

Australian Standard[®]

AS 4276.23:2016

Water microbiology

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

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.

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.

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 ± 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.