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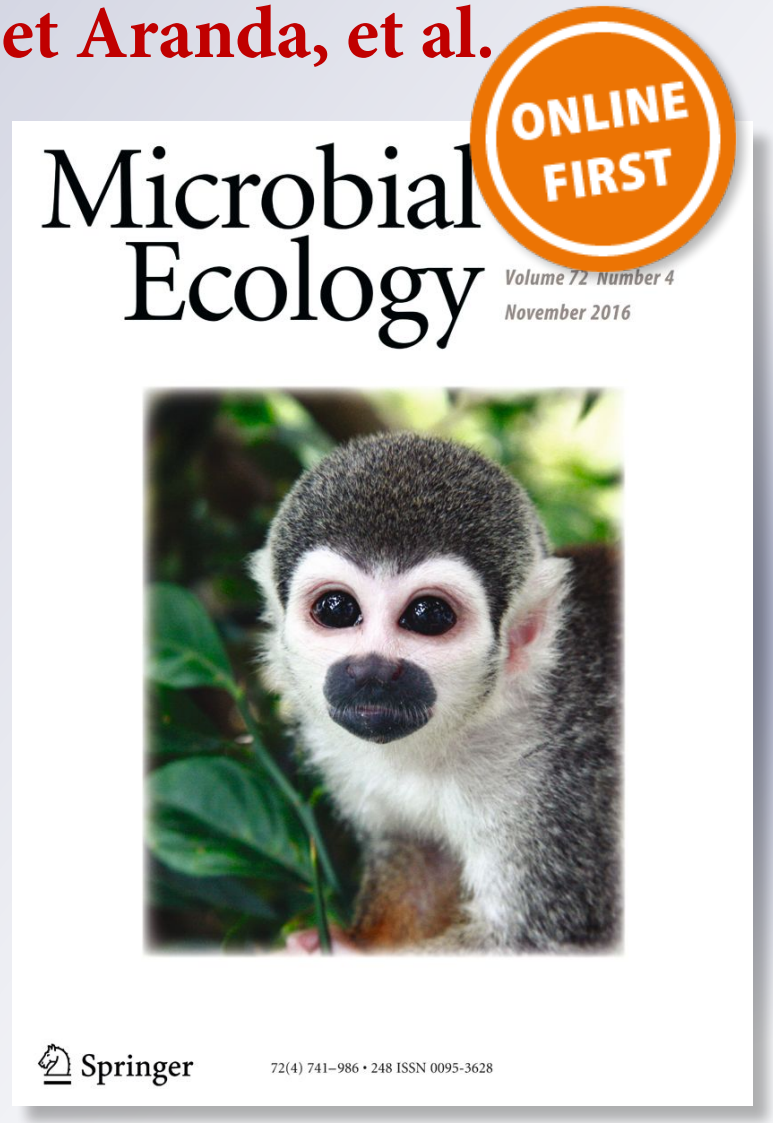


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ENVIRONMENTAL MICROBIOLOGY

# Effect of Composting Under Semipermeable Film on the Sewage Sludge Virome

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## Abstract

The addition of compost from sewage sludge to soils represents a sustainable option from an environmental and economic point of view, which involves the valorisation of these wastes. However, before their use as a soil amendment, compost has to reach the quality levels according to the normative, including microbial parameters. Viruses are not included in this regulation and they can produce agricultural problems and human diseases if the compost is not well sanitised. In this study, we carried out the analysis of the viral populations during a composting process with sewage sludge at an industrial scale, using semipermeable cover technology. Viral community was characterised by the presence of plant viruses and bacteriophages of enteric bacteria. The phytopathogen viruses were the group with the highest relative abundance in the sewage sludge sample and at 70 days of the composting process. The diversity of bacterial viruses and their specificity, with respect to the more abundant bacterial taxa throughout the process, highlights the importance of the interrelations between viral and bacterial communities in the control of pathogenic communities. These results suggest the possibility of using them as a tool to predict the effectiveness of the process.

**Keywords** Composting process · Virome · Sewage sludge · Electron microscopy · Ion torrent

## Introduction

Composting is a sustainable strategy commonly used in the treatment of sewage sludge, which involves the participation of different microbial communities. Through this technology, the valorisation of this waste is possible; obtaining the compost that is frequently used as a soil amendment due to its high content in organic matter and the capability of improving soil properties, with a positive effect on crop productivity. Despite that the majority of studies are focused on physical-chemical variables, analysing the properties of composted sewage sludge and the suitability of composted sewage sludge to be used as organic amendment, in recent decades, the number of studies related to microbial communities has increased, both related to culturable techniques [1–4] and no culturable techniques [5, 6]. Formerly, the studies of microbial parameters in composting have been mainly focused on bacterial community and/or enzymatic activities, due to the broad diversity and the possibility to use enzymes as an indicator of microbial activity [7–10]. The metagenomic tools have allowed the study of microbial diversity involved in these processes, providing new data on the diversity of the main bacterial taxa [5, 6, 11–13]. The interest in fungal communities has also increased, accompanied by the development of fungal databases. These studies have shed light on the role of this community in the biotransformation of the organic matter during composting and the involvement of the hydrolytic activity of these microorganisms by enzyme production [12, 14, 15].

In the last decade, viral metagenomic has achieved great progress in the field of environmental virology, even in virus taxonomy [16], saving the limitations of traditional methods

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and allowing the identification of new types of virus. However, it is considered that more than 50% of the sequences identified have no similarity in the GenBank and most of the data come from studies of environmental viral metagenomic [17]. The viral community has obtained a great interest due to the lack of normative in Europe, regarding total viral load in the final compost as well as specific viral groups. With regard to the composting of sewage sludge, this interest lies in the possible adverse effects of the final compost on the soil to which it should be applied. Among viruses, pathogens for plants and humans are the biggest concern for the further application of compost as a soil amendment. In addition, there is little information about the resistance of viral populations to the treatment processes used for the stabilisation and sanitisation of sewage sludge [18]. There are few studies on this community in the composting processes. However, several authors have characterised viral communities in biosolids and the results have revealed that bacteriophages are the most abundant viruses, with 60% of total predominance, while potential human pathogen viruses were detected in lower percentages [19]. On the other hand, the effect and interaction of viruses on the microbial communities during the composting process can affect the predominance of different groups of bacteria and fungi, having a positive effect if they are specific to bacterial or fungal pathogens [20]. This study was undertaken to gain knowledge on the changes of the viral population from sewage sludge to maturation composting. For this, a sample corresponding to sewage sludge and a sample corresponding to maturation composting phase were analysed under electron transmission microscopy and the isolated nucleic acids were used for next-generation sequencing (NGS) with the Ion PGM platform.

## Material and Methods

### Sampling

The samples were collected from a full-scale composting pile performed in the Biosolid Plant called '*El Salao*' located in Granada, Spain, as it was previously described by González et al. [21] and Robledo-Mahón et al. [22]. The pile was built using sewage sludge from a wastewater treatment plant (WWTP), previously stabilised by anaerobic mesophilic digestion and mixed with a vegetal bulking. Both materials were used in a volumetric proportion of 1:3 (sewage sludge: vegetal bulking). A semipermeable film membrane was used to cover the pile and the pile was connected to an aeration system on the floor during the first 30 days. Afterwards, the membrane was removed and the pile was kept under environmental conditions for 90 days. The first phase of the process was denominated 'composting phase' (first 30 days) and the second phase was denominated 'maturation phase' (from 30 to 120 days). In this study, sewage sludge samples (SS) and samples obtained after 70 days of composting, corresponding to the maturation phase (MP), were analysed. Composting samples were taken from five different points in the pile and mixed in one representative sample. The study was performed with analytical triplicates of each representative sample.

### Concentration of Viral Particles from Samples

Purification and concentration of viral particles were performed from 80 g of each sample. They were suspended in 200 mL of 1× phosphate buffer saline (PBS, pH 7.4) and homogenised using a vortex. The mixture was placed into an ultrasound bath Ultrasons-H 40 kHz (JP Selecta) for 10 min. The samples were centrifuged for 15 min at 5000 rpm at 4 °C using a centrifuge Beckman Avanti® J-25. This step was performed three times to obtain a representative volume of the supernatant. The resultant supernatant was filtered through a Whatman filter paper no. 2 and through a 0.45-μm membrane filter (Millipore®) [23]. The viruses were precipitated by adding 8% polyethylene glycol 8000 (PEG) (Thermo Fisher Scientific) and 0.3 M NaCl to the filtrate volume, homogenised by vortex and incubated for 12 h at 4 °C [24, 25]. After incubation, the samples were centrifuged at 7000 rpm for 20 min at 4 °C. The supernatant was discarded and the pellet was dissolved in 10 mL of PBS. Final elution containing virus-like particles (VLPs) was further concentrated using Amicon® Ultra—15 centrifugal filters (Merck) by centrifugation at 5000 rpm for 10 min at 4 °C in a centrifuge SL 16 (Thermo Scientific). The concentrated elution was collected in 1.5-mL tubes and stored at −20 °C. These steps were performed in triplicate [17].

### Transmission Electronic Microscopy

Each sample was prepared from 1 mL of the concentrated elution and placed in a carbon rack (300H Cu CF) for 5 min and negatively stained with 2% uranyl acetate. Samples were visualised using a Microscopy TEM, from Carl Zeiss Libra 120 Plus, at 120 kV.

### Isolation of Nucleic Acids

The DNA isolation was performed using the QIAamp® UltraSens® de QIAGEN Kit (Qiagen) following the manufacturer's instructions (Protocol: purification of viral RNA and DNA). The RNA isolation was performed using the Trizol® Kit (Invitrogen) following the manufacturer's instructions. RNA was used to synthetize the cDNA, using dsDNA synthesis kit (Roche, 11117831001) and primer random hexamers (Roche, 11034731001), in an Eppendorf Mastercycler Pro Thermocycler, following the protocol described in the cDNA Rapid Library Preparation Method Manual (Roche). To evaluate the presence of bacterial DNA, an end point PCR using rRNA 16S gene was

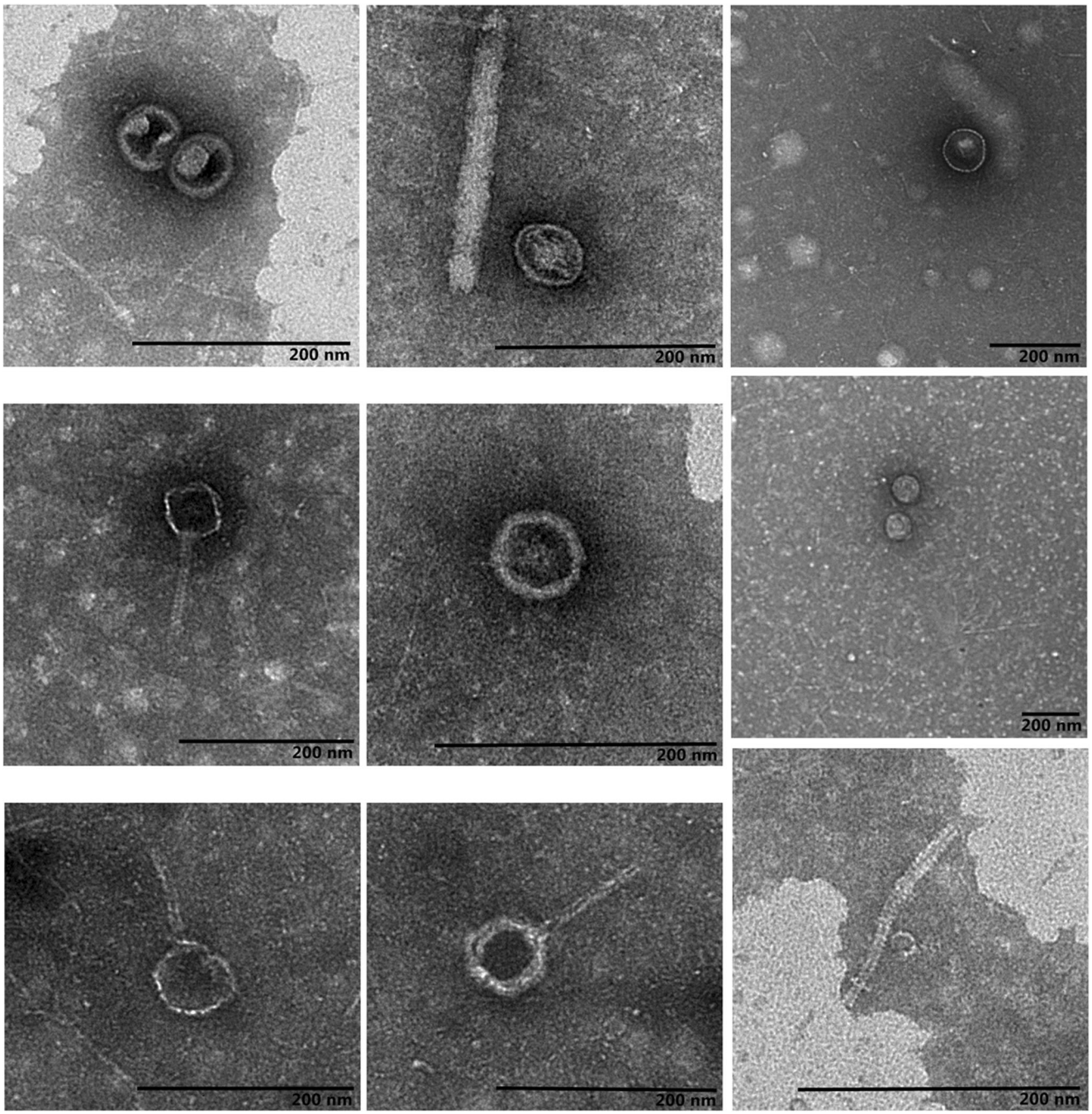


**Fig. 1** Morphologies of virus-like particles in sewage sludge (SS sample) by electron transmission microscopy (TEM). All black bars represent 200 nm

carried out using Horse-Power™ DNA Polymerase Kit (Canvax Biotech) according to Canvax Biotech manufacturer's instructions previously described in Robledo-Mahón et al. [22].

## Next-Generation Sequencing

A pool of DNA and cDNA for each sample in triplicate was prepared for the NGS library construction, resulting in 6 samples altogether. The pooled DNA was fragmented with Covaris M220, targeting peak fragment lengths of 400 bp. The barcoded libraries were prepared with the GeneRead DNA Library L Core Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Double size selection was performed with Agencourt AMPure XP Beads (Beckman Coulter). The libraries were quantified with the QIAseq Library Quant Assay Kit (Qiagen, Hilden, Germany) and with the Qubit version 3.0 fluorometer (Thermo Fisher Scientific, USA). Emulsion PCR and enrichment were carried out using the Ion PGM™ Hi-Q™ View OT2 Kit reagents (ThermoFisher Scientific—Ion Torrent, Carlsbad, CA, USA), according to the

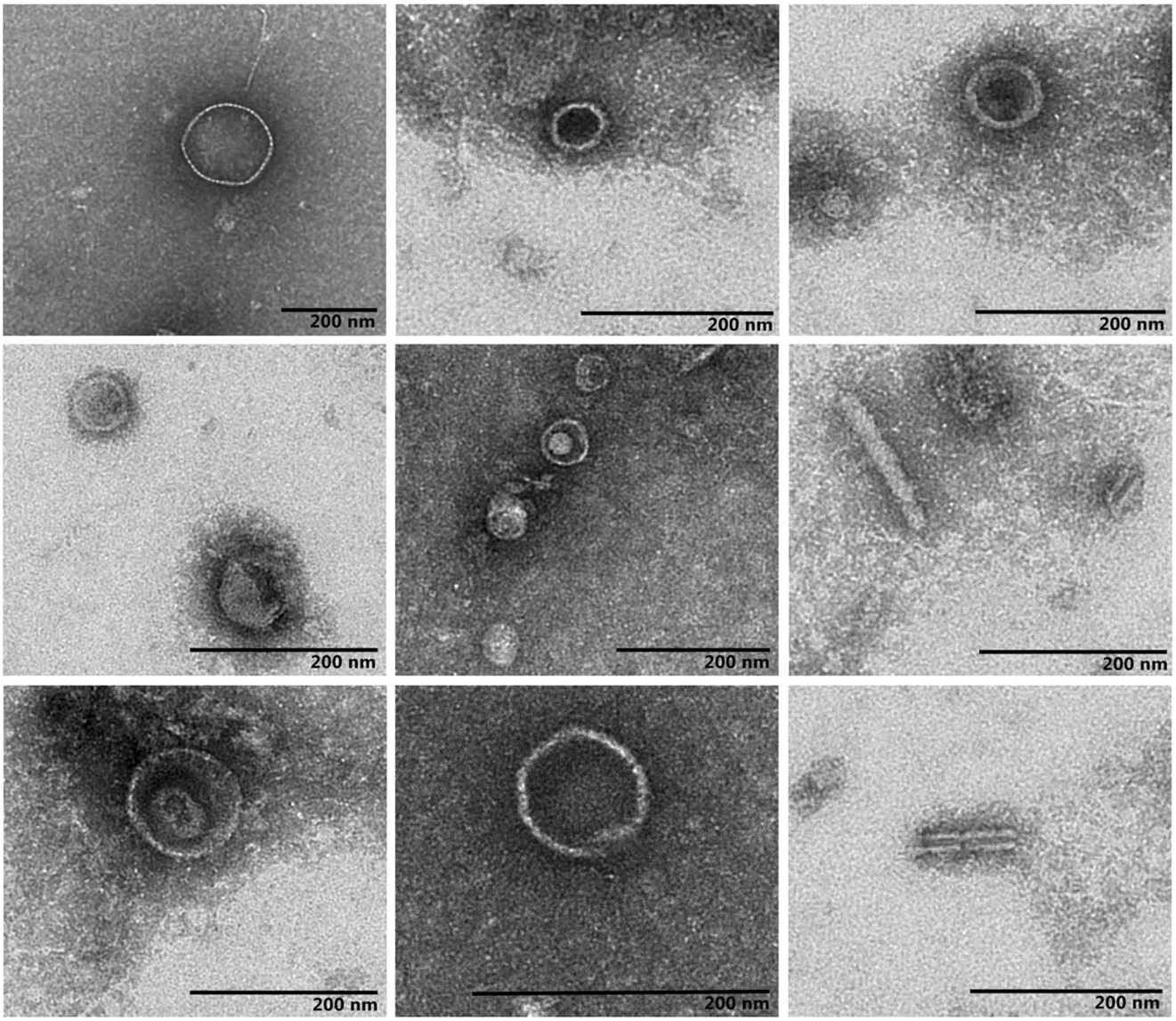


**Fig. 2** Morphologies of virus-like particles present in 70 days of composting (MP sample) by electron transmission microscopy (TEM). All black bars represent 200 nm

manufacturer's instructions. The library was sequenced on the Ion PGM platform using the Ion PGM™ Hi-Q™ View Sequencing Kit reagents (ThermoFisher Scientific—Ion Torrent, Carlsbad, CA, USA). Sequenced reads were quality checked and trimmed using the Ion Torrent Suite version 5.6.0. Additionally, low-quality bases were trimmed and duplicate reads removed with Geneious version 11.0.5 software suite (Biomatters Ltd., New Zealand). Reads from triplicates of one sample were grouped and analysed as one sample. Clean reads were subjected to a BlastN search. The BlastN results were analysed with MEGAN6 [26] for the taxonomic assignment of the reads using the lowest common ancestor (LCA) algorithm with default settings. Krona plot was used to visualised taxonomic abundances [27].

## Results and Discussion

### Transmission Electronic Microscopy Studies

In the first part of the study, the presence of virus-like particles (VLPs) in the SS sample and MP sample were visualised by TEM. The results obtained demonstrated the presence of VLPs in both samples, indicating the stability of this biological entity to both, the anaerobic mesophilic digestion previously applied to sewage sludge and to the composting treatment.

Figures 1 and 2 shown a wide diversity of viral morphologies, circular, filamentous and icosahedral, being the last one the most abundant morphology observed. These kinds of morphologies are in agreement with the results obtained in other studies, such

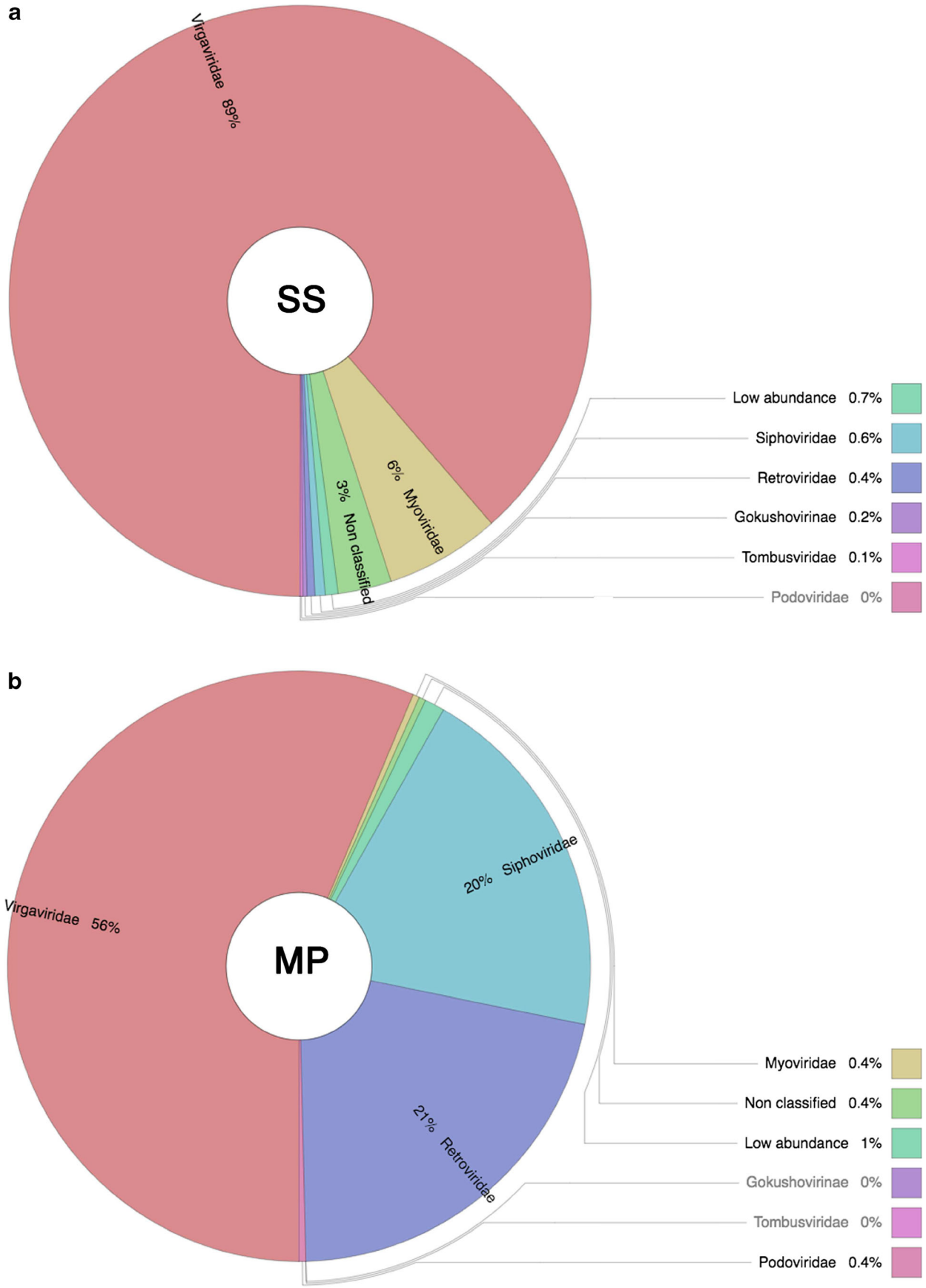


**Fig. 3** Percentage of the relative abundance of viral community, **a** in sewage sludge (SS sample) and **b** in 70 days of composting (MP sample) obtained by NGS

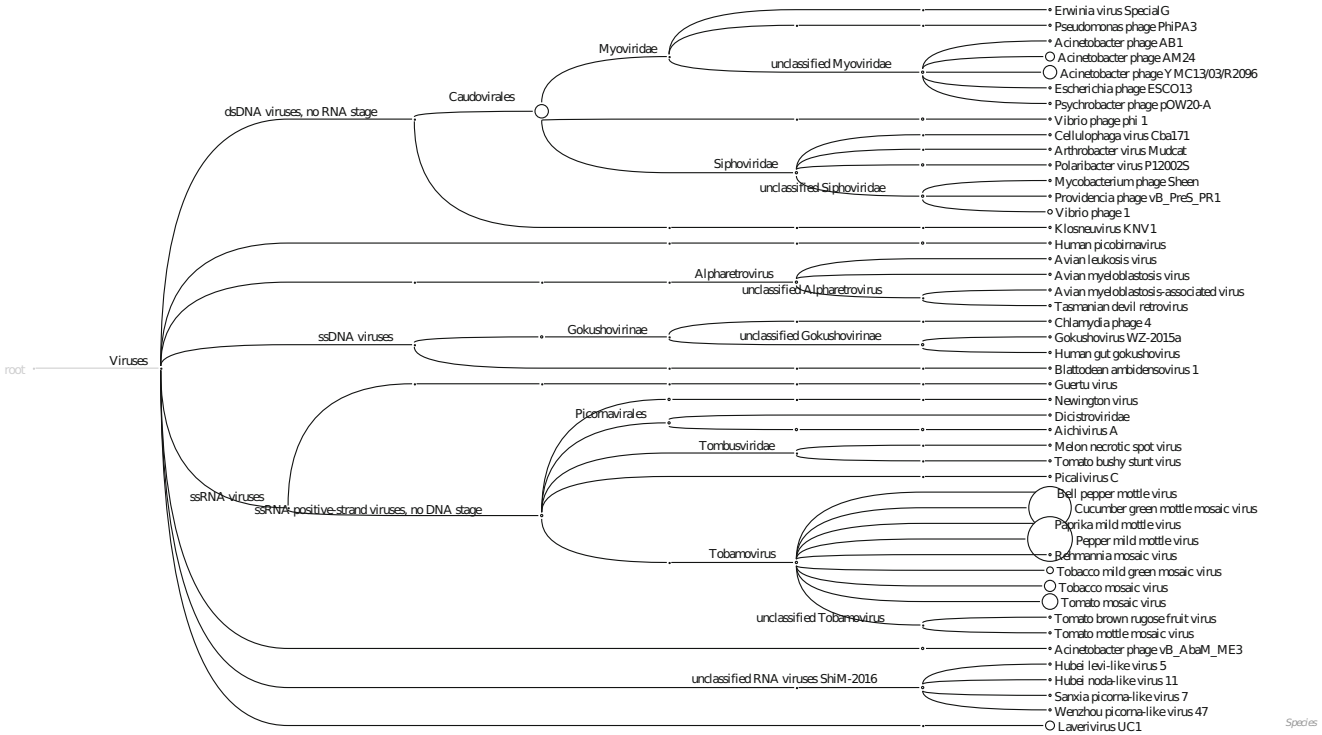


**Fig. 4** Taxonomic tree of viruses present in sewage sludge by NGS

as the study reported by Cantalupo et al. [28] in which, virus diversity was analysed in activated sludge. The icosahedral and circular morphologies were also similar to the morphologies reported by several authors in wastewater and aquatic systems [17, 29, 30]. In both studies, these morphologies have been described like morphology compatible with bacteriophages (tailed viruses) and plant viruses (filamentous viruses). Furthermore, the structures of bacteriophages, which are characterised by a binary structure, were observed in all the samples analysed.

## Viral Diversity Analysed by NGS

A total of 1,418,385 (mean length of 293 nt) and 2,624,174 (mean length of 315 nt) reads were obtained for the SS sample and the MP sample, respectively, after initial quality control trimming. After additional trimming and duplicate reads removal, there were 527,003 (mean length of 294 nt) and 292,084 (mean length of 324 nt) reads, obtained for the SS sample and the MP sample, respectively, which were used for further analysis. The

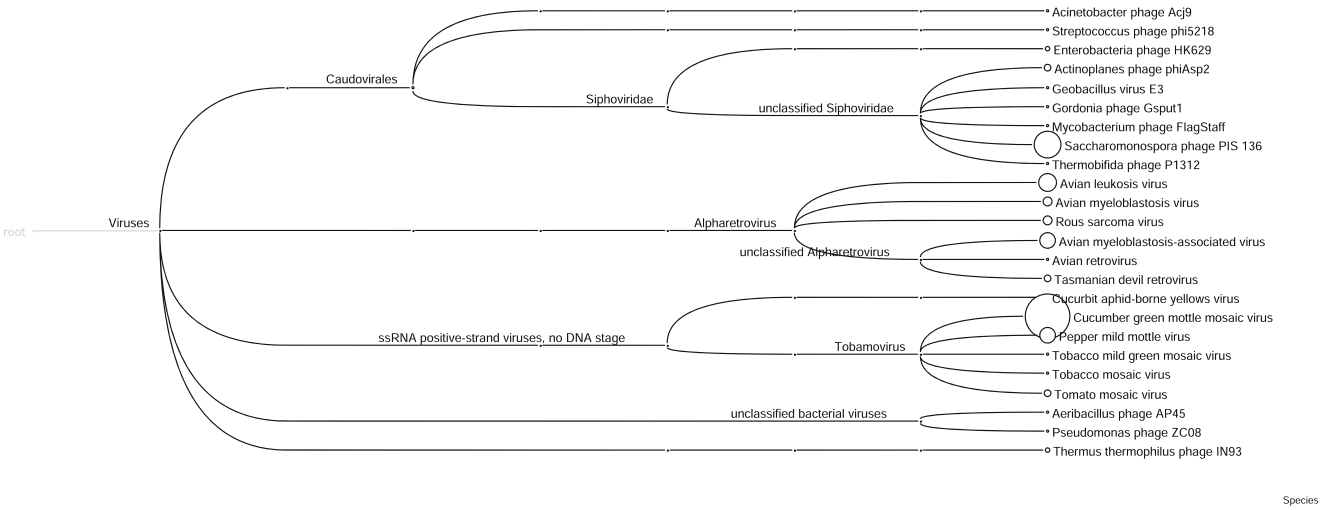


**Fig. 5** Taxonomic tree of viruses present in 70-day sample of composting by NGS

BlastN search identified 5015 (0.95%) of virus reads in the SS sample and 1353 (0.46%) of the virus reads in the MP sample. Other reads belonged to cellular organisms (371,983 (70.58%) reads for the SS sample and 193,782 (66.34%) reads for the MP sample); unclassified and other sequences (60 reads for the SS sample) and unassigned sequences (73,284 (13.91%) reads for the SS sample and 19,679 (6.74%) reads for the MP sample). Additionally, 76,661 (14.55%) reads for the SS sample and 77,270 (26.45%) reads for the MP sample had no hits.

Figure 3 a and b show the relative abundance of viruses obtained by NGS in SS and MP samples. In both samples, *Virgaviridae* was the most abundance family, but the trend on the abundance of this family was decreased during the composting process from SS to MP (from 89 to 56%). In SS sample, the second family in abundance was *Myoviridae* (6%); meanwhile, in MP sample, *Siphoviridae* (20%) and *Retroviridae* (21%) families were the most representative after *Virgaviridae* family. *Myoviridae* and *Siphoviridae* are families who included bacteriophages. The high increase in the abundance of *Siphoviridae* suggests the proliferation of bacteriophages during the composting. Species belonged to *Siphoviridae* family are associated with samples of faecal origin, due to the high number of these phages infecting enteric bacteria. Thus, it has been proposed as a useful bioindicator of faecal pollution [17, 29]. Moreover, these viruses, DNA viruses, have been previously described as more prevalent than RNA viruses in wastewater [31]. Viruses belonged to *Myoviridae*, *Siphoviridae* and *Podoviridae* were detected in both samples and this is in agreement with previous studies in raw sewage performed by Cantalupo et al. [28] within the most representative families.

A detailed virus characterisation is shown in the taxonomic assignation of each sample analysed of SS (Fig. 4) and MP (Fig. 5). The size of the circles indicates the number of sequences corresponding to each species. The SS sample had high virus diversity than MP sample.

As it has been mentioned before, *Virgaviridae* was the family more abundant in both samples. Tobamovirus was the genus more representative of this family, as can be seen in Figs. 4 and 5. In SS sample, the high number of sequences was identified as Tobamovirus. Particularly, cucumber green mottle mosaic viruses (CGMMV), pepper mild mottle virus (PMMoV), tomato mosaic virus (ToMV), tobacco mosaic virus and tobacco mild green mosaic virus were the most abundant viruses according to circle sizes (Fig. 4). Tobamovirus are ssRNA-positive strand viruses with circular or filamentous morphologies, being mostly phytopathogens. The presence of these viruses was also detected in MP sample (Fig. 5), but with a reduction in the number of sequences of all of them, except for CGMMV. The CGMMV is considered a major pathogen in *Cucurbitaceae* family crops in the world. The main crops affected by these viruses are cucumber, tomato and pepper, being insects the more common vectors [32]. The results obtained seem to indicate that the composting process could favour the removal of plant viruses, with the exception of CGMMV, as it was maintained in stable levels. The decrease of this plant viruses could be an indicator of the effectiveness of the composting, since PMMoV has been proposed as a potential faecal indicator in water, and it is considered as an abundant virus in human faeces, reaching $10^5$–$10^{10}$ copies $g^{-1}$ [33, 34]. Nonetheless, further studies will be necessary to validate the presence of this virus as an indicator. Previous studies by Cantalupo et al. [28] have reported the elimination of these viruses in the composting process through inactivation by temperature. This is in agreement with the results obtained in this study, in which a decrease of abundance was detected in MP samples compared to SS samples.

Bacteriophages were also present in both samples, as have been mentioned before. Most of them belonged to *Caudovirales* order, dsDNA viruses. *Caudovirales* were represented by families *Myoviridae* (6%) and *Siphoviridae* (0.6%). The morphology of this family was according to morphologies observed by TEM, tailed phages with contractile tail morphology, compatible with the family *Myoviridae* and long no contractile tail phage morphologies, compatible with *Siphoviridae* family (Figs. 1 and 2). The SS sample was represented by phages of enteric bacteria and bacteriophages which affect the *Acinetobacter* and *Mycobacterium* genus. ssDNA viruses, namely *Gokushovirinae*, were detected in this sample with low abundance (0.2%). The phage community might have a positive effect on the control of pathogen bacterial population. However, it should be also considered the exchange of genes involved in pathogenicity. For example, it has been demonstrated that the ability of phages for transforming non-virulent bacterial strains into virulent strains throughout the exchange of genes involved in the exotoxins production [29].

The bacterial diversity analysed in this composting process was governed mainly by *Bacillales*, *Actinomycetales* and *Pseudomonadales*, as it has been previously reported by Robledo-Mahón et al. (2018). These data fit well with the specificity of bacteriophages detected in MP samples. Most of them were phages specific of *Acinetobacter*, *Streptococcus*, *Geobacillus* or *Mycobacterium* among other species, which belonged to the abovementioned orders (Fig. 5) and identified by culture and non-culture techniques. In this sense, it is noteworthy the great abundance of Saccharomonospora phage in MP sample.

In consequence, the high temperatures reached in this kind of process using semipermeable cover film [21] could be an advantage for both bacterial and phages populations aforementioned. Human or animal viruses, as well as plant viruses belonged to the *Tombusviridae* family, were not detected at this phase of the composting process. Therefore, the results obtained suggest that, under the experimental conditions of this study, the composting process using semipermeable membrane could be effective for the removal of human or animal viruses, and this process achieves a reduction of the majority of phytopathogen viruses. In contrast, the abundance of bacteriophages was maintained in

similar percentages in both SS and MP samples, suggesting that this community could be useful tools as pollution indicators to monitoring the effectiveness of the composting process in the elimination of pathogens [17, 28, 29, 35].

The results of the distribution of viruses in the sequenced samples are according to the results reported by Bibby et al. [19], where the contributions of eukaryotic viruses were higher than bacteriophages in relative abundance. It is interesting to note that no fungal viruses were detected in the analysed samples.

In summary, the analysis of the viral community showed high diversity in the samples analysed by TEM, corresponding to the sewage sludge. The representative morphologies are according to the results obtained in similar studies in biosolids and in wastewater. The changes in the viral community showed a predominance of plant viruses and a reduction of diversity in composting. It was distinguished that human enteric viruses involved had lower representation at the end of composting (MP sample). However, in spite of the obtained results, further research is required.

## Conclusions

Viral community analysis shows a high abundance of phytopathogen viruses and a broad diversity of bacteriophages. The results obtained suggest the suitability of considering the study of possible toxic effects of compost for both animals and plants, including simple toxicity tests of filtrates from compost material, with the aim to avoid negative effects in the use of final compost as a soil amendment.

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