Desk study on European standard for the enumeration of viable helminth ova in sludge, soil and solid waste

Authors
Simonart T., Roussel S., Gireaudot-Liepman MF

Institut Pasteur de Lille – France
1, rue du Pr Calmette – BP 245 – F-59019 Lille cedex
Tel : +33 3 20 87 77 30 – Fax : +33 3 20 87 73 83

Acknowledgement

This work has been carried out with financial support from the following EU Member States: UK, Germany, France, Italy, Spain, Nordic countries, Netherlands, Denmark, Austria, EU DG XI and JRC, Ispra.

CONTENTS

SUMMARY

As a consequence of the increasing number of sewage treatment plants in Europe, there is a significant increase in sewage sludge production and significant increase in sludge management. Recycling on agricultural lands is a convenient and economically viable alternative to storage, discharge in landfills or in seas.

Microbiological risk assessment evaluation includes the evaluation and enumeration of viable Helminth eggs in sewage sludge. This evaluation needs to rely on standard detection methods including the determination of eggs viability.

At the European level, 3 methods have been described:

- the US EPA modified method (Annex 1)
- the Triple Flotation method (Annex 2)
- the Norwegian method (Annex3)

To date, no final standard method is available. Available standards (US EPA modified method, Triple Flotation method, Norwegian method) exist but need to be fully described. The following considerations need to be more clearly defined :

1) Agreement on target parameters to be selected: *Ascaris* sp. and/or *Toxocara* sp. and/or *Trichuris* sp. and/or *Taenia* sp.
2) Viability detection or not?
3) Detection limit ?

No method has been demonstrated to be capable of combining specific, quick and viability detection of Helminth eggs. For the detection, the most simple method and easiest to implement remains the US EPA method and the Norwegian method (the Triple Flotation method is rather complex and time consuming).

For the viability, fully and comprehensive characterisation of the techniques is needed. Developments are also needed.

1. INTRODUCTION

Waste water treatment leads to the production of two by-products:

- waters, discharged in high flow rivers and/or seas and oceans,
- sewage sludge.

The increasing number of sewage treatment plants at the European level, is linked to a significant increase in sewage sludge which is produced (ex: in France: 800 000 tonnes of sewage sludge expressed in Dry Weight, production expected to double by 2010, information from OTV, 1994).
The current issue is the final use or recycling of sewage sludge: storage (banned by European Directive), recycling on agricultural lands, discharge in landfills or in seas.

At the moment, the preferred solution is recycling of sewage sludge (raw or after treatment, liming, composting or other) on agricultural soils because of their high content in organic matters and fertilisers (Gaspard and Schwartzbrod, 1993 ; Gaspard *et al.*, 1997).
This agricultural use exists in the world and in particular in Europe: the percentage of sewage sludge recycled this way resumes: 45% in the UK, 40% in Germany, 70% in Denmark, 60% in France (speech of French Minister of Environment, IWA congress, 3-7 July 2000, Paris). This treatment is often considered a good way to combine management of natural resources and economic strategy due to its modest cost of implementation.

The absence of accidents affecting public health since this kind of sewage treatment exists (www.ademe.fr) should not occult the two main risks of such a recycling:

- chemical risks,
- microbiological risks (Elliott and Ellis, 1977 ; Boutin, 1982 ; Elissalde, 1994)

Microbiological risks are due to the presence of pathogens, microorganisms including enteroviruses (Schwartzbrod and Albert, 1988), bacteria (Jones, 1985 ; Schwartzbrod *et al.*, 1989 ; Squinazi *et al.*, 1989), protozoa and Helminths parasites (Gaspard *et al.*, 1997 ; Schwartzbrod and Strauss, 1989 ; Schwartzbrod *et al.*, 1990a, 1990b), fungi.

Among those parameters, viable Helminth eggs probably represent an underestimated factor of risk to public health: digestive Helminthiasis, diseases regularly caused by the ingestion of infective Helminth eggs, are worldwide ailments.

In contrast to the protozoa, helminths (including nematodes and cestodes) are multicellular with complex reproductive systems and life cycles involving intermediate hosts for the development of larval stages and a definitive host for the adult form.

The helminths are a group of parasites often referred to as worms. There are many species that infect humans. In the case of human cestodes, the adults inhabit the gut often fixing themselves with specific organs to the gut wall, laying thousands of eggs a day. Occasionally parts of the adult will break off and be visible in the stool as a white or creamy brown segment.

Diagnosis of helminth infection is normally through the detection of the eggs in the stool. These eggs are highly infectious, and very robust. The pig tapeworm has been shown to survive for a decade and still re-infect a new host when eaten. Worms will live in the gut for many years, reproducing, reducing the absorption of nutrients and generally affecting the host's health (Suzuki, 1980).

Symptoms of helminth infection are widespread, and may include gut pain, fatty or watery stools, anaemia and weight loss. Other potential dangers from helminth infection include the formation of cysts in the muscle, eye and brain of the host, and complete blockage of the gut.

Soil-transmitted helminthic infections are of two types: the hookworms, which undergo a cycle of development in the soil (the larvae being infective), and a group of nematodes that survive in the soil merely as eggs that have to be ingested in order for the cycle to continue (Kagei, 1983).

These ailments affect men and animals following a species' specificity and are directly linked to faecal contamination.

Ascariasis.
Adult worms of *Ascaris lumbricoides* live in the small intestine where they lay large numbers of eggs that are passed out with the faeces. The eggs are the infectious form in which the larvae develop. When ingested, the eggs hatch in the jejunum, penetrate the mucosa and are carried through the hepatic circulation to the heart and lungs. They again enter the stomach via the tracheae and oesophagus before growing to adulthood in the small intestine. Pneumonitis and intestinal obstruction may accompany heavy infestations (from Fueki, 1952).

Toxocariasis.
The disease results from the accidental infection of man with eggs of the ascarid roundworm of the dog, *Toxocara canis*, and cat, *T. cati*. The life cycle is the same as that of Ascaris but the invasive larvae become arrested in various tissues where they are phagocytosed. In the process they induce marked eosinophilia and local tissue reaction commonly involving the liver and eye.

Trichurias.
Trichuris trichiura ("whipworm") inhabits the caecum where they attach to the mucosa. Eggs from the mature worms are passed with the faeces and develop in the soil. When swallowed, the eggs hatch in the small intestine and the developing larvae pass directly to their attachment sites in the large intestine. Heavy infections can cause abdominal pain and chronic bloody diarrhoea that may result in rectal prolapse.

Taeniasis.
Taenia solium (pork tapeworm). The adult lives in the small intestine of man that is the definitive host. Segments of the worm pass through the anus and release large numbers of eggs that can survive for long periods outside of the body. When ingested by pigs, the eggs hatch and each releases an onchosphere that migrates through the intestinal wall and blood vessels to reach striated muscle where encystment occurs. When inadequately cooked pig meat is eaten by man, excystment occurs in the small intestine and an adult cestode (worm) develops. If the eggs are released into the upper intestine of man (e.g. through regurgitation) they can invade the host setting up a potentially dangerous larval infection known as cysticercosis in muscle and other sites. *T. saginata* (beef tapeworm) also infects man through cattle to develop into an adult tapeworm responsible for teniasis. The life cycle is similar to *T. solium* and in both species the adult tapeworm can grow up to 10 meters in length (Wang *et al*., 1997).

Risk assessment evaluation resulting from the presence of helminth eggs in sewage sludge needs to rely on standard detection methods including the determination of eggs viability.

2. EXISTING STANDARDS OR DRAFT STANDARDS

At the European level, at least 3 methods for the detection and enumeration methods for viable Helminth eggs in sewage sludge have been described:

- US EPA modified method (Annex 1)
- Triple flotation method (Annex 2)
- Norwegian method (Annex 3)

Based on their protocols, a comparison of these 3 methods has been summarised in the following Table 1:

Table 1 : Comparison of methods

	EPA modified	Triple Flotation (TF)	Norwegian method
Field of application	All sludge and sediments	Sludge (beware of thick and liquid sludge)	Compost – solid sludge
Target parameters	Helminths including	Helminths including cestodes (mainly Taenia)	Helminths including
Sampling weight	10g (dry)	3x0.5g (dry)	10-25g (raw)
Flotation solution	$NaNO_3$	$ZnSO_4$	Sucrose
Density	1.30	1.38	-
Homogenisation	Rotary agitator or Bag Mixer	Manual and Pasteur pipettes	Rotary agitator
Number of filtration	1	-	3
Number of steps	3 main steps		Undefinite (many)
Analytical duration (enumeration)	2h30	7h	2 to 3h
Microscope's reading time	reasonable	rather long	reasonable
Detection limit	<1 helminth/10g (dry)	<7 helminths/10g (dry)	<1 helminth/10g (raw)
Ease-of-use	++	+/-	++
Reproducibility	Robust	undefinite	undefinite
Viability	Larva development	MTT dye (blue)	Larva development

2.1 Detection and enumeration

Norwegian and EPA methods are relatively similar in their detection and enumeration principles. Indeed, in the Norwegian method, after filtration, the diphasic step is followed by the flotation using sucrose. In the US EPA method, after the straining, the flotation step is followed by the diphasic step using alcohol/ethanol.

Note: the size of the strains seems questionable and restrictive with risks of losing big eggs such as *Toxocara* eggs which size can reach 95µm.

In case of clogging with the Norwegian method, some eggs might be lost, which supports the use of the EPA method (160µm). Besides, the 38µm sieve might be too large and could loss smaller eggs such as *Tænia*, *Trichuridae* passing through. The use of sucrose as flotation solution presents the inconvenience to be syrupy (→ adhesion to surfaces). The recovery at the

water-sucrose interface seems to be a delicate handling and the whole steps should lead to a low yield.

Both French methods (US EPA modified method and TF method) have been evaluated (Guarini *et al.*, 2001). It seems that, as they are described now, the TF method has a higher yield due to a higher yield for cestodes (tænias).
On the other hand, laboratories questioned on the practicability of the methods, answered more favourably for the US EPA modified method than for the TF one. For the TF method, the number of tubes should be higher to be more reliable and the reading time at the microscope is rather long.

Due to the sample size (10-25g vs 1.5 g respectively), the detection limits of the EPA modified and Norwegian methods are much lower than the detection limit of the TF method.

From an economical point of view, the US EPA modified method seems to be cheaper (less time consuming), followed by the Norwegian method. Due to the reading of 18 slides per sample, the TF method is much more time consuming than the 2 latter methods and therefore more expansive.

2.2 Viability

The viability approach of both Norwegian and US EPA modified methods is based on the observation of larva development (nematods). Larva development is a true confirmation of viability. This is easy for *Toxocora* and *Ascaris* (because of their size and microscopic visibility), but rather difficult for *Trichuris* and *Capillaria*.

For *Tænia*, the Norwegian method does not propose a viability dye test whereas the US EPA modified method proposes the 'typical blue' method.

Regarding the TF method, the use of the MTT dye described as an indicator of viability by Owen (1985) leads to reliable results in aqueous solution. However, the use of a zinc sulphate solution during the flotation steps might be causing interferences and might be not compatible with the pH of the slide (precipitation).

3. EVALUATION OF DRAFTING A HORIZONTAL STANDARD

The following questions need to be resolved before any standardisation of techniques:

- target parameters: Nematodes (*Ascari*s, …) and/or Cestodes (*Taenia*…)?
- Detection or enumeration?
- Which detection limit is needed?
- Viability assessment?

To date, no final standard method is available. Available draft standards (US EPA modified method, Triple Flotation method, Norwegian method) exist but need to be fully described and adapted to the answers to the above questions.

No method is capable of combining quick detection and viability of helminth eggs. For the detection, the most simple method and easiest to implement remains the US EPA modified method and the Norwegian method (TF method is rather complex and time consuming).

For the viability, no fully and comprehensive characterisation of the techniques is available.

4. CRITICAL POINT AND RECOMMENDATIONS

See Chapter 3. Evaluation of drafting a horizontal standard.

5. DRAFT STANDARD (CEN TEMPLATE)

See Chapter 3. Evaluation of drafting a horizontal standard.

REFERENCES

Anonymous, 1997, Traiter et valoriser les boues (ouvrage collectif) - OTV – Eds L'Aquarène, Saint-Maurice (France, 94), 458p.

Boutin P., 1982, Risques sanitaires provenant de l'utilisation d'eaux polluées ou de boues de station d'épuration en agriculture, *TSM - L'eau*, 77 (12) : 547-557.

Elissalde N., 1994, Les germes pathogènes dans les boues résiduaires des stations d'épuration urbaines, Contract ADEME/ENSP/ENVN, 90p.

Elliott L.F. and Ellis J.R., 1997, Bacterial and viral pathogens associated with land application of organic wastes, *J. Environ. Qual.*, 6 (3) : 245-251.

Fueki K., 1952, On the modes of Ascaris infection, *Keio J. Med.*, 1 : 21-34.

Gaspard P.and Schwartzbrod J., 1993, Irrigation with waste water : parasitological analysis of soil, *Zentralbl Hyg. Umweltmed.*, 193 (6) : 513-520.

Gaspard P, Ambolet Y., Schwartzbrod J., 1997, Valorisation des boues de stations d'épuration en vue de l'amélioration des sols destinés à l'agriculture : contamination parasitaire et modélisation en vue de la gestion du risque sanitaire, *Bull. Acad. Nat. Med.*, 181 : 43-57.

Guarini P., Gireaudot-Liepman M-F, Simonart T, Roussel S, Cabon A. Validation de méthodes microbiologiques applicables aux boues.. PHASE II : essais interlaboratoires. Final report, June 2002. ADEME, France.

Jones P.W., 1985, Sewage sludge as a vector of salmonellosis, In proceedings of CEC COST 68/681, Seminar in Metz, France, 13p.

Kagei N., 1983, Techniques for the measurement of environmental pollution by infective stage of soil transmitted helminthes, In M. Yokogawa, *Collected Paper on the Control of Soil Transmitted Helminthiases*, Vol II, APCO, Tokyo, p. 27-46.

Owen R.R., 1985, Improved in vitro determination of the viability of *Taenia* embryos - *Annals of tropical Medecine and Parasitology*, 79 (6) : 665-656.

Schwartzbrod L. and Albert M., 1988, Analyse virologique des boues résiduaires : évaluation de protocoles d'extraction-concentration des virus, Report of the laboratory of virology, Faculté de pharmacie of Nancy, France, March 1998, 57p.

Schwartzbrod J., Papadopoulos O. and Burdin J.C., 1989, Détection et comportement des *Listeria* dans les boues d'épuration, In : *Microbiologie-Aliments-Nutrition*, 7 : 225-232.

Schwartzbrod J. and Strauss S., 1989, Kystes de *Giardia* au cours d'un cycle d'épuration, *TSM – L'eau*, 84 (6) : 331-334.

Schwartzbrod J., Stien J.L., Thevenot M.T. and Strauss S., 1990a, Sludge parasitological contamination, In L'Hermitte P. Ed., Treatment and use of sewage sludge and liquid agricultural wastes, Congress of Athens (Greece), 1-4 October 1990, Elsevier Applied Science, London.

Schwartzbrod J., Stien J.L., Thevenot M.T. and Strauss S., 1990b, Contamination parasitaire de boues résiduaires, composts et sédiments marins, *Journal français d'Hydrobiologie*, 21 (2) : 285-295.

Squinazi F., Lagneaux K., Nahapetian K., Marin M. and Festy B., 1989, Etude bactériologique des boues résiduaires des stations d'épuration des eaux usées : mise au point de techniques d'analyse, *Journal Français d'Hydrobiologie*, 20 (1) : 77-88.

Suzuki N., 1980, Diagnostic methods in intestinal helminth infections, In M. Yokowaga, *Collected Paper on the Control of Soil Transmitted Helminthiases*, Vol. I, APCO, Tokyo, p. 25-33.

Wang TC., Ma YX., Kuo Ch. and Far PC., 1997, A comparative study on egg hatching methods and oncosphere viability - Determination for *Taenia solium* eggs, *Intl. J. Parasitol*., 27 (11) : 1311-1314.