Somatic coliphages as surrogates for enteroviruses in sludge hygienization treatments

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ABSTRACT

Conventional bacterial indicators present serious drawbacks giving information about viral pathogens persistence during sludge hygienization treatments. This calls for the search of alternative viral indicators. Somatic coliphages’ (SOMCPH) ability for acting as surrogates for enteroviruses was assessed in 47 sludge samples subjected to novel treatment processes. SOMCPH, infectious enteroviruses and genome copies of enteroviruses were monitored. Only one of these groups, the bacteriophages, was present in the sludge at concentrations that allowed the evaluation of treatment’s performance. An indicator/pathogen relationship of 4 log10 (PFU/g dw) was found between SOMCPH and infective enteroviruses and their detection accuracy was assessed. The obtained results and the existence of rapid and standardized methods encourage the inclusion of SOMCPH quantification in future sludge directives. In addition, an existing real-time quantitative polymerase chain reaction (RT-qPCR) for enteroviruses was adapted and applied.

Key words | enterovirus, hygienization, indicator microorganism, quantitative polymerase chain reaction (qPCR), sludge treatment, somatic coliphages

INTRODUCTION

High amounts of hazardous sewage sludge are generated worldwide. In the European Union, total sewage sludge production was estimated at about 10 Mt of dry matter in 2007 (Mininni et al. 2014). Different approaches are being accomplished to properly manage this situation as landfilling, incinerating or land spreading. The reuse of biosolids through land spreading may result in important benefits to soil, by means of fertilizing. Nevertheless, the management and recycling of this residue without the proper preventive measures involves an evident risk for human and animal health due to the high levels of pathogen microorganisms present. Bacteria, viruses and parasites are detected at elevated concentrations throughout the sewage sludge treatment (Sidhu & Toze 2009). In particular, enteric viruses are of major importance in developed countries due to their low minimal infective doses, the prevalence and their persistence in the environment. These facts make necessary the application of hygienization treatments before the utilization of sludge as land fertilizer.

The European Directive 86/278/EEC regulates the reuse of sludge by land spreading. In spite of the evident health risk associated with the management of this residue, the directive does not contain specific regulation with respect to microbiological parameters. To complement this directive, the European Union developed the EU Working Document on Sludge, 3rd draft (EC 2000), which constituted a suggestion for the updating of the directive. This document states that the sludge to be used without restrictions in agriculture must be subjected to an advanced treatment. This treatment shall be initially validated through a 6 log10 reduction of a test organism such as Salmonella enterica serotype Senftenberg W 775. Likewise, the treated sludge shall not contain Salmonella spp. in 50 g and the treatment shall achieve at least a 6 log10 reduction in Escherichia coli to <5 × 102 CFU/g. However, inoculation of complex matrices, like sludge, with allochthonous microorganisms for validation is not easy to carry out in terms of a homogeneous distribution of the inoculum, as well as in terms of standardization. Besides, the initial levels of the proposed bacteria in different types of sludge are, frequently, <6 log10 (Mocé-Llivina et al. 2003; Ruiz-Hernando et al. 2014). Therefore, the
feasibility and reliability of this procedure are seriously questioned.

Moreover, conventional bacterial indicators present limitations predicting the fate of pathogenic viruses because such viruses survive better in the environment than bacteria (Grabow 2001). As a result, there is an urgent necessity of new indicator microorganisms as surrogates of pathogenic viruses. Bacteriophages of enteric bacteria (as somatic coliphages, SOMCPH) have been proposed as indicators of waterborne viruses in water quality control processes, as well as in different types of sludge (Grabow 2001; Jofre 2003; Mocé-Llivina et al. 2003; Guzmán et al. 2007a). Although there is not a clear consensus on the relationship between SOMCPH and enteric viruses in different types of water and sludge, the concentration of SOMCPH may help to predict the potential risk associated to enteric viruses (Guzmán et al. 2007b; Rezaeinejad et al. 2014). The genus Enterovirus (family Picornaviridae, order Picornavirales) is one of the principal groups of pathogens of concern in sewage sludge and are causative agents of many gastrointestinal and respiratory tract infections (other than those caused by bacteria), as well as other diseases such as meningitis, encephalitis, myocarditis or acute flaccid paralysis.

In this context, the aim of the present research was to assess the suitability of SOMCPH as surrogates for enteroviruses throughout the new sludge hygienization treatments that were proposed in the Routes European Project (2011–2014). To accomplish this objective, reduction in the levels of infectious SOMCPH, infectious enteroviruses (INFEV) and genome copies of enteroviruses (GCEV) were monitored in different steps of the sludge treatment processes. Additionally, an existing real-time quantitative polymerase chain reaction (RT-qPCR) for enteroviruses (Monpoeho et al. 2000) was reviewed, adapted and applied.

**METHODS**

**Sewage sludge samples**

Forty-seven single sludge samples were analyzed. They were produced in laboratory scale prototypes of four different treatment processes. In the first treatment process, secondary sludge was subjected to thermal hydrolysis pre-treatment and to thermophilic digestion. In the second treatment process, secondary sludge was subjected to sonication pre-treatment and to a two-phase anaerobic digestion (mesophilic and thermophilic conditions). In the third treatment process, mixed or secondary sludge was subjected to sequential digestion (mesophilic anaerobic digestion and aerobic digestion at room temperature). Finally, in the fourth treatment process, mixed sludge was subjected to hydrodynamic cavitation pre-treatment and to a two-phase anaerobic digestion (mesophilic conditions and thermophilic conditions). Detailed protocols of the first, second and third treatment processes were described in Levantesi et al. (2014). Samples were taken at different time intervals and were divided into the three following groups, depending on treatment intensity: ‘untreated or pre-treated’ for 20 samples that were not subjected to any digestion, ‘conventional treatment’ for 11 samples subjected to a single digestion and ‘intensive treatment’ for 16 samples that were subjected to the entire treatment processes.

**Bacteriophages extraction and enumeration**

SOMCPH were extracted from a 5–10 g sludge sample as described by Guzmán et al. (2007a), method in process of standardization by the European Committee for Standardization. Viral extracts were analyzed for the presence of SOMCPH as indicated in the ISO 10705-2 standard (Anonymous 2000). The detection limit of this technique was 1 PFU/g ww (wet weight).

**Enteroviruses extraction**

Enteroviruses were extracted from a 100 g sample following the US EPA standard (EPA 2005). Viruses were finally resuspended in 25 mL of 0.15 M Na₂HPO₄.

**Enteroviruses cell culturing**

Twenty mL of the viral extracts were analyzed for the enumeration of total culturable enteroviruses following the US EPA standard (EPA 2005) by the plaque assay system. The detection limit of this technique was 0.01 PFU/g ww.

**Viral RNA extraction and RT-qPCR for enteroviruses**

Viral RNA extractions were made with the QIAAamp viral RNA mini kit (Qiagen, Germany), following the manufacturer’s instructions. An aliquot of 140 μL of each viral extract was used for this purpose, obtaining a final volume of 60 μL of purified RNA solution that was stored at −80 °C.

An adaptation of a previously described RT-qPCR method (Monpoeho et al. 2000) was used for the detection of total genome copies (GC) of enteroviruses. The target
region was a 148 bp fragment of the conserved 5′ non-coding region (5′NCR) of enteroviruses. The following oligonucleotides were used: primer Ev1 (5′-GATTGTACCA-TAAAACGAC-3′), primer Ev2 (5′-CCTGTGACGCT- TATGAT-3′) and Ev-probe (5′-TAGGTTTCTG TGTCG-3′). The specificity of primers and hydrolysis probe was tested in silico through sequence alignment with the human Enterovirus (A, B, C and D) genomes obtained from the GenBank database (accession numbers NC_001859.1, NC_001612.1, NC_002058.3 and NC_001450.1, respectively). Likewise, the lack of a correct alignment with the rest of Enterovirus species (E, F, G and H) was confirmed (accession numbers NC_001859.1, NC_021220.1, NC_004441.1 and NC_003988.1, respectively).

RNA samples were run using the RNA Ultrasense™ One-step Quantitative RT-PCR System kit (Life Technologies, CA, USA). A final reaction volume of 25 μL was achieved, comprising 5 μL of test sample and 20 μL of reaction mixture. The reaction mixture was prepared as follows: 500 nM of Ev1 primer, 400 nM of Ev2 primer, 120 nM of Ev probe, 1.25 μL of RNA UltraSense™ Enzyme Mix, 5 μL of RNA UltraSense™ 5X Reaction Mix, 0.5 μL of ROX Reference Dye, 2% of polyvinylpyrrolidone, PVP-40 (Alfa Aesar, Germany), 1.5 μg of T4 gene 32 protein (New England BioLabs, MA, USA) and DEPC-treated water to complete total volume. Reactions were performed by the Step One™ Real-Time PCR System (Applied Biosystems, CA, USA) using the following thermocycling conditions: 50 °C for 45 min; 95 °C for 2 min; and 45 cycles of 94 °C for 15 s, 60 °C for 1 min.

The main difference with the method previously described by Monpoeho was the utilization of a cDNA standard, instead of RNA. The target sequence was amplified through RT-PCR from a human coxsackievirus B3 stock (species Enterovirus B), using the primers described above. The PCR product was purified with the DNA Pure Link™ Quick Gel Extraction & PCR Purification Combo Kit (Life Technologies, CA, USA), cloned into the pGEM T-Easy plasmid (Promega, WI, USA) and transformed into JM109 High Efficiency Competent Cells (Cat.# L2001) following manufacturer’s instructions. The plasmid was extracted and purified using the DNA Pure Link™ HiPure Plasmid Midi-prep Kit (Life Technologies, CA, USA). Then, the plasmid was cut with the restriction enzyme GsuI (BpmI) (Thermo Fisher Scientific, MA, USA) and purified again with the QIAquick PCR purification kit (Qiagen, Germany). Finally, the plasmid solution was fluorometrically quantified with a Qubit® 3.0 Fluorometer (Life Technologies, CA, USA). Taking in account the molecular mass of the plasmid-target sequence construct and the Avogadro’s number, the concentration in GC/μL was calculated. Serial decimal dilutions were made in Tris–EDTA buffer, pH 8.0 to the final concentrations of: 1 × 10^0 to 1 × 10^7 GC/μL. Standard dilutions were aliquoted to avoid freeze-thaw cycles and stored at −80 °C.

**Determination of limit of detection and limit of quantification of the RT-qPCR**

The limit of detection (LOD) of a qPCR is the smallest number of nucleic acid targets in a given template volume that was detected in at least 95% of the replicas. Limit of quantification (LOQ) refers to the lowest concentration of nucleic acid targets that can be determined with acceptable precision and accuracy. The method used by Blanchard et al. (2012) was followed to calculate the LODPCR and the LOQPCR. For the LODPCR, dilutions of cDNA standard were made to obtain the following concentrations: 50, 20, 10, 5, 2, 1 and 0.1 GC/μL of standard analyzed in the RT-qPCR. Twenty-four replicas of each dilution level were tested, in four independent trials (six replicas per trial). For the LOQPCR, the cDNA standard was used to create calibration curves as follows. Three independent trials were performed on three independent standard dilution series, ranging from 5 to 5 × 10^7 GC/μL of standard dilution analyzed in the qPCR. Two replicas were tested for each dilution level in each trial. Thus, three calibration curves, with their corresponding linear regression lines were created. Linearity was assessed through the calculation of mean bias, standard deviation (SD) and linearity uncertainty (ULINi) for each dilution level. Finally, combined linearity uncertainty (ULINi) was obtained. The amplification efficiency of the PCR was calculated with the slope of the linear regression lines using the equation E = 10^{−1/slope}.

**RT-qPCR samples analysis**

For quantifying samples, two replicas of the standard dilutions were added to each qPCR plate with at least five dilution levels, including the LOQ. Only regression lines with R^2 > 0.990 and efficiencies between 90 and 110% were accepted for calculations. Threshold was automatically set by software in each trial. A positive control and a non-template control were included. Direct samples and ten-fold dilutions were tested in order to avoid possible PCR inhibitors.
RESULTS AND DISCUSSION

Total solids content of samples was comprised between 0.92 and 4.98%. These data were used to express results per gram of dry weight (dw).

Somatic coliphages and infectious enteroviruses

The effect of treatments in the initial levels of SOMCPH and INFEV was calculated. For INFEV, concentrations were very low, as much in untreated/pre-treated sludge as in samples subjected to conventional or intensive treatments. Anyway, these results were in accordance with previous studies (Monpoeho et al. 2001; Lucena et al. 2005; Sidhu & Toze 2009). Fifty-seven percent of samples were below the detection limit and the total mean value was 0.16 log_{10} (PFU/g dw). This fact made it impossible to evaluate the hygienization performance of the assayed treatments by means of this pathogen. Nevertheless, 100% of untreated/pre-treated sludge samples contained SOMCPH at concentrations over 5 log_{10} (PFU/g dw), with a mean value (±SD) of 5.96 ± 1.29 log_{10} (PFU/g dw), which was also in accordance with previous works (Lucena et al. 2005; Sidhu & Toze 2009). Eighty-five percent of the treated sludge samples were positive for SOMCPH, with values of 4.95 ± 1.46 and 3.13 ± 0.84 log_{10} (PFU/g dw) for conventional and intensive treatments, respectively. Thus, reductions caused by treatments could be measured using the SOMCPH naturally present in sludge without the need of spiking allochthonous bacteria. The removal obtained was dependent on the treatment intensity and fluctuated from 1.53 to 3.72 log_{10} (PFU/g dw).

RT-qPCR performance

For the LOD_{PCR}, 100% of replicas of 5 GC/reaction dilution level gave a positive result. Thus, LOD_{PCR} of the quantitative real time RT-PCR was determined in 5 GC/reaction. The efficiency of the 3 standard curves tested was very high, from 94.22 to 97.56% and R^2 was between 0.999 and 1.00. For each dilution level, SD was below 0.5 log_{10} (PFU/g dw) and U_{INJ} was below 0.25 log_{10} (PFU/g dw) and U_{INJ} was below 0.25 log_{10} (PFU/g dw) and U_{INJ} was below 0.25 log_{10} (PFU/g dw) and U_{INJ} was below 0.25 log_{10} (PFU/g dw) and U_{INJ} was below 0.25 log_{10} (PFU/g dw). The latter is especially important in the sewage sludge treatment processes, as the capability of PCR of detecting even partially damaged genomes.

Genome copies of enteroviruses

In terms of data processing, the value corresponding to the quantification limit of the method (5 GC/reaction) was assigned both for samples that gave a negative result as well as for those that gave an undetermined result. This value corresponds with 1.07 × 10^2 GC/g ww and with variable numbers per g dw, depending on each sample.

Few differences in GCEV concentrations were found throughout the phases of the treatment processes assayed, ranging from 4.46 ± 0.77 log_{10} (GC/g dw) in untreated/pre-treated sludge samples to 4.05 ± 0.55 log_{10} (GC/g dw) and 4.40 ± 0.55 log_{10} (GC/g dw), in samples subjected to conventional treatments and samples subjected to intensive treatments, respectively. In addition, percentages of positive samples were not good indicators of treatments efficacy either: 75% for untreated/pre-treated samples, 50% for conventional treatments and 69% for intensive treatments. Therefore, monitoring of GCEV was not a useful tool to assess hygienization performances of the sludge treatment processes studied. The described GCEV values were always above INFEV levels and were similar to data corresponding to other enteric viruses in sludge (Sidhu & Toze 2009). However, studies have pointed out that is difficult to establish a constant relationship between infectious viral particles and GC detected by qPCR (Monpoeho et al. 2001). There are many affecting variables and environmental conditions, including time from the initial release from the host (EFSA 2012). The latter is especially important in the sewage sludge treatment processes, as is the capability of PCR of detecting even partially damaged genomes.

Comparison of infectious enteroviruses vs. somatic coliphages

From the average numbers of SOMCPH and INFEV obtained in this research, the following ratio indicator/pathogen of 4.60 ± 1.63 log_{10} was found. In addition, it was detected that INFEV levels were very low or below detection limits when SOMCPH numbers were below 4 log_{10} (PFU/g dw), Figure 1(a). This result agrees with a previous work in which Gantzler et al. (1998) found a threshold of 10^4 PFU/L below which no INFEV were detected in wastewater samples. The result obtained also agrees with a thorough study conducted previously in our laboratory (Lucena et al. 2005) within the European Project Horizontal. Large amounts of data from various publications were analyzed and an average ratio of 10^4–10^5 was constantly found in sewage, primary sludge and
secondary sludge. In contrast, no threshold was found for GCEV.

**Somatic coliphages as model microorganisms for monitoring sludge treatment processes and as sludge quality indicators**

A large number of studies suggest that conventional bacterial indicators are not the most adequate as surrogate for viral pathogens. Bacteriophages have been repeatedly proposed as microbiological quality indicators and for the evaluation of hygienization treatment processes in different matrices, as food, natural or treated waters, sludge and biosolids (Gantzer et al. 1998; Grabow 2001; Jofre 2005; Guzmán et al. 2007b; Sidhu & Toze 2009; Bertrand et al. 2012). Phages have only two physiological stages, active and inactive, and this supposes an advantageous characteristic over bacteria when assessing hygienization performances. The use of bacteriophages as indicators avoids problematic bacterial stages as the viable but non-culturable state, which could lead to possible regrowth or misleading results (Pascual-Benito et al. 2015).

SOMCPH is a group of bacteriophages that has proved to be a good viral indicator. Relationships were even found between them and viral pathogens (Costán-Longares et al. 2008). The structure, morphology and size of these phages are similar to those of enteric viruses. Simple and rapid standardized detection methods have been developed to date (Anonymous 2000; Guzmán et al. 2007a). Furthermore, this group has been shown to behave well as model microorganisms in sludge treatment processes, in particular when intensive thermal treatment is applied (Mocé-Llivina et al. 2003; Ruiz-Hernando et al. 2014).

Despite the fact that qPCR sensitivity and specificity are clearly higher to those of cellular culture based techniques, this molecular approach still has important drawbacks, as the inability to distinguish between infectious and non-infectious viral particles. Some progress has been made in this area through the use of viability dyes as propidium monoazide (PMA) and ethidium monoazide (EMA), but

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**Figure 1** | Evaluation of indicators' performances in the 47 sludge samples analyzed. (a) SOMCPH vs. INFEV. (b) Distribution of true positive (TP), true negative (TN), false positive (FP) and false negative (FN) for the three proposals ‘SOMCPH as surrogates for INFEV’, ‘GCEV as surrogates for INFEV’ and ‘SOMCPH as surrogates for INFEV including absence of INFEV when SOMCPH levels < 10⁴ PFU/g dw’.
more research is needed. Great efforts are needed to accomplish standardization, including the validation technique as well as the interpretation of results (Bustin et al. 2009).

Three indicator-pathogen proposals were evaluated in order to choose an adequate option for further consideration in sludge guidelines. Percentage of true negative (TN), true positive (TP), false negative (FN) and false positive (FP) samples were obtained taking into account the indicator ability to detect pathogen. The proposals were: ‘SOMCPH as surrogates for INFEV’, ‘GCEV as surrogates for INFEV’ and ‘SOMCPH as surrogates for INFEV including absence of INFEV when SOMCPH levels <10^4 PFU/g dw’ (Figure 1(b)). On the one hand, higher percentage of TN will indicate better detection of negative samples, whereas a higher percentage of TP will mean a higher capability for detecting positive samples. On the other hand, it is desirable to have a lower percentage of FN, in order to avoid underestimation of the risk, whereas a lower percentage of FP will mean reduction of risk overestimation. ‘SOMCPH’ obtained the best results for FN, but the worst results for TN. ‘SOMCPH <10^4’ kept a good result for FN while giving the best percentages for FP and TN. Nevertheless, ‘GCEV’ behaved worse than ‘SOMCPH <10^4’ in all cases. Then, the following characteristics of SOMCPH <10^4 proposal were calculated: sensitivity, Se = TP/(TP + FN), specificity, Sp = TN/(TN + FP) and negative predictive value, NPV = TN/(TN + FN). The results obtained, expressed in percentage, were: Se = 90%, Sp = 59%, NPV = 89%.

With this in mind, a limit of <10^4 PFU SOMCPH/g dw for unrestricted use of sludge in agriculture will be in agreement with current regulations (EPA 2005) that establish a limit for enteroviruses of <1 PFU/4 g dw. Thus, in the present study, 100% of samples subjected to intensive treatments would fulfill the SOMCPH restriction proposed, while only 18% and 10% of samples subjected to conventional treatments and untreated/pre-treated samples, respectively, would fulfill.

Finally, the achievement of E. coli and Salmonella limits pointed out in the European regulations (EC 2000) was assessed in a previous study (Levantesi et al. 2014). The 6 log_{10} E. coli removal proposed for the evaluation of advanced treatment performances could not be determined due to the low levels of this microorganism found in the untreated/pre-treated samples.

Taking into consideration the results obtained in this research as well as the results obtained in previous studies, we suggest SOMCPH being included as indicator microorganisms to complement the European regulations (EC 2000) for sludge quality. Other national regulations already include SOMCPH in sludge hygienization treatments (Anonymous 2012, 2014). Therefore, an alternative text for future EU regulations supported by the Routes project results could be the following: ‘sludge to be used in agriculture without restrictions, should comply with these requirements: … being treated by an advanced process that achieves 6 log_{10} reduction of a test organism such as Salmonella Senftenberg, or 4 log_{10} reduction of indigenous SOMCPH’, ‘… and fulfilling the limits of E. coli <500 CFU/g, Salmonella <1/50 g and SOMCPH <10,000 PFU/g’.

CONCLUSIONS

The levels of SOMCPH, INFEV and GCEV were monitored throughout different hygienization treatment processes for sewage sludge. The high SOMCPH concentration present in the analyzed sludge samples allowed the evaluation of the hygienization ability of all the treatment processes assayed. Reductions were greater when increasing the treatment intensity. On the contrary, INFEV levels were low in treated samples as well as in non-treated samples. In addition, an existing RT-qPCR for human enteroviruses was revised and adapted satisfactorily. GC data obtained clearly exceeded those of INFEV and they remained at similar levels in different samples. It was found that sludge with SOMCPH levels lower than 10^4 PFU/g dw contained INFEV levels that were very low or below detection limits. Ability of SOMCPH for acting as model microorganisms in sludge treatment processes was assessed. The obtained results encourage the inclusion of SOMCPH quantification in future sludge directives. This study suggests that new regulations should consider a 4 log_{10} reduction of indigenous SOMCPH. Our results also support the implementation of the European Directive by adding to the current limits established in the sanitized sludge regulation (E. coli at <500 CFU/g and Salmonella at <1 CFU/50 g) a limit for SOMCPH of <10,000 PFU/g.

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