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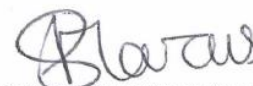
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1. INTRODUCTION

This document describes methods for the detection and enumeration, in potable water, cooling water and sewage water, of total coliform bacteria, faecal coliform bacteria, E.coli, and heterotrophic bacteria. It also refers to a method for the detection and enumeration of Legionella.

2. SUPPORTING CLAUSES

2.1 SCOPE

This guideline provides power plant staff with a compact comprehensive document, which specifies the microbiological methods to be followed for analysis of potable water, sewage effluent, cooling water and transformer oils/diesels as required.

2.1.1 Purpose

To define the minimum microbiological methods to be followed, as required, for the analysis of potable water, sewage effluent, cooling water and transformer oils/diesels.

2.1.2 Applicability

The guideline is Eskom specific, referring to the potable water, sewage effluent, cooling water and transformer oil/diesel systems.

2.2 NORMATIVE/INFORMATIVE REFERENCES

2.2.1 Normative

ISO 17025 standard.

Eskom's Standard 240-55864764.

Chemistry and Microbiology Standard for Condenser Cooling Water, Rev3, 240-55864767

2.2.2 Informative

- [1] SABS Method 221-2001. Microbiological analysis of water-General test methods, 4th edition
- [2] Standard Methods for the examination of water and waste water, 1989 17th edition, pp. 4-79.
- [3] Slanetz, L.W. and Bartley, C.H. (1957) J. Bacteriolog vol 74, pp 591-595

2.3 DEFINITIONS

None

Definition	Description
Planktonic	Bacteria which are free-living within the medium
Sessile	Bacteria which adhere to various surfaces to form a biofilm

2.3.1 Classification

- a. **Controlled Disclosure:** Controlled Disclosure to External Parties (either enforced by law, or discretionary).

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2.4 ABBREVIATIONS

Abbreviation	Description
CFU	Colony Forming Units
SRB	Sulphate reducing bacteria

2.5 ROLES AND RESPONSIBILITIES

- The Chemical Services Manager shall be responsible for ensuring that the requirements of this document are taken into account.
- Microbiologists shall make sure that all analysis carried out meet the ISO 17025 standard requirements.
- Chemical CoE shall be accountable for periodic revision of this document under the SCOT processes.
- Senior consultant on microbiology shall be responsible for consultation on application of this document.

2.6 PROCESS FOR MONITORING

None

2.7 RELATED/SUPPORTING DOCUMENTS

- Legionella standard
- Cooling water standard
- Chemistry

3. MICROBIOLOGICAL METHODS GUIDELINES

3.1 BASIS OF METHOD

- a. These microbiological methods are to act as a standardisation tool between the various microbiology laboratories within Eskom. This will allow comparison between different analyses completed in the laboratories.
 - Only a qualified microbiologist or similarly trained person i.e. analyst should complete the analyses.
 - All analyses should be conducted in duplicate and an average of the two counts reported. If the difference in counts (pre calculation for dilution) is more than 100, redo the analysis.
 - A qualified microbiologist must validate?conduct all the relevant calculations.
 - All filter techniques require that the filters be placed grid side up on the agar.
 - When flame sterilizing, all safety precautions must be observed.
 - When conducting multiple tests, petri-dishes can be stacked to a max of 5 and should be taped, for ease of handling.
- b. The analyses included in this guideline are of the following samples:
 - Potable Water
 - Sewage Effluent

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- Cooling Water
- Transformer oil/Diesels/Gearbox & Lubricating Oils

3.1.1 Potable water

Potable water is classified as water purified to standards that render it fit for human consumption. The methods followed for this analysis are specified in SANS Method 221-2001, 4th edition. The standard specifies that

- Total aerobic bacteria (1),
- Total coliforms (2) and
- Faecal coliforms (E coli) (3)

are enumerated. These methods are shown in the Appendix A, as Methods 1-3. In addition Eskom's Standard 240-55864764 for potable water specify that a free chlorine residual of less than or equal to 5mg/kg be maintained throughout the distribution system; chlorine concentration can be measured as described in Method 4, but any approved DPD (**N,N Diethyl-1,4 Phenylenediamine**) colorimetric method may be applied.

3.1.2 Sewage Effluent

- a. Sewage effluent quality is regulated by legislation which specifies that any sewage effluent being released into the environment contain less than 1 E coli CFU per 100ml and no trace of residual chlorine.
- b. Within Eskom the analyses completed are;
 - Total aerobic bacteria (1),
 - Total coliforms (2),
 - Faecal coliforms (E coli) (3) and
 - Faecal Streptococci (5),
- c. These methods are shown in the Appendix A as Methods 1-3 and 5. The analyses for free and total chlorine are imperative for sewage effluent analysis and are shown in Method 4 in Appendix A.
- d. In the case of severely contaminated sewage effluent it may be necessary to complete dilutions to obtain a true indication of the total aerobic bacteria load within the sample. The method for dilutions is shown in Method 6 in the Appendix A.

3.1.3 Cooling water

- a. The Chemistry and Microbiology Standard for Condenser Cooling Water, Rev3, 240-55864767, specifies that the following analyses shall be completed:
- b. Planktonic analyses for:
 - Total aerobic bacteria (1)
 - Total anaerobic bacteria (7)
 - Hydrogen sulphide producers (8)
- c. Sessile analyses for:
 - Total aerobic bacteria (1)
 - Total anaerobic bacteria (7)

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- Hydrogen sulphide producers (8)
- d. These methods are expanded in Methods 1, 7 and 8 in the Appendix. Sessile samples are obtained by sampling a Robbins Device stud, or similar biofilm monitoring device, into 100ml of sterile diluent, containing glass beads and gently shaking the container to disperse the biofilm into the water. This is then considered as the 10⁻² dilution.

3.1.4 Transformer oil/Diesels/Gearbox & Lubricating Oils

- a. These hydrocarbon substrates are susceptible to contamination by bacteria, fungi and moulds if water is present within the system. The water allows the microbes to grow on the interface, drawing sustenance from the hydrocarbon source and living within the water phase. Although there are several fungi that contaminate hydrocarbons, one of the most problematic is *Hormoconis resinae* which secretes a resin as one of its by-products. This resin blocks filters and sticks to pipe surfaces, reducing the heat transfer. Although there are no Eskom standards for oil microbiology it is suggested that either
- b. Fungi, (9 or 10) and
- c. Bacteria, (11) (optional, in case of contamination), or
- d. Yeasts and (9)
- e. *Hormoconis resinae* (9)

are enumerated as well as the water content. These methods are shown in Method 9, 10 and 11 of the Appendix.

3.1.5 Legionella

- a. Legionella analysis cannot be conducted at any of the Eskom power station laboratories until the laboratory is ISO 11731 accredited to carry out the analysis. Accurate sampling however is essential. Legionella samples are collected in sterile one litre containers.
- b. If the sample is taken from a large water body then a grab sampling technique is used. The use of this technique prevents contamination of the bottle. The bottle is filled by plunging it into the water a few centimetres below the surface and held there with its mouth directed towards the current. If there is no current, the bottle should be pushed in a direction away from the hand so as to create a current.
- c. If the sample is taken from a tap or valve, the water must first be allowed to run for at least two minutes or the point can be swabbed with ethanol and flamed to ensure that a representative sample is obtained. It is important to observe safety precautions eg. wear latex gloves and wash your hands after sampling with antibacterial soap.
- d. If the sample cannot be tested immediately, then it must be stored at 4°C either in a fridge or a cooler box and must be tested within twenty four hours.

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March 2017	2	M Ndwambi	Final Rev 2 Document for Authorisation and Publication

6. DEVELOPMENT TEAM

The following people were involved in the development of this document:

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7. ACKNOWLEDGEMENTS

- None

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APPENDIX A

1. DETERMINATION OF THE TOTAL AEROBIC BACTERIA IN WATER

1.1 METHOD NO 1

1.2 BASIS OF METHOD

The water is analysed using an agar medium specific for the growth of bacteria

1.3 INTERFERENCES

Inadequate neutralisation of the chlorine present in the sample.

Uncontrolled temperature during sample transportation(must be between 2°C-8°C during transportation)

1.4 RANGE OF APPLICATION

Not applicable

1.5 SAMPLING PROCEDURE

Samples of water should be taken in sterile 500ml screw capped glass bottles. The bottles must contain 1ml of 10% sodium thiosulphate solution, to neutralise the chlorine that may be present. The bottles will be prepared and used within the following week. If the sample is taken from a tap or valve, the water must be first allowed to run for at least two minutes to ensure that a representative sample is obtained. If the sample cannot be tested immediately, then it must be stored at 4°C either in a fridge or cooler box, and must be tested within twenty four hours. Chlorine determinations must be carried out at the time of sampling.

1.6 TIME REQUIRED FOR ANALYSIS

- Preparation and sterilisation of media - 45 mins.
- Analysis of sample - 2mins.
- Counting of colonies - 5 mins.

1.7 TOLERANCES

Not applicable

1.8 SPECIAL APPARATUS

- Sterile petri dishes.
- Sterile pipettes.
- Bunsen burner.
- Sterile tips

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1.9 SPECIAL REAGENTS

- Sterile Nutrient Agar/Plate Count Agar.
- Sterile Ringers solution (if necessary)

1.10 ANALYTICAL PROCEDURES

- a. Using a 1ml pipette, aseptically transfer 1ml of the water sample into petri dishes that have been marked with the date of the test and the origin of the sample.
- b. In the case of sewage or raw water, dilutions of the sample must be carried out. The dilution factor must also be marked on the petri dish.
- c. Melt the agar and allow to cool to approximately 47°C.
- d. Flame the mouth of the agar bottle and add approximately 10ml of agar to the petri dish. Mix the sample and agar thoroughly and allow to cool until the agar has set. (Do not expose the plates to the air as this will lead to contamination of the agar.)
- e. Invert the plates. Incubate for 48 hours at 35°C ($\pm 1^\circ\text{C}$).
- f. Count the colonies that form inside the agar and report count CFU/ml. If there are dilutions, the technician will multiply the number of colonies by the dilution factor and report the count CFU/ml.

NOTE: It may be necessary to make more than one dilution, to ensure that the correct number of colonies are obtained on one of the plates.

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2. DETERMINATION AND CONFIRMATION OF TOTAL COLIFORM BACTERIA IN WATER

2.1 METHOD NO 2

2.2 BASIS OF METHOD

The water is analysed and confirmed using an agar medium specific for growth of coliform bacteria.

2.3 INTERFERENCES

After addition of the ethanol, the medium must be allowed to stand for 10 minutes to ensure complete solution of basic fuchsin.

2.4 RANGE OF APPLICATION

Not applicable.

2.5 SAMPLING PROCEDURE

Samples of water should be taken in sterile 500ml screw capped glass bottles. The bottles must contain 1ml of 10% sodium thiosulphate solution, to neutralise the chlorine that may be present. The bottles will be prepared and used within the following week. If the sample is taken from a tap or valve, the water must be first allowed to run for at least two minutes to ensure that a representative sample is obtained. If the sample cannot be tested immediately, then it must be stored at 4°C either in a fridge or cooler box, and must be tested within twenty four hours. Chlorine determinations must be carried out at the time of sampling.

2.6 TIME REQUIRED FOR ANALYSIS

- Preparation and sterilisation of equipment - 20 mins.
- Analysis of one sample - 2 mins.
- Counting of colonies - 5 mins.

2.7 TOLERANCES

Not applicable

2.8 SPECIAL APPARATUS

- Sterile petri dishes
- Sterile membrane filtration units
- Forceps
- Sterile, gridded membrane filters - 45mm diameter and a 0.45µm pore size

2.9 SPECIAL REAGENTS

Sterile M- Endo Agar LES

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2.10 ANALYTICAL PROCEDURES

- a. Prepare M-Endo Agar LES as per instructions on the bottle.
- b. Allow to cool to approximately 47°C. Note that the medium must NOT be autoclaved. Add approximately 10ml of cooled agar to a petri dish and allow to set.
- c. Sterilise the filter apparatus by dipping in alcohol or methylated spirits and igniting.
- d. Sterilise the forceps by dipping in alcohol or methylated spirits and igniting.
- e. Aseptically transfer a sterile membrane filter to the filtration apparatus using the sterile forceps.
- f. Filter a known volume of water through the filter, usually 100ml in the case of potable water. Sewage water may need to be diluted. If less than 1ml or a dilution is to be used, then sterile Ringers solution must be added to the filter apparatus before filtration, to allow an even distribution of the sample over the surface of the filter.
- g. Transfer the filter to the petri dish using sterile forceps. Place the filter grid side up on the surface of the agar using a rolling action to eliminate any air bubbles.
- h. Invert the petri dishes and incubate at 35°C ($\pm 1^\circ\text{C}$) for 24 hours.
- i. Count those plates containing between 20 and 80 colonies. All colonies with a golden-green metallic sheen are regarded as total coliforms.

2.11 CONFIRMATION AND REPORTING

CONFIRMATION (ON POTABLE WATER ONLY)

2.12 SAMPLING PROCEDURE

Confirmatory tests will be conducted on each suspect colony (a maximum of 10), into tubes of lactose peptone water.

2.13 TIME REQUIRED FOR ANALYSIS

- Preparation and sterilisation of media - 45 mins.
- Analysis of one sample - 1min.

2.14 TOLERANCES

Not applicable

2.15 SPECIAL APPARATUS

- Test-tubes
- Durham tubes
- Inoculation loop

2.16 SPECIAL REAGENTS

- Lactose peptone water

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- Lactose peptone water may be prepared as follows:
- Peptone 10g
- Lactose 10g
- Sodium chloride 5g

Dissolve the ingredients in distilled water and dilute to 1L. Add the phenol red indicator. Dispense 10ml volumes into tubes or bottles each containing an inverted fermentation (Durham) tube. Sterilise by autoclaving at 121°C for 10 mins.

2.17 ANALYTICAL PROCEDURES

- a. Subculture each suspect colony (min of 5 colonies) into tubes of lactose peptone water and incubate at 35°C ± 1°C for 24 hours. NOTE: Prior to incubation, ensure that the fermentation (Durham) tubes are completely filled with medium and contain no air. Examine for gas formation, this is indicated by the formation of gas at least sufficient to fill the cavity of the top of the fermentation (Durham) tube. The formation of gas confirms the presence of coliforms.
- b. The samples must be done in duplicate to compensate for any operator errors.
- c. Positive and negative controls must be conducted on the lactose peptone solution.

2.18 REPORTING

2.19 PROCEDURE

- a. Confirmatory tests will be conducted on each suspect colony (maximum of 10).
- b. The count reported is the number of positive results to a maximum of 10.
- c. Thereafter the positive results obtained are related to a percentage of the count.
- d. I.e. if only 3 of the 10 confirmations are positive only 30% of the total count is reported as positive.
- e. $\frac{3}{10} = 0.3 \times 100 = 30\%$ if the initial count was 35
- f. $\frac{\text{Count obtained (35)} \times \text{percentage acceptable (30\%)}}{100} = \text{Count} \times \text{dilution factor} = \text{Count}$
- g. The results must be reported as total coliforms per 100ml of sample eg. if 25 colonies are counted on the plate with a 10⁻³ dilution of 1ml, the result is calculated as follows:
- h. $\frac{25}{0.001} = 25\,000 \times 10^6$ total coliforms per 100ml

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3. DETERMINATION OF THE NUMBER OF FAECAL COLIFORM BACTERIA AND CONFIRMATION OF E COLI

3.1 METHOD NO 3

3.2 BASIS OF METHOD

The water is analysed using an agar medium specific for the growth of faecal coliform bacteria

3.3 INTERFERENCES

After the addition of Rosolic acid, boil the agar for 1 minute then allow it to cool approximately to 47°C.

3.4 RANGE OF APPLICATION

Not applicable

3.5 SAMPLING PROCEDURE

Samples of water should be taken in sterile 500ml screw capped glass bottles. The bottles must contain 1ml of 10% sodium thiosulphate solution, to neutralise the chlorine that may be present. The bottles will be prepared and used within the following week. If the sample is taken from a tap or valve, the water must be first allowed to run for at least two minutes to ensure that a representative sample is obtained. If the sample cannot be tested immediately, then it must be stored at 4°C either in a fridge or cooler box, and must be tested within twenty four hours. Chlorine determinations must be carried out at the time of sampling.

3.6 TIME REQUIRED FOR ANALYSIS

- Preparation and sterilisation of equipment - 45 mins.
- Analysis of one sample - 2 mins

3.7 TOLERANCES

Not applicable

3.8 SPECIAL APPARATUS

- Sterile petri dishes
- Sterile membrane filtration units.
- Vacuum pump.
- Forceps.
- Sterile, gridded membrane filters - 47mm diameter and a 0,45µm pore size.

3.9 SPECIAL REAGENTS

- Sterile M-FC Agar.

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3.10 ANALYTICAL PROCEDURE

- a. Prepare the M-FC Agar as per instructions on the bottle.
- b. Allow to cool to approximately 47°C. Note that the medium must NOT be autoclaved. Add approximately 10ml of the cooled agar to a petri dish and allow to set.
- c. Sterilise the filter apparatus by spraying with ethanol or methylated spirits and igniting.
- d. Aseptically transfer a sterile membrane filter to the filtration apparatus using the sterile forceps.
- e. Filter a known volume of sample through the filter, usually 100ml in the case of potable water. Sewage and raw water may need to be diluted. If less than 1ml or a dilution is to be used, then sterile Ringers solution must be added to the filter apparatus before filtration, to allow an even distribution of the sample over the surface of the filter.
- f. Transfer the filter to the petri dish using sterile forceps. Place the filter grid side up on the surface of the agar using a rolling action to eliminate any air bubbles.
- g. Invert the petri dishes.
- h. Incubate in an incubator at 44.5°C (±1°C) for 24 hours.
- i. Count all colonies that are dark blue as faecal coliforms.
- j. The samples must be done in duplicate to compensate for any operator errors.
- k. The results must be reported as faecal coliforms per 100ml.

3.11 CONFIRMATION ON POTABLE WATER ONLY

3.12 BASIS OF METHOD

- a. There are two confirmatory tests that should be conducted on faecal coliforms. The confirmation tests are the Indole Test and Lactose fermentation.

NOTE: Both tests must be carried out for positive identification. Only 1 colony needs to be checked as the SABS standard requires less than 1 faecal coliform in order to be positive.

3.12.2 Indole test

- a. Tryptone water has been specially evolved as a substrate for the production of indole.
- b. The faecal coliforms are able to break down the amino acid tryptophan to form indole.

3.12.3 Lactose Fermentation

The medium utilised for this test is Brilliant Green Bile (2%) Broth. This medium may be used to confirm the presence of members of the coli-aerogenes group of organisms. The brilliant green content suppresses anaerobic lactose fermenters such as *Clostridium perfringens* which may give rise to false positive results at 44.5°C.

3.13 SAMPLING PROCEDURE

3.13.1 Indole test

Pick off the colony to be tested from the agar plate with a sterile inoculation loop and inoculate into 5ml of the sterile Tryptone water.

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3.13.2 Lactose fermentation

Pick off the colony to be tested from the agar plate with a sterile inoculation loop and inoculate into 10ml of Brilliant Green Bile (2%)

3.14 SPECIAL APPARATUS

- Durham tubes.
- Bunsen burner.
- Test tubes.
- Inoculation loop.

3.15 SPECIAL REAGENTS

3.15.1 Indole test

- a. Tryptone water is commercially available or may be prepared as follows:
 - 500 ml of sterile demin water
 - 5g of Sodium chloride
- b. Make up the Tryptone Water using the commercial product or as described above. Distribute 5ml into capped test tubes and sterilise.
- c. Kovac's reagent can be obtained commercially or prepared as follows:
 - 5g of paradimethylaminobenzaldehyde
 - 75ml of amyl alcohol
 - 25ml of concentrated hydrochloric acid

3.15.2 Lactose fermentation

- a. The medium is commercially available or may be prepared as follows:
 - 10g of Peptone
 - 10g of Lactose
 - 20g of Ox bile (purified)
 - 0.0133g of Brilliant green
 - pH \pm 7.4 must be obtained
- b. Add 40g of the above mentioned medium to 1 litre of distilled water and mix well. Dispense 10ml of the broth into capped test tubes containing Durham tubes and sterilise.

3.16 ANALYTICAL PROCEDURE

3.16.1 Indole test

- a. Incubate the test tube for 24 hours at 44.5 °C (\pm 1°C).

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- b. After incubation, add 0.5ml of Kovac's reagent.
- c. Allow to stand for 10 minutes. A dark red colour in the amyl alcohol surface layer constitutes a positive indole test. No colour change constitutes a negative indole test.

3.16.2 Lactose fermentation

- a. Inoculate the sterile test tube with the colony to be tested using a sterile inoculation loop.
- b. Incubate at 44.5°C ($\pm 1^\circ\text{C}$) for 24 hours.
- c. Examine for turbidity and the production of gas (the gas will be entrapped in the Durham tube). If both these reactions are observed, this constitutes a positive result. If turbidity is observed without gas production, this constitutes a negative result. Positive and negative control samples will be run with each set of confirmations.

3.17 REPORTING

3.18 PROCEDURE

- a. Confirmatory tests will be conducted on each suspect colony to a maximum of 10.
- b. The count reported is the number of positive results to a maximum of 10.
- c. Thereafter the positive results obtained are related to a percentage of the count.
- d. If only 3 of the 10 confirmations are positive only 30% of the total count is reported as positive.
- e. $\frac{3}{10} = 0.3 \times 100 = 30\%$ if the initial count was 35
- f. Count obtained (35) x percentage acceptable (30%) = Count x dilution factor = Count
100
- g. The results must be reported as total coliforms per 100ml of sample eg. if 25 colonies are counted on the plate with a 10^{-3} dilution, the result is calculated as follows:
- h. $\frac{25}{0.001} = 25\,000$ or $\times 10^6$ total coliforms per 100ml

4. TEST FOR FREE AND TOTAL RESIDUAL CHLORINE VALUES

4.1 METHOD NO 4

4.2 BASIS OF METHOD

- a. To determine free and total chlorine values in water samples by means of a colorimetric test.
- b. Sewage water samples need to be tested for free and total residual chlorine. Potable water samples need to be tested for free residual chlorine only.

4.3 INTERFERENCES

None

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4.4 RANGE OF APPLICATION

Not applicable

4.5 SAMPLING PROCEDURE

- a. Fill one 10ml cell to the mark with sample taken directly from the sample point tap or valve and place it in the left hand compartment of the comparator (the blank).
- b. Rinse the other cell with some of the sample also taken from the sample point and fill it up to the 10ml mark.

4.6 TIME REQUIRED FOR ANALYSIS

- Analysis of one sample – 3 min.

4.7 TOLERANCES

Not applicable

4.8 SPECIAL APPARATUS

- Lovibond “1000” comparator
- Lovibond “1000” 10ml cells
- Standard Lovibond discs (3/40 A, 3/40 B).

4.9 SPECIAL REAGENTS

- DPD Number 1 tablets
- DPD Number 3 tablets

4.10 ANALYTICAL PROCEDURE

- a. Place one DPD Number 1 tablet in the second cell and allow it to dissolve. If necessary assist by pre-crushing the tablets.
- b. Place the cell in the right hand compartment of the comparator and face a sunlight source.
- c. Revolve the appropriate disc until colours in the two windows match.
- d. Record the figure in the indicator window.
- e. The figure indicated in the window represents the free residual chlorine in mg/l-1 or ppm.
- f. Place a crushed DPD Number 3 tablet into the cell already containing the DPD number 1 tablet and allow to dissolve.
- g. Place the cell in the right hand compartment and face a white light source.
- h. Revolve the disk until the colours in the two windows match.
- i. Record the figure indicated in the indicator window.
- j. The figure represents the total chlorine in mg-1.

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5. DETERMINATION OF THE NUMBER OF FAECAL STREPTOCOCCI

5.1 METHOD NO 5

5.2 BASIS OF METHOD

The water is analysed using an agar medium specific for the growth of faecal streptococci.

5.3 INTERFERENCES

None

5.4 RANGE OF APPLICATION

SEWAGE ANALYSIS ONLY.

5.5 SAMPLING PROCEDURE

- a. Samples of water should be taken in sterile 500ml screw capped glass bottles. The bottles must contain 1ml of 10% sodium thiosulphate solution, to neutralise the chlorine that may be present. The bottles will be prepared and used within 7 days.
- b. If the sample is taken from a large water body then a grab sampling technique is used. The use of this technique prevents contamination of the bottle. The bottle is filled by plunging it into the water a few centimetres below the surface and held there with its mouth directed towards the current. If there is no current, the bottle should be pushed in a direction away from the hand so as to create a current.
- c. If the sample is taken from a tap or valve, the water must first be allowed to run for at least two minutes or the point can be swabbed with ethanol and flamed to ensure that a representative sample is obtained. It is important to take care when handling sewage samples and observe safety precautions, e.g. wear rubber gloves and wash your hands after sampling.
- d. If the sample cannot be tested immediately, then it must be stored at 4°C either in a fridge or a cooler box and must be tested within twenty four hours. However, chlorine determinations should be carried out at the time of sampling.

5.6 TIME REQUIRED FOR ANALYSIS

- Preparation and sterilisation of equipment - 45 mins
- Analysis of one sample - 5 mins

5.7 TOLERANCES

Not applicable

5.8 SPECIAL APPARATUS

- Sterile petri dishes (65mm).
- Sterile membrane filtration units.

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- Forceps.
- Sterile, gridded membrane filters - 47mm diameter and a 0.45µm pore size.

5.9 SPECIAL REAGENTS

Sterile Slanetz and Bartley Agar

5.10 ANALYTICAL PROCEDURE

- a. Prepare the Slanetz and Bartley Agar as per instructions on the bottle.
- b. Allow to cool to approximately 47°C. Note that the medium must not be autoclaved.
- c. Add approximately 10ml of the cooled agar to a 65mm petri dish and allow to set.
- d. Sterilise the filter apparatus by spraying with alcohol or methylated spirits and igniting.
- e. Sterilise the forceps by dipping in alcohol or methylated spirits and igniting.
- f. Aseptically transfer a sterile membrane filter to the filtration apparatus using the sterile forceps.
- g. Filter a known volume of sample through the filter. If less than 1ml or a dilution is to be used, then sterile Ringers solution must also be added to the filter apparatus before filtration, to allow an even distribution of the sample over the surface of the filter.
- h. Transfer the filter to the petri dish using sterile forceps. Place the filter grid side up on the surface of the agar using a rolling action to eliminate any air bubbles.
- i. Invert the petri dishes.
- j. Incubate at 44.5 °C (±1°C) for 48 hours.
- k. Count those plates. All colonies that are reddish-brown to pink in colour are regarded as faecal streptococci.
- l. The results are reported as faecal streptococci per 100ml.

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6. DILUTION TECHNIQUE

6.1 METHOD NO 6

6.2 BASIS OF METHOD

The water is diluted in sterile Ringers or other diluent solution in order to obtain counts for bacteria in poor quality waters.

6.3 INTERFERENCES

- Inadequate neutralisation of chlorine.

6.4 RANGE OF APPLICATION

Not applicable

6.5 SAMPLING PROCEDURE

Not applicable

6.6 TIME REQUIRED FOR ANALYSIS

- Preparation and sterilisation of media - 45 mins.
- Dilution of sample - 2mins

6.7 TOLERANCES

Not applicable

6.8 SPECIAL APPARATUS

- Sterile pipettes
- Bunsen burner

6.9 SPECIAL REAGENTS

- Sterile Ringers solution

6.10 ANALYTICAL PROCEDURE

Using a 1ml pipette, aseptically transfer 1ml of the sample water into 9ml of sterile Ringers solution in a sterile test-tube (giving a tenfold dilution). Mix by inverting the test-tube once only or by using a Whirli-mixer. Using a clean, sterile pipette, transfer 1ml of the mixed solution into a further 9ml of sterile Ringers solution. Continue in this way until the desired dilution is achieved. 1ml of the desired dilution is then placed in a sterile petri dish and the dilution is marked on the plate. When the plates are counted the results are recorded as the actual count.

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7. TOTAL ANAEROBIC BACTERIA

7.1 METHOD NO 7

7.2 BASIS OF METHOD

The bacteria are cultured on an agar medium specific for bacterial growth and are grown under anaerobic conditions.

7.3 INTERFERENCES

Inability to maintain anaerobic conditions.

7.4 RANGE OF APPLICATION

Not applicable

7.5 SAMPLING PROCEDURE

- a. Liquid samples must be taken in a sterile screw capped bottle.
- b. Solid samples must be taken in a sterile container eg. Whirl-pak bag.
- c. If the sample is not tested immediately then the container must be completely filled with water or evacuated to exclude as much air as possible.
- d. Nitrogen can be bubbled through the sample (using sterile tubing) to render the sample as anaerobic as possible.
- e. The sample can be stored for a maximum of 24 hours at 4°C in either a fridge or a cooler box.
- f. A representative sample must be collected according to sampling procedures mentioned in previous methods.

7.6 TIME REQUIRED FOR ANALYSIS

- Preparation and sterilisation of equipment - 45 mins.
- Analysis of one sample - 5 mins.

7.7 TOLERANCES

Not applicable

7.8 SPECIAL APPARATUS

- Sterile petri dishes
- Sterile pipettes
- Sterile tips
- Bunsen burner
- Anaerobic cabinet or jar

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- Anaerobic indicator strips
- Gas generating kits

7.9 SPECIAL REAGENTS

- Sterile Nutrient Agar/Plate Count Agar
- Sterile Ringers solution (if necessary)

7.10 ANALYTICAL PROCEDURE

- a. Dilute the sample if necessary.
- b. Using a pipette, transfer aliquots of the sample into petri dishes that has been marked with the date of the test, the origin of the sample and the dilution factor or volume of sample tested. For a solid sample 1g of sample should be placed in 9ml of Ringers solution and considered as 10⁻¹ dilution. Shake the mixture to dissociate the solid sample.
- c. Melt the agar and allow to cool to approximately 47°C.
- d. Flame the mouth of the agar bottle and add approximately 10-15ml of agar to the petri dish.
- e. Mix the sample and the agar thoroughly and allow to cool until the agar is set.
- f. Invert the plates
- g. Transfer to an anaerobic cabinet/jar as quickly as possible.
- h. Place an anaerobic indicator strip in the jar to ensure that anaerobic conditions are achieved.
- i. Incubate for 48 hours at 35°C ($\pm 1^\circ\text{C}$ in anaerobic jar and $\pm 2^\circ\text{C}$ in the anaerobic cabinet).
- j. Count the colonies that form and multiply the number of colonies by the dilution factor if necessary.
- k. Results are reported as colony forming units/ml (CFU's/ml)

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8. HYDROGEN SULPHIDE PRODUCING BACTERIA

8.1 METHOD NO 8

8.2 BASIS OF METHOD

The sample is cultured in media specific for the growth of Hydrogen Sulphide producing bacteria. The bacterial colonies which produce (H₂S) are black in colour. Only the black colonies are counted.

8.3 INTERFERENCES

None

8.4 RANGE OF APPLICATION

Not applicable

8.5 SAMPLING PROCEDURE

- a. Samples are taken in sterile containers (glass bottles or Whirl-pak bags)
- b. All samples should be handled aseptically.

8.6 TIME REQUIRED FOR ANALYSIS

- Preparation and sterilisation of equipment – 45 mins.
- Analysis of one sample – 5 mins

8.7 TOLERANCES

Not applicable

8.8 SPECIAL APPARATUS

- Sterile pipettes and tips
- Sterile test tubes and caps
- Bunsen burner
- Incubator
- Anaerobic cabinet/jar
- Anaerobic indicator strips
- Oxygen absorber

8.9 SPECIAL REAGENTS

- Sterile Iron Sulphite Agar
- Sterile Ringers solution (if necessary)

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8.10 ANALYTICAL PROCEDURE

- a. Add an aliquot of the sample to the sterile capped test tubes using a sterile pipette or sterile forceps if testing a deposit or add 1g of sample to 9ml of Ringers solution to conduct dilutions. (NB this will be a 10-1 dilution).
- b. Melt the agar and allow to cool to approximately 47°C.
- c. Completely fill the test tube with cooled agar.
- d. Replace the cap and invert the test tube once to mix the sample and the agar.
- e. Allow the agar to set with the test tube in an upright position.
- f. Incubate the test tubes in an anaerobic cabinet/jar, at 35°C ($\pm 1^\circ\text{C}$ in anaerobic jar and 2°C in anaerobic cabinet), for a period of up to one week. Ensure that anaerobic indicators are used.
- g. Check the test tube after 48 hours and record the number of black colonies that form.
- h. Results are reported as colony forming units per 1ml (CFU's/ml).

SAFETY NOTE: After the results have been recorded, if the agar is remelted to clean the test tubes, this must be carried out in a fume hood. This is due to the fact that hydrogen sulphide is produced, which is a toxic gas.

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9. EXAMINATION FOR THE PRESENCE OF YEAST, FUNGI AND HORMOCONIS RESINAE IN OILS AND HYDROCARBON FUELS COMPILED BY MRS L MOTSHANE

9.1 METHOD NO 9

9.2 BASIS OF METHOD

Used for the detection, isolation and counting of yeasts, fungi and H. resinae, a resin producing fungus in diesel/jet fuel samples.

9.3 INTERFERENCES

None

9.4 RANGE OF APPLICATION

None

9.5 SAMPLING PROCEDURE

Ensure that the points at which samples are taken are such that the samples are as representative as possible of the fuel throughout the fuel handling system-storage tanks, delivery lines, and fuel tanks.

9.6 TIME REQUIRED FOR ANALYSIS

- Media sterilisation: 15 min
- Filtration and rinsing: 20 min

9.7 TOLERANCES

Not applicable

9.8 SPECIAL APPARATUS

- Sterile glass funnels
- Sterile petri dishes
- Bunsen burner
- Sterile forceps
- Vacuum pump

9.9 SPECIAL REAGENTS

- Malt Extract Agar
- Sterile filter membranes (0.45 µm pores)
- 0.1% Triton X

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- Add 1ml of Triton to 1l of sterile distilled water.
- Filter the mixture into a second sterile container through silicone treated filter paper.
- Store the solution in the fridge.
- Sterile demineralized water

9.10 ANALYTICAL PROCEDURE

- a. Aseptically mount a sterile filtering apparatus on the manifold.
- b. Place a sterile membrane filter aseptically on the sinter glass base.
- c. Pour 100 ml of sample into the funnel. Switch on the vacuum pump and filter the sample.
- d. Rinse the funnel with 100 ml of 0.1% Triton X, followed by 100 ml of demineralised water.
- e. Make sure that the membrane is dry before the vacuum pump is switched off.
- f. Remove the membrane aseptically and place on malt extract agar plate. Complete each test in duplicate.
- g. Incubate the plates between 28°C ($\pm 1^\circ\text{C}$).and 35°C ($\pm 1^\circ\text{C}$).for 120 hours.
- h. Examine the plate every 24 hours for the slightest growth.
- i. Pay attention to any typical growth of *Hormoconis resinae* and note its intensity.
- j. If no growth is observed after 120 hours, the results are considered to be negative.
- k. Regard any other growth as “other fungi” and make a note to that effect.
- l. Yeasts are seen as single, independent microscopic round or oval colonies.
- m. The intensity of growth is recorded as follows:
 - + = Slight contamination = (<25% of the plate covered)
 - ++ = Moderate contamination = (25<50% of the plate covered)
 - +++ = Heavy contamination = (50<75% of the plate covered)
 - ++++ = Gross contamination = (75<100% of the plate covered)
- n. Appearance of growth: *H. resinae* Brown-grey powdery mat growth.
Other fungi A tuft of cotton, branching filaments (mycelium).
Yeasts Colourless or cream, oval or round bodies about 10-15 μm in diameter.

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10. DETERMINATION OF THE NUMBER OF TOTAL FUNGI IN OIL

10.1 METHOD NO 10

10.2 BASIS OF METHOD

The oil is cultured on an agar medium specific for the growth of fungi

10.3 INTERFERENCES

None

10.4 RANGE OF APPLICATION

Not applicable

10.5 SAMPLING PROCEDURE

- a. Label the bottle with a permanent marker, so that it corresponds to the sample being tested.
- b. Open the sampling valve and allow approximately 5 litres of the oil to run out into the bucket. This is to allow any water or sludge which has collected at the valve to flush out.
- c. Close the valve and place the sterilised bottle at the valve, taking care not to touch the mouth of the bottle.
- d. Open the valve and allow the bottle to fill (not to overflowing).
- e. The sample must be taken in a sterile screw capped glass bottle.
- f. It is important to sample at various points in an oil system so that a clear picture of the microbiological state of the system is obtained.

10.6 TIME REQUIRED FOR ANALYSIS

- Preparation and sterilisation of media - 45 mins.
- Analysis of sample - 2 mins.
- Counting of colonies - 5 mins.

10.7 TOLERANCES

Not applicable

10.8 SPECIAL APPARATUS

- Sterile 90mm petri dishes
- Sterile pipettes
- Bunsen burner

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10.9 SPECIAL REAGENTS

- Sterile Malt Extract Agar

10.10 ANALYTICAL PROCEDURE

- Melt the agar and allow to cool to approximately 47°C.
- Flame the mouth of the agar bottle, add approximately 15ml of cooled agar to a sterile petri dish and allow to set.
- Place an aliquot of the sample on the surface of the set agar (not more than 1ml).
- Spread the sample evenly over the surface of the agar using a glass spreader sterilised by dipping in ethanol or methylated spirits and igniting.
- Incubate the petri dish the upside down (inverted position), at 30°C ($\pm 1^{\circ}\text{C}$) for three days.
- Record the number of fungal colonies.
- The result are reported as colony forming units per ml (CFU ml^{-1})

NOTE : The petri dishes are incubated the right way up to prevent any of the oil from dripping off the surface of the agar, resulting in inaccurate results.

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11. DETERMINATION OF THE NUMBER OF TOTAL BACTERIA IN OIL

11.1 METHOD NO 11

11.2 BASIS OF METHOD

The oil is cultured on an agar medium specific for the growth of bacteria

11.3 INTERFERENCES

None

11.4 RANGE OF APPLICATION

Not applicable

11.5 SAMPLING PROCEDURE

- a. Label the bottle with a permanent marker, so that it corresponds to the sample being tested.
- b. Open the sampling valve and allow approximately 5 litres of the oil to run out into the bucket. This is to allow any water or sludge which has collected at the valve to flush out.
- c. Close the valve and place the sterilised bottle at the valve, taking care not to touch the mouth of the bottle.
- d. Open the valve and allow the bottle to fill (not to overflowing).
- e. The sample must be taken in a sterile screw capped glass bottle.
- f. It is important to sample at various points in an oil system so that a clear picture of the microbiological state of the system is obtained.

11.6 TIME REQUIRED FOR ANALYSIS

- Preparation and sterilisation of media - 45 mins.
- Analysis of sample - 2 mins.
- Counting of colonies - 5 mins.

11.7 TOLERANCES

Not applicable

11.8 SPECIAL APPARATUS

- Sterile 90mm petri dishes
- Sterile pipettes
- Bunsen burner

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11.9 SPECIAL REAGENTS

- Sterile Nutrient Agar

11.10 ANALYTICAL PROCEDURE

- Melt the agar and allow to cool to approximately 47°C.
- Flame the mouth of the agar bottle, add approximately 15ml of cooled agar to a sterile petri dish and allow to set.
- Place an aliquot of the sample on the surface of the set agar (not more than 1ml).
- Spread the sample evenly over the surface of the agar using a glass spreader sterilised by dipping in ethanol or methylated spirits and igniting.
- Incubate the petri dish the RIGHT WAY UP, at 35°C (\pm 1°C) for three days.
- Record the number of bacterial colonies.
- The result are reported as colony forming units ml⁻¹ (CFU ml⁻¹)

NOTE : The petri dishes are incubated the right way up to prevent any of the oil from dripping off the surface of the agar, resulting in inaccurate results.

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