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Removal of Enteric Pathogens from Real Wastewater Using Single and Catalytic Ozonation

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Abstract: Water scarcity is one of the main problems of this century. Water reclamation appears as an alternative due to the reuse of treated wastewater. Therefore, effluents treatment technologies (activated sludge, rotary biological discs, percolating beds) must be improved since they are not able to remove emerging contaminants such as enteric pathogens (bacteria and virus). These pollutants are difficult to remove from the wastewater and lead to adverse consequences to human health. Advanced oxidation processes, such as single and catalytic ozonation, appear as suitable complements to conventional processes. Catalytic ozonation was carried out using a low-cost material, a volcanic rock. Single and catalytic ozonation were capable of promoting total *Escherichia coli* removal from municipal wastewater after 90 min of contact. The presence of volcanic rock increases disinfection efficiency since *E. coli* regrowth was not observed. The identified viruses (*Norovirus* genotype I and II and *JC* virus) were completely removed using catalytic ozonation, whereas single ozonation was not able to eliminate *JC* virus even after 150 min of treatment. The higher performance of the catalytic process can be explained by the formation of hydroxyl radicals, proving that disinfection occurs in the liquid bulk and not due to adsorption at the volcanic rock.

Keywords: *JC* virus; *Norovirus*; Catalytic ozonation; Volcanic rock; *E. coli*; Wastewater disinfection

1. Introduction

The conventional wastewater treatment reveals difficulty in removing both chemical and biological emerging contaminants from water [1–7]. Among these pollutants, pharmaceutical and personal care products, as well as enteric pathogens [8–12], are of concern.

The traditional municipal wastewater treatment processes are usually based on biological technologies. These encompass microorganisms' usage for wastewater treatment. However, in these plants, enteric pathogens find the desirable conditions to proliferate and can be a source of water-borne disease epidemics [13]. Pathogens comprise three main groups that can be identified as a potential threat to human health: viruses, bacteria, and protozoa [14]. Some of them are used to monitor and evaluate the quality of wastewater and drinking water such as the bacteria *Escherichia coli* [14]. *E. coli* is the most common bacteria used as an indicator of faecal contamination. Moreover, some human enteric viruses can also be used for wastewater quality testing [13]. Different serotypes of *E. coli* can be found in water with different consequences on human health. The most problematic is the enterohemorrhagic *E. coli*, so-called O157-H7, that can cause bloody diarrhea and abdominal cramps,

as well as scarcer hemolytic uremic syndrome, which is life-threatening [15]. The enteric viruses are listed as emerging biological contaminants on the United States Environmental Protection Agency Contaminant Candidate List [16]. Nevertheless, the enteric viruses are widely present on discharged treated municipal wastewater [17,18]. Even so, there is still no regulation to enforce the monitoring of their concentration in treated wastewater discharges [8,9,13]. The main viruses found in these streams include human norovirus, polyomavirus, and Hepatitis A and E viruses. These pathogens, when in contact with individuals via the fecal–oral route or by food and environmental contamination, can cause multiple infections such as gastroenteritis and conjunctivitis, along with respiratory and liver problems [13]. Taking this into consideration, the presence of these pathogens in the natural sources can constitute an environmental and human health concern. Thus, the precautionary principle dictates that their effective removal from treated wastewater should be ensured before their discharge into natural courses. Due to the inefficiency of the traditional processes, complementary disinfection methodologies must be applied to promote the complete abatement of these pathogens [19,20].

Chlorine and chlorine dioxide are the most widely used disinfection methodologies. However, these processes can lead to harmful by-products, such halogenated organic compounds, that may bring adverse health effects [21]. Another methodology typically used is ozonation, which presents the strongest disinfection capacity while avoiding toxic by-products [20,21].

Ozone is a powerful oxidant capable of degrading emerging chemical contaminants [11,22,23]. This oxidant is widely used for drinking water and wastewater disinfection [20,24]. Its oxidizing capacity is proven to be efficient for bacteria, viruses, and protozoan pathogen destruction [25,26]. With respect to bacteria, ozone destroys the cell wall due to the protoplasmic oxidation, resulting in the cell lysis and leakage of cellular organelles [25,27]. The disinfection can occur via a direct attack of the molecular ozone or indirectly through hydroxyl radicals resulting from ozone decomposition [20,26]. Hydroxyl radical's production can be enhanced using heterogeneous catalysts due to the decomposition of ozone in the active sites [22,28]. The limiting step in the heterogeneous catalysis may be the catalyst material cost. However, this can be minimized using low-cost materials such as volcanic rock [29]. Gomes et al. [29] proved that the catalytic ozonation through volcanic rock is efficient for parabens decontamination.

Studies can be found in the literature with good results on pathogens disinfection with low ozone dosages and short contact periods [25]. Ozone was very quick at removing *E. coli* from ultrapure water using 0.16 mg O_3/L of transferred ozone dose (TOD) without regrowth [26]. Regarding studies involving viruses, Shin and Sobsey [30] verified a reduction of about 3 log of the *Norwalk* virus after 10 s of ozone contact time. Moreover, Schaar et al. [31] verified a reduction of 4–5 log for bacteriophage *MS2* (model virus) spiked in tertiary effluent in an ozonation pilot plant using 5 to 7 mg/L of ozone dosage. The presence of substances in the real wastewater, such as bromide and iodide, reduced the *MS2* removal and inactivation. However, removal can be completed by increasing ozone dose [32].

The aim of this work is attesting the capacity of single and catalytic ozonation (using a low-cost material as a catalyst) to remove bacteria and viruses from a secondary municipal wastewater. Since the effluent contained a significant amount of human *JC polyomavirus*, as well as genotypes I and II of human norovirus, these were the target viruses evaluated. Moreover, *E. coli* was selected as the target bacteria. To the best of our knowledge, this is the first work dealing with virus and bacteria removal from real municipal wastewater through catalytic ozonation using low-cost catalysts.

2. Materials and Methods

2.1. Chemicals and Volcanic Rock

Lauryl sulphate agar, tryptic soy agar, buffered peptone water, Bactident® Oxidase, and Bactident® KOVACS Indol reagent purchased from Merck KGaA (Darmstadt, Germany) were used for identification and quantification of *E. coli*. Real-time PCR protocols were used for studying viruses. All samples were processed in order to concentrate viral particles and were submitted

to nucleic acid extraction using a QIAmp® Viral RNA Mini Kit purchased from QIAGEN® (VWR, Lisbon, Portugal). According to the virus type, different reagents were used in the amplification and quantification steps, such as Maxima Probe qPCR Master Mix (2X) from Thermo Fisher Scientific (Lisbon, Portugal), SuperscriptTM III RT/Platinum Reaction Mix (2X), Enzyme Mix and Taq enzyme purchase from Invitrogen®, and specific primers and probes acquired from Thermo Fisher Scientific (Lisbon, Portugal).

The catalyst used in this study was a volcanic rock collected from São Miguel (Azores Islands, Portugal). This is an abundant natural material resulting from a volcanic magma eruption. It presents a high porosity and metal richness. The main characterization of the catalyst was previously carried out by Gomes et al. [29]. Briefly, the pHpzc (point zero charge) was 5.7, the BET surface area was 28.3 m^2/g, and the presence of two minerals of augite and diopside was detected through XRD. These minerals were characterized by a high percentage of silica. Moreover, the presence of Fe and Al was confirmed using Scanning Electron Microscopy- Energy Dispersive X-Ray analysis (SEM-EDS) a TESCAN VEGA 3 SBH - Easy Probe equipped- Bruker Nano XFlash®detector (Brno, Czech Republic) [29].

2.2. Experimental Procedures for Single and Catalytic Ozonation

Single and catalytic ozonation were carried out in a 2 L glass reactor with a thermostatic water-bath to maintain the temperature (25 ± 1 °C) and continuous stirring using a magnetic stirrer at 700 rpm [33,34]. Moreover, when a solid catalyst was used, the size of particles used was lower than 105 µm. Under these conditions, the chemical regime was guaranteed [28].

The reactor was covered with aluminum foil to inhibit the interference of visible and sunlight radiation since it can affect disinfection [35]. Ozone was produced from pure oxygen (99.9%, Praxair) using an ozone generator (802 N, BMT). The inlet and outlet concentrations of this oxidant were measured using gas ozone analyzers (BMT 963 vent and BMT 964 vent, respectively). With the values measured by the gas ozone analyzers, transferred ozone dose (TOD), expressed in mgO_3/L, could be determined using Equation (1), where the volume of effluent (2 L) used in the reactor is represented by V_{liquid}, Q_{Gas} is the gas flow rate (0.2 L/min), and $[O_3]^{in}$ and $[O_3]^{out}$ are the inlet and outlet ozone concentrations in the reactor, respectively [23].

$$TOD = \int_0^t \frac{Q_{Gas}}{V_{liquid}} \times ([O_3]^{in} - [O_3]^{out}) \times dt \quad (1)$$

As for the catalytic experiments, the catalyst load was 0.5 g/L. These conditions were selected based on the good performance obtained in a previous work related with parabens degradation [29]. Moreover, the catalyst was previously stirred with the effluent before feeding the ozonated gas to guarantee the particles suspension. Samples were withdrawn and rapidly filtered using a 0.45 µm filter in order to remove the catalyst.

Samples for bacteriological analysis were processed immediately after collection. As for the samples aimed for virological studies, they were filtered using a 0.2 µm filter to remove bacteria and suspended soils, and the filtrated samples were stored at −20 °C until analysis.

2.3. Municipal Wastewater Sampling

The municipal effluent provided from the secondary settler of a Portuguese wastewater treatment plant was used. Briefly, in that plant, the wastewater first suffers a pre-treatment (grading and iron chloride addition) and primary settling. Then, the wastewater is directed to a trickling bed biological reactor to remove most of the organic matter. A secondary settler is then used before the wastewater is discharged. Samples used were collected just before the discharge and used within 24 h.

2.4. Quantification of Culturable Bacteria—*E. coli*

E. coli quantification was carried out following the membrane filtration method (ISO 9308-1). Each sample underwent serial decimal dilutions (from 10^{-1} to 10^{-4}), which were filtered using a 0.45 µm cellulose membrane, in duplicate. Each membrane was inoculated in lauryl sulphate agar, and incubated for 24 ± 2 h at 37 ± 0.2 °C. After incubation, yellow colonies developed within the membrane comprising *E. coli* and other coliforms bacteria, which were counted and reported as colony forming units (CFU) per mL. To estimate how many of the counted yellow colonies corresponded to *E. coli*, confirmation tests were done. For such a purpose, five colonies of each considered plate (performing 10 colonies for each analyzed sample) were selected for inoculation in tryptic soy agar and incubated for 24 h at 37 °C. An oxidase test was performed for each obtained culture, and all oxidase-negative bacteria were further inoculated in buffered peptone water, and incubated at 44 °C for 24 h for an indol test. All selected yellow colonies in lauril-sulfate agar, with an oxidase-negative test and indol-positive results were considered as *E. coli*, and extrapolation for all counted yellow colonies were performed considering the proportion of *E. coli* confirmed colonies in the 10 selected ones.

2.5. Regrowth

When the *E. coli* quantification results are negative, it is important to guarantee that bacteria were really removed, so a regrowth test is executed. For such purpose, the sample was incubated at 37 °C for 24 h before being processed as described before (Section 2.4). If the following result is negative, it can be considered that *E. coli* was effectively removed.

2.6. Viruses Analysis

The virus studies were performed through real-time polymerase chain reaction (PCR) protocols, with virus identification and quantification being performed with a basis in their specific genome nucleotide sequence. Apart from the existent viruses in the samples, *Mengo* virus was spiked in collected samples in order to evaluate the viral recovery efficiency. Viral particles present in each sample were concentrated through an ultracentrifugation protocol [36]. Briefly, 75 mL of the sample, spiked with 0.5 mL of *Mengo* virus, were submitted to an ultracentrifugation (152,783 RCF/g) for 90 min at 18 °C for pellet formation. The supernatant was removed, and the pellet was resuspended in 0.5 mL of supernatant. Chloroform (0.5 mL) was added, and after convenient homogenization, centrifugation (405 RCF/g for 10 min) was carried out for phase separation (aqueous and organic phases), with selection of the liquid phase for analysis.

The liquid phase (0.140 mL) was submitted to nucleic acids extraction through the commercially available kit QIAmp® Viral RNA Mini Kit (QIAGEN®, Izasa, Carnaxide, Portugal) according to the manufacturer's instructions. The viral genome was eluted in 60 µL of elution buffer and stored at −20 °C until further analysis.

Amplification reactions were carried out in a final volume of 25 µL containing 7.5 µL of the viral genome. Primers and probes used for detection and quantification of the different virus were in accordance with literature [37–43].

For *JC polyomavirus* detection and quantification, 17.5 µL of Maxima Probe qPCR Master Mix (2X) (Thermo Fisher Scientific, Lisbon, Portugal), containing 300 nM of each primer, and 200 nM of TaqMan probe were used. The thermal cycling protocol included an initial 2 min incubation at 50 °C, followed by 10 min at 95 °C, and 45 cycles of 95 °C for 15 s, and 60 °C for 60 s.

Regarding noroviruses, both genotypes I and II, and hepatitis A virus, amplification was conducted in a master mix containing 12.5 µL of SuperScript III RT reaction mix (Invitrogen®), 0.5 µL of SuperScript III RT/Taq Mix (Invitrogen®), 500 nM of forward primer, 900 nM of reverse primer, and 250 nm of TaqMan probe. The thermal cycling protocol included an initial 1 h incubation at 55 °C for reverse transcription, followed by 5 min at 95 °C, and 45 cycles of 95 °C for 15 s, 60 °C for 60 s, and 65 °C for 60 s.

For hepatitis E virus (HEV) detection, the amplification reaction was carried out in a total volume of 25 µL, containing 400 nM of each primer, 120 nM of HEV probe, and 0.8µL of Superscript™ III RT/Platinum® Taq enzyme (Invitrogen®, Alfagene, Portugal). RT-PCR was carried out on the BIORAD CFX96® under the following temperature conditions: 50 °C for 30 min, 95 °C for 2 min, and 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. Negative and positive controls were used for each set of amplification reactions.

The construction of the standard curves for the viral load quantification was based on serial decimal dilutions of plasmids containing viral genome amplified regions, within the range of 1–10^5 genome copies per PCR reaction. The virus concentration expressed as $\log_{10}$ genome copies per L of wastewater ($\log_{10}$GC/L) was calculated based on the cycle threshold (Ct) values and the standard curves.

3. Results

3.1. E. coli Removal from Municipal Wastewater

Both ozonation processes, single and catalytic, were carried out for 150 min aiming toward the disinfection of secondary municipal effluent. Samples were withdrawn at the start of the experiments and at different time intervals, such as 30, 60, 90, 120, and 150 min. Then, the quantification and confirmation tests of culturable bacteria were carried out.

E. coli removal was expressed as a function of TOD values since in this kind of process, the limiting step is ozone production. Therefore, the TOD was analyzed to make conclusions about the economic viability of processes. Moreover, the amount of ozone required is a more reliable way to compare different ozone-based processes [44]. Table 1 reveals that the TOD was slightly lower when the catalyst was used. This means that the catalytic ozonation needed a lower amount of ozone than single ozonation for the disinfection. The TOD values required to achieve total *E. coli* removal were much higher for the actual wastewater compared to the results achieved when ultrapure water spiked with 3 log of *E. coli* was used. In that previous case, the maximum value of 0.16 mgO_3/L corresponding to 30 s of treatment was required to achieve total *E. coli* depletion [26]. The presence of real wastewater and its constituents such as organic matter and ionic species affects significantly the disinfection treatment [20,25,32], which will entail a higher ozone dose required for an effective disinfection.

Table 1. TOD value through each treatment (single and catalytic ozonation).

	Ozonation	**Catalytic Ozonation**
Time (min)	**TOD (mgO_3/L)**	**TOD (mgO_3/L)**
0	0.00	0.00
30	14.4	12.3
60	25.0	21.3
90	34.8	29.9
120	44.7	39.0
150	54.7	48.5

The initial *E. coli* concentration determined to be in the secondary municipal wastewater was of about 4 log which is not significantly different from the load tested with the spiked ultrapure water [26]. The resulting *E. coli* concentration using both processes as a function of TOD is shown in Figure 1.

As can be seen in Figure 1, both treatments were able to remove all *E. coli*. In the first 30 min, almost 2 logs of *E. coli* were removed, which proved the oxidative capacity of ozone. Total *E. coli* removal was achieved after 90 min, which corresponds to a TOD value of 29.9 mgO_3/L for catalytic ozonation and 34.8 mgO_3/L for single ozonation. Therefore, the presence of a catalyst allows a reduction of TOD required, maybe due to the formation of hydroxyl radicals, which increases the disinfection rate. In a previous work, the hydroxyl radical activity was checked with the same catalyst for parabens

mixture detoxification [29] through the usage of a radical scavenger (Isopropanol). The results obtained shown that a hydroxyl radical seems to be the predominant species for parabens degradation with catalytic ozonation with this very same catalyst. Thus, probably hydroxyl radicals are the main species responsible for the disinfection enhancement observed. Moreover, after 120 min, almost all coliforms bacteria were removed using catalytic ozonation, whereas single ozonation only reached these results after 150 min of oxidation corresponding a TOD value of 54.7 mgO_3/L.

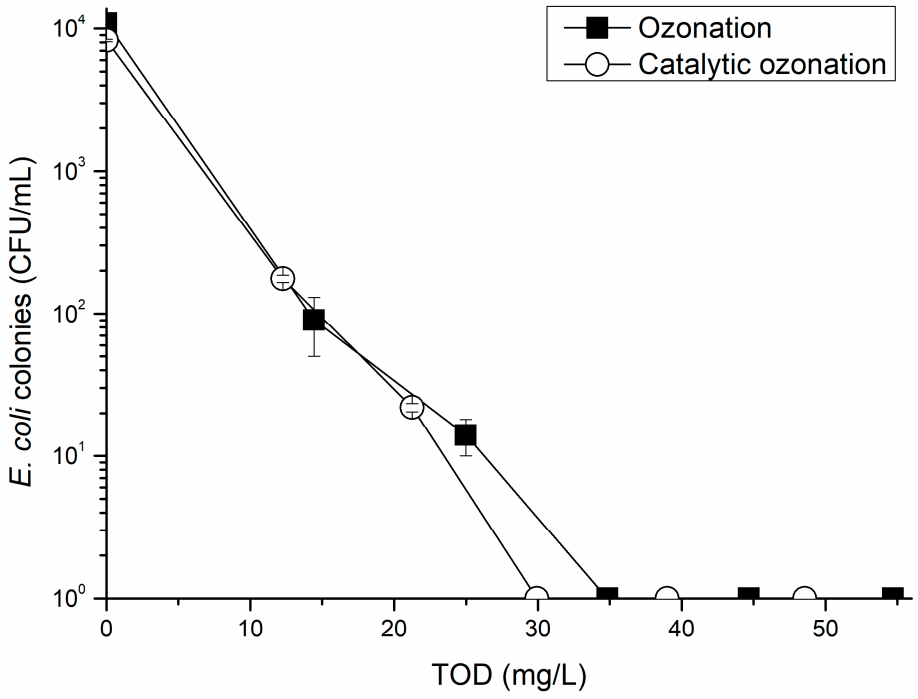

Figure 1. *E. coli* concentration using single and catalytic ozonation as function of TOD (using log_{10}scale).

The samples collected at 90, 120, and 150 min from the catalytic process were submitted to regrowth analyses. Bacteria regrowth was observed, but mostly as pink colonies, which are not characteristic of *E. coli*. However, for the single process, the samples collected at those very same reaction times presented pink and yellow colonies, which means coliforms and *E. coli* regrowth occurred. The number of regrown yellow colonies decreased between the 90 and 150 min samples. These results attest the efficiency of the catalytic ozonation regarding the complete removal of *E. coli*. Despite this, the catalytic ozonation result was not different from the single ozonation, since the dissolved molecular ozone had an important role on the *E. coli* inactivation [45,46].

In order to understand if some *E. coli* can be removed through adsorption onto the catalyst surface during the catalytic process, the wastewater was continuously stirred with the volcanic rock using the same solid load that was used during catalytic ozonation. During the process, oxygen was bubbled into the reactor instead of the ozonated gas mixture. The procedure was carried out for 150 min. Samples were taken at the start without any catalyst or oxygen, 20 min after stirring the effluent with the catalyst without oxygen, and 150 min after the feeding the oxygen to effluent/catalyst. Figure 2 shows the *E. coli* concentration as a function of time.

It is noted that the catalyst itself is not able to remove *E. coli*. In this experiment, the quantity of bacteria increased during the initial 20 min of stirring with the catalyst and without O_2. Over the next 150 min there was no significant variation in bacterial concentration. This increasing quantity of bacteria may be related to the effluent temperature in the reactor (25 ± 1 °C), which is favorable for bacteria growth. Thus, these results confirm that it is the combination of ozone and the catalyst that makes the treatment effective. As no adsorption was verified on the catalyst surface, it can be concluded that the disinfection occurs on the liquid bulk and not on the volcanic rock surface.

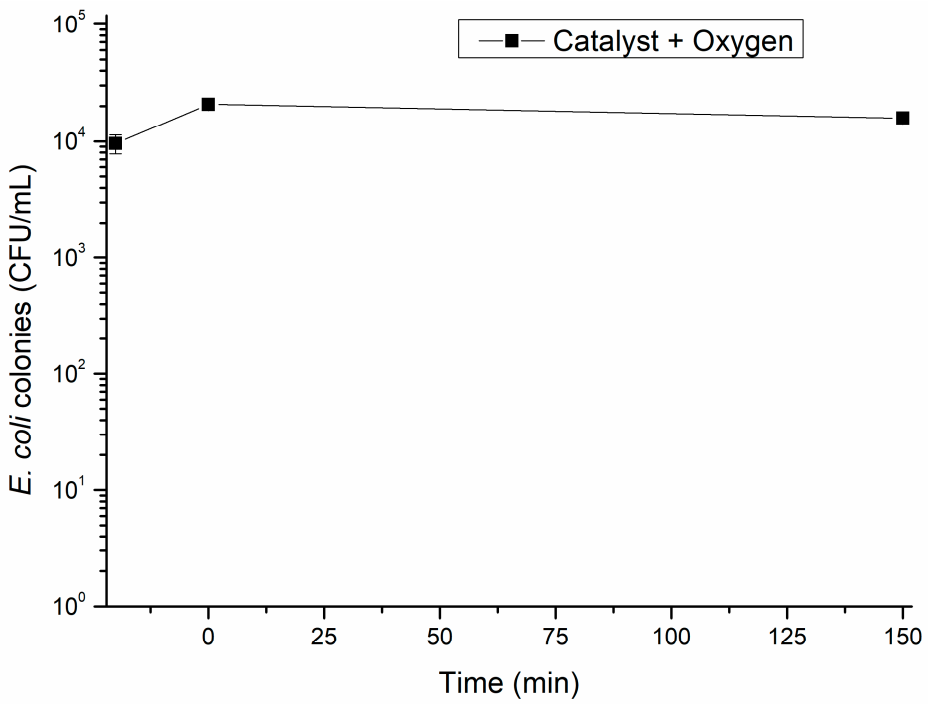

Figure 2. *E. coli* concentration using wastewater and volcanic rock as a function of time (−20 to 0 min without oxygen and from 0 min bubbling O_2, using $\log_{10}$ scale).

3.2. Virus Removal from Municipal Effluent

The initial and final samples collected from each treatment, were submitted to different PCR protocols in order to identify and quantify some viruses.

Different viruses were identified in the effluent, such as *JC polyomavirus* and *Norovirus* genotype I and II, with high initial concentrations ranging from 3000 to 945,000 copies per liter. Hepatitis A and E viruses were not detected in the effluent sample analyzed. Figure 3 summarizes the results of the amount of each virus in the initial wastewater and after 150 min of single and catalytic ozonation processes, as well as after 150 min of bubbling the effluent with oxygen and volcanic rock.

Among the identified viruses, the virus with the highest initial concentration in all experiments was *JC polyomavirus*, a human virus with a circular dsDNA genome, widely present in urban sewages [47–49], due to its continuous excretion by infected individuals [50]. The pathogenicity and virulence of this type of virus is mostly associated with immunocompromised states, such as those observed in patients with advanced HIV infection or leukemia.

The complete removal of *JC polyomavirus* was only achieved using the catalytic ozonation process, even if a higher initial virus concentration was detected at the beginning of the catalytic process compared with single ozonation. The lower efficiency of the treatment processes regarding this virus may be related with its higher initial concentration. Moreover, since it is a DNA virus, it should be more resistant to disinfection. In the experimental procedure using the catalyst bubbled with oxygen, only the concentration of *JC polyomavirus* slightly decreased. Thus, adsorption into the catalyst does not explain the efficiency of catalytic ozonation for virus removal. The combination between ozone and the volcanic rock potentiate disinfection.

The experimental results confirm the efficacy of the catalytic ozonation process when compared with the single ozone system and the catalyst itself. This positive performance can be justified once more with the assumed formation of highly reactive species when the low-cost catalyst is used in the ozonation process [29].

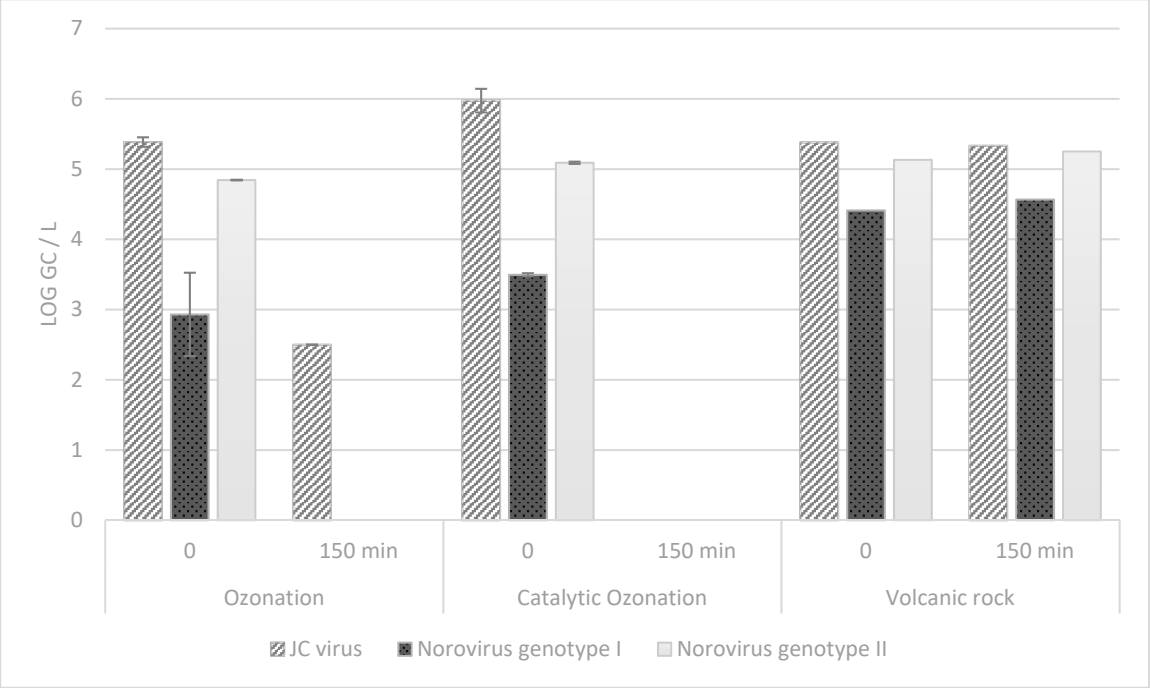

Figure 3. Initial and final virus concentration after single ozonation, catalytic ozonation, and contact with the catalyst.

3.3. Economic Implications

It can be observed that for the same reaction time, the transferred ozone dose was lower when the catalyst was used. This leads to economic benefits, since less ozone used implies less energy consume for ozone production and process.

The costs associated with ozone production can be estimated considering a value of 12 kWh/kgO_3 as the base, which is the average energy consumption in the ozone production in wastewater treatment plants [51]. The virus disinfection over 150 min of treatment for the single ozonation process consumed 0.66 kWh/m^3, while for the catalytic treatment, 0.58 kWh/m^3 was required. Nevertheless, it should be reminded that *JC polyomavirus* was only totally depleted when catalytic ozonation was applied. Thus, it would be necessary to use more energy so that single ozonation treatment be able to totally remove *JC polyomavirus*. On the other hand, in the case of catalytic ozonation, it was possible to achieve the total *E. coli* removal using a TOD value of 29.9 mg/L, which means an energetic consumption of 0.36 kWh/m^3. It must be pointed that no regrowth was found afterwards. Contrarily, for single ozonation, total *E. coli* removal was achieved using a TOD value of 34.8 mg/L, which represents an energy consumption of 0.42 kWh/m^3. Moreover, for this treatment stage, *E. coli* regrowth was still found. For no regrowth to be detected, a higher amount of ozone was required, which represented an energetic consumption above 0.66 kWh/m^3.

Regarding the real application of catalytic ozonation for enteric pathogens removal, it seems that the low-cost material usage and the improvement on the efficiency of the ozonation process regarding energy consume can be considered a suitable option to promote the disinfection of actual wastewater.

The volcanic rock is considered a low-cost catalyst since can be found abundantly in volcanic areas and no sophisticated preparation procedures are required such as those necessary for the preparation of traditional catalysts. While for the typical catalysts, precursors and solvents are needed—besides washing, drying and calcination steps (involving energy costs)—in this case, only a washing procedure would be needed. Of course, the environmental damage due to the rock's extraction should be considered. However, all catalysts based on metals require metal precursors that are produced using mining products as raw materials. Also, mining presents an important environmental impact. Still,

since volcanic areas are specific and usually protected, the full-scale application of this technology will require an environmental impact assessment.

4. Conclusions

Single and catalytic ozonation using a low-cost volcanic rock catalyst were tested to promote the disinfection of *E. coli*, norovirus genotype I and II, and *JC polyomavirus* from a secondary municipal wastewater. Disinfection of *E. coli* was efficient for both treatments, which revealed an ozone disinfection character. Moreover, the presence of a low-cost catalyst allowed for the reduction in the TOD required for *E. coli* abatement compared to the single ozonation process. In fact, for single ozonation, it was possible to verify some fecal bacteria regrowth after the treatment, whereas for catalytic ozonation, *E. coli* regrowth was not detected. This means that the low-cost material allowed more efficient disinfection and reduced the required ozone amount. The main reason for this improvement is the formation of hydroxyl radicals during catalytic ozonation. Regarding the removal of the identified virus in the municipal wastewater, norovirus genotype I and II were removed in both conditions after 150 min of treatment. However, the *JC polyomavirus*, which is characterized by its circular dsDNA genome, was only completely removed by the catalytic ozonation process. Thus, the combination of the catalyst provided from a natural source and ozone potentiate disinfection.

Regarding the economic analysis, the presence of a catalyst allows a reduction in terms of ozone amount, which will represent a lower impact in terms of ozone production costs. Thus, the low-cost catalyst makes this an interesting process for wastewater disinfection and water reclamation.

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