Soils, sludges and treated bio-wastes — Detection and enumeration of *Escherichia coli* in sludges, soils, and biowastes – Part 1: Membrane filtration method for quantification

Boden, Schlamm und behandelte Bio-abfälle — Quantitativer Nachweis von Escherichia coli aus Schlämmen, Böden, sowie Bioabfällen – Teil 1: Membranfiltrationsverfahren

Sols, boues et bio-déchets traités — Détection et dénombrement de Escherichia coli dans les boues, les sols, et les biodéchets – Partie 1 : Méthode par filtration sur membrane

ICS:

Descriptors: *E.coli*, sludges, soils, biowastes.

Document type: European Standard
Document subtype:
Document stage: Working Document
Document language: E

STD Version 2.2

Contents

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Foreword

This document (has been prepared in the framework of the Horizontal project.

This document is a working document.

The following TC's have been involved in the preparation of the standard:

The standard is divided into three parts:

- part 1 gives a membrane filtration for quantification,
- part 2 gives a miniaturised semi-quantitative MPN method,
- part 3 gives a semi-quantitative macromethod.

This standard is applicable and validated for several types of matrices. The table below indicates which ones.

Material	Validated	Document
Soil	Not validated yet	
Sludge	Not validated yet	
Biowaste	Not validated yet	
Soil improvers	Not validated yet	

Introduction

This document is developed in the framework of the project 'Horizontal'. It is the result of a desk study "Hygienic Parameters Feasibility of Horizontal Standards for *Escherichia coli* and *Salmonella* spp. In sludges, soils, and biowastes" and aims at evaluation of the latest developments in assessing *E.coli* in sludge, soil, treated biowaste and neighbouring fields. After discussion with all parties concerned in CEN and selection of a number of test methods described in this study the standard has been developed further as a modular horizontal method and has been validated within in the project 'Horizontal'.

Sludges, soils, and biowastes can contain pathogenic micro-organisms such as *Salmonella spp.* which occur mainly in the intestinal tract of humans and animals and are transmitted through faecal contamination. The use of such contaminated materials in agriculture may cause outbreaks of infection due to the production of contaminated food and animal foodstocks. They may also be transmitted to wild animals. There is a need to monitor the efficacy of the storage and treatment processes to control pathogens such as *Salmonella* spp., and application rates to land.

Escherichia coli is a non-pathogenic, Gram negative bacterium with a faecal origin. Consequently, it can be used as an indicator of faecal contamination. It can also be used to monitor the effectiveness of pasteurisation or disinfection treatments but it is comparatively sensitive (to heat, high pH) and cannot therefore reflect the behaviour of all pathogens in these materials.

Suitable quality control procedures, at least those described in ISO 8199:2005, have to be applied.

WARNING — "Waste and sludge samples can contain hazardous and inflammable substances. They can contain pathogens and be liable to biological action. Consequently, it is recommended that these samples should be handled with special care. The gases which can be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided wherever possible. National regulations should be followed with respect to microbiological hazards associated with this method".

The texts of the chapters 1 to 13 are normative; annexes are normative or informative, as stated in the top lines of the annexes.

1 Scope

This part of the European standard describes a membrane filtration procedure for the quantitative detection, by culture of individual colonies on chromogenic agar media, of *Escherichia coli*. in sludges, soils, and biowastes. This part of the standard is not suitable for materials whose treatment will significantly reduce bacterial levels to less than 10 viable *E. coli* per g wet weight, such as lime addition, drying or pasteurisation. A liquid enrichment and most probable number estimation method may be suited for such purpose.

NOTE The objective is to cover untreated and treated sludges, soils, and biowastes.

This membrane filtration method is not appropriate for enumeration and detection of other coliform bacteria without modifications to the chromogenic agar media.

It is suitable to evaluate the log reduction of *E.coli* through treatment, as well as the quality of the end product.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

EN 12880:2000, *Characterisation of sludges — Determination of dry residue and water content*.

EN ISO 5667-13:1997, *Water quality — Sampling — Part 13: Guidance on sampling of sludges from sewage and water treatment works*.

ISO 8199:2005, *Water quality — General guidance on the enumeration of micro-organisms by culture*.

3 Definitions

For the purposes of this European Standard, the following terms and definitions apply.

3.1
Escherichia coli
Escherichia coli, belongs to the family of *Enterobacteriaceae*, is Gram-negative, non-sporulating, rod-shaped, lactose positive and able to grow at 44 °C. Most *E. coli* strains are able to produce indole from tryptophan and are β-glucuronidase-positive

3.2
method definition
β-glucuronidase-positive and able to hydrolyse 5-bromo-4-chloro-3-indolyl-β-glucuronide (BCIG) when growing on an MLGA medium at the temperature of 44 °C

3.3
cfu, colony forming unit
growth of individual bacterial cells into visible colonies on agar media, including on membrane filters overlaying the agar media

3.4
vegetative bacteria
those bacteria which are capable of normal growth in broth or on agar media without pre-culture resuscitation

3.5
sub-lethally damaged bacteria
those bacteria which have been stressed but not killed by storage or subsequent treatment by, for example, mesophilic anaerobic digestion, lime stabilisation or composting

3.6
resuscitation
stimulation to vegetative growth of sub-lethally damaged bacteria previously incapable of growth on agar media

3.7
quantitative resuscitation
stimulation to vegetative growth of sub-lethally damaged bacteria recovered discretely on a membrane filter, prior to transfer to chromogenic medium for growth of individual colonies

3.8
presumptive positives
isolates which are believed to be *E. coli.*, but not yet confirmed

3.9
dry residue
the dry mass portion of the material obtained after the specified drying process. It is expressed as percent or in grams per kilogram [EN 12880:2000, 3.1]

4 Symbols and abbreviations

API – Analytical Profile Index

BCIG – 5-bromo-4-chloro-3-indolyl-β-glucuronide

DS – Dry Solid

MLGA – Membrane Lactose Glucuronide Agar

5 Principle

The homogenised diluted sample is filtered, the membrane filter recovered aseptically and incubated on membrane lactose glucuronide agar (MLGA), initially at (30 ± 1) °C for (4 ± 0.5) h. Subsequently, the temperature is increased to (44 ± 1) °C for (14 ± 2) hours. The presence of *E. coli* is indicated by presumptive green colonies resulting from the hydrolysis of BCIG.

The method has a limit of detection of approximately 27 cfu.g^{-1} wet weight sample, dependent on the solids content which at high concentrations (> 10 % (w/v)) may restrict filtration of the sample volume through the membrane if not first diluted.

6 Verification of interferences

7 Reagents, diluents and culture media

7.1 General instructions

To ensure reproducible results, prepare culture media and diluents using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare them with demineralised or distilled water free from substances capable of inhibiting growth under the test conditions (ISO 8199:2005). If the media are not used immediately, preserve them in the dark at (5 ± 3) °C for a maximum of one month in conditions avoiding any alterations in their composition.

NOTE The use of chemicals of other grades is permissible providing that they are shown to be of equivalent performance in the test.

7.2 Peptone saline solution

— Bacteriological peptone 1 g

— Sodium chloride 8.5 g

— Demineralised or distilled water to 1 000 mL

Make up following manufacturer's instructions. Sterilise in the autoclave (8.1) at (121 ± 3) °C for (15 ± 1) min. Store at (5 ± 3) °C for a maximum of 3 months.

7.3 MLGA chromogenic medium (Oxoid CM 1031 or equivalent)

— Membrane lauryl sulphate broth 76.2 g

— Sodium pyruvate 0.5 g

— *BCIG, monohexylammonium salt 0.2 g

— Bacteriological agar 10.0 g

— Demineralised or distilled water to 1 000 mL

*5-bromo-4-chloro-3-indolyl-β-glucuronide

Mix all ingredients, except the BCIG, and bring to the boil. Dissolve 200 mg BCIG in a combined solution of 2.5 mL of 95 % aqueous ethanol and 0.5 mL 1 M sodium hydroxide before adding to the molten base agar medium. Sterilise by autoclaving at (121 ± 3) °C for 15 ± 1 min. Pour into 50 mm Petri dishes in volumes of approximately 7 mL. Allow to set and store refrigerated at 5 °C in the dark. Use within 7 days.

7.4 MacConkey agar (Oxoid CM 0007 or equivalent)

— Peptone 20.0g

— Lactose 10.0g

— Bile Salts 5.0g

— Sodium Chloride 5.0g

— Neutral red	0.075g
— Agar	12.0g
— Demineralised or distilled water	to 1000mL

Make up following manufacturer's instructions. Sterilise in the autoclave (8.1) at (121 ± 3) °C for 15 ± 1 min. Store at 5 ± 3°C for a maximum of 1 month.

7.5 Demineralised or distilled water

Free from substances inhibiting growth under the test conditions. (ISO 8199:2005)

Sterilise in the autoclave (8.1) at (121 ± 3) °C for 15 ± 1 min unless sterilised in the preparation process.

8 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilised in accordance with the instructions given in ISO 8199.

It should be ensured that microbiological laboratory equipment of proven performance and suitability for the method should be used. Where ever possible the product specified should be used, if this is not possible a product of proven equivalent performance must be used.

8.1 Apparatus for sterilisation by dry heat (oven) or steam (autoclave).

8.2 Thermostatic incubator(s) regulated at (30 ± 1) °C and/or (44 ± 1) °C.

8.3 Homogeniser (e.g. Stomacher®, Seward Laboratories or equivalent).

8.4 Centrifuge capable of centrifuging 50 mL at 200*g* to 300*g*.

8.5 Membrane filters (0.45 μm gridded, cellulose nitrate)

8.6 Glass fibre pre-filter discs (47 mm diameter, e.g. Whatman GF/D pore size 2.7 μm or equivalent).

8.7 Vacuum pump (e.g. Neuberger Model N726-3FT-18 or equivalent).

8.8 Vacuum manifold (e.g. Millipore or equivalent) to hold filter units.

8.9 Sterile homogeniser bags, 250 mL volume, with or without integrated mesh to exclude large particulate matter (e.g. Stomacher®, Seward Laboratories 6041, 6041/STR or equivalent).

8.10 Sterile Petri dishes, 50 mm in diameter, for holding MLGA medium.

8.11 Sterile universals of 20 mL volume, or containers with similar capacity.

8.12 Sterile pipettes, glass or disposable plastic ware, capable of dispensing 1 and 10 mL volumes.

8.13 Sterile conical centrifuge tubes, 50 mL volume, disposable plastic.

8.14 Tweezers, capable of sterilisation by immersion in ethanol and subsequent flaming.

8.15 Scale, Ohaus precision standard balance or equivalent,

8.16 Refrigerator, capable of maintaining 5 ± 3°C

8.17 Vortex, Heidolph REAX top 100-2400 1/min or equivalent

8.18 pH meter, Jenway pH meter 3320 or equivalent

8.19 Beakers or Containers 100mL, 250mL and 1000mL

8.20 Laboratory spatula

8.21 Boiling bath, Clifton or equivalent

8.22 Bunsen Burner

8.23 Sterile Forceps

8.24 Nalgene filter holder with receiver Cat no: 300-4000 or equivalent

9 Sampling

Take samples of at least 100 g wet weight and deliver them to the laboratory as quickly as possible (within 24 hours). In order to prevent propagation or inactivation of *Salmonella* during transport to the laboratory and subsequent storage, the necessary precautions depending upon the matrix shall be taken.

NOTE Generally chilling the sample to (5 ± 3) °C is recommended.

9.1 General

Samples are liable to ferment and can contain pathogenic micro-organisms. It is essential to keep them away from any food or drink, and to protect any cuts. When transporting and handling samples, it is essential that national and international regulations relating to bio-hazardous samples are followed.

See also the Warning note in the introduction.

9.2 Storage

It is not advisable to store samples in the open laboratory. If samples are to be stored, store them at (5 ± 3) °C for a maximum period of 36 hours.

9.3 Handling

Cleanliness when working is essential. When handling sludge samples, it is necessary to wear gloves, a face and eye protection, and ensure adequate protection against bottles bursting. The gas evolved is flammable.

See also the Warning note in the introduction.

10 Procedure

10.1 Sample preparation

— Weigh 10 g (wet weight) of as received sample into a 100mL container (8.19).

— Add an appropriate volume of peptone saline solution (7.2) so that the final weight is 100g using a 100mL container (8.19) and mix thoroughly using a vortex mixer (8.17) (dilution A).

— Place in homogeniser bag (8.9) and place in the homogeniser (8.3) and homogenise for 2 min.

For samples with a dry solid content >20% a homogeniser bag with an integrated mesh should be used. For samples with dry solid content <20% a homogeniser bag without integrated mesh can be used.

For lime-treated materials:

adjust the pH to 7.0 ± 0.5 with 1 mol/L hydrochloric acid. The sample is transferred to a sterile 100mL container and tested using a pH meter (8.18).

NOTE 1 **If the pH drops below 4.5 during the neutralisation process, start a new analysis with a fresh test portion.**

NOTE 2 **For other relevant treatment chemicals (e.g. peracetic acid), a suitable oxidant neutralisation procedure must be used (see for example EN 1040:1997)[(1)].**

After neutralisation place the homogeniser bag (8.9) in a homogeniser (8.3) and homogenise for 1 min.

— Transfer the homogeniser bag contents to two disposable centrifuge tubes (8.13) and centrifuge the two 50 mL aliquots at 200*g* to 300*g* for 3 min.

— Decant the supernatant from the tubes in a beaker and filter through a glass-fibre pre-filter (8.6) using a Nalgene Filter holder with receiver (8.24) to remove fine debris.

The Nalgene filters should have been sterilised in a boiling bath (8.21) prior to analysis, the filters are removed from the boiling bath using sterile forceps (8.23) and attached to the vacuum pump (8.7).

The glass fibre filter (8.6) is placed on the Nalgene filter using sterile tweezers (8.14) before the Nalgene filter cup is secured in position. The vacuum may now be used to draw the sample through

the filter; it is recommended that the sample is not all introduced to the filter cup at the same time because blockages may occur.

The Nalgene filters should be returned to the boiling bath and be sterilised for a minimum of 5 minutes before being used again.

10.2 Sample dilution

The number of dilutions to subsequently filter varies according to the presumed level of contamination of the material to be tested. Typically, dilution A (the filtrate) should be serially diluted 10^{-1}-10^{-3} with peptone saline solution (7.2). This will permit the enumeration of up to 10^4 *E. coli* per g wet weight sample. Higher bacteria levels will require additional dilutions of the filtrate to 10^{-8} (for example, untreated sludge may contain 10^8 -10^9 *E. coli* per g wet weight).

Prepare the relevant number of sterile universals (8.11) according to the number of selected dilutions; add 9 mL of sterile peptone saline solution (7.2) to each.

Using a sterile pipette (8.12); transfer 1 mL of the filtrate to the first universal containing 9 mL of peptone saline solution and mix thoroughly using a vortex mixer (8.17).

Using a fresh pipette (8.12), transfer 1 mL of the diluted sample to the second universal containing 9 mL of peptone saline solution and mix thoroughly using a vortex mixer (8.17).

Continue as above until all the dilutions have been prepared.

10.3 Membrane Filtration

10.3.1 Transfer the filter base (8.8) from the boiling water bath (8.21) to the manifold using sterilised forceps (8.23). Take care not to contaminate its surface by touch or clothing.

10.3.2 Membranes (8.5) are removed from their packets with sterilised tweezers (8.14) and placed grid side up on to the filter base. Membranes that have torn are dropped or which touch any object must be discarded. Membranes should only be handled by the edge with tweezers designed for that purpose. The tweezers must be sterilised first heating in a Bunsen burner (8.22), and then dipping directly into boiling water.

10.3.3 The filter cup is then removed from the water bath and attached to the base taking care not to wrinkle the membrane. Filter funnels should only be removed from the water bath with disinfected forceps and placed directly onto bases. Hands may be used to transfer funnels back to the water bath. Neither forceps nor tweezers should be placed directly onto the bench. When not in use they should be placed on a specified rack to prevent contamination. If the filtration equipment is left for any significant length of time, the bases must be returned to the water bath. Bases left unused for short periods can be covered with the base or lid of a sterile Petri dish until filtration recommences.

10.3.4 Add a sufficient volume of peptone saline solution (15 ± 5 mL) into the filter cup, pipette 1mL of the diluted sample into the filter cup. Replace the top on the universal. Place the used universal back into the rack. The universal must not be placed on the filtration bench.

10.3.5 The sample may now be drawn through the filter by vacuum and only when filtration is complete should the vacuum be turned off. The magnetic filter cup is lifted off, and returned to the boiling water bath.

10.3.6 The membrane is carefully removed using sterile tweezers and transferred to the MLGA 50mm Petri dish (7.3). The membrane should be 'rolled' into the plate to prevent air bubbles becoming trapped between the growth medium and the membrane, and the lid of the Petri dish is replaced. Air bubbles will prevent growth medium getting to the surface of the membrane and thus restrict the growth of any isolated bacteria.

10.3.7 Any wrinkled or torn membranes discovered after filtration must be discarded. The filter base must then be re-sterilised and the dilution filtered again.

10.3.8 Once filtration of samples is complete, the filter funnels are placed in to the boiling water bath for disinfection. They must be totally immersed in boiling water for at least two minutes before being removed to continue filtration.

10.3.9 Before filtration of the first dilution (10^{-8}) add a negative control, i.e. 1 mL peptone saline solution. After the last dilution (10^{0}), add a negative control (1 mL peptone saline solution) and a positive control (i.e. 1 mL suspension of *E. coli* NCTC 9001).

10.4 Resuscitation and enumeration of colonies on chromogenic agar

Remove the filter from the housing using sterile tweezers (8.13) and transfer to the surface of a 50 mm diameter MLGA plate (7.3). Incubate plates initially at (30 ± 1) °C for (4 ± 0.5) h. Subsequently, increase the temperature to (44 ± 1) °C for (14 ± 2) h.

Enumerate presumptive typical dark green colonies by eye, only plates within the range 10-100 colonies should be considered for the expression of results. If quantification of colonies within the range is not possible, counts outside the range 10-100 are considered for the expression of results.

10.5 Confirmation of colony identity

The typical presumptive colonies are sub-cultured onto non-selective MacConkey agar (7.4). It is important to subculture any colonies suspected of being *E.coli* regardless of colour alone. A minimum of two colonies per plate, per sample; and a maximum of each morphological type per plate and per sample should be taken for confirmation using API 20E. The MacConkey subculture plates are then incubated at (36 ±2) °C for (21 ± 3) hours.

Confirmation of typical colonies is achieved by API 20E biochemical test strips.

Preparation of the strip - Prepare an incubation box (tray and lid), and pipette using a pastette approximately 5mL of sterile water into the wells in the plastic tray. This creates a humid atmosphere and stops the strip drying out during incubation.

Preparation of the inoculum - There are three types of inoculum available for use; an ampoule of 0.85% NaCl medium (5mL), suspension medium (5mL) or sterile saline/distilled water without additives (5mL). 0.85% NaCl is the preferred inoculum. Using a sterile swab, remove a single well isolated colony from a sub-culture plate and carefully emulsify to achieve a homogeneous bacterial suspension.

Inoculation of the strip - Using a pastette, Fill both the tube and the cupule of the tests CIT, VP and GEL with the bacterial suspension. Fill only the tube and not the cupule of all the other tests. Create an anaerobic environment in the tests ADH, LDC, ODC, H_2S and URE by overlaying the tube by placing mineral oil in the cupule.

Reading the strip - After the incubation period, read the strip by referring to the reading table.

If three or more tests (GLU test + or -) are positive after the incubation period, record all of the spontaneous reactions on the result sheet and reveal the tests which require the addition of reagents.

- TDA test: add 1 drop of TDA reagent
- IND test: add 1 drop of JAMES reagent
- VP test: add 1 drop of each VP1 and VP2 reagents.

NOTE: If the number of positive tests after the incubation period is less than three; re-incubate the test strip for a further 24hrs.

Interpretation - Determination of numerical profile; on the results sheet the tests are separated into groups of three and a value, 1, 2 or 4 is indicated for each. A 7 digit profile is produced when all the corresponding results for each group are combined together.

The 7 digit profile can be translated into a profile using either the electronic software available or by looking the result up in the profile index book.

10.6 Determination of the dry residue content

The numbers of *E. coli* may be calculated per wet weight or dry weight. For the latter, it is necessary to determine the dry residue of the sample using the method described in EN 12880:2000. This shall be performed in parallel with the microbiological analysis.

11 Expression of results

Calculation of the number of *E. coli* (present per g wet weight of the original sample) is by dividing the total number of typical colonies (n) on the filter of the selected plates by the total volume filtered of the initial sample. The confirmation rate of the colonies must be taken into account when calculating the final result according to ISO 8199 (2005).

$$c = \frac{n}{v}$$

where:

c = original wet weight concentration of *E. coli* no. per g,

n = total number of typical *E. coli* colonies on the selected membranes

v = total volume filtered through the selected membrane (from 1 ml)

NOTE: The dilution factor of the dilution A taken for filtration step should not be forget in the final calculation.

Example:

If the volume of the test dilution used (v_i) is 1 mL of dilution A and the following counts are obtained at the respective dilutions:

Dilution	Counts
10^{-2}	81 colonies
10^{-3}	15 colonies

Then:

n= 81+15 = 96

v= (0.1 x 1 x 0.01) + (0.1 x 1 x 0.001)

$c = 96 / 0.0011$ = 8.7 10^4 cfu/g ww

Numbers present per g dry weight of sample are calculated according to:

$$c = \frac{n}{ve} \times 100$$

e = % dry residue of the original wet sample.

12 Performance data

Information concerning the repeatability and reproducibility of the procedure, obtained using interlaboratory tests, is given in Annex A (informative).

This annex A will be completed by the results of the validation study (European scale Interlaboratory trial) that will be performed during the FP6 EU Horizontal-Hyg project.

See Annex A.

13 Test report

The test report shall contain the following information:

a) reference to this part of this European Standard;

b) all information necessary for complete identification of the sample;

c) details of sample pre-treatment, if carried out;

d) results of the determination according to Clause 9; and

e) any detail not specified in this part of this European Standard and any other factor which may have affected the results.

Annex A
(informative)

Performance data of the interlaboratory comparison

The comparability exercise took place during week starting 30th November 1998. Laboratories were notified in advance of the date of the exercise and the analysis required. A bulk sample of digested sludge (mesophillic anaerobic digestion) was collected from a participating survey site.

The bulk sample was used to prepare individual samples of approximately 200 g. These were sent out by courier to each of the participating laboratories to arrive by 12 noon the following day. Laboratories were requested to commence analysis on the day of arrival. The sample was analysed for:

- *E. coli* by means of membrane filtration on MLGA;

- dry solids.

A standard pro-forma was included with the sample for data reporting.

A.1 Results

Results were obtained from all participating laboratories (n=11). Summary results are shown in Table 1.

Table A.1 — Summary of results of inter-laboratory comparison

Parameter	Mean	Range	SD	Organiser's reference result
Log_{10} *E. coli*/100g	7.29	5.3-7.5	0.66	7.68
Dry solids %	4.42	4.07-4.6	0.14	4.45

The frequency distribution of results for both parameters was calculated and the results plotted in Figures 1 and 2.

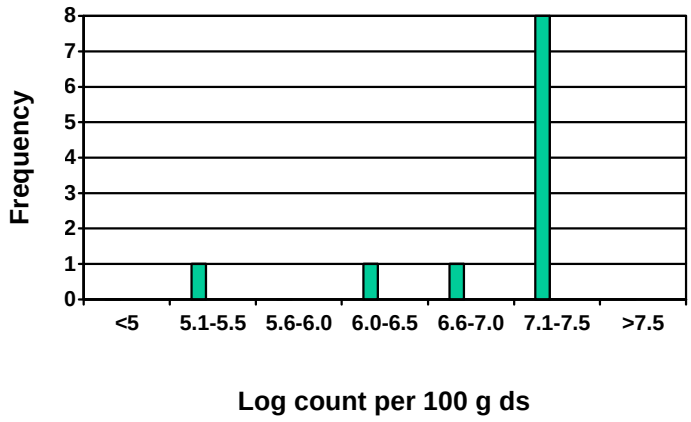

Figure A.1 — Frequency distribution of *E. coli* counts

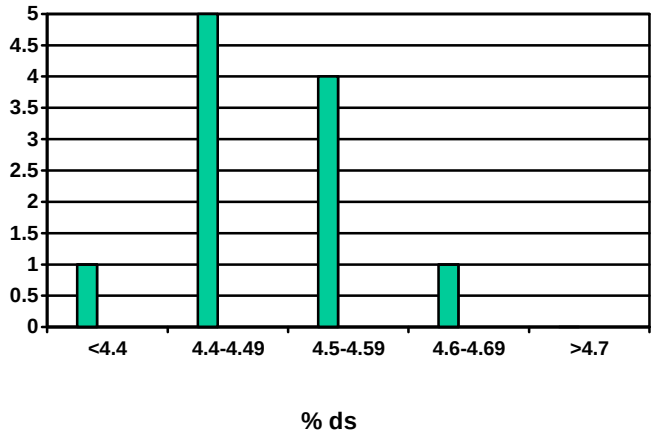

Figure A.2 — Frequency distribution of dry solids data

A.2 Discussion

The findings from this inter-laboratory exercise were encouraging. The results for the dry solids showed an approximate normal distribution, with the majority of laboratories (n=9) reporting results between 4.4% and 4.6% ds content. There was one result (4.07%) which could be described as an outlier.

Microbiological data are expected to be more variable than results of physicochemical analysis. This was evident in the exercise. Counts of micro-organisms tend to be log normally distributed and to account for these values were log transformed. The range of counts spanned 2 logs, although a single outlier was responsible for this. The large majority of labs (n=9) reported results within one order of magnitude.

Overall, it is concluded that the variability between laboratories is no greater than expected for microbiological analysis.

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