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

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Abstract

Extraction of viruses and bacteriophages from sludge, soil and treated biowaste requires homogenization, elution, clarification and detoxification–decontamination steps. Seeding these matrixes with bacteriophages does not reproduce what happens in nature. Therefore, naturally occurring matrixes, raw sludge, digested and dewatered sludge and compost, containing high numbers of somatic coliphages, and soils contaminated with wastewater or raw sludge were used in the extraction assays. Based on eluting the bacteriophages with beef extract, a feasible method in which the different steps had been optimized has been established. The method is feasible, repeatable, robust and applicable in routine laboratories. Digested and dewatered sludge has been probed to be useful as a reference material for validation studies and for "in lab" quality control. The established method includes homogenization by magnetic stirring, elution (which is performed at the same time that homogenization) with 10% beef extract at neutral pH, clarification by centrifuging at 4000 × *g* and decontamination by filtration through low protein binding 0.22 μm diameter pore size membrane filters.

Keywords: Extraction method; Coliphages; Sludge; Soil; Biowaste

1. Introduction

This study was done in the framework of a wide project, launched by the European Union, aimed to establish standard methods for testing sludge, soil and treated biowaste (Horizontal-HYG, 2005). An exhaustive literature review indicated that, as in the case of human viruses, the extraction of bacteriophages from these matrixes 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 et al., 2004; Arraj et al., 2005).

For the sampling, transportation and conservation of these matrices the same principles as for bacteria apply (ISO 5667-13, 1997; ISO 5667-15, 1999; Standard Methods, 2001; USEPA 625/R-92/013, 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 and Sorber, 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 they are found in biosolids, when the soil has been amended with this matrix. 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 bacteriophage content are available. This enables the comparison of recovery efficiencies of different methods and consequently to choose the best procedure. Many results on bacteriophage elution efficiencies by different procedures had been reported. Most of the available methods for extracting viruses from biosolids and sediments have also been assayed with

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bacteriophages (Jofre et al., 1989; Albert and Schwartzbrod, 1991; Soares et al., 1994; Ahmed and Sorensen, 1995; Lasobras et al., 1999; Mignotte et al., 1999), but as stated earlier, most of the different studies results are not comparable because of the unfeasibility of seeding biosolids. In addition, methods had not been compared in most of the reports; however, some of these elution methods had been compared in two studies (Lasobras et al., 1999; Mignotte et al., 1999). In which, the efficiencies of elution of somatic coliphages, F-specific RNA phages and bacteriophages infecting *Bacteroides fragilis* with different elution methods from the same samples were tested and compared. A simple method based on elution with 10% beef extract provided recoveries similar or higher to recoveries obtained with all the other methods. This procedure (based on the method first described by Ahmed and Sorensen, 1995) was selected as the starting point for this study because of its efficiency of recovery and simplicity. Moreover, this eluting approach is very similar to that used in the USEPA 625/R-92/013 (2003, Appendix H) 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 virus recovery include a first step aimed to adsorb free viruses to solids, followed by centrifugation and elution of viruses from pelleted solids (USEPA 625/R-92/013, 2003, Appendix H; Mignotte et al., 1999). Nevertheless, no information on this step is available for bacteriophage 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 obtaining a method feasible for implementation in routine laboratories. Naturally occurring somatic coliphages were selected to be studied, because their abundance in naturally occurring samples (Lasobras et al., 1999; Jiménez 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 (2000), Standard Methods (2001), USEPA 821-R-01-030 (2001) and USEPA 821-R-01-029 (2001) 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 *B. 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 matrixes; and soil experimentally contaminated with sludge or sewage were studied. The first three matrixes 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 of comparable contamination levels were available.

2. Materials and methods

2.1. Bacteriophages detection

Plaque forming units (PFU) of somatic coliphages were counted by the double agar layer technique on strain WG5 of *E. coli* following the ISO 10705-2 standard (2000). F-specific RNA bacteriophages PFU numbers were determined on strain WG49 of *Salmonella typhimurium* (now *S. enteritidis*, var Typhimurium), following the ISO 10705-1 standard (1995).

2.2. Naturally occurring matrices

Raw and digested–dewatered sludge samples were collected at municipal activated sludge sewage treatment plant that serves a population of about 400 000 inhabitants. Raw primary sludge (about two thirds) and secondary sludge (about one-third) were mixed. This mixture (referred to herein as raw sludge) contained about 3.6% of dry matter.

After thickening, the raw sludge was subjected to anaerobic-mesophilic (35 °C) digestion for 20–25 days. It was then mixed with a solution of synthetic organic polyelectrolyte flocculating agent before mechanical dewatering by means of centrifugation. The final digested–dewatered sludge contained about 25% of dry matter.

Samples collected were transported to the laboratory, kept at 4 °C and tested within the following 12 h.

Several commercial compost matrices were tested for the occurrence and levels of somatic coliphages. Two compost matrixes (reported as B and C) consistently gave numbers of somatic coliphages high enough to avoid uncertainty in the measurements. These were used in the experiments. The compost matrixes studied contained about 60% of dry matter.

Numbers of samples tested for each matrix are those indicated in the headings of each figure at Section 3.

2.3. Experimentally contaminated soil

Soil previously sieved through a 2 mm pore size mesh was contaminated with raw sewage or raw sludge at a proportion 75:25 (w/v). After vigorous mixing, the mixture was allowed to dry at room temperature for 72 h. It was then stored at 4 °C for a maximum of 15 days until testing. The dry matter content of this sewage and sludge amended matrices was about 53%.

2.4. Elution procedure

The basic selected method according to the available information was that described by Lasobras et al. (1999) with minor modifications. Twenty five millilitres of liquid samples (raw sludge) or 25 g of solid samples (dewatered sludge, compost