Soils, sludges and treated biowastes – Detection and enumeration of viable helminth ova in sludge soils and biowastes - Flotation method on Natrium nitrate $NaNO_3$ solution, specific gravity 1.35

Boden, Schlamm und behandelte Bio-abfälle -

Sols, boues et bio-déchets traités – Détection et dénombrement des oeufs d'helminthes viables dans les boues, les sols et les bio-déchets – Méthode de flottation en solution de nitrate de sodium, densité 1.35

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Contents

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Foreword

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

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

This document does not replace any existing CEN method.

The standard is divided into three parts:

- part 1 describes a flotation method on calcium nitrate solution, sp. gr. 1.35,
- part 2 describes a flotation method on sodium nitrate solution, sp. gr. 1.35,
- part 3 describes a flotation method on zinc sulphate solution, sp. gr. 1.35.

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

Material	Validated	Document
Soil	Not validated yet	[reference]
Sludge	Not validated yet	
Biowaste	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 the enumeration of viable helminths ova in sludge, soil and treated biowastes" and aims at evaluation of the latest developments in assessing viable helminths ova 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, soil improvers, growing media and biowastes can contain pathogens 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 and application rates to land.

Helminths are intestinal parasites, infecting human and animals. In the simplest cycle, a parasite stage from human is immediately infective for other humans. In other infections such as ascariasis or trichuriasis, a maturation period outside the body is required before the parasite become infective. However, for many parasite infections, a second or even or third host is required for completion of the life cycle. The infective stages are usually within ova, which are excreted in fresh faeces of infected individuals and may survive for weeks to months in the environment. Parasitic infections present a potential health risk associated with use of sludge, soil and treated biowastes due to the existence of these highly resistant helminth ova and their low infective doses.

WARNING – "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 pressurise 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".

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 draft European Standard describes a procedure for the detection and enumeration of viable pathogenic helminths ova in soils, sludge, and treated biowastes.

The user should, prior to analysis, validate the method for the particular type of sample they wish to analyse: soils, sludges and treated biowastes.

This method is of particular use to determine the efficiency of treatment process as outlined in the revision of Directive 86/278/EEC (3rd draft, CEN/TC 308-doc 525 [1]) and the Regulation (EC) No 208/2006 [2] for the elimination of pathogens in treated substrates sewage sludge. The treatment processes are validated through to a defined Log reduction with a resistant test organism such as *Ascaris* ova.

2 Normative references

These normative references are cited at appropriate places in the text and the publications are listed hereafter. For dated references, only the edition cited applies. For undated references the latest edition of the publication referred to applies (including any amendments).

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

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

ENV ISO 13843:2001, *Water quality — Guidance on validation of microbiological methods*.

3 Definitions

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

3.1
helminth ova
helminths are parasitic worms. Helminths typically enter the body through the mouth and lodge in the intestines where they hatch, grow into adult worms, and begin producing eggs or ova. The ova are then passed out of the body with feces. Some helminths can infect areas of the body other than the intestines.
Helminth ova were commonly found in relatively large numbers in environment.

There are three main classes of helminths that are important from a human health standpoint: nematodes (roundworms), cestodes (tapeworms), and trematodes (flukes).
The helminth ova are among the more particularly targeted in Europe for the sludge monitoring, the nematodes ova as Trichuridae (Trichuris, Capillaria,…) and as Ascaridiae (Ascaris, Toxocara,…) and the cestodes ova as Taeniidae (Taenia) and as Hymenolepidiae (Hymenolepis).

3.2
viable ova
ova are considered as being viable or potentially viable when the integrity of their structure can be observed or if a larval development is detected inside

3.3
non viable ova
Any absence or disorganisation of internal/external structures is criteria attesting the non viability of ova

3.4
method definition
nematodes or cestodes ova able to be isolated by flotation solution and their viability estimated by respectively microscopic examination of their integrity and the exclusion of blue trypan

3.5
dry residue
the dry mass portion of the sample 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

5.1 Detection and enumeration of helminth ova

The detection and enumeration of helminths ova requires five successive steps:

- dissociation of the helminth ova from the organic matter;
- separation of helminth ova from the medium by flotation and filtration;
- concentration of the helminth ova by settling;
- second flotation to bring the helminths ova at the top of the solution by flotation to be fixed onto the cover glass.
- microscopic examination to detect and enumerate helminth ova.

5.2 Evaluation of the viability of Nematodes ova

The evaluation of the nematodes ova viability is based on the microscopic examination of the internal and external ova structure.

5.3 Evaluation of the viability of Cestodes ova

The evaluation of the cestodes ova viability is based on the exclusion of the trypan blue dye by living cells according to the AFNOR PR XP X 33-031 experimental standard [3]. The viable ova keep their initial colour while dead ova are blue stained.

6 Reagents

To ensure reproducible results, prepare solutions using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent 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].

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

6.1 Natrium nitrate $NaNO_3$ solution, sp.gr. 1.35

$NaNO_3$	615.5 g
Distilled water	1000 mL

Dissolve $NaNO_3$ in distilled water, using a magnetic stirring bar. Check the specific gravity (sp.gr.1.35 ± 0.1) with a densitometer (7.1.1). Adjust the specific gravity by the addition of either $NaNO_3$ or water.

The solution may be stored at room temperature up to 6 months.

6.2 Sodium hypochlorite (NaOCl) solution (containing 1% active chlorine)

NaOCl solution 1%	50 mL
Distilled water	700 mL

Dissolve Sodium hypochlorite (NaOCl) solution 1 % in distilled water, using a magnetic stirring bar.

6.3 Sodium hypochlorite (NaOCl) solution 0.5 %

NaOCl solution 12%	2 mL
Distilled water	13 mL

Dilute sodium hypochlorite solution 12% in distilled water.

The solution is unstable. It should be prepared just before its use.

6.4 Sodium chloride (NaCl) solution, 1 M

NaCl	58.5 g
Distilled water	1000 mL

Dissolve NaCl in distilled water.

The solution may be stored at room temperature up to 1 year.

6.5 Trypan Blue solution 0,4%

Trypan blue	0.4 g
Distilled water	100 mL

Dissolve Trypan blue in distilled water.

The solution may be stored at room temperature up to 1 year.

7 Apparatus

In addition to usual microbiological laboratory equipment:

7.1 Equipment for sample preparation

7.1.1 Densitometer

7.1.2 pH meter with accuracy of ± 0.1

7.1.3 Analytical balance

7.1.4 Metal spatula

7.1.5 Centrifuge with mobile rotor capable of centrifuging at 450g and 660g

7.1.6 Conical centrifuge tubes, 10 mL

7.1.7 Test tubes rack

7.1.8 Graduated cylinder, 100 mL, 1000 mL volume

7.1.9 Beakers, 100 mL, 250 mL, 500 mL volume

71.10 Erlenmeyer-container, 1000 mL volume

7.1.11 Conical centrifuge tubes, 50 and 10 mL volume

7.1.12 Glass rods

7.1.13 1 mm mesh metallic sieve

7.1.14 25 µm mesh plastic/metal sieve

7.1.15 Vortex mixer

7.1.16 Vacuum pump

7.1.17 Washing bottle

7.1.18 Refrigerator capable of maintaining (5 ± 3)ºC

7.2 Equipment for microscopic examination

7.2.1 **Adjustable pipette** for measuring and distributing from 100 µL to 1000 µL

7.2.2 **Sterile tips** for pipette

7.2.3 **Standard light microscope**, with x10 and x50 magnification

7.2.4 **Sedgwick-Rafter cell**

7.2.6 **Thermostatic incubator(s)** regulated at (28 ± 1)ºC

8 Sampling

Take samples of at least 100 g wet weight and deliver them to the laboratory as quickly as possible (within 24 h). In order to prevent inactivation of helminth ova during transport to the laboratory and subsequent storage, the samples should be stored in suitable containers in the dark at (5 ± 3)ºC.

8.1 General

Samples are liable to ferment and can contain pathogens. 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.

8.2 Storage

It is not advisable to store samples in the open laboratory. If samples are to be stored, store them in the dark at (5 ± 3)℃ for no more than 1 week after receipt.

8.3 Handling

Cleanliness when working is essential. It is necessary to wear gloves, face and eye protection, and sufficient body protection to guard against bottles bursting. The gas evolved is usually flammable, so all equipment in the vicinity shall be flame proof to avoid any source of ignition.

See also the Warning note in the introduction.

9 Procedure

9.1 Sample preparation

9.1.1 Dissociation/separation of helminths ova from the organic matter

Mix sample and weigh out a representative sub-sample of 25 g (wet weight) into a 500 mL beaker.

Add sodium hypochlorite separating solution containing 1% active chlorine (6.2), into the beaker to make a paste with the sample. The volume of sodium hypochlorite solution to be added depends on the type of sample.

Homogenize by mixing the sample with the sodium hypochlorite solution using a glass rod, and allow settling during 10 min. Mix with a glass rod several times during this time period.

9.1.2 First flotation

Add natrium nitrate flotation solution (6.1) to the sample paste (9.1.1) so as to obtain a final volume of 150 mL. Homogenise the suspension by mixing thoroughly using a glass rod and allow settling during 30 min.

Pour carefully the supernatant through a 1 mm mesh metallic sieve (7.1.13) held on a large 250 mL beaker, and discard the remaining sediment. Pour the obtained filtrate through a 25 µm mesh plastic/metallic sieve (7.1.14). The 250 mL beaker is thoroughly rinsed with tap water, using a washing bottle, and the backwash water is also poured through the 25 µm mesh plastic/metallic sieve.

Wash the residue retained by the 25 µm mesh plastic/metallic sieve, with tap water, using a washing bottle. Pour and flush the collected material on the 25 µm mesh plastic/metallic sieve into a 10 mL conical centrifuge tube placed into a test tube rack. If there is a lot of residue, then pour the residue into 2 conical centrifuge tubes.

Complete the conical centrifuge tubes up to 10 mL with distilled water.

Centrifuge the conical tubes 10 min at 450 *g*.

Pour the supernatant off the tubes while leaving few millilitres of liquid above the sediment pellet, and proceed to a second flotation (9.1.4).

If the pellet is thin, then the supernatant should be carefully removed using a pipette. Resuspend the sediment by mixing thoroughly with the remaining water, and adjust the volume to 1 mL with distilled water. Then proceed to the microscopic examination (9.2).

9.1.3 Second flotation

Add 2 ml natrium nitrate flotation solution (6.1) to the sediment pellet in the conical centrifuge tube (9.1.3). Homogenise by mixing thoroughly with a vortex mixer (7.1.15). Complete the volume to 10 mL with natrium nitrate flotation solution (6.1).

Centrifuge 10 min at 450 g.

Allow settling during 30 min. Pour carefully the supernatant into a 50 mL new centrifuge tube. Complete the conical centrifuge tubes up to 50 mL with distilled water.

Centrifuge the conical tubes 10 min at 450 *g*.

Siphon carefully the supernatant off with a vacuum pump. Resuspend the sediment by mixing thoroughly with the remaining water, and adjust the volume to 1 mL with distilled water. Then proceed to the microscopic examination.

9.2 Microscopic examination for the detection and enumeration of helminths ova

Proceed to the microscopic examination of the 1 mL suspension using a Sedgwick-Rafter cell at low magnification (x10, x20) field by field.

The type and the number of helminths ova found are noted.

9.3 Nematodes ova viability evaluation

The evaluation of the viability of nematode ova is performed in the same time as the enumeration of helminth ova, by microscopic examination of the nematode ova structure at a higher magnification (x50):

- potentially viable ova:	- integrity of structures observed - unembryonated ova with non-visible defects
- viable ova:	- motile larva detected inside
- non viable ova:	- any absence of internal structures - or any disorganisation of the internal structure - and/or any damage of the external shell

9.4 Cestodes ova viability evaluation

Following the enumeration of viable and non viable nematodes ova (9.3) pour the analysed sample from the Sedgwick-Rafter cell into the same conical centrifuge tube than the one previously used (9.1.2). Rinse the Sedgwick-Rafter cell and the cover glass slide with up to 10 mL of distilled water, pouring the washing water off the same conical centrifuge tube to avoid any lost of ova.

Centrifuge the suspension 5 min at 660 g.

Siphon carefully the supernatant off with a vacuum pump. Resuspend the sediment pellet in 1 mL of sodium hypochlorite (NaOCl) 0.5 % solution (6.3) and mix during 1 min with a vortex mixer.

Add 5 mL of sodium chloride (NaCl) 1 M solution (6.4) and mix with a vortex mixer. Centrifuge 5 min at 660 g.

Siphon carefully the supernatant off with a vacuum pump. Rinse the sediment pellet with 5 mL of NaCl 1M solution (6.4) and mix with a vortex mixer. Centrifuge 5 min at 660 g.

Siphon again carefully the supernatant off with a vacuum pump. Rinse the sediment pellet with 5 mL of NaCl 1M solution (6.4) and mix with a vortex mixer. Centrifuge a last time 5 min at 660 g.

Discard carefully the supernatant with an automatic pipette while leaving around 900 µL (sediment pellet + remaining supernatant). Resuspend the sediment with the remaining supernatant.

Add 100 µL of Trypan Blue 0.4% solution (6.5). Allow settling 10 min at room temperature, time for the dye to react.

Proceed to the second microscopic examination of the 1 mL suspension using a Sedgwick-Rafter cell, at low magnification (x10, x20) field by field, and then at a higher magnification (x50) to examine all the cestodes ova.

All viable cestodes ova remain uncoloured while dead cestodes ova become blue coloured.

9.5 Determination of the dry residue content

The numbers of viable helminth ova can 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.

10 Expression of results

10.1 Number of viable helminths ova

The number of viable helminths ova corresponds to the number of viable nematodes ova and the number of viable cestodes ova observed and identified during the two microscopic examinations of the whole concentrate using the Sedgwick-Rafter cell.

Report the result as being the number (n) of viable helminths ova in 25 g (wet weight) sample.

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

$$N / 25 \times e$$

N = number of viable helminth ova in 25g (wet weight)

e = the dry residue (%) of the original wet sample

10.2 Total number of helminths ova

The total number of helminths ova corresponds to the number of viable and non viable nematodes and cestodes ova observed and identified during the two microscopic examinations of the whole concentrate using the Sedgwick-Rafter cell.

Report the result as being the total number of viable and non viable helminths ova in 25 g (wet weight) sample.

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

$$N_t / 25 \times e$$

N_t = total number of helminth ova in 25g (wet weight)

e = the dry residue (%) of the original wet sample

11 Performance data

Not available.

12 Test report

The test report shall contain the following information:

a) a reference to this European Standard including its date of publication;

b) sampling report including precise identification of the sample;

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

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

e) expression of results, according to 10.

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

Bibliography

[1] CEN/TC 308 - doc 525:2001, Revision of Directive 86/278/EEC (3rd Draft).

[2] Regulation (EC) No 208/2006 of February 2006 amending Annexes VI and VIII to Regulations (EC) No 1774/2002 of the European Parliament and of the Council as regards processing standards for biogas and composting plants and requirements for manure.

[3] Pierzo V., E. Pierlot, F.Le Broc, S. Roussel and T.Simonart (2004). Final Desk Study Report on Feasibility of Horizontal Standards for the enumeration of viable helminth ova in sludge, soil and treated biowastes - Annex 2. EC-FP6-project Horizontal-Hyg contract n° SSPI-CT-2003-502411.

[4] AFNOR PR XP X 33-031 - Characterisation of sludge - Enumeration of viable helminth eggs in sludges - Double flotation method in a sodium nitrate solution.