonies is considered to be such that they may obscure lactose-fermenting colonies, this should be noted on the Sample Record Form. Count all of the yellow and green colonies irrespective of size within 15 minutes of being removed from the incubator as the yellow colouration may change on cooling and standing. All yellow colonies are presumptive non-*E. coli* coliform

bacteria and green colonies are presumptive *E. coli*.

6.8 Select five typical presumptive coliform colonies and five typical presumptive *E. coli* colonies

for confirmation. (Yellow colonies may confirm as *E. coli* as some strains do not express  $\beta$ -glucuronidase and other strains appear negative when first isolated. Occasionally green colonies may not confirm as *E. coli* but may confirm as coliform bacteria). For this reason, both yellow and green colonies must be confirmed for both coliforms and *E. coli*. The Coliform

& *E. coli* Confirmation proforma, EE, should be used to record the confirmation results.

Confirmation for coliform bacteria

6.9 Sub-culture each colony into a universal of LPW (5.2). At the same time, streak the same colony onto a plate of NA (5.4) and MA (5.6).

6.10 Incubate the LPW, NA and MA in an appropriately labelled incubator at  $37 \pm 1^\circ\text{C}$  (4.2) for 24

hours.

6.11 Examine the LPW after 24 hours for acid production (positive reaction is a colour change from

red to yellow) and if the results are negative, re-examine after a further 24 hours of incubation.

6.12 Examine the NA plate after 24 hours to check for purity and carry out an oxidase test (5.5)

following the manufacturer's instructions. Coliforms are oxidase-negative.

6.13 Examine the MA plate after 24 hours for typical coliform bacteria. Coliform colonies will

appear pink to red, mucoid or non-mucoid often with a halo of precipitation. See appendix C for more details on colony morphology.

6.14 Coliform colonies are confirmed if oxidase-negative and produce acid in LPW at  $37^\circ\text{C}$ , see

appendix D for confirmation summary.

Confirmation of *E. coli* bacteria

6.15 Sub-culture each colony into two universals of LPW (5.2) and one tube of TW (5.7). At the

same time, streak the same colony onto a plate of NA (5.4) and MA (5.6).

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6.16 Incubate one LPW and the TW in an appropriately labelled incubator at  $44 \pm 1^\circ\text{C}$  (4.7) for 24

hours.

6.17 Incubate the other LPW, NA and MA in an appropriately labelled incubator at  $37 \pm 1^\circ\text{C}$  (4.2)

for 24 hours.

6.18 Examine the universals of LPW after 24 hours for acid production (positive reaction is a colour

change from red to yellow) and if the results are negative, re-examine after a further 24 hours of incubation at the relevant temperature.

6.19 Examine the NA plate after 24 hours to check for purity and carry out an oxidase test (5.5)

following the manufacturer's instructions. *E. coli* are oxidase-negative.

6.20 Examine the MA plate after 24 hours for typical *E. coli* bacteria. *E. coli* colonies will appear

pink to red, mucoid or non-mucoid often with a halo of precipitation. See appendix C for more

details on colony morphology.

6.21 Add 0.2 - 0.3ml of Kovacs' reagent to the TW after 24 hours of incubation at 44°C.

Indole

production is demonstrated by the rapid appearance of a deep red colour to the upper nonaqueous

layer.

6.22 *E. coli* colonies are confirmed if oxidase-negative, produce acid in LPW at 37°C & 44°C, and

indole in TW at 44°C, see appendix D for confirmation summary.

6.23 When calculating results from serial dilutions, select the plate with the number of typical colonies formed on the membrane between 10-100 (ideally count plates containing between 20-80 colonies). Typical and atypical colonies must not exceed 200 per plate. If there are <10 typical colonies on the lowest serial dilution plate count the number of colonies. If there are >100 typical colonies on the highest serial dilution plate record this as greater than (>) 100.

6.24 The total number of yellow and green colonies is regarded as the presumptive coliform count

and the number of green colonies is the presumptive *E. coli* count.

The number of confirmed coliform bacteria is calculated by multiplying the number of presumptive coliform bacteria by the proportion of the isolates that are both lactose-positive (in LPW) and oxidase-negative.

The number of confirmed *E. coli* bacteria is calculated by multiplying the number of presumptive *E. coli* by the proportion of the isolates that are lactose-positive (in LPW at 37°C and 44°C), produce indole from TW at 44°C and are oxidase-negative, combined with any proportion of yellow colonies isolates that subsequently confirm as *E. coli*.

For more information on plate counting see SOP 15.

## **7.0 CALCULATION**

7.1 If the whole plate can be accurately counted:

count x 100

CFU in 100ml = volume of sample filtered (ml) x dilution factor

## **8.0 EXPRESSION OF RESULTS**

8.1 The coliform and *E. coli* count is rounded to two significant figures and expressed as colony

forming units in 100ml of sample at the temperature and time of incubation.

8.2 If no colonies are isolated (i.e. 100ml of 100 = 0) express the results as Not Detected (ND)

1 x 100

less than (<)

volume of sample filtered (ml) x dilution factor = CFU in 100ml

## **9.0 THEORETICAL LIMIT OF DETECTION**

**FOODLABS LABORATORY TEST METHOD M016  
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9.1 Sensitivity (CFU/100ml) is dependent on the lowest dilution made:

100ml of 100 = Not Detected

100ml of 101 = <10

100ml of 102 = <100

100ml of 103 = <1000

100ml of 104 = <10000

**10.0 QUALITY CONTROL**

10.1 Include an uninoculated plate, two positive controls of *Escherichia coli* and *Klebsiella aerogenes* and a negative control of *Pseudomonas aeruginosa*.

For full information on quality control see SOP 14.

**11.0 HEALTH & SAFETY**

11.1 Normal aseptic precautions for handling micro-organisms should be taken.

11.2 Follow manufacturer's guidelines for preparation of media.

For information on handling micro-organisms see SOP 2 and any relevant risk assessments and COSSH assessment forms.

**12.0 DISPOSAL**

12.1 For more information on waste disposal see SOP 11.

**13.0 REFERENCES**

13.1 BS ISO 8199:2007 Incorporating amendment No. 1 (2008) Water Quality – General Guidance

on the Enumeration of Micro-organisms by Culture.

13.2 The Microbiology of Drinking Water 2002 Part 4B – The Enumeration of Coliform Bacteria and

*Escherichia coli* by a Single Membrane Filtration Technique.

**Appendix A**

Preparation of Lactose Peptone Water

Peptone 10g

Sodium chloride 5g

Lactose 10g

Phenol red 2.5ml of 0.4% m/v aqueous solution

Deionised Water 1litre

Dissolve the ingredients in the water and adjust to pH 7.5 + 0.2. Add the phenol red indicator

and distribute in 5ml volumes into tubes. Autoclave at 115°C for fifteen minutes. Store for 1 month at 2-8°C

## **Appendix B**

Preparation of Tryptone Water

## **FOODLABS LABORATORY TEST METHOD M016 ENUMERATION OF TOTAL COLIFORMS AND ESCHERICHIA COLI IN WATERS USING MLGA**

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Tryptone 20g

Sodium chloride 5g

Deionised Water 1litre

Dissolve the ingredients in the water and adjust to pH 7.5 + 0.2. Distribute in 5ml volumes into

tubes. Autoclave at 115°C for ten minutes. Store for 1 month at 2-8°C

Appendix C

Colony morphology on MacConkey agar.

### **Organism Colour Remarks**

*Escherichia coli* red non-mucoid

*Aerobacter aerogenes* pink mucoid

*Enterococcus* species red minute, round

*Staphylococci* pale pink opaque

*Pseudomonas aeruginosa* green-brown fluorescent growth

Taken from: The Oxoid website on the 19/08/08.

[http://www.oxoid.com/UK/blue/prod\\_detail/prod\\_detail.asp?pr=CM0007&c=UK&lang=EN](http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0007&c=UK&lang=EN)

Appendix D

Confirmation summary

Purity check

on NA

Colony growth

on MA

Oxidase test LPW @ 37°C LPW @ 44°C Indole test

COLIFORM OK Typical Negative Positive Negative Negative

COLIFORM OK Typical Negative Positive Positive Negative  
E. COLI OK Typical Negative Positive Positive Positive

**FOODLABS LABORATORY TEST METHOD M027**  
**DETECTION AND ENUMERATION OF *LEGIONELLA***  
MEMBRANE FILTRATION TECHNIQUE

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**1.0 INTRODUCTION**

*Legionella* species are commonly found in domestic and industrial water systems, including hot water

systems, cooling water systems for air conditioning and industrial purposes, spas, whirlpools, industrial coolants used for machine lubrication and in respiratory therapy equipment. These systems

produce favourable conditions for the growth of *Legionella*.

Some species of *Legionella* are pathogenic and infections manifest in two forms;

Legionnaires

disease or 'legionella pneumonia', and its milder form, pontiac fever. Infection is caused by inhalation of airborne droplets containing the organism. *Legionella pneumophila* is usually the

causative species, but less commonly *Legionella micdadeiae*, *Legionella bozemanii*,

*Legionella feeleii*

and *Legionella dumoffii* have been reported, amongst others as the causative species.

Members of the genus *Legionella* are gram-negative rods which are catalase positive, and are weakly

motile by one or two polar flagella. They have an absolute requirement for L-cysteine and will not

grow in media deficient of this nutrient.

**2.0 SCOPE**

This method is for the isolation of *Legionella* organisms and estimation of their numbers in environmental samples. The method is applicable to all kinds of environmental samples including

potable, industrial and natural waters. This method is based on BS 6068-4.12:1998 ISO 11731:1998

Detection and enumeration of *Legionella*.

Limitations: A negative latex agglutination test does not mean that the culture is not a *Legionella*

species. It only indicates that the culture is not *Legionella pneumophila* serogroups 1 through to 14

nor *L. longbeachae* 1 and 2, *L. bozemanii* 1 and 2, *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei*, *L.*

*anisa*. A cross reaction may occur between *L. pneumophila* serogroup 1 and serogroup 9 due to

naturally occurring group antigens. If both the *L. pneumophila* serogroup 1 and 2-14 reagents agglutinate with the isolate then this cross reaction should be suspected. Cross reactions with the

*Legionella* Species Test Reagent have been reported to occur occasionally with certain serotypes of

other Legionellae (e.g. *L. parisiensis*, *L. sainthelensi*, *L. steigerwaltii*, *L. wadsworthii*, *L. santicrucis*, *L.*

*tusconensis*, *L. gratiana*, *L. cincinatiensis*).

Deviation: ISO 11731:1998 states incubation temperature should be 36 + 1°C, we incubate at 37°C ±

1°C.

### 3.0 APPLICATION

Bacteria, including *Legionella* organisms, in the water sample are concentrated by membrane filtration. To reduce the growth of unwanted bacteria, aliquots of the concentrated sample are subjected to treatment with acid and heat. Treated and untreated test aliquots are then inoculated

onto plates of agar medium selective for *Legionella*, and incubated. Samples containing sufficient

numbers of *Legionella* need not be subject to concentration prior to culture. Plates are incubated and

observed at intervals for the presence of characteristic colonies. Any suspect isolates are subjected to

confirmation and identification tests.

### FOODLABS LABORATORY TEST METHOD M027

### DETECTION AND ENUMERATION OF *LEGIONELLA*

#### MEMBRANE FILTRATION TECHNIQUE

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### 4.0 APPARATUS

4.1 Incubator 37°C ± 1°C

4.2 1 ml pipette

4.3 0.250ml pipette

4.4 Sterile pipette tips

4.5 Smooth Tipped Tweezers

4.6 Water Bath 50°C ± 1°C

4.7 Laboratory Timer

4.8 Sterile gridded cellulose acetate 47mm diameter membranes 0.45µm nominal pore size

4.9 Lidded, white vented plastic containers

4.10 Sterile 30ml universals

4.11 5ml pipette

4.12 Vortex Mixer

4.13 Binocular Plate Microscope

4.14 Sterilised Membrane Filtration Apparatus

4.15 90mm sterile Petri dishes, triple vent

4.16 Sterile disposable spreaders

4.17 Ultra Violet Lamp (360 + 20 nm)

4.18 Safety Cabinet

### 5.0 MEDIA AND REAGENTS

5.1 Legionella CYE Agar Base Oxoid CM655

5.2 Legionella BCYE Growth Supplement Oxoid SR110C

5.3 Legionella GVPC Selective Supplement Oxoid SR152E

5.4 Nutrient Agar Oxoid CM3 Merck 1.05450

5.5 25ml volumes of sterile de-ionised water

5.6 10x Concentrated Acid Buffer pH2.2 (see Appendix A)

5.7 Legionella Latex Kit Oxoid DR800

5.1 + 5.2 + 5.3 will hereafter be referred to as GVPC.

5.1 + 5.2 will hereafter be referred to as BCYE.

For more information on media preparation see SOP 3.

### 6.0 PROCEDURE

For more information on sample processing and aseptic techniques see SOP 2

For the full procedure of membrane filtration techniques see SOP 7.

For more information on pour plate technique see SOP 5.

For more information on the *Legionella* latex kit see SOP 8.

6.1 Liquid samples may be plated directly if the number of *Legionella* is expected to exceed 105.

Because the number of *Legionella* in any given sample is not known, concentration techniques are usually performed. Dilutions (if necessary) should be prepared using sterile de-ionised water.

6.2 Before filtering, invert the sample container gently five times to ensure homogeneous distribution of the micro-organisms.

6.3 Using sterile smooth-tipped tweezers (4.5), place a sterile membrane filter (4.8), grid side up

onto the filtration apparatus (4.14) and secure the sterile filtration funnel.

6.4 Filter the desired volume of sample and/or dilution and, taking care not to damage the membrane, remove the funnel and again using the sterile smooth tipped tweezers (4.5), remove the membrane. Place all membranes from one sample into a universal containing 25ml sterile de-ionised water, taking care to avoid any loss of deposit. A maximum of four membranes may be put in 25ml of sterile water, a maximum of eight membranes may be put in a 90ml bottle containing 25ml of sterile water. Not more than eight membranes may be

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used for any one sample. Where less than 1 litre of water is filtered this must be clearly stated on the SRF and final test certificate. For storage instructions of the concentrate, see Appendix B.

6.5 Shake concentrates vigorously or place on a vortex mixer (4.12) for between 2 to 5 minutes to remove bacterial deposits from the membrane.

6.6 This suspension is the concentrated sample and is analysed using the three separate procedures below. For each treatment, a colour coded sticky label is attached to the plate: white = untreated, pink = heat treatment and blue = acid treatment. If there is any delay between shaking/vortexing, ensure the concentrate is shaken 5 times immediately prior to the following treatments.

**6.7 Untreated Sample**

Plate out 0.25ml of the concentrated sample with no additional treatment onto a pre-poured and dried plate containing GVPC.

**6.8 Heat Treated Sample**

Aseptically add 1ml of the concentrated sample to an empty, sterile universal and place in a water bath (4.6) at 50 + 1°C for 30 + 2 minutes using the laboratory timer (4.7). Remove from

the water bath and plate out 0.25 ml onto a pre-poured and dried plate containing GVPC agar.

**6.9 Acid Treated Sample**

Aseptically pipette (4.11) 10ml of the concentrated sample to an empty, sterile universal and add 1ml of pH 2.2 acid buffer. Allow to stand at ambient temperature for 5 + 0.5 min. Plate out

0.25ml onto a pre-poured and dried plate containing GVPC agar.

6.10 Spread the inoculum from each treatment carefully until it is absorbed, taking no more than

one minute and using a new sterile spreader (4.16) for each dilution. Allow the inoculated media to stand until the inoculate has been completely absorbed and attach the lid to each plate using a blank sticky label. Invert the plates and place in lidded, white vented plastic containers (4.9). Label these with the dates that the plates are to be examined and incubate in one of the appropriately labelled incubators (4.1) making sure to record the incubators' laboratory reference number (LRN) on the *Legionella* day sheet for complete traceability.

6.11 The plates should be examined at three intervals between 2 -12 days during the incubation

period. Colonies visible at <72 hours should be marked with a red pen. Thereafter, additional colonies should be marked at the second examination (6-8 days) with a green pen and at the third examination (10-12 days) with a blue pen.

6.12 Colonies of *Legionella* are often white-grey-blue-purple in colour, but may be brown, pink,

lime-green or deep red. They are smooth with an entire edge and exhibit a typical ground-glass appearance (opalescence), which can be visualised under the binocular plate microscope (4.13) with oblique halogen lighting. Under Ultra Violet light (4.17) colonies of several species (*L. bozenanii*, *L. gormanii*, *L. dumoffii*, *L. anisa*, *L. cherrii*, *L. steigerwaltii*, *L. gratiana*, *L. tucsonensis* & *L. parisiensis*) will autofluoresce brilliant white. *L. rubrilucunc* & *L.*

*erythra* appear red. Colonies of *L. pneumophila* appear dull green often tinged with yellow. The colour of fluorescence can help to differentiate colonies in samples containing different species of *Legionella*. To avoid the possibility that *Legionella* cells could be killed, plates should not be exposed to UV light for longer than necessary. Confirmation of these species is subject to the limitations of the confirmation kit (see section 2.0 scope limitations). If experienced staff suspect a presumptive colony outside the scope of the kit, if required by the customer, these may be sent to a reference lab for further analysis.

6.13 Record the number of each characteristic colony type present. On GVPC agar, colonies may

develop in less than three days. Large colonies (>5mm in diameter) visible at less than 72 hours should be marked with a red pen and are then not considered to be *Legionella* spp. Small colonies (0.1mm-5mm in diameter), visible at less than 72 hours should be examined for morphology typical of *Legionella* spp. All suspect colonies should be confirmed as described in section 6.15.

6.14 Heterotrophic bacteria, fungi and protozoa will grow on selective media, therefore it is important that a distinction is made between non-*Legionella* and *Legionella* colonies. If there is heavy growth of heterotrophic organisms or fungi which obscures plate reading, this should be recorded with a letter C on the sample record form. If heavy growth is observed on all three of the GVPC plates, the Technical Manager should be informed.

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6.15 Select three colonies characteristic of *Legionella* from the GVPC plate. Streak each colony

onto both Nutrient agar and BCYE agar using the same 1µl loop for both medias but separate loops for each colony. This ensures the same colony is tested throughout.

6.16 Incubate aerobically for at least 48 hours at 37°C in the Legionella confirmation incubator,

LRN 48. Those colonies that grow on the BCYE agar but not on the Nutrient agar must be confirmed by using a *Legionella* latex kit, following the manufacturer's instructions (see Appendix C). This enables a confirmed count of each *Legionella* colony type to be made. Care should be taken when interpreting growth on Nutrient agar plates as a heavy streak can be interpreted as bacterial growth when it is not.

## **7.0 CALCULATION**

7.1 Regard each treatment as a separate test and calculate the level of confirmed organisms of each type in each sample using the formula below.

Highest confirmed count of 3 treatments x volume(ml) of re-suspension diluent  
Volume (ml) plated x No. of litres filtered

7.2 Calculate confirmed counts for each treatment by multiplying the suspect count by the proportion of colonies confirmed as *Legionella*.

## **8.0 EXPRESSION OF RESULTS**

8.1 If present, report the highest confirmed count as the level of *Legionella* in the sample. Depending on the serotyping results, report as *Legionella* serogroup 1, *Legionella* serogroup 2-14 or *Legionella* species present in the sample volume and include the calculated count. Report absence as 'Not Detected' in the sample volume.

## **9.0 THEORETICAL LIMIT OF DETECTION**

9.1 If one litre of sample is filtered and re-suspended in 25ml and 0.25ml is plated, the test sensitivity is 100 CFU/litre.

## **10.0 QUALITY CONTROL**

10.1 Include an uninoculated plate, a positive control of *Legionella pneumophila* and a negative

control of *E.coli* in the analysis. Control plates should be checked at <72hrs and recorded on the *Legionella* day sheet at the 6-8 day and 10-12 day examinations.

For full information on quality control see SOP 14.

## **11.0 HEALTH & SAFETY**

11.1 Normal aseptic precautions for handling micro-organisms should be taken.

11.2 Follow manufacturer's guidelines for preparation of media.

11.3 *Legionella* spp. are highly pathogenic organisms if inhaled; liquid cultures and suspensions of organisms must be handled in the Safety Cabinet (4.18).

11.4 The following stages of this method, where possible, take place in the Safety Cabinet:

Plating

Counting and confirmation

For information on handling micro-organisms see SOP 2 and any relevant risk assessments and COSSH assessment forms.

## **12.0 DISPOSAL**

12.1 For more information on disinfection and waste disposal see SOP 11.

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## **13.0 REFERENCES**

13.1 BS 6068-4.12:1998/ISO 11731:1998 Water Quality- Detection and enumeration of *Legionella*.

13.2 Legionnaires Disease. The control of *Legionella* bacteria in water systems. Approved Code of

Practice and Guidance L8. 2000

13.3 BS ISO 8199:2007 Incorporating amendment No. 1 (2008) Water Quality Guidance on the enumeration of Micro-organisms by Culture

**Appendix A**

10x Concentrated Acid Buffer

Reagent A is 17.4g of concentrated hydrochloric acid in 100ml distilled water (sp gr 1.18, minimum assay 35.4%).

Reagent B is 14.9g of potassium chloride in 100ml of distilled water.

Mix 3.9mls of 2.0mol/l hydrochloric acid (Reagent A) with 25mls of 2.0mol/l potassium chloride

(Reagent B). Adjust to pH  $2.2 \pm 0.2$  by addition of 1.0mol/l potassium hydroxide. Sterilise by autoclaving at 121oC for 15 minutes. Acid buffer can be stored at room temperature for no longer

than 1 month.

**Appendix B**

Storage Instructions

The time interval between sample date and its concentration should ideally be less than 2 days but

must not exceed 5 days. Concentrated samples may be stored in the dark at  $6 \pm 2^{\circ}\text{C}$ . The time interval from date concentrated to date plated should not exceed 9 days.

**Appendix C**

Legionella Sample Record Form Nomenclature

1. Typical 2. Atypical 3. No Growth C. Confluent Growth

BCYE NA LATEX TEST KIT

1 3 Requires Latex

3 1 No further work required

3 3 Requires re-streaking onto BCYE and NA

2 1 No further work required

2 3 Requires Latex