

**Desk Study
Feasibility of Horizontal Standards
for the enumeration of Viable Helminth Ova
in Sludge, Soil Treated Biowastes**

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CONTENTS

SUMMARY	4
1. INTRODUCTION	6
2. EXISTING STANDARDS OR DRAFT STANDARDS	10
2.1 Selection of standards, draft standards and/or into practice methods	10
2.1.1 <i>Request for information</i>	10
2.1.2 <i>Questionnaire on the EU practices on helminth monitoring in sludges and similar matrices</i>	11
2.2 Presentation of identified standards, draft standards and/or into practice methods	15
2.2.1 <i>Double flotation method in a sodium nitrate solution (AFNOR PR XP X 33-031 experimental standard)</i>	15
2.2.2 <i>Triple flotation method in a zinc sulphate solution (AFNOR XP X 33-017 experimental standard)</i>	16
2.2.3 <i>Norwegian flotation in sucrose method</i>	17
2.2.4 <i>Austrian flotation in a sugar solution method</i>	18
2.2.5 <i>German flotation in a zinc sulfate solution method</i>	19
2.2.6 <i>Hungarian double flotation in a calcium nitrate solution method</i>	19
2.3 Comparison of identified standards, draft standards and/or into practice methods	20
2.3.1 <i>Enumeration of helminth eggs</i>	20
2.3.2 <i>Viability evaluation</i>	22
3. EVALUATION OF DRAFTING A HORIZONTAL STANDARD	24
4. CRITICAL POINT AND RECOMMENDATIONS	25
5. DRAFT STANDARD (CEN TEMPLATE)	26
REFERENCES	27
ANNEX 1 – LETTER SEEKING INFORMATION AND OUTLINING THE HORIZONTAL PROJECT	29
ANNEX 2 – AFNOR PR XP X33-031	30
ANNEX 3 – AFNOR XP X 33-017	40
ANNEX 4 – ISOLATION OF HELMINTH EGGS FROM COMPOSTED SLUDGE	47
ANNEX 5 – HORIZONTAL DESK STUDY 5 – HELMINTH OVA IN SLUDGES QUESTIONNAIRE	49
ANNEX 6 – SUMMARY OF THE PRINCIPLE OF THE METHODS USED BY THE RESPONDENTS TO THE QUESTIONNAIRE WHO PERFORM HELMINTH ANALYSIS IN SLUDGES AND SIMILAR MATRICES	52

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 resulting from the presence of Helminth eggs in sewage sludge as well as in other treated and untreated sludge, soil and biowastes in European Union needs to rely on a European standard method including the determination of eggs viability.

At the European level, 6 methods have been identified :

- 1) A modified US/EPA method (1992) based on a double flotation in a sodium nitrate solution, under French standardisation process (AFNOR PR XP X33-031), see the protocol in **Annex 2**;
- 2) A Triple flotation in a Zinc sulphate solution method, to be printed as French experimental standard in April 2004 (AFNOR XP X33-017), see the protocol in **Annex 3**;
- 3) A Norwegian method based on a flotation in sucrose, see the protocol in **Annex 4**;
- 4) An Austrian method based on a flotation in a sugar solution, see the short summary protocol in **Annex 6**.
- 5) A German method based on a flotation in a zinc sulphate solution, see the short summary protocol in **Annex 6**.
- 6) An Hungarian method based on a double flotation in a calcium nitrate solution, see the summary protocol in **Annex 6**.

To date, no method to be used as draft horizontal standard is available.

Two French available experimental standards (AFNOR PR XP X33-031 and XP X33-017) exist but need to be further studied for their performance in terms of viability, organisms to be identified and fit for purpose to all sludges, soil and biowastes matrices. More particularly, they need to be validated at a European scale.

The four other identified European methods seem not to be in a standardisation process. The Norwegian method allows the enumeration of viable helminth ova.

Three other methods from Austria, Germany and Hungary, have been identified thanks to a (non exhaustive) questionnaire but do not evaluate viability. They all need to be fully described and further studied for their relevance to helminth eggs enumeration in all sludge, soil and biowastes matrices, and especially for eggs viability evaluation.

No method has been demonstrated to be capable of combining specific, quick and viability detection of all Helminth eggs.

The following considerations need to be more clearly defined before drafting a horizontal standard on viable helminth ova in sludge, soil and biowastes :

- 1) European agreement on the more relevant helminth target to be monitored considering the risk in all the European Union countries (including assessing countries) : the whole viable Helminth group, or only viable Nematodes and

- especially *Ascaris* spp. and/or *Toxocara* spp. and/or only viable Cestodes and especially *Taenia* spp. , etc. ;
- 2) The European acceptable contamination level of those identified target helminth in all sludge, soil and biowastes matrices, and as a consequence, the detection limit required for the horizontal standard.

Such considerations need to be discussed during an European expert workshop. Such a workshop on the viable helminth ova topic has been proposed in the FP6 proposal on “Horizontal Standards on Hygienic microbiological Parameters for Implementation of EU Directives on Sludges, Soils, Soil Improvers, Growing Media and Biowastes”. The conclusion of the workshop would be discussed in a critical review that would allow to identify and propose the most fit for purpose method(s) applicable to European sludges, soils and treated Biowastes. Those identified draft standards would be evaluate for their suitability in a intra-laboratory work involving some European expert laboratories.

1. INTRODUCTION

This report is one of the five Project Horizontal desk study reports that attempt to assess hygienic parameters (WP 3), which may be needed to assure the sanitation of sludges, soils, soil improvers, growing media and biowastes. The five desk studies reports highlight draft potential methods for the hygienic parameters likely to be included in future sludge and biowaste Directives :

Desk study report 3A “Feasibility of horizontal standards for *Escherichia coli* and *Salmonella* in sludges, soils, soil improvers, growing media, and biowastes” deals with *Escherichia coli* and *Salmonella* spp.,

Desk study report 3B “Rapid Methods for detection of *E. coli* (including *E. coli* O157) and *Salmonella* in sludges, soils, soil improvers, growing media, and biowastes”, deals with rapid methods available for *E. coli* (including *E. coli* O157) and *Salmonella*,

Desk study 4 “Feasibility of Horizontal standard methods for detection of *Clostridium perfringens* and *Enterococci* in sludges, soils, soil improvers, growing media, and biowastes” deals with *Clostridium perfringens* and *Enterococci*,

Desk study 5 “Feasibility of horizontal standards for the enumeration of viable helminth ova in sludge, soil and treated biowaste”, deals with viable helminth ova,

Desk study 6 “Literature review on levels of pathogens and their abatement in sludges, soils, soil improvers, growing media, and biowastes” deals with the occurrence of pathogens and their abatement.

It is not only necessary to make methods available to determine specific micro-organisms, but also to provide a detailed protocol for sampling heterogeneous matrices such as sludges, soils, soil improvers, growing media, and biowastes to obtain fit for purpose results. Results are needed for validating plant performance (percentage pathogen reduction) and end product specification in terms of hygienic microbiological parameters (e.g. EU 2000). This will include co- and pre-normative research, including consideration of carrying out method validation for complementary bacterial indicators (e.g. *Enterococci* and *Clostridium perfringens*), and viable helminth ova (cestodes and nematodes). For parameters likely to be included in future Directives (i.e. *E. coli* and *Salmonella* spp.), the selected methods will be assessed in large Europe-wide interlaboratory trials involving many European countries. For other parameters, there is a need to develop preliminary standards in order to carry out the relevant research. In the sludge and Biowaste draft directives (EU 2000 and EU 2001), *E. coli* and *Salmonella* are specifically mentioned. This leads to the logical choice to start the work on these organisms as one of the parameters in phase 1 of project Horizontal. For the other parameters, Project Horizontal desk studies 4 and 5 of WP3 to prepare draft potential protocols for CEN and ISO discussion are also being prepared.

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 (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 ; ADEME, 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* (nematode) 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 (nematode) 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.

Toxocariasis and other ascarids from animals are unfrequent and are present as larvae migrans and the risk is constituted by playground in schools and other children leisure places.

Trichuriasis.

Trichuris trichiura ("whipworm", nematode) 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 cestode). 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 taeniasis. 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). *Taenia solium* is extremely rare in western European Union, whereas *Taenia saginata* (cattle as intermediate host) is not so unfrequent.

The Helminth ova are relevant to sludge hygiene, because they are present in sludge and they are reputed to be very resistant to treatments. The USA, France, Germany and Hungaria have included this parameter in their national Directives on sludge hygienisation.

Risk assessment evaluation resulting from the presence of viable helminth eggs in sewage sludge as well as in other treated and untreated sludge, soil and treated biowastes in European Union needs to rely on a European standard method including the determination of eggs viability.

However, such an European standard method has to investigate the whole range of helminth target organisms regarding risk assessment in all European Union countries (including European assessing countries) and its performance in terms of viability, organisms to be identified and fit for purpose to all sludge, soil and treated biowastes matrices have to be demonstrated at a European scale.

2. EXISTING STANDARDS OR DRAFT STANDARDS

2.1 Selection of standards, draft standards and/or into practice methods

2.1.1 *Request for information*

No European standard on helminth ova being available, a request for information about European methods used on helminth ova was needed.

Following a meeting of the members of the Horizontal work package 3: Hygienic Parameters, it was decided that a letter outlining the project be sent to all member states. The aim of the letter was to obtain feedback from the member states through TC308 regarding all accepted national or international standards on the five microbiological parameters to be studied in this work package 3 : *Escherichia coli*, *Salmonella* spp., intestinal *Enterococci*, *Clostridium perfringens* and helminth ova in sludge, soil, compost, and biowastes. Any validated standards for water and wastewater on those parameters were also requested.

In order not to exclude the smaller European countries from the study, effort was also made to contact all those members listed on the CEN / ISO voting list. This enabled the any validated methods from those countries to be included in the report. The letter sent out to member states included an introduction to the project “Horizontal” so that it gave members an understanding of what the objectives were. The request for method information also included background on Work Package 3.

A copy of the letter sent out is given in **Annex 1**.

Responses were requested before the meeting of TC308 in Oslo on 29th March 2003 so that all submitted methods from the member states could be discussed at the meeting. From the letter sent out after the meeting requesting information on WP3: Hygienic Parameters, there was very few responses, and only for *E. coli* and *Salmonella* spp. parameters (see Horizontal Desk study report 3A “Feasibility of horizontal standards for *Escherichia coli* and *Salmonella* in sludges, soils, soil improvers, growing media, and biowastes”).

Without any return information from all member states and all members listed on the CEN / ISO voting list concerning any methods for helminth ova (nematodes and cestodes) in waters, wastewaters, sludge, soil, compost or biowaste, only three methods have been identified for the detection and enumeration of Helminth eggs in sewage sludge, each based on a flotation principle, both of them in standardisation process in France :

- 1) A modified US/EPA method (1992) based on a double flotation in a sodium nitrate solution, under French standardisation process (AFNOR PR XP X33-031), see the protocol in **Annex 2**;
- 2) A Triple flotation in a Zinc sulphate solution method, to be printed as French experimental standard in April 2004 (AFNOR XP X33-017), see the protocol in **Annex 3**;
- 3) A Norwegian method based on a flotation in sucrose, see the protocol in **Annex 4**.

Those three methods have been presented and compared in a previous draft of this report, leading to the conclusion that there were a need to validate those three methods for their relevance for the enumeration of viable helminth ova in sludge, soil and treated biowastes at a European scale.

But more specifically, it was underlined that there was a real need to have an European agreement among specialists on the more relevant Helminth target to be monitored in sludge, soil and treated biowastes, etc... (Helminth or more specifically *Ascaris* spp. or *Taenia* spp....?).

During the 3rd and 4th December 2004 meeting in Brussels, the Steering Committee therefore asked the Horizontal work package 3 leader and writer of the desk study on helminth ova to organise an expert workshop drawing from a list of names to be provided by the Steering Committee members. The desk study should then be reviewed in light of the results of this expert consultation and submitted to the Steering Committee by March 2004.

Despite a remind message sent on 19th January 2004 to all the Steering Committee members, only 6 experts of 3 EU countries (Austria, Spain and Finland) were identified (2 experts per country) by the middle of February 2004. Due to the very few identified European experts on helminth in sludge, soil and treated biowastes, and the very short remaining time to achieve the work, it was impossible to organise the workshop.

2.1.2 *Questionnaire on the EU practices on helminth monitoring in sludge and similar matrices*

In view of the impossibility to organise the workshop due to the lack of identified EU experts on the topic, the desk study writer decided however to send a short (non exhaustive) questionnaire in order to try to obtain more information concerning the EU practices on helminth monitoring in sludge and similar matrices, thanks (i) to the 6 identified experts list obtained from three horizontal Steering Committee members, and (ii) to the internal network of EU colleagues contacts of the Water and Environment Dpt of the Institut Pasteur de Lille.

A questionnaire was therefore elaborated and sent to around 25 EU contact colleagues assumed to perform (routine) analysis in sludge, soil and biowastes, and distributed in 16 different EU countries (including assessing countries).

The 16 different countries were (in alphabetic order) Austria, Belgium, Cyprus, Denmark, Estonia, Finland, Germany, Greece, Hungary, Ireland, Italy, the Netherlands, Norway, Portugal, Romania, Spain and the United-Kingdom.

In the message sent together with the questionnaire, it was asked to the recipient to forward if possible the message to any other national colleague being involved in the topic and being then able to full-fill the questionnaire.

This questionnaire was not exhaustive but should allow to collate a first set of interesting information about European practices on the topic.

The questionnaire is presented in **Annex 5**.

A total number of 17 questionnaires were returned (more than 60% of responses).

Question 1a :

Among them, 13 respondents distributed in 10 different European countries (from Estonia, Finland, Greece, Hungary, Ireland, The Netherlands, Norway, Portugal, Spain and the United-Kingdom) did not perform helminth analysis in sludge or similar matrices (question 1a) and so did not answer the following questions of the questionnaire.

One was in the process to built up a diagnostic laboratory for parasite diagnostics for warm/blooded animals and was working with several Ph.D. students on various parasite projects and was projecting to handle a specific project to test the efficacy of treatment of sludge.

Among those 13 respondents :

- 3 respondents forwarded the questionnaire to an assumed expert national colleague. One of them answered to the questionnaire as performing routinely the helminth analysis. The two others did not answer.
- 1 respondent from Ireland outlined that neither his Public laboratory, his Environmental Protection Agency and a local Authority laboratory dealing with sewage were aware of any laboratory in Ireland doing this work.
- 1 respondent from Portugal outlined that he did not know any laboratory in Portugal performing helminth ova analyses in sludge regularly. However, the respondent precised that such analysis was performed in wastewater for use in agriculture, due to a Portuguese legislation requiring the analysis of “ova of intestinal parasite” in wastewater for use in agriculture.

Question 1b :

Only 4 respondents from 4 different countries (Austria, Germany, Hungary and Italy) perform helminth analysis in sludge and similar matrices and have full-filled the whole analytical part of the questionnaire (question 1a to 1h).

For only 2 out of those 4 respondents (Germany and Hungary), the helminth eggs enumeration in sludge and similar matrices is required in their national legislation (question 1b).

Question 1c to 1h :

The results of the analytical practices (questions 1c to 1h) of those 4 respondents are summarized in **Table 1**, together with the practices of the microbiological laboratory of the Water and Environment Dpt of the Institut Pasteur de Lille (France).

Question 1c :

The methods presented by 3 out of the 4 respondents who perform the helminth analysis in sludge and similar matrices are 3 flotation method based on a different principle. They are shortly described in **Annex 6** and listed below :

- 1) Austrian respondent’s method is based on a flotation in a sugar solution adapted by Hejny/Brandl (1995) from an originally method described by Horn *et al.* (1990).
- 2) German respondent’s method is based on a flotation in a zinc sulphate solution as described in Thienpont *et al.* (1979).
A modified “Allen and Ridley” method is used for the *Ascaris* enumeration for the main work on investigations about the tenacity of *Ascaris* ova in

biotechnological plants for the treatment of biological wastes, slurry and sewage sludge (method not described and reference not detailed in the questionnaire).

- 3) Hungarian respondent's method is based on a double flotation in a calcium nitrate solution (following an internal method with no literature reference).

The method used by the Italian respondent is not specified in the questionnaire. However, none of those methods appears to be in a standardisation process.

Question 1d & 1e:

The Italian method allow the enumeration of helminth (no precision about the specificity for nematodes and/or cestodes).

The German method allow the enumeration of both nematodes and cestodes (including *Taenia* sp.).

Both Austrian and Hungarian methods focused specifically on Nematodes (including *Ascaris* sp.) enumeration. The Austrian method allows in addition the enumeration of *Coccidia* oocysts (question 1e).

The Austrian method has been used for the helminth ova enumeration in soil and sand, but the respondent precised that they have never been able to detect Taeniid or any other cestodes eggs with it and never have evaluated the method for this purpose. The respondent added that the method should however be applicable to cestodes.

None of those methods allows a viability evaluation, except for *Ascarids* (and especially *Toxocara*) or Trichuids for the Austrian method. Those latest helminth nematods eggs were regularly detected with the Austrian method and the viability of such nematodes can be evaluated by the presence of potentially infectious larvae.

Question 1f and 1g:

The Austrian respondent analyses sand and soil samples in the frame of research projects (10 studies in the last 10 years, 350-500 samples / study), and sludge/slurry sporadically (on demand).

The Hungarian respondent who performs 10-50 analysis of helminth eggs per month , does analyse sewage sludge, soil and wastes in 30% of the cases for each of those three matrices, and sand in 10% of the cases.

The German respondent perform less than 10 analyses per month on anaerobic treated sludge and anaerobic stabilized sludge.

The Italian respondent perform the analysis of helminth eggs in raw sewage sludge and in compost but only 1 times per 3 months for both type of matrices.

Question 1h :

The German respondent who applies a method allowing the enumeration of both nematodes and cestodes had unfortunately no comparative results available.

The Austrian and Hungarian respondents who both apply a method which allows the enumeration of only nematodes found more often *Toxocara* spp. (Austrian and Hungarian respondents) and *Ascaris* spp. (Hungarian respondent) in soil, sand and sludge.

The Italian respondent found more often Nematodes in raw sewage sludge (no precision about nematodes and/or cestodes specificity of the method used in question 1d).

Table 1 – Results of the analytical practices of the four respondents who perform helminths analysis in sludges and similar matrices (analytical practices of the Water & Environment Dpt of Institut Pasteur de Lille (F) given as an indication).

Respondent	1	2	3	4	Indicative		
Country	Austria	Germany	Hungary	Italy	France		
Method	Flotation in a Sugar solution	Flotation in a Zinc Sulfate solution	n.s.	Double Flotation in a Calcium Nitrate solution	n.s.	Double Flotation in a Natrium Nitrate solution	
Method's Reference	Hejny-Brandl, 1995 (Mod.Horn <i>et al.</i> , 1990)	Thienpont <i>et al.</i> , 1979	Mod."Allen and Ridley" method	n.s.	n.s.	AFNOR PR XP X33-031 Mod.US/EPA, 1992 Mod.Gaspard and Schwartzbrod, 1993	
Targeted Helminth	Helminth						
	V&NV	X	X	-	X ^c	X	
	Viable	-	-	-	-	X	
	Nematods						
	V&NV	X	X	-	X ^c	X	
	Viable	-	-	-	-	X	
	Ascaris						
	V&NV	-	-	X	X ^c	X	
	Viable	X ^d	-	-	-	X	
	Cestodes						
	V&NV	-	X	-	-	X	
	Viable	-	-	-	-	X	
Taenia							
V&NV	-	X	-	-	X		
Viable	-	-	-	-	X		
Other	Coccidia oocysts	-	-	-	-	X	
Analysis Frequency	<10 / month	-	X	-	X	-	
	10-50/month	X	-	X	-	X	
Matrices & Frequency of analysis	Sludge/slurry	Sporadic (on demand)	-	See ^b	-	-	-
	Sewage sludge	-	-	See ^b	30%	-	XXX
	Raw Sewage Sludge	-	-	-	-	1 / 3 months	XXX
	Lime stabilized Sewage Sludge	-	-	-	-	-	XXX
	Pelleted Air-Dried Sewage Sludge	-	-	-	-	-	XX
	Industrial Sewage Sludge	-	-	-	-	-	XXX
	Anaerobic Treated Sludge	-	X	-	-	-	-
	Anaerobic Stabilized Sludge	-	X	-	-	-	-
	Composted Sewage Sludge	-	-	-	-	-	XXX
	Compost	-	-	-	-	1 / 3 months	-
	Sand	In research projects	-	-	10%	-	-
	Soil	^a	-	-	30%	-	-
Wastes	-	-	See ^b	30%	-	XXX	
More often Helminth eggs observed	Nematods	-	No comparative results.	-	-	In raw sewage sludge	n.s.
	<i>Toxocara</i> spp.	Predominantly in both sludge and soil/sand		-	In soil, sand and sewage sludges	-	19%
	<i>Ascaris</i> spp.	-		X	-	-	37%
	<i>Trichuris</i> spp.	-		-	-	-	75%
	<i>Capillaria</i> spp.	-		-	-	-	12%
	<i>Hymenolepis</i> spp.	-		-	-	-	23%
<i>Taenia</i> spp.	-	-	-	-	25%		

Legend : n.s. : Not specified V&NV : no viability evaluation Mod. : Modified

^a : "10 studies in the last 10 years, 350-500 samples / study".

^b : Mainly investigations on the tenacity of *Ascaris* ova in biotechnological plants for the treatment of biological wastes, slurry & sewage sludge

^c : "Number of all recovered helminth eggs/100 g sample reported with the number of damaged eggs detected by the microscope examination

^d : "The viability can be evaluated by the presence of potentially infectious larvae of Ascarids (especially *Toxocara*) or *Trichuris*."

Conclusion on the questionnaire :

The results of this non exhaustive questionnaire on the EU practices of the helminth analyses in sludge and similar matrices are promising, taking into account the very small number of questionnaires initially sent (around 25 in 16 different EU countries including assessing countries) and the fact that the recipients were not always assumed to perform helminth analyses in sludge.

Four different additional methods from 4 different EU countries have been identified (Austria, Germany, Hungary, Italy) and three of them shortly described. However, none of them seems to be in a standardization process.

Such a questionnaire approach appears to be very fruitful to obtain information about the analytical practices in EU countries.

The main limiting factor of such an approach remains however the identification of the national experts including not only research institute type laboratories but also routine sludge, soil and biowastes analyses laboratories. Such information would be obtained thanks to the collaboration of relevant national organisation in each EU countries, such as standardisation bodies and Ministry of Health and/or Environment, and thanks to the networks of European contacts of laboratories specialised in sludge analyses.

2.2 Presentation of identified standards, draft standards and/or into practice methods

A total of 6 methods for the detection and enumeration of Helminth eggs in sludge, soil and/or biowastes have been identified. Two of them are in French standardisation process.

Those 6 methods are presented and discussed for their possible relevance as being a draft Horizontal standard in this chapter.

2.2.1 Double flotation method in a natrium nitrate solution (AFNOR PR XP X 33-031 experimental standard)

This method has been adapted by the Water and Environment Dpt of the Institut Pasteur de Lille (France) from the method described by Gaspard and Schwartzbrod (1993), based on a modification of the US/EPA method (1992).

The method is presented in **Annex 2**.

The principle is based on a double flotation in a natrium nitrate solution (sp.gr 1.30) and is proposed for the enumeration of viable helminth eggs in sludge, including nematodes and cestodes eggs. The helminth eggs are enumerated in a subsample of 10 g (dry weight).

The method used to detect the viability of cestodes eggs is based on the exclusion of the trypan blue dye by living cells. The colour of the viable eggs does not change while dead eggs are blue stained.

The method used to detect the viability of nematodes eggs is based on the structure observation. Nematodes eggs are considered as viable eggs when the integrity of their structures can be observed or if a larva is detected inside. However, any absence of

internal structures or any disorganisation of the internal structures are criteria attesting that the eggs are non viable eggs.

This method is routinely used to analyse untreated and treated sewage sludge (urban and industrial sewage sludge including raw sewage sludge, lime stabilized sewage sludge, pelleted air-dried sewage sludge, composted sewage sludge, etc...) and wastes (around 200 samples analysed in 2003).

This method has been proposed as French AFNOR experimental standard (AFNOR PR XP X33-031) by the end of 2003.

A previous version of this method has been tested for its performances in interlaboratory trials, in comparison with the French Triple flotation to be printed as French experimental standard in April 2004 (ADEME, 2002 and see §2.3). A summary of the main conclusion of this study is presented in the proposed experimental standard (see §9). The main results of this study will be fully described as informative Annex B before the end of 2004. However, the method has been improved afterwards (addition of a second flotation) and the protocol proposed in the PR XP X 33-031 is not the same than the one tested during this interlaboratory trials study.

Some additional performances data are therefore needed and will be provided to AFNOR before the end of 2004 as intra-laboratory data in order to go on with the standardisation process. Correlation data between the internal structure observation for the evaluation of the nematodes viability and the larvae development and data justifying the addition of a second flotation step will be presented.

This draft experimental standard lacks as well validation data that would only be possible to obtain while organising a large-scale European round-robin exercise, including assessing countries and testing a large scale of European representative matrices.

2.2.2 Triple flotation method in a zinc sulphate solution (AFNOR XP X 33-017 experimental standard)

This method has been developed by the Faculty of Pharmacy of the University of Caen (France) and will be printed as French AFNOR experimental standard XP X 033-017 in April 2004.

An English version of the French experimental standard XP X 033-017 to be printed is presented in **Annex 3**. This English version, provided by the AFNOR in the frame of the comments process of this horizontal desk study in September 2004, is not exactly the same than the French experimental standard XP X 033-017. More particularly the informative annex A presenting the interlaboratory trials results in the French version is replaced by other performances results in this English version.

The method is based on three successive centrifugation-flotation steps while resuspending the sludge homogenate suspension in a zinc sulphate solution. After each flotation step, one cover glass is placed on the top of the centrifugation tube on the surface of the meniscus formed by the flotation solution, and observed on microscope to identify and to count the viable/non viable helminth eggs. The helminth eggs are enumerated in 3 subsamples of 0.5 g (dry weight).

The viability test is based on the activity of malate dehydrogenase in eggs by means of an oxydo-reduction transforming the soluble, weakly yellow coloured tetrazolium into insoluble blue formazan.

The method is presented as allowing the investigation of *Ascaris*, *Toxocara*, Trichurids and Taeniids eggs.

Performance data presented in the informative Annex A in the French AFNOR XP X33-031 experimental standard refers to interlaboratory trials results. Three different parts are presented.

The first one corresponds to a short experimental description of the protocol used for the implementation of 3 different interlaboratory trial studies. Two of the interlaboratory trials described involved 13 and 15 different laboratories who analysed 2 different stabilized sludge samples. The third one involved 18 laboratories who analysed 2 different sludge samples (the same sludge before and after liming). The two French methods proposed as French AFNOR experimental standard were compared during those 3 interlaboratory trials. This part lacks crucial information such as the date of the interlaboratory trials and the version of the protocol of the tested methods (at least a reference), the scale of the interlaboratory trials (only French laboratory involved?), the number of replicates analysed per sample. The interlaboratory trials performed in the frame of the ADEME study (ADEME, 2002, see §2.2.8) are what is more not presented.

The second part of this informative Annex A deals with repeatability data of the triple flotation method. Those data concern only Taeniids eggs enumeration without any specification on the viability evaluation, while the method is presented as enumerating viable helminth ova (nematodes and cestodes). The results seem what is more to be intralaboratory results on only 3 different samples (2 stabilized sludge and 1 lime stabilized sludge). No discussion and conclusion are however presented. Some additional information are so needed to explain the presented results and additional data on the other targeted eggs are therefore needed.

The third part of this informative Annex A presents results of the third interlaboratory trial involving 18 laboratories. As for repeatability data, the presented data concern only Taeniids eggs without any specification on the viability evaluation, while the targeted parameter of the method is helminth eggs (nematods and cestods). No results on the two other interlaboratory trials are however presented.

In addition to the lack of performance data available, and as for the AFNOR PR XP X 033-031 experimental standard, validation data on the viable helminth eggs investigated in this method are needed. Such validation data could only be obtained while organising a large-scale European round-robin exercise, including assessing countries and testing a large scale of European representative matrices.

2.2.3 Norwegian flotation in sucrose method

This method entitled “Isolation of helminth eggs from composted sludge” has been described by Bjørn Gjerde from the Norwegian School of Veterinary Science (Oslo, Norway); This method is mainly based on a method developed by cand.med.vet. Karl Bergstrøm during his research work at the Norwegian School of Veterinary Science from 1977 to 1979 on the occurrence of parasite eggs in sewage sludge. Bergstøm, for his part, modified a method published in 1977 by RJ Jørgensen as a stencil entitled

'Isolation of nematode eggs in soil and sediments. Principles and exercises' at the Royal Veterinary and Agricultural University, Copenhagen, Denmark.

The method is based on a flotation in a sucrose solution. The analysis is performed on 10 subsamples of 10 g of raw sample, the final result being expressed as the average number of eggs of the 10 subsamples.

This method has been described for composted sludge but is presented as being also used for isolation of helminth eggs from samples of raw sludge (using subsamples of 25 grams), dewatered sludge, and soil.

The viability is evaluated based on a structure observation while classifying eggs as dead (clearly damaged), potentially alive (unembryonated, with no visible defects), or alive (moving larvae inside). However, because unembryonated nematode eggs (usually ascarids eggs) found by this method in fresh samples of composted sludge were sometimes difficult or impossible to classify with regard to viability, a larva development test is then performed (analysis of another series of 10 subsamples of 10 g after an incubation at 27°C for 4 weeks). If unembryonated live eggs had been present in the fresh sample, live eggs containing larvae should now be found in the incubated sample.

No performance data have been collated on this method in the frame of this desk study.

2.2.4 Austrian flotation in a sugar solution method

This method has been identified thanks to the questionnaire sent in the frame of this horizontal desk study elaboration. The respondent who informed us about this method is one of the both Austrian experts identified by the Austrian Horizontal Steering Committee member.

The method, summarised in **Annex 6**, is based on a flotation in a sugar solution adapted by Hejny/Brandl (1995) from an originally method described by Horn *et al.* (1990). It allows the analysis of a subsample of 15 ml of wet sample or 50 ml of dry sand or soil.

This method does not appear to be in a standardisation process.

This method has been used for the helminth ova enumeration in soil/sand in general, but the respondent precised that they have never been able to detect Taeniid or any other cestodes eggs with it and never have evaluated the method for this purpose, but it should be applicable to cestodes. They predominantly found *Toxocara* spp. In both sludge and soil/sand with this method.

Viability can be evaluated by the presence of potentially infectious larvae of ascarids (or *Trichuris*), but not for other nematodes or cestodes. The target organisms of the respondent's laboratory were ascarids (especially *Toxocara*) which have been detected regularly with this method, and they then have been able to distinguish between infectious and not infectious larvae.

This method has been applied on sand and soil samples in the frame of research projects (10 studies in the last 10 years, 350-500 samples / study), and on sludge/slurry sporadically (on demand).

No performance data have been collated on this method in the frame of this desk study.

2.2.5 German flotation in a zinc sulfate solution method

This method has been identified thanks to the questionnaire sent in the frame of this horizontal desk study elaboration.

German respondent's method is based on a flotation in a zinc sulphate solution as described in Thienpont *et al.* (1979). This method is shortly summarised in **Annex 6** and does not appear to be in a standardisation process. The analysis is based on a sludge subsample of 5 g material (wet or dry weight not precised).

This method is presented as allowing the enumeration of both nematodes and cestodes (including *Taenia* sp.) without any viability evaluation.

This method has been mainly applied in the frame of investigations on the tenacity of *Ascaris* ova in biotechnological plants for the treatment of biological wastes, slurry and sewage sludge. The samples analysed were anaerobic treated sludge and anaerobic stabilized sludge.

This method uses the same flotation solution than the one used for the French AFNOR XP X 33-031 experimental standard. However, it seems that only one step flotation is used instead of three for the French XP X 33-031 method.

No performance data have been collated on this method in the frame of this desk study.

2.2.6 Hungarian double flotation in a calcium nitrate solution method

This method has been identified thanks to the questionnaire sent in the frame of this horizontal desk study elaboration.

The principle of this method is based on a double flotation in a calcium nitrate solution (sp.gr. 1.35) following an internal method (no literature reference). This method is shortly summarised in **Annex 6** and does not appear to be in a standardisation process. The subsample to be analysed is 100 g (no precision on wet or dry weight).

This method investigates specifically Nematodes (including *Ascaris* sp.) without any viability evaluation. However, the number of detected dead eggs based on the observation of the eggs structure is indicated.

The helminth eggs more often found are *Toxocara* spp. and *Ascaris* spp. in soil, sand and sludge.

The Hungarian respondent's laboratory performs 10-50 analysis per month according to this method on sewage sludge, soil and wastes (in 30% of the cases for each of those three matrices), and on sand (in 10% of the cases).

No performance data have been collated on this method in the frame of this desk study.

2.3 Comparison of identified standards, draft standards and/or into practice methods

Based on the (available) protocols, a comparison of the 6 methods described in §2.2 has been summarised in **Table 2**.

2.3.1 Enumeration of helminth eggs

Norwegian and French AFNOR PR XP X33-031 (modified US/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 modified US/EPA method, after the straining, the flotation step is followed by the diphasic step using alcohol/ethanol.

The size of the sieves seems questionable and restrictive for the Norwegian method due to the risks of losing big eggs such as *Toxocara* eggs which size can reach 95µm.

For the Norwegian method, in case of clogging, some eggs might be lost, which supports the use of the modified US/EPA method (160µm). Besides, the 38µm sieve might be too large and could lose smaller eggs such as *Taenia*, *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 proposed experimental standards (modified US/EPA PR XP X33-031 and Triple Flotation XP X33-017 methods) have been evaluated in the frame of interlaboratory trials at a French scale (ADEME, 2002).

Two successive interlaboratory trials involving 14 French laboratories have been organised in 2001. For each trial, each laboratory has to analyse 5 sludge samples randomly distributed from a set of six naturally contaminated sludge samples (3 non lime stabilized sewage sludge and 3 lime stabilized sewage sludge tested in each trial). In parallel to the interlaboratory trial, a homogeneity control was performed by the laboratory in charge of the preparation of the 6 batch to be tested.

The conclusion of this study was that the Triple Flotation method had a higher yield than the modified US/EPA method due to a higher yield for cestodes (*Taenia*). The US/EPA method (AFNOR PR XP X33-031) has been improved afterwards by adding a second step flotation, in order to increase the yield of the method.

However, it was concluded that the modified US/EPA method allowed to recover a higher number of viable eggs with a better accuracy than the triple flotation method.

On the other hand, laboratories questioned on the practicability of the methods, answered more favourably for the modified US/EPA method than for the Triple Flotation one. For the Triple Flotation method, the number of tested tubes should be higher to be more reliable and the reading time at the microscope is rather long.

Due to the sample size (10-250g vs 1.5 g dry weight respectively), the detection limits of the modified US/EPA and Norwegian methods are much lower than the detection limit of the TF method. For other described methods, it's difficult to compare due to the fact that it was not specified if the subsample weight was dry or wet weight.

Table 2 : Descriptive comparison of the 6 standards/methods identified

	French AFNOR PR XP X33-031 (modified US/EPA)	French AFNOR XP X33-017 (Triple flotation)	Norwegian method	Austrian method	German method	Hungarian method
Field of application	Sewage sludge Other Treated and Untreated Sludge	Sludge	Composted sludge Raw sludge, Dewatered sludge, soil	Soil, Sand, Sludge/slurry	Slurry, Sewage Sludge, Wastes	Sewage Sludge, Soil, Sand, Wastes
Target parameters	Helminths including nematodes and cestodes	Helminths including nematodes and cestodes (mainly Taeniids)	Helminths	Helminths including nematodes (not tested for cestodes)	Helminths including nematodes and cestodes	Helminths including nematodes
Sampling weight	10g (DW)	3 x 0.5g (DW)	10 x 10g (WW) (10 x 25g (WW) for raw sludge)	15 ml (W) 50 ml of dry sand or soil	5g (not specified)	100g (not specified)
Flotation solution	NaNO ₃	ZnSO ₄	Sucrose	Sugar	ZnSO ₄	CaNO ₃
Density (sp.gr)	1.30	1.38	-	-	-	1.35
Viability evaluation	- <u>Nematodes</u> : Structure eggs observation - <u>Cestodes</u> : Blue trypan exclusion	- MTT dye (blue)	- Structure eggs observation - Larvae development for nematodes (in some cases)	- <u>For ascarids and Trichuris only</u> : Detection of infectious larvae	-	(Number of dead eggs specified)

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

The comparison with the Austrian, German and Hungarian methods identified thanks to the questionnaire sent in the frame of this desk study is not feasible due to the fact that the protocols are not fully detailed (not required in the questionnaire in this first step).

2.3.2 Viability evaluation

Only the two French proposed experimental standards (modified US/EPA AFNOR PR XP X33-031 and Triple Flotation AFNOR XP X33-017) and the Norwegian method allow the enumeration of viable helminth eggs.

The modified US/EPA method uses two different techniques :

1) *For nematodes*, the detection of viable eggs is based on the structure observation. Nematodes eggs are considered as viable eggs when the integrity of their structures can be observed or if a larva (moving or not moving) is detected inside. However, any absence of internal structures or any disorganisation of the internal structures and/or any damage of the external shell (irregularity, deformation, broken shell, ...) are criteria attesting that the eggs are non viable eggs.

The results of a study comparing this viability approach with the larvae development approach will be added in the proposed AFNOR PR XP X33-031 experimental standard before the end of 2004.

2) *For cestodes*, the detection of viable eggs is based on the exclusion of the trypan blue dye by living cells. The viable eggs retain their initial colouration while dead eggs are blue stained.

For the Triple Flotation method, the viability test is based on the activity of malate dehydrogenase in eggs by means of an oxydo-reduction transforming the soluble, weakly yellow coloured tetrazolium (MTT) into insoluble blue formazan. Blue stained eggs are considered as viable, while dead eggs retain their initial brown coloration.

This coloration is presented as a good estimate of the infectivity for Taeniid eggs. However, the coloration is not reliable to other nematodes eggs than Trichurids.

This leads to the conclusion that the viability approach used in this standard allows the detection of viable eggs only for the Taeniids and the Trichurids, but not for the whole investigated helminth group.

For the Norwegian method, the viability is evaluated based on a structure observation while classifying eggs as dead (clearly damaged), potentially alive (unembryonated, with no visible defects), or alive (moving larvae inside).

A larva development is however performed for nematodes eggs due to the fact that unembryonated nematode eggs (usually ascarids eggs) found by this method in fresh samples of composted sludge were sometimes difficult or impossible to classify with regard to the viability approach based on the structure observation. If unembryonated

live eggs had been present in the fresh sample, live eggs containing larvae should now be found in the incubated sample.

The three Austrian, German and Hungarian methods identified thanks to the questionnaire do not allow the viability evaluation of the investigated helminth eggs. However, for the Austrian method, the viability can be evaluated by the detected of the presence of potentially infectious larvae of ascarids (especially *Toxocara* spp.) or *Trichuris*, but not for other nematodes or cestodes.

In the Hungarian method the number of detected dead eggs is specified in addition to the number of total nematodes helminth eggs.

3. EVALUATION OF DRAFTING A HORIZONTAL STANDARD

No European standard on viable helminth eggs exist.

At the European level, 6 methods have been identified :

- 7) A modified US/EPA method (1992) based on a double flotation in a sodium nitrate solution, under French standardisation process (AFNOR PR XP X33-031), see the protocol in **Annex 2**;
- 8) A Triple flotation in a Zinc sulphate solution method, to be printed as French experimental standard in April 2004 (AFNOR XP X33-017), see the protocol in **Annex 3**;
- 9) A Norwegian method based on a flotation in sucrose, see the protocol in **Annex 4**;
- 10) An Austrian method based on a flotation in a sugar solution, see the short summary protocol in **Annex 6**.
- 11) A German method based on a flotation in a zinc sulphate solution, see the short summary protocol in **Annex 6**.
- 12) An Hungarian method based on a double flotation in a calcium nitrate solution, see the summary protocol in **Annex 6**.

To date, no method to be used as draft horizontal standard is available.

Two French available experimental standards (AFNOR PR XP X33-031 and XP X33-017) exist but need to be further studied for their performance in terms of viability, organisms to be identified and fit for purpose to all sludge, soil and treated biowastes matrices. More particularly, they need to be validated at a European scale.

The four other identified European methods seems not to be in a standardisation process at a national level.

The Norwegian method allows the enumeration of viable helminth ova.

Three other methods from Austria, Germany and Hungary have been identified thanks to a (non exhaustive) questionnaire but do not evaluate viability. They all need to be fully described and further studied for their relevance to helminth eggs enumeration in all sludge, soil and biowastes matrices, and especially for eggs viability evaluation.

No method has been demonstrated to be capable of combining specific, quick and viability detection of all Helminth eggs. For viability, no fully and comprehensive characterisation of the techniques is available.

Further investigation are therefore needed before proposing a draft horizontal standard on viable helminth eggs enumeration, and more particularly performance data and validation data for fit for purpose to all sludges, soil and treated biowastes at a European level.

4. CRITICAL POINT AND RECOMMENDATIONS

Regarding the conclusion of § 3. Evaluation of drafting a horizontal standard, the following considerations need to be more clearly defined before drafting a horizontal standard on viable helminth ova in sludge, soil and biowastes :

- 1) European agreement on the more relevant helminth target to be monitored considering the risk in all the European Union countries (including assessing countries) : the whole viable Helminth group, or only viable Nematodes and especially *Ascaris* spp. and/or *Toxocara* spp. and/or only viable Cestodes and especially *Taenia* spp. , etc. ;
- 2) The European acceptable contamination level of those identified target helminth in all sludge, soil and biowastes matrices, and as a consequence, the detection limit required for the horizontal standard.

Such considerations need to be discussed during an European expert workshop.

Such a workshop on the viable helminth ova topic has been proposed in the FP6 proposal on “Horizontal Standards on Hygienic microbiological Parameters for Implementation of EU Directives on Sludges, Soils, Soil Improvers, Growing Media and Biowastes”. The questionnaire approach applied in the frame of this desk study is highly recommended as a preliminary step in order to well identify the European experts that could participate in such a workshop. The targeted expert would be identified in all the EU countries (including assessing countries) in Research laboratories but also in routine sludge analyses laboratories.

The conclusion of the workshop would be discussed in a critical review that would allow to identify and propose the most fit for purpose method(s) applicable to European sludge, soil and treated Biowastes. Those identified draft standards would be evaluated for their suitability in an intra-laboratory work involving different European representative helminth expert laboratories.

5. DRAFT STANDARD (CEN TEMPLATE)

As regard to the conclusion of the precedent chapters 3 and 4, no method to be used as a draft horizontal standard is available.

Two French available experimental standards (AFNOR PR XP X33-031 and XP X33-017) exist but need to be further studied for their performance in terms of viability, organisms to be identified and fit for purpose to all sludge, soil and treated biowastes matrices. More particularly, they need to be validated at a European scale.

The four other identified European methods (Norwegian, Austrian, German and Hungarian methods) seems not to be in a standardisation process at a national level and all need to be fully described and further studied for their relevance to helminth eggs enumeration in all sludge, soil and biowastes matrices, and especially for eggs viability evaluation.

Therefore, no draft standard can be proposed as CEN template in the frame of this desk study on viable Helminth ova enumeration in sludge, soil and treated biowastes.

It is however hoped that the work proposed in the FP6 proposal on “Horizontal Standards on Hygienic microbiological Parameters for Implementation of EU Directives on Sludges, Soils, Soil Improvers, Growing Media and Biowastes” would allow to identify and propose the most fit for purpose method(s) applicable to European sludge, soil and treated Biowastes, thanks to a critical review including a European expert workshop on the topic.

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ANNEX 1

Letter Seeking Information and Outlining the Horizontal Project

ALcontrol Laboratories
Templeborough House
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8th March 2003

Ref: L250-GA14424

Dear Sir/Madam,

Re: - Information needed for the Oslo meeting of TG5 27th March 2003 for the Horizontal Project

With respect to the project “HORIZONTAL”, we are presently carrying out desk studies in order to gather the relevant information on existing data concerning microbiological parameters in sludge, soil and biowaste. You will find below in the appended Annex some information about the project and the specific objectives of Work Package 3: “Hygienic parameters”.

Please could you kindly provide information on any or all of the following: -

1. Established validated national or international standards methods for *E. coli*, *Salmonella* or *Clostridium perfringens* in sludge, soil, compost and biowaste
2. Established validated national or international standards methods for *E. coli*, *Salmonella* or *Clostridium perfringens* in water/wastewater
3. Any methods for helminth ova (nematodes and cestodes) in waters, wastewaters, sludge, soil, compost or biowaste
4. Any established or novel rapid method (significantly more rapid than the relevant culture method) for *E. coli* or *Salmonella*
5. Any local or national study on the typical levels of pathogens or indicators in sewage sludge, soil compost or biowaste.
6. Laboratories (including appropriate contact) to take part in interlaboratory trials to validate the proposed *E. coli* and *Salmonella* standards for sludge, soil, compost and treated biowaste.

We would appreciate your E-mail response to the undersigned by Monday 24th March 2003, so that it can be discussed at our Oslo meeting of CEN/TC308/WG1 that will take place from March 26th to 28th.

Thanking you in anticipation

Yours faithfully

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ANNEX 2

AFNOR PR XP X 33-031 – Characterisation of sludge - Enumeration of viable helminth eggs in sludges - Double flotation method in a natrium nitrate solution

English Version to the French AFNOR PR XP X 33-031 proposed as experimental standard.

Introduction

Helminth (nematodes and cestodes) are intestinal parasites, infecting human and animals. In the simplest cycle, a parasite stage from human is immediately infective for other humans. In other infections such as ascariasis or trichuriasis, a maturation period outside the body is required before the parasite is infective. However, for many parasite infections, a second or even or third host is required for completion of the life cycle.

The infective stages are usually within eggs, which are excreted in fresh faeces and may survive for weeks or months in the environment.

Sludges, soils, soil improvers, growing media and biowastes can therefore contain pathogenic helminth eggs. The use of such pathogen-contaminated materials in agriculture can cause outbreaks of infection due to the production of contaminated food or animal feedstocks and may also be transmitted to wild animals, consequently, there is a need to monitor rates to land.

Examination for helminth eggs should only be carried out in laboratories competent for carrying out work involving pathogens.

1 Scope

This standard specifies a method for the enumeration of viable pathogenic eggs (nematodes and cestodes) in sewage sludge and other treated and untreated sludge.

2 Normative References

This document incorporates by dated and undated references, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For subsequent amendments to or revisions of any of these publications apply to this document only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

- NF EN ISO 3696 – *Laboratory water for analytical use - Specification and methods.*

- NF EN 12880 : 2000 - *Characterisation of Sludges - Determination of dry residue and water content.*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply :

- Parasites Helminth presenting a potential risk (non exhaustive list) :

The Helminth eggs more particularly targeted in Europe for the sludges monitoring, are the following :

- Nematodes eggs :
 - as Trichuridae (ex : *Trichuris trichura*, *Capillaria aerophila*,...)
 - as Ascarididae (ex : *Ascaris lumbricoides*, *Toxocara canis*,...)
- Cestodes eggs :
 - as Taeniidae (ex : *Taenia solium*, *Taenia saginata*,...)
 - as Hymenolepididae (ex : *Hymenolepis nana*, *Hymenolepis diminuta*,...)

4 Principle

4.1 Detection and enumeration of parasites helminth eggs

N.B : This method was developed to obtain an optimal recovery of both nematodes and cestodes.

The enumeration of helminth eggs requires five successive steps :

- Parasites dissociation from organic matter,
- Flotation using a sodium nitrate solution,
- Sedimentation,
- Concentration,
- Microscopic detection and enumeration (before and after staining).

4.2 Viability of *Taeniidae* and *Hymenolepididae* eggs (Cestodes)

The method used to detect the viability of cestodes eggs is based on the exclusion of the trypan blue dye by living cells. The viable eggs retain their initial colour while dead eggs are blue stained.

4.3 Viability of Nematodes eggs

The method used to detect the viability of nematodes eggs is based on the observation of the eggs structure. Nematodes eggs are considered as viable eggs when the integrity of their structures can be observed (see Table in Annex A) or if a larva (moving or not moving) is detected inside. However, any absence of internal structures or any disorganisation of the internal structures are criteria attesting that the eggs are non viable eggs.

5 Reagents

Reagents of recognised analytical grade and water of quality 3 as required in EN ISO 3696 have to be used.

5.1 Sodium nitrate (NaNO_3) solution, sp.gr 1.30, pH 7.0

Dissoudre 615 g de NaNO_3 dans 1 litre d'eau à l'aide d'un agitateur magnétique. Vérifier la masse volumique (1,30) à l'aide d'un densimètre. Laisser reposer une nuit et vérifier le pH à l'aide d'un pH mètre étalonné et l'ajuster si nécessaire à $\text{pH}=7 \pm 0,2$.

Stabilité : 6 mois à température ambiante.

Dissolve 615g of NaNO_3 in 1 L of distilled water using magnetic stirring. Check the specific gravity to (sp.gr $1.30 \pm 0,1$). Allow to settle overnight and adjust the pH to $7 \pm 0,2$ if necessary.

5.2 Sulfuric acid (H_2SO_4) solution, 0.1N

Dilute 3mL of H_2SO_4 95% ($D_{4}^{20}=1,83$) in 997 mL of distilled water.

Stable 1 year at room temperature in the dark.

5.3 Alcohol-Acid solution, 33 %

Prepare 1 litre of the solution while mixing 650 mL of H_2SO_4 - 0,1N solution with 350 mL of ethanol solution 95°.

Stable 6 months at room temperature in the dark.

Attention : This reagent is volatile.

5.4 Stabilized di-ethyl ether ($\text{C}_4\text{H}_{10}\text{O}$) solution

For example ACROS ORGANICS – Ether, stabilized c.p.* or equivalent.

Attention : This reagent is volatile.

5.5 Sodium hypochlorite (NaOH) solution, 0.5 % (p/v)

Dilute 2 mL of sodium hypochlorite 12° solution in 13 mL of distilled water.

Unstable solution, to be prepared just before use.

5.6 Sodium chlorure (NaCl) solution, 1M

Dissolve 58,5 g of NaCl in 1 litre of distilled water.

Stable 1 year at room temperature.

5.7 Trypan blue solution, 0,4 %

Dissolve 0,4 g of Trypan blue in 100 mL of distilled water. Stable 1 year at room temperature.

6 Apparatus

Usual microbiological laboratory equipment, and in particular :

- 6.1 Homogeniser** (e.g. Stomacher®, Seward Laboratories or equivalent).
- 6.2 Sterile homogeniser bags** (e.g. Stomacher®, Seward Laboratories or equivalent), 300 mm x 150 mm.
- 6.3 Centrifuge with non mobile rotor adapted for 500 ml to 1 litre conical or bottom round flasks.**
- 6.4 Centrifuge with mobile rotor adapted for 50ml conical tubes.**
- 6.5 Conical or bottom round centrifuge flaks**, 500ml to 1 litre volume, polypropylene plastic.
- 6.6 Conical centrifuge tubes**, 50ml volume.
- 6.7 160 µm mesh metallic sieve.**
- 6.8 Plastic Beaker** of 5 litre volume.
- 6.9 Hydrometer**, capable to measure 1,3 g/mL.
- 6.10 Vacuum pump.**
- 6.11 Rotary mixer for 500ml to 1 litre flasks.**
- 6.12 Vortex mixer** suitable for tubes.
- 6.13 Sedgewick-Rafter cell for microscope examination.**
- 6.14 Microscope with x10 and x20 magnification.**

7 Sample preparation

Determine the dry residue of the sample using the method described in EN 12880 : 2000 (2) prior to the microbiological analysis.

8 Procedure

8.1 Sample preparation

For liquid sludge (dry residue < 10%), pour a sample portion corresponding to 10 g dry weight into a centrifuge flask (500 ml to 1 litre, 6.5) and centrifuge at 3 000 g

(29400m.s^{-2}) for 15 minutes (6.3). The sediment is then treated with the flotation solution (see 8.3).

For solid and thick sludges (dry residue > 10%), weigh a representative 10 g dry weight of the as received sludge into a homogeniser bag (6.2). Proceed as described in 8.2.

8.2 Dissociation of parasites from organic matter (sludges with dry residue>10%)

- a) Add 200 ml of tap water and homogenise for 90 seconds in a homogeniser (6.1) in order to obtain a homogeneous suspension. If the sample contains large debris (wood or stone), use a manual homogenisation with a glass rod in order not to break the homogeniser bag.
- b) The resulting mixture is then poured through a 160 μm diameter sieve (6.7) held on a large 5 L beaker (6.8). Thoroughly rinse the blender bag with tap water (around 500 ml) and pour the resulting washing fluid through the sieve (6.7), and flush it in the beaker (6.8)
- c) Wash the material retained in the sieve with around 4 litre of tap water while carefully mixing it with a spatula. The washing water is then poured into the 5 L beaker (6.8). Discard the remaining material embedded on the sieve
- d) Allow the washed poured sample to settle in the 5 L beaker (6.8) at room temperature (for at least 3 hours) while covering the beaker with an aluminium foil.
- e) Siphon off the supernatant while just leaving few millimetres of fluid above the sediment layer, while using a vacuum pump (6.10). The obtained sediment is usually of around 300 ml volume (maximum 500 ml). Mix the settled material by swirling and then pour off the sediment in a centrifuge flask (6.5).
- f) Centrifuge at minimum 800 g (7900 m.s^{-2}) with a centrifuge (6.3) for 5 min.
- g) Siphon off the supernatant. Treat the sediment (around 50 ml to 100 ml) with the flotation solution (see 8.3).

8.3 Flotation

- a) Resuspend the sediment thoroughly in 200 ml of sodium nitrate (NaNO_3) solution sp.gr. 1.30 (5.1) or in 250 ml if the volume of the sediment is important. Homogenise for 5 min with a rotary mixer (6.11).
- b) Centrifuge (6.3) for 3 min at 800 g (7900 m.s^{-2}) (for solid sludges) or at 2000g (19600 m.s^{-2}) (for liquid sludges).
- c) Carefully pour the sodium nitrate supernatant into the same 5 L beaker (6.8) than the one used in 8.2. Keep the sediment in the centrifuge flask (6.5).

- d) Resuspend the remaining sediment in 200 ml of sodium nitrate (NaNO_3) solution sp.gr. 1.30 (5.1) or in 250 ml if the volume of the sediment is important. Homogenise for 5 min with a rotary mixer (6.11) (second flotation step).
- e) Centrifuge (6.3) for 3 min at 800 g (7900 m.s^{-2}) (for solid sludges) or at 2000g (19600 m.s^{-2}) (for liquid sludges).
- f) Carefully pour the sodium nitrate supernatant into the same 5 L beaker (6.8) than the one used c).

8.4 Sedimentation

- a) Dilute the sodium nitrate (NaNO_3) supernatant obtained in the 5L beaker in 8.3 with tap water in order to decrease the specific gravity from 1.30 to around 1.00 : add around 5 L of tap water to the volume of sodium nitrate supernatant. Cover the beaker and allow to settle for 3 h.
- b) Siphon off carefully the supernatant while leaving few millimetres of fluid above the settled material.
- c) Resuspend the sediment obtained in the 5L beaker (6.8) by swirling, and pour into one 50 ml conical centrifuge tube (6.6). Centrifuge (6.4) the tube at 660 g (6500 m.s^{-2}) for 3 min. Discard the supernatant and keep the sediment.

Repeat the 8.4c) step until the whole sediment obtained in the 5 L beaker (6.8) could be concentrated in the conical centrifugation tube (6.6).

- d) Rinse the 5 L beaker (6.8) with distilled water. Pour the washing liquid in the 50 ml conical centrifuge tube (6.6) containing the centrifuged sediment. Centrifuge (6.4) 3 min at 660 g (6500 m.s^{-2}).
 - If the volume of the sediment $\leq 0,25$ ml, resuspend it with around 1 ml of H_2SO_4 solution (5.2), put the whole volume in a Sedgewick-Rafter cell (6.13) and proceed with the microscope examination (8.6).
 - If the volume of the sediment $> 0,25$ ml, proceed to a concentration step (8.5).

8.5 Concentration

- a) Resuspend the sediment in 15 ml of acid alcohol solution 33% (5.3) and homogenise with a vortex mixer (6.12).
- b) Add 10 ml of ethyl ether solution (5.4), close the capped centrifuge tube (6.6) and homogenise with a vortex mixer (6.12). Allow the gas to let off while lightly opening the cap.
- c) Centrifuge (6.4) the tube at 660 g (6500 m.s^{-2}) for 3 min.

- d) Siphon off the supernatant while leaving few millimetres of fluid above the sediment layer and keep the sediment.
- e) If too numerous debris are observed between the organic and the aqueous phases, proceed another time with 8.5a), 8.5b), 8.5c), 8.5d).
- f) In order to eliminate the remaining ethyl ether solution, resuspend the sediment in 10ml of H₂SO₄ solution 0.1 N (5.2).
- g) Centrifuge (6.4) at 660 g (6500 m.s⁻²) for 3 min.
- h) Siphon off the supernatant while leaving few millimetres of fluid above the sediment layer and add H₂SO₄ solution (5.2) to the sediment in the centrifuge tube to obtain a final volume of around 1 ml-1,5 ml.
- i) If the microscope examination is not possible immediately, it's possible to store the sediment in the H₂SO₄ solution (5.2) for several days at room temperature.
- j) Distribute the whole sediment volume in one to two Sedgewick-Rafter cell(s) (6.13) (around 1-1,5 ml per cell) with an automatic pipette, while well homogenising the sediment in the cell.
- k) Proceed to the microscope examination of the whole surface of the Sedgewick-Rafter cell(s) (6.13) (8.6).

If the volume of the sediment exceeds 2 ml or if the sediment is too dense (more than 2 Sedgewick-Rafter cells to examine), proceed another time with the flotation step (8.3) but add 30 ml of natrium nitrate (NaNO₃) solution sp.gr. 1.30 instead of 200 ml (8.3 a). Continue with the sedimentation step (8.4) while diluting the supernatant in order to decrease the specific gravity from 1.30 to around 1.00 while adding around 1 litre of water instead of 5 litre (8.4a).

8.6 Enumeration of viable helminth eggs.

8.6.1 Enumeration of viable and non viable helminth eggs

Proceed to the microscope examination of the whole surface of the Sedgewick-Rafter cell(s) containing the sediment sample (vertical or longitudinal scanning) on a microscope with x10 or x20 magnification (6.14). The identification of the helminth eggs depends on the size and on the morphology of the observed parasite eggs, as described in the table in Annexe A.

This first examination allows the enumeration of the nematodes including non viable and viable eggs (see 8.6.2) together with the enumeration of the cestodes eggs without any viability evaluation. If cestodes eggs are detected, proceed with the viability step as described in 8.6.3.

8.6.2 Evaluation of the viability of nematodes eggs

The enumeration of the viable nematodes is performed in the same time that the one for the enumeration of viable and non viable helminth eggs (see 8.6.1).

The method used to detect the viability of nematodes eggs is based on the observation of the eggs structure. Nematodes eggs are considered as viable eggs when the integrity of their structures can be observed (see Table in Annex A) or if a larva (moving or not moving) is detected inside. However, any absence of internal structures or any disorganisation of the internal structures and/or any damage of the external shell (irregularity, deformation, broken shell, ...) are criteria attesting that the eggs are non viable eggs.

8.6.3 Evaluation of the viability of cestodes eggs

- a) After the enumeration of viable and non viable helminth eggs (8.6.1), pour the sediment from the Sedwick-rafter cell(s) (6.13) into the same 50 mL conical centrifuge tube (6.6) than the one from 8.4.d or 8.5.h.. Rinse the Sedgwick-Rafter cell(s) with 10 ml of distilled water and pour off the washing water in the centrifuge tube, in order not to loose any egg.
- b) Centrifuge (6.4) the suspension in the centrifuge tube at 660 g (6500 m.s^{-2}) for 5 min.
- c) Siphon off carefully the supernatant. Resuspend the sediment in 1 ml of Natrium hypochlorite NaOH solution 0.5 % (5.5) and mix during 1 min with a vortex mixer (6.12).
- d) Add 5 ml of natrium chloride (NaCl) solution 1 M (5.6) and mix with a vortex mixer (6.12). Centrifuge (6.4) at 660 g (6500 m.s^{-2}) for 5 min.
- e) Siphon off carefully the supernatant with a vacuum pump (6.10). Rinse the sediment with 5 ml of natrium chlorite (NaCl) solution 1M (5.6) and mix with a vortex mixer (6.12). Centrifuge (6.4) at 660 g (6500 m.s^{-2}) for 5 min. repeat this step 8.6.3.e) another time.
- f) Discard carefully the supernatant with an automatic pipette while leaving around 1 mL (sediment + remaining supernatant). Resuspend the sediment with the remaining supernatant.
- g) Add 100 μl of Trypan Blue solution 0,4% (5.7) and allow to react 10 min at room temperature. Proceed to a second microscope examination to enumerate viable cestodes eggs (as in 8.6.1), based on the exclusion of the trypan blue dye by living cells : the colour of the viable eggs does not change while dead eggs are blue stained.

9 Expression of results

9.1 Viable and non viable helminth eggs

The number of viable and non viable helminth eggs corresponds to the total number of nematodes and cestodes eggs observed and identified during the first microscope examination (8.61).

Report the result as the number of helminth eggs in 10 g dry weight.

9.2 Viabl helminth eggs

The number of viable helminth eggs corresponds to the total number of viable cestodes eggs (8.6.3) and the number of viable nematodes eggs (8.6.2).

Report the result as the number of viable helminth eggs in 10 g dry weight.

10 Performance data

An Interlaboratory trial performed with 13 to 18 French laboratories in 2001, allows to evaluate the repeatability and the reproducibility of this method in comparison with the Triple flotation mehtod described in the AFNOR XP X 33-017 experimental standard (see ADEME, 2002).

The conclusion of this interlaboratory trial is that the Triple Flotation AFNOR XP X 33-017 method allows to detect more Taenidae eggs than the method described in this document (adapted from Gaspard and schwartzbrod (1993), which is a modified US EPA method (1992)). No significant difference was observed with the other helminth eggs. However, the method described in this document appeared more efficient for the viable helminth eggs enumeration, in the experimental conditions used. “The uncertainty of the viability rate produced by the triple flotation method is significantly higher than the one observed with the modified EPA method (...). What is more, it seems that the modified EPA method provide a result higher than the one provided by the Triple flotation method (...). The modified EPA method allow to find a higher number of parasites helminth eggs with a better accuracy for a given tested sample.”

During this study, the modified US EPA method was based on a single Natrium nitrate (NaNO_3) flotation. It was decided to add a second flotation step in the method described in this standard, in order to improve the number of parasites helminth eggs enumerated (see Annexe B to be added).

11 References

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Annexe A
(Normative)

Characterization of helminth eggs

	Eggs	Size (µm) length / width	Form	Shell	Internal structure	Colour
NEMATODES	<i>Ascaris</i>	60 / 40	Ellipsoidal, Symmetric	External: hillocky-mammillated layer Internal: smooth and thick	Only 1 central germinative cellule	Brown
	<i>Toxocara</i>	75-85 / 65-75	Spherical	External : smooth and thick Internal: granule (golf ball like)	1 central germinative cellule	Brown
	<i>Trichuris</i>	50-55 / 20-25	Oval, small citrus aspect	Brown thick shell with clear polar plugs	1 rounded cellule with a granule aspect	Yellow-brown
	<i>Capillaria</i>	51-68 / 30-35	Ovoid	Thick shell with stria breadthwise and polar plugs	1 rounded cellule with a granule aspect	Brown
CESTODES	<i>Taenia</i>	35-40 / 30-35	Rounded - ellipsoidal	External: slim, colourless, albumin like, shell rarely present, Internal : radially striated, thick, which delimit the embryonated oncosphere (30µm)	Six hooked oncosphere – Hexacanth embryo	Brown
	<i>Hymenolepis</i>	<i>nana</i> : 40-50 / 30-40	Rounded - ellipsoidal	External: slim, smooth, vitreous Internal : presence of polar filaments	Oncosphere with six hooks – Hexacanth embryo	Transparent
		<i>diminuta</i> : 60-80 / 70-85		No polar filaments		Yellowish

ANNEX 3

AFNOR XP X 33-017 – Characterisation of sludge - Enumeration and viability of parasites helminth eggs : Triple flotation method

Revised version 9th October 2003

Highly similar Version to the French AFNOR XP X 33-017 experimental standard to be printed in April 2004.

1 Helminth eggs investigated: Ascaris, Toxocara, Trichurids and Taeniids

2 Eggs identification : (not in the AFNOR proposition of norm)

Identification of eggs based on measures and morphometry of eggs (see key in Cabaret et al, 2002) and an Atlas available on the web for identification of eggs and false parasites to come.

3 Aim of the technique

Identify, count and evaluate indirectly viability of the helminth eggs above mentioned. The method is based on three successive centrifugation-flotation steps while resuspending the sludge homogenate suspension in a zinc sulphate solution. After each flotation step, one cover glass is placed on the top of the centrifugation tube on the surface of the meniscus formed by the flotation solution, and observed on microscope to identify and to count the viable/non viable helminth eggs. The viability test is based on the activity of malate dehydrogenase in eggs by means of an oxydo-reduction transforming the soluble, weakly yellow coloured tetrazolium into insoluble blue formazan.

3 Apparatus

The following apparatus shall be used:

- a) Centrifuge with rotor adapted for tubes described below (mobile pots).
- b) 20mm diameter and 11 ml conical glass centrifuge tubes.
- c) 40 ml bottom round plastic centrifuge tubes.
- d) Glass slides.
- e) 20 mm x 20 mm cover glass.
- f) Microscope with x 10 and x 40 magnification.
- g) 90 mm or 100 mm diameter Petri dish
- h) Absorbent paper.

4 Reagents

4.1 Zinc sulphate $ZnSO_4 \cdot 7H_2O$, solution with volumic mass 1.38 g/ml

Dissolve 727.5 g of $ZnSO_4$ in 1 L of water, using magnetic stirring. Solution is stable at 22°C +/- 5 °C.

4.2 Malic acid solution, 50 g/L (solution A)

Dissolve 2.5 g of malic acid in 50 ml of water pH and adjust to 7.0,2. Neutralise with a sodium hydroxide solution and then adjust to pH 8 with a diluted sodium hydroxide solution (0.2 mole/L). Solution stable at (22+/- 5) °C.

4.3 Beta-nicotinamide adenine dinucleotide solution, 25 g/L (solution B)

Dissolve 0.025 g of β -nicotinamide adenine dinucleotide in 1 mL of water. Stable one month at 5+/- 3 °C in the dark.

4.4 Magnesium chloride solution, 48 g/L (solution C)

Dissolve 0.048 g of magnesium chloride in 1 ml of water. Stable one month at 5+/- 3 °C.

4.5 (3- 4,4-dimethylthiazol-2-yl -2,5-diphenyltetrazolium) bromide solution, 6g/L (solution D).

Dissolve 0.006g of (3- 4,4-dimethylthiazol-2-yl -2,5-diphenyltetrazolium) bromide in 1 mL of water. Stable one month at 5+/- 3 °C in the dark

4.6 Phenazine methosulphate solution, 5 g/l (solution E)

Dissolve 0.005g of phenazine methosulphate in 1 mL of water. Stable one month at 5+/- 3 °C in the dark

4.7 Oxydoreduction solution

Prepare 30 microL of oxydoreduction solution while adding 6 microL of each solution A, B,C,D and E.

5 Sample preparation

Dry weight measurement.

6 Procedure

For solid sludge samples, pour a sample portion corresponding to 0.5 g (dry weight) into a 40 mL bottom round plastic centrifuge tube.

For liquid sludge samples, pour a sample portion corresponding to 0.5 g (dry weight) of sample into a 40 mL bottom round plastic centrifuge tube, and centrifuge at 700G for 5 minutes. The obtained sediment corresponds to the analytical aliquot.

6.1 Suspension preparation

Homogenise very carefully the analytical aliquot in a 40 mL bottom round plastic centrifuge tube, using a glass agitator or a Pasteur pipette regarding the censity, while adding successive volumes of a total volume of 22 mL of zinc sulphate solution.

Immediately separate the homogenate suspension into two 11 mL conical glass centrifuge tubes.

6.2 Determination

First flotation

Place both 11 mL conical glass centrifuge tubes in the centrifuge.

a) Helminth eggs enumeration without any viability evaluation

Centrifuge at 180G for 3 minutes at room temperature. Fill each centrifuge tube with zinc sulphate solution to obtain a meniscus on the top of which a 20 mm x 20 mm cover glass is placed.

Wait 3 minutes, carefully take out cover glasses and put them on a glass slide for immediate microscope examination. Proceed to the second flotation.

b) Helminth eggs enumeration with viability evaluation

Centrifuge at 180 g for 3 minutes at room temperature. Fill each centrifuge tube with zinc sulfate solution in order to obtain a meniscus on the top of which a 20 mm x 20 mm cover glass is placed.

Wait 3 minutes and carefully take out cover glasses. Return them and add 15 microL of oxydoreduction solution. Homogenise with a buttoned Pasteur pipette in order to distribute the solution on the surface of the cover glass and put them on glass slides for microscope examination (glass slide 1). Proceed to the second flotation.

Second flotation

Immediately after the first flotation, rehomogenise both centrifuge tubes with a Pasteur pipette by up and down movements.

a) Helminth eggs enumeration without any viability evaluation

Proceed as described for the first flotation. The glass slide 2 is so obtained. Proceed to the third flotation.

b) Helminth eggs enumeration with viability evaluation

Proceed as described for the first flotation. The glass slide 2 is so obtained. Proceed to the third flotation.

Third flotation

Repeat the previous protocol. The glass slide 3 is so obtained.

6.3 Enumeration and viability of helminth eggs.

a) Helminth eggs without any viability evaluation.

Proceed to the microscope examination of the whole surface of each cover glass at low magnification (x 10) in order to detect and enumerate helminth eggs.

If the examination under a microscope is not immediately performed, prepared slides must be stored (maximum 30 minutes) in a wet atmosphere (For example in a Petri dish with a saturated water absorbent paper inside) in order to avoid the crystallization of the zinc sulfate.

b) Helminth eggs enumeration with viability evaluation.

Proceed to the examination under a microscope of the whole surface of each cover glass at low magnification (x 10) in order to detect and enumerate helminth eggs.

If the examination under a microscope is not immediately performed, prepared slides must be stored (maximum 30 minutes) in a wet atmosphere (For example in a Petri dish with a saturated water absorbent paper inside) at 39 °C in order that the enzymatic reaction could occur.

Put the three glass slides into Petri dishes at 39°C at constant humidity during 5h30min +/- 30min.

Observe again helminth eggs and detect the coloration : blue stained eggs are considered as viable, while dead eggs retain their initial brown coloration. This coloration is a good estimate of infectivity for Taeniid eggs; coloration is not reliable in other nematode eggs then Trichurids.

7 Expression of results

a) Helminth eggs

Add helminth eggs recovered at each flotation, identify them and report the result in number of viable helminth eggs in x g dry weight.

At least 3 analytical aliquots per sample are necessary for the expression of results.

b) Viable helminth eggs

Report the number of eggs which present a blue stain coloration. These eggs are considered as viable, while dead eggs keep their initial brown coloration.

Report the result in number of viable helminth eggs in x g dry weight.

At least 3 analytical aliquots per sample are necessary for the expression of results.

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Annex 1 to XP X 33-017

A triple flotation technique for evaluating helminth contamination of urban sewage sludge: from danger to risk.

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1) Is examination of 1.5 g of dry matter enough with TF technique?

Table 1: Quantity of sludge processed and helminth eggs recovered (in 1.5 g) in sewage sludge (126 samples examined from liquid-1 site, paste-2 sites, or pastesewage sludge treated by lime-1 site)

Number of triple floatations	Ascaris	Toxocara	Trichuris	Taenia
3 (1.5 g)	0.12* (0.33)** 88***	0.48 (0.63) 59.5 ^a	1.60 (1.81) 28.6 ^a	6.00 (5.04) 9.5 ^a
6 (3 g)	0.12 (0.26) 81	0.55 (0.57) 40.5 ^a	1.36 (1.34) 11.9 ^b	6.30 (5.61) 2.4 ^b
8 (4 g)	0.09 (0.20) 81	0.61 (0.53) 23.8 ^b	1.39 (1.09) 9.5 ^b	6.32 (5.64) 2.4 ^b

*arithmetic average, ** standard-deviation, *** percentage of negative results

Statistical significant differences (P<0.05) indicated by different superscripts

No significant differences on means and percentages with 3, 6, and 8 samples per sewage sludge for all four groups of parasites;

Significant differences in percentages of negative results using Mantel-Haenszel test : Taenia (3> 6 and 8, p=0.03), Trichuris (3> 6 or 8, p=0.03, 3> 6 and 8, p=0.0001), Toxocara (3>8 p=0.002).

Examination of 1.5 g dry-matter yield similar results to those obtained with processing a larger amount of sludge

2) is the classical TF technique as good as US EPA ?

Table 2 : Helminth eggs recovered using triple flotation, US EPA, and EPA modified (d=1.30 and d=1.38)

Technique Number of triple floatations	Ascarids	Toxocara	Trichurids	Taenids
Triple flotation n=16	0.13* (0.34)** 88***	0.25 (0.45) 75	0.69 (0.87) 56	3.81 ^a (3.58) 44 ^a
US EPA n=14	0 (-) 100	1.01 (1.27) 43	1.57 (2.1) 50	0 ^b (-) 100 ^b
EPA d=1.30 n=13	0 (-) 100	0.38 (0.87) 77	1.38 (1.76) 54	0.62 ^b (1.04) 69 ^a
EPA d=1.38 n=11	0 (-) 100	0.45 (1.04) 82	2.27 (3.00) 36	0.55 ^b (1.04) 73 ^a

*arithmetic average, ** standard-deviation, *** percentage of negative results
 Statistical significant differences (P<0.05) indicated by different superscripts

TF yielded strikingly more Taenia eggs and was as good as other technique for nematodes.

3) How long can we store the samples when using TF technique

Table 3: Preservation at 4-6°C of sewage sludge and triple floatation technique recovery of total eggs and viable eggs as assessed from MTT and PMS blue coloration (4sites of sewage sludge).

Preservation (days at 4- 6°C) before use of triple floatation	Trichurids	Viable Trichurids	Taenia	Viable Taenia
Day 0 n=13	2.77* (1.4-4.7)**	1.1 (0.2-1.9)	8.1 (5.5-10.6)	2.15 (1.2-3.0) ^a
Day 15 n=13	1.5 (1.0-2.2)	0.5 (0.2-0.9)	8.9 (5.5-11.3)	1.10 (0.5-1.6) ^a
Day 30 n=13	1.2 (0.5-1.7)	0.2 (0-0.5)	7.2 (4.4-10.1)	0.5 (0.2-0.9) ^b
Day 45 n=9	1.8 (0.8-2.8)	0.2 (0-0.4)	5.2 (2.8-8.0)	0.3 (0-0.7) ^b
Day 60 n=7	0.86(0.3-1.7)	0.3 (0-0.6)	4.1 (2.1-6.1)	0 ^b

* arithmetic average and ** confidence interval at p=0.95 using 500 bootstraps

Up to 15 days for all cases.

4) Are the eggs reliably recognised on morphometry ?

Table 4 Helminth eggs morphometry in seven types of sludge

Egg	Ascarids	Toxocara	Trichurids	Taenids
Width (microns)	50* (8)**	71 (7)	29 (5)	31 (1)
	35-69***	57-77	23-49	28-41
	24****	7	140	29
Length (microns)	63 (7)	82 (7)	58 (9)	36 (2)
	56-80	68-96	44-84	30-43
	24	68	140	456

*arithmetic average, ** standard-deviation , *** range, **** number of eggs

The eggs have different size and characteristic in sludge and a key to assist individual identification is in construction.

5) Relationship between modified MTT dye, viability and infectivity ?

Table 5 : Relationship between viable *Taenia saginata* eggs and actual infection of young cattle (essay undertaken at INRA Nouzilly, unpublished data, available at AFNOR)

The eggs come from stools and were stored at 7 °C for 5 months prior use.They were given once. The difference in viability is due to the fact that the 3000 egg group was at room temperature (19°C) and the 3022 egg group was maintained for 5 days at 30°C in anaerobic conditions. The larvae were counted at

necropsy, performed 60 days after infection. Masseters, tongue, diaphragm, and heart were carefully examined.

Eggs given/ 4 months old cattle (no of cattle)	Viability using MTT dye (%)	Taenia larvae (range)	Viable Taenia larvae (range)
0 (4) Negative control	-	0	0
3000 (4) room temperature	a 47	a 1 (0-2)	a 0,75 (0-2)
3022 (6) 5 days at	b 12	a 0,5 (0-2)	b 0,17 (0-1)

The different letters (a, b) indicates according to the column significant differences using the Mantel-Haenszel test at p=0.08

Conclusions

- 1) cattle used was uninfected previously
- 2) the "fresh" eggs are 3.9 more viables, and they are transformed after ingestion into larvae, with an efficacy 4.4 times increased compared to the eggs stored anaerobically at 30 °C.

The MTT dye appeared as a good indicator of infectivity

GENERAL CONCLUSIONS BASED ON THE PRESENTED RESULTS

TF is as efficient as modified US EPA for nematode eggs, and much better for Taenids. It has been tested in a wide range of sludge type (and even composted sludge, data not presented here). TF is associated with a viability technique based on a modified MTT dye which seems to correspond to infectivity in cattle for Taenia.

Annex 2 to XP X 33-017

Main recent references on TF method in sludge and related subjects by INRA in Nouzilly -F., Faculty of Pharmaco of Caen-F and by the Institute of tropical Medicine in Antwerpen B.

-Cabaret J., Geerts S., Madeline M., Ballandonne C., Barbier D. The use of urban sludge on pastures: the cysticercosis threat. *Veterinary Research*, 2002, 33, 575-598.

-Barbier D., Perrine D., Duhamel C., Doublet R., Georges P. Parasitic hazard with sewage sludge applied to land. *Applied and Environmental Microbiology*, 1990, 56, 1420-1422.

-Madeline M, Ballandonne C., Guyon R., Malas J.P., Vilagines P., Cabaret J., Barbier D. Mise en évidence de la viabilité des œufs de taenidés dans les boues résiduaires urbaines. *Journal Européen d'Hydrologie*, 2001, 31, 85-90

-Madeline M., Ballandonne C. , Vilagines P., Malas J.P., Cabaret J., Barbier D.. Boues résiduaires : difficultés d'évaluation du risque parasitaire. *Journal Européen d'Hydrologie*, 2003, in press.

-Moussavou-Boussougou M., Madeline M., Ballandonne C, Dorny P, Mouline C., Roy X., Guiard P., Chaumeil T., Barbier D., Cabaret J. Use of urban sewage sludge and cattle slurry on pastures : parasitism, paddock fertilisation and cattle growth. 10^{ème} Rencontres Recherches Ruminants. 2003 in press.

ANNEX 4

Isolation of helminth eggs from composted sludge

by Bjørn Gjerde, the Norwegian School of Veterinary Science, Oslo, Norway

From each sample of composted sludge, 10 subsamples of 10 grams each were examined as described below. Weighed samples were transferred to the container of a blender, and a few drops of detergent and 300-400 ml of tap water were added. The subsample was homogenised by running the blender for at least 15 seconds. The resulting mixture was poured through three metal test sieves (diameter 200 mm; height 50 mm) stacked in a sink. The sieves at the top, in the middle and at the bottom of the stack had mesh sizes of 70 mesh (aperture 212 µm), 140 mesh (aperture 106 µm), and 400 mesh (aperture 38 µm), respectively. The container of the blender was rinsed with 200-300 ml of tap water, and the resulting washing fluid was also poured through the sieves.

The material that was retained in the bottom sieve was collected in one corner of the sieve by gentle flushing with water from a tubing connected to a tap. The material collected was poured and flushed into a 100 ml centrifuge tube. The container of the blender and the sieves were then thoroughly flushed before processing the next subsample.

After centrifugation at 2000 rpm for 10 minutes, the supernatant was siphoned off, leaving about 5 mm of fluid above the sediment. Twenty millilitres of 10% formalin were added to the tube, and thoroughly mixed with the sediment and remaining supernatant with a glass rod. Five millilitres of ether were then added to the tube, forming a discrete layer on top of the suspension. The centrifuge tube was capped and vigorously agitated for about 30 seconds. The tube was then left in a rack for at least 1 h to allow sedimentation and separation of fat from the suspension. After sedimentation, three layers had formed. The top layer consisted mainly of fat, and formed a plug in the tube, the middle layer contained a mixture of water and formalin, and the bottom layer was the sediment. The fat-rich plug was separated from the tube wall by means of a needle and poured out and collected together with the middle layer.

One or two drops of detergent were added to the centrifuge tube along with approximately 70 ml of saturated sucrose solution (1400 grams sucrose pr. 1000 ml of water). The sediment was mixed with the sucrose solution and additional sucrose solution was added to 2-3 cm below the top of the tube. Then 20 ml of water was gently placed on top of the sucrose layer by means of a 20 ml syringe.

After centrifugation at 2000 rpm for 10 minutes, the interface between the sucrose layer and the water layer was gently siphoned off by means of a 20 ml syringe (without a needle). A total of 14-15 ml of fluid was withdrawn from the interface by moving the tip of the syringe in circles across the whole area. The syringe was connected to a membrane filter holder containing a nitrocellulose filter (diameter 25 mm, pore size 8 µm), and the contents of the syringe were gently pressed through the filter. The syringe was detached, 10 ml of water was drawn into it from a beaker and pressed through the filter after the syringe had been reconnected to the filter holder. The syringe was detached again, and filled with air, which was pressed through the filter after reconnection of the syringe to expel water.

The filter was transferred to a microscope slide using forceps with flat ends and left to air dry at room temperature. Four to five drops of immersion oil were added to the filter

and a cover slip placed upon it. The filter was then systematically examined for parasite eggs under a microscope at 100× magnification.

Eggs were identified to genus, family or other grouping, depending on type. The eggs were classified as dead (clearly damaged), potentially alive (unembryonated, with no visible defects), or alive (moving larvae inside). Eggs of different types and condition were enumerated. After examination of all 10 subsamples in the manner described, the average number of eggs of different types per 10 gram of composted sludge was calculated.

Unembryonated nematode eggs, usually ascarid eggs, found in fresh samples of composted sludge, were sometimes difficult or impossible to classify with regard to viability. In these instances, another portion of the original sample was incubated at 27°C for 4 weeks. After incubation, another series of 10 subsamples of 10 grams each were examined as described above. If unembryonated live eggs had been present in the fresh sample, live eggs containing larvae should now be found in the incubated sample.

This method can also be used for isolation of helminth eggs from samples of raw sludge (using subsamples of 25 grams), dewatered sludge, and soil.

Acknowledgement

This method is mainly based on a method developed by cand.med.vet. Karl Bergstrøm during his research work at the Norwegian School of Veterinary Science from 1977 to 1979 on the occurrence of parasite eggs in sewage sludge. Bergstrøm, for his part, modified a method published in 1977 by RJ Jørgensen as a stencil entitled 'Isolation of nematode eggs in soil and sediments. Principles and exercises' at the Royal Veterinary and Agricultural University, Copenhagen, Denmark.

ANNEX 5

**HORIZONTAL DESK STUDY 5
HELMINTH OVA IN SLUDGES
Questionnaire**

Office Use
Only

Section A: Helminth Short questions

1a. Do you perform helminth ova analyses in sludges or similar matrices?

Yes	No
<input type="checkbox"/>	<input type="checkbox"/>

1a

1b. If yes in 1a, is helminth ova enumeration in sludges and similar matrices required in your national legislation?

Yes	No
<input type="checkbox"/>	<input type="checkbox"/>

1b

1c. If 'yes' in 1a, please precise the reference of the method used and/or the principle :

1c

1d. If 'yes' in 1a, does the method applied allow the enumeration of :

Helminth ova (no viability evaluation)	<input type="checkbox"/>
Viable helminth ova	<input type="checkbox"/>
Nematods ova (no viability evaluation)	<input type="checkbox"/>
Viable nematods ova	<input type="checkbox"/>
Ascaris ova (no viability evaluation)	<input type="checkbox"/>
Viable Ascaris ova	<input type="checkbox"/>
Cestodes ova (no viability evaluation)	<input type="checkbox"/>
Viable Cestodes ova	<input type="checkbox"/>
Taenias ova (no viability evaluation)	<input type="checkbox"/>
Viable taenias ova	<input type="checkbox"/>
Other	<input type="checkbox"/>

1d

1e. If 'other' in 1d, state which target parameter (helminth group/viability or not) :

1e

1f. If yes in 1a, how many helminth ova analyses on sludges or similar matrices per month do you perform on ...

- <10
- 10-50
- 51-100
- 100-500
- >500

1f

1g. If yes in 1a, state on which types of sludges or similar matrices (please precise the frequency of analyses per type) :

1g

1h. If yes in 1a, which helminth ova do you more often find and in which type of sludge or similar matrice?

1h

General Remarks...

Section B: Comments and Background

2a. What type of lab do you work in?

- State lab
- Water Company
- Research lab
- Routine lab
- Private lab
- Other

2a

2b. If 'other', please specify

2b

3a. What is your role in the laboratory?

- Quality Manager
- Technician
- Head of Laboratory
- Head of Department
- Head of Institution / Company
- Other

3a

4b. If 'other', please specify

4b

5a. What type of organisation do you work for?

- Public
- Private
- Other

5a

5b. If 'other', please specify

5b

6a. Who do you test for (customers)?

- Public Authority
- Own Organisation
- Private Clients
- Research
- Other

6a

6b. If 'other', please specify?

6b

7. What is your country of origin?

7

Thank you for completing the questionnaire

ANNEX 6

Summary of the principle of the methods used by the respondents to the questionnaire who perform helminth analysis in sludges and similar matrices. (copy of the full-filled question 1c of the questionnaire)

A – Austrian respondent's method

Sludge, slurry and soil samples are being examined according to a standard protocol adapted from KRAUTHAUF and SEILER 1994 (originally published by HORN; 1986).

15 ml of wet sample/50 ml of dry sand or soil are mixed with alkaline antiformin solution or Tween solution (for sand), sieved and sedimented (1 h).

The sediment is pelleted by centrifugation and the pellet is mixed with flotation solution (sugar), centrifuged, covered $\frac{3}{4}$ with a cover slip and filled to the top with flotation solution.

After O/N flotation the cover slip was removed and placed on a glass slide for microscopical examination at 100x magnification.

B – German respondent's method

5 g material are mixed with zinc sulfate solution.

Subsequently, we put in 50-100 ml of saturated NaCl-solution and/or 33 % Zinksulfat solution.

The mixture is then filled over a filter in a glass tube.

After 20-40 minutes a cover slip is removed and placed on a glass slide for microscopical examination at 50-100x magnification.

C – Hungarian respondent's method

1./ Dissociation/separation

100 g sample with sodium hypochlorite solution (1 p.c.) thoroughly mixed by a glass rod manually until it reaches a pulpous consistency.

Wait 10 minutes while 3-4-times mixed up by a glass rod manually.

2./ Flotation (first)

Dilute the mixture with flotation solution (calcium nitrate solution sp.gr. 1.35) to 500 millilitres. Thoroughly mixed up by a glass rod manually.

Wait 30 minutes.

3./ Filtration

Pour off the supernatant through an 1 mm mesh metallic sieve to a 15 • m mesh plastic sieve. The retained material in the bottom of 15 • m mesh plastic sieve pour off and thoroughly rinse by tap-water into a 10 ml centrifuge tube.

4./ Sedimentation

Pour up to 10 millilitres by tap-water and centrifugation at 450 g for 10 minutes.
After centrifugation the supernatant pour off (discard) leaving a few mm of fluid above the sediment .

5./ Flotation (second)

Pour up to 10 millilitres by flotation solution, thoroughly mixing the sediment with the flotation solution by a glass rod , then centrifugation 450 g for 10 minutes.
After centrifugation fill the centrifuge tube with flotation solution to obtain a positive meniscus on the top of which a cover glass is placed.
Wait 30 minutes, carefully take out the cover glass and put them on a glass slide for microscopic examination.

6./ Microscope examination

Proceed to the microscopic examination of the whole surface of the cover glass at 50x - 100x magnification in order to detect and enumerate helminth eggs.

(Noted dead eggs are detected/characterized only on the observation of the eggs structure.)