



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

Critical review on:

**Methods for Bacteriophages (and viruses) to be monitored in EU
in sludges, soils and treated biowastes**

(DL 2/4.2)

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: University of Barcelona
(partner 11)**

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)	

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October 2005

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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 workshop 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 bacteriophages (and viruses) to be monitored in EU in sludges, soil and treated biowastes that should be produced in the frame of the SubWP2/4. It 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 between 4 selected laboratories (pre-normative work).

1. Introduction

For this critical review we have tried to collect all the available information from three different sources: 1) in scientific literature by consulting the data bases (PubMed, Sciendirect), 2) the sources of standardised methods as well as 3) some consultations to experts.

From the scientific literature consultation, one hundred sixty two scientific publications about bacteriophages in biosolids had been detected and consulted; 36 of them contained some information on detection and quantification methods for bacteriophages. One hundred and fifty scientific publications about viruses in biosolids had been detected and consulted; of them 86 contained some information on methods.

From the available standardised methods research it arised that there are not standardised method for sample preparation and phage extraction from the sample. On the contrary well standardised methods are available for determination and enumeration of bacteriophages (ISO, EPA and Standards Methods).

The third source of information was the consultation of European experts through one questionnaire and one European Workshop organised in Lille on 18th-19th-20th April 2005 both in the frame of the Horizontal-Hyg project. The conclusions of the questionnaires (see Annexe 1) and of the workshop discussion between experts are included in this critical review.

2. Background

2.1. - Adsorption of viruses to solids.

Viruses had been described to have a tendency to adsorb to solids (18, 30, 89, 142, 149), though it seems that there are some differences between different viruses regarding adsorption efficiency, solids to which they adsorb better and conditions that favour adsorption (17, 24, 53, 90, 110). Also, it seems that there is no doubt that once adsorbed to solids viruses persist longer than when they are free (20, 50, 135, and 147).

Consequently viruses are expected to concentrate in sludges, biowastes, sediments and soils, where they will persist longer than in the contaminated waters.

2.2. - Viruses in sludges, biowastes and soils.

2.2.1.- Viruses reported in sludges and biowastes All viruses excreted by man and animals are expected to be found in sludges and biowastes, and consequently in soils amended with these materials and irrigated with faecally contaminated water.

Table 1 summarises the different viruses and their densities reported for different sludges and biowastes and soils. First difficulty in interpreting the results is that there is a great diversity of sludges with a very poor definition of their characteristics. Second is that methods used to recover and enumerate the human viruses are very diverse and that results are expressed in very different ways. Therefore it is difficult to establish significant comparisons.

Table 1.- Enteroviruses in sludges and biowastes

Enterovirus (units as indicated)		References
Primary sludges		
500-2800	PFU ¹ /100 ml	(21)
910-2320	PFU/100 ml	(19)
140000	PFU/100 ml	(26)
890-200000	PFU/100ml	(81)
67-850	PFU/l	(124)
8-1728	PFU/100 ml	(54)
50-4100	PFU/100 ml	(150)
66-483	PFU/100ml	(122)
85-4300	PFU/100g	(126)
23-135	PFU/100ml	(2)
85-492	PFU/100 ml	(136)
70000	PFU/100 g DM ²	(131)
110-10670	MPNCU ³ /100 g DM	(93)
Activated sludge		
125-821	PFU/100 ml	(19)
64-320	PFU/100 ml	(124)
140-207	PFU/100 ml	(122)
70-701	PFU/100 ml	(126)
<30-2780	MPNCU/100 g DM	(93)
Settled (thickened activated sludge)		
1190-3780	PFU/100 ml	(122)
< 30 – 720	PFU/100 g DM	(121)
Settled (thickened primary sludge)		
250-259	PFU/100 ml	(122)
Anaerobic mesophilically digested		
500-8500	PFU/100 ml	(89)
63-377	PFU/100 ml	(19)
50-1000	PFU/100 ml	(81)
<10-735	PFU/1000 ml	(56)
16-607	PFU/100 ml	(124)
12-48	PFU/100 ml	(122)
Aerobically digested sludge		
1,7-84	TCID ₅₀ ⁴ /g	(43)
<10-110	PFU/1000 ml	(56)
<0,1-135	TCID /g	(124)
82-249	PFU/100 ml	(122)
Dewatered digested sludge		
<10-1022	PFU/1000 ml	(56)
160	MPNCU/100g	(131)
Lagooned sludge		
0,02-4,6	TCID /g	(43)

Legend: 1: PFU: plaque forming units ; 2: DM: dry matter ; 3: MPNCU: most probable number of cytopathogenic units ; 4: TCID₅₀: tissue culture infectious dose (50%).

Most data reported are on enteroviruses. Only a few data on adenoviruses and rotaviruses had been reported (table 2). A few data of presence/absence of astroviruses, detected by CC-PCR (cell-culture followed of nested PCR) are also available (31).

Table 2. - Adenoviruses and rotaviruses in comparison to enteroviruses in primary sludges.

Viruses in primary sludges			
Adenoviruses (FFU/litre)	Rotaviruses (FFU/litre)	Enteroviruses (PFU/litre)	References
5800-32500	-	1320	(150)
-	30-260000	850-43000	(25)

Legend: FFU = fluorecent foccus units ; PFU = plaque forming units.

In any case the numbers of enteroviruses detected in sludges are not negligible, with the greatest values found in primary (raw) sludges. In industrialised countries the values of enteroviruses reported range from 5000 to 20000 PFU per 100 g (dry weight) of sludge. Values of adenovirus are 3 or 4 times higher as well as numbers of rotaviruses. Values of viruses in primary sludge referred to dry weight range from one to two orders of magnitude higher than the values found in sewage.

Numbers of viruses in biowastes (more or less processed sludges) are only available for enteroviruses, and are decreasing according to the completeness of the treatment. Astroviruses had also been detected by CC-PCR, though non-quantified, in lime treated primary sludge (31). No naturally occurring human viruses had been reported in sludges that have undergone thermal treatments, including compostage.

Data on quantitative PCR are available for enteroviruses (97, 98). In both reports the numbers of genomes clearly exceed the number of infectious viruses by between 2 and 3 orders or magnitude in primary sludge and sludge

after mesophilic-anaerobic digestion. Numbers of genomes in sludge after lime or thermal treatments, including compostage (except one composted sample taken in the winter period) are below the detection limit of the technique at the present stage.

Table 3.- Quantitative data of genome copies of enteroviruses in sludges

Cytopathogenic enteroviruses versus genomes (PCR)	MPNUC/100 g dry weight	Genomes/100 g dry weight	References
Primary sludge	4570	1370000	(98)
Activated sludge	290	93600	(98)
Thickened sludge	90	106000	(98)
Digested sludge	70	480000	(98)

Legend: MPNUC = more probable number of cytopathogenic units

2.2.2.- Viruses in soils and sediments

Information about the occurrence of naturally occurring viruses of human origin in soils is null, though information about the behaviour of viruses in soil, after inoculation of viruses into experimental soil columns (112, 132), or application of sludges to soil (15, 17, 20, 51, 102) is relatively abundant. As well, information on viruses infiltration through soil (22, 123, 148); inactivation in soil (23, 52, 64, 77, 115, 133, 137), wetlands (96) or interaction with vegetables (40, 140, 141) and methods to detect them (65) is available. This information is useful since it provides information on transport and survival of viruses in soils, but nothing on levels of naturally occurring viruses.

Information of naturally occurring viruses in river, lake and sea sediments is more abundant (39, 87, 114, 120, 126, and 130), (table 4). From this sort of data it can be deduced that the concentrations of viruses in sediments are higher than in the water columns.

Table 4.- Cytopatogenic enteroviruses in sediments

Sediments	Mean	Range	Expression of results	References
Marine	14	0-120	PFU/1000g	(126)
River	1800	0-10000	PFU/100g	(126)
Marine	4	0-40	TCID ₅₀ /100ml	(126)
Marine (Antarctica)	0	-	-	(120)
Marine	-	7-10	PFU/1000g	(39)
Fluffy-sediments	-	39-398	PFU/ 379liter	(39)

Legend: PFU = plaque forming units ; TCID₅₀ = total cytopatogenic infectious dosis 50

2.2.3. Persistence of viruses during treatments of sludges and biowastes.

According to their destination sludges receive different treatments, the most frequent being storage, digestion (aerobic and anaerobic, mesophilic and thermophilic) and, to a lesser extent, disinfection by pasteurization, irradiation, lime treatment and composting. Table 5 summarises different data on the persistence of virus to anaerobic mesophilically digested treatments. Information available refers mostly to cytopathogenic enteroviruses, with a single report for astroviruses (31), which has the inconvenient that they were not quantified. Effects of treatment with lime, composting, and heat treatments are also available (99). Naturally occurring viruses are not further detected after these treatments.

Table 5.- Enteroviruses in different sludges and biowastes after treatment

Primary sludge	Activated sludge	Settled-thickened primary sludge	Anaerobically mesophilically digested sludge	Log ₁₀ -reduction	References
2320	817	-	220	~ 1	(19)
1520	140	-	80	~ 1-2	(19)
-	-	250	28	~ 1	(122)
42000	-	-	510	~ 2	(81)
-	3290	-	161	~ 1	(131)
4570	290	-	70	~ 1-2	(98)
1.4 x 10 ⁶ *	9.4 x 10 ⁴ *	-	4.8 x 10 ⁵ *	~ 1	(98)

Legend: Values of enteroviruses expressed as MPNCU or PFU /100ml or 100 g;

* copies genomes /100g.

Again, information is extremely diverse regarding kinds of sludges studied, description of samples, extraction method, virus detection method and expression of results. As well some results correspond to experiments done with spiked viruses and others with naturally occurring viruses (37, 134). Consequently, it is very difficult to take out many conclusions. However, two or three conclusions arise. First is that viruses seem to survive better than bacteria, and consequently bacterial indicators are not suited to follow the fate of viruses in sludges, biowastes and soil. Second that the numbers of naturally occurring viruses are too low to determine the extent of inactivation (except for mesophilic-anaerobic digestion), since the detection limit (including detection of genomes) is such that inactivations higher than between 2 or 3 log₁₀ units obtained with the different treatments can not be quantified.

2.2.4.- Need of indicators

The low number of human viruses detected in untreated sludges and the relative difficulty of the methods needed to detect them requires the use of indicators, since the low numbers of viruses will make very difficult, if not unfeasible, the validation of sludge treatment processes, the monitoring of the performance of treatment plants and the determination of virological quality of biowastes, where the expected numbers of human viruses are very low.

Though there is no information, the same constrains can be supposed regarding the presence of viruses in animal biowastes.

With the present knowledge, though imperfect, bacteriophages seem to be the group of organisms better suited as indicators of viruses.

This does not mean that investigation in prevalence of human viruses in biowastes using the best methods available should be abandoned. On the contrary, a good knowledge of the prevalence of pathogenic viruses will facilitate the estimation of risks and determine the requirements regarding the elimination of viral indicators and their limit number in the biowastes to be used for different purposes.

2.3. Bacteriophages as potential indicators (model organisms) of viruses

2.3.1. Bacteriophages as indicators

Three main groups of bacteriophages infecting enteric bacteria have so far been considered as potential model microorganisms for various aspects of water, and consequently sewage and biowastes, quality assessment: somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *Bacteroides fragilis*. Table 6 summarizes some of the features of the three groups of bacteriophages that will be more extensively described below.

Table 6.- General features of the three groups of bacteriophages proposed as model microorganisms for water quality assessment.

Feature	Somatic coliphages	F-specific RNA-phages	Phages of <i>Bacteroides fragilis</i>
Homogeneity of the group	+	+++	+++
Availability of standardized methods	+++	+++	+++
Occurrence and levels in human feces	++	+	+
Occurrence and levels in animal feces	+++	+	+
Occurrence and levels in municipal sewage	+++	+++	+++
Occurrence and levels in abattoir sewage	+++	+++	+
Probability of replication in environment	+/-	+/-	-
Resistance to inactivation in environment	++	+	+++
Resistance to disinfection			
Chlorination	+	++	+++
Ozonisation	ND	+++	ND
UV irradiation	+	+++	++
High energy radiation	++	+	++
Thermal treatment	+++	+	+++
High pH	+++	+	+++

Legend : +++, high ; ++, intermediate ; +, low ; -, very low ; ND, no data available.

Information extracted from reviews cited as references 12, 55, 69,70.

Somatic coliphages

Somatic coliphages are bacteriophages which consist of a capsid containing single- or double-stranded DNA as the genome. The capsids may be of simple cubic symmetry or complex structures with heads, tails and tail fibers. They are classified into the following families: *Myoviridae* (dsDNA, long contractile tails, capsids up to 100 nm), *Siphoviridae* (dsDNA, long non-contractile tails, capsids 50 nm), *Podoviridae* (dsDNA, short non-contractile tails, capsids 50 nm) and *Microviridae* (ssDNA, no tail, capsid 30 nm). All types are found in sewage, although the most abundant are *Myoviridae* and *Siphoviridae* (1, 105). Somatic coliphages attach to the bacterial cell wall and may lyse the host cell in 20-30 min under optimal conditions. They produce plaques of widely different size and morphology. The methodology to detect them is very simple and results may be obtained in 4-6 hours.

Natural host strains of somatic coliphages include, besides *Escherichia coli*, other closely related bacterial species. Some of these may occur in pristine waters, so somatic coliphages may also multiply in these environments even in *E. coli* (152). Indeed, one of the drawbacks of somatic coliphages is their replication potential outside the gut. However, the contribution of this potential replication outside the gut to their occurrence in natural environments seems, if any, rather low (104, 106).

The term somatic coliphages covers many types of phage with a wide range of characteristics, including differential resistance to inactivating factors. Moreover, different strains of *E. coli* as well as different assay media count different numbers and types of somatic coliphages (70). Consequently, the information available on both the presence and the behavior of somatic coliphages in water environments has to be interpreted very cautiously, since the data reported in the literature had been obtained with different host strains of *E. coli*, media and assay conditions. To avoid confusion, the term somatic coliphages, unless otherwise indicated, will be restricted to phages infecting *E. coli* C as established by Standard Methods (3).

Standardised methods for detecting and enumerating somatic coliphages are available (Standard Methods (3), ISO (5) and USEPA (142)).

Bacteriophages frequently used to study somatic coliphages behavior are T-even and T-odd, ϕ X174 and PRD-4.

F-specific RNA bacteriophages

F-specific RNA bacteriophages consist of a simple capsid of cubic symmetry of 21-30 nm in diameter and contain single-stranded RNA as the genome. They belong to the family *Leviviridae*. They infect bacteria through the sex pili, which are coded by the F plasmid first detected in *E. coli* K12. The F plasmid is transferable to a wide range of Gram-negative bacteria. The F plasmid does not form below 25 °C (61). Therefore the probability of these phages replicating in the environment is small (152). The infection process is inhibited by the presence of RNase in the assay medium, which can be used to distinguish between the F-specific RNA bacteriophages and the rod-shaped F-specific DNA bacteriophages of the family *Inoviridae*, which also infect the host cell, through the sex pili.

Strains (*Salmonella typhimurium* WG49 and *Escherichia coli* HS) tailored to detect F-specific bacteriophages also detect small percentages of somatic phages. All phages detected by the tailored strains are usually referred to as F-specific bacteriophages. The number of F-specific RNA bacteriophages is the difference between the number of phages counted in the presence and in the absence of RNase in the assay medium (61). More than 90% of the phages detected in sewage by tailored strains are F-specific RNA bacteriophages. This percentage may be lower in receiving waters.

A ISO standardized method for the detection and enumeration of F-specific RNA bacteriophages is now available (4). Results can be obtained in 12 hours. Bacteriophages frequently used to study F-specific RNA bacteriophages behavior are F2, MS2 and Q β .

Bacteriophages infecting *Bacteroides fragilis*

The most abundant bacteriophages infecting *Bacteroides fragilis*, one of the most common bacteria in the gut of warm blooded animals, belong to the family *Siphoviridae*, with flexible tails (dsDNA, long non-contractile tails, and capsids

up to 60 nm). Phages infecting *B. fragilis* attach to the cell wall of the host bacteria and may lyse the host cell in 30-40 minutes under optimal conditions. They produce clear plaques, which do not differ very much in size or morphology. *B. fragilis* strains differ widely in the numbers of phages that they recover from municipal sewage, but most strains recover bacteriophages that are very similar and belong to the family *Siphoviridae*. Bacteriophages infecting *B. fragilis* have not been reported to replicate under environmental conditions.

The method for detecting these bacteriophages is slightly more complex than methods for detecting the other groups because of the anaerobic nature of the host bacteria. However, a relatively simple ISO standardized method is now available (6). Results can be obtained in 18 hours. Recommended strain in the standardised method is strain RYC2056.

Bacteriophages frequently used to study *Bacteroides fragilis* bacteriophages behavior are B40-8 and B56-3.

2.3.2. Bacteriophages in raw sewage

Bacteriophages expected to be present in sludges and biowastes should be those present in raw sewage.

Bacteriophages of the three groups mentioned above are consistently present in raw sewage and sewage effluents. Consequently, sewage pollution will lead to contamination by the three groups.

Most reports indicate that somatic coliphages are the most abundant in raw municipal sewage, with values ranging from 10^6 to 10^7 PFU/100 ml, approximately less than one order of magnitude lower than the numbers of fecal coliforms or *E. coli* (10, 12, 13, 61). In addition, they are the most abundant in abattoir wastewater, with values that are similar to those found in municipal wastewater or values that keep the proportion to fecal coliforms as in municipal

wastewater (94, 109). Values from slurries of different animals are also similar (24).

Numbers in sewage effluents depends on the wastewater treatment. However, they are present in the great majority of sewage effluents.

F-specific RNA bacteriophages rank second in abundance in both municipal raw sewage and raw wastewater from abattoirs. The most frequent average values range from $5 \cdot 10^5$ to $5 \cdot 10^6$ PFU/100ml, usually about one order of magnitude lower than values for somatic coliphages (35, 36, 69, 78, 123, 128). The ratios between F-specific RNA bacteriophages and somatic coliphages are of the same order of magnitude in both raw municipal sewage and wastewater from most abattoirs. In some slurries (e.g. cattle) and specific abattoir wastewater, their relative abundance may be lower. Numbers in sewage effluent depend on the treatment, but these phages are always found in wastewater effluents.

Bacteriophages infecting *Bacteroides fragilis* RYC2056 are found in municipal sewage (Europe, Africa and America), frequently ranging from 10^4 to 10^5 PFU/100 ml, usually one order of magnitude less than F-specific RNA bacteriophages. Their ratio with respect to somatic coliphages and F-RNA bacteriophages is remarkably constant in municipal sewage (78, 88). Host strain VPI 3625 has been shown to recover approx. 10^4 PFU per 100 ml in the USA (55) while strain HSP40 detected approx. 10^4 PFU per ml in some geographic areas, but much lower values in other areas (9, 55, 56, 138).

In abattoir effluents, phage numbers detected by host strain RYC2056 range from 0 to 10^4 PFU/100 ml, but their ratios to somatic coliphages and to F-RNA bacteriophages are significantly lower compared to urban sewage (24, 116, 119).

Numbers in municipal sewage effluents depend on the treatment, but these phages are regularly found in wastewater effluents.

Numbers of the three groups of bacteriophages are fairly constant in raw sewage throughout the world, as are the numbers of bacterial indicators. Furthermore, bacteriophages of the three groups are consistently found in sewage effluents. On average, all the groups of phage are more abundant in raw sewage than most pathogens.

2.3.3.- Adsorption of bacteriophages to solids.

Bacteriophages had also been described to have a tendency to adsorb to solids (10, 11, 12, 47, 75). Also, it seems that there is no doubt that once adsorbed to solids they persist longer than when they are free (28, 29, 80).

Consequently, as viruses do bacteriophages are expected to concentrate in sludges sediments and soils, where they will persist longer than in the contaminated waters.

2.3.4.- Bacteriophages in sludges and biowastes

Studies on the abundance of bacteriophages infecting enteric bacteria in wastewater sludges (biosolids) are relatively scarce. Table 7 summarises the different bacteriophages and their densities reported for different sludges, biowastes and soils. First difficulty in interpreting the results is that there is a great diversity of sludges with a very poor definition of their characteristics. Second is that methods used to recover and enumerate the human viruses are very diverse and that results are expressed in very different ways. Therefore it is difficult to establish significant comparisons.

Table 7.- Bacteriophages in sludges and biowastes

	Primary sludge		References
Somatic coliphages	10^6 - 10^7	PFU ¹ /100 g DM ²	(93)
	10^6 - 10^7	100 g sludge	(78)
	10^7	100 g DM	(94)
	10^5 - 10^8	PFU/ 100 g TS ³	(67)
	10^7 - 10^8	PFU/100 g	(108)
F-specific RNA phages	10^4 - 10^5	PFU/100 g DM	(93)
	10^4 - 10^6	100 g sludge	(78)
	10^{3-4}	100 g DM	(94)
Phages of <i>B. fragilis</i>	10^6 - 10^8	PFU/100 g	(108)
	10^2 - 10^5	PFU/100 g DM	(93)
	10^4 - 10^5	100 g sludge	(78)
Activated sludge			
Somatic coliphages	10^5 - 10^7	PFU/100 g DM	(93)
	10^5 - 10^6	PFU/100 g	(78)
F-specific RNA phages	10^2 - 10^5	PFU/100 g DM	(93)
	10^4 - 10^5	100 g	(78)
Phages of <i>B. fragilis</i>	10^3	PFU/100 g DM	(93)
	10^3	PFU/100 g	(78)
Thickened sludge (primary + activated sludge)			
Somatic coliphages	10^4 - 10^7	PFU/100 g DM	(68)
F-specific RNA phages	10^2 - 10^3	PFU/100 g DM	(68)
Phages of <i>B. fragilis</i>	10^3	PFU/100 g DM	(68)
Dewatered digested sludge			
Somatic coliphages	10^5 - 10^6	100 g	(78)
F-specific RNA phages	10^3 - 10^4	100 g	(78)
Phages of <i>B. fragilis</i>	10^3	100 g	(78)
Compost (static pile)			
Somatic coliphages	120 (7 week)	PFU/100 g	(129)
	50 (10 week)	PFU/100 g	(129)
Compost (windrow composting system)			
Somatic coliphages	240 (3 week)	PFU/100 g	(129)
	500 (4 week)	PFU/100 g	(129)
Compost (natural draft system)			
Somatic coliphages	218 (7 week)	PFU/100 g	(129)
	0 (10 week)	PFU/100 g	(129)
Lime treated sludge			
Somatic coliphages	Control $1.5 \cdot 10^6$	PFU/10 g DM	(94)
	$1.3 \cdot 10^3$ (pH: 10.0)		
	$7.4 \cdot 10^2$ (pH: 11.5)		
	< 12 (pH: 12.0)		
	< 12 (pH: 12.0)		
F-specific RNA phages	Control $7.1 \cdot 10^4$	PFU/10 g DM	(94)
	< 12 (pH: 10.0)		
	< 12 (pH: 11.5)		
	< 12 (pH: 12.0)		

Legend: 1: PFU: plaque forming units ; 2: DM: dry matter ; 3: TS: total solids

Most data are on somatic coliphages, though there are some data regarding F-specific RNA bacteriophages and bacteriophages infecting *Bacteroides fragilis* as well. Phages of all the three groups are found in all samples of raw (primary) sludges, though the numbers given by the different authors are significantly different.

The accumulation of bacteriophages in both primary and secondary sludges is similar to that of bacterial indicators, and the ratios of such indicators to phages do not differ significantly from those in sewage (27, 92, 93, 109) as do not the ratios to viruses (Table 8). The three groups of bacteriophages seem to accumulate similarly (10, 105, 138).

Table 8. - Range of ratios between the densities of somatic coliphages and cytopathogenic enteroviruses reported in different publications

Sample	Enteroviruses PFU per 100 ml	Somatic coliphages PFU per 100 ml	Ratio
Sewage	10^{1-3}	10^{5-8}	10^4-10^5
Primary sludge	10^{2-5}	10^{5-8}	10^4-10^5
Activated sludge	10^{1-3}	10^{5-7}	10^4-10^5

However, sludges are usually subjected to additional treatment before released to the environment as biowastes. Phage concentrations in treated sludges depend on the treatment (94, 95).

2.3.5.- Bacteriophages in soils and sediments.

The tendency to be adsorbed to particles has been described to be similar for viruses and somatic coliphages (114). At least in waters with a high fecal pollution, the three groups of bacteriophages suggested as model microorganisms settle similarly (10). However, their survival in sediments is expected to be similar to that of viruses. Occurrence of phages in sediments

and their numbers suggest that as occurs with viruses they persist better adsorbed than free (10, 35, 36, 87, 111, 138).

As well, phages may adsorb to soil particles, when this has either been irrigated with contaminated water or amended with sludges or biowastes (49, 53). Data about adsorption of phages to soil are available, but they correspond to inoculated soil columns in the laboratory (145, 151). We have not found data on the occurrence of naturally occurring bacteriophages in soils.

2.3.6.- Persistence of bacteriophages during sludge or biowastes treatments

According to their destination, sludges receive different treatments, the most frequent being storage, digestion (aerobic and anaerobic, mesophilic and thermophilic) and, to a lesser extent, disinfection by pasteurization, irradiation, lime treatment or composting. Data on reductions of numbers of phages in sludges and biowastes after different treatments is summarized in table 9.

The survival of bacteriophages in sludge during storage depends on the temperature (72), as occurs for human viruses. At 4 °C all survive quite well, whereas at higher temperatures F-specific bacteriophages inactivate more rapidly than somatic coliphages and bacteriophages infecting *Bact fragilis*. The difference in decay rates becomes significant at 37 °C (95).

Different kinds of digestion processes are used to reduce the amount and improve the quality of sludges. Bacteriophage f2 has been reported to survive more successfully than enteroviruses and rotaviruses to both mesophilic and thermophilic digestion.

Table 9.- Elimination of bacteriophages during sludge treatments

Treatment		Group of bacteriophages	Log ₁₀ reduction	References
Anaerobic mesophilic digested		Somatic coliphages	~ 1	78
		F-specific RNA phages	~ 3	78
		Phages of <i>B. fragilis</i>	1 - 2	78
Composting		Somatic coliphages	4 - 6	
Lime treatment	pH 10.0	Somatic coliphages	~ 3	94
	pH 11.5		~ 4	
	pH 12.0		4.7	
	pH 12.0		~ 5	
	pH 10.0	F-specific RNA phages	> 4	
	pH 11.0		> 4	
	pH 12.0		> 4	
Thermal treatment 80°C	30 min	Somatic coliphages	0.6	95
	90 min		3.0	
	30 min	F-specific RNA phages	1.3	95
	90 min		> 3.4	
	30 min	Phages of <i>B. fragilis</i>	0.3	95
	90 min		>2.7	

2.4.- Most suitable bacteriophages to be used as indicators in sludges, biosolids and soils

According to the previous information and the feasibility of the detection methods described below it seems that at present the best candidate group of phages is the group somatic coliphages, being somatic coliphages those detected by the host strains used in the ISO and USEPA methods.

Table 10 summarises some of the properties that make the somatic coliphages the best option.

Table 10.- Summary of characteristics of the different groups of bacteriophages

	Somatic coliphages	F-specific RNA phages	Phages of <i>Bact. fragilis</i>
Abundance in sludges	+++	++	+
Standardized methods	+++	+++	+++
Feasibility of detection	+++	++	++
Time needed for results	4- 6 h	8-12 h	18-24 h
Resistance to heat	+++	++	+++
Resistance to high pH	+++	++	+++

Legend: +: bad; ++: regular ; +++: good

3. Methods for detecting and enumerating phages (viruses) in sludges, biowastes and soils

Thirty six papers dealing with methods for detecting bacteriophages in sludge, biosolids, soil and sediments had been detected. Most of these papers contain very little information regarding many of the questions that we need to address in order to fix the protocols that we should settle. This is probably the results of the pressure that the authors of scientific papers receive to make the publications as short as possible. As well the present information indicates that most of the methods are based in the same principles, but that there is a lot of variability in the details.

3.1. Aspects that need to be addressed

There is a need of exact (high trueness) (84), precise (84), robust (84) and feasible methods for detecting bacteriophages in different kinds of sludges, biowastes and soils. The scientific literature reviewed reveals a great dispersion of methods in the studies performed so far. However, as stated above there are some trends common to most of the methods used so far. Generally speaking

the processes followed to detect bacteriophages follow the same steps as methods described for detecting viruses.

The steps for phages (and virus) detection from sludges, biowastes and soils are the following: sampling, conditioning of samples, extraction (elution), concentration, decontamination and detoxification and detection. Though there are some major issues as for example phage extraction and phage detection, all the above mentioned steps have to be considered when establishing a method for detection of phages (and viruses).

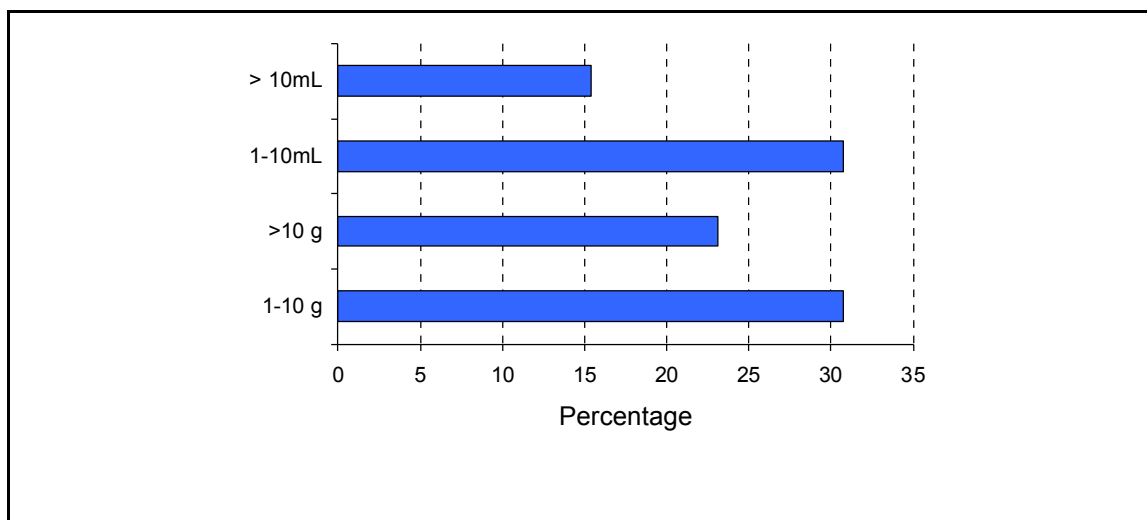
For cytopathogenic enterovirus a standardized method has been approved by USEPA (142) and a AFNOR draft is being prepared in France (7, 8).

3.1.1. Sampling and handling samples

Sampling. Sampling sludges, biowastes and soil is of major importance taking into consideration the heterogeneity of the materials sampled. Sampling should theoretically be identical for phages and viruses. Scientific information about the sampling for testing phages (viruses) in sludges, biowastes and soil is very scarce. However, it is assumable that the same sampling methods as for other parameters should be applied. Assuming this, several standardised methods are available (ISO5667-13.1997; ISO5667-17, 2000; ISO 5667-19, 2004; EPA/625/R-92/013).

Size of the sample. Regarding the amount of sample that is representative it arises that even when a smaller sub-sample will be processed further, it seems advisable to take samples big enough to be representative (10 to 25 g). The scientific literature for both bacteriophages and viruses offers quite disperse data. For viruses the amount indicated in the only standardised method available so far USEPA (142) states 1000 ml (joining ten samples of 100 ml each). For bacteriophages there is some dispersion in the scientific literature reviewed (Figure 1). In any case 10 g (151) seems to be the minimum amount of the subsample to be tested.

Figure 1.- Amounts of sludge samples processed in the different reports (data expressed as % of publications, number of publications studied= 23)



It seems essential to determine how to minimize the heterogeneity of the sample or how to determine the representative sub-sample. However, this is a problem concerning all microbial parameters.

Preservation of the samples. Preservation and transport of the samples should be similar for viruses and phages and also for other microbial parameters and there are some standardised methods (ISO 5667-15, 1999). However, from the desk study it arises that bacteriophages survive quite well in natural samples at 4°C for extended periods of time (up to 72 to 96 hours) (11, 20, 33, 41, 65, 76, 78, 125, 138). This is an additional advantage of bacteriophages and it is recommended to study this extended survival, since it adds extra value to bacteriophages as potential indicators.

3.1.2. Amendment of the sample.

Obviously sludges, biowastes and soils require a suspension in a buffer plus homogenization prior to determining the amounts of microorganisms to be studied, both by means of cultural and molecular methods.

The review of the scientific literature regarding methods used to determine bacteriophages and viruses reveals that there are some differences between the methods to determine bacteria and the methods to determine

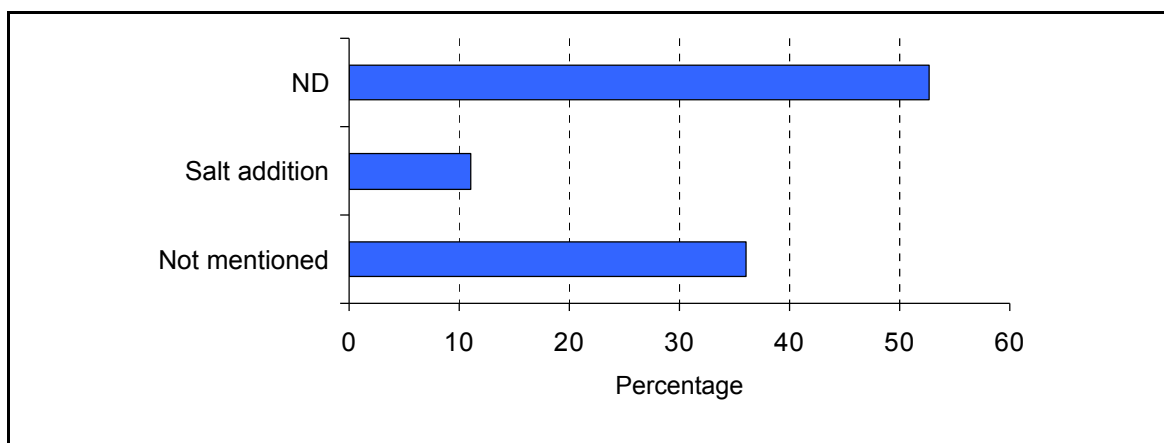
bacteriophages and viruses. Bacteriophages and viruses had been described to be adsorbed to solids, and consequently they should be extracted from solids before analysis (10, 41, 53, 78, 93, 101, and 151). Two steps are needed prior to extraction. These are salting and separation of the solids.

Salting.

This concept refers to the addition of mono, di or trivalent cations to the sludge to favour the adsorption of the viruses suspended in the water to the solid fraction before the extraction step. Salting, and addition of cations has been extensively studied for the recovery of enteroviruses from sludges (28, 65, 78, 94). It has been included in the USEPA standardised method for recovery of viruses from sludges (142). In spite of the wide acceptance of salting for the recovery of viruses, it is difficult to determine whether salting improves substantially the method since because of the low numbers it is difficult to precise which percentage of viruses are in the aqueous and solid phages of sludges, and because studies performed with spiked viruses will not reflect the real world.

For bacteriophages, salting has not been extensively used. Only a 11 % of the consulted publications had used this approach, and in the different papers they have used different cations, with non conclusive results on the need of salting, figure nº 2 (10, 78, 94).

Figure 2.- Salting procedures in the different reports (data expressed as % of publications, number of total publications studied = 36)



Legend: ND: information regarding solid separation non specified .

In a preliminary study done in our laboratory to gain information in this topic we have detected about only a 10% of the somatic coliphages present in a primary sludge to be non-adsorbed to particles (data not shown).

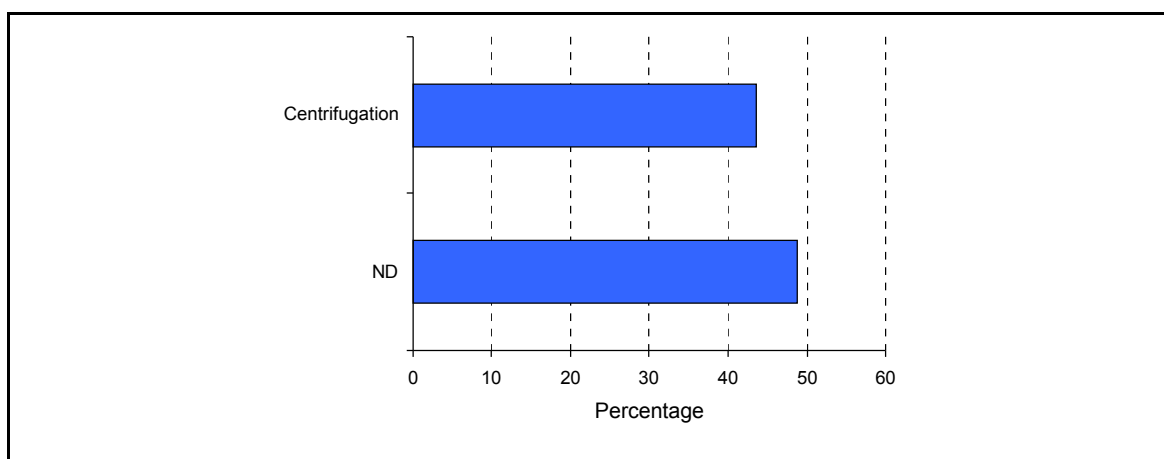
Separation of solids previous to the bacteriophages (viruses) extraction.

The information on viruses indicates that most methods include this step (10, 34, 41, 59, 78, 93, 101); and consequently this step has been included in the standardised methods for enteroviruses USEPA (142).

Regarding bacteriophages (figure 3), 45% of papers related to this topic report a separation of the solids before the extraction procedure (10, 11, 14, 33, 34, 48, 64, 73, 76, 78, 93, 94, 95, 137, 138), though comparative studies are not available. In all the reports the separation was done by centrifugation, ranging from 1.000 to 15.300 *g*.

In the same preliminary study mentioned above most somatic coliphages in a primary sludge were in the solid fraction. Then, to equal the processing of these samples to the non-dewatered sludges, biowastes and soils, it seems recommendable to centrifuge the sludge in order to separate the solid fraction, which will then be processed for the extraction of bacteriophages. Centrifugation ranging from 1.500 *g* to 5.000 *g*.

Figure 3.- Separation of solids (applies to non-dewatered sludges) (data expressed as % of publications, number of total publications studied = 35)



Legend: ND: information regarding solid separation non specified .

3.1.3. Extraction of bacteriophages (and viruses) from sludges, biosolids and soil

All the procedures reported regarding procedures to detect and enumerate viruses and bacteriophages from sludges, biosolids and soil include an extraction procedure. This extraction implies suspension or re-suspension of the solid sample in a convenient eluent solution, homogenization-elution and clarification (separation of solids).

No information has been detected about tries to detect either viruses or phages directly from homogenized material. It may be interesting to make a try for direct detection of bacteriophages without the elution step, since it will facilitate the methodology, though experience with water with high contents of particulate material and background bacteria seem to indicate that direct detection without extraction will not be a good approach.

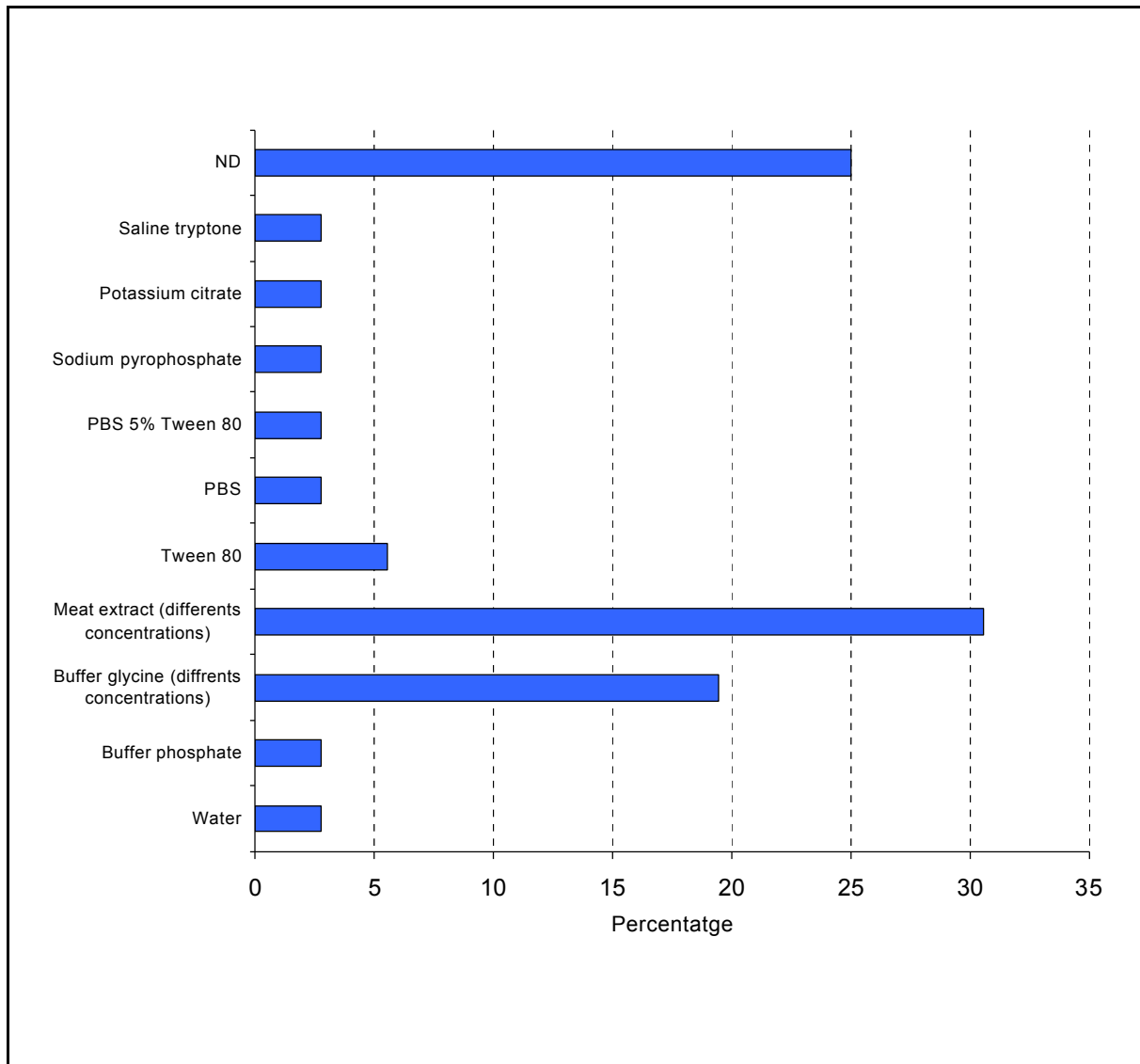
Suspensions in the eluent solution.

For both bacteriophages and viruses there are two major issues to be considered. First, the eluent solution and second the ratio solids suspended to eluent solution.

Eluent solution. For both viruses and bacteriophages there is a great dispersion of eluents used, though with variations all of them are based on either a solution of beef extract, or a solution of glycine buffer. However as said before there is a great variation regarding concentration of these two compounds, pH and accompanying substances.

Figure 4 summarizes the variability found in the literature regarding the eluents used for the recovery of bacteriophages.

Figure 4. Different eluents used for the recovery of bacteriophages (data expressed as % of publications, number of total publications studied = 36)



Legend: ND: information regarding eluent non specified.

All this information has lead to the recommendation of 10 % beef extract and pH 7 in the standardised methods available for viruses USEPA (142).

Ratio solids suspended to eluant solution.

As with the eluent solutions used there is a great variability in the ratio solid sample: eluent solution used for both the elution of viruses and bacteriophages. In both cases the ratio 1 (wet weight):10 (volume) is the most frequently reported.

Time of contact.

Again in the literature studied there is a great variability of the time of contact between the eluent solution and the sample, with times ranging from 10 minutes to 30 minutes (11, 14, 41, 76, 78, 93, 108, 138, 145, 151). Frequently the time of contact depends on the pH of the eluent solution. Neutral eluents allow longer contact times.

The USEPA standardised method for viruses fixes a 30 minutes contact time (142).

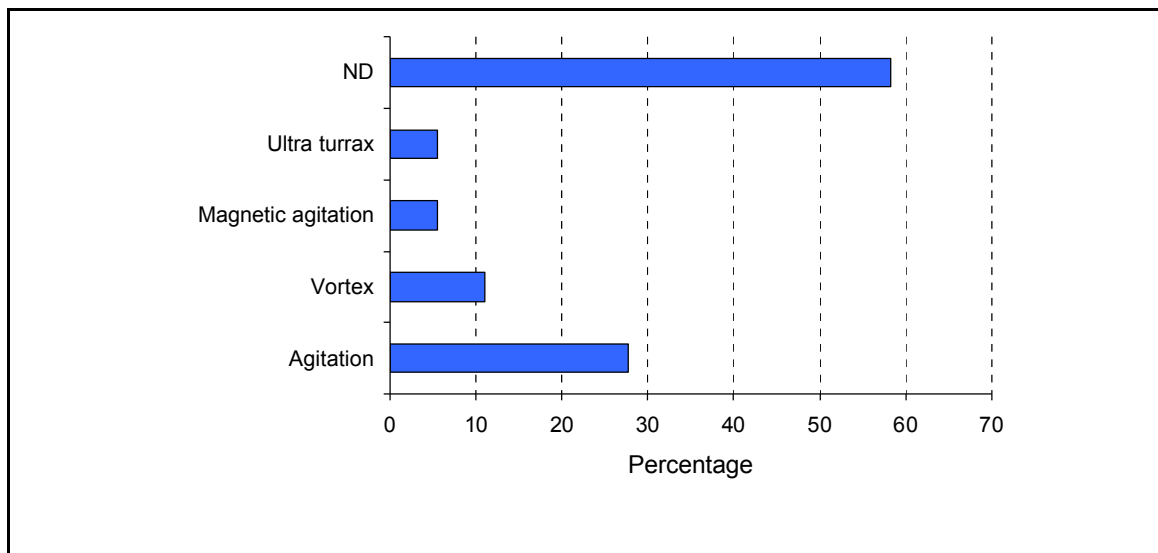
Homogenization

Sludge, treated biowastes and soils contain particles that differ in size and composition. Pathogens and indicators may be either adsorbed to the surface of these particles or included in the particles. The question arising is whether after suspension of the solids in the eluent solution we should intend to minimize the heterogeneity of the size of the particles, and if so how this homogenization should be done and whether a maximum size of the particles after homogenization be guaranteed. In this case the homogenization coincides with the elution period.

The critical review reveals again for both viruses and bacteriophages a great variability in this process. Moreover, many publications do not give many details about this phase of the process. Magnetic stirring, blending, orbital shaking, homogenization using ultraturrax, etc, had been reported (11, 14, 41, 76, 93, 108, 137, 145, and 151).

Magnetic stirring is the homogenization procedure indicated in the USEPA standardised method available for viruses (142). Figure 5 gives an indication of the diversity of procedures reported for phages.

Figure 5. Different kind of homogenization used for bacteriophages extraction (data expressed as % of publications, number of total publications studied =36)



Legend: ND: information regarding homogenization non specified .

In some procedures, ultrasonication is applied as an additional treatment to the sample to improve phage recovery (14, 93, 94, 151), although this seems a procedure that may make difficult the standardisation of the procedures (60).

Clarification (separation of solids)

The great majority of described methods for detection of viruses and bacteriophages include a separation of solids from the eluent solution where the bacteriophages and viruses are expected to be after the extraction step.

For viruses, decantation, centrifugation and filtration had been reported though centrifugation is the most frequently used 1.500 – 5.000 *g*. The standardised method (USEPA) for viruses includes centrifugation at 10.000 *g*, 4 °C, and 30 minutes (142).

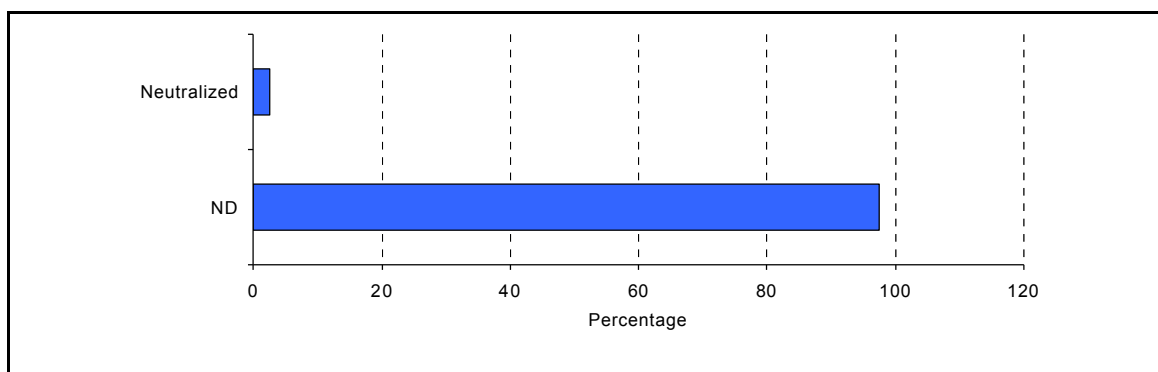
For bacteriophages only centrifugation has been reported. There is again a great variation regarding *g* and time. Centrifugation speeds ranging from 1.000 *g* to 15.300 *g*, had been reported and times ranging from 3 to 30 minutes had been reported (10, 11, 14, 33, 48, 73, 78, 93, 94, 95, 137, 138). The centrifugation speed may be a limiting factor depending on the centrifuges available in the laboratories. It seems that 4.500-5.000 *g* is the maximum speed

that low grade centrifuges can reach. Time does not seem to be an important factor if the sample is at neutral pH and the centrifuge is refrigerated and consequently centrifugation time may be increased.

Neutralization

Articles which describe neutralization concentrate at pH 7 (10, 14, 93 and 137). However, only 5% of the publication studied report a neutralization step (see figure 6).

Figure 6. - Neutralization used for bacteriophages concentrate (data expressed as % of publications, number of total publications studied = 36)



Legend: ND: information regarding neutralization non specified.

3.1.4. Secondary concentration

All methods described for viruses include a concentration of the viruses present in the clarified eluent solution containing the eluted viruses. Organic flocculation (74), adsorption-elution (44), ultracentrifugation (42, 46) and PEG (poly-ethyleglycol) precipitation (82, 83) had been reported. The standardised method USEPA (142), recommends organic flocculation (74).

Methods described so far for bacteriophages obviate this step. According to the numbers of phages (somatic coliphages) reported in different biosolids described above, this step does not seem necessary. However, if necessary, methods to concentrate bacteriophages from aqueous solutions are available (76).

3.1.5. Decontamination and detoxification

Decontamination appears as an indispensable step in the process of detection of both infectious viruses or viral genomes (genomic methods). Filtration, through 0.22 or 0.45 μm pores size membrane filters (34, 95, and 151), treatment with chloroform (93, 108), contact with antibiotics (146), ultracentrifugation (62) and dialysis (66) had been reported.

Filtration through membranes of 3.0, 0.45 and 0.25 μm pore size is the method indicated in the USEPA standardised method, for decontamination of the viruses suspensions after the extraction and secondary concentrations steps (142).

Regarding bacteriophages, this step is not mentioned in the great majority of reports, most probably because decontamination was not done. Standardised methods for the detection of bacteriophages, mostly settled for waters, can be applied without decontaminations of the sample. However, some reports describe the use of trichlorotrifluoroethane (14), chloroform (93, 108) or membrane filtration (34, 95, 149) to decontaminate the suspensions of phages extracted from sludges before phage analysis.

It seems that the need of decontamination will depend on the biosolid tested and on the extraction method used. Probably, an extraction method performed nearby neutral pH will make recommendable a decontamination step.

Summary on methods for extracting phages from sludges and biowastes.

the desk search for methods to detect bacteriophages in sludges, only two reports compare methods (78, 93), though considering the methods tested in the 2 papers almost all methods described so far were compared. The best performing methods in the two studies are relatively similar, and both use a solution of beef extract as eluting solution. These methods can be summarised as follows:

Method 1

Described by Ahmed and Sorensen (1995) for the extraction of *B. fragilis* phages from sediments. A sludge volume providing 5 g (dry matter) was added with 45 ml 10% beef extract (OXOID, LP029B) at pH 9 and stirred at 500 rpm for 30 min. The mixture was sonicated on ice (100 W; 0.9 s) for 5 min-1 min steps, mixed again for 5 min and then centrifuged at 5000 g for 1 h at 4°C.

The supernatant, neutralized at pH 7.2, constituted the extract that was assayed for phages without any further decontaminating treatment.

Method 2.

Modification of that of Williams & Hurst (1988) for the elution of somatic coliphages from sludges. Briefly, each sample was centrifuged at 1.400 g for 15 min and the supernatant fluid discarded. The sludge was resuspended using a volume of a solution of 10% beef extract in water, pH 7.0. The volume of beef extract solution used for suspending the sediment was equal to 10 times the sediment volume. After 15 min of magnetic stirring, the suspension was centrifuged at 10.000 g at 4 °C for 30 min. The supernatant fluid was then assayed for phages without any further decontaminating treatment.

3.1.6. Detection

Whereas the methods to extract bacteriophages and viruses from biosolids result to be very similar the detection methods are different as a consequence of the need of specific hosts for growing animal viruses, which need animal cells to grow, and bacteriophages that need bacteria.

In the other hand the potential use of genomic methods will equalize the methods. However at this stage and taking into consideration the need to detect infectious microbes in order to evaluate the effect of treatments it seems not recommendable to opt for genomic methods.

Viruses

Methods to detect culturable viruses, had only been reported for enteroviruses (16, 38, 58, 63, 79, 96, 107, 113, 127), rotaviruses (25, 82), hepatitis (71, 103) and adenoviruses (32, 79, 139). The great majority of descriptions refer to cytopathogenic enteroviruses, usually detected on BGM cells (38). Reported results for enteroviruses in sludges refer to presence/absence methods (25,93), presence/absence applied to the most probable number format (25, 93) and plaque forming test measured by the monolayer technique (38). In fact they are the same methods used for water analysis.

The standardised methods available for enteroviruses, USEPA and AFNOR, include the plaque assay on a cell monolayer (7, 8, 142).

A few reports have been detected that apply genomic methods for the detection of enteroviruses (57, 97, 99), rotaviruses (71) and astroviruses (31). The application of the CC-PCR approach may circumvent the problem of detection of infectious viruses. However up to now all the CC-PCR approaches are complex and long lasting (many days) approaches (32).

Bacteriophages

All the published investigations on bacteriophages in sludges, biowastes and soils have used the quantitative approach, applying the double layer technique.

In this case we have not found a simple report where the presence/absence approach (frequently used for drinking waters) has been applied.

Though there is some variation in the methods used in different investigations, it is becoming more and more frequent and recommendable to use standardised methods for the evaluation of bacteriophages in aqueous solutions. Table 11 summarises the available standardised methods for the detection and evaluation of phages.

Table 11. - Standardised methods for detection of bacteriophages

	US EPA		Standard methods ³		ISO ⁴	
	P/A ¹	Quantification ²	P/A	Quantification	P/A	Quantification
Somatic coliphages	+	+	-	+	+	+
F-specific RNA phages	+	+	-	-	+	+
Phages infecting <i>Bacteroides fragilis</i>	-	-	-	-	+	+

Legend :

1. USEPA method 1601, (143).
2. USEPA method 1602, (144)..
3. Standard methods for the examination of water and wastewater. 20th ed., 2001, (3).
4. ISO 10705-2 (2000) (4), ISO 10705-1 (1995) (5), ISO 10705-4 (2001) (6).

Both the USEPA and the ISO methods give similar results. However attending to the international character of ISO and the strong contact with CEN, we recommend the ISO methods.

Regarding genomic methods, the great heterogeneity of somatic coliphages (105) may difficult the application of genomic methods to somatic coliphages. At the present stage RT-PCR has been applied to detect F-specific RNA phages in water (121), and their application to *Bacteroides fragilis*, taking into consideration the apparent homogeneity of this group of bacteriophages (119) and reported results on the detection of a precise group of phages infecting *Bacteroides* (117, 118), seems feasible to apply genomic methods. However as in the case of viruses it does not seem that the results contributed by genomic methods will be useful for many purposes. Moreover in the case of bacteriophages the simplicity of methods detecting infectious phages supports their use. Some of them, mostly the method for the detection of somatic coliphages, indeed provide results in 4 to 6 hours.

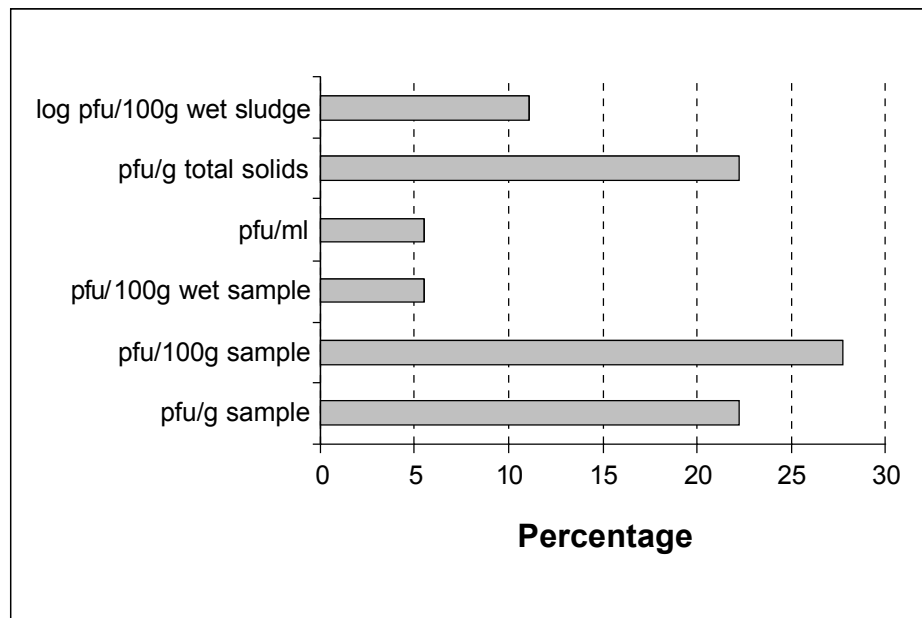
3.1.7. Expression of results

Regarding viruses in the literature reviewed there is a mix of results given in terms of presence/absence and plaque forming units (PFU), or the most probable number of cytopathogenic units (MPNCU) or tissue culture infectious dose (TCID₅₀) when numbers are given. The standardised method (USEPA) requires the results to be expressed as PFU per 4 g of dry solids.

For phages, only quantification by PFU is reported in the literature reviewed.

In the other hand both for viruses and bacteriophages there is a great diversity with regard to the amount and characteristics of the sample, since some refer to mass or volume of the sample, but when they refer to mass they do not indicate whether they refer to wet or dry mass. In figure 7, it is shown the diversity detected in the expression of the amount of sample.

Figure 7. - Different results expressions (data expressed as % of publications, number of total publications studied = 18)



It seems convenient to reach an agreement as how to express the results for all the microbial parameters studied in the HORIZONTAL (perhaps per g of dry matter or per g of dry mass).

3.1.8. Conclusions

3.1.8.1. Conclusions of the desk study

The conclusion of the desk study is that at this moment there is not any available standardised method for the study of bacteriophages in sludges, soil and treated biowastes. On the contrary standardised methods for the detection and enumeration of bacteriophages as well as the most fitting bacteriophages had been clearly identified.

However different methods had been found in the scientific literature regarding the extraction of phages from sludges, soil and treated biowastes. All methods share the same steps: Conditioning of the sample, elution, concentration (optional), decontamination (optional) and titration.

Only two papers comparing methods were found (78, 93). The best performing methods in the two studies are summarised below. They are based in the same principles and are quite similar.

Method 1

Described by Ahmed and Sorensen (1995) for the extraction of *B. fragilis* phages from sediments. A sludge volume providing 5 g (dry matter) was added with 45 ml 10% beef extract (OXOID, LP029B) at pH 9 and stirred at 500 rpm for 30 min. The mixture was sonicated on ice (100 W; 0.9 s) for 5 min-1 min steps, mixed again for 5 min and then centrifuged at 5000 g for 1 h at 4°C.

The supernatant, neutralized at pH 7.2, constituted the extract that was assayed for phages without any further decontaminating treatment.

Method 2.

Modification of that of Williams & Hurst (1988) for the elution of somatic coliphages from sludges. Briefly, each sample was centrifuged at 1.400 g for 15 min and the supernatant fluid discarded. The sludge was resuspended using a volume of a solution of 10% beef extract in water, pH 7.0. The volume of beef

extract solution used for suspending the sediment was equal to 10 times the sediment volume. After 15 min of magnetic stirring, the suspension was centrifuged at 10.000 g at 4 °C for 30 min. The supernatant fluid was then assayed for phages without any further decontaminating treatment.

However, the desk study revealed that there are still several aspects regarding conditioning of the sample, elution, clarification and decontamination that need to be addressed.

3.1.8.2. Conclusions of the workshop

Taking into consideration the conclusions of the desk study and the results of the enquiry-questionnaire performed among the participants to the European Horizontal-Hyg workshop, (annex 1), the conclusions that follow in table 12 were agreed during the workshop held in Lille (France) in April 2005.

Table 12. – Conclusions on the draft standardised method to be applied for the enumeration of bacteriophages in sludges, soils and treated biowastes.

ISSUE	QUESTIONS / PROPOSALS	AGREEMENTS
Identification of suitable target bacteriophages	Somatic coliphages F-specific RNA bacteriophages Bacteriophages of <i>Bact fragilis</i>	Somatic coliphages However, in our opinion we should consider whether we settle a method for somatic coliphages or for all bacteriophages.
Analysis	Presence/absence ? Quantitative ?	Quantitative
Sample preparation	10- 20 g (wet matter)? Estimation?	10 g of wet weight
Sample storage and transport (conservation)	Temperature: From 5 ± 3 °C Storage: From 24 to 96 hours.	Temperature: From 5 ± 3 °C Storage: <48 hours with exception for chemical treatment (lime) which should be neutralised but following recommendations of working groups.

Treatment of the sample before extraction (?)	Addition of mono, di or trivalent cations (applies to non-dewatered sludges)? Separation or not of solids (applies to non-dewatered sludges)?	No addition of salts Centrifugation (liquid samples)
Extraction of bacteriophages	Suspend or re-suspend the solid fraction in eluting solution (which and ratios ?) Different degrees of homogenization of the suspension (which homogenization and time of contact?)	Suspend the solid fraction in eluting solution Agitation
Eluting solution	Beef extract and glycine buffer? Beef extract at two pHs? Which pHs?	Beef extract 10% pH 7 (to be compared to pH 9)
Ratio sample:eluent (w/v)	Ratio 1: 10?	Ratio 1:10
Agitation	The same homogenization as for bacteria? Time of homogenization-elution?	The same homogenization as for bacteria: stomacher (to be compared to orbital or wrist shaking) Time of homogenization-elution? To be tested
Solid separation	Decantation or centrifugation?	Centrifugation (several assays to be done at different <5000 g)
Sample neutralization	If necessary	If necessary (<u>only if elution at pH9, not if elution at pH7, yes at pH9</u>)

Concentration	Yes or not? If yes, should we do P/A tests	No
Sample decontamination	Yes or not? If yes, how?	Filtered and non-filtered will be compared
Detection methods	ISO quantitative?	Yes
Analytical method and results for bacteriophages	Somatic coliphages: ISO 10705-2:2000. F-specific RNA bacteriophages: ISO 10705-1:1995. Bacteriophages of <i>Bact. fragilis</i> : ISO 10705-4:2001.	ISO 10705-2:2000
Expression of results - Sludge	Referred to dry weight? Referred to 1, 10 or 100 g	To be discussed
Samples to test	Spiked "versus" non spiked. Spike does not seem adequate (different reasons). The most varied possible, and containing enough amount of naturally occurring bacteriophages.	Non spiked Sludge, dewatered sludge, compost, contaminated soils with sewage
Is it feasible to prepare reference materials for quality control	Yes for methods of quantification of phages. (?) for extracting methods. Can we?	Yes Yes

3.1.8.3.- Proposed methods

Only a main method arised. However variations in several steps were seen as necessary. Summarising, the method and variations to be tested are:

1. Take the sample (10 g of wet weight) : For samples with low content of dry matter (non-dewatered sludges) centrifuge and discard the supernatant.
2. Suspend or resuspend the sample or the sediment (1/10 w/v) in 10% beef extract
 - 2.1. pH 7.0
 - 2.2. pH 9.0
3. Homogenization
 - 3.1. Magnetic stirring
 - 3.2. Ultraturrax
 - 3.3. Laboratory shaker. Flask Shaker with two side-arms.
 - 3.4. Stomacher
4. - Neutralize to pH 7.0 (this step only in the case of eluting at pH 9.0)
5. - Clarification step.
 - 5.1. Decantation
 - 5.2. Centrifugation at 5000 x g . Discard sediment
6. - Decontaminations step
 - 6.1. Without any decontamination
 - 6.2. Filtration through 0.45 pore size non-protein binding membranes.
7. Titration. Titration of somatic coliphages following the ISO 10705-2 (2000) standard.

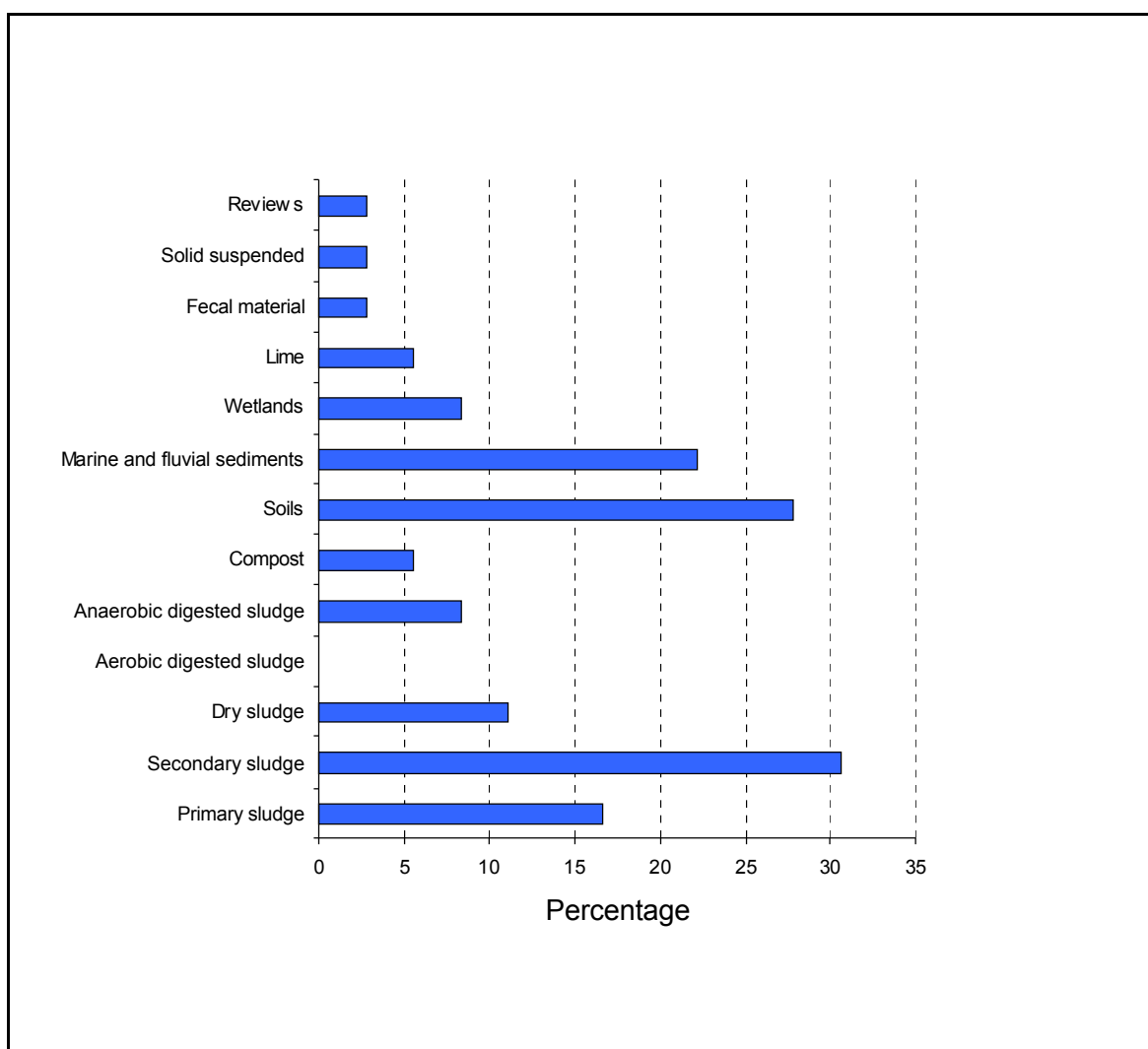
The still open four steps will be tested before and during the suitability study (ruggedness trial) scheduled as following step of the Horizontal-HYG project, in order to identify the optimal procedure to be proposed as horizontal draft standard.

3.2.- Kind of samples to be used to settle the method

First emerging question is whether it will be acceptable to spike samples as it is possible to do with water in order to settle the method. Though there are a few reports in which bacteriophages had been spiked, it does not seem recommendable to use spiked samples after considering all the information available regarding the adsorption of the viruses and phages to solids and to the complexity of the matrixes found in different sludges, biowastes and soils.

Studies to determine the presence of viruses and bacteriophages reveal a great diversity of samples studied. Figure 8 shows the distribution of the studies done with phages.

Figure 8.- Distribution of the studies done with bacteriophages (data expressed as % of publications, number of total publications studied = 58)



Considering this information and the numbers of phages reported to be present in different kinds of treated and untreated sludges and biowastes as well as in soil, we have detected a few sludges and biowastes (untreated sludge, treated sludge, compost) that contain a number of phages great enough for these studies and that represent the different matrixes that can be found among sludges and biowastes.

In the case of soil, it seems possible to make an experimental contamination with sewage that will allow having contaminated soil.

3.3.- Is it possible to prepare reference material for the validation of the methods?

The question is whether it will be possible to use reference materials while working with bacteriophages and viruses in sludges, biowastes and soils.

It is possible to prepare reference material for the quality assurance of the methods to detect bacteriophages (9, 91, 100), and in fact they have been extensively used (88). There are also reference materials for bacteria (85).

However, we have not found any report with information referring to the use or availability of material for the full process of detection of bacteriophages (viruses) in sludges, biowastes and soils.

Some preliminary results in our laboratory (data not published) indicate that it is feasible to prepare some biosolid samples containing naturally occurring somatic coliphages to be used as references materials for a few laboratories and a short period of time. These materials will only be useful for comparing the performance of the different extraction methods in different laboratories. However these materials will not allow determining the efficiency of the method.

NOTE. - Without the possibility to spike, it will not be possible to establish the efficiency of the methods; we will only have the best method available. However the ratios among the numbers of bacteriophages found in sewage and sludges seem to indicate that the efficiency of the method is quite high.

4. - CONCLUSION

The critical review work, based on the numerous scientific publications studies, the available standardised methods identified and the consultation of experts (questionnaire and workshop) allowed to make some fruitful conclusion and to identify methods to be applied during the following step of suitability study of the Sub WP2/4 of the HORIZONTAL-Hyg project: Methods for bacteriophages (and viruses) to be monitored in EU in sludges, soils and treated biowastes.

Somatic coliphages detected by the ISO standard methods were identified as the target bacteriophages. A method based suspension of the biosolids in 10% beef extract ~~for~~ as eluent, homogenization, and neutralization if necessary, clarification, decontamination and phage titration will be fixed once different variations in several critical steps (pH of the eluting solution, homogenization, and need of neutralization, clarification and decontamination steps) identified as the best.

As well the feasibility of preparing homogeneous natural (non-spiked) reference material to be used in the following suitability study (ruggedness test) step of the study horizontal-HYG project to be performed in different laboratories will be tested.

ACKNOWLEDGEMENTS

The authors thank to the collaborators Andrey PAYAN, Michel MONTEMAYOR, Ana COSTAN and Carolina GUZMAN for their collaborations in this study.

Annex I

Synthesis of the responses received from European experts to the Horizontal-Hyg Questionnaire sent before the Horizontal-Hyg Workshop of 19-21st April 2005 in Lille - Bacteriophages and viruses in sludge, soils and treated biowastes

(7 Full filled Questionnaires received from 4 EU countries: Cyprus (1 laboratory), France (3 laboratory), Germany (1 laboratory) and Spain (1 laboratory); and South Africa, (1 laboratory).

REGULATION

A1. Is the enumeration of bacteriophages and viruses in sludges, soils and treated biowastes required by your national legislation?

Enumeration of viruses (enteric viruses) is only required in the French legislation

A2. If yes, is there a (standard) method required by your legislation?

No standard methods are required

A3. If yes, is there a maximum level required by your legislation?

Maximum level required by French legislation is <3 MPNCU / 10 gr dry matter

SAMPLE STORAGE AND TRANSPORT

B1. Is there a regulation for the maximum length of time allowed before analysis commences? Temperature of storage etc. before analysis ?

- Temperature: From 5 ± 3 °C
- Storage: From 24 to 96 hours

PREPARATION OF SAMPLE

C1. Which amount of sub-sample do you analyse (specify equivalent dry weight or wet weight)?

- 10g dry matter (4/7)
- 25g wet weight (1/7)
- 20 g wet weight (1/7)
- 1-10 mL (1/7)

C2. Which method do you use to homogenize the sample (please include a brief description of methodology)?

- Shaking + Sonication (1/8)
- Ultraturrax + Sonication (1/8)
- Stomacher homogenization (1/8)
- Rotative homogenization (1/8)
- Shaking (from 30 min to 20 hours) (2/8)
- Vortex mixer (3 to 5 min) (2/8)

C3. Is the pH checked during this preparation? If yes, any special treatment in case of low or high value?

- Use of an elution buffer at pH 9.6, and further adjustment to pH 7,2 (1/7)
- No answer (6/7)

ANALYTICAL METHOD AND RESULTS FOR BACTERIOPHAGES

D1. Which method do you use to extract the bacteriophages?

- Beef extract 10%, pH 9 (2/7)
- Peptone 0.01% + homogenize + centrifugation (1000g 5 min) (1/7)

D2. Which type of bacteriophages do you evaluate?

- Somatic coliphages (6/7)
- F-specific RNA bacteriophages (5/7)
- Bacteriophages of *B. fragilis* (5/7)

D3. Which method do you use to evaluate the extracted bacteriophages?

- Somatic coliphages: ISO 10705-2:2000 (6/7)
- F-specific RNA bacteriophages: ISO 10705-1:1995 (5/7)
- Bacteriophages of *B. Fragilis*: ISO 10705-4:2001 (5/7)

D4. What is the total analysis time of the method?

Depending on bacteriophages from 24 to 48 hours

D5. What media do you use for analysis?

- ISO mediums (3/7)
- Tryptone soya agar-CMO131 (1/7)
- No answer (3/7)

Is the media ready made from the manufacturer or is it prepared in-house?

- Ready made media: 2 laboratories (2/7)
- In house media: 4 laboratories (4/7)

ANALYTICAL METHOD AND RESULTS FOR VIRUSES

E1. Which method do you use to extract the viruses?

- Beef extract 10% pH 9 (2/6)
- Beef extract 10% pH 9 + PEG concentration (1/6)
- Sonication + Centrifugation + PEG 6000 concentration (1/6)
- Freezing/thawing + Sonication + PEG 6000 concentration (1/6)
- Sonication + Centrifugation + Chloroform (1/6)

E2. Which type of viruses do you evaluate?

- Enterovirus (4/6)
- Parvovirus (1/6)
- ECBO virus (1/6)
- Cytopathogenic viruses (1/6)

E3. Which method do you use to evaluate the extracted viruses?

- Cell Culture (BGM, PLC/PRF/5)(5/6)
- RT-PCR (1/6)
- TEM (Negative staining) (1/6)
- Sequencing (1/6)

E4. What is the total analysis time of the methods?

- Cell Culture 1-3 weeks (depending on method)
- PCR and RT-PCR 24 hours

E5. What media do you use for analysis? Is the media ready made from the manufacturer or is it prepared in-house?

- | | |
|-------------------|----------------------|
| Ready made media: | 2 laboratories (2/7) |
| In house media: | 4 laboratories (4/7) |
| No answer | (1/7) |

TYPICAL RANGE OF LEVELS FOUND AND FREQUENCY OF ANALYSES

F1. In which type of sludge or similar matrix do you find more often bacteriophages and viruses?

- Sludges (3/5)
- Soil(1/5)
- Treated biowaste (1/5)
- Slurry (1/5)
- Compost (1/5)

F2. How many analyses of bacteriophages and viruses in sludges, soils and treated biowastes do you perform per month?

- <10 analysis per month (3/3)
- 10-50 analysis per month (1/3 for viruses)

QUALITY CONTROL**G1. Do you use standard control strains? If yes, which ones?**

- **Phages:** ISO 10705-2:2000 *E.coli* WG5 (4/6)
ΦX154 phage (4/6)
ISO 10705-1:1995 *Salmonella typhimurium* WG49 (4/6)
MS2 phage (4/6)
ISO 10705-4:2001 *Bacteroides fragilis* RYC 2056 (4/6)
B 56-3 phage (4/6)
- **Viruses:** Attenuated Polio virus (1/6)
Others(1/6)

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