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E. coli CB390 as an Indicator of Total Coliphages for Microbiological Assessment of Lime and Drying Bed Treated Sludge

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Abstract: The use of a single host strain that allows for an evaluation of the levels of total coliphages in any type of environmental sample would facilitate the detection of and reduction in complexity and costs, favoring countries or areas with technical and economic limitations. The CB390 strain is a candidate for this type of simultaneous determinations, mainly in water samples. The objective of the study was to establish the recovery capacity of the CB390 strain in solid and semi-solid samples and to evaluate the microbiological quality of the sludge generated and stabilized by lime and drying beds in two WWTPs in Colombia. The results of both matrices indicated that CB390 recovered similar numbers of total coliphages ($p > 0.05$) against the two host strains when evaluated separately. Only the drying bed treatment was able to reduce between 2.0 and 2.9 Log_{10} units for some microorganisms, while the addition of lime achieved a maximum reduction of 1.3 Log_{10} units for *E. coli*. In conclusion, the CB390 strain can be used in solid and semi-solid samples, and the treatment in a drying bed provided a product of microbiological quality. However, the results are influenced by the infrastructure of the WWTP, the treatment conditions, and the monitoring of the stabilization processes.

Keywords: biosolids; domestic wastewater; heavy metals; microbiological indicators; sewage sludge; total coliphages; wastewater treatment plant

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1. Introduction

Heightened food demand due to an increase in world population has resulted in excessive water use increasing in sewage waters. These waters must be treated in wastewater treatment plants (WWTP), and reutilized or discharged into bodies of water under better conditions [1]. According to the United Nations World Water Assessment Programme (WWAP), more than 80% of the world's wastewaters and over 95% of emerging countries dispose of their waters without previous treatment [2].

In Colombia, the treatment of domestic urban wastewater reached between 42 and 42.9% during 2017 and 2018. However, the Colombian government has a projected coverage of 54.3% for 2022 and 68.6% for 2030 [3–5]. Nevertheless, in less favored rural or urban areas, basic sanitation coverage rates are lower [6–8].

As a result of wastewater treatment, liquids are separated from solids, and sludge is obtained from the sedimentation process [9]. Sludge can be stabilized through different

technologies, generating a product known as biosolid, presenting a lower load pathogenic microorganism [10,11]. Every year tons of sludge and biosolids are produced worldwide [11–15]. Due to the low rates of domestic water treatment in Latin America, the generation of sludge or biosolids is low [2,16–19]. However, 250,172 and 134,900 tons of biosolids were produced in seven Colombian cities and municipalities in 2018 and 2019, respectively [20]. This is a higher level of production in comparison to those generated in 2003 and 2007 [17,21].

Class B biosolids contain limited pollutants. Therefore, they must be handled with minimal public contact. They can be used in farms, forestry, and land recovery [22,23]. Due to the presence of pathogenic microorganisms and heavy metals, the inappropriate use of biosolids represents a potential risk to public health and the environment [9,24–26]. The presence and levels of pathogens and chemical compounds depend on the source of the wastewater and the efficiency of the treatment [27–30].

Despite different sludge stabilization processes, the complete elimination of pathogens and heavy metals cannot be guaranteed. Heavy metals may require another additional treatment to improve the characteristics of the sludge [29,31,32] or the review of conditions or factors that can determine the efficiency of the presence of other microorganisms or consortiums such as sulfate reducing bacteria (SRB), to allow for the removal of heavy metals in sludge [33–35] . Therefore, it is necessary to evaluate the quality of the sludge before it is utilized or disposed of. Their use is determined by the regulations of each region or country [15,22,29,36–50]. In the case of Colombia, this activity is ruled by decree 1287 of 2014 [42].

Quality determination, chemical status, or microbiological evaluation of these types of waste are mainly carried out in the WWTPs of the main cities of Colombia. As a result, most plants in different municipalities ignore the sludge quality and efficiencies of the stabilization treatment.

Enteric viruses are among the different groups of microorganisms that can be found in sludge and stabilized sludge. These are considered a high-risk group due to their resistance to inactivation, prolonged survival, and low infective dose [51]. Therefore, their determination becomes relevant. However, due to the costly and time-consuming detection processes, bacteriophages are alternative indicators of the presence of fecal viral pollution [52–55]. This proposal is based on the fact that bacteriophages have similar or close characteristics concerning their biology, morphology, similar structures, fate, infection, transport, and similar survival patterns against enteric viruses, providing more detailed information on the presence of viral pathogens in liquid, solid or semi-solid environmental samples [54,56–60]. Somatic and F-specific coliphages have been proposed as indicators of pathogens and sanitation efficiency [58,61–64]. Somatic coliphages present a higher count and resistance to treatments, followed by F-specific phages [53,60–62,65].

The independent detection and enumeration of these types of viral indicators are even more wasteful and expensive when used to evaluate the microbiological quality of any type of matrix. Therefore, it is necessary to have a single host strain capable of determining total bacteriophage levels regardless of the type of matrix and that its recovery levels are similar compared to other host strains that were traditionally used (*E. coli* WG5 and *Salmonella enterica serovar typhimurium* WG49).

According to the above, the proposed strain for simultaneous detection of total bacteriophages corresponds to *E. coli* CB390 against other evaluated strains (C-3000, C3322, and CN13 plus HS) [64,66]; however, its evaluation has mainly been carried out in different types of water sample [66–70]. Therefore, it is necessary to have data from other types of solid and semi-solid samples because the extraction processes, geographical conditions, or the culture media used could somehow influence their behavior. According to Jebri et al. [71] in activated sludge samples, strain CB390 obtained better total bacteriophage recoveries with some changes in the series of media used compared to that which is traditionally used [67–69].

The evaluation of this strain with several possible environmental samples from different areas and countries and according to the procedure described by Guzmán et al. [69] can help one to obtain a better knowledge of its behavior, efficiency, and possible limitations.

Finally, evaluating and determining e the total bacteriophage count in sludge, in addition to reducing the costs and laboratory analysis times for treatment plants, would allow evaluating the reduction or elimination of a greater number of viruses present in the samples [72,73]. The levels of recovery that are presented in this study could expand the use of the host strain internationally. Regarding Colombia, the evaluation of viral indicators in biosolids complements this being a regulatory requirement [42].

Many of these plants do not have a defined operating procedure. In most cases, the WWTPs in Colombia do not know the microbiological quality of the generated sludge and the efficiency of the stabilization process. This investigation focuses on two objectives. First, to evaluate the ability of the *E. coli* CB390 strain to simultaneously detect somatic coliphages and F-specific phages in semi-solid or solid sludge samples, based on the satisfactory results that this strain has had in liquid samples, described above. Second, to publicize the microbiological quality of the sludge before and after its stabilization in two municipal WWTPs in Colombia.

2. Materials and Methods

2.1. WWTPs Location and Treatment Plant Description

Chiquinquira's WWTP is located in the Boyacá department. It is located approximately 136 km from La Calera's WWTP in the Cundinamarca department (Figure 1). Both types of plants receive water collected by the sewerage network of the municipal seat. It is a combined sewer system whose wastewaters mainly come from domestic, commercial, and industrial sources with the respective stormwater input (Table 1). For both WWTPs, the treated sludge is buried within the same treatment plant facilities to avoid any contact.

2.2. Sampling

A total of 24 and 27 samples of untreated and treated sludge from the Chiquinquirá WWTP were analyzed to determine the concentration of microorganisms and heavy metals. About 800 g of sludge was collected in sterile Ziploc bags and sampling within the first four months of 2020. Nine and ten samples of treated and untreated sludge from La Calera were also evaluated, which were sampled between March and April 2020. Table 1 describes the types of treatments carried out at each of the plants. From the total of the samples collected from WWTP of La Calera, three samples of sludge without and with treatment were chosen to evaluate the levels of heavy metals under Colombian decree 1287 of 2014 [42].

Furthermore, eight affluent and effluent wastewater samples from La Calera and 24 from Chiquinquirá were analyzed for WWTPs microbiological quality evaluation and determination. The samples were collected in sterile 500 mL plastic bottles. In the WWTP of Chiquinquirá, the UV disinfection system was damaged.

All liquid and solid samples were taken at different times and days of the week within the time mentioned above. All samples were collected and maintained at <10 °C until processed. For microbiological analysis, samples were analyzed within 12 (±8) h after their collection, whereas for helminth egg and heavy metals a maximum of 16 days after their sampling was allowed.

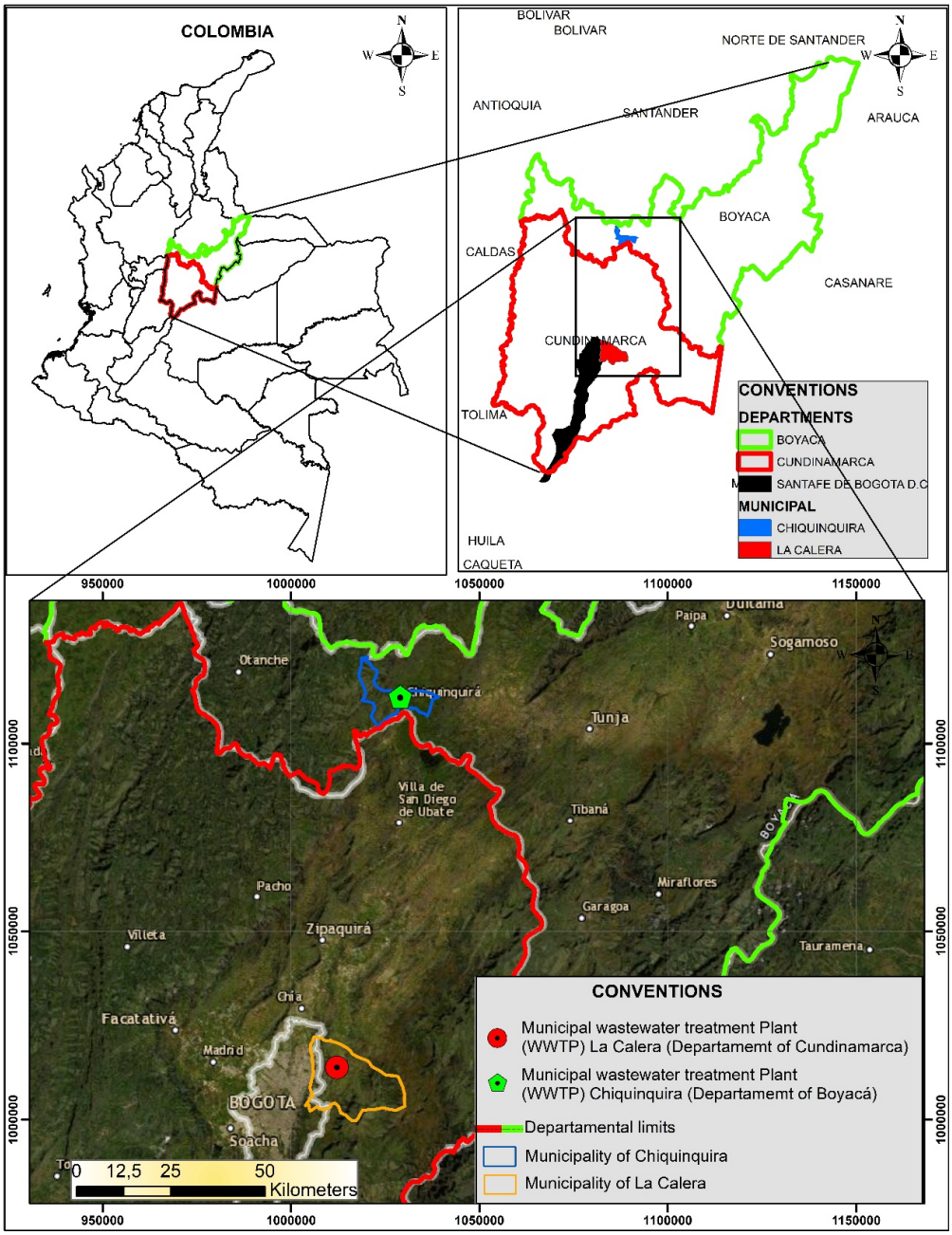

Figure 1. La Calera and Chiquinquirá, Colombia WWTPs localization.

Table 1. Municipalities of La Calera and Chiquinquirá, Colombia WWTPs sludge treatment and condition description.

WWTPs Flow Treatment	Population Served	Water Line	Sludge Treatment	Type of Sludge Stabilization	Time of Treatment or Stabilization	Quantity of Treated Sludge Generated
La Calera 32 L/s	~18,000 people	Pretreatment Primary treatment	Digester Drybeds	Drybed	~2 months	~4 to 7 Ton/year
Chiquinquirá 240 L/s to 252 L/s	~72,770 people	Secondary treatment	Thickeners and Dewatering	Lime-treated	~1 month	~480 Ton/year

~: Approximately.

2.3. Microbiological Analysis of Sludge and Wastewater Samples

Microbiological (thermotolerant coliforms and *Salmonella* spp, somatic coliphage, total helminth eggs, and viable helminth eggs.) and chemical evaluation of untreated and

treated sludge were based on that Decree 1287 of 2014 to evaluate their quality [42], as described below.

2.4. Thermotolerant Coliform (TTC)

To quantify thermotolerant coliforms or fecal coliforms, the EPA/625/R-92/013 method was used (Annex F) [74]. A total of 30 mL or g of sludges were mixed with 270 mL of sterile Phosphate Buffered Saline (PBS) and suspended by magnetic stirring at room temperature for 15 min. This suspension was used to prepare decimal dilutions, and then thermotolerant coliform was quantified by the membrane filtration procedure. For filtration, 0.45 µm × 47 mm cellulose acetate membranes (Sartorius) were used, and a vacuum filtration system, Sartorius. The blue-colored colonies on the membrane filter and M-FC medium (Merck) supplemented with 1% solution of rosolic acid were counted as the thermotolerant coliform. The results of thermotolerant coliforms are expressed as plaque-forming units per grams of dry weight basis (CFU/g dwb) [42,74]

2.5. Salmonella spp.

The most probable number of *Salmonella* spp. was determined according to EPA, Method 1682 [75]. Briefly, a given volume of sample was inoculated into the enrichment medium Tryptic Soy Broth (TSB) and incubated for 24 h at 37 °C. After incubation, a series of aliquots of the enrichment culture were inoculated in modified semi-solid Rappaport Vassiliadis (MSRV, OXOID) supplemented with novobiocin 2% (OXOID) and malachite green to inhibit the growth of non-*Salmonella* species while allowing most Salmonella species to grow. Presumptive *Salmonella* colonies were isolated on xylose-lysine desoxycholate agar (XLD) and confirmed using lysine-iron agar (LIA), triple sugar iron agar (TSI), and urea broth. The results of *Salmonella* spp. are expressed as most probable number per 25 g of dry weight basis (MPN/25 g dwb) [42,75]

2.6. Helminth Eggs (HE)

Viable and total helminth eggs were detected and quantified according to NOM-004-SEMARNAT-2002 (Annex V) [45]. In summary, this technique consisted of mixing and shaking the equivalent of 2.0 g of total solids or dry weight sample with 1 L of Tween 80 solution (0.1%). A 24 h sedimentation was performed then, the supernatant was discarded, and the pellet was filtered through a 160 µm sieve to remove the largest particles. The solids retained on the sieve were washed with 2 L of distilled water; this wash was collected in a clean 5 L plastic container.

Subsequently, the sample was subjected to the second sedimentation for 6 h, the supernatant was discarded, and the sediment was placed in 250 mL conical tubes to centrifuge at 660× *g* for 5 min. Previously the conical tubes had approximately 150 mL of $ZnSO_4$ (density 1.3). At the end of the centrifugation process, the supernatant was recovered in a clean 2.5 L plastic container, 1 L of distilled water was added, and the sediment was discarded.

Finally, sedimentation was carried out of 8 h discard the supernatant and 15 mL of acetoacetic buffer and 10 mL of ethyl acetate was added to this, followed by a gentle homogenization. The resulting pellet was mixed with 5 mL of H_2SO_4 (0.1 N) and then incubated at approximately 26° C for 4 weeks, allowing for air exchange. Finally, the sample was examined under a light microscope, eggs were counted, and viability was determined based on the formation of developing larvae. The results are expressed as Total Helminth Eggs (HET/4 g) and viable Helminth Eggs (VHE/4 g) [42,45].

In addition to that described in Decree 1287 of 2014 [42], the detection of other bacteriological indicators such as *E. coli* (CFU/g dwb) and total coliforms (CFU/g dwb) [76] was performed. For viral indicator detection, two additional indicators were detected: F-specific coliphages (F-specificPH) and CB390 phages (CB390PH).

2.7. Total Coliforms (TC) and E. coli

To quantify the total coliforms and *E. coli* the EPA/625/R-92/013 method (Annex F) [74] and ISO 9308-1 method were used [74,76]. A total of 30 mL or g of sludges were mixed with 270 mL of sterile Phosphate Buffered Saline and suspended by magnetic stirring at room temperature for 15 min. This suspension was used to prepare decimal dilutions, and then the bacterial were quantified by the membrane filtration procedure. For filtration, 0.45 µm × 47 mm cellulose acetate membranes (Sartorius) were used, and a vacuum filtration system, Sartorius. The dark blue/violet colonies were enumerated as *E. coli*. Additionally the sum red and dark blue/violet colonies on Chromocult agar (Merck) were enumerated as Total Coliform. The results are expressed as plaque-forming units per grams of dry weight basis (CFU/g dwb).

2.8. Somatic Coliphages, F-Specific Coliphages and CB390 Phage Analysis

Bacteriophages were isolated from solid and semisolid samples through the method described by Lasobras et al. [61]. In brief, samples were mixed with 10% beef extract at a 1:10 (*w*/*v*) ratio and homogenized through magnetic agitation for 30 min at room temperature. Following this, the suspension was centrifuged at 4000× *g* for 30 min. Subsequently, the supernatant was filtered through 0.22 µm syringe filters polyethersulfone (PES) membrane (Sartorious). Subsequently, somatic coliphages (SOMCPH) were detected according to the ISO 10705-2 (2000) method [77] using the *E. coli* WG5 (ATCC 700078) strain. F-specific phages were detected according to the ISO 10705-1 (1995) method [78] using the *Salmonella enterica serovar typhimurium* WG49 (ATCC 700730) strain.

The protocol described by Guzmán (2008) was used for CB390 (CB390PH) strain detection [69], where a series of additives, antibiotics and a combination of two types of media are used for the growth of bacteria, and the double layer agar described by the ISO procedure [77,78]. The strain *E. coli* CB390 (CECT9198) was grown on modified Scholten agar (MSA) (OXOID) with 100 µg/mL ampicillin (Sigma-Aldrich). For the double-layer agar technique, TYG agar and semisolid agar (OXOID) were supplemented with ampicillin (100 gmL^{-1}), Ca^{2+} and Mg^{2+}. The sum of somatic coliphages and F-specific phages were considered as total coliphages (TCPH). The results are expressed as Plaque Forming Units per gram of dry weight basis (PFU/g dwb).

2.9. Chiquinquirá WWTP Sludge Chemical Analysis

According to Decree 1287/2014 [42] the following 10 heavy metals were evaluated: A arsenic (As), cadmium (Cd), copper (Cu), chromium (Cr), mercury (Hg), molybdenum (Mo), nickel (Ni), lead (Pb), selenium (Se) and zinc (Zn) according to USEPA (2001) [79] by an inductively coupled plasma-optical emission spectrophotometer (ICP-OES Horiba Jobin-Yvon Ultima 2 CE). A total of 20 g of the sludge samples were weighed and dried at 60 °C for 12 h. To achieve homogeneity, the dried samples were sieved using a 5-mesh polypropylene sieve and ground in a mortar and pestle. Approximately one gram of dry material sample was refluxed for one hour with mass grade nitric acid and chlorohydric acid. The samples were transferred to a 100 mL volumetric flask using water type I grade. The extract solution was filtered before analysis in the instrument. The sample was analyzed by direct analysis verifying that the turbidity was below 1 NTU. The results were reported as mg/kg.

2.10. Evaluation of Microbiological Indicators in Wastewater Samples

To evaluate the influent and effluent water's microbiological quality, the presence of total coliforms (CFU/100 mL) and *E. coli* (CFU/100mL) was found, according to the ISO 9308-1 method [76]. Samples were collected and stored established according to the Standard Methods for the Examination of Water and Wastewater [80]. For filtration, 0.45 µm × 47 mm cellulose acetate membranes (Sartorius) were used, and a vacuum filtration system, Sartorius. Moreover, the dark blue/violet colonies were enumerated as *E. coli*.

Additionally, the sum of red colonies and *E. coli* colonies on Chromocult agar (Merck) was enumerated as total coliform.

To detect CB390 (CB390PH) phages in water, samples were filtered through 0.22 µm syringe filters PES membrane (Sartorious), according to Guzmán et al. [69], ISO 10705-2 [77] and ISO 1705-1 [78], as previously described. The results are expressed as Plaque Forming Units in 100 mL analyzed water (PFU/100 mL).

2.11. Data Analysis

All statistical analyses were carried out using IBM SPSS Statistics v26 software. Normal distribution was evaluated using the Kolmogorov-Smirnov test. Data without a Gaussian distribution were analyzed using Wilcoxon (Mann-Whitney U) and Kruskal-Wallis tests non-parametric tests. All tests of significance were two-tailed and p values of <0.05 were considered statistically significant (sludge without and with treatments).

3. Results

3.1. Sludge Bacterial and Viral Indicators

E. coli CB390 concentrations in sludge samples with and without treatments were similar to the counts obtained for the sum of total coliphages phages (somatic and F-specific coliphages); no significant differences ($p > 0.05$) were observed in the recoveries presented from *E. coli* CB390 compared to *E. coli WG5* and the sum of total coliphages (Figure 2).

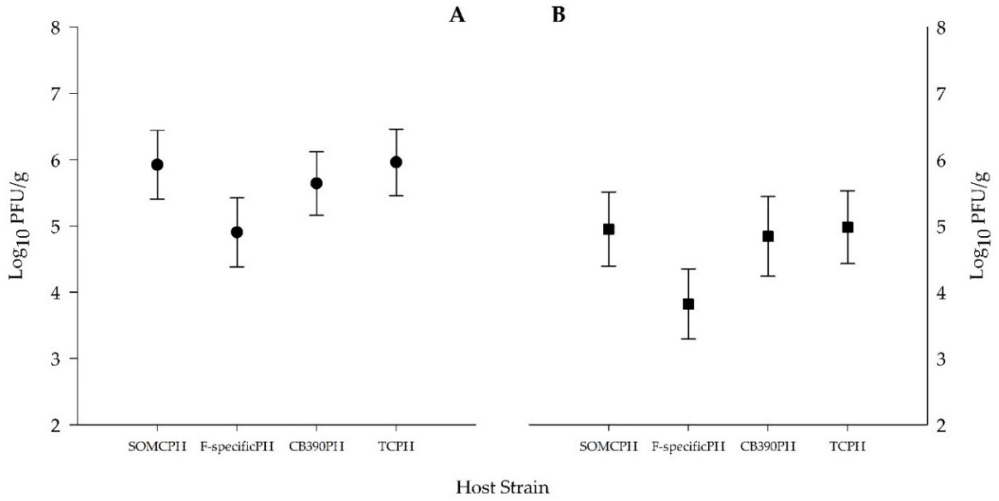

Figure 2. SOMCPH, F-specificPH, CB390PH, and TCPH concentrations in sludge samples without treatment (**A**) and with treatment (**B**). SOMCPH: Somatic Coliphages, F-specificPH: F-specific Coliphage, PHCB390: CB390 phages, TCPH: Total Coliphages.

The concentration of total coliforms, thermotolerant and *E. coli* in sludge samples without treatment from the La Calera WWTP was 6.2, 5.8, and 5.3 Log_{10} CFU/g while the values for these same indicators in the Chiquinquirá WWTP were higher with reported values between 6.7 and 7.6 Log_{10} CFU/g (Figure 3).

Regarding WWTP La Calera, drying bead sludge treatment results at week four for total coliform concentrations were 4.9 Log_{10} CFU/g, C. thermotolerant of 4.4 log_{10} CFU/g, and *E. coli* of 3.8 Log_{10} CFU/g, whereas at week eight bacterial levels were lower, reducing an additionall 1.5 Log_{10} units for a maximum total of 2.9 log_{10} units.

On the other hand, total coliform, thermotolerant, and *E. coli* levels in WWTP Chiquinquirá were 6.4, 5.9, and 5.4 Log_{10} CFU/g respectively, with a maximum reduction of 1.2 Log_{10} unit.

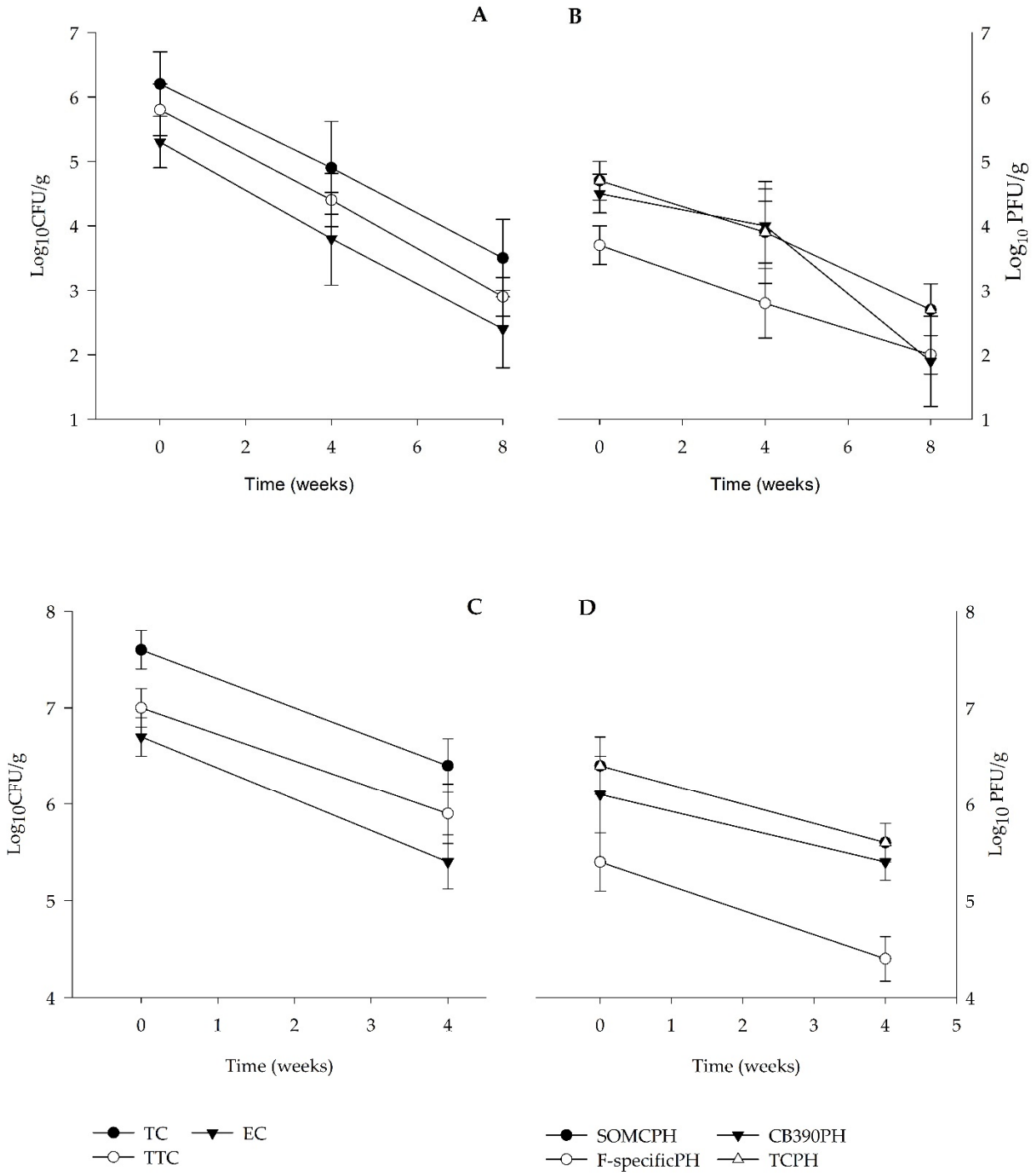

Figure 3. La Calera (**A**,**B**) and Chiquinquirá (**C**,**D**) WWTPs microbiological indicators in untreated and treated sludge as a function of time (weeks). TC: Total Coliforms, TTC: Thermotolerant Coliforms, EC: *E. coli*, SOMCPH: Somatic Coliphages, F-specificPH: F-specific Coliphages, PHCB390: CB390 phages, TCPH: Total Coliphages.

Concentrations of the viral indicators (SOMCPH, F-specificPH, CB390PH, and TCPH) in untreated samples of WWTP La Calera were 3.7 and 4.7 Log_{10} PFU/g. These results contrast with the values found in the WWTP of Chiquinquirá, where the observed levels were from 5.4 to 6.4 Log_{10} PFU/g (Figure 3) (Appendix A—Table A1).

The concentration of viral indicators at week four of treatment in sludge samples from WWTP Chiquinquirá for SOMCPH, F-specificPH, CB390PH, and TCPH corresponded to 5.6, 4.4, 5.4, and 5.6 Log_{10} PFU/g, respectively, thus, reaching a maximum reduction in viral indicators of 1.0 Log_{10} units. The levels of these indicators in WWTP La Calera were 3.9, 2.8, 4.0, and 3.9 Log_{10} PFU/g, reaching reductions between 1.2 y 1.4 Log_{10} units at week eight (Figure 3).

These results were obtained in La Calera WWTP after approximately 30 days of stabilization by lime addition (Figure 3); the pH reached during the study was less than 9.0. Other variables and conditions are not controlled or monitored (proportion of lime, time, humidity) by the WWTP which may affect the levels of microbiological reduction.

3.2. Concentration of Salmonella spp and Helminth Egg in Sludge

For the WWTPs Chiquinquirá and La Calera, the concentration of *Salmonella* spp. in the sludge was 3.4 MPN/25 g and 2.5 MPN/25 g respectively. The total helminth egg values for both plants were 75.3 and 109 HET/4 g; however, viable egg concentration was lower, with concentrations of 22.4 and 43.8 VHE/4 g, respectively (Figure 4) (Appendix A—Table A1).

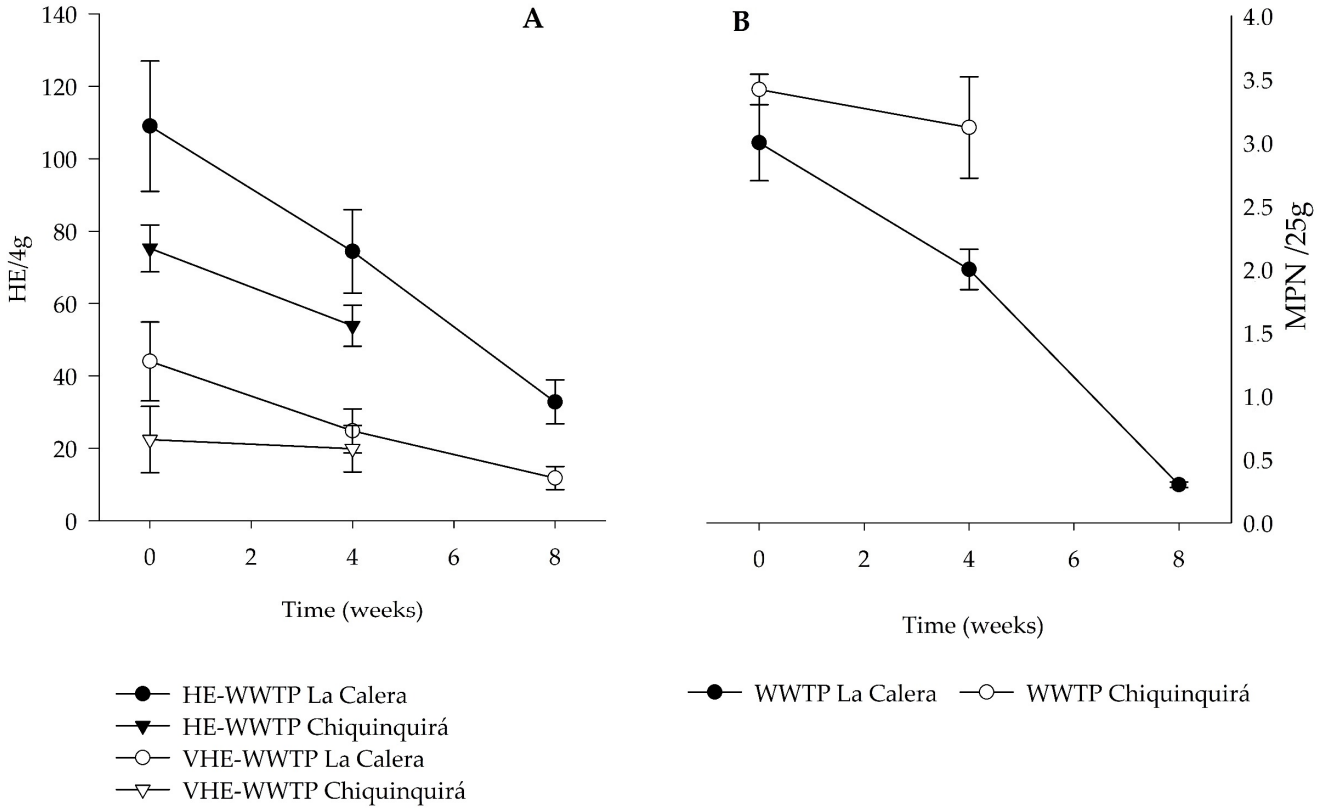

Figure 4. Chiquinquirá and La Calera's WWTPs helminth egg concentration (**A**) and *Salmonella* spp. (**B**) in sludge with and without treatment as a function of time (weeks). HE: Helminth Eggs, VHE: Viable Helminth Eggs.

In the fourth week of treatment of the sludge from the Chiquinquirá plant, the values were 3.1 MPN/25 g for *Salmonella* spp., total helminth eggs 53.9 HET/4 g, and viable 19.9 VHE/4 g. In contrast to La Calera's plant, at week four and week eight *Salmonella* spp. and helminth eggs obtained better results. The reduction was closed to one logarithmic unit for *Salmonella*, while for helminth eggs decreased by 0.6 Log_{10} units obtained final concentrations of *Salmonella* spp. of 0.3 MPN/25 g; for total and viable eggs 32.8 HET/4 g and 11.8 VHE/4 g were obtained after eight weeks of treatment (Figure 4) (Appendix A—Table A1).

3.3. Chiquinquirá WTTP Heavy Metal Concetration

Sludge Cd, Cu, Cr, Hg, Mo, Ni, Pb, and Zn concentrations corresponded to 43.8, 57.6, 10.7, 0.5, 4.8, 24.0, 15.6, and 1.1 mg/kg, respectively; however, according to the method's limit of detection, Ar and Se were not detected in any analyzed sample (<4.0 mg/kg). Mercury was only detected in one sample with a concentration of 0.6 mg/kg (Figure 5).

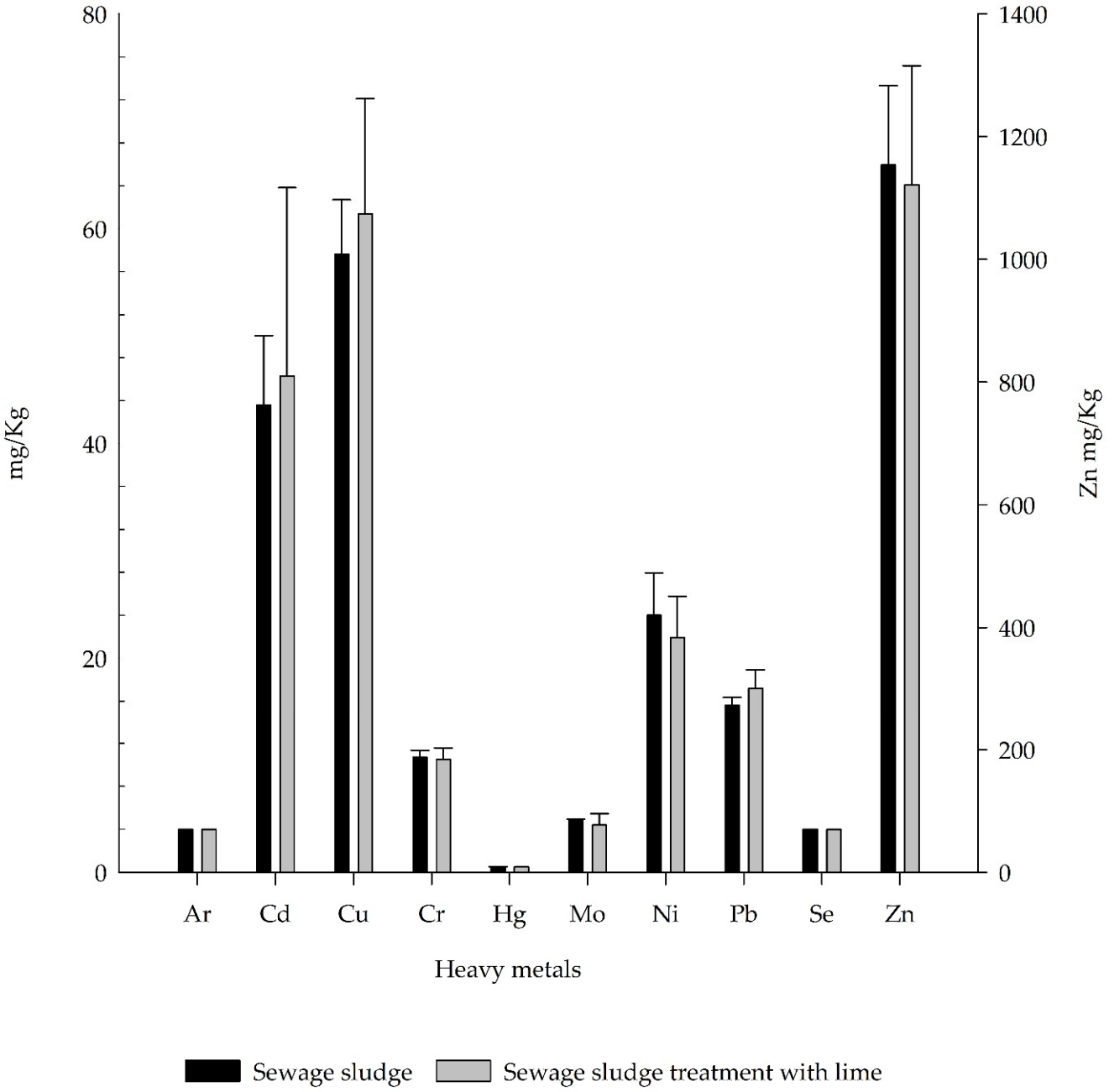

Figure 5. Chiquinquirá, Boyacá (Colombia) WWTP heavy metal concentration in treated and untreated sludge.

Average heavy metal concentration in lime-treated sludge for Cd, Cu, Cr, Mo, Ni, Pb, and Zn were 46.3, 61.4, 10.5, 4.4, 21.9, 17.2, and 1.1 mg/kg, respectively. Selenium was only detected in one sample, which presented a maximal concentration of 4.0 mg/kg. Furthermore, Ar (<4 mg/kg) and Hg (<0.5 mg/kg) were not detected in the analyzed samples (Figure 5).

3.4. Bacterial and Viral Indicators in Domestic Waste Water

Total coliforms at the entry for both La Calera's and Chiquinquirá's WWTPs had an average concentration of 7.6 Log_{10} CFU/100 mL, whereas for *E. coli*, the average concentration was 6.5 and 6.2 Log_{10} CFU/100 mL, respectively. In La Calera's WWTP effluent water samples, average counts of 6.6 and 5.4 Log_{10} CFU/100 mL were observed for total coliforms and *E. coli*. On the other hand, for the WWTP Chiquinquirá, concentrations of 6.3 and 5.4 Log_{10} CFU/ 100 mL were identified (Figure 6).

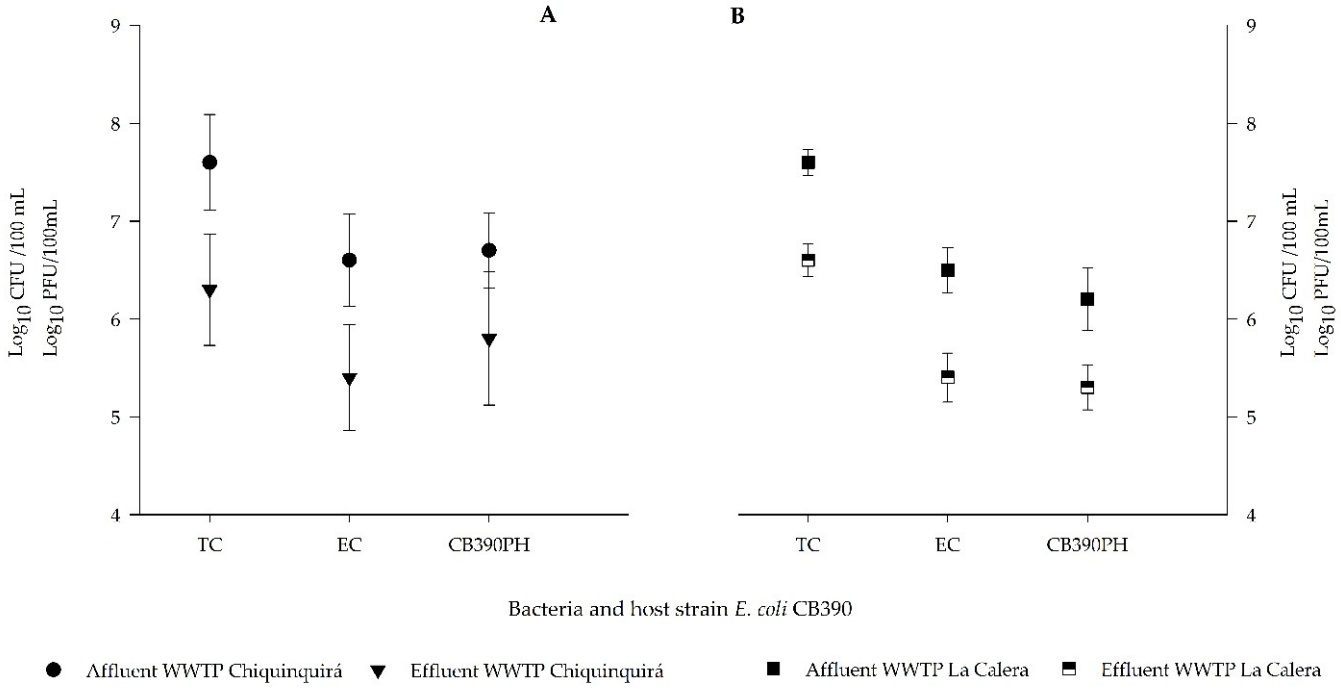

Figure 6. Affluent and effluent total C, *E. coli* and C390 phage concentration in samples collected from Chiquinquirá (**A**) and La Calera (**B**) WWTPs. TC: Total Coliforms, EC: *E. coli*, PHCB390: CB390 phages.

At La Calera´s plant, CB390PH concentrations for entry and effluent water samples were 6.2 and 5.3 Log_{10} PFU/100 mL, respectively. On the other hand, the observed values for the Chiquinquirá WWTP were 6.7 and 5.8 Log_{10} CFU/100 mL, respectively (Figure 6).

4. Discussion

E. coli CB390 coliphage recovery compared to total coliphages in untreated and treated sludge did not present significant differences ($p > 0.05$, Kruskal Wallis). The value of total coliphages is equivalent to the independent count of somatic and F-specific coliphages. Reported concentrations for somatic coliphage WG5 strain and total coliphages in all solid and semi-solid samples were slightly higher than those reported for CB390; however, these differences were not statistically significant ($p > 0.05$, Kruskal Wallis) (Figure 2). These results are consistent with the recovery levels of the same strain in different types of liquid matrices, regardless of geographic location [66–70].

The recovery values of the CB390 strain reported here suggest the possibility of its use in solid or semi-solid matrices for simultaneous somatic and F-specific coliphage detection (Figure 2), expanding its microbiological evaluation in a larger type of environmental samples.

Although the recovery results do not present significant differences, it is important to note that most of the CB390 counts are always below *E. coli* WG5 and above WG49. These levels could be improved by using the series of double-layer agar media described by ISO 10705-1 [78] standard allowing higher and closer values to each other, according by to Jebri et al. [71]. Therefore, it is necessary to continue evaluating this strain with a greater number of samples from different sites and types of treatments.

A total reduction of 1.2 Log_{10} units was observed for *E. coli* microbiological counts in Chiquinquirá' s WWTP at the fourth week of treatment, followed by thermotolerant and total coliforms; nevertheless, reduction differences among them were not significant. Likewise, the same was observed for reduced levels of different viral indicators ($p > 0.05$). Nonetheless, total coliphages, somatic and CB390 phages were the least affected by lime supplementation, compared with low phage detection by *Salmonella* WG49 (Figure 3).

Concerning *Salmonella* spp. the reductions in total and viable helminth eggs were 0.04, 0.15, and 0.1 Log_{10} units, respectively (Figure 4). It is worth noting that the results obtained in this study could be due to the mixture of sludge and lime because as the WWTP has not established the ratio of lime that needs to be added, the mixture is done manually, there is no pH control, and the arrangement in the cells is outdoors. The above experiment was probably carried out in the face of adequate control factors, such as the homogenization of compounds, temperature, pH, and humidity, which could allow for the survival of the evaluated indicators [74,81].

Regarding lime-treated sludge, the data obtained in this study are quite different from those of other studies [65,82–84], because in those studies, the results showed that microorganism levels were very low or undetectable after treatment with lime. Thermotolerant and not thermotolerant, as well as pathogenic bacteria were unappreciable, despite the very short time to lime exposure. The behavior was similar for somatic and F-specific coliphages: despite a reduction in these microorganisms, somatic coliphages were the most resistant to the treatment [61,65,84]. In contrast, helminth eggs were unaffected by lime addition in a short period of time, hence, their reduction could require more than three weeks [81,83,84]. Collectively, these results confirmed that lime treatments produce biosolids of sufficient microbiological quality, which can be used for agricultural purposes without restrictions [82,85].

Concerning WWTP La Calera reductions in comparison with drying beds at the fourth and eight week of treatment by different bacteriological and viral indicators, the evaluated *Salmonella* spp. and helminths eggs were higher compared to the lime-treatment performed in WWTP Chiquinquirá (Figures 3 and 5). Both types of treatments presented significant differences for each evaluated indicator ($p < 0.05$).

Bacteria reduction results for WWTP La Calera drying beds at the fourth week were between 1.3 and 1.5 Log_{10} PFU/g; whereas, for phages, they were between 0.5 and 0.8 Log_{10} units. At the eighth week of drying, reduction levels were even greater for bacteria 2.7 and 2.9 Log_{10} units. In contrast, phage reduction was lower and ranged between 1.9 and 2.1 Log_{10} PFU/g. *E. coli* was the indicator that presented the highest reduction, followed by thermotolerant and total coliforms. F-specific phages presented the greatest reduction, followed by total coliphages, somatic and CB390 (Figure 3). F-specific phage reduction in this type of drying against other viral indicators presented a discrete significant difference ($p < 0.05$). The reduction levels here reported were similar to those reported under the same type of stabilization, where a decrease in indicators, pathogenic bacteria and parasites were observed [74,82,86,87].

Concerning heavy metal concentration in WWTP Chiquinquirá sludge, no changes were observed after treatment ($p > 0.05$) (Figure 5). For La Calera's heavy metal concentration, García and Díaz described low detected values [88]. On the other hand, sludge treatment by the addition of lime or by the drying bed process did not affect possible sample heavy metal concentration [86]. Therefore, it is important to highlight that the presence of these compounds is related to wastewater origin, in addition to the control of industrial activities carried out in the zone [27–29,89]. For both evaluated WWTPs, water was mainly from domestic, commercial, and institutional sources with respective stormwater.

According to the microbiological results obtained in this study, lime-treated sludge cannot be used for the described uses in decree 1287 of 2014 [42]. Inadequate sludge use represents a possible public health and environmental risk due to the presence of pathogenic microorganisms [9,24–26]. These results could be mainly due to the state of the WWTP infrastructure, technical, operational, and economic limitations. The absence or weakness of the control, follow-up and monitoring processes within the stabilization process is another factor to consider.

In contrast, drying bed treated sludge generated a product of better quality, which can be utilized according to decree 1287 of 2014 [42]

Finally, in domestic wastewater total coliform, *E. coli*, and CB390 phage detected that the concentrations from the evaluated plant affluent and effluent were similar between plants. However, a higher level of phages detected was recognized in the Chiquinquirá plant compared to La Calera (Figure 6). Concerning both plants, *E. coli* and CB390 detected similar phage levels compared to the concentrations reported by other treatment plants in Colombian municipalities [67,90]. Regarding bacteria and phage reduction after treatment, their values were between 1.3 and 0.8 Log_{10} units, respectively (Figure 6). The reductions in bacteriological and viral indicators could be greater; however, the UV light disinfection process in the Chiquinquirá WWTP was damaged. Bacteria reduction levels were similar to those reported in internal reports available on the Espucal E.S.P website [91] and Empochiquinquirá E.S.P. [92].

5. Conclusions

According to the technical settings and sludge stabilization process conditions for each evaluated WWTPs, it was observed drying bed treatment resulted in a higher quality product in comparison with lime treated sludge. The WWTP La Calera must establish the operational parameters for stabilization by lime, where an adequate reduction in microorganisms is ensured.

Heavy metal levels in sludge samples with or without treatment were unaffected, regardless of the type of treatment performed.

In conclusion, the results obtained in this investigation suggest that *E. coli* CB390 could be used to detect somatic and F-specific coliphages (total coliphages) simultaneously in semi-solid and solid samples.

It is necessary to collect a greater amount of microbiological data from sludge to continue evaluating the efficiency and limitation of the strain in different types of stabilization treatments and WTTPs from Colombia or other sites. On the other hand, This allows for the determination of the type of treated sludge, its possible uses or reuses in accordance with Colombian regulations.

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Appendix A

Table A1. Bacterial concentration, viral indicators, *Salmonella* spp. and helminth eggs in untreated and treated sludge samples collected from La Calera and Chiquinquirá WWTPs.

Indicator	WWTP La Calera						WTTP Chiquinquirá			
	Sludge n: 9		Sludge Dryingbed Treatment n: 5 (4 Weeks)		Sludge Dryingbed Treatment n: 5 (8 Weeks)		Sludge n: 24		Lime-Terated Sludge n: 27 (4 Weeks)	
	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range	Average (SD)	Range
TC Log_{10} (UFC/g)	6.2 (0.5)	5.7–6.9	4.9 (0.7)	4.1–5.7	3.5 (0.6)	2.5–4.0	7.6 (0.2)	7.2–8	6.4 (0.3)	5.7–6.8
TTC Log_{10} (UFC/g)	5.8 (0.4)	5.3–6.4	4.4 (0.4)	3.9–4.9	2.9 (0.3)	2.3–3.1	7.0 (0.2)	6.5–7.4	5.9 (0.3)	5.2–6.3
E. coli Log_{10} (UFC/g)	5.3 (0.4)	4.7–5.9	3.8 (0.7)	3.0–4.6	2.4 (0.6)	1.4–3.0	6.7 (0.2)	6.2–7.1	5.4 (0.3)	4.9–5.8
SOMCPH Log_{10} (UFC/g)	4.7 (0.3)	4.3–5.1	3.9 (0.5)	3.4–4.3	2.7 (0.4)	2.1–3.1	6.4 (0.3)	5.9–7.1	5.6 (0.2)	5.3–6.0
F-specificPH Log_{10} (UFC/g)	3.7 (0.3)	3.3–4.1	2.8 (0.5)	2.2–3.4	1.6 (0.3)	1.1–2.0	5.4 (0.3)	4.8–6.1	4.4 (0.2)	4.0–4.9
PHCB390 Log_{10} (UFC/g)	4.5 (0.3)	3.9–5	4.0 (0.6)	3.3–4.6	2.6 (0.7)	1.4–3.1	6.1 (0.4)	5.6–7.0	5.4 (0.2)	5.2–5.9
TCPH Log_{10} (UFC/g)	4.7 (0.3)	4.4–5.2	3.9 (0.5)	3.4–4.4	2.7 (0.4)	2.1–3.1	6.4 (0.3)	6.0–7.1	5.6 (0.2)	5.4–6.1
Salmonella MPN/25 g	2.5 (0.3)	2.1–2.9	2.0 (0.2)	1.9–2.2	0.3 (0.0)	0.3–0.3	3.4 (0.1)	3.2–3.6	3.1 (0.4)	2.4–3.5
HE/4 g	109 (18)	82–136	74.4 (11.5)	56–84	32.8 (6.1)	24–40	75.3 (6.5)	64–88	53.9 (5.7)	40–66
VHE/4 g	43.8 (10.9)	24–58	24.8 (6.1)	20–34	11.8 (3.2)	8.0–16	22.4 (9.2)	4.0–34	19.9 (6.4)	8.0–34

TC: Total Coliforms, TTC: Thermotolerant Coliforms, SOMCPH: Somatic Coliphages, F-specificPH: F-specific Coliphages, PHCB390: CB390 phages, TCPH: Total Coliphages, HE: Helminth Eggs, VHE: Viable Helminth Eggs, SD: Standard Deviation.

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