

# Australian Standard<sup>®</sup>

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## Water microbiology

### Method 23: Soils, sediments, sludges, slurries and bio-solids—Procedures for sample preparation

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AS 4276.23:2016

#### PREFACE

This Standard was prepared by the Standards Australia Committee FT-020, Water Microbiology.

The objective of this Standard is to provide procedures for preparation of high-particulate-liquid (slurries), solid and semi-solid environmental matrices for microbiological examination. These matrices are frequently analysed for a range of microorganisms, particularly faecal indicators and pathogens. In particular, biosolids are tested to determine fitness-for-purpose for various end uses. There is a lack of peer-reviewed procedures for the preparation of these matrices for microbiological examination and a demand exists by laboratories seeking technical accreditation for analysing them.

The main use of this Standard will be for the testing of biosolids for various end uses as part of the process of ensuring that public health is not compromised through the use of biosolids with inappropriate pathogen levels. The procedures are also applicable for preparation of soils, sediments and sludges for microbiological analysis. Examples of matrices of interest are those that relate to soil irrigation using reclaimed and agricultural wastewater, bacterial partitioning into sediments in aquatic environments, and waste treatment efficacy with respect to reduction of microbial/pathogen loads in sludges.

The terms ‘normative’ and ‘informative’ have been used in this Standard to define the application of the appendix to which they apply. A ‘normative’ appendix is an integral part of a Standard, whereas an ‘informative’ appendix is only for information and guidance.

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#### FOREWORD

Biosolids are regarded as a valuable resource. The National Water Quality Management Strategy publication, *Guidelines for Sewerage Systems—Biosolids Management*, states that there is a ‘need to ensure that public health is not compromised through using biosolids with inappropriate pathogen levels’. This new Standard would facilitate more consistent and effective testing of biosolids nationally for pathogenic and indicator microorganisms.

Microorganisms in solid and semi-solid matrices are often adsorbed onto, or absorbed within, particulate material. This may produce uneven physical distributions, resulting in decreased subsample representativeness, and decreased overall test precision and accuracy. The goal of this Standard is effective sample homogenization, allowing subsequent accurate and reproducible quantitative dispensing and/or dilution. Results in subsequent analyses may be semi-quantitative or quantitative.

Most Probable Number (MPN), presence-absence (PA) and direct-agar plate-based techniques require homogenous sample suspensions for representative analysis. MPN and PA techniques allow for the presence of substantial amounts of matrix material in the growth medium. Therefore, physical separation of target organism from matrix is not necessary.

Direct-agar plating techniques such as spread-plate or membrane filtration are subject to matrix interference such as membrane filter and/or pipette clogging. The inability to distinguish target colonies from particulate matrix material is another potential interference. Mitigation of such interference may be achieved through physical separation of microorganisms from the matrix and/or dilution of sample such that matrix no longer impedes or confounds the analysis.

Analysis of samples containing large-size interfering particles (>1–2 mm diameter) may require continuous resuspension during liquid handling to prevent differential particle settling; and dilution or separation of target microorganisms from particles where such particles interfere with culture based enumeration.

Separation of target microorganisms from matrix particles is promoted through the use of physical disruption in the presence of a homogenization solution containing a surfactant and dispersing agent.

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## METHOD

### 1 SCOPE

This Standard sets out a method for the preparation of soils, sediments, sludges [including high-particulate-content liquids (slurries)] and biosolids for culture-based bacterial analysis utilizing selective growth media and/or conditions, including most probable number (MPN), presence/absence (PA) and agar plating techniques. Examples of analytes include (but are not limited to): *E. coli*, thermotolerant coliforms, enterococci, *Salmonella* spp., *Vibrio cholerae*, *Clostridium perfringens*, *Campylobacter jejuni/coli*, *Burkholderia pseudomallei*, iron and sulphur bacteria and hydrocarbon-degrading bacteria.

NOTE: Flow diagram of the procedure for sample preparation for soils, sediments, sludges [including high-particulate-content liquids (slurries)] is given in Appendix A.

Although sample preparation for protozoans, helminths and viruses in these matrices is not within the scope of this Standard, some guidance on sample preparation is given in Appendix B.

### 2 REFERENCED DOCUMENTS

The following documents are referred to in this Standard:

AS

2031 Water quality—Sampling for microbiological analysis (ISO 19458:2006, MOD)

AS/NZS

4276 Water microbiology

4276.1 Method 1: General information and procedures (ISO 8199:2005, MOD)

### 3 DEFINITIONS

For the purposes of this Standard the following definitions apply:

#### 3.1 Analyte

The chemical or biological entity that is of interest and intended to be measured in an analytical procedure. For example, *E. coli* is an analyte.

#### 3.2 Biosolids

Treated organic solids produced by wastewater treatment processes that in most cases can be beneficially recycled. The term biosolids does not include untreated wastewater sludges, industrial sludges or the product produced from the high temperature incineration of sewage sludge. Biosolids are typically thickened and dewatered and include blended materials such as soil improvers, fertilizers, composts and worm castings.

NOTE: The terms 'sludge' and 'biosolid' are often used synonymously, however biosolids are treated sludge. For the purposes of this Standard, either term may be used, with that preferred by the end-user recommended, with the exception of untreated wastewater sludges, industrial sludges and the product produced from the high temperature incineration of sewage sludge.

#### 3.3 Cell calibrator equivalent (CCE)

A quantity of nucleic acid equivalent to a cell. Obtained by calibration studies using nucleic acid-based techniques.

#### 3.4 Homogenate

A preparation of the sample that has been made uniform or similar in composition by the mixing or blending of unlike components of the sample.

#### 3.5 Limit of detection (LoD)

The lowest quantity of an analyte that can be distinguished from the absence of that analyte (a blank value or negative control) within a stated confidence limit.

#### 3.6 Matrix

Matrix refers to the components of a sample other than the analyte of interest. The matrix can have a considerable effect on the way the analysis is conducted and the quality of the results obtained.

#### 3.7 Measurand

The quantity per unit of the analyte intended to be measured. For example, 'most probable number of *E. coli* per gram' is a measurand.

#### 3.8 Sample

A limited quantity of a product that is intended to be similar to and represent a larger amount of that same product.

#### 3.9 Sediment

Solid fragmented material, such as silt, sand, gravel, chemical precipitates and organic particles, transported and deposited by water, ice, or wind and which has settled to the bottom of liquid suspension. Sediments are typically saturated or semi-saturated.

#### 3.10 Sludge

Soft, thick material produced from the accumulation of solids in a variety of industrial processes such as chemical coagulation, flocculation and sedimentation during wastewater treatment. Sludges are otherwise untreated and have generally higher water content than biosolids (typically <25% solids).

NOTE: The terms 'sludge' and 'biosolid' are often used synonymously, however biosolids are treated sludge. For the purposes of this Standard, either term may be used, with that preferred by the end-user recommended, with the exception of untreated wastewater sludges, industrial sludges and the product produced from the high temperature incineration of sewage sludge.

### **3.11 Slurry**

A thin mixture of an insoluble substance with a liquid. Generally free flowing.

### **3.12 Soil**

The top layer of the earth's surface, consisting of rock and mineral particles mixed with organic matter, with a range of water content up to saturated. This includes subsoils.

### **3.13 Subsample**

A portion of the original sample that is representative of the original sample, thereby assuring equivalency in analytical measurements. This may be created by trimming, subdividing, splitting of, or discrete collection from, the original sample.

## **4 PRINCIPLE**

Solid, semi-solid and viscous liquid (i.e. high moisture content sludge) samples are mixed as much as practicable. Subsamples as necessary are quantitatively suspended in a homogenization buffer or an enrichment medium. Sample homogenization procedures are based on whether the sample is liquid or solid. The suspension is subjected to vigorous mixing to produce a uniform homogenate by any of several methods (shaking, blending, stomaching) and pH adjusted to 7.0–7.5 or the pH of the applicable growth medium. The homogenate may then be used directly as sample material for MPN-based determinations and serially-diluted for both MPN, spread-plate and membrane filtration methods. Liquid enrichment media are the starting point for both MPN and presence/absence analyses.

For reporting of results on a dry weight basis, a subsample is dried at 103°C–105°C to constant weight for gravimetric determination of percent total solids, with the result being used to calculate the MPN or cfu (colony forming units)/g dry weight.

#### **NOTES:**

- 1 A 10-fold w/v dilution results from the solids homogenization process. Such dilution can reduce interference by matrix materials on growth media, including matrix-associated potentially toxic materials such as metals and antimicrobials. The impact of such interferences should be considered if homogenized sample is analysed directly without dilution; such as for liquid samples, or homogenates produced with a <10-fold concomitant dilution.
- 2 Sample must be homogenized in order to effectively adjust pH.

## **5 REAGENTS (See Appendix C)**

### **5.1 Soil extraction buffer (SEB)**

### **5.2 Diluents**

As specified in AS/NZS 4276.1.

### **5.3 1 N and 10 N HCl solution, as required**

For homogenate pH adjustment.

### **5.4 1 N NaOH solution**

For homogenate pH adjustment.

### **5.5 pH calibration buffers**

4, 7 and 10.

## 6 APPARATUS

### 6.1 Apparatus and materials for homogenization

#### 6.1.1 *Sterile containers*

Suitable for weighing samples.

#### 6.1.2 *Sterile container*

Such as a stainless steel or plastic bucket suitable for sample collection.

#### 6.1.3 *Sterile wide bore pipettes or equivalent measuring devices*

#### 6.1.4 *Sterile beakers*

Glass or plastic, assorted sizes.

#### 6.1.5 *Sterilizing equipment*

Such as autoclave or dry heat sterilizer.

#### 6.1.6 *Magnetic stirrer and stir bars (magnetic fleas)*

#### 6.1.7 *Sterile blender jars or blender homogenizer*

Capable of being disinfected between samples.

#### 6.1.8 *Vortex mixer*

#### 6.1.9 *pH meter*

#### 6.1.10 *Gloves for handling samples and extraction equipment*

#### 6.1.11 *Sample handling apparatus*

Sterile spatulas, spoonulas, funnels, or other equipment for transfer and manipulation of sample.

#### 6.1.12 *Mechanical shakers*

#### 6.1.13 *Stomacher (peristaltic blender) and sterile bags*

#### 6.1.14 *Forceps*

#### 6.1.15 *80% (v/v) or 70% (w/w) ethanol, or 70% (v/v) or 60% (w/w) isopropanol*

For disinfecting forceps.

### 6.2 Apparatus for percent solids by drying to constant weight

#### 6.2.1 *Evaporating dishes*

Minimum 100 mL capacity. Dishes may be made of porcelain, high-silica glass, aluminium or platinum.

#### 6.2.2 *Desiccator*

Moisture concentration in the desiccator should be monitored by an instrumental indicator or with a colour-indicator desiccant.

#### 6.2.3 *Drying oven*

Thermostatically-controlled, capable of maintaining a uniform temperature of 103°C to 105°C throughout the drying chamber.

#### 6.2.4 *Balance*

#### 6.2.5 *Container handling apparatus*

Gloves, tongs, or a suitable holder for moving and handling hot containers after drying.

#### 6.2.6 *Sample handling apparatus*

Spatulas, spoonulas, or other equipment for transfer and manipulation of sample.

#### 6.2.7 *Watch glass*

Capable of covering evaporating dishes (see 6.2.1).

## 7 SAMPLES

The sampling plan shall be in accordance with end-user requirements. Sterile resistant-glass or plastic bottles shall be used to collect samples for analysis, provided that the material in suspension does not adhere to container walls. Containers should be capable of being tightly sealed. Sterile or disinfected sampling equipment shall be used such that the sampling surfaces in contact with sample are sterilized or disinfected between samples (example: sampling-surfaces of a soil auger; inner surfaces of a push- or piston-corer; spatulas, corers, trowels, shovels). If the end-user requirements in terms of sampling equipment are more stringent, these shall be followed.

To optimize the recovery of the target microorganisms, transportation conditions and holding times should be in accordance with AS 2031, unless end-user requirements are more stringent. Sample storage conditions prior to analysis should be those indicated in the applicable analytical procedure utilizing the homogenate produced in this method. Analysis shall commence as soon as possible after collection, with samples being brought to room temperature before commencing the analysis.

### NOTES:

- 1 See references [3], [5] and [6] for additional guidance on sampling.
- 2 Samples are generally classified as liquid when they contain approximately <7% total solids.

## 8 PROCEDURES

### 8.1 Mixing

Sub-sampling for analysis is a process by which an analytical unit is obtained from a sample. Samples shall be well mixed prior to obtaining subsamples for processing. It is essential to ensure that any subsamples are representative. When assessing the homogeneity of a sample, bear in mind that this will vary depending on the matrix, which can be solid, semi-solid or high-particulate-liquid in nature.

Liquid and semi-liquid samples shall be mixed thoroughly by shaking, stirring or mechanical agitation/mixing in order to re-suspend any settled or floating material. It may be necessary to transfer the sample to a larger, or more suitable container. Visually assess the resultant sample for homogeneity. Some liquid samples may contain aggregates or other material capable of being disrupted. If such samples cannot be adequately mixed by the shaking, stirring or mechanical agitation/mixing, then stomaching should be used.

Wet sludges may be mixed by stirring the entire sample thoroughly with a sterilized spatula. Alternatively, a blender may be used.

For solid samples, such as caked, thickened or thermally dried sludges and composts, a variety of measures may be needed to ensure that adequate mixing is achieved. These sludges vary in nature, with some being relatively moist and others being hard and unyielding. Composts are usually moist and friable, however some may contain hard materials such as rocks, twigs, and other plant matter. For semi-solid and hardcaked sludges and composts, it is particularly important for the sample to be well mixed. Soft caked sludges and soft composts may be transferred to a stomacher bag placed inside a second blender bag. This can be crushed or ground using a suitable heavy object to aid the mixing process. Hard caked sludges, pressed sheet sludges and granules and pellets may need to be aseptically macerated, crushed or ground to facilitate mixing. If using a laboratory mill, the manufacturer's instructions should be followed to ensure that microorganisms are not damaged due to mechanical action.

## 8.2 Subsampling and homogenization

### 8.2.1 General

If results are to be reported on a dry weight basis, the percent solids of subsamples from the thoroughly mixed sample shall be determined as per the procedure in Appendix D.

The homogenization procedure, including pH neutralization, should be performed in less than 60 min. and the homogenate diluted and/or placed directly into growth media within this time period. This does not include the time required for treating difficult matrices so that they are in a suitable condition for complete homogenization. For example, particularly hard matrices requiring soaking and very low moisture content matrices which are difficult to wet.

If a non-selective medium is used in the applicable method, the receptacle used in homogenization shall be sterile rather than disinfected.

Spiking of samples with positive control microorganisms shall occur after pH neutralization.

### 8.2.2 Liquid samples ( $\leq$ approx. 7% solids)

An adequate degree of homogenization of liquid samples should have been achieved in the initial mixing step. If not, a subsample is homogenized using stomaching or blending. The minimum subsample volume required for a liquid sample is 300 mL. Check the pH of homogenized liquid samples. Sample pH shall be adjusted to 7.0–7.5 by addition of 1.0 N HCl or NaOH prior to removal of further subsamples.

### 8.2.3 Solid samples ( $>$ approx. 7% solids)

The procedure for solid samples shall be as follows: weigh out  $30 \pm 0.1$  g of well-mixed sample into a receptacle to be used for homogenization (blender jar, stomacher bag, sample container) and add 270 mL SEB. Cover or otherwise seal the sample in the container and homogenize. It may be beneficial, especially if the sample is particularly hard or dry, to allow it to soak in the SEB prior to homogenization (0.5–1.0 h). Check pH and adjust to 7.0–7.5 by addition of 1 N HCl or NaOH. This homogenate represents 0.1 gram wet weight sample per millilitre.

### 8.2.4 Direct addition to enrichment broths

When well mixed sample is directly added to enrichment broths, the sample is in effect being homogenized in the broth rather than extraction buffer. The same procedural details apply except that the weight of sample added to an enrichment broth is according to the applicable method, with pro rata adjustments as necessary. The tolerance on any respective masses and volumes is  $\pm 5\%$ . Liquid samples will have been pH adjusted prior to addition and require no pH adjustment unless a different pH is specified in the applicable method. For solid samples, adjust the pH only after the subsample is added to the enrichment broth.

## 8.3 pH neutralization

For non-alkaline stabilized liquid and solid samples, 1 N HCl or NaOH is used for pH neutralization. The procedure shall be as follows: actively mix or stir samples during pH adjustment. A sterile magnetic stir bar and a magnetic stir plate combination can be used for this activity. Add the HCl or NaOH while stirring to adjust pH to 7.0–7.5. Ensure pH is stable at 7.0–7.5 by observing for several minutes after final acid or alkali addition. Either remove a small aliquot of the sample or ethanol sterilize the pH probe (for direct sample application) before pH measurements. Calibrate pH meter with pH 4, 7 and 10 buffers.

For alkaine-stabilized samples (biosolids, sludges or industrial liquids) with high pH (up to pH 12), 10 N HCl is used for pH neutralization. The procedure shall be as follows: slowly add the HCl, constantly stirring samples during acid addition as previously described, and neutralize to pH 7.0–7.5. Exercise particular care and use a fume hood for strong acid additions. Increase observation of final mixture to 15 minutes to ensure pH remains stable at pH 7.0–7.5.

When solid subsamples are to be taken directly from the original well mixed sample into enrichment broths, pH shall be checked and adjusted to 7.0–7.5 in the enrichment broth under constant stirring, unless a different pH is specified in the applicable method. Liquid samples that have been pH adjusted will require further adjustment if the applicable method specifies a different pH to 7.0–7.5.

During pH adjustment, the volume of added HCl or NaOH shall not exceed 5% of the homogenized sample volume (15 mL). The pH of samples shall not be allowed to fall below 5 during pH adjustment, unless the pH of the sample was already less than 5.

## **8.4 Homogenization techniques**

### **8.4.1 *Manual and/or mechanical mixing***

It is common in sludge matrices for aggregation to occur. Therefore simple manual or mechanical mixing, as generally employed to prepare microbiological matrices, may not be sufficient to achieve a homogenous sample. More robust containers may be required to adequately and safely process these samples. Take care when opening receptacle lids as foaming can occur during mixing. Consider aerosol minimizing strategies to avoid biological safety concerns, including inhalation.

Manual mixing may involve shaking the sample, or diluted sample, by hand in a suitable sealed receptacle. Mechanical mixing automates the process in an instrument dedicated to the purpose. Examples of mechanical mixers include orbital shakers, vortex mixers and stomachers. Follow the manufacturer's instructions during use.

### **8.4.2 *Blending***

Laboratory blenders consist of sterilizable vessels with lids. Blenders provide a rapid and effective means of homogenizing samples which contain particulate, aggregated or fibrous material. It is important to sterilize blender-vessels between uses when different samples are homogenized. The vessel should be thoroughly cleaned and washed before being autoclaved. The blender speed and blending time settings should be varied to suit individual samples. These settings should be verified to establish optimum conditions for each particular type of matrix tested.

### **8.4.3 *Stomaching (peristaltic blending)***

Stomachers are instruments that are commercially available in several different types. They are commonly used for homogenizing food samples. Homogenization is achieved by the rhythmic operation of paddles against the sample contained in a strong sterile plastic stomacher and sterile bag. Speed and timing options may be available, but it is not possible to specify these variables for this work. However, lower speeds for short time periods are usually adequate. Adherence to manufacturer's instructions is necessary.

A problem that may be encountered is puncturing of the plastic bag due to the presence of grit, twigs and other sharp objects. Although the use of two or more bags may overcome this, blending or manual and/or mechanical homogenization methods rather than stomaching is recommended in such cases.

After stomaching has been completed, the bag is removed from the instrument and supported in such a way that the contents are not spilled. Stomacher bag supports are commercially available. The homogenized sample is now available for transfer to a sterile container.

Stomacher bags are available with filters for straining out larger particles after homogenization. There are also bags with an internal mesh insert which aid in the removal of particulate matter that might otherwise puncture the bag. This might be important for procedures such as membrane filtration where high levels of particulates can cause significant interference.

NOTES:

- 1 Samples may contain certain large debris objects such as stones and pieces of wood that are not able to be disrupted by blender based grinding or may puncture stomacher bags. These objects may be removed using sterile or ethanol disinfected implements such as forceps. However, rinsing of these objects is necessary in order to avoid the loss of microorganisms associated with biofilms and specific matrices such as wood. This is achieved by rinsing with a minimal volume of SEB (<5% total volume) prior to homogenization. Pooled rinsate is added to the material to be homogenized.
- 2 Particular care regarding safety is necessary with samples of high pH such as alkaline-stabilized materials, where 10 N HCl is used for the pH adjustment.
- 3 Very low moisture content samples (<17%) may be difficult to wet due to hydrophobic characteristics. Wetting can be achieved by aseptic kneading in a sterile plastic bag or by grinding with a disinfected mortar and pestle. Repeated mixing of the material with SEB may be necessary. Adequate wetting may take 30–60 min prior to further physical homogenization (mechanical mixing, blending or stomaching).
- 4 Sampling, subsampling, and pipetting multi-phase samples may introduce significant errors. Use special handling techniques to ensure that sample homogeneity and integrity is maintained during subsampling. For example, continuous stirring with a magnetic stirrer will reduce settling-out of suspended particles, although this should be avoided if paramagnetic particles are present. The use of wide-bore pipettes is necessary when visible suspended particles are present. Some components of samples may adhere to the sample container. This requires intensive homogenization and focus on sample container rinses in order to ensure accurate results.
- 5 During moisture content determinations, crust formation may occur in some samples during drying of biosolids and other matrices. The crust may inhibit evaporation, requiring extended drying times.

### 8.5 Post-homogenization analytical techniques

Guidance on post-homogenization analytical techniques is given in Appendix E.

## 9 QUALITY CONTROL (QC)

The laboratory shall have a clearly defined quality control system to ensure that the apparatus, reagents, media and techniques are suitable for the testing undertaken, and shall be as follows:

- (a) Controls designed to detect contamination shall include use of all apparatus, homogenization and pH-adjustment reagents, and growth media used for processing of non-QC samples.
- (b) Spike pH-neutralized homogenate with applicable target microorganisms introduced at levels close to the lower limit of detection such that recovery is demonstrated. Homogenate used is prepared from negative control matrix, which is the same matrix class being tested and previously determined to have <1 cfu, MPN or CCE of the target test microorganism per reporting unit. If a suitable matrix is not available, material of the same matrix may be treated to achieve the same for the respective test microorganism using sterilization or disinfection (e.g. gamma irradiation, pasteurization, autoclaving). The use of sterilized matrix is sub-optimal because the impact of competing microflora has been removed.
- (c) Positive reference cultures shall be those specified in the applicable microbiological methods.

## 10 TEST REPORT

The report shall contain the following information:

- (a) Date of sample preparation.
- (b) Reference to this Australian Standard, i.e. AS 4276.23.
- (c) The measurand result reported as required in the applicable method(s) used to test the subsample or the homogenate produced using the procedure in this Standard. Results are reported on a wet weight and/or dry weight basis, as required.

## 11 BIBLIOGRAPHY

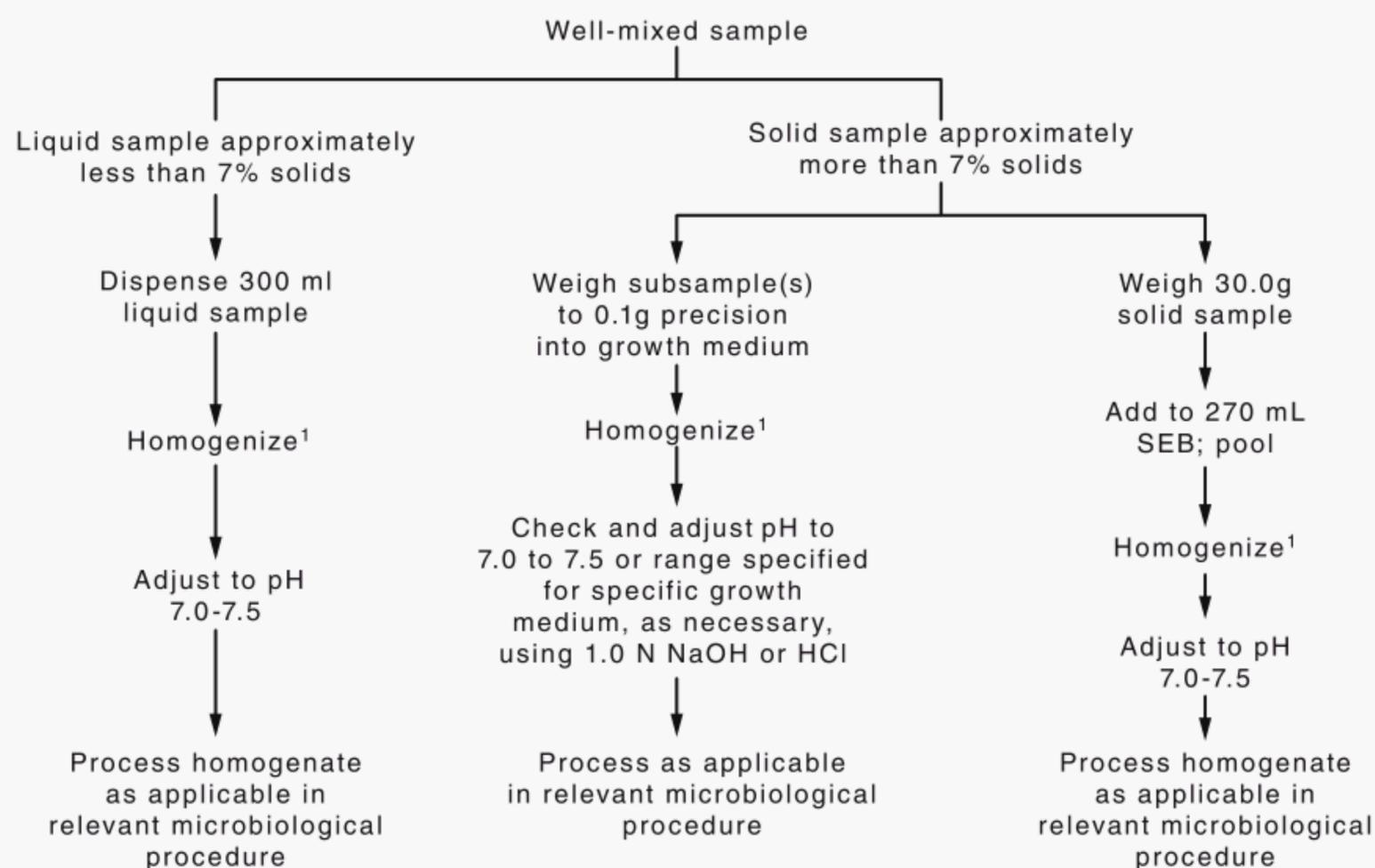
Information given in the following literature was taken into account in the preparation of this Standard:

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- [2] United States Environmental Protection Agency (USEPA) (2001) Method 1684: *Total, fixed, and volatile solids in water, solids and biosolids*. EPA-821-R-01-015. USEPA Office of Water, Washington, DC, USA.
- [3] United States Environmental Protection Agency (USEPA) (2005) Method 1680: *Fecal coliforms in sewage sludge (biosolids) by multiple-tube-fermentation using lauryl tryptose broth (LTB) and EC medium*. EPA-821-R-04-026. USEPA Office of Water, Washington, DC, USA.
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- [9] US Food and Drug Administration (FDA). (2001) *Evaluation and Definition of Potentially Hazardous Foods—Chapter 6. Microbiological Challenge Testing*, Report of Institute of Food Technologists for FDA of US Department of Health and Human Services. IFT/FDA Contract No. 223-98-2333, Task Order No. 4. <http://www.fda.gov/Food/FoodScienceResearch/SafePracticesforFoodProcesses/ucm094154.htm> (accessed March 2015).

## APPENDIX A

FLOW CHART OF THE PROCEDURE FOR SAMPLE PREPARATION FOR  
SOILS, SEDIMENTS, SLUDGES (INCLUDING HIGH-PARTICULATE-  
CONTENT LIQUIDS AND SLURRIES)

(Informative)



<sup>1</sup> Manual or mechanical mixing; blending or stomaching.

APPENDIX B  
GUIDANCE ON SAMPLE PREPARATION FOR PROTOZOAN, HELMINTHS  
AND VIRUSES  
(Informative)

## B1 PROTOZOANS AND HELMINTHS

### B1.1 General

The following reagent is recommended for matrix homogenization for protozoans and helminth analysis. Reference should be made to original methods for further details of associated post-homogenization procedures.

### B1.2 Protozoan extraction buffer

#### References:

LARSEN, E, SMITH, J, NORTON, R and CORKERON, M. *Appl. Environ. Microbiol.* 2013, Vol. 79, pp. 2424-2427.

PEZZANA, A, VILAGINÉS, Ph, BORDET, F, COQUARD, D, SARRETTE, B and VILAGINÉS, R. *Water Science and Technology.* 2000, Vol.41, pp. 111-117.

#### B1.2.1 Preparation of complete buffer

Phosphate buffered saline (PBS), (single strength) .....	1.0 L
Tween 80 .....	0.15 mL
Antifoam B .....	0.15 mL
Sodium hexametaphosphate (NaPO <sub>3</sub> ) <sub>n</sub> (65–70%) .....	2.0 g

Add the Tween 80, Antifoam B and sodium hexametaphosphate to the PBS. Mix well. Store in a plastic or glass container at room temperature or under refrigeration at 5 ± 3°C.

#### B1.2.2 Phosphate buffered saline

NaCl .....	8.0 g
KCl .....	0.2 g
Na <sub>2</sub> HPO <sub>4</sub> , anhydrous .....	1.44 g
KH <sub>2</sub> PO <sub>4</sub> .....	0.24 g
Water .....	800 mL

Dissolve the ingredients in the water. Adjust pH to 7.4 with HCl or NaOH. Make up to a final volume of 1000 mL with water. Store in a plastic or glass container at room temperature or 5 ± 3°C. PBS is available commercially as a concentrate.

## B2 VIRUS ELUTION BUFFER

Reagents for elution of virus particles from solid matrices vary widely dependent on target virus and detection method (cell culture and/or nucleic acid-based techniques). Reference should be made to original methods for further details of the associated post-homogenization procedures. The following references provide guidance regarding selection of virus homogenization and elution reagents.

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APPENDIX C  
REAGENTS  
(Informative)

### C1 SCOPE

This Appendix sets out the formulation and procedure for preparing the buffer specified in this Standard.

### C2 SOIL EXTRACTION BUFFER

Ref. LARSEN, E, SMITH, J, NORTON, R and CORKERON, M. *Appl. Environ. Microbiol.* 2013. Vol. 79, pp. 2424–2427.

The following formulation is modified to that given in the reference by the addition of potassium dihydrogen phosphate for additional buffering.

#### C2.1 Preparation of complete buffer

NaCl .....	8.5 g
Sodium hexametaphosphate (NaPO <sub>3</sub> ) <sub>n</sub> (65%–70%) .....	2.0 g
Antifoam A .....	0.1 mL
Tween 20 <sup>®</sup> stock solution or equivalent .....	10.0 mL
Potassium dihydrogen phosphate stock solution .....	1.25 mL
Laboratory reagent grade water .....	985 mL

Dissolve all ingredients, except Tween 20<sup>®</sup> stock solution, in 985 mL laboratory reagent grade water. Check pH and adjust to 7.2 ± 0.2, if necessary, using 0.1 N HCl or NaOH. Autoclave at 121°C (15 PSI) for 15 min. Cool to <50°C and aseptically add 10 mL Tween 20<sup>®</sup> stock solution. Mix and store at room temperature away from light.

#### C2.2 Tween 20<sup>®</sup> stock solution or equivalent

Tween 20 <sup>®</sup> or equivalent .....	20.0 g
Laboratory reagent grade water .....	80 mL

Using continuous stirring, dissolve Tween<sup>®</sup> in reagent grade water. This may be aided through continuous stirring using a stir-bar and magnetic stirring apparatus with application of low heat. Do not boil or overheat. Filter (0.45 µm pore size) sterilize. Store in the dark.

#### C2.3 Potassium dihydrogen phosphate stock solution

Potassium dihydrogen phosphate (KH <sub>2</sub> PO <sub>4</sub> ) .....	34.0 g
Laboratory reagent grade water .....	500.0 mL

Dissolve KH<sub>2</sub>PO<sub>4</sub> in 500 mL laboratory reagent grade water. Adjust pH to 7.2 ± 0.5 with 1 N NaOH, and bring volume to 1 L with laboratory reagent grade water. Autoclave at 121°C (15 PSI) for 15 min. Store tightly sealed at 1–8°C.

APPENDIX D  
DETERMINATION OF PERCENT SOLIDS  
(Normative)

### D1 PERCENT SOLIDS

If results are to be reported on a dry weight basis, percent solids shall be determined on a representative sample of the original liquid, semi-solid or solid sample material. Determinations may be made on homogenized liquid samples prior to pH adjustment or addition of diluent or large-debris rinse water. The procedure shall be as follows:

- (a) Heat evaporating dish and watch glasses at 103–105°C for 1 h in an oven. Cool and store the dried equipment in a desiccator.
- (b) Weigh each evaporating dish and watch glass prior to use (record combined weight as ‘ $w_d$ ’).
- (c) *Fluid samples* If the sample contains enough moisture to flow readily, stir to homogenize, and place a 25 g to 50 g sample aliquot on a prepared evaporating dish. Spread each sample so that it is evenly distributed over the evaporating dish. Cover each sample with a watch glass, and weigh to the nearest 0.01 g (record weight as ‘ $w_s$ ’).

*Solid samples* If the sample consists of discrete pieces of solid material (biosolids sludge, for example), take cores from each piece with a cork borer or pulverize the entire sample coarsely on a clean surface by hand, using rubber gloves. Place a 25 g to 50 g aliquot of the pulverized sample on a prepared evaporating dish. Spread each sample so that it is evenly distributed over the evaporating dish. Cover each sample with a watch glass, and weigh (record weight as ‘ $w_s$ ’).

- (d) Dry the samples at 103–105°C for a minimum of 12 h, cool to balance temperature in a desiccator, and weigh.
- (e) Heat the residue for 1 h, cool it to balance temperature in a desiccator, and weigh. Repeat this heating, cooling, desiccating, and weighing procedure until the weight change is less than 4% or 50 mg, whichever is less. Record the final weight as ‘ $w_t$ ’.

NOTE: Samples should be weighed as quickly as possible to reduce evaporation from wet samples and adsorption of water from the environment by dried samples. If past performance has verified that drying to constant weight (less than 4% or 50 mg change) is reproducibly achieved for a particular matrix with drying for a fixed period, then the respective drying time may subsequently be used without the need to re-weigh samples.

### D2 CALCULATIONS

#### D2.1 Percent total solids

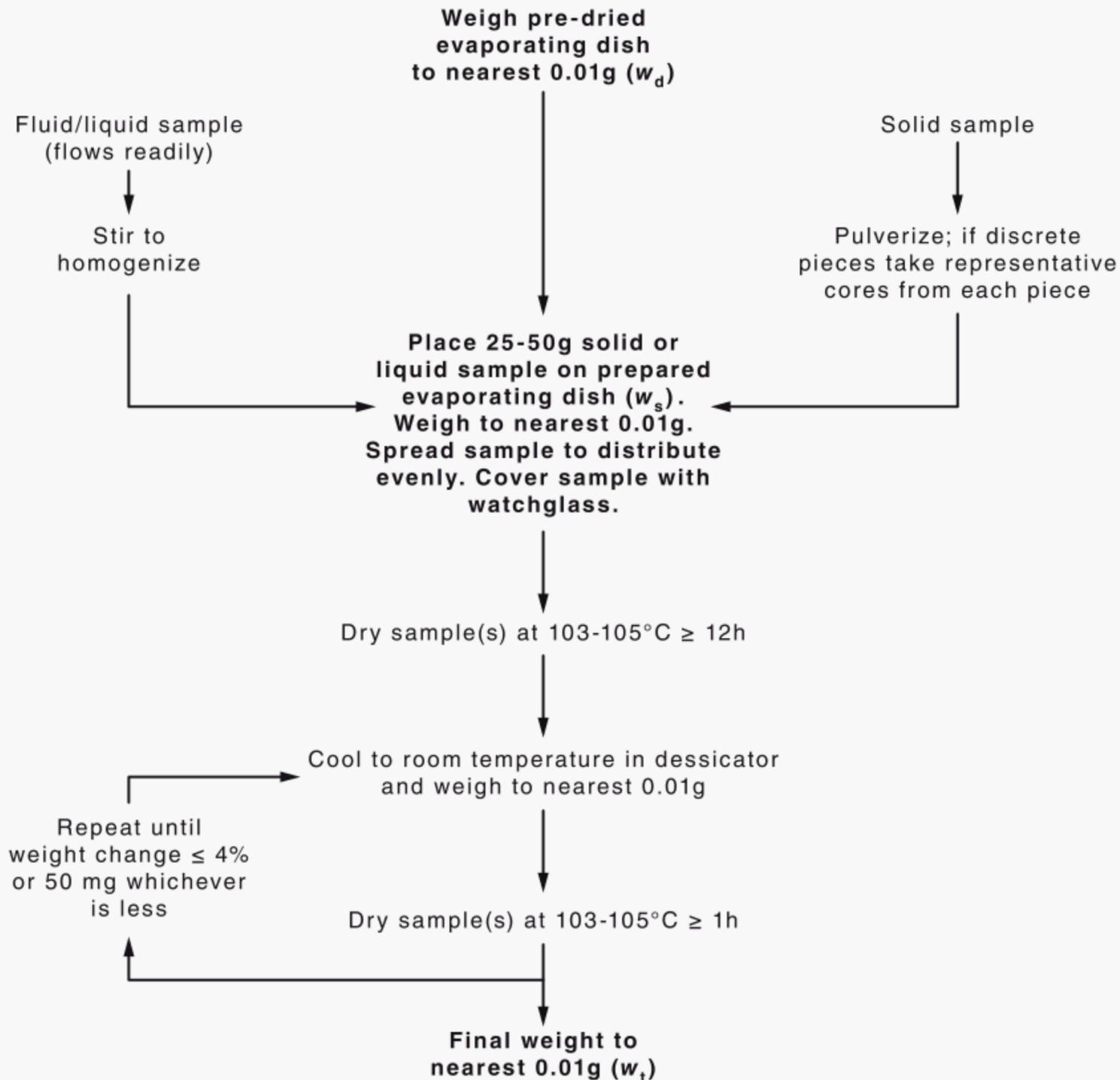
$$\% \text{ total solids} = \frac{w_t - w_d}{w_s - w_d} \times 100$$

where

- $w_t$  = weight of dried residue and dish (nearest 0.01 g)  
 $w_d$  = weight of dish (nearest 0.01 g)  
 $w_s$  = weight of wet sample and dish (nearest 0.01 g)

**D2.2 Target microorganism per gram dry weight**

$$\text{Target microorganism per gram dry weight} = \frac{\text{cfu target microorganism/g wet weight}}{\% \text{ total solids}} \times 100$$

**D3 FLOW CHART**

$$\% \text{ total solids} = \frac{w_t - w_d}{w_s - w_d} \times 100$$

## APPENDIX E

## GUIDANCE ON POST-HOMOGENIZATION ANALYTICAL TECHNIQUES

(Informative)

**E1 DILUTIONS**

Homogenates may be used directly for inoculation of growth media and/or further diluted. In order to retain a representative amount of sample in dilution series, the first in a series of 10-fold serial dilution of homogenates should utilize either 10 mL homogenate in 90 mL diluent; or 11 mL homogenate in 99 mL diluent. Subsequent dilutions should use similar volumes to increase representativeness of dilutions. If not prescribed in the applicable microbiological method used to analyse the homogenate, the diluent used should be either phosphate buffered water or peptone water. All dilutions should be very vigorously mixed to promote homogeneity. Dilutions of 1:2, 1:5 or other ratios may also be used dependent on the requirements of the microbiological procedure used to analyze the homogenate, as well as ultimate reporting requirements.

**E2 METHODS****E2.1 MPN**

For analyses using the MPN technique, undiluted homogenates may be inoculated. Referring to the subsamples used in this Standard, liquid (undiluted) and solid homogenates (1/10), Table E1 provides examples of wet weight sample mass represented by various subsample volumes as dispensed into various strengths of a liquid medium.

**TABLE E1****WET WEIGHT SAMPLE MASS RELATIVE TO SUBSAMPLE VOLUME**

Sample mL	Volume of medium mL	Strength of medium	Mass of liquid sample/container mL or g wet weight	Mass of solid sample/container g wet weight
50	50	Double	50.0	5.0
20	10	Triple	20.0	2.0
10	10	Double	10.0	1.0
1	10	Single	1.0	0.1

Amount of sample added and numbers of replicates at each dilution are dependent on the level of sensitivity and precision required. Generally, the greater the amount of sample introduced into the growth medium, the greater the sensitivity compared to lower amounts. Similarly, the greater the number of tubes per dilution (commonly 3, 5 or 10), the greater the precision.

**E2.2 Presence-absence**

For microbiological analytes for which a presence/absence determination is required, one may generally add the amount of homogenate corresponding to the mass or volume of sample defining the desired limit of detection (LoD). For example, in the table above, to achieve a LoD of 1 cfu target microorganism per 2 g wet weight solid, one may dispense 20 mL sample homogenate into 10 mL triple strength growth medium.

### **E2.3 Membrane filtration and spread plating**

For microbiological analyses utilizing membrane filtration or spread-plating, the homogenate should be diluted sufficiently to reduce interference by matrix materials. Such interference may include filter clogging, or an inability to distinguish distinct target colonies from one another or from matrix material.

### **E2.4 Molecular biological techniques**

As permitted in the applicable analytical procedure for analysis of the respective target microorganism, analysis of homogenates using molecular biological techniques, such as polymerase chain reaction (PCR) for detection, may employ either an enrichment-PCR approach using any of the above techniques, or direct extraction of nucleic acid from homogenate. Such techniques should include a quantified internal positive control microorganism in each analysis, with cells spiked into samples after pH neutralization.

#### **NOTES:**

- 1 During re-suspension and dilution, rapid settling of some higher-mass particles may occur, such as gravels and sands. If it is not possible to resuspend these such that a homogenous suspension is obtained containing these particles, allow them to settle (<1 min) and withdraw the supernatant, avoiding withdrawing liquid from <1 cm deep in the suspension. As silts and particularly clays may promote sedimentation of suspended bacteria, withdrawal of aliquots for dilution should occur prior to their settling as observed visually.
- 2 Where required reporting LoDs are based on dry weights and an estimate of dry weight cannot be made, it is recommended an amount of sample be analysed at approximately 0.1 and 0.5 of the wet weight LoD. That is, the LoD is being lowered to compensate for the fact that the dry weight is likely to be in the range of 10% to 50%.
- 3 Nucleic acid based techniques (NABTs) are subject to substantial and variable matrix interference. Materials present in solid samples such as metals, clays and particulate organics are known to interfere with nucleic acid recovery and PCR reactions. Determination of LoD and use of whole-cell-based positive control materials is therefore strongly recommended.

### **E2.5 Choice of microbiological analytical procedure**

The choice of microbiological methodology following homogenate preparation will depend on the purpose of the analysis/analyses, the expected count of target microorganism(s) and the physical characteristics of the material analysed (for example, percent solids, particle size distribution). These will affect the method of sub-sampling, amount of sample analysed, homogenization and dilution procedures.

To achieve the most sensitive analysis (lowest limit of detection of measurand), the maximal amount of starting material is tested. This is often the case where anticipated target microorganism numbers are low (<10 cfu per gram dry weight). For microbiological tests this is often a representative subsample(s) of the well-mixed sample itself, or the undiluted homogenate produced using the procedure in this Standard. MPN-based procedures are often optimal for growth-based analyses of samples containing substantial amounts of particulates. Such undiluted material is more likely to contain substances that may exert toxicity and/or interfere with subsequent detection, such as metals; surface-active, antimicrobial, or pH-altering compounds; competing microorganisms. Interfering substances should be neutralized by addition/application of neutralizing substances to sample-growth medium, or homogenate [8]. Low recoveries in samples spiked with applicable target microorganisms is indicative of interference. Challenge testing is recommended [9].

Sample components that impart colour or fluorescence to growth media may also interfere with growth-based procedures using chromogenic and/or fluorogenic media. Such interference can most often be determined by examination for interfering colour or fluorescence upon mixing of sample with medium, i.e. before incubation. Where such interference is observed, it needs to be neutralized through sample dilution or other means prior to incubation.

NOTES:

- 1 Interference via inhibition is often evidenced in MPN-format procedures by a lower number of target-microorganism-positive subsamples at a lower dilution, or conversely higher numbers of positive subsamples at a higher dilution.
- 2 Common neutralizing agents/methods for interfering substances include: sodium bisulfite (gluteraldehyde, mercurials); dilution (phenolics, alcohol, aldehydes, sorbate); glycine (aldehydes); lecithin (quaternary ammonium compounds, parahydroxybenzoates, bisbiguanides); polysorbate (quaternary ammonium compounds, iodine, parabens); thioglycollate (mercurials); thiosulfate (mercurials, halogens, aldehydes); and EDTA (magnesium or calcium).

Some samples may contain target microorganisms at up to  $10^6$  per gram wet weight of sample. These may include high organic content soils and sediments, crude sludges and those from low microbial reduction treatment processes. Membrane filtration or defined substrate MPN techniques may be utilized for such samples. Solids in the homogenate and at lower dilutions may preclude filtration due to clogging of the filter and/or inhibition or obscuring of colonies on the filter. This is typically not a problem at sample dilutions greater than  $10^{-2}$  to  $10^{-3}$ .

For high microbial reduction sludges, or samples where low target microorganism counts (<100 cfu per 10 g dry weight) are anticipated, the MPN technique as described above may be applied.

Chemically treated or highly dried samples may require sample pre-treatment and preparation, such as lime neutralization or re-hydration. These may impose additional constraints on subsequent procedures. For presence-absence or MPN analyses, particularly those where a specific diluent and/or medium pH is specified (e.g. outside the range pH 7.0–7.5), different approaches during sample pre-treatment and preparation may be required.

NOTES:

- 1 Due to potential toxicity, inhibition and interference, enrichment culture of solids are optimally performed at ratios of greater than 1:10 w/v solids to enrichment medium.
- 2 Substantial numbers of physiologically stressed and sub-lethally injured target microorganism(s) can occur in solid and semi-solid matrices. This is particularly the case in samples which have been subjected to heat, drying and/or chemical treatment. Detection using growth-based procedures may need to be specifically designed with reference to maximizing sub-lethally damaged microorganism recovery. This typically includes use of liquid resuscitation growth media rather than membrane filtration. Use of pre-enrichment media, particularly for microorganisms such as *Salmonella* and *E. coli* O157, promotes recovery of these injured microorganisms. Some defined substrate chromogenic/fluorogenic media are also specifically formulated to recover sub-lethally injured microorganisms.

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