UNIVERSITY OF KWAZULU-NATAL	*Standard Operating Procedure* PRG pollution research group	**Effective Date:** 14 April 2015	**Version:** ***002***
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Standard Operation Procedure – Helminth Testing

1. Scope and Application

The prevalence of helminth infections in people living with basic water and sanitation in third-world countries, is generally high. Due to the extreme hardiness of the eggs of the roundworm, *Ascaris lumbricoides*, they are used in the waste and sanitation field as a 'marker' for the safe re-use of human waste. It is generally accepted that if any of the various waste treatments used are successful in inactivating *Ascaris* eggs, then all harmful bacteria and viruses should also be killed.

Other commonly found helminths are *Trichuris trichiura*, *Taenia* spp. and in areas with very sandy soils, hookworm spp. and *Strongyloides stercoralis*. Various animal parasites are also commonly encountered. In countries where piped water is not chlorinated, the presence of free-living soil and water organisms are also encountered and need to be differentiated from pathogens.

2. Summary

Helminth eggs are thought to adhere to soil particles, possibly as a result of charge interactions with or adsorption of eggs to the particles. Many waste samples, even if they are not from Urine-Diversion Toilets, are often contaminated with silica particles, hence the use of ammonium bicarbonate as a wash solution.

Water samples that have a high fat content, such as is commonly found in places like India, need to be treated differently from black-water and other water and wastewater samples. Here, it is suggested that a surfactant such as Tween 20 or 7X is used to break up the fats, rather than ammonium bicarbonate.

Laboratory testing for helminths is based on four main principals: washing, filtration, centrifugation and flotation of the eggs to remove them from the various waste mediums.
Ammonium bicarbonate is used as both a wash solution and also to dissociate the eggs from the soil particles. (Tween 80 or 7X should be used for fatty samples.)

Filtration, using 100µm and/or 20µm sieves is used to separate larger and smaller particles from the eggs both after washing and after flotation.

Centrifugation to 1. Sediment the deposit and remove the water before flotation, 2. Aid the separation process during flotation and 3. Sediment the final sieved and washed eggs retrieved during flotation.

Flotation, using a solution of zinc sulphate at a specific gravity of 1.3 is used to float eggs (with a relative density of <1.3) out of the matter retained with them on the 20µm sieve (this is called the retentate).

3. Apparatus and Glassware

- Compound microscope with 10x and 40x objectives (and preferably, a camera)
- Bench-top centrifuge with a swing-out rotor that can spin a minimum of 8 x 15ml plastic conical test tubes (Falcon tubes) and, if possible, buckets that can also spin a minimum of 4 x 50ml Falcon tubes
- Sink with hose attached to tap for washing using high water pressure
- Top-pan balance (scale, for weights up to 200gm and accurate to 2 decimal places)
- Magnetic stirrer and bar magnets
- Vortex mixer
- Hydrometer that can measure SG between 1.2 and 1.3
- 100µm mesh stainless steel flat sieve, diameter 200mm
- 20µm mesh stainless steel flat sieve, 200mm
- 20µm mesh stainless steel flat sieve, 100mm
- Plastic test tube racks to hold the 15ml Falcon tubes (and if using 50ml tubes, one for those)
- Plastic 200ml beakers
- Plastic “hockey-stick ” shaped spreaders
- Plastic 3ml Pasteur pipettes (non-sterile)
- Non-sterile gloves
- Applicator sticks and wooden tongue depressors
- Microscope slides (76 x 26 x 1.2mm)
- Cover glasses (22 x 40mm)

4. Collection, Preservation and Storage

- After taking samples from the various waste materials, these should be stored as is at approximately 4 - 10^{o}C. Processing is always best as soon after sampling as possible, but providing that there is sufficient moisture and the samples are fairly large, the eggs should be unharmed and development will be arrested at these low temperatures.

5. Safety Precautions

- Always wear gloves and laboratory coat or plastic apron while processing the samples
- After testing, wash and rinse sieves and beakers and leave to drain on draining rack
- Dispose of used gloves after completion of processing the samples
- Wash hands using antiseptic soap.

6. Reagents

- **Physiological Saline (8.5g/lt NaCl)**

  Dissolve 8.5 g sodium chloride in distilled or de-ionized water. Make small amounts to use up at one time or if large amounts are made, it is preferable to decant them into smaller containers and autoclave for 15 min at 121°C. Cool to room temperature and store.

- **Ammonium Bicarbonate (AmBic)**

  Dissolve 119gm of ammonium bicarbonate in 1lt de-ionized water (use a magnetic stirrer and bar magnet) – store in a glass jar.

- **Tween 80 or 7X**

  Use neat – see method

- **Zinc Sulphate ($ZnSO_4$ $7H_2O$)**

  Dissolve 500gm zinc sulphate in approximately 800ml de-ionized water (use magnetic stirrer and bar magnet) and adjust SG using more of the chemical or water to raise or lower the SG to 1.3

- **0.1N Sulphuric acid (H_2SO_4)**

  Add 500ml de-ionized water to a 1 litre plastic bottle, pour 3ml concentrated sulphuric acid into a 10ml graduated cylinder, then pour the H_2SO_4 into the plastic bottle containing the water, re-cap and shake. Un-cap, add 497ml of de-ionized water to the plastic bottle, re-cap and shake.

7. Procedure VIP, UDDT, Thick Sludges

- Place a 200ml plastic beaker (labelled with sample number) on top-pan balance, zero balance, weigh 10 or 20gm of sample into beaker.

  NOTE: IF waste material is very dry (e.g. pelletised or totally dessicated), then soak for 12 – 24 hours in ±80ml physiological saline to soften. Next, break up and mix sample well in the saline. Stand to sediment solids for 4 hours. Remove as much supernatant as possible without disturbing deposit, continue with next step below.

- Add 50-80ml AmBic and a magnetic stirring bar, mix on magnetic stirrer for 10 minutes.
- Pour this mixture over 100µm sieve which fits on top of 20µm sieve (wet sieves with tap H_2O first).
- Rinse beaker with tap H_2O and pour over sieves.
- Wash magnet over sieves and remove, wash 100µm sieve well (using "hockey-stick" spreader, or preferably, gloved hand) over 20µm filter, regularly checking bottom sieve for fluid build-up. Use same hockey stick to stir sample on 20µm sieve while holding 100µm sieve directly above so as not to lose any sample. When 20µm sieve has drained sufficiently, place the 100µm sieve back on top and continue washing. Repeat this until sample on 100µm sieve is sufficiently well washed.
- Separate sieves and then rinse 20µm sieve well. Use water-pressure to wash the material to one side of sieve to make collection easier.
- Rinse all material off 20µm filter into original rinsed-out labelled beaker.
- Pour beaker contents into 4 x 15ml conical test tubes labelled with sample number or if retentate is large, use 50ml labelled tubes. (The aim after the next step is to have ≤1ml deposit in a 15ml tube and ≤5ml in a 50ml tube.)
- Centrifuge at 3000rpm (1389g) in centrifuge with swing-out rotor for 10 minutes.
- Pour off supernatant, leaving deposits in test tubes.
- Place test tubes in rack with applicator stick in each (as a stirring rod) and pipette in $ZnSO_4$, 3ml at a time, vortexing in between addition of the chemical, until tubes are filled to 14ml mark for 15ml tubes / 45ml mark for 50ml tubes.
- Centrifuge at 2000rpm (617g) for 10 minutes.

- Pour supernatant flotation fluid over smaller diameter 20µm sieve. Wash remaining deposits out of test tubes and keep one aside for re-use.
- Wash material on sieve well with tap water and rinse it down to one side of the sieve for collection. Using a 3ml plastic pipette, transfer the material back into the test tube kept aside.
- Centrifuge at 3000rpm (1389g) for 10 minutes to obtain the final deposit.
- Pour off supernatant water and pipette up the deposit, place it on one or more microscope slides (but make one slide at a time so they don't stand for long periods and dry out), place a 22x40mm coverslip on top, examine and count every *Ascaris* egg, classifying them as viable, potentially viable or dead. *Trichuris*, *Taenia*, hookworm spp. eggs can also be counted and assessed simply as potentially viable or dead.

Liquid Samples

- If the water is effluent from a waste-water treatment plant and is fairly clean with low suspended solids, then it is preferable to use a large sample of 5 – 10lt, measured out using a 1lt measuring cylinder.

  NOTE 1: IF sample is black water with a high concentration of solids, then use amounts of 250 - 1000ml. The sample should be measured out and then stood for 4 hours or overnight to sediment the solids. Then, remove the supernatant fluid and treat as in second step above of: **7. Procedure – VIP, UD, and thick sludges**

  NOTE 2: IF sample is fatty, then measure out selected sample size (from 250 – 1000ml), pour into plastic beaker large enough to contain the sample with at least 5-10cm above the sample, so that it does not spill when mixing on magnetic stirrer. Add 1ml per litre of neat Tween 80 or 7X directly into the sample (so as to make a ± 0.1% solution). Mix well using magnetic stirrer and magnet in beaker for 20 minutes. Then proceed as for next step below.

- The measured sample is poured slowly through a 100µm sieve which fits on top of a 20µm sieve and is well washed, checking bottom sieve for fluid build-up.
- Separate sieves and then rinse 20µm sieve well and wash material to one side of sieve for collection.
- Rinse all material off 20µm filter into 2 - 4 x 15ml conical test tubes.
- Centrifuge at 3000rpm (1389g) in centrifuge with swing-out rotor for 10 minutes.
- Pour off supernatant and retain deposits left in 2 - 4 x 15ml test tubes.

- Place test tubes in rack with applicator stick in each (as a stirring rod) and pipette in $ZnSO_4$, 3ml at a time, vortexing in between addition of the chemical, until tubes are filled to 14ml mark.
- Centrifuge at 2000rpm (617g) for 10 minutes.
- Pour supernatant flotation fluid over smaller diameter 20µm sieve. Wash out test tubes and keep one aside for re-use.
- Wash material on sieve well with tap water and rinse down to one side of sieve for collection. Using a 3ml plastic pipette, transfer material back into test tube kept aside.
- Centrifuge at 3000rpm (1389g) for 10 minutes to obtain the final deposit.
- Pour off supernatant water and pipette up the deposit, place it on a microscope slide, place a 22x40mm coverslip on top, examine and count every *Ascaris* egg, classifying them as viable, potentially viable or dead. *Trichuris*, *Taenia*, hookworm spp. eggs can also be counted and assessed simply as potentially viable or dead.

Procedure for incubating samples for viability testing

- Weigh 10 or 20gm into a 200ml plastic beaker, on a top-pan balance.
- Add approximately 10 – 20 ml deionized water; 0.1N H_2SO_4, or 1% formalin to sample.
- Cover with parafilm, prick holes in parafilm to allow air into sample, or place a plastic petri dish lid on top.
- Incubate for 21 - 28 days at 25-28°C (Check regularly to see that the sample has not dried out and add more water, 0.1N H_2SO_4 or 1% formalin as necessary to keep sample moist. Aerate the samples daily by swirling or vortexing carefully.)
- After 28 days, remove from incubator, stand for 4 hours or overnight to sediment the sample, remove the supernatant fluid, and then proceed as for step 2 onwards described above in: **7. Procedure – VIP, UD, and thick sludges**