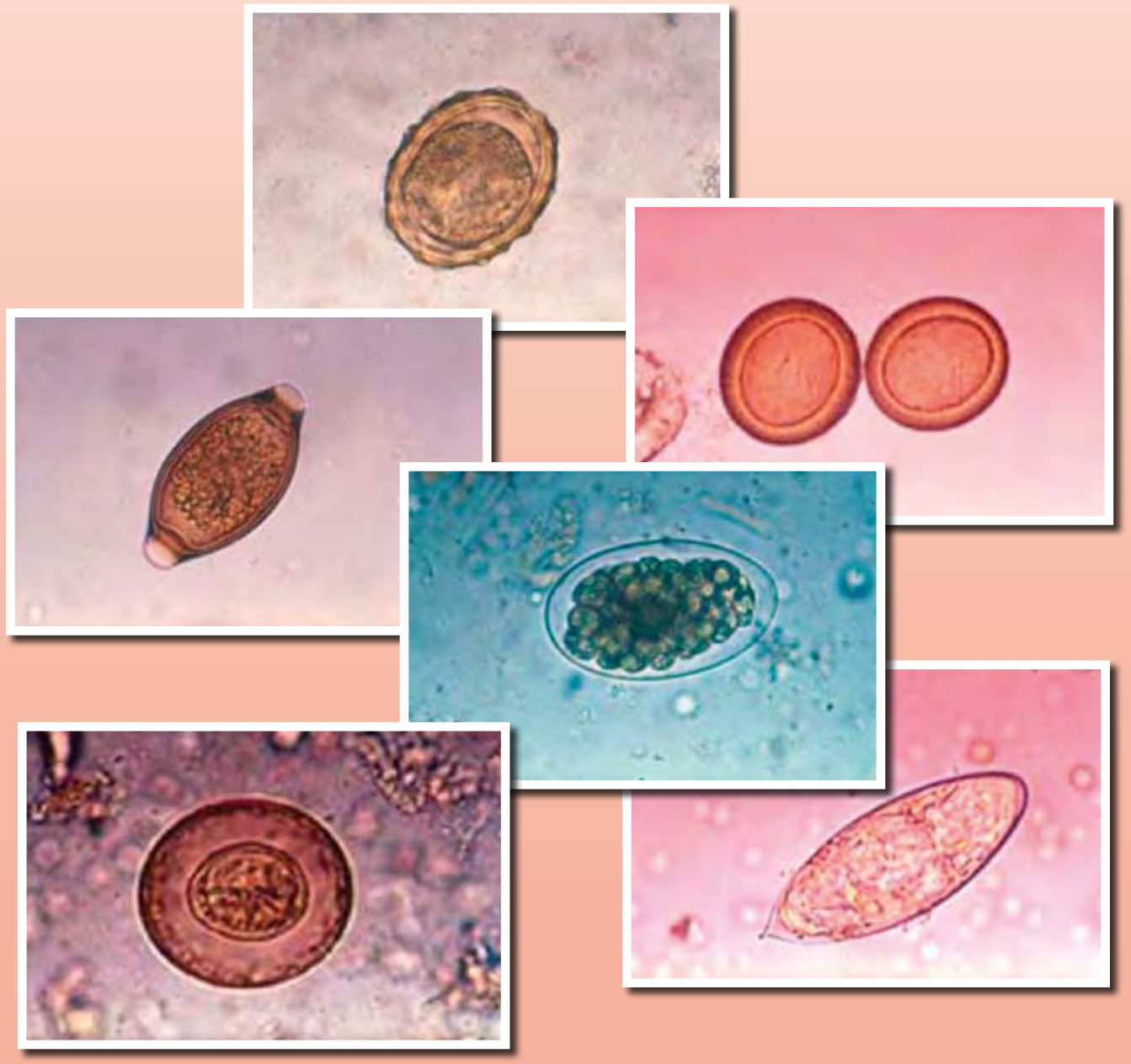
Standard Methods for the Recovery and Enumeration of Helminth Ova in Wastewater, Sludge, Compost and Urine-Diversion Waste in South Africa

Priya Moodley, Colleen Archer & David Hawksworth
in association with Lizette Leibach



TT 322/08
Water Research Commission

STANDARDS METHODS FOR THE RECOVERY AND ENUMERATION OF HELMINTH OVA IN WASTEWATER, SLUDGE, COMPOST AND URINE-DIVERSION WASTE IN SOUTH AFRICA

Report to the
Water Research Commission

by

Priya Moodley[1], Colleen Archer[2] and David Hawksworth[2]
in association with
Lizette Leibach[3]

[1]Zitholele Consulting
[2]University of KwaZulu-Natal, Pollution Research Group
[3]ERWAT

WRC Report No. TT322/08
March 2008

Obtainable from

Water Research Commission
Private Bag X03
GEZINA
0031

The publication of this report emanates from a project entitled: *Adopting internationally acceptable methods and building capacity to measure Helminth Ova in wastewater and sludge samples* (WRC Project No. K5/1662)

This report has a CD attached consisting of:

- 2 Posters: (1) The methods and (2) Photographs of helminth ova commonly seen,
- The approach to the development of the methods, as well as the procedure followed, supporting information and a list of the trainees,

ISBN 978-1-77005-648-0

Printed in the Republic of South Africa

PREFACE

This manual has been developed to specify a standard analytical method for water and wastewater laboratories to recover and enumerate helminth ova in wastewater, sludge, compost and urine-diversion waste. The manual serves to guide microbiologists and laboratory technicians by means of step-by-step procedures to analyse various types of waste samples and to recognize different helminth species found in South Africa. It has been developed in a manner that will allow both the experienced practitioner and the trainee technician to understand, interpret and carry out the method. It also includes a collection of photographs to assist the laboratory practitioner with the microscopic analysis of the helminth ova that are recovered.

The adoption of this method as a standard by the various laboratories around South Africa will assist in creating the much needed capacity and expertise in the recovery, identification and enumeration of helminth ova. It will also enable the determination of a set of prevalence statistics on helminth ova, which is currently lacking for the country.

While this manual has been developed as a stand-alone document, supporting posters outlining the method and depicting the photographs of helminth ova commonly seen have also been developed. These are available on the CD attached to this document. In addition, the approach to the development of the method, as well as the procedure followed and supporting information were also documented as a separate document on the CD attached to this document.

The method documented in this manual is an attempt at documenting a standard, simple and cost effective analytical method for South Africa for the recovery and enumeration of helminth ova. It is thus considered to be a living publication, and will be reviewed periodically based on local and international scientific advancements and analytical developments that may take place. All users are urged to take a critical view regarding the manual in terms of usefulness, suitability and applicability.

ACKNOWLEDGEMENTS

This training manual emanated from a Water Research Commission funded project entitled: *"Adopting internationally acceptable methods and building capacity to measure Helminth Ova in wastewater and sludge samples".*

The contribution of the reference group, listed below is gratefully acknowledged:

Mrs APM Moolman	Water Research Commission (Chairperson)
Mrs W Moolman	Department of Water Affairs and Forestry
Mr I Bailey	Water Environment
Mrs S Jackson	eThekwini Municipality: Water & Sanitation

Project team:

Mrs P Moodley	Zitholele Consulting (Project Manager)
Mrs L Leibach	ERWAT (method development, training session and baseline prevalence analysis)
Mrs CE Archer	University of KZN – Pollution Research Group (method development, training manual and training session)
Mr D Hawksworth	University of KZN – Pollution Research Group (method development and training session)
Ms C Maya-Rendon	National Autonomous University of Mexico (method development)
Ms Lee-Ann Boyd	Golder Associates Africa (Pty) Ltd

The following organisations/persons are gratefully acknowledged:

- The Water Research Commission for the funding of the project.
- The World Health Organisation for use of photographs.
- C.E. Archer & C.C. Appleton of the University of KwaZulu-Natal for use of photographs and the life cycle diagram of *Ascaris lumbricoides*.
- ERWAT and University of KwaZulu-Natal Pollution Research Group for permitting use of their laboratories.
- Dr Heidi Snyman and Dr N Rodda for their guidance.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

CD CONTENTS

- Procedure and approach followed for methods development and information on training sessions
- 2 Posters

1 INTRODUCTION

The helminths are a very large and varied group of multicellular parasitic worms. Some infect humans, others animals or plants, while many may be free-living in the soil. Some produce male and female worms, others are hermaphrodites. All have differentiated organs, and their life cycles include the production of ova (eggs) or larvae as well as the complex alternation of generations which can include up to three different hosts. There are three major groups of helminths containing members that have man as their host *viz*. flukes (Trematoda), tapeworms (Cestoda), and roundworms (Nematoda). Helminth infections are spread through the ingestion or inhalation of their ova, some of which can survive outside the host for long periods of time, or via larvae/cercariae penetrating skin exposed to infected soil/water. Once inside the body, helminth ova hatch and many undergo maturation in the tissues before re-entering the gut and lodging in the intestines. Here they grow and undergo sexual reproduction, resulting in the production of eggs or larvae which are passed out via the faeces to the environment (Figure 1).

Helminths may damage tissues (e.g. visceral larva migrans caused by the dog and cat round worm, genus *Toxocara*), cause blood-loss (e.g. hookworm species) and result in more serious effects like epilepsy when man becomes an accidental intermediate host (e.g. the pork tapeworm, *Taenia solium*). Contamination of crops with helminths can take place through direct faecal contamination with both human and animal excreta, or through the use of contaminated sludge or wastewater for agriculture. While worldwide the prevalence of helminth ova and the incidence of infections are well documented, in southern Africa research on the prevalence of the different helminth species in wastewater and sludge samples is severely lacking.

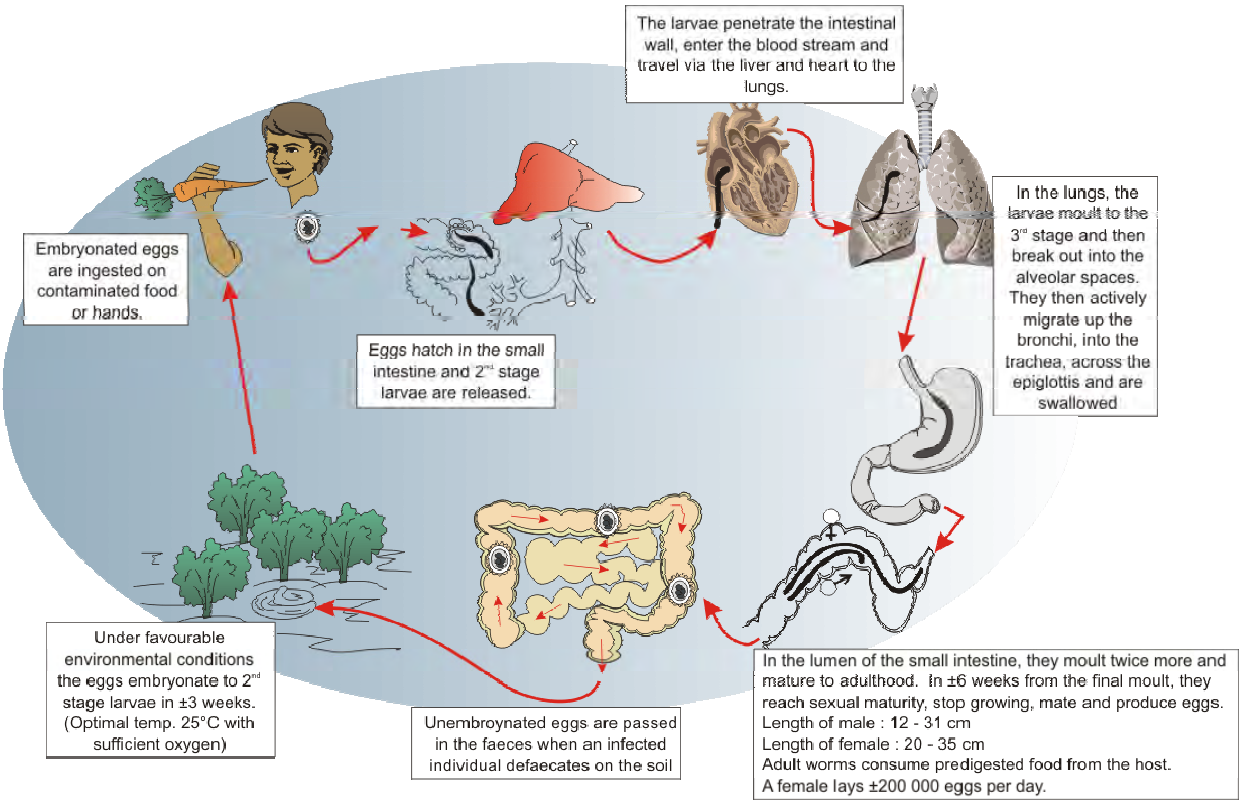


Figure 1: Diagram of the life cycle of *Ascaris lumbricoides*, the most common human nematode infection worldwide (*Adapted from C.E. Archer & C.C. Appleton, Unpublished ©*).

2 THE PROBLEM

To date in South Africa, no standard method exists for measuring helminth ova in wastewater, sludge or faecal samples mixed with soil. Helminth ova concentrations in wastewater and sludges have thus been largely underestimated or never reported, as the presence of *Ascaris lumbricoides* eggs is frequently the only standard that has been used. While reporting numbers of viable *Ascaris* ova in sludges is a requirement in terms of certain regulatory obligations, no analytical method has been specified. As a result, different laboratories in South Africa have adopted different methods. Several studies done internationally however, show evidence that *Ascaris* ova are often not the most prevalent of the helminths in wastewater samples. For example, while in the USA, the relative prevalence of different helminth species is *Ascaris* > *Toxocara* > *Toxascaris* > *Trichuris* > *Hymenolepis*; in France the relative prevalence of helminth species is *Taenia* > *Trichuris* > *Toxocara* > *Ascaris* (Cabaret *et al.,* 2002). Research on the prevalence of the different helminth species in South African wastewater and sludge samples could not be located. It should be mentioned, though, that there is a reasonable amount of data on the prevalence of these infections in humans, especially school children. According to Cabaret et al. (2002), the prevalence of human taeniasis (*Taenia spp*.) in Africa is 2-10% while Belgium, France, Germany, Hungary, Italy, Montenegro, Poland, Spain and the Netherlands show a prevalence of 0.01-2%. This demonstrates that the continued reliance on the *Ascaris* standard could seriously underestimate the total helminth ova concentrations in South Africa. Consequently due to the lack of expertise and of no standardised method being available to do total helminth ova counts, their true prevalence in human waste in South Africa remains unknown. With the revised sludge guidelines soon to be implemented, total helminth ova counts will be a requirement of the sludge classification system. The viable helminth ova count will not only give an indication of the effectiveness of the disinfection process of the wastewater treatment facility but will also generate data on the prevalence of the different species, as well as provide some indication of the incidence of infection in communities serviced by these treatment plants. A standard method will also allow helminth ova counts to be reliably compared between laboratories and to regulatory guidelines.

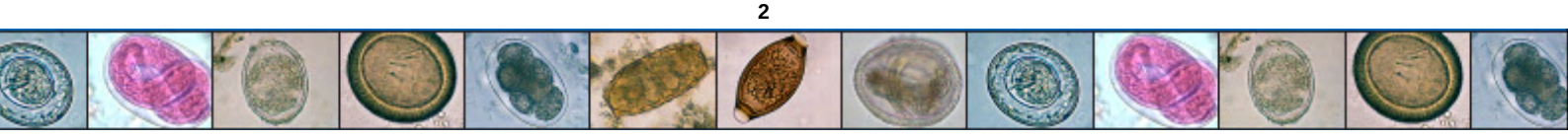
3 THE APPROACH FOLLOWED TO DEVELOP A STANDARD METHOD

A method similar to the one currently in use in a several laboratories in South Africa was compared to other internationally used methods. Recommendations made were that an adapted Environmental Protection Agency (EPA) method be adopted to measure helminth ova based on the fact that this technique demonstrated a greater recovery rate than the rest of the procedures. While the original intention was to adopt this adapted EPA method for South Africa, with some refinement to suit the local context, a local group in South Africa also recently reported the development of an improved recovery protocol, the Ammonium Bicarbonate/Zinc Sulphate (AmBic/$ZnSO_4$) method for the detection of helminth ova (particularly *Ascaris*).

The method documented in this manual is based on an adapted EPA method which allows a better recovery of helminth ova using the relative specific gravities of the ova of different helminth species, and the AmBic/$ZnSO_4$ method for the detection of helminth ova in soil-based samples. The resulting method is based on the strengths of both methods and has been tested to determine its suitability and appropriateness for the South African context.

The method, which is based on a combination of washes, filtrations and flotations, has been adapted into three separate procedures to suit the main groups of waste products, *viz*.

- wastewater or effluent,
- wet sludge, and
- dry or composted sludge and UD waste.



4 FIELD OF APPLICATION

The method describes the detection, identification and quantification of total and viable helminth ova in wastewater or effluent, sludge and dry or composted sludge and UD waste.

5 PURPOSE OF THIS MANUAL

The purpose of this manual is:

- to promote the use of a standard method for the recovery and enumeration of total helminth ova in wastewater, sludge, compost and UD waste by all laboratories in South Africa;
- to encourage the analysis of waste and wastewater samples for helminth ova by providing a simple, cost-effective and accurate method suitable for the South African context;
- to create an understanding and provide guidance on performing the method for the recovery and enumeration of helminth ova;
- along with training workshops and quality control, to create much needed expertise and skills in the application of the methodology and in the recovery and enumeration of total helminth ova.

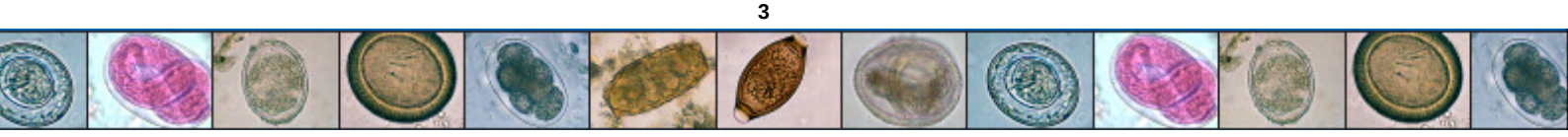
6 WHO SHOULD USE THIS MANUAL?

This manual has been developed to provide a standard method to support various types of water and wastewater laboratories in South Africa undertake analysis of wastewater or effluent, sludge and dry or composted sludge and UD waste for the recovery and enumeration of total helminth ova. The manual is therefore targeted at:

- Water treatment service providers
- Wastewater treatment service providers
- Local authorities and town/city councils that own and operate their own water/wastewater laboratories
- Water/wastewater scientists
- Research organisations/institutions
- Regulators

7 SUPPORTING INFORMATION

In addition to the step by step methodology that is provided, some photographs have been included in the manual to enable the untrained eye to examine samples and recognize different helminth species found in South Africa. Posters outlining the method and depicting these photographs have also been developed and are available from the WRC.



8 METHODS FOR THE DETERMINATION, IDENTIFICATION AND QUANTIFICATION OF HELMINTH OVA

The method has been adapted into three separate procedures to suit specific types of waste samples. Thus this manual includes a separate procedure for the analysis of each of the following:

- **wastewater or effluent**,
- **wet sludge**, and
- **dry or composted sludge and UD waste.**

Also included is an example of the **manipulation of the method** to accommodate **variations in sludge types.**

9 THE PRINCIPLE BEHIND THE METHOD

The method is based on three fundamental processes viz. (1) washing, (2) filtering one or more times, and then (3) floating and sedimenting of the retrieved parasites. A flotation step is used for the isolation of helminth ova using density gradient centrifugation and a chemical solution that is saturated at a specific gravity of 1.3 so that all helminth ova having relative densities that range from 1.13 (e.g. *Ascaris*) to 1.27 (e.g. *Taenia*) are able to float in that solution.

10 PERFORMING THE METHOD

The following components with regard to performing the method are described in detail in the sections that follow:

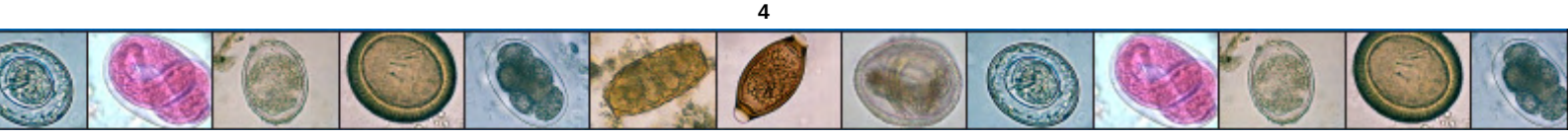
- **Equipment required**
- **Reagents required**
- **Solution preparation**
- **Step by step method for each waste type:**

    Analysis of wastewater or effluent

    Analysis of wet sludge, and

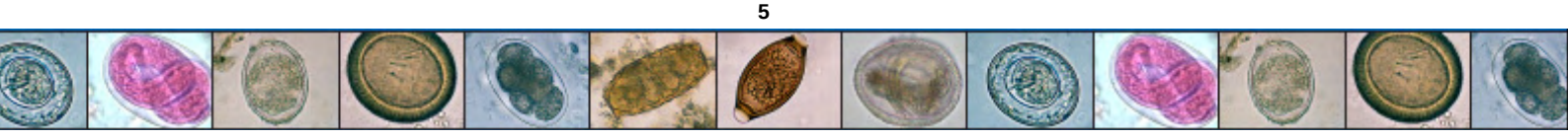
    Analysis of dry or composted sludge and UD waste

    Analysis of sludges that may vary from the above waste types



10.1 EQUIPMENT REQUIRED

- A centrifuge with a swing-out rotor and buckets that can take 15 mℓ and/or 50 mℓ plastic conical test tubes
- Vortex mixer
- Retort stand with at least 2 clamps on it
- Large plastic funnels to support the filters (± 220 mm diameters)
- Filters / sieves: 1x 150 µm; 1x 100 µm; 1x 20 µm mesh sizes
- Approximately. 6 plastic beakers (500 mℓ volume)
- Plastic wash bottles
- At least 4 glass (Schott) bottles: 1 ℓ, 2 ℓ and 5 ℓ sizes, for make-up and storage of the chemical solutions and de-ionized water
- Magnetic stirrer and stirring magnets
- 15 mℓ and 50 mℓ plastic conical test tubes
- 3 x small glass beakers (100 mℓ)
- Plastic Pasteur pipettes and plastic stirring rods
- Glass microscope slides (76 x 26 x 1.2 mm)
- Square and rectangular cover-slips (22 x 22 mm and 22 x 40 mm)
- A binocular compound microscope with 10x eyepieces, a 10x objective and a 40x objective



WORKING OUT THE G-FORCE OF THE CENTRIFUGE:

G-force (or g) = (1.118 x 10 $^{-5}$) r s^2 = 0. 00001118 x r x s^2

***where**:*

s = revolutions per minute (i.e. the speed you spin at)

r = the radius (the distance in centimetres from the centre of the rotor to the bottom of the bucket holding the tubes, when the bucket is in the swing-out position)

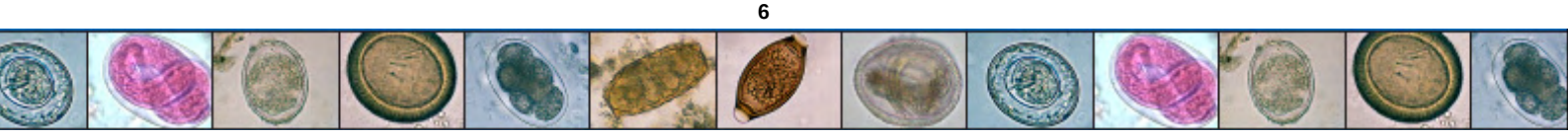
Calibration and Standardization

All equipment used **must be** calibrated with certified or adjusted standards according to the manufacturer's specifications.

10.2 REAGENTS

- **Zinc Sulphate ($ZnSO_4$)**
- **Ammonium Bicarbonate (AmBic) or Tween80**

The make up of the above reagents is described in Section 10.3.



10.3 SOLUTION PREPARATION

Zinc Sulphate

1. $ZnSO_4$ (heptahydrate) is made up by dissolving 500 g of the chemical in 880 mℓ de-ionised or distilled water.
2. A **hydrometer must be used** to adjust the specific gravity (SG) to 1.3, using more chemical if the SG is too low or more water if it is >1.3.

This high specific gravity facilitates the floating of heavier ova such as *Taenia* sp. (SG = 1.27). It is not critical if the SG of the $ZnSO_4$ solution is just over 1.3 but it should **never** be below!

Ammonium Bicarbonate (AmBic)

The AmBic solution is essentially a saturated ammonium bicarbonate solution made up as follows:

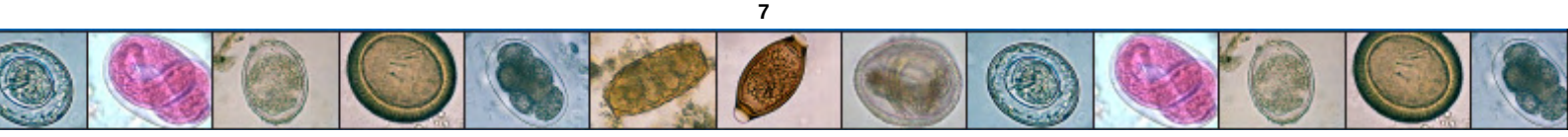
1. Ammonium bicarbonate* is made up by dissolving 119 g of the chemical in 1000 mℓ of de-ionised water.

**can be obtained from Sigma Aldrich*

0.1% Tween80

1. 1 mℓ of Tween80 is measured out using a pipette and placed in 999 mℓ of de-ionized or distilled water to give a 0.1% wash solution.

Note: Tween80 is extremely viscous and it is necessary to wash all of it out into the water in which it is made up, by alternately sucking up water and blowing it out using the same pipette.



10.4 STEP BY STEP METHOD

Note: If more than one water, sludge, compost or UD sample is to be processed simultaneously, label the samples, any corresponding beakers, test tubes and microscope slides with an appropriate laboratory number so as not to mix up results.

PROCEDURE FOR EFFLUENT OR WASTEWATER

1. An amount of 5 ℓ of wastewater, or the total volume received from the Plant, can be used. Write down the volume used for testing.

2. Supporting the 150 µm and 20 µm filters in 2 funnels one beneath the other in a retort stand, filter the sample through the 150 µm filter onto the 20 µm filter, swirling the 20 µm filter to facilitate draining of the fluid. If preferred, a plastic stirring rod can be used instead, to stir the filtrate on the filter, which also helps the fluid to pass through.

3. Any matter held back by the 150 µm filter is discarded, whilst the solids collected on the 20 µm filter are kept and rinsed off into a plastic beaker. Make sure to rinse off everything on this filter into the plastic beaker so as not to lose any of the parasites.

4. Pour the contents of the beaker into as many test tubes as are needed to accommodate the whole filtrate, and centrifuge at 1389 g (±3000 rpm) for 3 min. Pour, or suction off, the supernatant fluid and discard it.

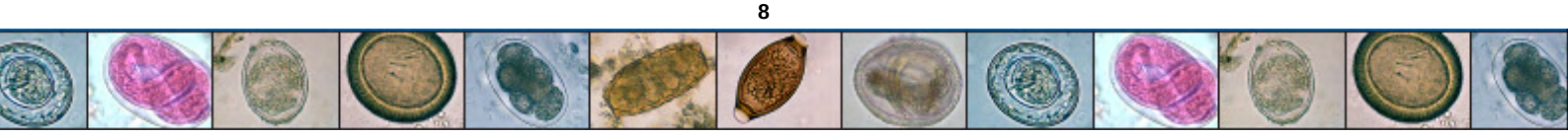
5. Combine the deposits into a suitable number of tubes so that there is not more than 1 mℓ in a 15 mℓ test tube or 5 mℓ in a 50 mℓ test tube.

6. Re-suspend each deposit in a few millilitres of $ZnSO_4$ and vortex well to mix. Keep adding more $ZnSO_4$ and mixing until the tube is almost full.

7. Centrifuge the tubes at 617 g (±2000 rpm) for 3 min. Carefully remove the tubes from the centrifuge and, using a plastic Pasteur pipette, transfer the supernatant to 3 or 4 test tubes. Fill the tubes with distilled water to **reduce** the SG of the $ZnSO_4$ so as not to damage the eggs and also to allow them to **deposit** upon centrifugation.

8. Centrifuge the tubes at 964 g (±2500 rpm) for 3 min; remove & discard the supernatant fluid. Combine the deposits into one test tube, using water to recover all the eggs from the other tubes. Then centrifuge again at 964 g for 3 min to get one deposit.

9. The deposit is transferred to one or more microscope slides, each one is covered with a coverslip, and examined under the microscope to enumerate each species of helminth ova using the 10x objective and the 40x objective to confirm any uncertainties. A calculation is performed so that the ova count can be expressed as eggs per litre of effluent, for each parasite species. (E.g. If 215 *Ascaris* eggs were found in 5 ℓ of effluent, then there are 215/5 = 43 *Ascaris* eggs/ℓ).



PART 2: THE METHOD

TO TEST FOR VIABILITY

- Perform steps 1 to 8 of procedure above, then continue as follows

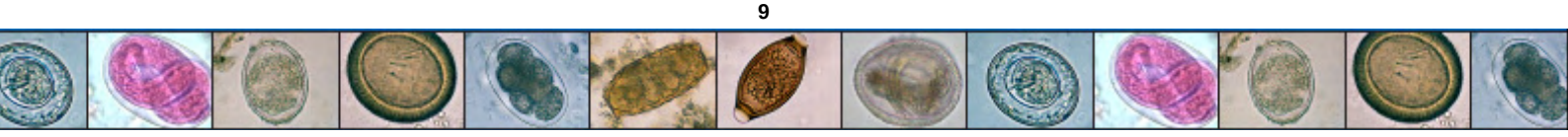
10. Once there is a final deposit in the test tube and re-suspend it in 4 mℓ of 0.1 H_2SO_4. Before incubating mark the test tube with the level of liquid and incubate at a temperature of 26ºC for three to four weeks. Check the level of liquid in each one of the test tubes and add the reagent every time that is necessary, compensating for any evaporation that may occur.

11. Once the incubation time is over, homogenize the deposit and proceed to quantify the eggs. Remove all of the deposit using a plastic Pasteur pipette and place it onto one or more microscope slides. Place a cover slip over each deposit and examine microscopically using the 10x objective and the 40x objective to confirm any unsure diagnoses. Only those ova where the larva is observed are considered viable.

Note: Viability of *Ascaris* ova may be determined at step 9 when enumeration is being done. However this requires experience in microscopy and a trained eye. While the *Ascaris* ova are being examined and counted they may be categorized as follows:

- Undeveloped
- Containing a motile larva
- Containing an immotile larva (in good condition)
- Containing an immotile larva (necrotic i.e. dead)
- Egg broken, dead

Only those *Ascaris* ova with motile and immotile larva in good condition are considered viable.



PROCEDURE FOR WET SLUDGE/SLUDGE TO LAND

Note: It is always preferable to work with small sub-samples as eggs may not be as easily released from a large sample to float out of the sludge when doing the $ZnSO_4$ flotation technique. Rather increase the number of sub-samples than overload each test-tube in order to keep the number of tubes down.

The number of sub-samples will also be dependent on the helminth ova load expected. This will require knowledge of the epidemiology of helminths in the particular area in South Africa. Consequently, more sub-samples must be done in an area of low endemicity and less in a highly endemic area.

1. Mix the sludge sample well by swirling and stirring with a plastic rod. From the total sample take 4 x 15 mℓ sub-samples and place them into 4 x 50 mℓ test tubes. (If the solid content is high this should be sufficient sample. If it is low, take more 15 mℓ sub-samples).

2. Add either a few millilitres of 0.1% Tween80 **or** AmBic solution to the samples, vortex and add more wash solution. Repeat this procedure until the tubes are filled to approximately a centimetre from the top.

3. Place the 150 µm sieve in a funnel in a retort stand with a plastic beaker underneath to catch the filtrate. Filter the well-mixed contents of the tubes one at a time, rinsing out each tube and washing this water through the sieve as well.

4. Pour the filtrate into test tubes and centrifuge at 1389 g (±3000 rpm) for 3 minutes. Suction off the supernatant fluids and discard. Combine the deposits into a suitable number of tubes so that there is not more than 1 mℓ in a 15 mℓ tube or 5 mℓ in a 50 mℓ tube

5. Re-suspend each of these deposits in a few millilitres of $ZnSO_4$ and vortex well to mix. Keep adding more $ZnSO_4$ and mixing until the tube is almost full.

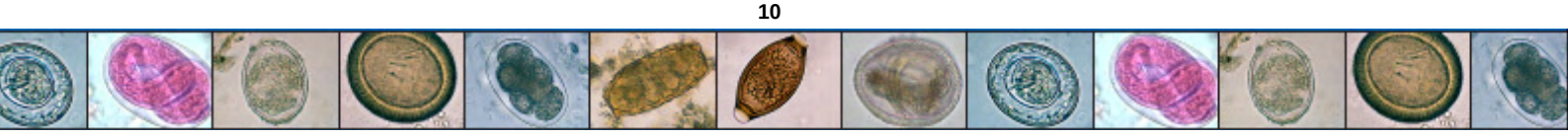
6. Centrifuge the tubes at 617g (±2000 rpm) for 3 minutes. Remove from the centrifuge and pour the supernatant fluids through the 20 µm filter, washing well with water.

7. Collect the matter retained on the sieve and wash it into test tubes.

8. Centrifuge the tubes at 964 g (±2500 rpm) for 3 minutes; remove & discard the supernatant fluid. Combine the deposits into one test tube, using water to recover all the eggs from the other tubes. Then centrifuge again at 964 g for 3 minutes to get one deposit.

9. Once there is one final deposit, remove all of it using a plastic Pasteur pipette and place it onto one or more microscope slides. Place a coverslip over each deposit and examine microscopically using the 10x objective and the 40x objective to confirm any unsure diagnoses.

10. Each species of helminth ova is enumerated separately and reported as eggs per gram of wet sludge.



PART 2: THE METHOD

Note: If reporting needs to be specified in number of ova/dry weight the following is required:

- Take a sub-sample from the original sample prior to step 1.
- Weigh the sub-sample and dry in an oven. Weigh the sample again after drying. This will indicate the moisture content of the wet sludge sample.
- Analyse the sludge sample (wet sludge) as described above from steps 1 to 9.
- The result can then be calculated back to dry weight as follows: Viable helminth ova count in wet sample * (100/moisture content) / wet weight

TO TEST FOR VIABILITY

- Perform steps 1 to 8 of procedure above, then continue as follows:

11. Once there is a final deposit in the test tube and re-suspend it in 4 mℓ of 0.1 H_2SO_4. Before incubating mark the test tube with the level of liquid and incubate at a temperature of 26ºC for three to four weeks. Check the level of liquid in each one of the test tubes and add the reagent every time that is necessary, compensating for any evaporation that may occur.

12. Once the incubation time is over, homogenize the deposit and proceed to quantify the eggs. Remove all of the deposit using a plastic Pasteur pipette and place it onto one or more microscope slides. Place a cover slip over each deposit and examine microscopically using the 10x objective and the 40x objective to confirm any unsure diagnoses. Only those ova where the larva is observed are considered viable.

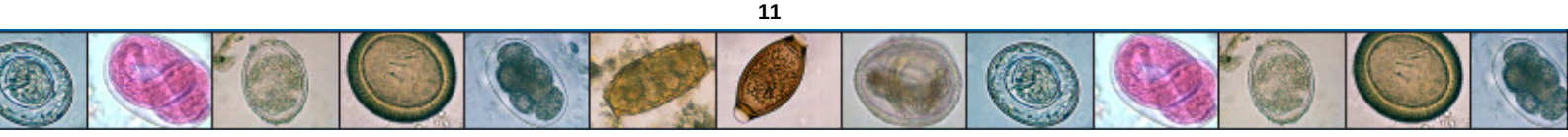
Note: Viability of *Ascaris* ova may be determined at step 9 when enumeration is being done. However this requires experience in microscopy and a trained eye. While the *Ascaris* ova are being examined and counted they may be categorized as follows:

- Undeveloped
- Containing a motile larva
- Containing an immotile larva (in good condition)
- Containing an immotile larva (necrotic i.e. dead)
- Egg broken, dead

Only those *Ascaris* ova with motile and immotile larva in good condition are considered viable.

Note: Samples may be examined slightly differently from that described in step No. 9 above by doing the following:

The deposits are filtered through a 12 µm ISOPORE membrane, which is then rinsed with distilled water. The membrane is air-dried, cut in half and placed on a microscope slide. Immersion oil is used to clear the membrane before examining under the microscope.



PROCEDURE FOR COMPOSTED SLUDGE OR URINE DIVERSION WASTE

1. Weigh out 2 or more 1 g samples into 15 mℓ test tubes. 50 mℓ test tubes may be used if a centrifuge that can take these large tubes is available. If using 50 mℓ tubes, weigh out 3 g maximum per tube. See note at beginning of WET SLUDGE method for reasons.

2. Add a few millilitres of AmBic **or** 0.1% Tween80 and vortex well. Add more solution to about 6 mℓ (in a 15 mℓ tube) or 20 mℓ (in a 50 mℓ tube) and vortex on and off, repeating the addition of solution and vortexing until the tubes are filled to 10 mℓ / 40 mℓ and have been vortexed over a period of about 30 minutes in total.

3. Centrifuge the tubes at 1389 g (±3000 rpm) for 3 min and discard the supernatant. Re-suspend in de-ionized water and vortex to wash off the AmBic or Tween80, and centrifuge again at 1389 g for 3 minutes. Discard the supernatant.

4. Re-suspend each deposit in a few millilitres of $ZnSO_4$ and vortex well to mix. Keep adding more $ZnSO_4$ and mixing until the tube is almost full.

5. Centrifuge the tubes at 617 g (±2000 rpm) for 3 minutes. Carefully remove the tubes from the centrifuge and, using a plastic Pasteur pipette, transfer the supernatant to 3 or 4 test tubes. Fill these tubes with distilled water to **reduce** the SG of the $ZnSO_4$ so as not to damage the eggs and also to allow them to **deposit** upon centrifugation.

6. Centrifuge at 964 g (±2500 rpm) for 3 minutes. Remove tubes and discard the supernatant fluid. Combine the deposits into one test tube, using water to recover all the eggs from the other tubes. Centrifuge again at 964 g for 3 minutes to get one deposit.

Note: At this point if the sample contains a lot of large particles of light debris that floated with the eggs e.g. grass, filter the deposit through a 100 µm filter, collect the filtrate in test tubes and centrifuge again to get a deposit for microscopy.

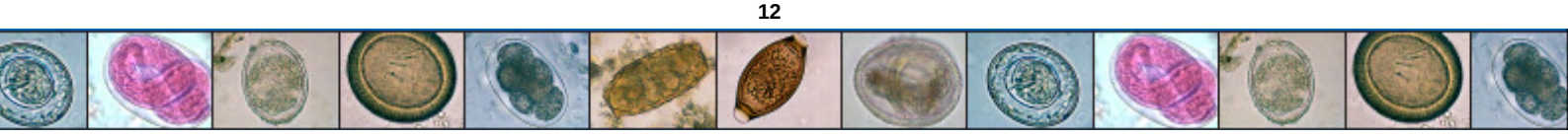
7. Once there is one final deposit, remove all of it using a plastic Pasteur pipette and place it onto one or more microscope slides. Place a coverslip over each deposit and examine microscopically using the 10x objective and the 40x objective to confirm any unsure diagnoses.

8. Each species of helminth ova is enumerated separately and reported as eggs per gram of compost or UD waste.

TO TEST FOR VIABILITY

- Perform steps 1 to 6 of procedure above, then continue as follows:

9. Once there is a final deposit in the test tube and re-suspend it in 4 mℓ of 0.1 H_2SO_4. Before incubating mark the test tube with the level of liquid and incubate at a temperature of 26ºC for three to four weeks. Check the level of liquid in each one of the test tubes and add the reagent every time that is necessary, compensating for any evaporation that may occur.

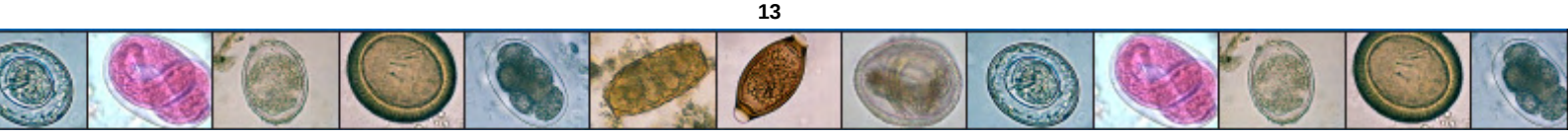


10. Once the incubation time is over, homogenize the deposit and proceed to quantify the eggs. Remove all of the deposit using a plastic Pasteur pipette and place it onto one or more microscope slides. Place a cover slip over each deposit and examine microscopically using the 10x objective and the 40x objective to confirm any unsure diagnoses. Only those ova where the larva is observed are considered viable.

Note: Viability of *Ascaris* ova may be determined at step 9 when enumeration is being done. However this requires experience in microscopy and a trained eye. While the *Ascaris* ova are being examined and counted they may be categorized as follows:

- Undeveloped
- Containing a motile larva
- Containing an immotile larva (in good condition)
- Containing an immotile larva (necrotic i.e. dead)
- Egg broken, dead

Only those *Ascaris* ova with motile and immotile larva in good condition are considered viable.

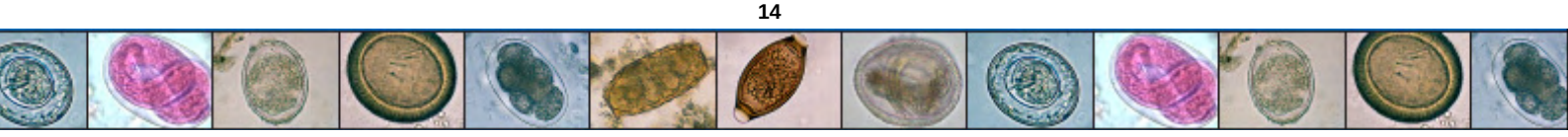


PROCEDURE FOR SLUDGES THAT VARY IN CHARACTERISTICS

Due to variations in sludges from different wastewater treatment plants, one may be required to manipulate the method slightly to accommodate these differences in characteristics. However the three basic principles (washing, filtering, floating and sedimenting) are always maintained. Here is an example for Dry Sludge/"Biltong" Sludge.

EXAMPLE: DRY COMPOST / "BILTONG" SLUDGE

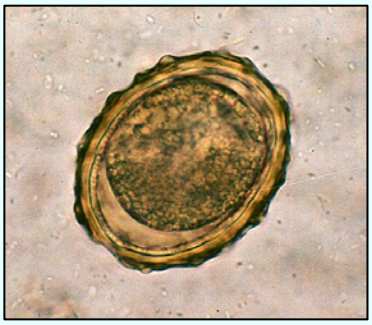
1. Weigh out 10 g of the **very** dried out pieces of compost in a 500 mℓ plastic beaker.
2. Add a measured amount (probably 150 mℓ should be sufficient) of either 0.1% Tween80 or distilled or deionised water and allow to soak overnight.
3. The next morning, break up the softened sample and blend well with a plastic stirring rod.
4. Place the 150 µm sieve in a funnel in a retort stand with a plastic beaker beneath to catch the filtrate. Filter the well-mixed softened compost through this filter, stirring it and using distilled water to make sure all parasite eggs are sieved through the filter into the beaker.
5. Pour the filtrate into test tubes (15 mℓ or 50 mℓ tubes) and centrifuge at 1389 g (±3000 rpm) for 3 minutes. Suction off the supernatant fluids and discard. Combine the deposits into a suitable number of tubes so that there is approximately 1 mℓ in a 15 mℓ tube or 5 mℓ in a 50 mℓ tube.
6. Re-suspend each of these deposits in a few millilitres of $ZnSO_4$ and vortex well to mix. Keep adding more $ZnSO_4$ and mixing until the tube is almost full.
7. Centrifuge the tubes at 617 g (±2000 rpm) for 3 minutes. Remove from the centrifuge and pour the supernatant fluids through the 20 µm filter, washing well with water.
8. Collect the matter retained on the sieve. Using a squeeze bottle of distilled water, rinse everything off the top of the filter into the test tubes. (Squirt the water from the underside of the filter as well to make sure that any eggs that may be a bit stuck on the filter will also wash off into the test tubes.)
9. Centrifuge the tubes at 964 g (±2500 rpm) for 3 minutes; remove & discard the supernatant fluid. The deposits can then be combined into one test tube, using water to rinse out all the eggs from the other tubes and then centrifuge again at 964 g for 3 minutes to get one deposit.
10. Once there is one final deposit, remove all of it using a plastic Pasteur pipette and place it onto one or more microscope slides. Place a coverslip over each deposit and examine microscopically using the 10x objective and the 40x objective to confirm any unsure diagnoses.
11. Each species of helminth ova is enumerated separately and reported as eggs per gram of dry sludge.



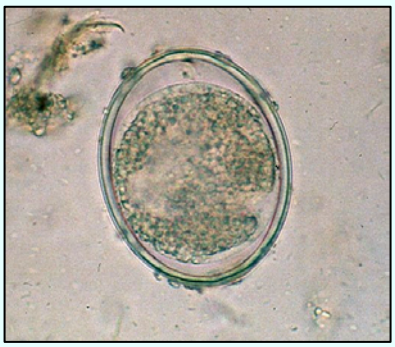
11 REFERENCE PHOTOGRAPHS

The photographs in this section have been included as a source of reference to aid in microscopic analysis for the identification of helminth ova species.

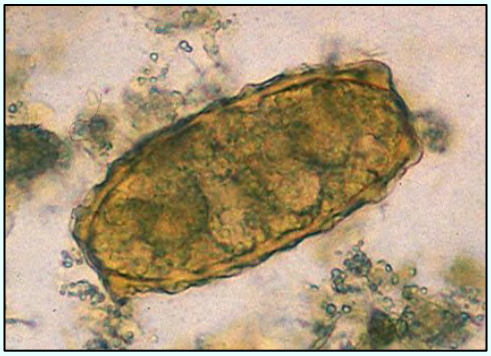
THE MOST COMMON HELMINTH OVA



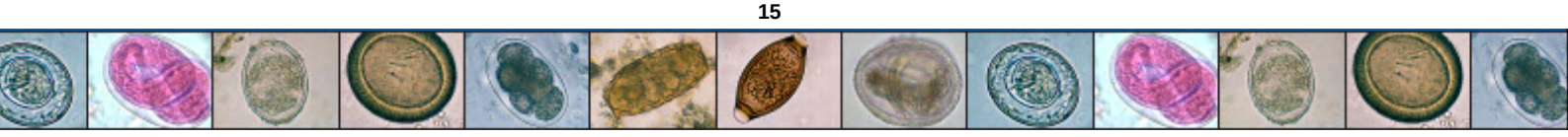
Normal fertile *Ascaris lumbricoides* ovum showing brownish coloured mamillated outer shell.
Size : 55 – 75 x 35 – 50 µm.
(WHO, 1994)



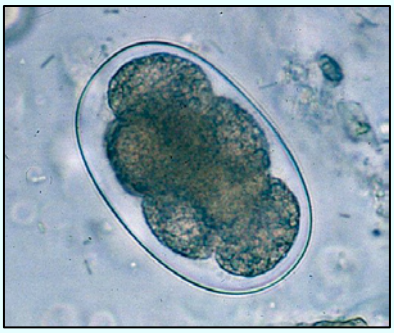
Normal fertile *Ascaris* ovum but without the mamillated layer - referred to as decorticated.
Size : 55 – 75 x 35 – 50 µm.
(WHO, 1994)



Infertile *Ascaris* ovum – It is longer and thinner than a fertile egg. The brownish coloured mamillated layer is irregular and the egg contents are granular and disorganized.
Size : 85 – 95 x 43 – 47 µm.
(WHO, 1994)

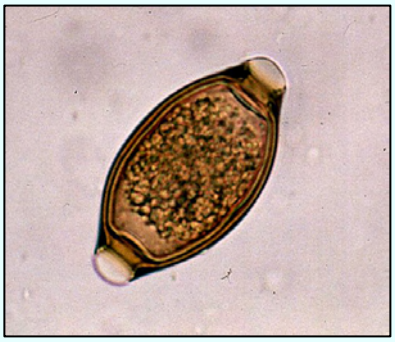


PART 3: PHOTOGRAPHS OF HELMINTH OVA



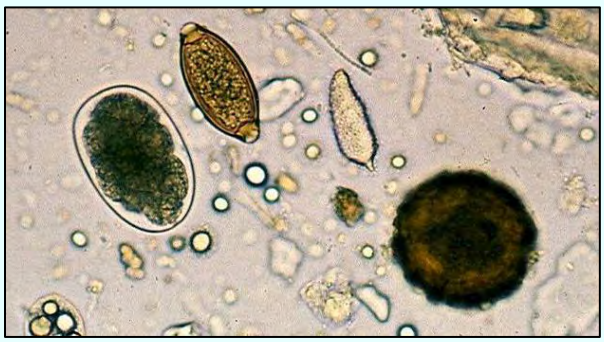
Hookworm sp. ovum has a thin wall and is usually seen in the 4 – 8 cell stage in fresh faeces.
Size: 60 – 75 x 36 – 40 µm.

(WHO, 1994)



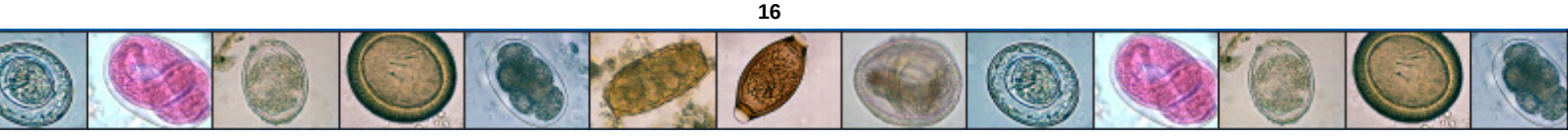
Trichuris trichiura ovum has a smooth brown coloured shell with bipolar "plugs".
Size: 50 – 55 x 22 – 24 µm

(WHO, 1994)

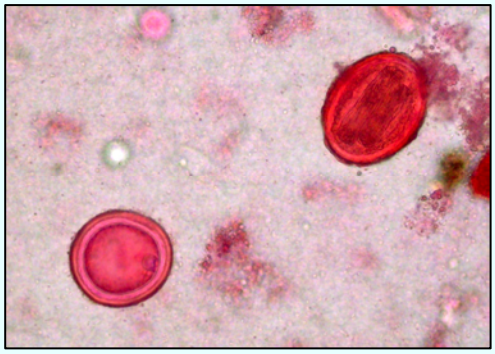


A combination photo to show the different egg sizes of *Ascaris* (right) *Trichuris* (middle) and Hookworm sp. (left).

(WHO, 1994)



PART 3: PHOTOGRAPHS OF HELMINTH OVA



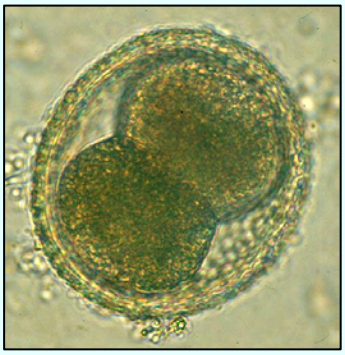
(Top Right) : *Ascaris* ovum

(Bottom Left): Spore that can be confused with *Ascaris*

(Archer and Appleton, Unpublished)

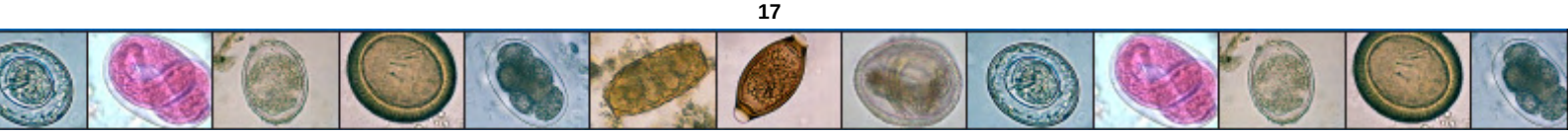
Taenia sp. ova – the pork and beef tapeworm eggs are identical. Each has a thick striated egg wall containing a 6-hooked oncosphere.
Size : 31 – 43 µm in diameter.

(WHO, 1994)

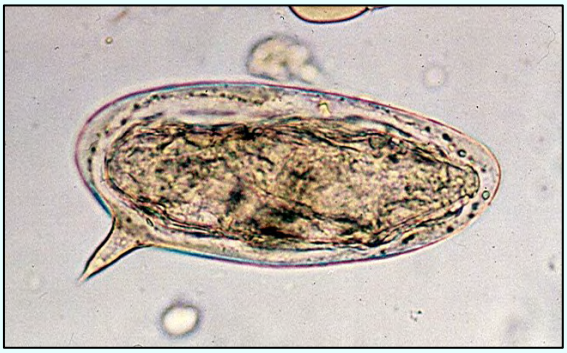


(Left) *Toxocara cati* ovum in 2-celled stage. Size: 65 x 75 µm
(Right) *Toxocara canis* ovum, fully embryonated. Size: 85 x 75 µm

(Archer and Appleton, Unpublished)

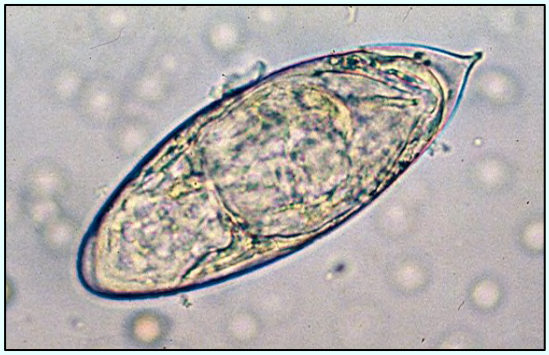


LESS COMMON HELMINTH OVA, LARVAE AND CILIATE CYSTS



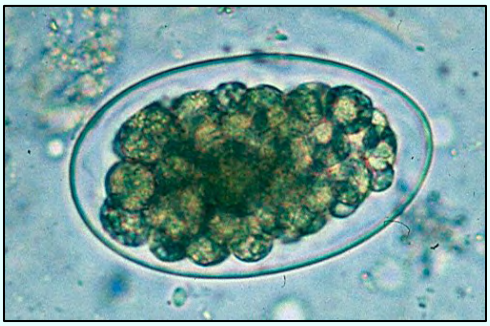
Schistosoma mansoni ovum has a thin transparent shell with a lateral spine and a miracidium inside.
Size: 114 – 175 x 45 – 70 µm.

(WHO, 1994)



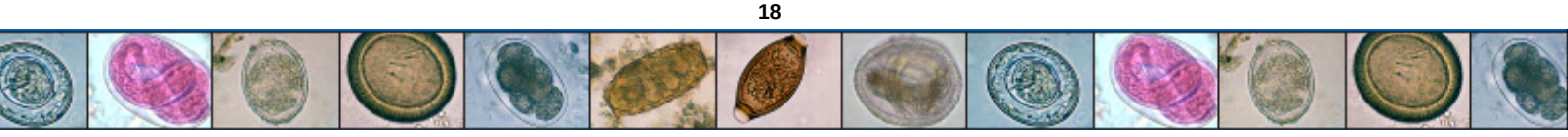
Schistosoma haematobium ovum, normally found in urine, but also in faeces. It has a thin shell, a terminal spine and contains a miracidium.
Size: 112 – 170 X 50 – 70 µm.

(WHO, 1994)



Trichostrongylus ovum resembles hookworm but is larger with one end more tapered than the other.
Size: 75 – 95 x 40 – 50 µm.

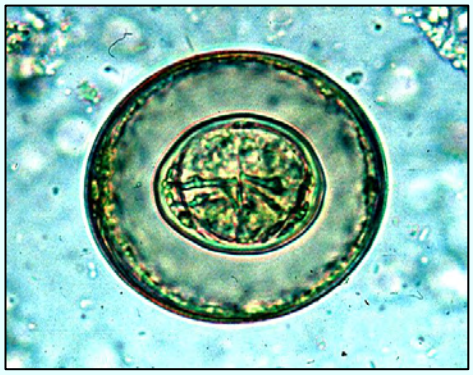
(WHO, 1994)



PART 3: PHOTOGRAPHS OF HELMINTH OVA

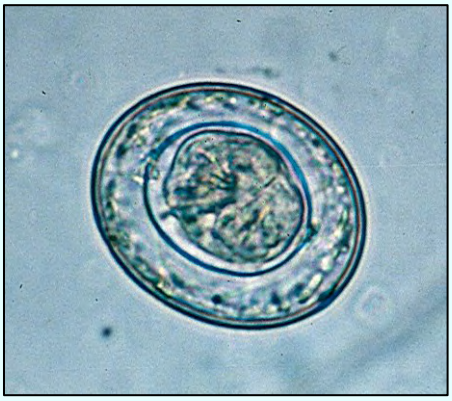
Fasciola hepatica ovum – very large with an operculum at one end.
Size: 130 – 150 x 63 – 90 µm.

(WHO, 1994)



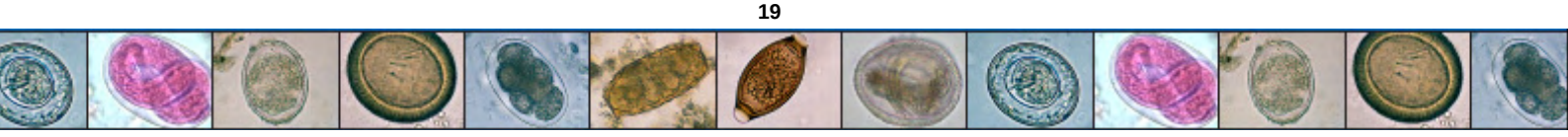
Hymenolepis diminuta ovum is spherical, yellowish-brown in colour, with polar filaments that lie just inside the shell wall. In the middle of the egg is a 6-hooked oncosphere (embryo).
Size: 70 – 85 µm in diameter.

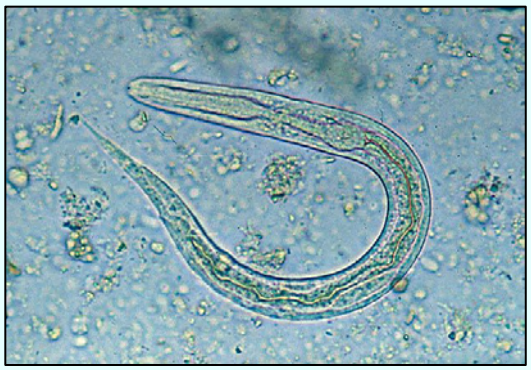
(WHO, 1994)



Hymenolepis nana ovum is slightly more oval than spherical and has a thin clear shell. Polar filaments fill the area between the oncosphere and shell.
Size: 30 – 47 µm in diameter.

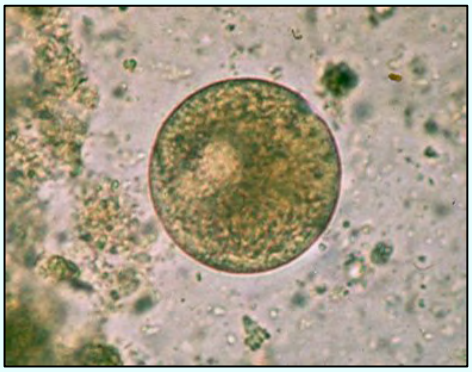
(WHO, 1994)





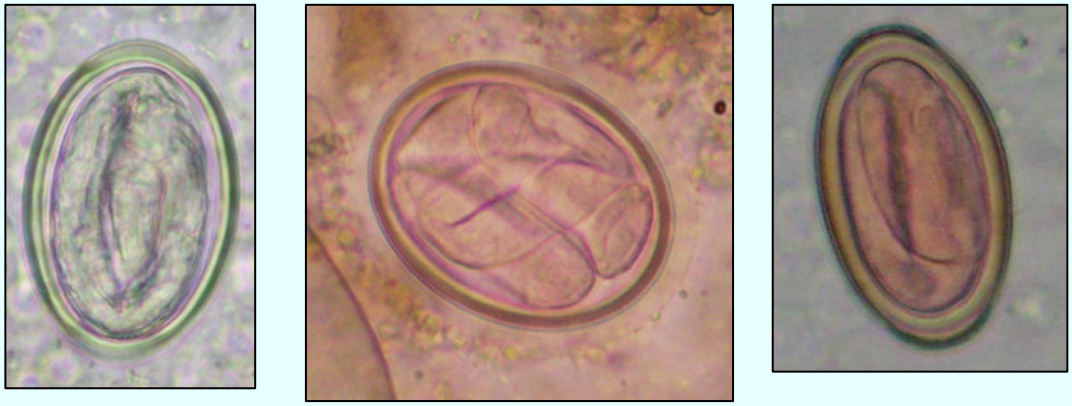
Strongyloides stercoralis rhabditiform larva. This is the first stage rhabditoid larva passed in faeces. However other stages of the free-living soil dwelling life-cycle may be seen in sewage and UD waste.
Size: 180 – 380 x 14 – 20 µm.

(WHO, 1994)



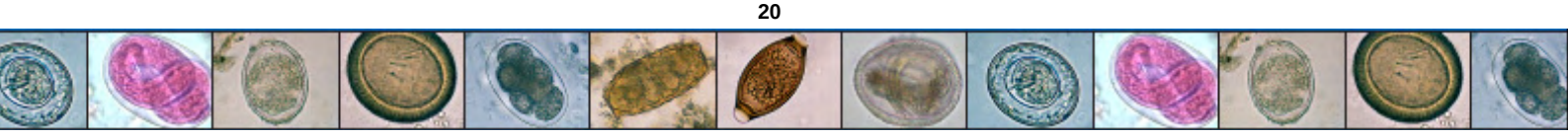
Balantidium coli cyst – this is NOT a helminth egg, BUT a protozoan cyst.
Size: 45 – 75 µm in diameter.

(WHO, 1994)

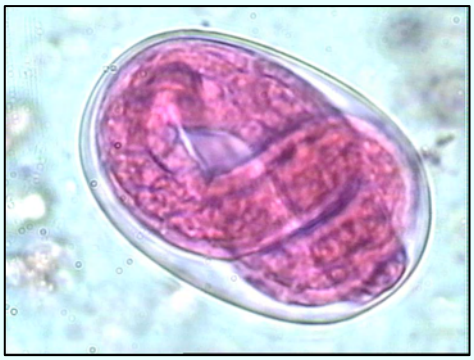


Three spirurid nematode ova: (left) *Streptopharagus* sp. found in rat faeces; (middle) *Physaloptera caucasia* from baboon faeces: 44 – 65 x 32 – 45 µm and (right) *Streptopharagus* sp. From baboon faeces: 37,9 – 41,7 x 19,0 – 28,6 µm

(Archer and Appleton, Unpublished)



PART 3: PHOTOGRAPHS OF HELMINTH OVA

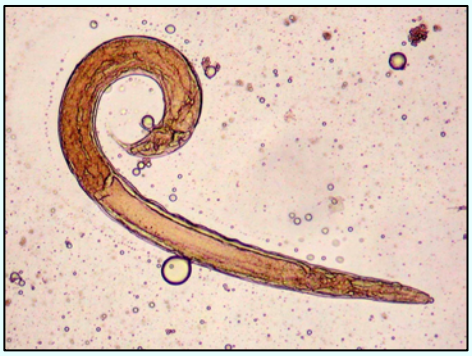


Strongyloides sp. ovum from rat faeces.
Size: 50 – 58 x 30 – 34 µm

(Archer and Appleton, Unpublished)

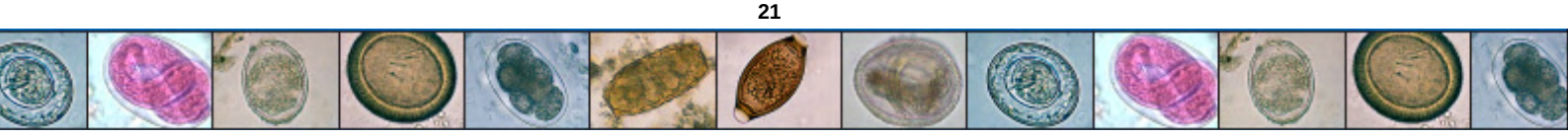
Strongyloides stercoralis – Free-living gravid female worm:
±1,2 mm x 65 µm

(Archer and Appleton, Unpublished)

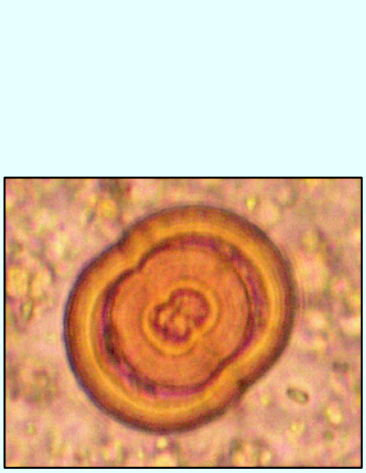


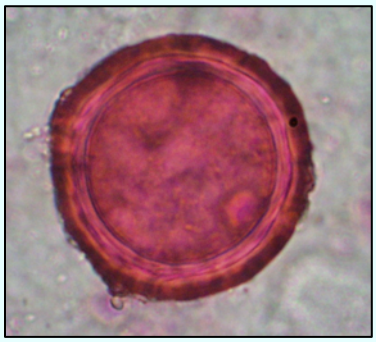
Strongyloides stercoralis - Free-living male worm:
±0,7 mm x 40 – 50 µm

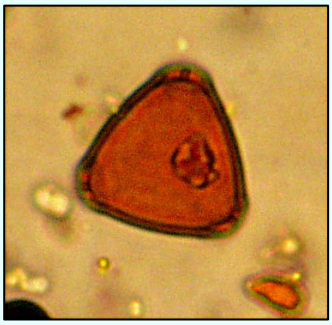
(Archer and Appleton, Unpublished)



PSEUDO-PARASITES

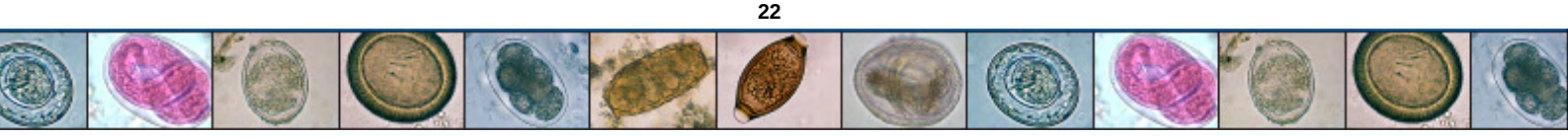






Photographs of "Pseudo-Parasites" or Artefacts – *i.e.* fungal, plant material or pollen that looks parasite ova to the untrained eye!

(Archer and Appleton, Unpublished)

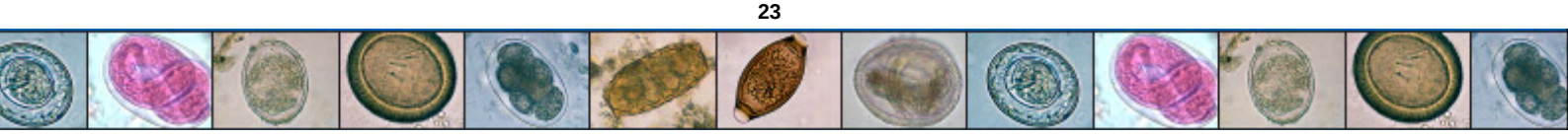


12 CONCLUSION

The method documented above is considered to be efficient and cost-effective for the measurement of helminth ova burdens in wastewater, sludge, compost and UD waste. It is proposed that it be adopted as the standard methodology for South Africa. However it is also known that sample qualities and characteristics vary throughout the country and thus it is up to the laboratory practitioner to include additional wash and filtration steps as required. Thus the laboratory practitioner should adapt the method accordingly to suit his or her sample qualities as well as the existing laboratory facilities/equipment at his/her disposal. It is important however to maintain the principle behind the method so that comparable results are obtained.

This manual presents an attempt to document a standard and simple analytical methodology for South Africa for the recovery and enumeration of helminth ova. It is thus considered to be a living publication, and will be reviewed periodically based on local and international scientific advancements.

A contact list is provided in Appendix 1, of the persons that have been involved in the development of the method. This serves as a support base to users of this manual, so that guidance and assistance can be elicited from them when the need arises. In addition, the report submitted to the WRC as part of the project deliverable (available on CD from the WRC) also includes the contact information of persons from laboratories throughout South Africa that have been trained on the method as part of the capacity building component of the project. These persons could offer assistance to users of the manual in resolving teething problems through information sharing and skills transfer between laboratories, as well as sharing experiences and daily realities.



13 REFERENCES

ARCHER C, MAYA-RENDON C, HAWKSWORTH D, LEIBACH L, RODDA N, SMITH MT, APPLETON CC, BUCKLEY C and MOODLEY P (2006). University of KwaZulu-Natal Pollution Research Group / Catalina Maya collaboration in association with Zitholele Consulting to find the simplest, most cost-effective methods for determining helminth ova burdens in wastewater, sludge, compost and urine-diversion waste in South Africa. Report to the Water Research Commission. Project Number K5/1662.

CABARET J, GEERTS S, MADELINE C and BARBIER D (2002). The use of urban sewage sludge on pastures: the Cysticercosis threat. *Vet*. *Res*. **33**: 575-597.

EPA Environmental Regulations and Technology: Control of Pathogens and Vector Attraction in Sewage Sludge. (Revised October 1999). EPAl625/R-921013.

HAWKSWORTH D, ARCHER C, RODDA N, SMITH M, APPLETON C and BUCKLEY C (in preparation). Method for the recovery of *Ascaris* ova from solid waste from urine diversion toilets. University of KwaZulu-Natal. Pollution Research Group. Unpublished.

JIMÉNEZ B and MAYA C (2006). Techniques for determining Helminth ova in wastewater and sludge. Instituto de Ingeniería, UNAM, Mexico.

W.H.O. (1998). Training manual on diagnosis of intestinal parasites based on the WHO Bench Aids for the Diagnosis of Intestinal Parasites. World Health Organization, Geneva. WHO/CTD/SIP/98.2

PHOTOGRAPHS ARE COURTESY OF:

Mrs C.E. Archer and Prof. C. C. Appleton: University of KwaZulu-Natal. Unpublished ©.

World Health Organization, Geneva, 1994: Bench Aids for the Diagnosis of Intestinal Parasites.

LIFE CYCLE DIAGRAM IS COURTESY OF:

Mrs C.E. Archer and Prof. C. C. Appleton: University of KwaZulu-Natal. Unpublished ©.

APPENDIX 1

USEFUL CONTACTS:

1. **TECHNICAL QUERIES ON METHOD**

Mrs Colleen Archer TEL 031 260 3233
Pollution Research Group
School of Biological and Conservation Sciences (Howard College Campus)
University of KwaZulu-Natal
Durban
4041

Email 1: archerc@ukzn.ac.za
Email 2: psarcher@mweb.co.za

Ms Lizette Leibach TEL 011 929 7058
Control Microbiologist FAX 011 929 7065
ERWAT
P O Box 13106
Norkem Park
1631

Email: lizette@erwat.co.za

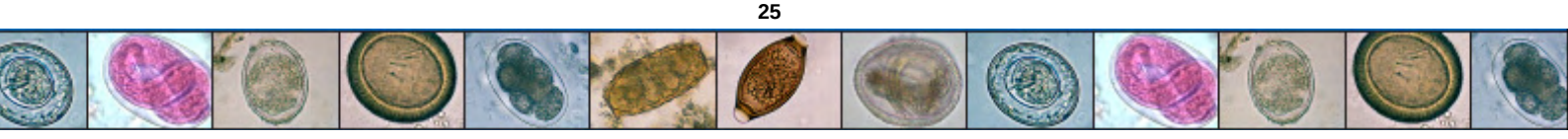
Mr David Hawksworth TEL 031 260 3233
Pollution Research Group
School of Biological and Conservation Sciences (Howard College Campus)
University of KwaZulu-Natal
Durban
4041

Email: 200275860@ukzn.ac.za

International Contact (Mexican Group):

Ms Catalina Maya-Rendon
Institute of Engineering
National Autonomous
University of Mexico
Mexico City
Mexico

Email: CMayaR@iingen.unam.mx



2. PROJECT/METHOD RELATED INFORMATION

Ms Priya Moodley TEL 011 254 4895
Zitholele Consulting
PO Box 6002,
Halfway House
Midrand
1685

Email: Priyam@zitholele.co.za