SSPI-CT-2004- 513660

HORIZONTAL - HYG

HORIZONTAL STANDARDS ON HYGIENIC PARAMETERS FOR IMPLEMENTATION OF EU DIRECTIVES ON SLUDGE, SOIL AND TREATED BIO-WASTE

Instrument: STREP

Thematic Priority: PRIORITY 8.1 STREP
Topic 1.5 Environmental assessment

D 2/3.2
Critical review on:
Viable helminth ova methods in sludges, soils and treated biowastes

Due date of deliverable:

Actual submission date: 8-1-2006

Start date of project: 1-10-2003

Duration: 3 years

Organisation name of lead contractor for this deliverable: NIEH (partner 16)

DRAFT, Revision, Final

Project co-funded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	PU
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

Authors: Zoltan Szabo, Bela Vargha
National Institute of Environmental Health, Budapest
Correspondence address: szaboz@okk.antsz.hu

October 2005

TABLE OF CONTENT

NB : please add a table of content.

0. GENERAL INTRODUCTION

The European STREP "HORIZONTAL-HYG" project to develop "Horizontal Standards on Hygienic Microbiological parameters for implementation of EU Directives on sludge, soil and treated biowaste" started on 1st December 2004. This project is carried out under the umbrella of the main project HORIZONTAL "Development of horizontal standards for soil, sludge and biowaste".

The strategic objectives of this HORIZONTAL-HYG project focus on the development of reliable and harmonised European standards for sampling and hygienic microbiological parameters in the field of sludge, soil and treated biowastes and similar matrices. These methods are of fundamental importance to properly evaluate the environmental problem they may pose and to facilitate regulation of these parameters related to different uses and disposal governed by EU Directives. The Working document on revision of the Sewage Sludge Directive (86/278/EEC; draft April 2000) and the Working Document on Bio-waste (draft February 2001) called for standards on sampling, and analysis of hygienic and biological parameters, inorganic parameters and organic pollutants.

This project is concentrated only on the development of horizontal standards (if possible) for **microbiological parameters**, including **sampling and sample handling** taking into account the limited stability of microbiological parameters. Defining test organisms and test methods for the validation of safe treatment processes (biotechnological, chemical and physical treatment) forms part of the project.
Besides sampling and sample handling (WP 1) and process control and process validation (WP3), the central work package (WP 2) deals with methods by which microbiological parameters describing the microbiological quality of the final product or applicable for the re-isolation of test organisms applied in validation procedures shall be determined in a reliable way :
For *Salmonella* spp. and *Escherichia coli* (SubWP2/1) drafted CEN standards are available and therefore a co-normative work will be performed consisting in the validation of those methods (performance data). This work will consist in three main steps : (i) a training in a central laboratory of 16 EU laboratories for methods to be validated, (ii) an intralaboratory suitability study of methods to be validated (fit for purpose on the nine different matrices that are to be targeted) and finally (iii) an interlaboratory round robin test with selected laboratories to validate the methods.
For Enterococci and *Clostridium perfringens* (SubWP2/2), **viable helminth ova** (SubWP2/3) and **bacteriophages** (SubWP2/4), all relevant from the point of view of human and animal health as well as plant protection and environmental safety, only a pre-normative work will be performed (no validation study). This will consist in two main steps : (i) a critical review including an European workhop with experts first leading to a decision if and for which substrates standards shall be drafted and (ii) an intralaboratory suitability study of identified draft standards (fit for purpose on the nine different matrices that are to be targeted).
For plant pathogens (SubWP2/5), only a 12 months desk study will be performed.

This report corresponds to the Critical review report on methods for enumeration of viable helminth eggs to be monitored in EU in sludges, soil and treated biowastes that should be produced in the frame of the SubWP2/3. This report includes the conclusions of the European Horizontal-Hyg Workshop on this topic held in Lille (France) on April 2005. This report identifies draft horizontal methods for the targeted parameter that have to be studied for fit for purpose on sludge, soil and treated biowastes in the frame of the ~~intralaboratory~~ suitability study (rugedness trial) between 4 selected laboratories (pre-normative work).

~~Introduction~~1. INTRODUCTION

In May 2004 a desk study ~~(Work Package 2/3)~~ was conducted in the frame of the Horizontal project (Work Package 2/3 on microbiological hygienic parameters) by Partner 3 (Institut Pasteur de Lille, France) titled 'Desk Study Feasibility of Horizontal Standards for the enumeration of Viable Helminth Ova in Sludge, Soil and Treated Biowastes' (Pierzo *et al.*, 2004). NB : Please add the reference in your literature references at the end of the report)
The main outcome of this study was that while several methods exist for the enumeration of viable helmonth ova, ***no method to be used as draft horizontal standard is available***.

This critical review will complete the discussions and conclusions of this preliminary desk study while adding further data based on (i) informations obtained on the EU practical uses on viable helminth ova from EU experts thanks to both the Horizontal-Hyg questionnaire and Workshop held in Lille in April 2005, and (ii) on additional literature data thanks to 36 analysed literature references on the subject.
The aim of this critical review is to propose one (or maximum three) draft horizontal standard method(s) for the enumeration of viable helminth ova in sludges, soils and treated biowastes.
Those methods will have then to be tested in a suitability study (rugedness trial) in order to check their fit for purpose on several matrices representative of sludges, soils and treated biowastes.

~~At the European level, 6 methods have been identified and reviewed:~~

~~1) A modified US/EPA method (1992) based on a double flotation in a natrium nitrate solution, under French standardisation process (AFNOR PR XP X33-031),~~

~~2) A Triple flotation in a Zinc sulphate solution method, to be printed as French experimental standard in April 2004 (AFNOR XP X33-017),~~

~~3) A Norwegian method based on a flotation in sucrose,~~

~~4) An Austrian method based on a flotation in a sugar solution,.~~

~~5) A German method based on a flotation in a zinc sulphate solution,~~

~~6) An Hungarian method based on a double flotation in a calcium nitrate solution.~~

2. ~~Comparison of identified methods~~SUMMARY OF THE HORIZONTAL DESK STUDY

At the European level, 6 methods have been identified and reviewed in the frame of the Horizontal desk study (Pierzo *et al.*, 2004) :

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

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

2.1. Enumeration of helminth eggs

Norwegian and French AFNOR PR XP X33-031 (modified US/EPA) methods are relatively similar. 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 is restrictive for the Norwegian method due to the risks of losing big eggs. 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 ~~at a French scale. of Interlaboratory~~ interlaboratory trials ~~(ADEME, 2002)~~ at a French scale (ADEME, 2002) NB :

Not in your literature list at the end of the report, please, refer it either as ADEME project, or as authors (Guarini Ph., ...). The results of those interlaboratory trials showed that the Triple Flotation method had a higher yield than the modified US/EPA method due to a higher yield for cestodes (*Tænia*) but the US/EPA method (AFNOR PR XP X33-031) by adding a second step flotation allowed to recover a higher number of viable eggs than the Triple Flotation Method.

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 (NB : precise the respective detection limits of both methods, please). than the detection limit of the Triple Flotation method (NB : precise the detection limit, please). For other described methods it was not specified if the subsample weight was dry or wet weight.

From an economical point of view, the modified US/EPA method seems to be faster and cheaper followed by the Norwegian method.

2.2. Viability evaluation

The modified US/EPA (AFNOR PR XP X33-031), Triple Flotation (AFNOR XP X33-017) and the Norwegian method allowed 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. Nematode eggs are considered as viable eggs when the integrity of their structures can be observed or if a larva is detected. Any absence of internal structures or any disorganisation of the internal structures and/or any damage of the external shell are criteria that the eggs are non viable eggs.
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.

In the Triple Flotation method the activity of malate dehydrogenase in viable eggs transformes the soluble, yellow coloured tetrazolium (MTT) into insoluble blue formazan. Blue stained eggs are considered as viable, while dead eggs retain their initial brown

coloration. The inconvenience of the v~~V~~iability 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).

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.

2.3. Conclusion and perspectives

The m~~M~~ain conclusion of the desk study was that no method has been demonstrated to be capable of combining specific, quick and viability detection of all Helminth eggs.

According the Desk Study the following considerations were proposed to be more clearly defined:
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 *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.

~~This~~ Those considerations were discussed for agreement between EU experts during ~~an~~ the Horizontal-Hyg European expert workshop ~~in~~ held in Lille in April 2005.

3. EUROPEAN WORKSHOP, ~~orkshop~~ Lille ~~18th 20th~~ April 2004~~5~~

~~The workshop summary can be found in Appendix 1.~~

NB : Please, withdraw this workshop summary from the appendix (it was a preliminary internal wummary made by Karine Vidor in April 2005).

Copy from the Final workshop report sent by Karine Vidor last 18 October the specific parts corresponding to :

1) The summary/intro of the workshop (place, dates, number of participants, etc…)

2) The main objectives of the workshop for your helminth section

The aim of the Viable helminth ova workshop was to identify the more suitable target organisms (Helminths or only *Ascaris* or *Taenidae* ova, …) regarding risk assessment in EU countries, and to evaluate the performances of the available methods in terms of viability, organisms to be identified and most fit for purpose to sludges, soils and treated biowastes.

3) The aim of the sending of the questionnaires prior to the workshop.

3.1. Information on EU practical uses obtained from the questionnaires

The Questionnaire sent to all the EU experts who will participate to the Workshop is presented in Appendix 1. The ~~Questionnaire responses (~~detailed responses received from this~~to~~ questionnaire~~s~~ are in Appendix ~~2~~2~~)~~.

A total of 17 answers were received for the Viable Helminth ova part, representing 11 EU countries (including 4 new EU countries) and 1 from South Africa~~responded~~ :

- Austria(2)
- Cyprus
- Czech Republic
- Denmark
- Estonia
- France (5)
- Germany (1)
- Hungary

- Italy
- Portugal
- South Africa
- Spain

The ~~Summarized~~ main information obtained from those questionnaire answers are summarized below~~:~~.

1. Regulation		
1.1. Required by legistation	Does not know	1
	Yes	10
	No	6
1.2. Standard method	No answer	9
	Yes	4
	No	4
1.3. Maximum level	No answer	9
	No	1
	< 1 ova/ 1000 ml	2
	< 3 ova/10 g dry weight	3
	max. 3 ova / 10 g dry weight	1
	1 ova / 10 g dry weight	1
2. Sample storage and transport		
2.1. Max. lenght of time and tamperature of storage	No answer	4
	Yes	3
	No	10
3. Preparation of sample		
3.1. Amount	~~Nincs válasz~~Does not know	4
	0,2 ml	1
	200 ml	1
	5 g wet weight	1
	5-10 g wet weight	1
	10 g wet weight, 0.5 g dry weight	1
	15 or 100 g wet weight	1
	50 g wet weight	1
	?	1
	1.5 g dry weight	1
	1.5 g dry weight	4
3.2. Method to homogenize	No answer	5
	Suspensation	1
	Shaken, homogenisation	4
	Mixing	3
	Sieving	1

	Centrifugation	2
	To bray in an agate mortar	1
3.3. pH checked	No answer	7
	No	10

Analytical method and results

4.1. 4.1. Method	No answer	5
	Standard method	?
	Microscopy	?
	Flotation	2
	Separation and flotation	1
	Sedimentation + concentration + microscopical examination	1
	Sedimentation + flotation + microscopical examination :	3
	Sedimentation + flotation + filtration/sieving + microscopical examination :	2
	Filtration + concentration + flotation + microscopical examination :	1
4.2. Flotation solution / spec. gravity	Sucrose / 1.3,	2
	$ZnSO_4$ / 1.3 , 1.38	2
	$Ca(NO_3)_2$ / 1.35	1
	$NaNO_3$ / 1.3	1
	$NaNO_2$ / ?	1
4.3. Analysis time	No answer	5
	15 min	1
	30 min	1
	5 h	1
	6-8 h	3
	20 h	2
	1 day	2
	1-2 day	1
	3 day-3 week	1

		Viable	V/NV
4.4. Targeted helminths	Helminth	3	4
	Nematodes	3	2
	Ascaris	5	3
	Cestodes	2	5
	Taenia	2	4
	Other viable	3	2

5. ~~5.~~ Typical range of levels

NB : Summarised information maybe more readible if the table is presented in this way…

5.1. Matrix where higher frequency	Sludge	Waste water	Treated biowastes	Compost	Extrement	Soils	Sand	Shurry
Helminth Viable	1	1	1	1				
Helminth V/NV	1	1		1	1			
Nematodes Viable	1	1	1	1				
Nematodes V/NV	1		1	1	1			
Ascaris Viable	3	1	1	1		1	1	
Ascaris V/NV	3		4	1	1	1	1	
Cestodes Viable	2		1	1				1
CestodesV/NV	1		1	1	1			
Taenia Viable	2		1	1				1
Taenia V/NV	2		1					1

5.1. Matrix where higher frequency

Helminth Viable	sludge, waste water, treated biowastes,compost
Helminth V/NV	treated biowastes, excrement, sludge, compost
Nematodes Viable	sludge, waste water, treated biowastes,compost
Nematodes V/NV	treated biowastes, excrement, sludge, compost
Ascaris Viable	sludge (3), waste water , treated biowastes, compost, soils, sand
Ascaris V/NV	sludge (3), treated biowates (4), compost, extrement, soils, sand
Cestodes Viable	sludge (2), shurry, treated biowastes, compost

CestodesV/NV	sludge, treated biowastes, compost, extrement
Taenia Viable	sludge (2), shurry, treated biowastes, compost
Taenia V/NV	sludge (2), shurry, treated biowastes, compost

5.2.Frequency of analyses

sludge	No answer	8
	<10	7
	0-50	1
	No	1
soil	No answer	10
	<10	6
	No	1
Treated biowaste	No answer	11
	<10	4
	0-50	1
	No	1
Other	No answer	11
	<10	5
	No	1

6. Qality control

6.1. Standard control strains	No answer	5
	Used to use Ascaridia	1
	Not yet	1
	No	10

3.2. Discussions of the workshop

~~The aim of the Viable helminth ova workshop was to identify the more suitable target organisms (Helminths or only *Ascaris* or *Taenidae* ova, …) regarding risk assessment in EU countries, and to evaluate the performances of the available methods in terms of viability, organisms to be identified and most fit for purpose to sludges, soils and treated biowastes.~~

After the presentation of some European experiences by experts and the presentation of the summary of the answers obtained from the questionnaires, the main focused critical issues were discussed between experts participating to the workshop.

Discussion about the choice of target helminths focused ~~to~~ on the following questions~~,~~ :

- Epidemiological importance

- Cultivation conditions
- Resistance properties

The amount of ideal sample size – wet or equivalent dry weight - was not solved because of the different matrices involved at a horizontal scale.
Due to the high infection rate recently relatively small sludge samples produce pozitiv results, although the lime or heat treated sludge and biowaste samples need bigger sample sizez for safety reasons.

There were no large differences in sample storage and transport (conservation) condition practices.

Several experts proposed special pretretment step for dissociation of helminth eggs from organic matter in solid and liquid matrices.

Applicability of flotation solutions was discussed in detail, experiences were different depending on composition/density, tested species and matrices. Other question is how the flotation solutions influence the viability of helminth eggs.

Viability procedure optimisation needs more research in the future to find appropriate fast biochemical or stainings method to detect viable eggs other than Taeniids and Trichurids.

Some interesting comments were made regarding to the parasitological aspects of sludge treatment process validation. It seems that Ascaris eggs are appropriate for this application.

3.3. Conclusions of the workshop

NB : please insert here the part of the final workshop report of 18October2005 on your section (intro to the ccls table and table with agreed ccls).

The main conclusion agreed are the following : (NB : or copy the § after the ccls table in the final workshop report).

1. Sample size (wet weight) will be discussed later.
2. Samples to be stored at 5±3ºC prior to analysis and ~~in~~ during transport
3. Samples to be analysed within 1 week.

4. pH modification of samples is necessary in case of chemical treatment processes (eg. Lime).
5. There is a need to find more relevant literature search for
   - Pretreatment methods
   - Actions of flotation solutions
6. Uniform density of flotation solution (1,35) was decided.
7. Application of 20-25µm filter / sieve diameter depends on commercial availability.
8. It was agreed that basic steps of proposed standard method are the following,
   - Pretreatment
   - Flotation
   - Microscopical examination
   - Extra-viability testing (staining)

Outlook and perspectives

~~1.~~1 For the viable helminth ova, experts agreed on a list of target organisms which are: Nematodes (*Trichuris, Ascaris, Toxocara, Strongyloides* like), Cestodes (*Taenia* like).

2. Some additional analytical developments are still to be studied to identify the final test method. Like viability procedure optimisation adoption of double staining development if it is feasible and testing other Tetrazolium salts for nematodes in parallel to cestodes staining (MTT staining)

4. ~~Other literature references~~DISCUSSION BASED ON ADDITIONNAL LITERATURE DATA

In the "Desk Study Feasibility of Horizontal Standards for the Enumeration of Viable Helminth Ova in Sludge, Soil and Treated Biowastes" (~~Authors : Pierzo V., Pierlot E., Le Broc F., Roussel S., Simonart T.) [Horizontal – WP3 : Helminth ova – Final Desk Study Report, 23 April~~ *et al.*, 2004~~)]~~ features of 6 investigation methods (~~Double flotation – – mod.~~ US/EPA, Triple flotation, Norwegian, Austrian, German and Hungarian method) ~~are~~ were compared.

~~In this Final Report,~~However, in the reviewed six methods, the amount of the survey sample, the used flotation solutions and their density, the assessment of the viability, the distinction tool of the living/not living ova were contrasted, among others.

The desk study and the Lille Workshop conclusions ~~feel it necessary~~well identified the necessity to perform further investigations to establish an horizontal draft ~~-~~standard method.

We kept it useful the elaboration of ~~the available literature data concerning the main questions (survey sample amounts, used flotation solutions and their density, methods serving the investigation of the viability of the helminth ova)~~.

~~For that,~~Therefor, thanks to ~~-~~the study of the available literature on the topic, we completed the summation of the data concerning the main critical issues (survey sample amounts, used flotation solutions and their density, methods serving the investigation of the viability of the helminth ova) ~~above mentioned features of the 6 evaluated methods~~ by further literature data~~.~~. Those additional data are listed here below by theme points.

4.1. Comparison of sample amounts

Nb : Short description on information (for exple, number of literature ref found & analysed / mainly information on soil (13 ref among 15 in total) and only & on compost and 2 on sludge / No mention on WW or equivalent DW???, etc…)?
Reference to tables?

Table 4.1.a – Information from the 6 methods identified by the horizontal desk study (Pierzo *et al.*, 2004)

Method	Quantity of the samples *(DW = Dry wet ; WW = Wet weight)*
Modified US/EPA	10 g (equivalent DW-)
French AFNOR (Triple flotation)	3 x 0.5 g (-equivalent DW-)
Norwegian	10 x 10 g (-WW-) composted sludge
	10 x 25 g (-WW-) raw sludge
Austrian	15 ml (-WW-) wet sample
	50 ml dry sand or soil
German	5 g
Hungarian	100 g

Table 4.1.b – Information from the analysis of the available literature on the topic

~~Further data~~Literature references	Quantity of the samples (in DW or WW????)
Alf (1952)	5-10 g soil
Ash et Orihel (1987)	20-30 g soil
Beawer (1952)	20-25 g soil
Borg et Woodruff (1973)	15-20 g soil

Caveness et Jensen (1955)	15 ml soil
Dubin et al. (1975)	40 ml soil
Dunsmore et al. (1984)	25 g soil
Kazacos (1983)	5-40 g soil
Meyer et al. (1978)	75 g sludge
O'Donnell et al. (1984)	100 g soil or sludge
Quinn et al (1980)	25 g soil
Povum (1875)	10 g soil
Spindler (1929)	5-10 g soil
Steer et al. (1974)	30 g compost
Woodruff et Shah (1976)	15-20 g soil

4.2. Comparison of the used flotation solutions

NB : Short description on information (for exple, number of ref literature study found and analysed / mainly information on soil (13 ref among 15 in total) and only & on compost and 2 on sludge / No mention on WW or equivalent DW???, etc…)?

Reference to tables?

Table 4.2.a – Information from the 6 methods identified by the horizontal desk study (Pierzo *et al.*, 2004)

Method	Applied flotation solution	Density (sp.gr.)
Modified US/EPA	$NaNO_3$	1.30
French AFNOR (Triple flotation)	$ZnSO_4$	1.38
Norwegian	Sucrose	?
Austrian	Sugar	?
German	$ZnSO_4$	?
Hungarian	$Ca(NO_3)_2$	1.35

Table 4.2.b – Information from the analysis of the available literature on the topic

Method	Applied flotation solution	Density (sp.gr.)
Aradi (1962)	$NaNO_3$ + NaCl (1:2) NaCl K_2CO_3 Sugar (55 p.c.) $MgSO_4$ + K-bichromate(2 p.c.)	1.25 1.18–1.20 1.45 1.235 1.35
Ash et Orihel (1987) /a	$ZnSO_4$ NaCl $MgSO_4$ Sugar	1.18–1.20 1.20–1.26 " "

Bailenger (1979)	$ZnSO_4$	1.18
Bánki (1979)	$NaCl + NaNO_3$	1.30
Borg et Woodruff (1973)	$ZnSO_4$	1.18
Dada et Lindquist (1979) /b	$ZnSO_4$ HgI	1.20 1.63
Dubin et al. (1975)	$NaNO_3$	?
Dunsmore et al. (1984)	$NaNO_3$	1.22
Faus et al. (1938) /c	Saccharose	?
Fox et al. (1981)	Succrose $ZnSO_4$ NaCl Na-dichromate	1.40 1.18 1.20 1.20
Gaspar et al. (1996)	NaCl	1.19
Griffiths et al. (1990)	Ludox (colloidal silica)	1.16
Jankó et Lengyel (1983) /d	$Ca(NO_3)_2$	1.30
Kassai (2003) /e	NaCl $NaNO_3$ $ZnSO_4$ $MgSO_4$ Sugar $NaCl + NaNO_3$ (1:1) $Ca(NO_3)_2$	1.20 1.18–1.20 1.30–1.47 1.28–1.32 1.20–1.28 1.27 ?
Kassai et al. (1988) /f	NaCl $ZnSO_4$ K_2CO_3 $MgSO_4$ + K-bichromate $MgSO_4 + Na_2S_2O_3$	1.20 1.30 1.45 1.32 1.30
Kazacos (1983) /g	$NaNO_3$ $ZnSO_4$	1.35 ?
Majoros (1985) /h	$ZnSO_4$ + NaCl $ZnSO_4 + MgSO_4$ $ZnSO_4$ + KCl	1.40 1.40 1.40
Makara (1966)	$NaNO_3$ + NaCl (2:1)	1.25–1.28
Meyer et al. (1978) /i	$ZnSO_4$	1.20
Nelson et Darby (2001) /j	$ZnSO_4$ $MgSO_4$ NaCl	1.20 1.20 1.20
Nemeséri et Holló (1972) /k	NaCl Glicerine K_2CO_3 $MgSO_4$ Na-hyposulphite	? ? 1.45 1.25 1.37–1.4

	Sugar $ZnSO_4$ $MgSO_4 + Na_2S_2O_3$	1.235 1.18 1.30
O'Donnell et al. (1984)	Succrose	1.26
Quinn et al. (1980) /l	$ZnSO_4$ $MgSO_4$ $MgSO_4$ NaCl $MgSO_4 + KI$	1.09, 1.27 1.07, 1.14 1.275 1.205 1.33
Ruiz de Ybanez et al. (2000) /m	Saccharose	1.27
US EPA (1992)	ZnSO4	1.20
Woodruff et Shah (1976)	ZnSO4	1.18

Legend / Remarks :

/a The too high density of $ZnSO_4$ can harm the ova with very thin yolk-bag

/b $ZnSO_4$ gave a better result than HgI

/c The saturated saccharose solution deforms rapidly the ova

/d $Ca(NO_3)_2$ gave the best results

/e NaCl crystallizes too fast, the sugar solution is gluey,
$Ca(NO_3)_2$ has a smaller tendency to crystallization

/f $MgSO_4$ + K-bichromate crystallizes slowerly

/g $NaNO_3$ gaves a better result than $ZnSO_4$; $NaNO_3$ crystallizes fastly,
more bubbles were formed in $ZnSO_4$

/h $ZnSO_4$ may deform the ova and crystallizes fastly,
the K_2CO_3 solution has air bubbles because of the detergents,
$ZnSO_4$ is agressive, hygroscopic,
bromides and heavy metal salts are expensive and toxic,
the iron(III) salts are brown, hygroscopic and they do denaturate the proteines,
beetsugar and glycerine are expensive

/i the used flotation solution never killed the Ascaris specimens

/j the 1.20 sp.gr. $ZnSO_4$, $MgSO_4$, NaCl flotation solution does not inaktivates the Ascaris ova

/k NaCl crystallizes too fast, glycerine is expensive,
the K_2CO_3 solution deformes the ova,
$MgSO_4$+$Na_2S_2O_3$ solution does not crystallize

/l The $ZnSO_4$ solution gave a poor result,
$MgSO_4$+KI is more effective then $MgSO_4$ itself,

the physico-chemical properties and density of the flotation solution are equally important and significant for a greater efficacy

/m From among the used solutions of density of 1.20 to 1.35, the saccharose solution of density of 1.27 gave the best result

4.3. ~~Further notes on the effects of O~~other treatments steps ~~of the methods~~ (e.~~-~~g. chemical separation of ~~the~~ ova and ~~the~~ medium~~-~~)

After Barrett (1976), keeping the ova of Ascaris during 24 hours in 7.4% hypochlorite solution does not influence the viability of them. Meyer (Meyer et al., 1978~~-~~) stated that 2.62% hypochlorite solution did not kill the Ascaris specimens.

Organic solvents (formaldehyde-ether) are letal to the helminth ova (Barrett , 1976~~-~~).

Bouhoum & Schwartzbrod (1989) comparing a wide range of flotation solutions tested, found that iodomercurate (Janeckso & Urbanyi, 1931) concentrated the greatest range of species of parasitic helminth eggs, but concluded that the reagent was too corrosive and expensive for routine use. Arthur's method (described in Faust et al., 1938), in which saturated saccharose is used as a flotation solution, was found to deform eggs rapidly, while zinc sulfate solution (Faust et al., 1938) did not concentrate *Trichuris* spp. or *Capillaria* spp. very well. Acid-alcohol or diethyl-ether itself did not diminished the viability of Ascaris ova under the investigation circumstances; the acetoacetic buffer had not harmful effect, too. None of the reagents used for the flotation step of the sample cleaning procedure ($ZnSO_4$, $MgSO_4$, and NaCl) or during incubation (0.1 N H_2SO_4 and 0.5% formalin) inactivated the ova (Nelson et Darby, 2001~~-~~).

4.4. Investigation of viability of the ova of parasitic helminths

Dead Nematoda ova can be stained by 0.1 % methylen blue solution, for the yolk-bag will be permeable for the stain, so they will be stained (however, usually, they will be distorted also), but the living ova does not stain (Majoros, 1985~~-~~).

Alf (1952) cites Sulz and Sikobalova (1947), Heller (1935) and Mireckij (1942), that after their data on the staining of helminth ova and larvas, the dead wurms can be stained well with gentiana violet and methylen blue solutions, but they do not stain the living larvas (neither he reviews the exact investigation methods nor he writes that if this is concerning ova with thick yolk-bag, too) .

Autoclaving the ova of Ascaris suum (30 minutes long, on 120 ^{o}C) does kill them, and, due to this treatment, the ova will be permeable to the concentrated Nile-blue sulphate solution, therefore they will be stained (Meyer et al., 1978) .

The reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny–l_tetrazolium_–bromide) bymitochondrial enzymes to its blue formazan product is ~~a~~ widely used to determine the viability of parasites (Cabaret et al., 2002~~-~~). Some caution must remain especially when using a complex media such as sludge: it was shown that plant catechols and flavoinoids reduce MTT through iron ions (Habtemariam S., ~~(~~1995).

The ova of parasitic helminths found in the soil, sand, waste and compost samples (~~-~~generally in a small number) and ~~they~~ found in sewage sludges the most often (Ascaris, Toxocara, Trichuris spp.), have a thick yolk-bag~~,~~. Regarding ~~and, after~~ the recently available data, assessing the viability of these ova with a necessary confidence, can be done based only on the morphological feature (the injured, deformed ova are probably dead) or on the appear of embryonized state, followed by incubation, having greater amount of ova (in the case of living ova).

~~**Comparative evaluation of the investigated national parasitological investigation methods and literature data at some important points of the investigation methods**~~

~~**Summary**~~

5. GENERAL CONCLUSION

Regarding the ~~By the comparative evaluation, the~~ data analysed from ~~of~~ the six ~~national parasitological investigation~~ E~~-~~uropean national methods identified by the Horizontal desk study (Pierzo et al., 2004) and ~~those~~ the ~~of~~ data obtained from the 36 litera~~r~~ture~~y~~ sources ~~work~~, some conclusions can be proposed on the critical issues and the main steps of a horizontal draft standard on viable helminth ova methods in sludges, soils and treated biowastes can be identified~~were used~~.

~~In the following, we make some remarks on the unique national methods, after, the literary data will be summarized concerning the sample amounts, the used flotation solutions and their density, the effects of the unique treatments during the apply of the methods, and, the investigation of viability of the ova of parasitic helminths, finally, we make proposals to the implementation of the investigation methods.~~

1./ ~~Some remarks~~Main remarks and comments ~~-~~on the ~~unique~~ 6 national methods identified by the horizontal desk study (Pierzo *et al.*, 2004) :

Method	Remarks/comments
Modified US/EPA	- time- and work efficient
French AFNOR (Triple flotation)	- time- and work consuming - very small sample amount (0.5 g)
Norwegian	- the small ova can penetrate through the filter of aperture diameter 38 µm and can be lost
Austrian	- few data are available on the method
German	- few data are available on the method - sample amount is too small (5g) - investigation is oversimplified
Hungarian	- the high sample amount (100 g) makes heavy performance of the investigation - the ova can be lost during manipulation

2./ Analytical Sample amount

The analytical sample amounts has a great variety between 5 and 100 g ~~on~~ wet weight, or between 15–40 ml. In the majority of the methods a sample amount of 20–30 g is the most common.

3./ Flotation solutions

Similarly, lot of type of the flotation solutions are used by the different methods. In some cases, not only one but even two different chemicals are used to produce a flotation solution of a given density.

The l~~L~~ist of chemicals used ~~in the~~as flotation solutions, ranged by ~~the~~ frequency of their mention in the literature sources analysed (number of reference in the literature sources ~~–~~in parentheses ~~the number of allusion is to be found~~).

- $ZnSO_4$ (17)
- NaCl (14 – including ~~-~~five times used ~~not only in itself~~with another flotation solution)
- $MgSO_4$ (12)
- $NaNO_3$ (8)
- Sugar (4)
- K_2CO_3 (3)
- $Ca(NO_3)_2$, K-bichromate, $Na_2S_2O_3$, Saccharos and Succrose (2)
- Glycerine, HgI, KI, KCl, Ludox, Na-dichromate, Na-hyposulphite (1)

~~2- 2 times are mentioned: $Ca(NO_3)_2$, K-bichromate, $Na_2S_2O_3$, Saccharose, Succrose.~~

~~1-1 is mentioned : glycerine, HgI, KI, KCl, Ludox, Na-dichromate, Na-hyposulphite~~

~~.~~NB : Could you check if I well understood?…

4./ Density of the flotation solution

Very various ~~The~~ densities~~y~~ of the used flotation solutions are found in the literature sources~~is very different, too, after the literary data~~ :

The analysis of the literature sources shows that the following densities are mentioned at the here below precised frequency :

~~Literature mentions it~~ 10 times: 1.20 sp.gr.

5 times: 1.18 sp.gr. , 1.30 sp.gr.

4 times: 1.40 sp.gr.
3 times: 1.18–1.26 sp.gr., 1.45 sp.gr.
2 times: 1.18-1.20 sp.gr.
once: 1.07, 1.09, 1.14, 1.16, 1.19, 1.20–1.28, 1.205, 1.22, 1.25, 1.25–1.28, 1.235, 1.26, 1.27, 1.275, 1.28–1.32, 1.3–1.47, 1.32, 1.33, 1.35, 1.37–1.4, 1.63 sp.gr.

5./ Further informations

The effects of the chemicals and methods used by the investigation methods (the lesion and deformation of the yolk-bag, too fast crystallizing, bubbles, toxicoty, costs, efficacy, effect on viability) were reviewed in detail at the remarks.

6./ Viability protocol

The possible, recommended or described staining methods, used to differentiate the living and dead ova and larvas, were listed on the basis of the literature data at the investigation for the viability of helminth ova. However, it shall be underlined that the ova of the most frequent parasitic helminths (Ascaris, Toxocara, Trichuris spp.) have a thick yolk-bag, so the assessment of viability can be done with higher certainty only by the morphological feature and by the observation of the growth of the ova and of the state of embryonizatedness.
The inconvenience of evaluating the state of embryonizatedness is however highly time and place (in incubators) consuming.

7./ Finally, based on the available data and the conclusions of the April workshop and this critical review, the next following proposal shall be done for the main to the steps of the parasitological investigational methods of draft horizontal standard method for the enumeration of viable helminth ova in the samples of soil, sand, waste and compost, based on the available data:

- Precise please the targeted helminths proposed to be investigated.
- For preparation (for the separation of the ova from the media) – hypochlorite solution can be used.

- The recommended amount of the sample to be analysed is: 25–30 g (wet weight).

~~The minimal amount shall not be less than 10 gram, the maximal one shall not be more than 50 gram.~~ NB : You have to fixe the final analytical amount and not open this point. Please, take a decision between 25 and 30g, and fix only one amount.

- The 5 main steps of the method to be standardized shall be :
  1) Filtration/sieving,
  2) Sedimentation,
  3) Flotation,
  4) Viability evaluation
  5) Microscopical examination.

- A preliminary pre-treatment step for the peparation of the ova from the matrix can be added, using hypochlorite solution (NB : concentration?).

- Filtration/Sieving :

  The aperture of the filters used to retain the helminth ova shall not be more than 20–25 micrometer.

- Flotation :

  The used flotation solution shall be optional from among $ZnSO_4$, $NaNO_3$ and $Ca(NO_3)_2$ solutions, which shall have a recommended density between 1.30–1.40 sp. gr., as far as possible between 1.30–1.35 sp.gr. Those 3 open proposals will be tested during the suitability study by the 4 participant laboratories in order to identify the more fit for purpose solution for an application on sludges, soils and treated biowastes.

~~The aperture of the filters used to retain the helminth ova shall not be more than 20–25 micrometer, and, the basic components of the method to be standardized shall be the next ones: filtration/sieving, sedimentation, flotation, microscopical examination.~~

- Viability evaluation :

  To assess the viability of targeted helminth ova (Nb : remind the chosen targeted helminth ova to be investigate), two possibilities are proposed, but each being applicable only on one part of the targeted helminth ova to be investigated :

  1) a specific staining protocol seems to be able to carry out on Cestodes,

2) the ovum structure/morphology, and moreover, the embryonizatedness following incubation is applicable for the Nematoda. However, this last investigation is highly time and place consuming and fastiduous to implement in routine in a laboratory.

Therefore, we do recommend the research for further possible staining methods to assess the viability of the Nematoda ova, or better for developping one single double staining method to assess the viability of the whole targeted helmith to be investigated. However, this development which is highly time consumming was not scheduled in the frame of the Horizontal-Hyg project. This will only be possible thanks ro additional fundings allowing to imply researchers specifically on this development. Otherwise, the draft horizontal standard on viable helminth ova tested in the frame of the Horizontal-hyg project during the Suitability study will not imply a efficient viability evaluation.

Thanks to those main steps and specification, a draft horizontal draft standard will be written in order to be tested during the suitability study (rugedness trial) that will be performed by 4 identified EU laboratories from june to decemeber 2006. The three possible methods implying the three different flotation solutions will be tested in parallel for comparision on the different matrices representatives from sludges, soils and treated biowastes to be tested.~~We feel necessary the elaboration of such a method, which is equally suitable for the parasitological/helminthological investigation of samples of sludges, wastes, compost, sand and soil~~.

NB : The draft protocol should be annexed to this report.

6. REFERENCES~~Literature~~

NB : Please check carefully that all the cited references in your report are well listed here…).

ADEME (2002)…. Or Guarini, etc…..

Alf, Sz. L. (1952) : A helmintológiai talajvizsgálat módszerei. [The methods of the helminthological soil examination.] - Közegészségügy 1952, 2 , pp. 1–12. (manuscript)

Aradi, M. P. (1962) : Parazitológia. [Parasitology.] pp. 405-475. In : Az orvosi laboratóriumi asszisztensek kézikönyve. II. kötet. [Handbook of the medical laboratory assistents. Vol. 2.] Ed.: Ormay L. - Medicina Könyvkiadó. Budapest. p. 583.

Ash, L. R. et Orihel, T.C. (1987) : Parasites: A Guide to Laboratory Procedures and Identification. - ASCP Press, Chicago, p. 328.

Bailinger, J. (1979) : Mechanisms of parasitological concentration in coprology and their practical consequences. - J. Amer. Med. Technol. 41 : 65-71.

Bánki, Gy. (1979) : Parazitológia. [Parasitology.] pp.b83-106, - In: Jegyzet a mikrobiológiai laboratóriumi szakasszisztensek részére. [Notes for the microbiological laboratory assistents.] Ed.: Gy. Dóbiás, Budapest, l979.

Barrett (1976) : Parasitology 73 : 109-121. – cit. in: Meyer et al. (1978).

Beaver, P.C. (1952) : Observations on the epidemiology of ascariasis in a region of high hookworm endemicity. - J. Parasitol. 38 : 445-452.

Borg, O.A. et Woodruff, A.W. (1973) : Prevalence of Infective Ova of Toxocara Species in Public Places. - British Med. J. 4 : 470-472.

Bouhoum K, Schwartzbrod J. (1989.) Quantification of helminth eggs in wastewater. Zentbl Hyg Umweltmed. 188:322–330.

Cabaret J., Geerts S., Madeline M., Ballandonne C., Barbier D. (2002.) The use of urban sewage sludge on pastures: the cysticercosis threat.Vet. Res. 33 (2002) 575–59.

Caveness,F.E. et Jensen, H.J. (1955) : Modification of centrifugal-flotation technique for the isolation and concentration of nematodes and their ova from soil and plant tissues - Proc. Helminthol.Soc. Wash- 22: 87-89.

Dada, B.J.O. et Lindquist, W. D. (1979) : Studies on flotation techniques for the recovery of

helminth ova from soil and the prevalence of ova of Toxocara spp. in some Kansas public places. - J. Am. Vet. Med. Assoc. 174 :1208-1210.

Dubin, S. et al. (1975) : Contamination of soil in two city parks with canine nematode ova including Toxocara canis : A preliminary study. - Am. J. Public Health 65 : 1242-1245.

Dunsmore, J.D. et al. (1984) : Prevalence and survival of Toxocara canis ova in the urban environment of Perth, Australia. - Vet. Parasitol. 16 : 303-311.

Faust. E.C. et al. (1938) : A critical study of clinical laboratory technics for the diagnosis of protozoan cysts and helminth ova in feces. - Amer. J. Tropic. Med. Hyg. 18 : 169-183.

Fox, J. C. et al. (1981) : Sewage Organisms : A Color Atlas. - Metropolitan Sanitary District of greater Chicago, Illinois. p. 116.

Gaspard, P. et al. (1996) : A Method for Assessing the Viability of Nematode Ova in Sludge. - Environ. Technol. 17 : 415-420.

Griffiths, B.S. (1990) : The use of colloidal silica to extract nematodes from small samples of soil or sediment. - Nematologica 36 : 465-473.

Habtemariam S., (1995) Catechols and quercetin reduce MTTthrough iron ions: a possible artefact in cell viability assays, Phytother. Res. 9 603-605.

Heller (1935) : cit. In: Alf (1952).

Janeckso A, Urbanyi L (1931) Méthode d'enrichissement coprologique. *Revue générale de médecine vétérinaire,* **41**: 496-497.

Jankó, M. et Lengyel, A. (1983) : A parazitológiai vizsgálatok gyakorlatának jelen tapasztalatai és a jövő feladatai. [The present experiences and the future tasks of the praxis of the parasitological examinations.]- Egészségtud. 27 : 80-86.

Kassai, T. (2003) : Helmintológia. [Helminthology.]- Medicina Könyvkiadó Rt. Budapest, p.369.

Kassai, T. et al. (1988) : Állatorvosi parazitológiai diagnosztika. [Veterinary parasitological diagnostics.] - Állatorvostudományi Egyetem. Budapest, p. 182

Kazacos, K.R. (1983) : Improved method for recovering ascarid and other helminth ova from soil associated with epizootics and during survey studies. - Amer. J. Vet. Res. 44: 896-900.

Majoros, G. (1985) : Kombinált flotációs eljárás alkalmazása a kérődzők endoparazitáinak koprológiai rutinvizsgálatára. [The use of a combined flotation method for routine coprological examination for endoparasites of ruminants.] - Országos Állategészségügyi Intézet. Budapest, p. 99.

Makara, Gy. (1966) : Parazitás bélbetegségek. [Parasitic bowel diseases.] - Medicina Könyvkiadó. Budapest, p. 236.

Meyer, K.B. et al. (1978) : Recovery of Ascaris ova from sludge. - J. Parasitol.64 :380-383.

Mireckij (1942) : cit in: Alf (1952) .

Nelson, K.L. et Darby, J.L. (2001) : Inactivation of Viable Ascaris Ova by Reagents during Enumeration. - Appl.Environ.Microbiol. 67 : 5453-5459.

Nemeséri, L., Holló, F. (1972) : Állatorvosi parazitológiai diagnosztika. [Veterinary parasitological diagnostics.] – Mezőgazdasági Kiadó. Budapest, p. 469.

O'Donnell, C.J. et al. (1984) : Survival of Parasite Ova Upon Storage in Sludge. - Appl. Environ. Microbiol. 48 : 618-625.

Pierzo V. et al. (2004), Desk study etc…

Povum, E.J. (1975) : Dog roundworms and public health. - Vet. Rec. 97 : 78.

Quinn, R. et al. (1980) : Studies on the incidence of Toxocara and Toxascaris spp ova in the environment. I. A comparison of flotation procedures for recovering Tococara spp ova from soil. - J. Hyg. (Camb) : 84 : 83-89.

Ruiz de Ybanez, M.R. et al. (2000) : Improved methods for recovering ova of Toxocara canis from soil. - J. Helminthol. 74 : 349-353.

Spindler, L.A. (1929) : On the use of a method for the isolation of Ascaris ova from soil. - Am. J. Hyg. 10 : 157-164.

Steer, A.G. et al. (1974) : A modification of the Allen and Ridley technique for the recovery of Ascaris lumbricoides ova from municipal compost. - Water Res., 8: 851-853.

Sulc et Sikobalova (1947) : cit. in: Alf (1952).

U.S.EPA (1992) : Environmental regulations and technology. Control of Pathogens and Vector Attraction in Sewage Sludge. - EPA / 625 / R-92 / 013.

Woodruff, A.W. et Shah, A. I. (1976): Improved method of recovering Toxocara species ova from soil. - British Med. J. ii, 621-622.

ANNEX 1

Add HOR-HYG April Questionnaire

ANNEX 2

NB : Add the detailed responses on the HOR-Hyg questionnaire.

ANNEX 3

Nb : Add the draft proposed protocol as draft horizontal standard to be tested during the HOR-HYG suitability study of 2006…