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

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Final Report
Development of a standarised protocol for analyses somatic coliphages in sludge, soil and treated biowastes. (DL 3/4.2)

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1. Desk study. Bacteriophages (and viruses) to be monitored in EU in sludges, soils and treated biowastes

1.1. Introduction

For this desk study we have tried to collect all the available information both in scientific literature by consulting the data bases (PubMed, Sciendirect), the sources of standardised methods as well as some consultations to experts.

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.

1.2. 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 means 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.

1.3. 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*..

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

Thirty nine 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.

1.5. Aspects that need to be addressed

There is a need of exact (high trueness), precise, robust and feasible methods for detecting bacteriophages in different kinds of sludges, biowastes and soils (Lightfoot and Maier, 1998). 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 U.S. Environmental Protection Agency (USEPA, 1989) and an AFNOR draft is being prepared in France.

2. Development of a feasible method to extract somatic coliphages from sludge, soil and treated biowaste

2.1. Introduction

This study aimed to settle a standard method for testing sludge, soil and treated biowaste (Horizontal – HYG 2005). The performed exhaustive bibliographic revision indicated that, as in the case of human viruses, the extraction of bacteriophages from these matrices once in the laboratory requires the following steps: homogenization, elution, clarification and detoxification-decontamination (Straub et al., 1991; Gabrieli et al., 1997; Lasobras et al., 1999; Mignotte et al., 1999; Khan et al., 2002; Mignotte-Cadiergues et al., 2002; Mocé-Llivina et al., 2003a; Karima et al., 2004; Van, 2004; Arraj et al., 2005).

For the sampling, transportation and conservation of these matrices the same principles as for bacteria apply (Anon. 1997; Anon. 1999; Anon., 2001; USEPA, 2003), and consequently are not addressed.

The establishment of standard methods for recovering microorganisms, particularly viruses, and consequently bacteriophages from biosolids is problematic because of the great variability of matrices and the extreme difficulty to reproduce natural conditions by seeding viruses into the samples. Indeed, viruses in biosolids are either free, or included in particles or adsorbed to particles (Funderburg et al., 1985; Ketranakul et al., 1991; Armon and Kott,

1996; Araujo et al., 1997). In soils, these viruses are either free or adsorbed to soil particles or as in biosolids when the soil has been amended with biosolids. Consequently, spiking viruses into a sample will not reproduce natural conditions. Then, if it is not feasible to seed, it is not possible to directly quantify the efficiency of recovery of different methods in the traditional way that is adding viruses, extracting and counting them. However, at least for some bacteriophages, as for example somatic coliphages, biowastes with high bacteriophages content occur. This makes possible to compare the efficiency of recovery of different methods and consequently choose the best procedure.

Many results on bacteriophages elution efficiency by different procedures had been reported. Practically, all elution methods previously assayed for the extraction of viruses have also been assayed with bacteriophages (Mignotte et al., 1999). As well, several elution methods have been compared for the recovery of groups of bacteriophages proposed as potential viral indicators (somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *Bacteroides fragilis*) (Lasobras et al., 1999; Mignotte et al., 1999). A simple method based on elution with beef extract was defined and provided very good results for these three groups of bacteriophages (Lasobras et al., 1999; Mignotte et al., 1999). This procedure was selected as the starting point for this study. Moreover, this eluting approach is very similar to that used in the USEPA/625/R-92/013, Appendix H (USEPA, 2003) for the elution of enteroviruses from biosolids.

However, little effort has been focused so far to processes before and after the elution step, namely homogenization, clarification and decontamination steps. Furthermore, for liquid sludge samples, little effort has so far been done on determining the fraction (either the entire sample, the liquid or the solid fraction) of the sample to test. Methods for viruses recovery include a first step aimed to adsorb free viruses to solids, followed by centrifugation and elution of viruses from solids after pelleting them (USEPA/625/R-92/013 Appendix H, 2003). Nevertheless, no information on this step is available for bacteriophages recovery. Since the aim of this research was settling a simple and feasible method, it was also determined whether this additional step was necessary or not.

The objective of this work was to optimize the entire bacteriophages extraction procedure from sludge, soil and treated biowaste, with the final aim of settling up a method feasible in routine laboratories.

2.2. Experimental approach

Naturally occurring somatic coliphages were selected as those to study, because their abundance in naturally occurring samples (Lasobras et al., 1999; Jimenez et al., 2002; Mignotte-Cadiergues et al., 2002; Mocé-Llivina et al., 2003a) and their diversity in morphology. Consequently, they represent a set of viruses with different characteristics (Ackermann and Nguyen, 1983; Muniesa et al., 1999) and they seem to be a good candidate to be used as viral indicators in biosolids (Mocé-Llivina et al., 2003a). Moreover, ISO 10705-2 (Anom., 2000), Standard Methods (Anom., 2001) and USEPA 625/R-92/013 (USEPA, 2003) protocols for enumerating somatic coliphages in water can be used to count these microorganisms in the eluates. The scientific literature indicates that extraction methods that are suitable for somatic coliphages will also be adequate for F-specific RNA phages and phages infecting *Bacteroides fragilis*, which have also been studied as potential surrogate indicators (Lasobras et al. 1999; Mignotte et al., 1999). Raw sludge, digested-dewatered sludge, and compost, all of them naturally occurring matrices; and soil experimentally contaminated with sludge or sewage were studied. The first three matrices contained high enough numbers of phages to avoid uncertainty in the measurements due to low numbers of phages in the sample. In contrast, soil had to be contaminated since no naturally occurring soil samples with repetitive numbers of phages were available.

2.3. Discussion and Conclusions

Although no significant differences were found between extracting phages from the entire raw sludge or the pellet after centrifugation, it seems that processing the entire sample allows recovering about a 10% more of phages, which according to the phages recovered in the supernatant are not adsorbed

to solids going to the pellet. Because of this and to make the procedure easier and faster is then recommended to process the entire liquid sludge.

The homogenization procedures assayed show that all gave very similar extraction efficiencies. Given that magnetic stirring requires only a stirrer and a magnetic bar, equipment commonly found in all routine laboratories, magnetic stirring is the recommended homogenization procedure.

Elution at pH 7.2 or 9.0 did not show significantly different recoveries of somatic coliphages. Therefore, to avoid potential inactivation problems when the method is applied to other phages, such as F-specific RNA bacteriophages which might be sensitive to high pH, the recommended pH is 7.2.

Centrifugation at low speed (4000 x*g*) and also spontaneous settling for 150 min, gave the same results as centrifugation at 10000 x*g*. A standardized centrifugation speed can be achieved while spontaneous settling may depend on different variables such as the form and size of the container. Therefore, to ensure the robustness of the procedure, centrifugation is recommended. Given that low speed centrifuges are more commonly found in routine laboratories than those with the capacity to reach 10000 xg, the recommended procedure will be clarify by centrifugation at 4000 x*g.*

Although, a trained operator counted similar numbers of plaques with and without filtration of the clarified eluate, the method can be made more robust by including a filtration through low protein binding membranes step.

According to the results discussed above, the recommended procedure for extracting somatic coliphages from sludge, soil and biowaste is that outlined below:

1. Transfer 25ml or 25g of a representative (blended) sample to a sterile container with a screw top and add beef extract 10%, pH 7.2 at 1:10

2. Shake the sample by magnetic stirring for 20 min at room temperature at sufficient speed to develop vortex (500-900 rpm)

3. Centrifuge the sample at 4000 x*g* and 4ºC for 30 min

4. Recover the supernatant and filter through low protein binding 0.22 µm pore size membrane filters.

5. Harvest the filtered eluate in a sterile container with a screw top and keep at 4ºC until testing. Apply the ISO standard 10705-2 (2000) for detection and enumeration of somatic coliphages in water. Perform PFU/ml counts and adjust the results to final values of PFU/g d.m. (dry mass).

This defined protocol was applied to analyze the reference materials. The results obtained, besides the information provided regarding the suitability of the reference materials, show that the method is repetitive and that primary validation was achieved.

Though not long lasting, digested and dewatered sludge as reference material are possible for interlaboratory reproducibility studies in order to further validate the method, and for in lab quality control. This matrix, after one week of stabilization at 4ºC is stable enough to be used as reference material for more than 70 days. In contrast, raw and activated sludge performed poorly as reference materials.

The method is also feasible for most routine laboratories, since neither special equipment nor a specific training of personnel is required. The cost of the materials and reagents for extraction (including decontamination by filtration) and phage enumeration once extracted should be between 20 and 25 euros. Most of the cost is attributable to the membrane filters and beef extract reagent.

On the basis of information reported elsewhere (Lasobras et al., 1999; Mignotte et al., 1999), the procedure proposed here, should also be suitable for F-specific F-RNA bacteriophages and bacteriophages infecting *Bacteroides fragilis*, since with all extraction procedures that not include high pH the numbers of the three groups of bacteriophages being studied as potential indicators keep the proportions found in raw sewage.

3. Training session on the standardised protocol for analyses of somatic coliphages in biosolids

3.1. Development of the training session

A training session was hold at the University of Barcelona on July 10th to 13th 2006, in order to transfer the developed protocol for the extraction of bacteriophages from sludge, soils and treated biowastes. The aim of the training session was to take practice on the developed standardised protocol (see Annex on this document) by staff from two partners involved in the project HORIZONTAL-HYG (Institute Pasteur of Lille, France; and University of Hohenheim, Stuttgart, Germany). The research group of University of Barcelona (UB) was organising and leading the activity, providing facilities, protocols and previous expertise on the standardised protocol. Consequently, three participant laboratories were involved on this training session and trials for data comparison.

Initially, all the participants read and becoming familiar with the standardised protocol see, for extraction of bacteriophages from sludge, soils and treated biowastes. The first practice was focused on acquiring expertise on the application of new standardised protocol and the enumeration of bacteriophages according to ISO:10705-2 by participant staff. Two reference materials previously prepared by partner UB were assayed in this training session: sewage and ΦX174 phage solution. Control charts for each reference material were obtained in order to confirm participants were applying properly protocols and measuring parameters into the control limits.

However, previously to the training session, UB group was developing reference materials. They are based on naturally contaminated (non-spiked) materials, as indicated above. They do not need freezing (storage at 4°C). Optimal life-time was also evaluated and it is sufficient attending to HORIZONTAL-HYG purposes. Three kinds of matrices were: DHT (anaerobically digested and dehydrated sewage sludge) and RAW 1 (primary sludge), RAW 2 (activated sludge). This reference material was distributed in recipients (60 g each). Homogeneity and stability for these reference materials were analysed, and they fulfilled T1 (intravial) and T2 (intervial) requirements.

Two matrices were assayed at the training session: 10 samples of DHT/reference material and 5 samples of commercial compost. They were analysed by each participant in 5 independent sessions distributed during the work schedule.

3.2. Conclusions of Training Session

Staff participating in the training session got very successfully the required expertise to perform:

- The method for eluted bacteriophages in sludges, soils and treated biowastes.
- The enumeration according to ISO:10705-2

Trials with sewage reference material and ΦX174 phage solution, and later 5 assays using DHT and commercial compost lots were favourable. The suitability of the methods was well verified on the 2 matrices tested. Minor technical specifications were revised to amend the draft version of the standard before being proposed for the first CEN consultation on March 2007. The final version is included in this document (Annex).

4. Interlaboratory study

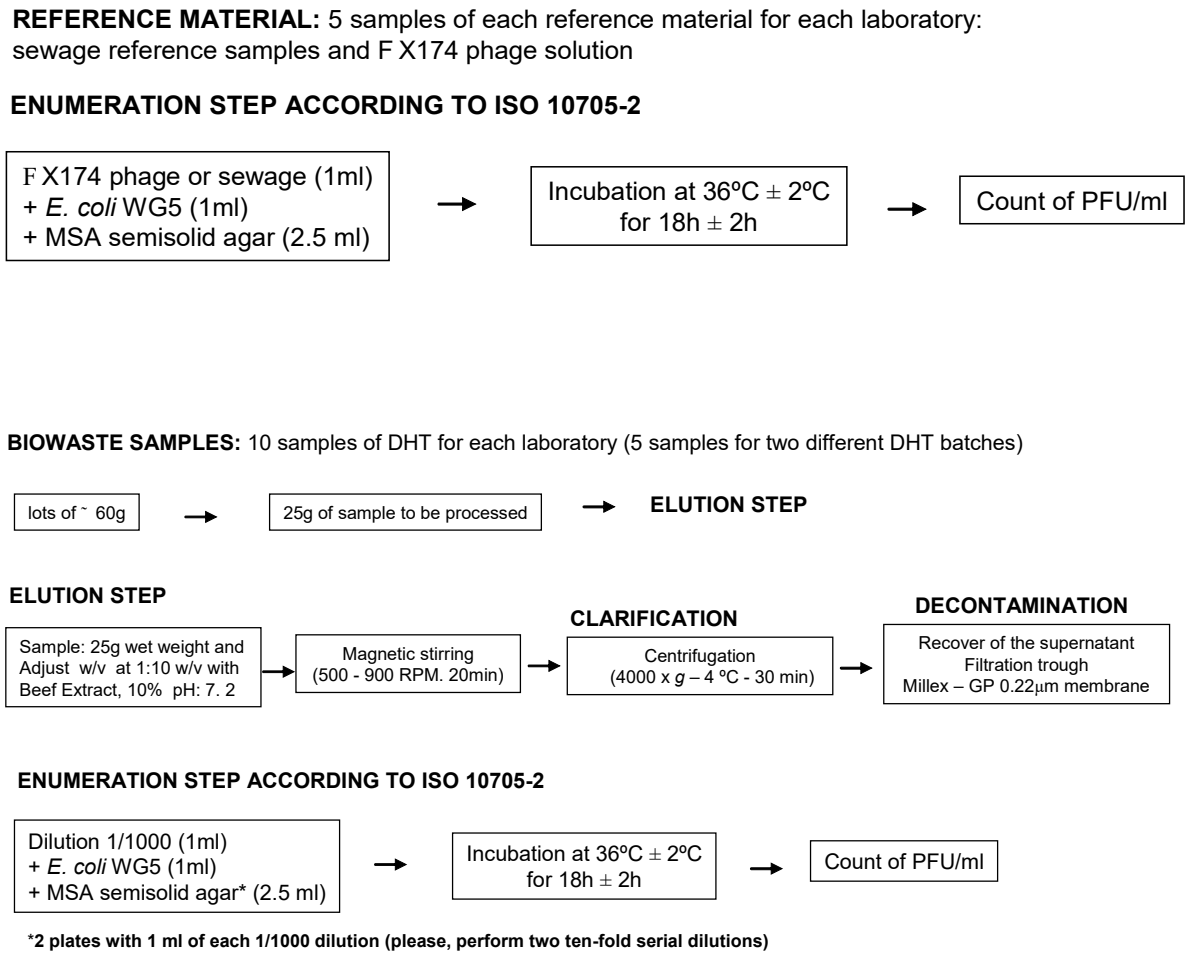
An interlaboratory study was performed. Three laboratories of HORIZONTAL-HYG partners were involved on the study: University of Barcelona (leader of the activity), Institut Pasteur of Lille and University of Hohenheim (Stuttgart).

4.1. Work schedule for interlaboratory assay

On November 27th, 2006, a delivery of 5 samples of reference material was provided to the French and German groups from UB. These reference materials were developed by UB and were based on naturally occurring phages in urban sewage and a calibrate ΦX174 phage solution. Later, two batches of reference biosolids (DBT: anaerobically digested and dehydrated sewage sludge) were prepared by UB as above explained (3. Training session). Five samples of each batch were sent to other participant partners on December 4th, 2006. Final delivery of results by all the participants was done by December 15th, 2006.

4.2. Protocols performed in the interlaboratory study

The protocols performed by participants in the study were those assayed during the training session. They are briefly explained in the following schemes:



4.3. Conclusions of the interlaboratory study

The interlaboratory study was performed successfully and the following conclusions were obtained:

1. No difficulty has been encountered on the performance of the planned interlaboratory study.
2. Participants implemented successfully to their laboratories the established protocol for eluted bacteriophages in sludges, soils and

treated biowastes. No problems have been indicated from the participant laboratories on the performance of interlaboratory study and the established protocol.

3. Participant laboratory 1 presented results fitting on the control chart values for reference materials (sewage and phage type) and for phages in biosolids.
4. Participant laboratory 2 presented results that indicated a problem on the sensitivity of the host strain used for reference materials and biosolids. This circumstance could be solved by changing the working culture of the host strains.
5. Final draft Standard procedure for extraction of bacteriophages from sludge, soils and treated biowastes was approved by the HORIZONTAL-HYG consortium.

5. Field studies: occurrence and levels of indicators and selected pathogens in different sludge and biosolids.

5.1. Development of field studies

The knowledge of occurrence and levels of indicators (fecal coliforms and sulphite reducing clostridia spores) and selected pathogens (*Salmonella* spp. and enteroviruses) in different sludge and biosolids respect to bacteriophages is necessary for further implementation of methods in real scenarios. Consequently, though field studies were not planned for bacteriophages in the project HOR-HYG, partners involved consider necessary to obtain and provide this data.

Four types of sludges (incoming and outgoing of two different sludge treatment utilities) were tested by the UB. Other partners have no chances to perform field studies because these were not considered by the planned work for the project. Six samples were collected from each sampling point. Each sample was transported to the laboratory, kept at 4ºC and tested within the next 12 hours.

To quantify faecal coliforms and sulphite reducing clostridia spores, sludges and biosolids (30 ml or g) were mixed with 270 ml of sterile phosphate buffer and suspended by magnetic stirring at room temperature for 30 minutes. This suspension was used to prepare ten-fold dilutions as described in USEPA standard procedures (Anon. 2003). Faecal coliforms were quantified both by Most Probable Number (MPN) analysis and membrane filter procedure, both according to the USEPA standard (Anon. 2003). Spores of sulphite-reducing clostridia (SSRC) were counted by the procedure described by Bufton (1959).

For *Salmonella* detection, samples were mixed with buffered peptone water (BPW) at a 1:10 (w/v) ratio and suspended by shaking for 1-2 hours at 37ºC. The presence/absence of *Salmonella* was then determined using the conventional enrichment method based on the international ISO standard (Anon. 1995a) slightly modified. Briefly, the modification consists of substituting the traditional enrichment in Rappaport-Vassiliadis broth by selection with a new commercial solid medium (SMS®), which ensures better *Salmonella* recovery than the conventional ISO standard method.

Bacteriophages were extracted from sludges and biosolids as described previously. Plaque Forming Units (PFU) of somatic coliphages were counted by the double agar layer technique on E. coli strain (WG5) following the ISO 10705-2 (Anon. 2000). PFU numbers of F-specific RNA bacteriophages were determined on *Salmonella* Typhimurium strain (WG49) (now *S. enteritidis*, var Typhimurium) in accordance with ISO 10705-1 (Anon. 1995b). PFUs of bacteriophages infecting *Bacteroides fragilis* strain RYC2056 were determined using the double agar layer method following the ISO method (Anon. 2001b). Enteroviruses were eluted from samples according to USEPA standard (Anon. 2003), and elution was done with 10% beef extract and viruses were concentrated by organic flocculation according to Katzenelson et al. (1976). Eluted viruses were enumerated by the double-layer plaque assay (Mocé-Llivina et al. 2004) using the Buffalo Green Monkey (BGM) cell line (European Collection of Animal Cell Cultures, accession number 90092601). Plaques were isolated, re-grown on BGM cells and tested by RT-PCR. For it, specific primers (EP1 - EP4) for the 5´ NCR region of the enteroviruses genome were used

(Gow et al. 1991). RNA extraction was performed with a QIAamp® Viral RNA Mini Kit (QIAGEN GmbH, Germany) following the manufacturer's instructions.

5.2. Discussion and conclusions

Bacterial and viral indicators, as well as enteroviruses were detected in high numbers in raw sludge. The ratios among their densities are similar to those found in the raw sewage of the study area (Blanch et al. 2004; Lucena et al. 2004; Mocé-Llivina et al. 2004; Montemayor et al. 2005). When densities in both sewage and raw sludge are referred to dry mass, the results prove quite similar for the studied parameters.

Anaerobic mesophilic digestion followed by dewatering proved the described low disinfecting power (about one $\log_{10}$ unit reduction for most microbial parameters) of this sludge treatment (Carrington et al. 1991; Lasobras et al. 1999; Gantzer et al. 2001). Two parameters exhibited significantly different behaviors: the sulphite reducing clostridia spores, whose numbers do not undergo any change; and the F-specific bacteriophages, which suffered a more than 2 $\log_{10}$ units reduction. The latter has already been observed and is probably linked to the sensitivity of this group of bacteriophages to temperatures over 25ºC (Lasobras et al. 1999; Mocé-Llivina et al. 2003; Mocé-Llivina et al. 2005; McLaughlin and Rose 2006).

The numbers for most indicators in the dewatered sludges resemble those found in the digested-dewatered sludge. This may indicate that sludge dewatering achieves similar reductions in the numbers of indicators and pathogens as does mesophilic digestion. In fact, it has been reported that sludge dewatering alone causes changes in the numbers of microorganisms recovered (Monteleone et al. 2004).

Composting greatly reduced the numbers of microorganisms. Most compost samples contained very low numbers of indicators and pathogens. *Salmonella* was isolated in one compost sample, which also exhibited unusually high counts of faecal coliforms. Aside from this particular sample, faecal coliform levels in the compost indicate a greater than 6 $\log_{10}$ reduction during the process. In addition, considering the numbers of *Salmonella* in sewage

reported in industrialized countries, including the area were the study was performed (Gale 2005; Muniesa et al. 2005), this bacterial pathogen underwent a more than 5 $\log_{10}$ reduction. In fact, while sample-to-sample variations with regard to microbial quality in the composting process are very clear, these differences do not exist in the studied digested-dewatered sludge. Such sample-to-sample variations are to be expected from a soft technology process like outdoor composting. Efforts should be undertaken to minimize the impact of climatic conditions on these processes.

Data reported here and by others show that, even in areas with high health standards, pathogens such as *Salmonella* (Yaziz and Lloyd 1979; Gale 2005), and enteroviruses (in this study, coxsackie B viruses) (Hurst 1989; Straub et al. 1993; Soares et al. 1994; Mignotte et al. 1999; Monpoeho et al. 2001; Monpoeho et al. 2004) are present in non-negligible amounts in raw or insufficiently treated sludges and should be regarded as a potential health risk. Ideally, their fate in treatments as well as their occurrence and levels in sludges should be determined. However, the low numbers of pathogens in raw sludges makes accurate counts in treated sludges extremely difficult. Consequently, it is impossible to precise the removal of these pathogens in such highly inactivating processes like thermal treatments, liming or composting. Molecular methods like nucleic acid amplification methods, as for example PCR, seem most promising, but are not devoid of difficulties. Aside from methodological problems, sludge matrices typically contain many inhibitors of amplification reactions. At the present stage of development, these methods do not allow to distinguish between live (infectious) and dead (non-infectious) pathogens. Moreover, even the numbers of genomes might not be high enough. Thus, Monpoeho et al. (2004) have studied the occurrence and fate of enterovirus genomes in raw and treated sludges. With the exception of anaerobically-mesophilically digested sludge, they were unable to detect any genomes in treated sludges. It appears then, that indicators are still needed not only to estimate the fate of pathogens in treatment processes, but also to evaluate the potential occurrence and levels of pathogens in the treated sludges. The question remains whether to take in account only the present indicator (faecal coliforms or *E. coli*) or if a small group of indicators will prove more adequate.

Results reported here on the occurrence and levels of faecal coliforms and *E. coli* (the only regulated microbial indicator thus far) as well as those reported elsewhere seem incongruent (Chauret et al. 1999; Monteleone et al. 2004). The biosolid matrix clearly exerted great influence over the results obtained using the present standardized methods for enumerating faecal coliforms bacteria and consequently for *E. coli*, which under current regulations are indicators contemplated for microbiological management of biosolids. Whereas some matrices favour those numbers obtained by MPN, the most predictable result based on basic principles of microbiology, other matrices favor those numbers obtained by membrane filtration. At present, we cannot explain these apparent incongruities. These results enforce that extra effort must be exercised regarding the standardization of microbiological methods applied to all types of sludges, biosolids and soils.

Sulphite reducing clostridia spores had been suggested as indicators for protozoan oocysts (Payment and Franco 1993). Based on the data obtained by the field studies and that from other authors, their numbers are not only high in raw sludge, but are also still found in numbers well over the detection limits of feasible procedures in treated sludges or biosolids (Chauret et al. 1999; Gantzer et al. 2001; Rimhanen-Finne et al. 2004; Pourcher et al. 2005).

In all the reports in which the three groups of phages were counted (Lasobras et al. 1999; Mignotte et al. 1999), including the present study, the highest counts corresponded to somatic coliphages, followed by F-specific RNA bacteriophages and bacteriophages infecting *Bacteroides fragilis*. Moreover, in compost only somatic coliphages were regularly found in numbers that are detectable by feasible procedures. Taking into account the data presented here on anaerobic-mesophilic digestion and composting and that reported elsewhere regarding pasteurization (Mocé-Llivina et al. 2003), composting (Shaban 1999) and liming (Mignotte-Cadiergues et al. 2002), somatic coliphages remain the most detectable indicator by feasible procedure in treated sludges where viruses may still persist. In this study, the methodology used to detect somatic coliphages is very reliable, as indicated by the low standard deviations observed in the raw, digested-dewatered and dewatered sludges. Unfortunately, the number of naturally occurring human viruses in raw sludge

proved too low for detection purposes following different sludge treatments. The only treatment that allows consistent quantification of human viruses is anaerobic-mesophilic digestion, in which the inactivation of enteroviruses and somatic coliphages is similar (Berman et al. 1981; Safferman et al. 1988; Monpoeho et al. 2004). Taking into consideration, all of the available results on the persistence of somatic coliphages in raw and treated sludges, as well as the feasibility of the procedures used to detect them, we recommend the inclusion of somatic coliphages within a small group of indicators for following biosolid quality. We have no doubt that quantifying somatic coliphages yields more precise information than bacterial indicators do regarding the potential presence of enteric viruses in treated sludges.

6. Executive summary

An initial desk study indicated that many biosolids contain human virus, but also that even in the more contaminated ones, they are not very abundant and that they are difficult to recover and quantify. Moreover, feasible methods for detecting infectious viruses are only available for enteroviruses, and it is well known that infectious viruses are needed for risk assessment.

The need of indicators seemed obvious, as it was clear that the traditional bacterial indicators are not a good option for predicting presence and behaviour of human viruses in biosolids, biowastes and soils.

Bacteriophages appeared as more suitable indicators, and among the proposed groups of phages, somatic coliphages aroused as those potentially more useful attending to their numbers in biosolids, feasibility of the detection methods and for sharing behaviour with viruses in biosolid and biowastes processing. Consequently, at present, somatic coliphages appear as a very useful indicator. Moreover, feasible standardised methods (ISO (adopted by CEN) and USEPA) for detection and quantification of somatic coliphages suspended in water, and consequently in aqueous solutions, are available.

As well, literature available indicated that as human viruses, bacteriophages present in biosolids tend to be either included in or adsorbed to particles. Consequently, an extraction steep is necessary. There is a relatively

abundant literature regarding extraction of human viruses and bacteriophages from solids.

The methods for extracting bacteriophages (and also human viruses) from solids require the following steeps: homogenization, elution, clarification and decontamination. Described methods mostly vary in the elution steep. In a pair of papers comparing several elution methods showed elution with beef extract as the more efficient one. However the described methods required optimization in the various steeps. The optimization of the various steeps was the main aim of this work.

Unfortunately, inoculation of biosolids with known concentrations of viruses (as it is feasible with water samples) are not mimicking what happens in the real world because of inclusion and adsorption of phages in/to solid particles. Therefore, it was decided to perform the experiments of optimization of the extraction method with matrixes containing concentrations as high as possible of naturally occurring somatic coliphages. The studied matrixes were raw sludge, digested and dewatered sludge, selected (for its content of somatic coliphages) compost and soil contaminated with raw sewage. Some of these matrixes contained very homogeneous and steady numbers of phages and consequently it was possible to compare modifications of each one of the steeps of the extraction method and how they affected the efficiency of recovery. The method was optimised, published in scientific literature (Guzman et al. 2007. J. Virol. Methods 144: 41-48) and a draft of a “standard method” for the extraction of somatic coliphages from biosolids, biowastes and soils has been presented for approval to the CEN/TC308/WG1/TG5.

The non-convenience of inoculating these sort of matrices, since as said previously, the inoculated material does not mimic what happens in nature, complicated the performance of validation studies. Reference materials are needed for validation studies. To get round this problem, a few natural biosolids were tested as potential reference materials. For it, the matrices were distributed in a great number of containers, and placed at 4ºC. Then, somatic coliphages were enumerated after different days of storage from two containers and also from two subsamples of each one of the containers. This allowed testing the intra and inter-container homogeneity and the time elapsed without

significant descent in the number of phages detected. Digested-dewatered sludge probed to be an excellent reference material lasting in perfect condition for at least 2 months. This will allow to make validation multilaboratory studies and have a reference material for "*in lab*" quality control. In fact, a small validation study of the extraction method with three laboratories was performed with satisfactory results.

A few experiments done with other phages, as for example F-specific RNA bacteriophages, indicate that the method will also be applicable to other phages. As well, it may be useful for extracting human and animal viruses, though this should be further verified.

In conclusion, a feasible, fast and low cost method for determining somatic coliphages from biosolids, biowastes and soils is available. Besides the feasibility of the methods for extraction, detection and enumeration, somatic coliphages have, in our opinion, several advantages to follow the higienization processes of sludges as well as to have an indication of the viral contamination of these solid matrixes. These additional advantages are:

i) their determination is based in infectivity, thus avoiding the problem that present the molecular methods

ii) to the present stage of knowledge there are infectious or not, thus avoiding the problems linked to "stressed" or "injured" individuals that frequently present bacteria

iii) in most of the matrixes studied they are present in concentrations high enough to quantitatively follow the efficacy of higienization processes

iv) no interferences by other microorganisms or toxicity of the extract impairing their detection had been detected.

Potential adoption of coliphages as viral indicators in biosolids, biowastes and soils will require further investigation, at least in the following aspects.

i) Extensive validation of the extraction method though multilaboratory studies

ii) Further verifying whether the method is applicable to human viruses

iii) Obtaining comparative data on somatic coliphages and infectious enteric viruses in different biosolid matrices and in different geographical areas. This information is necessary for validating and verifying biosolid higienization processes using bacteriophages and for risk assessment derived of different uses of biosolids, biowastes and soils.

7. References

Ackermann, H. W., Nguyen, T. M., 1983. Sewage coliphages studied by electron microscopy. Appl. Environ. Microbiol. 45, 1049-1059.

Anonymous 1995a. Water Quality. Detection and enumeration of *Salmonella*. Geneva, Switzerland: International Organization for Standardization. ISO 6340.

Anonymous 1995b. Water quality. Detection and enumeration of bacteriophages -part 1: Enumeration of F-specific RNA bacteriophages. International Organization for Standardization. Geneva, Switzerland.). ISO 10705-1.

Anonymous 2000. Water quality. Detection and enumeration of bacteriophages -part 2: Enumeration of somatic coliphages. Geneva, Switzerland: International Organization for Standardization. ISO 10705-2.

Anonymous 2001. Water quality. Detection and enumeration of bacteriophages -part 4: Enumeration of bacteriophages infecting *Bacteroides fragilis*. Geneva, Switzerland: International Organization for Standardization. ISO 10705-4.

Anonymous, 1997. Water quality – Sampling – Parte 13: Guidance on sampling of sludges from sewage and water treatments works. ISO 5667-13

Anonymous, 1999. Water quality – Sampling – Parte 15: Guidance on preservation and handling of sludges and sediment samples. ISO 5667-15.

Anonymous, 2000. Water Quality – Detection and Enumeration of bacteriophages – Part 2: Enumeration of somatic coliphages. International Organization for Standarization, Geneva, Switzerland. ISO 10705-2:2000 (E).

Anonymous. (2001). Standard Methods for the Examination of Water and Wastewater 20th edn. Washington, D.C. American Public Health Association.

Araujo, R. M., Puig, A., Lasobras, J., Lucena, F., Jofre, J., 1997. Phages of enteric bacteria in fresh water with different levels of faecal pollution. J. Appl. Microbiol. 82, 281-286.

Armon, R., Kott, Y., 1996. Bacteriophages as indicators of pollution. Crit. Rev. Envriron. Sci. Technol. 26, 299-335.

Arraj, A., Bohatier, J., Laveran, H., Traore, O., 2005. Comparison of bacteriophage and enteric virus removal in pilot scale activated sludge plants. J. Appl. Microbiol. 98, 516-524.

Berman, D., Berg, G. and Safferman, S. 1981. A method for recovering viruses from sludge. J Virol Methods 3, 283-291.

Blanch, A. R., L. Belanche-Muñoz, X. Bonjoch, J. Ebdon, C. Gantzer, F. Lucena, J. Ottoson, C. Kourtis, A. Iversen, I. Kühn, L. Moce, M. Muniesa, J. Schwartzbrod, S. Skraber, G. Papageorgiou, H. D. Taylor, J. Wallis, and J. Jofre. 2004. Tracking the origin of faecal pollution in surface water. An ongoing project within the European Union Research Programme. J. Wat. Health 2, 249-260

Bufton, A.W. 1959. A note on the enumeration of thermophilic sulphite-reducing bacteria. J Appl Bacteriol 22, 278-280.

Carrington, E.G., Pike, E.B., Auty, D. and Morris, R. 1991. Destruction of faecal bacteria, enteroviruses and ova of parasites in wastewater sludge by aerobic thermophilic and anaerobic mesophilic digestion. Water Sci Technol 24, 377-380.

Chauret, C., Springthorpe, S. and Sattar, S. 1999. Fate of *Cryptosporidium* oocysts, *Giardia* cysts, and microbial indicators during wastewater treatment and anaerobic sludge digestion. Can J Microbiol 45, 257-262.

Funderburg, S. W., Sorber, C. A., 1985. Coliphages as indicator of enteric viruses in activated sludge. Water Res. 19, 547-555.

Gabrieli, R., Divizia, M., Donia, D., Ruscio, V., Bonadonna, L., Diotallevi, C., Villa, L., Manzone, G., Panà, A., 1997. Evaluation of the wastewater treatment plant of Rome airport. Wat. Sci. Technol. 35, 193-196.

Gale, P. 2005. Land application of treated sewage sludge: quantifying pathogen risks from consumption of crops. J Appl Microbiol 98, 380-396.

Gantzer, C., Gaspard, P., Galvez, L., Huyard, A., Dumouthier, N. and Schwartzbrod, J. 2001. Monitoring of bacterial and parasitological contamination during various treatment of sludge. Water Res 35, 3763-3770.

Gow, J.W., Behan, W.M.H., Clements, G.B., Woodall, C., Riding, M. and Behan, P.O. 1991. Enteroviral RNA sequences detected by polymerase chain reaction in muscle of patients with postviral fatigue syndrome. Br. Med. J .302, 692-696.

Hurst, C.J. 1989. Fate of viruses during wastewater sludge treatment processes. Crit Rev Environ Control 18, 317-343.

Jiménez, B., Maya, C., Sanchez, E., Romero, A., Lira, L., Barrios, J. A., 2002. Comparison of the quantity and quality of the microbiological content of sludge in countries with low and high content of pathogens. Wat. Sci. Technol. 46, 17-24.

Karima M.R., Manshadia F.D., Karpiscakb M.M. and Gerba C.P. 2004. The persistence and removal of enteric pathogens in constructed wetlands. Water Res., 38, 1831-1837.

Katzenelson, E., Fattal, B. and Hostoversky, T. 1976. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. Appl Environ Microbiol 32, 638-639.

Ketranakul, A., Ohgaki, S., Yano, K., 1991. Comparative study of RNA-F-specific coliphages and polioviruses in activated sludge processes. Wat. Sci. Technol. 24, 407-412.

Khan M.A., Satoh H., Katayama H., Kurisu F. and Mino T. 2002. Bacteriophages isolated from activated sludge processes and their polyvalency. Water Res., 36, 3364-3370.

Lasobras J., Dellunde J., Jofre J. and Lucena F. 1999. Occurrence and levels of phages proposed as surrogate indicators of enteric viruses in different types of sludges. J. Appl. Microbiol., 86, 273-281.

Lightfoot N.F. F., Maier E. A. (1998). Microbiological Analysis of Food and Water: Guidelines for Quality Assurance. Publisher: Elsevier Science, 284pp.

Lucena, F., Duran, A. E., Morón, A., Calderón, E., Campos, C., Gantzer, C. and Skraber, S. 2004. Reduction of bacterial indicators and bacteriophages infecting faecal bacteria in primary and secondary wastewater treatments. J. Appl Microbiol 97, 1069-1076.

McLaughlin, M.R. and Rose, J.B. 2006. Application of *Bacteroides fragilis* phage as an alternative indicator of sewage pollution in Tampa Bay, Florida. Estuaries and coasts 29, 246-256.

Mignotte B., Maul A. and Schwartzbrod,L. 1999. Comparative study of techniques used to recover viruses from residual urban sludge. J. Virol. Methods, 78, 71-80.

Mignotte-Cadiergues B., Gantzer C. and Schwartzbrod L. 2002. Evaluation of bacteriophages during the treatment of sludge. Water Sci.Technol., 46, 189-194.

Mocé-Llivina L., Muniesa M., Pimenta-Vale H., Lucena F. and Jofre, J. 2003. Survival of bacterial indicator species and bacteriophages after thermal treatment of sludge and sewage. Appl. Environ. Microbiol., 69, 1452- 1456.

Mocé-Llivina, L. F. Lucena and J. Jofre. 2004. A double layer plaque assay for the quantification of enteroviruses. Appl Environ Microbiol 70, 2801-2805.

Monpoeho, S., Maul, A., Bonnin, C., Patria, L., Ranarijaona, S., Billaudel, S. and Ferré, V. 2004. Clearance of human-pathogenic viruses from sludge: Study of four stabilization processes by real-time reverse transcription-PCR and cell culture. Appl Environ Microbiol 70, 5434-5440.

Monpoeho, S., Maul, A., Mignotte-Cadiergues, B., Schwartzbrod, L., Billaudel, S. and Ferré, V. 2001. Best viral elution method available for quantification of enteroviruses in sludge by both cell culture and reverse transcription-PCR. Appl Environ Microbiol 67, 2484-2488.

Monteleone, M.C., Furness, D., Jefferson, B. and Cartmell, E. 2004. Fate of *E. coli* across mechanical dewatering processes. Environ Technol 25, 825-831.

Montemayor, M., Valero, F., Jofre, J. and Lucena, F. 2005. Occurrence of *Cryptosporidium* spp oocysts in raw and treated sewage and river water in north-eastern Spain. J Appl Microbiol 99, 1455-1462.

Muniesa, M., Blanch, A. R., Lucena, F. and Jofre, J. 2005. Bacteriophages may bias the outcome of bacterial enrichment cultures. Appl Environ Microbiol 71, 4269-4275.

Muniesa, M., Lucena, F., Jofre, J., 1999. Study of the potential relationship between the morphology of infectious somatic coliphages and their persistence in the environment. J. Appl. Microbiol. 87, 402-409.

Payment, P. and Franco, E. 1993. *Clostridium perfringens* and somatic coliphages as indicators of the efficiency of drinking water treatment for viruses and protozoan cysts. Appl Environ Microbiol 59, 2418-2424.

Pourcher, A.M., Morand, P., Picard-Bonnaud, F., Billaudel, S., Monpoeho, S., Federighi, M., Ferré, V. and Moguedet, G. 2005. Decrease of enteric micro-

organisms from rural sewage sludge during their composting in straw mixture. J Appl Microbiol 99, 528-539.
Rimhanen-Finne, R., Vuorinen, A., Marmo, S., Malmberg, S. and Hänninen, M.L. 2004. Comparative analysis of *Cryptosporidium*, *Giardia* and indicator bacteria during sewage sludge hygienization in various composting processes. 2004. Lett Appl Microbiol 38, 301-305.
Safferman, R.S., Rohr, M.E. and Goyke, T. 1988. Assessment of recovery efficiency of beef extract reagents for concentrating viruses from municipal wastewater sludge solids by the organaic flocculation procedure. Appl Environ Microbiol 54, 309-316.
Shaban, A.M. 1999. Bacteriological evaluation of composting systems in sludge treatment. Water Sci Technol 40, 165-170.
Soares, A.C., Straub, T.M., Pepper I.L. and Gerba, C.P. 1994. Effect of anaerobic digestion on the occurrence of enteroviruses and *Giardia* cysts in sewage sludge. J Environ Sci Health Part A 29, 1887-1898.
Straub, T.M., Pepper, I.L. and Gerba, C.P. 1993. Hazards from pathogenic microorganisms in land-disposed sewage sludge. Rev Environ Contam Toxicol 132, 55-91.
U.S. EPA. 1989. Manual of Methods for Virology - EPA600/4-84/013 (R-7). Chapter 7; Method for recovering viruses from sludges, soils, sediments and other solids.
USEPA, 2003. Control of pathogens and vector attraction in sewage sludge. Cincinnati, OH 45268. 625/R92/013
Yaziz, M.I. and Lloyd, B.J. 1979. The removal of salmonellas in conventional sewage treatment processes. J Appl Bacteriol 46, 131-142.

8. ANNEX. Extraction of bacteriophages from sludge, soil and treated biowastes (Version 5. April 17th, 2008)

Extraction of bacteriophages from sludge, soil and treated biowastes

Contents

Scope

This standard specifies a procedure for the elution of somatic coliphages from sewage sludge, compost and biowaste samples. The standard is mainly intended for somatic coliphages but can also be applied to other bacteriophages and viruses, which tend to adsorb to solids. The user should, prior to analysis, validate the method for the particular type of sample they wish to analyse: sludges, soils, soil improvers, growing media (i.e., compost) and biowastes.

The presence of somatic coliphages in a sewage sludge, compost or biowaste sample generally indicates pollution by faecal contaminants. Their survival in the environment, removal by sludge treatments and transport in soil resembles that of food-and waterborne human enteric viruses, for example the entero-, hepatitis A, and rotaviruses.

Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this draft CEN XXXX. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this draft CEN XXXX are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 31-0:1992, *Quantities and units — Part 0: General principles.*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods.*

ISO 5667-13:1997, *Water quality — Sampling — Part 13: Guidance on sampling of sludges from sewage and water treatment works.*

ISO 5667-15:1999, *Water quality — Sampling — Part 15: Guidance on preservation and handling of sludge and sediment samples.*

EN 12880:2000. *Characterisation of sludges - determination of dry residue and water content.*

ISO 6887: 1983. *Microbiology – General guidance to the enumeration of micro-organisms by culture.*

ISO 8199:2001. *Water quality - General guide to the enumeration of micro-organisms by culture.*

ISO 10705-1: 1995. *Water quality - Detection and enumeration of bacteriophages - Part 1: Enumeration of F-specific RNA bacteriophages.*

ISO 10705-2: 2000. *Water quality - Detection and enumeration of bacteriophages. Part 2: Enumeration of somatic coliphages.*

ISO 10705-4: 2001. *Water quality - Detection and enumeration of bacteriophages - Part 4: Enumeration of bacteriophages infecting* Bacteroides fragilis.

Terms and definitions

For the purposes of this draft CEN XXXXX, the terms and definitions given in ISO/IEC Guide 2 and the following apply:

3.1

Bacteriophages

Viruses which are capable of infecting selected strains of a host bacterium

NOTE Bacteriophages produce visible plaques (clearance zones) in a confluent lawn of the host strain grown under appropriate culture conditions.

3.2

Somatic coliphages

Bacterial viruses which are capable of infecting selected *Escherichia coli* host strains (and related strains) by attachment to the bacterial cell wall as the first step of the infection process.

3.3

Bacteriophage infecting *Bacteroides fragilis*

Bacterial viruses which are capable of infecting selected *Bacteroides fragilis* host strains by attachment to the bacterial cell wall as the first step of the infectious process

3.4

F-specific RNA bacteriophages:

Bacterial viruses which are capable of infecting a specified host strain with F-pili or sex-pili to produce visible plaques (clearance zones) on a confluent lawn grown under appropriate culture conditions, whereas the infectious process is inhibited in the presence of a concentration of 40 (occasionally 400) µg/ml of RNase in the plating medium.

3.5

Plaque-forming Units (pfu):

An entity - usually a virion, but also e.g. and “infectious centre” - which gives raise to a single plaque under appropriate conditions. The ISO standards for the determination of bacteriophages use the equivalent term “plaque forming particles” (pfp).

Safety precautions

Bacteriophages are non-pathogenic to man and animals, but some types are very resistant to drying. Appropriate precautions should therefore be taken to prevent cross-contamination of test materials, particularly when examining or handling samples of high titre or when inoculating cultures of the host strain. Such procedures shall be carried out in a biohazard cabinet or a separate area of the laboratory.

NOTE - "Waste and sludge samples may contain hazardous and inflammable substances. They may contain pathogens and be liable to biological action. Consequently it is recommended that these samples should be handled with special care. The gases which may be produced by microbiological activity are potentially inflammable and will pressurize sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided where possible. National regulations should be followed with respect to microbiological hazards associated with this method".

Principle

Bacteriophages are extracted from the sludge, soil and biowaste solid materials (biosolids since now) by homogenisation, elution, clarification and decontamination of samples. The extract is used for the enumeration of different types of bacteriophages according to the standard protocols ISO 10705-1, ISO 10705-2 or ISO 10705-4, or any other established or standardised method for enumeration of bacteriophages.

Diluent, buffers and reagents

Basic materials

Use ingredients of uniform quality and chemicals of analytical grade for the preparation of culture media and reagents and follow the instructions given in annex A. For information on storage see ISO 5667-15, except where indicated in this draft CEN XXXX. Alternatively, use dehydrated complete media and follow strictly the manufacturer's instructions.

For the preparation of reagents, use glass-distilled water or deionised water free from substances which might inhibit bacterial growth under the conditions of the test, and complying with ISO 3696.

NOTE Use of other grades of chemicals is permissible providing they are shown to be of equal performance in the test.

Eluting solution

Use beef extract solution (Annex A.2) to 10% at pH 7.2

Apparatus and glassware

Usual microbiological laboratory equipment, including

7.1 Hot-air oven for dry-heat sterilization and an autoclave. Apart from apparatus supplied sterile, glassware and other equipment shall be sterilized according to the instructions given in ISO 8199.

7.2 Magnetic stirrer and stir bars

7.3 pH meter with accuracy of ± 0.1

7.4 Bunsen burner

7.5 Class II safety cabinet

7.6 Refrigerated centrifuge capable of attaining 5,000 x g and screw-cap centrifuge bottles (500 ml of capacity) that can withstand 5,000 x g.

Usual sterile, microbiological laboratory glassware or disposable plastics ware according to ISO 8199 and including 7.11 Petri dishes of 9 cm or 14 cm to 15 cm diameter, vented.

7.6 Graduated pipettes of 0.1 ml, 1 ml, 5 ml and 10 ml capacity and Pasteur pipettes.

7.7 Glass bottles of suitable volume.

7.8 Culture tubes with caps or suitable alternative.

7.9 Measuring cylinders of suitable capacity.

7.10 Membrane filter for decontamination, having a pore size of 0.22 µm. Low protein-binding membranes, as for example, those composed of polyvinylidene difluoride or polyether sulphone.

7.11 Filtration equipment

7.11 Vials, lidded, of 1.5 ml to 3 ml capacity.

7.12 Refrigerator, temperature set at (5 ~ 3) °C.

Sampling

Take samples of at least 500 g wet weight and transport to laboratory as quickly as possible, chilled at 5 (±3) °C, in accordance with ISO 8199, ISO 5667-13 and ISO 5667-15.

General

As samples are liable to ferment and contain pathogenic microorganisms, it is of paramount importance to adhere to national and international regulations relating to bio hazardous samples when handling and transporting samples. It is essential to keep samples away from food or drink, and to protect any cuts.

Storage

When samples are not to be analysed immediately, store them at 5±3°C in well labelled containers, preferably plastic. At these condition, samples can be stored for a maximum period of 48 hours. Samples should not be stored on an open bench in the laboratory.

Handling

Good laboratory practice and cleanliness is essential. When handling sludge samples it is necessary to wear gloves, face and eye protection, and sufficient body protection to protect against spillages or bottles bursting. The gas evolved when opening sludge samples is flammable and so should be carried out away from naked flames and all equipment should be flame proof.

Procedure

Homogenisation

Mix (by shaking) the liquid samples or weigh out a representative (by blending smaller fractions) sub-sample of 25 g (wet weight) of the solid samples.

Transfer 25 ml of liquid samples or 25 g of solid samples to a sterile vessel of a minimum capacity of 500 ml and with screw threaded cap.

Elution

Add a volume of sterile beef extract solution to the vessel containing the biosolids up to a final volume of 250 ml.

Add a sterile stir bar into vessel containing the biosolids

Place vessel on magnetic stirrer and stir at a speed sufficient to develop a vortex and regulated to avoid the formation of foam for 15 - 20 min at room temperature.

Avoid the release of aerosols by using a vessel with a screw threaded cap.

NOTE: For lime treated sludge, adjust the pH of the elution mixture to 7.2 ±0.5 with M hydrochloric acid. If the pH drops below 4.5 whilst neutralizing the sample, a new sample should be prepared. If other chemical treatment changing the pH of the sludge samples has been used, a suitable neutralisation procedure should be adopted.

Clarification

Add the vortexed elution mixture to a sterile centrifuge tube appropriate for your centrifuge.

Centrifuge the vortexed elution mixture at 4,000 x *g* at 4° C (±2) for 30 min.

Recover the supernatant by decanting it into a beaker and discard the sediment.

Decontamination

Filter the supernatant through low protein-binding membranes, as for example, those composed of polyvinylidene difluoride or polyether sulphone of a pore size of 0.2 µm (7.10). Total volume to be decontaminated is related to the density of coliphages which varies for different biosolids (see Annex B). Taking into consideration that the final values are referred to 1 g, it is suggested taking at least 10 ml for primary sludge, activated sludge, thickened sludge and de-watered sludge; and at least 20 ml for different compost and lime-treated biosolids.

Harvest the filtrate in a sterile recipient with screw cap.

Refrigerate the decontaminated sample immediately at 5°C (±3), and maintain it at that temperature until it is assayed for the enumeration of bacteriophages within twelve hours.

Enumeration of bacteriophages

The enumeration of bacteriophages is undertaken in accordance to the ISO 10705-1, ISO 10705-2 or ISO 10705-4, or any other established or standardised method for enumeration of bacteriophages. If dilution are required for enumeration, use peptone-saline solution (Annex A.1) or another diluent complying with ISO 6887.

Expression of results

Determination of dry matter content

The dry matter content is measured using the method described in EN 12880:2000.

Enumeration of plaques

Select plates with well-separated, and preferably more than 30, plaques whenever present. If only counts below 30 plaques per plate are found, select plates inoculated with the largest volume of sample. From the number of plaques counted, calculate the number of plaque-forming particles (or plaque forming units) of bacteriophages in 1 ml of the diluted sample in accordance to ISO 10705-1, ISO 10705-2 or ISO 10705-4.

NOTE: No interference or toxicity effects from the biosolid matrix are observed

Adjustment of results to dry weight

Calculate the number of pfu/ml (or pfp/ml) taking into consideration the initial dilution performed to the biosolid sample with the beef extract solution.

Correct values considering the percentage of total solids in sludge, soil, compost or biowaste sample.

Express the result as pfu/g.dw or pfp/g.dw.

Summarising, the calculation is as follows:

$$\text{pfu/g.dw} = \text{N pfu/ml} \times \frac{250\text{ml}}{25\text{ ml or g.ww}} \times \frac{100\text{ g.ww.}}{\text{x g.dw}}$$

- N: Total number of bacteriophages in the extract expressed in plaque-forming units (pfu) per ml (also termed plaque-forming particles, pfp)
- g.dw: grams dry weight in 25 ml or 25 g of analysed sample.
- g.ww: grams wet weight.
- X: Percentage in whole numbers

Test report

The test report shall contain the following information:

- a reference to this draft CEN XXXX
- a reference to the standard method used to enumerate the type of bacteriophages
- all details necessary for complete identification of the sample
- the incubation time, if different from the standard time in clause 9.5
- the results expressed in accordance with clause 10 as pfu (or pfp) /g.dw
- any other information relevant to the method

Annex A

A.1 Saline peptone solution

Peptone	1.0 g
Sodium chloride	8.5 g
Distilled water	1000 ml

Dissolve the ingredients in hot water. Adjust the pH to 7.2 ± 0.2 at (45 ~ 3) °C so that after sterilization it will be 7.2 ± 0.5. Dispense in convenient volumes and autoclave at (121 ± 3) °C for 15 min. Store in the dark for not longer than 6 months.

A.2 Beef Extract solution (10% pH: 7.2)

Beef extract	10 g
Distilled water	100 ml

Dilute the beef extract in distilled water

Adjust the pH to 7.2 ± 0.2.

Autoclave at 121 ± 3°C for 15 min.

Store in the refrigerator. Check the solution and discard if bacterial contamination is observed

Annex B
(Informative; source Horizontal-Hyg SSPI-CT-2004- 513660)

Levels of enteroviruses and somatic coliphages in sewage and different biosolids matrixes

	Enteroviruses PFP/100ml	Somatic coliphages PFP/100ml	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}

Levels of bacteriophages in biosolids

	Somatic coliphages	FRNA-specific	*B.fragilis* bacteriophages
Primary sludge	10^5 - 10^9	10^3 - 10^6	10^2 - 10^5
Activated sludge	10^5 - 10^8	10^2 - 10^5	10^2 - 10^3
Thickened sludge	10^4- 10^7	10^2 - 10^3	10^2 - 10^3
De-watered	10^5 - 10^8	10^3 - 10^4	10^2 - 10^3
Compost (static pile)	10^2 (7 week)		
	10^1(10 week)		
Compost (windrow composting system)	10^2 (3 week)		
	10^2 (4 week)		
Compost (natural draft system)	10^2 (7 week)		
	<1 (10 week)		
After lime treatment	Control 1.5 10^6	<10 (pH: 10.0)	
	10^3 (pH: 10.0)	<10 (pH: 11.5)	
	10^2 (pH: 11.5)	<10 (pH: 12.0)	
	<10 (pH: 12.0)		

- **pfu/ 100g ; DW**

Bibliography

Guzman, C., J. Jofre, A.R. Blanch and F. Lucena. (2007). Development of a feasible method to extract somatic coliphages from sludge, soil and treated biowaste. Journal of Virological Methods 144, Vol 41-48.

Guzman, C., J. Jofre. M. Montemayor and F. Lucena. (2007). Occurrence and levels of indicators and selected pathogens in different sludges and biosolids. Journal of Applied Microbiology, Vol 103, pp 2420- 2429.

Lasobras, J., Dellundé, J., Jofre, J. and Lucena, F. (1999). Occurrence and levels of phages proposed as surrogate indicators of enteric viruses in different types of sludges. Journal of Applied Microbiology, Vol 86, pp 723-729.

Mingotte, B., Gantzer, C. and Schwartzbrod, L. (2002). Evaluation of bacteriophages during the treatment of sludge. Water Science and Technology, Vol 46, No 10, pp 189-194.

Mingotte, B., Maul, A. and Schwartzbrod, L. (1999). Comparative study of techniques used to recover viruses from residual urban sludge. Journal of Virological Methods, Vol 78, pp 71-80.