Soils, sludges and treated bio-wastes — Extraction of bacteriophages from sludge, soils and treated biowastes

Boden, Schlamm und behandelte Bio-abfälle —

S*ols, boues et bio-déchets traités — Extraction des bactériophage dans les boues, les sols et bio-déchets traités*

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Foreword

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

This document is currently undergoing ruggedness trials.

The document does not replace any existing CEN method.

The following TC's have been involved in the preparation of the standard: TC 308.

The standard describes an extraction method of bacteriophages,

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

Material	Validated	Document
Soil		
Sludge		
Biowaste		
Waste		

Introduction

This document is developed in the framework of the project 'Horizontal'. It is the result of a critical review "Methods for bacteriophages (and viruses) to be monitored in EU in sludges, soils and treated biowastes" and aims at evaluation of the latest developments in assessing bacteriophages in sludge, soil and biowastes. 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 will be validated within the project ”Horizontal”.

Sludges, soils and biowastes can contain pathogens such as Enteroviruses, *Salmonella* spp. Most occur in the intestinal tract of humans and animals and can be 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 storage and treatment processes to control pathogens, and application rates to land.

Viruses had been described to have a tendency to adsorb to solids. There are some differences between different viruses regarding adsorption efficiency, solids to which they can be adsorbed better and conditions that favour adsorptions. Once they are adsorbed to solids, they persist longer than when they are free. Consequently, viruses are expected to concentrate in sludges, biowastes, sediments and soils, where they will persist longer that in the contaminated waters.

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 12 are normative; annexes are normative or informative, as stated in the top lines of the annexes.

1 Scope

This European draft standard specifies a procedure for the elution of bacteriophages from sewage sludges, compost and biowaste samples. The user should, prior to analysis, validate the method for the particular type of sample they wish to analyse: sludges, soils and biowastes.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this HOR-HYG draft. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this HOR-HYG draft standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 31-0:1992, *Quantities and units — Part 0: General principles.*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods.*

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

ISO 5667-15:1999, *Water quality — Sampling — Part 15: Guidance on preservation and handling of sludge and sediment samples.*

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

ISO 6887: 1983. *Microbiology – General guidance to the enumeration of micro-organisms by culture.*

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

ISO 10705-1: 1995. *Water quality - Detection and enumeration of bacteriophages - Part 1: Enumeration of F-specific RNA bacteriophages.*

ISO 10705-2: 2000. *Water quality - Detection and enumeration of bacteriophages. Part 2: Enumeration of somatic coliphages.*

ISO 10705-4: 2001. *Water quality - Detection and enumeration of bacteriophages - Part 4: Enumeration of bacteriophages infecting* Bacteroides fragilis.

3 Definitions

For the purposes of this draft standard, the terms and definitions given in ISO/IEC Guide 2 and the following apply.

3.1
bacteriophages
Bacterial viruses which are capable of infecting selected host strains

NOTE Bacteriophages produce visible plaques (clearance zones) in a confluent lawn of the host strain grown under appropriate culture conditions.

3.2
somatic coliphages
bacterial viruses which are capable of infecting selected *Escherichia coli* host strains (and related strains) by attachment to the bacterial cell wall as the first step of the infection process

3.3
bacteriophage infecting *Bacteroides fragilis*
bacterial viruses which are capable of infecting selected *Bacteroides fragilis* host strains by attachment to the bacterial cell wall as the first step of the infectious process

3.4
F-specific RNA bacteriophages:
bacterial viruses which are capable of infecting a specified host strain with F-pili or sex-pili to produce visible plaques (clearance zones) on a confluent lawn grown under appropriate culture conditions, whereas the infectious process is inhibited in the presence of a concentration of 40 (occasionally 400) µg/ml of RNase in the plating medium

3.5
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]

4 Symbols and abbreviations

5 Principle

Bacteriophages are extracted from the sludge, soil and biowaste solid materials (biosolids since now) by homogenisation, elution, clarification and decontamination of samples. The extract is used for the enumeration of different types of bacteriophages according to the standard protocols ISO 10705-1, ISO 10705-2 or ISO 10705-4, or any other established or standardised method for enumeration of bacteriophages.

6 Reagents, diluents and culture media

6.1 General instructions

To ensure reproducible results, prepare culture media and diluents using constituents of uniform quality and chemicals of recognised analytical grade, and follow the instructions given in annex A. For information on

storage see ISO 5667-15, except where indicated in this HOR-HYG draft standard. Alternatively, dehydrated diluent or complete medium prepared following strictly the manufacturer's instructions.

For the preparation of reagents, use glass-distilled water or deionised water free from substances which might inhibit bacterial growth under the conditions of the test, and complying with ISO 3696.

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

6.2 Elution buffer

For making elution buffer, use beef extract (Annex A.2) to 10% at pH 7.2

7 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilised in accordance with the instructions given in ISO 8199:2005.

Usual microbiological laboratory equipment and in particular:

7.1 Hot-air oven for dry-heat sterilization and an autoclave

7.2 Magnetic stirrer and stir bars

7.3 pH meter with an accuracy of ± 0.1

7.4 Bunsen burner or working within a Class II safety cabinet

7.5 Refrigerated centrifuge capable of of attaining 5,000*g* and screw-cap centrifuge bottles (500 ml of capacity) that can withstand 5,000*g*

7.6 Usual sterile, microbiological laboratory glassware or disposable plastics ware according to ISO 8199 and including 7.11 Petri dishes of 9 cm or 14 cm to 15 cm diameter, vented

7.7 Graduated pipettes of 0,1 ml, 1 ml, 5 ml and 10 ml capacity and Pasteur pipettes

7.8 Glass bottles of suitable volume

7.9 Culture tubes with caps or suitable alternative

7.10 Measuring cylinders of suitable capacity

7.11 Membrane filter units for decontamination, having a pore size of 0.22 µm, low protein-binding membranes, as for example, those composed of polyvinylidene difluoride or polyether sulphone

7.12 Plastics vials, lidded, of 1,5 ml to 3 ml capacity

7.13 Refrigerator, temperature set at (5 ± 3) °C

8 Sampling

Take samples of at least 500 g wet weight and transport to laboratory as quickly as possible, chilled at (5 ± 3) °C, in accordance with ISO 8199, ISO 5667-13 and ISO 5667-15.

8.1 General

As samples are liable to ferment and contain pathogenic microorganisms, it is of paramount importance to adhere to national and international regulations relating to bio hazardous samples when handling and transporting samples. It is essential to keep samples away from food or drink, and to protect any cuts.

See also the Warning note in the introduction.

8.2 Storage

When samples are not to be analysed immediately, store them at (5 ± 3)°C in well labelled containers, preferable plastic. At this condition, samples can be stored for a maximum period of 48 h. Samples should not be stored on an open bench in the laboratory.

8.3 Handling

Good laboratory practice and cleanliness is essential. When handling sludge samples it is necessary to wear gloves, face and eye protection, and sufficient body protection to protect against spillages or bottles bursting. The gas evolved when opening sludge samples is flammable and so should be carried out away from naked flames and all equipment should be flame proof.

See also the Warning note in Introduction.

8.4 Safety precautions

Bacteriophages are non-pathogenic to man and animals, but some types are very resistant to drying. Appropriate precautions should therefore be taken to prevent cross-contamination of test materials, particularly when examining or handling samples of high titre or when inoculating cultures of the host strain. Such procedures shall be carried out in a biohazard cabinet or a separate area of the laboratory.

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

9 Procedure

9.1 Homogenisation

Mix sample and weigh out a representative sub-sample of 25 g (wet weight).

Transfer 25 ml or 25 g of representative (blended) biosolid sample to a sterile vessel of a minimum capacity of 500 ml and with screw top.

9.2 Elution

Add a volume of sterile elution buffer to the vessel containing the biosolids up to a final volume of 250 ml.

Add a sterile stir bar into vessel containing the biosolids

Place vessel on magnetic stirrer, and stir at speed sufficient to develop vortex for 15 - 20 min at room temperature.

Avoid the formation of foam by regulating the stirring speed and the formation of aerosols by screwing top the vessel.

NOTE **For lime treated sludge adjust the pH to (7.2 ± 0.5) with 1 M/1 hydrochloric acid. If the pH drops below 4.5 whilst neutralizing the sample, a new sample should be prepared. If other chemical treatment is used on sludge samples to be tested a suitable neutralisation procedure should be adopted.**

9.3 Clarification

Add the eluted biosolids to a sterile centrifuge tube appropriate for your centrifuge.

Centrifuge the biosolids-eluate mixture at 4,000 x *g* at (4 ± 2)℃ for 30 min.

Recover the supernatant by decanting it into beaker and discard the sedimented solids.

9.4 Decontamination

Filter the supernatant through low-binding membranes, as for example, those composed of polyvinylidene difluoride or polyether sulphone of a pore size of 0,2 µm (7.11). Total volume to be decontamined is related to the density of coliphages which varies from different biosolids (see Annex B). Attending to the final values are referred to 1 g and these estimated values, it is suggested taking at least 10 ml for primary sludge, activated sludge, thickened sludge and de-watered sludge; and at least 20 ml for different compost and lime-treated biosolids.

Harvest the filtrate in a sterile recipient with screw top.

Refrigerate the clarified-eluted sample immediately at (5 ± 3)℃, and maintain it at that temperature until it is assayed for the enumeration of bacteriophages within 12 h.

9.5 Enumeration of bacteriophages

The enumeration of bacteriophages is undertaken in accordance to the ISO 10705-1, ISO 10705-2 or ISO 10705-4, or any other established or standardised method for enumeration of bacteriophages. If dilution is required for enumeration, use peptone-saline solution (Annex A.1) or another diluent complying with ISO 6887.

9.6 Determination of the dry residue content

The dry matter content is measured using the method described in EN 12880:2000.

10 Expression of results

10.1 Enumeration of plaques

Select plates with well-separated, and preferably more than 30 plaques whenever present. If only counts below 30 per plate are found, select plates inoculated with the largest volume of sample. From the number of plaques counted, calculate the number of plaque-forming particles of bacteriophages in 1 ml of the diluted sample in accordance to ISO 10705-1, ISO 10705-2 or ISO 10705-4.

10.2 Adjustment of results to dry weight

Calculate the number of pfp/ml attending to the initial dilution performed to the biosolid sample with the elution buffer.

Correct values attending to the percentage of total solids in sludge, soil, compost or biowaste sample.

Express the result as pfp/g.dw or pfu/g.dw.

Summarising, the calculation is the next:

$$\text{pfp/g.dw} = \text{N pfp/ml} \times \frac{250\ \text{ml}}{25\ \text{ml or g.ww}} \times \frac{100\ \text{g.ww.}}{\text{X g.dw}}$$

N Total number of bacteriophages in the extract expressed in plaque-forming particles per ml (also termed plaque-forming units, pfu)

g.dw grams dry weight in 25 ml or 25 g of analysed sample.

g.ww grams wet weight.

X Percentage in entire numbers

11 Performance data

Information concerning the repeatability and reproducibility of the procedure, following the ruggedness study (European scale Intralaboratory trial) performed during the FP6 EU Horizontal-Hyg project are given in the corresponding Suitability Study report [4].

12 Test report

The test report shall contain the following information:

a) reference to this part of this European draft Standard;

b) a reference to the standard method used to enumerate the type of bacteriophages

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

d) the incubation time, if different from the standard time in clause 9.5

e) the results expressed in accordance with clause 10 as pfp/g.dw

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

Annex A
(informative)

Elution Buffer

A.1 Saline peptone solution

Peptone	1 g
Sodium chloride	8.5 g
Distilled water	1000 ml

Dissolve the ingredients in hot water. Adjust the pH to 7.2 ± 0.2 at (45 ± 3)℃ so that after sterilization it will be 7.2 ± 0.5. Dispense in convenient volumes and autoclave at (121 ± 3)℃ for 15 min. Store in the dark for not longer than 6 months.

A.2 Beef extract solution (10% pH 7.2)

Beef extract	10 g
Distilled water	100 ml

Dilute the beef extract in distilled water. Adjust the pH to 7.2 ± 0.2.

Autoclave at 121 ± 3℃ for 15 min. Store in the refrigerator. Check the solution and discard if bacterial contamination is observed

Annex B
(informative)

Levels of enteroviruses and somatic coliphages in different kind of waters and biosolids

	Enteroviruses PFP/100ml	Somatic coliphages PFP/100ml	Ratio
Sewage	10^{1-3}	10^{5-8}	10^{4}-10^{5}
Primary sludge	10^{2-5}	10^{5-8}	10^{4}-10^{5}
Activated sludge	10^{1-3}	10^{5-7}	10^{4}-10^{5}

Levels of bacteriophages in biosolids

	Somatic coliphages	F RNA-specific	*B. fragilis* bacteriophages
Primary sludge	10^5 - 10^9	10^3 – 10^6	10^2 – 10^5
Activated sludge	10^5 – 10^8	10^2 – 10^5	10^2 – 10^3
Thickened sludge	10^4 – 10^7	10^2 – 10^3	10^2 – 10^3
De-watered	10^5 – 10^8	10^3 – 10^4	10^2 – 10^3
Compost (static pile)	10^2 (7 week) 10^1 (10 week)		
Compost (windrow composting system)	10^2 (3 week) 10^2 (4 week)		
Compost (natural draft system)	10^2 (7 week) <1 (10 week)		
After lime treatment	Control 1.5 10^6 10^3 (pH 10.0) 10^2 (pH 11.5)	<10 (pH 10.0) <10 (pH 11.5) <10 (pH 12.0)	

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