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

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Draft Method I (US EPA modified method)

1 General information

Helminth 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 Field of application

The objective of this method is to detect and enumerate viable Helminth eggs in untreated sewage sludge or in sewage sludge treated by composting, heat drying, alkaline treatment, or other hygienic treatment.

3 Principle

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

Sampling and transport
Samples shall be collected, transported and stored as specified in CEN. In addition, all the apparatus and containers which come in direct contact with the sample shall be clean.

3.1 Enumeration of helminth eggs

The enumeration of Helminth eggs requires five steps:

a) parasites dissociation from organic mater
b) flotation using a natrium nitrate solution
c) sedimentation
d) concentration
e) miscroscopic examination using a Sedgwick-Rafter counting chamber

3.2 Viability of cestodes eggs

The method to detect the viability of cestodes eggs is based on the principle of exclusion of the trypan blue dye by living cells. The viable eggs do not change in colour when the dead ones are blue stained.

3.3 Viability of nematodes eggs

The method to detect the viability of nematodes eggs is based on the observation of the eggs structure including size, shape, thickness of the shell, presence of specific structures of the shell (mamillated covering, operculum, spine).

4 Apparatus

The following apparatus shall be used :

a) 5 L plastic beaker
b) 160 µm mesh metallic sieve
c) spatula
d) mechanical blender
e) Plastic bag (300 mm x 150 mm)
f) Suction pump
g) Centrifuge with rotor for 500 ml or 1 L capacity bottles and rotor for 50 ml conical tubes.
h) Vortex mixer
i) Refrigerator
j) pH meter, with temperature compensation and pH measuring cell.
k) Hydrometer
l) Sedgewick-Rafter cell for microscopic examination
m) Microscope with x10 and x40 objectives

5 Reagents

Unless otherwise specified, only analytical grade reagents shall be used, and the aqueous solutions shall be prepared with double-distilled or deionised water.
The following reagents shall be used.

5.1 Natrium nitrate solution, sp.gr. 1.30, pH 7.0

Dissolve 615g of $NaNO_3$ in 1 L of distilled water. Check the specific gravity with a hydrometer and the pH with a pH meter. Adjust specific gravity to 1.30 ± 0,1 and pH to 7.0 ± 0,2 if necessary.

5.2 35% acid alcohol solution

Mix 65 ml of 0.1 N H_2SO4 solution with 35 ml of ethyl alcohol solution.

5.3 Ethyl ether solution, reagent grade

5.4 Natrium hypochlorite solution

Dissolve 2 ml of natrium hypoclorite 12° solution with 13 ml of distilled water.

5.5 Natrium chlorure 1 M

Dissolve 58.5 g of NaCl in 1 litre of distilled water.

5.6 Trypan blue solution

Dissolve 40 mg of trypan blue solution in 10 ml of distilled water.

6 Sample preparation

Dry weight measurement.

7 Procedure

7.1 Dissociation of parasites from organic mater

a) For liquid sludges (dry weight < 10%), pour a sample portion corresponding to 10 g dry weight into a centrifuge bottle (500 ml to 1 litre) and centrifuge at 3 000 g for 15 minutes. The sediment is then treated with the flotation solution (see 7.2).
b) For solid and thick sludges (dry weight ≥ 10%), suspend a sample portion corresponding to 10 g dry weight with 150 ml of water. The subsample is then homogenised by running a blender for 90 seconds.
c) The resulting mixture is then poured through a 160 µm diameter sieve held on a large 5 litre capacity beaker. Thoroughly rinse the blender bag with water (around 500 ml) and pour the resulting washing fluid through the sieve, and flush it in the beaker.
d) Wash the material retained in the sieve with 4 litre of water while carefully mixing it with a spatula in order to release the possible parasites from the sludge aggregates. The material collected is then flushed and poured into the beaker. Discard the remaining material embedded on the sieve.
e) Allow the washed poured sample to settle overnight at room temperature while covering the beaker with an aluminium foil.
f) Siphon off the supernatant while just leaving few millimetres of fluid above the sediment layer.
g) Mix the settled material by swirling and then pour it into a 500 ml or 1 litre centrifuge bottle. Rinse the beaker and pour the resulting washing fluid into the centrifuge bottle.
h) Centrifuge at 800 g to 1500 g for 5 min.
i) Siphon off the supernatant. Keep the sediment (around 500 ml to 1 litre).

7.2 Parasites flotation

a) Resuspend the sediment thoroughly in around 200 ml of natrium nitrate solution sp.gr. 1.30. Homogenise for 5 min with a vortex mixer.
b) Centrifuge at 800 g for 3 min.
c) Carefully pour the natrium nitrate supernatant into the same beaker than the one used in 7.1.
d) Resuspend the remaining sediment in 200 ml of natrium nitrate solution sp.gr. 1.30, and proceed as for a) to c) (second flotation step).
e) The total natrium supernatant volume obtained in the beaker is then around 400ml.

7.3 Parasites sedimentation

a) Dilute the supernatant with around 5 litre of water in order to decrease the specific gravity from 1.30 to around 1.00. Cover the beaker and allow to settle for at least 3 h.
b) Siphon off the supernatant while leaving few millimetres of fluid above the settled material.
c) Resuspend the sediment by swirling, and pour into one or two 50 ml conical centrifuge tubes. Rinse the beaker two or three times with water and pour the resulting washing liquid into the centrifuge tube(s).
d) Centrifuge the tubes at 660 g for 3 min.
e) If two centrifuge tubes were used, put both sediment(s) together in one of both 50 ml conical centrifuge tube. Thoroughly rinse the other conical centrifuge tube with water.
f) Centrifuge at 660 g for 3 min. Siphon off the supernatant and then proceed to the concentration step (see 7.4).

7.4 Parasites concentration

a) Resuspend the pellet in 15 ml of acid alcohol solution and homogenise with a vortex mixer.
b) Add 10 ml of ethyl ether solution, close the capped centrifuge tube and homogenise with a vortex mixer. Allow the gas to let off while lightly opening the cap.
c) Centrifuge the tube at 660 g for 3 min.
d) Siphon off the supernatant while leaving few millimetres of fluid above the sediment layer.
e) Resuspend the sediment in 10ml of 0.1 N H_2SO4 solution in order to eliminate the ethyl ether solution and centrifuge at 660 g for 3 min.
f) Discard the supernatant while leaving few millimetres of fluid above the sediment layer.
g) If the volume of the sediment exceeds 1 ml or if the sediment is too dense (more than 2 slides to examine), proceed another time with the flotation step (7.2) but add 30 ml of natrium nitrate solution sp.gr. 1.30 instead of 200 ml. Continue with the sedimentation (7.3) while diluting the supernatant in order to decrease the specific gravity from 1.30 to around 1.00 while adding around 1 litre of water instead of 5 litre. Proceed then to the concentration (7.4).

7.5 Miscroscope examination

Examine the concentrate under a microscope using a Sedgwick-Rafter cell in order to enumerate the detected eggs.
Report the number of viable nematodes eggs regarding their internal structure (dead eggs are characterised by the absence of an organised internal structure, and the presence of tiny drops in the internal structure).

7.6 Cestodes viability

a) After examination under a microscope, pour the sediment from the Sedwick-rafter cell into the same centrifuge tube than the one used in 7.4 e or g. Rinse the Sedgwick-Rafter cell with 10 ml of water.
b) Centrifuge at 660 g for 5 min.
c) Resuspend the sediment in 0.5% natrium hypochlorite solution and allow to stand for 1 minute while shaking with a vortex.
d) Add 5 ml of NaCl 1 M solution and homogenise with a vortex mixer.
e) Centrifuge at 660 g for 5 min.
f) Wash the sediment with 5 ml of NaCl 1 M solution, homogenise and centrifuge at 660 g for 5 min.
g) Wash the sediment with 5 ml of distilled water, homogenise and centrifuge at 660 g for 5 min.
h) Add 100 µl of 0.4% trypan blue solution and allow to stand 10 minutes at room temperature.
i) Examinethe stained sediment under a microscope while using a Sedgwick-Rafter cell and enumerate the viable cestodes eggs : the viable eggs did not change in colour when the dead ones are blue stained.
j) Report the number of viable cestodes eggs.

8 Expression of results

The number of viable helminths eggs corresponds to the total number of viable nematodes eggs (7.5) and viable cestodes eggs (7.6j).

Express the result while reporting the number of viable helminths eggs per 10 g dry weight.

References

1 US/E.P.A. method for the detection and enumeration of Helminth eggs : December 1992, p147-152.

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