

Draft method for the detection and enumeration of viable Helminth eggs in bio-wastes (untreated or treated sewage sludge, compost, soil).

DRAFT METHOD II (TRIPLE FLOTATION METHOD)

1 General information

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 is 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 eggs, which are excreted in fresh faeces and may survive for weeks or months in the environment.

Parasitic infections present a potential sewage health risk associated with use of sludge for agricultural, to the existence of highly resistant stages of the organisms and low infective dose.

2 Principle

This method identifies, quantifies and determines the viability of several types of eggs from intestinal parasites: *Ascaridida*, *Trichuroidea* (nematodes) and *Taenidae*, *Hymelopididae* (cestodes).

2.1 Numeration of helminth eggs

The recheck of helminth eggs needs three successive centrifugation-flotation steps while resuspending the sludge homogenate suspension in a zinc sulfate solution. After each flotation step, one cover glass is placed on the top of the centrifugation tube on the surface of the meniscus formed by the flotation solution, and observed on microscope to identify and to count the helminth eggs.

2.2 Viability of helminth eggs

The method for the determination of the viability of helminth eggs is based on the oxydoreduction reaction to reveal the activity of the malate deshydrognase of a zygote, or of blastomers or embryo while obtaining blue stained eggs (non soluble formazan).

3 Apparatus

The following apparatus shall be used:

- a) Centrifuge with rotor for 11 ml and 40 ml conical tubes (mobile pots).
- b) 20mm diameter and 11 ml conical glass centrifuge tubes.

- c) 40 ml bottom round plastic centrifuge tubes.
- d) Glass slides.
- e) 20 mm x 20 mm cover glass.
- f) Microscope with x 10 and x 40 objectives.
- g) 90 mm or 100 mm diameter Petri dish
- h) Absorbent paper.

4 Reagents

4.1 Zinc sulphate $ZnSO_4 \cdot 7H_2O$,

Dissolve 727.5 g of $ZnSO_4 \cdot 7H_2O$ in 1 L of water. Check the specific gravity with a hydrometer. Adjust specific gravity to $1.38 \pm 0,1$ g/ml if necessary.

4.2 Malic acid solution, 50 g/l (solution A)

Dissolve 2.5 g of malic acid in 50 ml of pH $7 \pm 0,2$ water. Neutralise with a sodium hydroxide solution and then adjust to pH 8 with a diluted sodium hydroxide solution.

4.3 β -nicotinamide adenine dinucleotide solution, 25 g/l (solution B)

Dissolve 0.025 g of β -nicotinamide adenine dinucleotide in 1 ml of water.

4.4 Magnesium chloride solution, 48 g/l (solution C)

Dissolve 0.048 g of magnesium chloride in 1 ml of water.

4.5 (3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) bromide solution, 6g/l (solution D).

Dissolve 0.006g of (3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) bromide in 1 ml of water.

4.6 Phenazine methosulphate solution, 5 g/l (solution E)

Dissolve 0.005g of phenazine methosulphate in 1 ml of water.

4.7 Oxydoreduction solution

Prepare 30 μ l of oxydoreduction solution while adding 6 μ l of each solution A, B,C,D and E.

5 Sample preparation

Dry weight measurement.

6 Procedure

For solid sludge samples, pour a sample portion corresponding to 0.5 g (dry weight) into a 40 ml bottom round plastic centrifuge tube.

For liquid sludge samples, pour a sample portion corresponding to 0.5 g (dry weight) of sample into a 40 ml bottom round plastic centrifuge tube, and centrifuge at 700 g for 5 minutes. The obtained sediment corresponds to the analytical aliquot.

6.1 Suspension preparation

Homogenise very carefully the analytical aliquot in a 40 ml bottom round plastic centrifuge tube, using a glass agitator or a Pasteur pipette regarding the necessity, while adding successive volumes of a total volume of 22 ml of zinc sulfate solution.

Immediately separate the homogenate suspension into two 11 ml conical glass centrifuge tubes.

6.2 Determination

First flotation

Place both 11 ml conical glass centrifuge tubes in the centrifuge.

a) Helminth eggs enumeration without any viability evaluation

Centrifuge at 180 g for 3 minutes at room temperature. Fill each centrifuge tube with zinc sulfate solution to obtain a meniscus on the top of which a 20 mm x 20 mm cover glass is placed.

Wait 3 minutes, carefully take out cover glasses and put them on a glass slide for immediate microscope examination. Proceed to the second flotation.

b) Helminth eggs enumeration with viability evaluation

Centrifuge at 180 g for 3 minutes at room temperature. Fill each centrifuge tube with zinc sulfate solution in order to obtain a meniscus on the top of which a 20 mm x 20 mm cover glass is placed.

Wait 3 minutes and carefully take out cover glasses. Return them and add 15 µl of oxydoreduction solution. Homogenise with a buttoned Pasteur pipette in order to distribute the solution on the surface of the cover glass and put them on glass slides for microscope examination (glass slide 1). Proceed to the second flotation.

Second flotation

Immediately after the first flotation, rehomogenise both centrifuge tubes with a Pasteur pipette by up and down movements.

a) Helminth eggs enumeration without any viability evaluation

Proceed as described for the first flotation. The glass slide 2 is so obtained. Proceed to the third flotation.

b) Helminth eggs enumeration with viability evaluation

Proceed as described for the first flotation. The glass slide 2 is so obtained. Proceed to the third flotation.

Third flotation

Repeat the previous protocol. The glass slide 3 is so obtained.

6.3 Enumeration and viability of helminth eggs.

a) Helminth eggs without any viability evaluation.

Proceed to the microscope examination of the whole surface of each cover glass at low magnification (x 10) in order to detect and enumerate helminth eggs.

If the examination under a microscope is not immediately performed, prepared slides must be stored (maximum 30 minutes) in a wet atmosphere (For example in a Petri dish with a saturated water absorbent paper inside) in order to avoid the crystallization of the zinc sulfate.

b) Helminth eggs enumeration with viability evaluation.

Proceed to the examination under a microscope of the whole surface of each cover glass at low magnification (x 10) in order to detect and enumerate helminth eggs.

If the examination under a microscope is not immediately performed, prepared slides must be stored (maximum 30 minutes) in a wet atmosphere (For example in a Petri dish with a saturated water absorbent paper inside) at 39 °C in order that the enzymatic reaction could occur.

Put the three glass slides into Petri dishes at 39°C at constant humidity during 5h30min +/- 30min.

Observe again helminth eggs and detect the coloration : blue-grey stained eggs are considered as viable, while dead eggs keep their initial coloration.

7 Expression of results

a) Helminth eggs

Add helminth eggs recovered at each flotation, identify them and report the result in number of viable helminth eggs in x g dry weight.

At least 3 analytical aliquots per sample are necessary for the expression of results.

b) Viable helminth eggs

Report the number of eggs which have conserved their morphologic integrity and which present a blue stained zygote or blastomeres or embryo. These eggs are considered as viable, while dead eggs keep their initial coloration.

Report the result in number of viable helminth eggs in x g dry weight.

At least 3 analytical aliquots per sample are necessary for the expression of results.