Appendix I
Test Method for Detecting, Enumerating, and Determining the Viability of *Ascaris* Ova in Sludge

1.0 Scope

1.1 This test method describes the detection, enumeration, and determination of viability of *Ascaris* ova in water, wastewater, sludge, and compost. These pathogenic intestinal helminths occur in domestic animals and humans. The environment may become contaminated through direct deposit of human or animal feces or through sewage and wastewater discharges to receiving waters. Ingestion of water containing infective *Ascaris* ova may cause disease.

1.2 This test method is for wastewater, sludge, and compost. It is the user's responsibility to ensure the validity of this test method for untested matrices.

1.3 This standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use. For specific hazard statements, see section 9.

2.0 Referenced Documents

2.1 ASTM Standards:

- D 1129 Terminology Relating to Water[1]
- D 1193 Specification for Reagent Water[2]
- D 2777 Practice for Determination of Precision and Bias of Applicable Methods of committee D-19 on Water[3]

3.0 Terminology

(Definitions and Descriptions of Terms must be approved by the Definitions Advisor.)

3.1 Definitions - For definitions of terms used in this test method, refer to Terminology D 1129.

3.2 Descriptions of Terms Specific to This Standard:

3.2.1 The normal nematode life cycle consists of the egg, 4 larval stages and an adult. The larvae are similar in appearance to the adults; that is, they are typically worm-like in appearance.

3.2.2 Molting (*ecdysis*) of the outer layer *(cuticle*) takes place after each larval stage. Molting consists of 2 distinct processes, the deposition of the new cuticle and the shedding of the old one or exsheathment. The cuticle appears to be produced continuously, even throughout adult life.

3.2.3 A molted cuticle that still encapsulates a larva is called a *sheath*.

3.2.4 Ascarid egg shells are commonly comprised of layers. The outer tanned, bumpy layer is referred to as the *mammillated* layer and is useful in identifying *Ascaris* eggs. The mammillated layer is sometimes absent. Eggs that do not possess the mammillated layer are referred to as *decorticated* eggs.

3.2.5 A potentially infective *Ascaris* egg contains a third stage larva[4] encased in the sheaths of the first and second larval stages.

4.0 Summary of Test Method

4.1 This method is used to concentrate pathogenic *Ascaris* ova from wastewater, sludge, and compost. Samples are processed by blending with buffered water containing a surfactant. The blend is screened to remove large particulates. The solids in the screened portion are allowed to settle out and the supernatant is decanted. The sediment is subjected to density gradient centrifugation using magnesium sulfate (specific gravity 1.20). This flotation procedure yields a layer likely

[1]Annual Book of ASTM Standards, Vol 11.01.
[2]Annual Book of ASTM Standards, Vol 11.01.
[3]Annual Book of ASTM Standards, Vol 11.01.

[4]P.L. Geenen, J. Bresciani, J. Boes, A. Pedersen, L. Eriksen, H.P. Fagerholm, and P. Nansen (1999)The morphogenesis of Ascaris suum to the infective third-stage larvae within the egg, J. Parasitology 85(4):616-622.

to contain *Ascaris* and some other parasitic ova, if present, in the sample. Small particulates are removed by a second screening on a small mesh size screen.[5] The resulting concentrate is incubated at 26EC until control *Ascaris* eggs are fully embryonated. The concentrate is then microscopically examined for the categories of *Ascaris* ova on a Sedgwick-Rafter counting chamber.

5.0 Significance and Use

5.1 This test method is useful for providing a quantitative indication of the level of *Ascaris* ova contamination of wastewater, sludge, and compost.

5.2 This test method will not identify the species of *Ascaris* detected nor the host of origin.

5.3 This method may be useful in evaluating the effectiveness of treatment.

6.0 Interferences

6.1 Freezing of samples will interfere with the buoyant density of *Ascaris* ova and decrease the recovery of ova.

7.0 Apparatus

7.1 A good light microscope equipped with brightfield, and preferably with phase contrast and/or differential contrast optics including objectives ranging in power from 10X to 45X.

7.2 Sedgwick-Rafter cell.

7.3 Pyrex beakers, 2 L. Coat with organosilane.

7.4 Erlenmeyer flask, 500 mL. Coat with organosilane.

7.4 A centrifuge that can sustain forces of at least 660 X G with the rotors listed below.

7.4.1 A swinging bucket rotor to hold 100 or 250 ml centrifuge glass or plastic conical bottles.

7.4.2 A swinging bucket rotor to hold 15 ml conical glass or plastic centrifuge tubes.

7.5 Tyler sieves.

7.5.1 20 or 50 mesh.

7.5.2 400 mesh, stainless steel, 5 inch in diameter.

7.5.3 A large plastic funnel to support the sieve. Coat with organosilane.

7.6 Teflon spatula.

7.7 Incubator set at 26EC.

7.8 Large test tube rack to accommodate 100 or 250 mL centrifuge bottles.

7.9 Small test tube rack to accommodate 15 mL conical centrifuge tubes.

7.10 Centrifuge bottles, 100 or 250 mL. Coat with organosilane.

7.11 Conical centrifuge tubes, 15 mL. Coat with organosilane.

7.12 Pasteur pipettes. Coat with organosilane.

7.13 Vacuum aspiration apparatus.

7.13.1 Vacuum source.

7.13.2 Vacuum flask, 2 L or larger.

7.13.3 Stopper to fit vacuum flask, fitted with a glass or metal tubing as a connector for 1/4 inch tygon tubing.

7.14 Spray bottles (16 fl oz.) (2).

7.14.1 Label one “Water”.

7.14.2 Label one “1% 7X”.

8.0 Reagents and Materials

8.1 Purity of Reagents — Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society[6]. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

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[5]This method is based on a protocol published by Bowman, D.D., M.D. Little, and R.S. Reimers (2003) Precision and accuracy of an assay for detecting *Ascaris* eggs in various biosolid matrices. Water Research **37**(9):2063-2072.

[6]Reagent Chemicals, American Chemical Specifications, American Chemical Society, Washington, D.C. For suggestions on testing of Reagents not listed by the American Chemical Society, see Analar Standards for Laboratory Chemicals, BHD Ltd., Poole, Dorset, U.K. and the United States Pharmacopeia and National Formulary, U.S. Pharmaceutical Convention, Inc. (USPC).

8.2 Purity of Water — Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type I.

8.3 Preparation of Reagents — Prepare reagents in accordance with Practice E200.

8.3.1 Phosphate-buffered water (1 L = 34.0 g KH_2PO_4, pH adjusted to 7.2 ± 0.5 with 1 N NaOH).

8.3.2 1% (v/v) 7X ("ICN" laboratory detergent) (1 L = 999 mL phosphate-buffered water, 1 mL 7X "ICN", Adjust pH to 7.2 ± 0.1 with 1N NaOH).

8.3.3 Magnesium sulfate, sp. gr. 1.20. (1 L = 215.2 g $MgSO_4$, check specific gravity with a hydrometer; adjust as necessary to reach 1.20).

8.3.4 Organosilane. For coating glassware. Coat all glassware according to manufacturer's instructions.

8.3.5 Fresh *Ascaris* ova for positive control, purified from *Ascaris* infected pig fecal material.

9.0 Precautions

9.1 When handling *Ascaris* ova and biosolids, personal protective measures must be employed to prevent infection. Prevention of infection in humans is a matter of good personal hygiene. Wear a laboratory coat at all times in the laboratory. In addition, latex or nitrile gloves and splash protection safety glasses should always be worn in the laboratory. Mouth pipetting is strictly forbidden. Contaminated pipettes are never laid down on the bench top but are immediately placed in a pipette discard container which has disinfectant in it. Contaminated equipment is separated as it is used into containers for disposable materials and containers for re-cycling. After these containers which are always autoclave pans, are full, they are autoclaved for 30 minutes at 121EC and 15 pounds/in^2. Contaminated glassware is never washed until after it has been autoclaved. Eating, drinking, and smoking in the laboratory is not permitted. Likewise, refrigerators are not to be used for storing lunches or other items for human consumption. If infective *Ascaris* ova are ingested they may cause disease.

10.0 Sampling

10.1 Collect 1 liter of compost, wastewater, or sludge in accordance with Practice D 1066, Specification D 1192, and Practices D 3370, as applicable.

10.2 Place the sample container(s) on wet ice or around chemical ice and ship back to the laboratory for analysis within 24 hours of collection.

10.3 Store the samples in the laboratory refrigerated at 2 to 5EC. Do not freeze the samples during transport or storage.

11.0 Preparation of Apparatus

11.1 Test the centrifuge with a tachometer to make sure the revolution's per minute correlate with the speed gauge.

11.2 Calibrate the incubator temperature with a NIST traceable thermometer.

11.3 Microscope.

11.3.1 Clean the microscope optics.

11.3.2 Adjust the condenser on the microscope, so Köhler illumination is established.

12.0 Procedure

12.1 The percentage moisture of the sample is determined by analyzing a separate portion of the sample, so the final calculation of ova per gram dry weight can be determined. The concentration of ova in liquid sludge samples may be expressed as ova per unit volume.

12.2 Initial preparation:

12.2.1 Dry or thick samples: Weigh about 300 g (estimated dry weight) and place in about 500 ml water in a beaker and let soak overnight at 4 - 10EC. Transfer to blender and blend at high for one minute. Divide sample into four beakers.

12.2.2 Liquid samples: Measure 1,000 ml or more (estimated to contain at least 50 g dry solids) of liquid sample. Place one half of sample in blender. Add about 200 mL water. Blend at high speed for one minute transfer to a beaker. Repeat for other half of sample.

12.3 Pour the homogenized sample into a 1000 mL tall form beaker and using a wash bottle, thoroughly rinse blender container into beaker. Add 1% 7X to reach 900 ml final volume.

12.4 Allow sample to settle four hours or overnight at 4 - 10EC. Stir occasionally with a wooden applicator, as needed to ensure that material floating on the surface settles. Additional 1% 7X may be added, and the mixture stirred if necessary.

12.5 After settling, vacuum aspirate supernatant to just above the layer of solids. Transfer sediment to blender and add water to 500 ml, blend again for one minute at high speed.

12.6 Transfer to beaker, rinsing blender and add 1% 7X to reach 900 ml. Allow to settle for two hours at 4 - 10EC, vacuum aspirate supernatant to just above the layer of solids.

12.7 Add 300 ml 1% 7X and stir for five minutes on a magnetic stirrer.

12.8 Strain homogenized sample through a 20 or 50 mesh sieve placed in a funnel over a tall beaker. Wash sample through sieve with a spray of 1% 7X from a spray bottle.

12.9 Add 1% 7X to 900 mL final volume and allow to settle for two hours at 4 - 10EC.

12.10 Vacuum aspirate supernatant to just above layer of solids. Mix sediment and distribute equally to 50 mL graduated conical centrifuge tubes. Thoroughly wash any sediment from beaker into tubes using water from a wash bottle. Bring volume in tubes up to 50 ml with water.

12.11 Centrifuge for 10 minutes at 1000 X G. Vacuum aspirate supernatant from each tube down to just above the level of sediment. (The packed sediment in each tube should not exceed 5 mL. If it exceeds this volume, add water and distribute the sediment evenly among additional tubes, repeat centrifugation, and vacuum aspirate supernatant.)

12.12 Add 10 to 15 mL of $MgSO_4$ solution (specific gravity 1.20) to each tube and mix for 15 to 20 seconds on a vortex mixer. (Use capped tubes to avoid splashing of mixture from the tube.)

12.13 Add additional $MgSO_4$ solution (specific gravity 1.20) to each tube to bring volume to 50 mL. Centrifuge for five to ten minutes at 800 to 1000 X g. DO NOT USE BRAKE.

12.14 Allow the centrifuge to coast to a stop without the brake. Pour the top 25 to 35 mL of supernatant from each tube through a 400 mesh sieve supported in a funnel over a tall beaker.

12.15 Using a water spray bottle, wash excessive flotation fluid and fine particles through sieve.

12.16 Rinse sediment collected on the sieve into a 100 mL beaker by directing the stream of water from the wash bottle onto the upper surface of the sieve.

12.17 After thoroughly washing the sediment from the sieve, transfer the suspension to the required number of 15 mL centrifuge tubes, taking care to rinse the beaker into the tubes. Usually one beaker makes one tube.

12.18 Centrifuge the tubes for three minutes at 800 X G, then discard the supernatant.

12.19 If more than one tube has been used for the sample, transfer the sediment to a single tube, fill with water, and repeat centrifugation.

12.20 Aspirate the supernatant above the solids.

12.21 Resuspend the solids in 4 mL 0.1 N H_2SO_4 and pour into a 20-mL polyethylene scintillation vial or equivalent with loose caps.

12.22 Before incubating the vials, mark the liquid level in each vial with a felt tip pen. Incubate the vials, along with control vials containing *Ascaris* ova mixed with 4 mL 0.1 N H_2SO_4, at 26EC for three to four weeks. Every day or so, check the liquid level in each vial. Add reagent grade water up to the initial liquid level line as needed to compensate for evaporation. After 18 days, suspend, by inversion and sample small aliquots of the control cultures once every 2 - 3 days. When the majority of the control *Ascaris* ova are fully embryonated, samples are ready to be examined.

12.23 Examine the concentrates microscopically using a Sedgwick-Rafter cell to enumerate the detected ova. Classify the ova as either unembryonated, embryonated to the first, second, or third larval stage. In some embryonated *Ascaris* ova the larva may be observed to move. See Figure 1 for examples of various *Ascaris* egg categories.

13.0 Calculation

13.1 Calculate % total solids using the % moisture result:

$$\% \text{ Total solids} = 100\% - \% \text{ moisture}$$

13.2 Calculate catagories of ova/g dry weight in the following manner:

$$\text{Ova/g dry wt} = \frac{(NO) \times (CV) \times (FV)}{(SP) \times (TS)}$$

Where:

NO = no. ova
CV = chamber volume(= 1 mL)
FV = final volume in mL
SP = sample processed in mL or g
TS = % total solids

14.0 Report

14.1 Report the results as the total number of *Ascaris* ova, number of unembryonated *Ascaris* ova, number of 1st, 2nd, or 3rd stage larva; reported as number of *Ascaris* ova and number of various larval *Ascaris* ova per g dry weight.

15.0 Keywords

Ascaris, ova, embryonation, viability assay, helminth.

Notice

The PEC was consulted in a recent (1998-1999) pilot study by Lyonnaise des Eaux concerning the use of a microscope in making helminth ova counts for different types of sludge. Solids and debris present in the sludge being viewed with the microscope were found to impair ones ability to count. Dilution of raw sludge and digested sludge, however, with phosphate-buffered water prior to analyzing them significantly improved the number of ova that could be counted. Raw sludges were diluted by a factor of 20 and digested sludges by a factor of 5. QA/QC procedures were followed to validate this procedure. The PEC should be consulted for more details.

[revised May 15, 2003]

Figure A1.1. *Ascaris* ovum: potentially non-fertile, note bumpy mammilated outer layer.

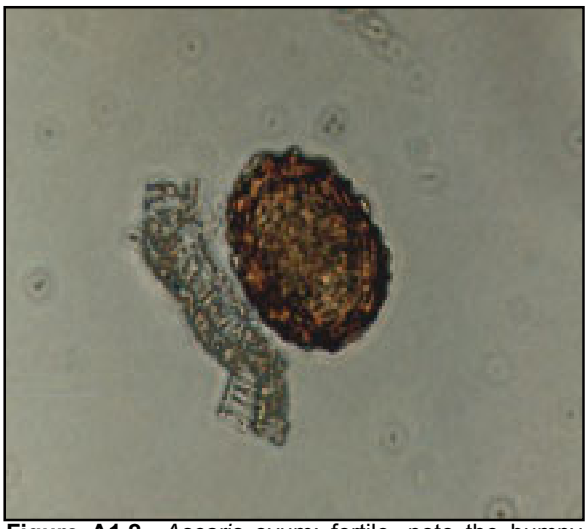

Figure A1.2. *Ascaris* ovum: fertile, note the bumpy outer mammilated layer.

Figure A1.3. *Ascaris* ovum: decorticated, unembryonated. Note the outer mammilated layer is gone

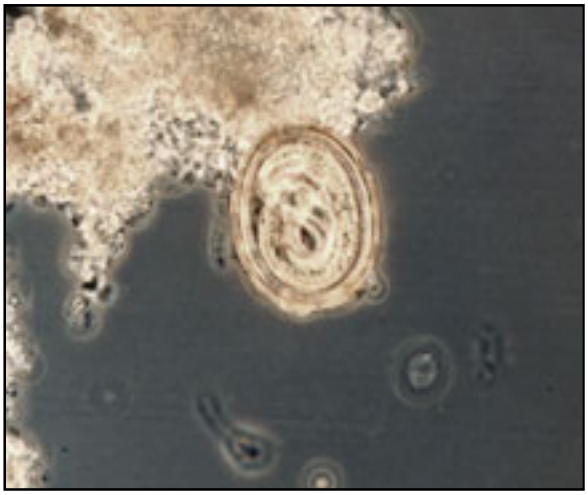

Figure A1.4. *Ascaris* ovum: decorticated and embryonated.

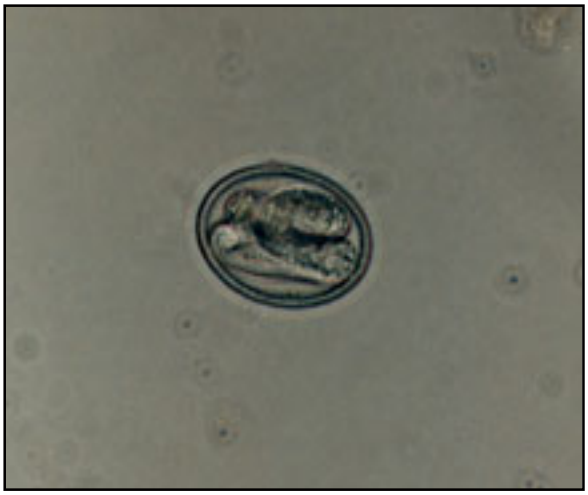

Figure A1.5. *Ascaris* ovum: decorticated, embryonated.

Figure A1.6. *Ascaris* ovum with second stage larva; note the first stage larval sheath at the anterior end of the worm