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RESEARCH

Synergistic effects of chlorine and ozone on ultraviolet disinfection of different microorganisms in secondary wastewater effluent

Raphael Corrêa Medeiros[1,2,3] · Kamila Jessie Sammarro Silva[2] · Maria Teresa Hoffmann[2] · Bárbara Luiza Souza Freitas[2] · Mariza de Camargo[1] · Patrícia Rodrigues Fortes[1] · Lyda Patricia Sabogal-Paz[2] · Luiz Antonio Daniel[2]

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Abstract
This study investigated the synergistic effects of chlorine or ozone application to secondary effluent prior to ultraviolet (UV) disinfection on the inactivation of *E. coli*, total coliforms, *Clostridium perfringens*, *Giardia* spp. cysts, and *Cryptosporidium* spp. oocysts. The physicochemical parameters remained statistically similar in the chlorine assays. In contrast, ozonation reduced the COD, solids, turbidity, and absorbance at 254 nm. The order of microorganism resistance was as follows: *E. coli* = total coliforms < *C. perfringens* across all treatments (both individual and sequential). The ozone dosage was more strongly correlated with microbial inactivation than was the applied CT (concentration × contact time), indicating greater efficacy with greater ozone consumption. Chick's kinetic model provided the best fit for UV radiation, whereas the Hom model was more suitable for chlorination. Standalone ozone treatment notably reduced *Giardia* cyst concentrations, and standard fluorescence reduction after sequential treatments suggested oxidative damage to cyst walls. The high viability of *Cryptosporidium* oocysts after disinfection raises significant public health concerns. Synergistic inactivation varied by treatment: ozone-UV (0.02 to 1.28 log) and chlorine-UV (0.07 to 0.82 log), depending on the target organism. These findings indicate that lower CT values for primary disinfectants can effectively reduce pathogen levels, offering a more sustainable approach to wastewater treatment.

Keywords Sequential disinfection · Wastewater treatment · Pathogenic protozoa · Indicator bacteria · Public health

1 Introduction

Wastewater discharges are a primary source of pathogenic microorganisms in receiving water bodies [1]. Even with conventional treatment, secondary effluents often contain high concentrations of microorganisms, posing significant public health risks [2, 3]. This is why implementing wastewater disinfection offers several benefits, including enhancing public health protection by acting as a barrier against environmental pathogens, reducing the risk of waterborne disease transmission, and promoting the safe reuse of treated water [4–6].

Chlorination is the most conventional disinfection method applied to water and wastewater and currently remains popular [7–9]. Chlorine inactivates microorganisms by oxidizing cellular membrane components, altering their permeability, or even causing cell rupture. It also precipitates proteins and may affect nucleic acids [4, 6]. However, chlorination is associated with the formation of disinfection byproducts (DBPs), which can be harmful to both water ecology and human health [10–13]. Hence, finding a balance between effective microorganism inactivation and the minimization of DBP formation has been a key area of extensive investigation of alternative disinfection processes [9, 14–16] from which ozonation [1, 17, 18] and ultraviolet (UV) radiation [19–22] stand out, especially for wastewater reclamation.

Ozone is a powerful oxidizing agent, and it has been applied in odor control and chemical oxidation of

✉ Raphael Corrêa Medeiros
medeiroscg@yahoo.com.br

[1] Department of Engineering and Environmental Technology, Federal University of Santa Maria, Frederico Westphalen, Rio Grande Do Sul State, Brazil

[2] Department of Hydraulics and Sanitation, University of São Paulo, São Carlos, São Paulo State, Brazil

[3] Environmental Academic Department, Federal University of Technology, Campo Mourão, Paraná, Brazil

complex organic molecules [18, 23, 24]. It also presents strong disinfectant action [25, 26], inactivating pathogens by destroying cellular integrity and damaging nucleic acids [27]. Accordingly, UV radiation serves as an effective alternative to chlorine, offering the advantage of not generating toxic byproducts, and compared to ozone, it requires minimal maintenance and is simpler to operate [6]. Like ozone, UV treatments leave no residuals, a key benefit in wastewater treatment, and their efficiencies remain unaffected by pH or temperature variations [28].

Notably, because different disinfection processes have intrinsically different mechanisms to inactivate microorganisms, they also present specific limitations [29], particularly when resistant organisms are targeted. This has driven growing interest in exploring potential synergistic effects among disinfectants [29–32]. Through synergistic interactions, the range of microorganisms targeted can be expanded and the overall efficiency can be increased [33]. Furthermore, such approaches can reduce disinfection costs by lowering chemical dosage requirements and may also decrease the DBP formation [34, 35].

In light of this, this study aimed to investigate the synergistic effects of applying chlorine or ozone prior to UV disinfection as a catalyst pretreatment. This was assessed on the basis of the inactivation of bacteria, *Escherichia coli* and total coliforms, and resistant organisms, *Clostridium perfringens*, *Giardia* spp. cysts, and *Cryptosporidium* spp. oocysts, from secondary effluent subjected to individual and sequential batch treatments.

2 Materials and methods

2.1 Sample collection and experimental scheme

The matrix used in this study was biologically treated secondary effluent from a small-scale wastewater treatment plant (WWTP) located at the University of São Paulo, São Carlos, Brazil, which treated approximately 37.6 m^3 of wastewater per day. This WWTP comprises a preliminary treatment, an upflow anaerobic sludge blanket (UASB) reactor, and an activated sludge system.

Disinfection assays were carried out in bench-scale batch experiments. A general scheme of the experimental design is shown in Fig. 1.

Experiments applying standalone chlorine, ozone, and ultraviolet radiation were performed in triplicate, considering total coliforms, *E. coli*, and *C. perfringens*. For the sequential tests, two doses of chlorine and ozone, and one dose of ultraviolet radiation were chosen. These assays were carried out in quadruplicate for total coliforms, *E. coli*, *C. perfringens*, *Giardia* spp. cysts, and *Cryptosporidium* spp. oocysts as target organisms.

2.2 Disinfection experiments

2.2.1 Chlorination

The experiments were performed in a jar test apparatus set to an agitation velocity gradient of 100 s^{-1}, equivalent to 100 rpm. The disinfectant applied was a sodium hypochlorite (NaOCl) solution with a concentration of 10 to 12% (Sigma-Aldrich®). Free and total chlorine concentrations

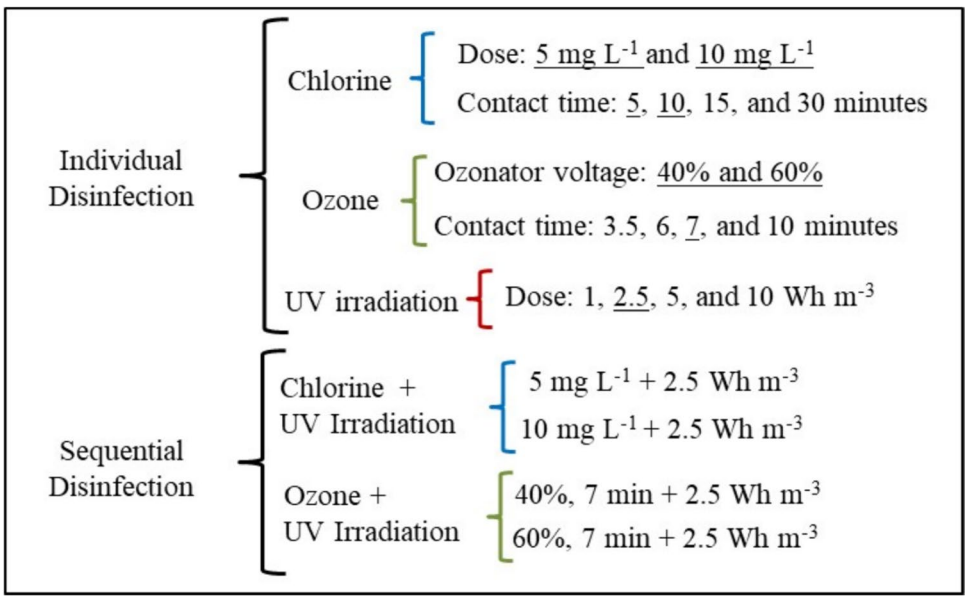

Fig. 1 Design of individual and sequential disinfection experiments. *Note*: Chlorine, ozone and UV doses applied in sequential disinfection tests are underlined

were determined via the DPD (N, N-diethyl-*p*-phenylenediamine) method via immediate reaction powder pillows (Hach®). Chlorinated samples were analyzed at 530 nm using a DR 2800 spectrophotometer (Hach®). Once the contact time was completed and immediately after residual chlorine was tested, 3% sodium metabisulfite was added to the samples for reaction quenching [36] so that interference with microbiological and physicochemical assays would be avoided.

2.2.2 UV irradiation

Tests were carried out in a stainless-steel reactor (40 cm × 45 cm × 10 cm) illustrated in Fig. 2a. An aluminum reflector dome (39.7 cm × 44.7 cm × 10 cm) containing six low-pressure mercury vapor lamps (electrical power consumption of 15 W) was attached to it. These germicidal lamps were evenly spaced to ensure uniform exposure and did not remain in contact with the liquid.

The exposure times needed to achieve the target doses (1, 2.5, 5, and 10 Wh m^{-13}) were determined on the basis of the effluent's absorbance at 254 nm, which was measured via a DR 5000 Hach® spectrophotometer. The mean intensity of ultraviolet radiation at 254 nm, incident on the liquid surface (I_o), was measured through actinometry [37]. The disinfection tests were carried out with 5.4 L of secondary effluent, resulting in a liquid layer height of 3 cm. The lamps were positioned 4 cm above the top of the liquid layer.

2.2.3 Ozonation

The pilot-scale experimental unit (Fig. 2b) applied for ozonation consisted of an oxygen generator using atmospheric air and the pressure swing adsorption (PSA) process method; an ozone generator (Eaglesat®); a flow meter; a ceramic diffuser to generate microbubbles; an ozonation column (acrylic, 0.5 thick, 5 cm internal diameter, and 150 cm height); and a gas washing bottle containing potassium iodide to capture any residual ozone that did not react during the process.

Prior to disinfection tests, the ozone generator was kept on for 10 min for stabilization. Two liters of effluent were used to fill the ozone column. Off-gas was analyzed via the iodometric method [37]. The samples from the ozone

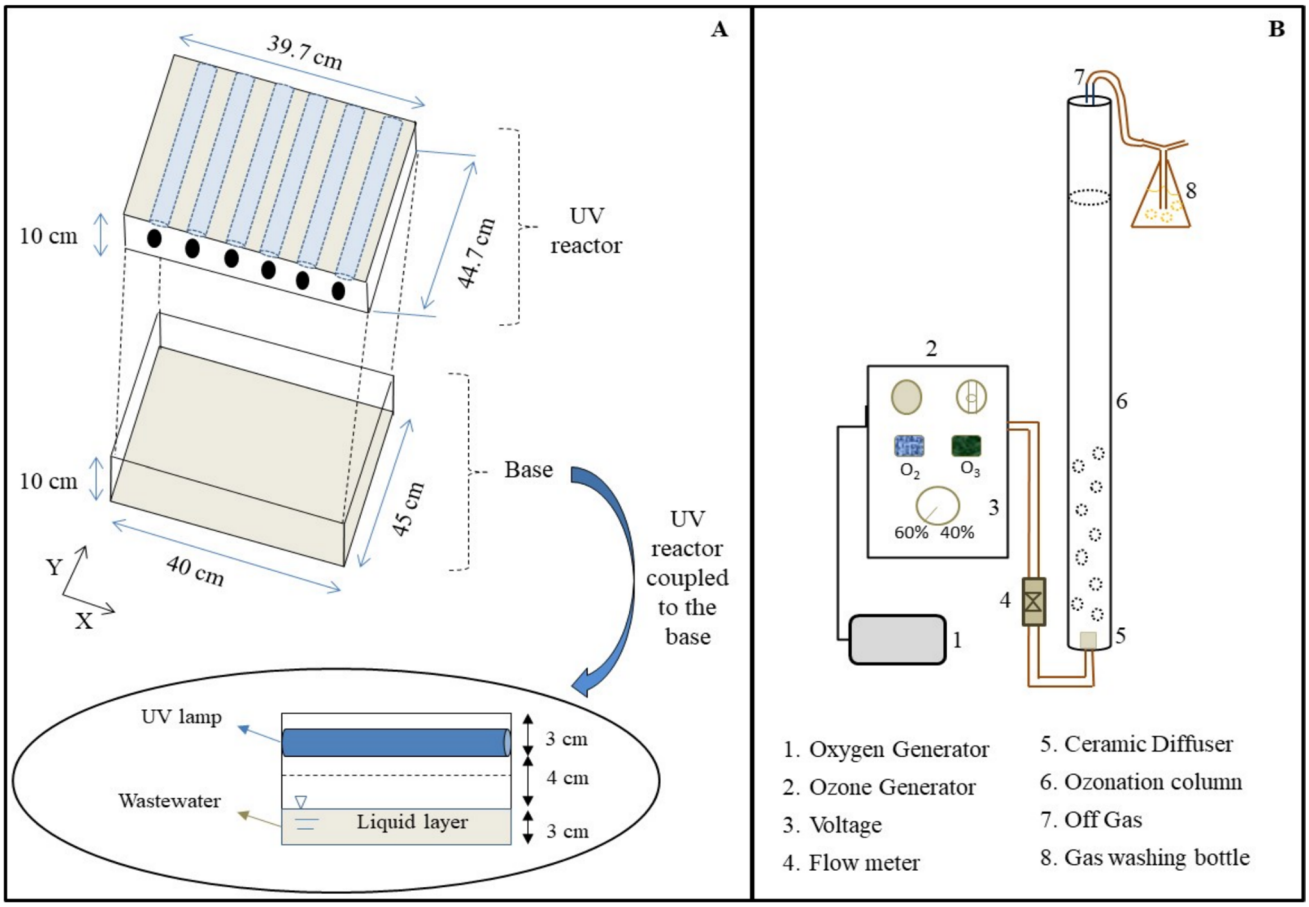

Fig. 2 Schematic representation of the experimental units used for: **a** UV disinfection and **b** ozonation

column were tested via the colorimetric method (Ozono HR AV®; DR 2800 Hach® spectrophotometer).

Two ozone productions were applied (0.11 and 0.46 g L^{-1}) at an air flow rate of 1 g.min^{-1} for different exposure times (3.5, 6, 7, and 10 min), resulting in low (3.3 mg L^{-1} to 9.4 mg L^{-1}) and high (13.5 mg L^{-1} to 38.5 mg L^{-1}) ozone dosages respectively. However, as the residual ozone concentration was substantially low, our study focused on the consumed ozone rate, defined as the mass of applied ozone divided by the contact time. The ozone application rate was assumed to be constant during the experiments. A detailed mass balance for quantifying the ozone mass and concentration deployed and consumed during the assays is provided in the supplementary material.

The product of the ozone concentration and the contact time, CT, was calculated according to Wu and Dan [38], as displayed in Eq. 1:

$$CT = \int_0^t C(t)dt \quad (1)$$

where C = consumed ozone rate (mg L^{-1} min^{-1}) and t = contact time (min).

2.2.4 Sequential disinfection

In sequential disinfection experiments, the effluents treated with either ozone or chlorine were collected and transferred to the UV radiation disinfection unit. These assays were carried out to evaluate synergistic effects of disinfectants in the inactivation of indicator microorganisms, as well as *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts.

2.3 Physicochemical analyses

The physicochemical quality of the effluent was monitored in terms of absorbance at 254 nm, total alkalinity, residual chlorine, chemical oxygen demand (COD), residual ozone, pH, total solids, total suspended solids, temperature, and turbidity. The testing procedures followed the Standard Methods for the Examination of Water and Wastewater [37].

2.4 Microbiological exams

Escherichia coli and total coliforms were quantified via the membrane filtration method [37] using Chromocult® Coliform Agar (Merck®) medium. *C. perfringens* detection and enumeration were performed via the membrane filtration technique detailed by Medeiros and Daniel [33]. In cases where there was 100% inactivation of total coliforms, *E. coli*, and *C. perfringens*, inactivation values were calculated as if there were 1 CFU remaining after disinfection.

For *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts, samples were concentrated by membrane filtration followed by immunomagnetic separation, as described by Medeiros et al. [39]. The recovery percentages were 67.5% for *Giardia* cysts and 22.5% for *Cryptosporidium* oocysts. Detection was carried out via an immunofluorescence assay (IFA), concomitant with a viability assessment, by analyzing inclusion/exclusion of the vital dye propidium iodide (PI) [40, 41]. Additionally, morphological damage was evaluated by alterations in typical fluorescence from IFA [42, 43], in order to back up inferences on viability. In this sense, (oo)cysts were classified into three categories: viable (oo)cysts with standard fluorescence; viable (oo)cysts with altered fluorescence; non-viable (oo)cysts (into which there was PI uptake). In this study, it was inferred that extensive wall damages would allow PI to be incorporated by the (oo)cysts; hence, these would be considered nonviable, whereas mild damage would alter fluorescence, but (oo)cysts would remain viable. The slides were examined under an immunofluorescence microscope (Olympus® BX51) at 400 to 800X magnification.

2.5 Disinfection kinetics

Depending on the disinfectant under test, kinetics were evaluated considering adherence to the model proposed by Chick (1908), Chick-Watson (1908), Collins (1970), and Hom (1972) [44]. Equations 2, 3, 4 and 5 represent these equations.

$$\frac{dN}{dt} = -kN \quad (2)$$

$$\frac{dN}{dt} = -k'C^nN \quad (3)$$

$$\frac{N}{No} = \left(\frac{Ct}{b}\right)^{-n} \quad (4)$$

$$\frac{dN}{dt} = -k''C^nt^{m-1}N \quad (5)$$

where k, k', k'' = the inactivation rate constant (t^{-1}); No = initial microorganism concentration (before disinfection); N = the microorganism concentration at time t; C = the disinfectant concentration (mg L^{-1}); t = time; b = the x intercept when N/No = 1; n, m = regression coefficients.

2.6 Data analysis

Statistical analysis was performed via STATISTICA 7.0 (StatSoft® Inc., 2004). Differences in the means of normally distributed data were tested by Student's t test or, for multiple comparisons, one-way ANOVA and Tukey's post hoc test. To investigate correlations in inactivation, the results of the ozonation experiments were subjected

to the Spearman rank test. *P*-values lower than 0.05 were considered statistically significant for all the aforementioned tests.

Table 1 Secondary effluent characterization for individual and sequential disinfection assays

Parameter	Secondary effluent
pH	7.16 ± 0.85
Temperature	24.1 ± 1.6
Total alkalinity (mg $CaCO_3$ L^{-1})	152 ± 104
Turbidity (NTU)	13.95 ± 19
Absorbance 254 nm	0.224 ± 0.03
COD (mg L^{-1})	66 ± 26
N–NH_3 (mg L^{-1})	26 ± 19
Total solids (mg L^{-1})	336 ± 47
Total suspended solids (mg L^{-1})	21 ± 15
Total coliforms (CFU 100 mL^{-1})*	2.2×10^5
Escherichia coli (CFU 100 mL^{-1})*	1.6×10^4
Clostridium perfringens (CFU 100 mL^{-1})*	6.7×10^3
Giardia spp. (cyst L^{-1})*&	1.4×10^3
Cryptosporidium spp. (oocyst L^{-1})*#	40

*geometric mean;& four assays.

#detected in just one assay.

Mean ± standard deviation.

3 Results and discussion

3.1 Characterization of the effluent

Table 1 displays the characteristics of the secondary effluent used in this research, which contained autochthone microorganisms targeted in the disinfection tests. Even in treated effluent, there were still high concentrations of microbiological contaminants.

3.2 Individual disinfection

3.2.1 Chlorination

The results for the physicochemical parameters are provided in the supplementary material (Table S1). Chlorination generally led to a pH increase at the highest applied dose, attributed to the reaction of sodium hypochlorite in aqueous solution, which releases OH^- ions [44]. Moreover, the observed increase in total solids, particularly in the dissolved fraction, may be associated with the addition of metabisulfite and the potential release of intracellular and extracellular materials during microbial cell oxidation.

As shown in Fig. 3, the resistance of *E. coli* and total coliforms was lower than that of *C. perfringens*, an anaerobic bacterium capable of forming spores (a resistance form). Inactivation of these organisms even after exposure to a CT of 300 mg min L^{-1}, was lower than one logarithmic unit.

In the assays with 5 mg L^{-1}, no statistically significant difference was observed between the inactivation of *E. coli*

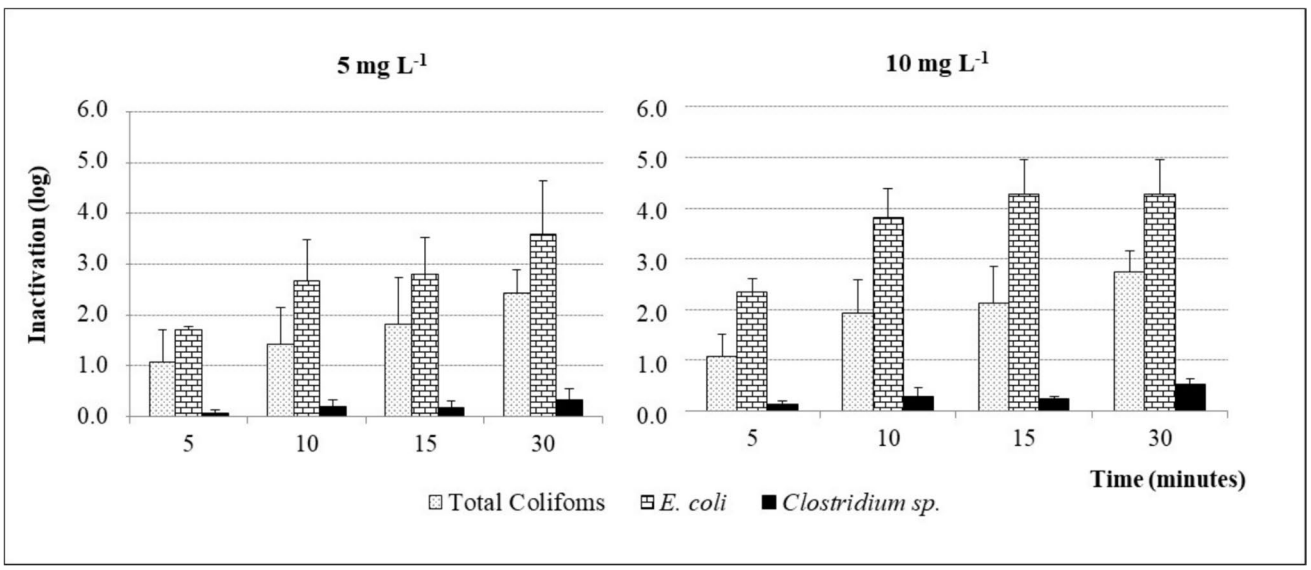

Fig. 3 Inactivation of indicator bacteria obtained by chlorination at 5 mg L^{-1} and 10 mg $L.^{-1}$ at different contact times (bars refer to standard deviation)

and total coliforms. However, at a dose of 10 mg L^{-1}, *E. coli* was the least resistant microorganism among the tested bacteria (*t*-test; $p < 0.05$), with CT values around 150 mg min L^{-1} achieving 100% inactivation efficiency (~4.20 log). The inactivation of total coliforms was lower than the 3-log reduction reported by Li et al. [45] for reclaimed water disinfection with a CT of 20 mg min L^{-1}. *Clostridium perfringens* demonstrated the highest resistance to chlorination (*t*-test; $p < 0.05$) at both doses, with a maximum inactivation of 0.62 log at a CT of 300 mg min L^{-1}. In contrast, Venczel et al. [46] reported a 1-log inactivation at the same CT (300 mg min L^{-1}).

One of the major challenges in comparing wastewater disinfection data with other studies in the literature lies in the variability of applied doses and contact times, often driven by differences in the physicochemical quality of the effluent, which might also vary. However, it is worth noting that, in this research, the high pH of the effluent (approximately 8.0) and high concentrations of ammoniacal nitrogen, with consequent formation of chloramines and decrease in the concentration of free chlorine, may have hindered microorganism inactivation by chlorine.

With respect to chlorine inactivation kinetics, the Collins, Chick, and Hom models provided a good fit, as shown in Table 2. The model fit varied depending on the microorganism; the Hom model demonstrated the best fit for total coliforms, the Collins model for *E. coli*, and the Chick model for *Clostridium* sp.

Table 2 Chick, Chick-Watson, Hom and Collins inactivation kinetics modeling parameters for chlorination

Target organism	Model	Constants/coefficients	Values	R^2
Total coliforms	Chick	*k* (5 mg L^{-1})	−0.2210	0.9277
		k (10 mg L^{-1})	−0.2550	0.9115
	Chick-Watson	*k'*	0.2172	0.0429*
		N	0.2062	
	Hom	*K*	0.7778	**0.9577**
		N	0.2062	
		m	0.4908	
	Collins	*n* (5 mg L^{-1})	1.0370	0.9326
		b (5 mg L^{-1})	1.3462	
		n (10 mg L^{-1})	1.2080	0.9311
		b (10 mg L^{-1})	1.3512	
E. coli	Chick	*k* (5 mg L^{-1})	0.3416	0.8899
		k (10 mg L^{-1})	0.4457	0.8294
	Chick-Watson	*k'*	0.2353	0.1223*
		n	0.4554	
	Hom	*K*	1.1473	0.8902
		n	0.4554	
		m	0.3677	
	Collins	*n* (5 mg L^{-1})	1.6030	**0.9666**
		b (5 mg L^{-1})	1.2128	
		n (10 mg L^{-1})	2.1028	**0.9553**
		b (10 mg L^{-1})	1.0767	
Clostridium sp.	Chick	*k* (5 mg L^{-1})	0.0268	**0.9665**
		k (10 mg L^{-1})	0.0409	**0.9707**
	Chick-Watson	*k'*	0.0105	0.4794*
		n	0.6535	
	Hom	*K*	0.0178	0.8951
		n	0.6535	
		m	0.7878	
	Collins	*n* (5 mg L^{-1})	0.1277	0.7289*
		b (5 mg L^{-1})	1.8394	
		n (10 mg L^{-1})	0.1933	0.7302*
		b (10 mg L^{-1})	1.7820	

*not significant; data in bold indicate the best fit

3.2.2 Ozonation

The results for physical and chemical parameters obtained after ozonation are provided in the supplementary material (Table S2). In short, the COD, solids, turbidity, and absorbance at 254 nm decreased after ozonation, whereas pH increased, mainly during high applied dosages.

Figure 4 shows that ozonation required higher CT values to inactivate *E. coli* and total coliforms than chlorination did. For *Clostridium* sp., ozonation resulted in an average CT of 126 mg min L^{-1} that was insufficient to achieve a 1-log inactivation.

Ozonation enhances the quality of the final effluent by generating nonspecific hydroxyl radicals, which are formed through the consumption of ozone and contribute to effective disinfection [47]. Furthermore, ozone undergoes rapid decomposition in wastewater, releasing less selective hydroxyl radicals that play a critical role in oxidation processes [24]. Like chlorination, ozonation is influenced by the physicochemical properties of the effluent, such as pH, temperature, and organic matter content, making direct comparisons with other studies in the literature challenging. For instance, Shi et al. [29] achieved 5-log *E. coli* reduction using only 5 mg L^{-1} of ozone in secondary wastewater effluent of higher quality, characterized by turbidity up to 4.3 NTU and DOC levels up to 11.1 mg L^{-1}.

Once again, *Clostridium perfringens* exhibited high resistance, achieving less than 1-log inactivation even in CT 126 mg min L^{-1}. This aligns with the findings of Gehr et al. [48], who reported a 2-log inactivation of fecal coliforms and close to 1-log for *Clostridium perfringens* with an ozone dose of 30 mg L^{-1} to 50 mg L^{-1}.

Like chlorination, the model fit varied depending on the microorganism, with the Chick, Collins, and Hom models providing the best fits (Table 3).

3.2.3 UV irradiation

To account for UV light attenuation in the 3 cm wastewater layer (absorbance of 0.224 cm^{-1} at 254 nm), the effective UV dose was calculated by integrating irradiance over the depth using the Beer-Lambert law, resulting in an average irradiance approximately 51% of the surface value. Consequently, surface doses of 1, 2.5, 5, and 10 Wh m^{-3} correspond to effective UV doses of approximately 10.8, 27, 54.1, and 108 mJ cm^{-2}, respectively.

The application of ultraviolet radiation did not result in significant changes in the physicochemical characteristics of the effluent (Table S4). UV radiation acts directly on the genetic material of the cells, causing dimerization of the thymine nitrogenous base; again, *E. coli* and total coliforms are less resistant than *C. perfringens* is (Fig. 5).

Similar to the tests with ozone and chlorine, *E. coli* was more susceptible to UV disinfection, although no significant difference was observed in the inactivation of total coliforms (*t*-test, $p > 0.05$). Moreover, *Clostridium perfringens* exhibited significant resistance to the applied doses, with an average inactivation of approximately 0.8 log only at a

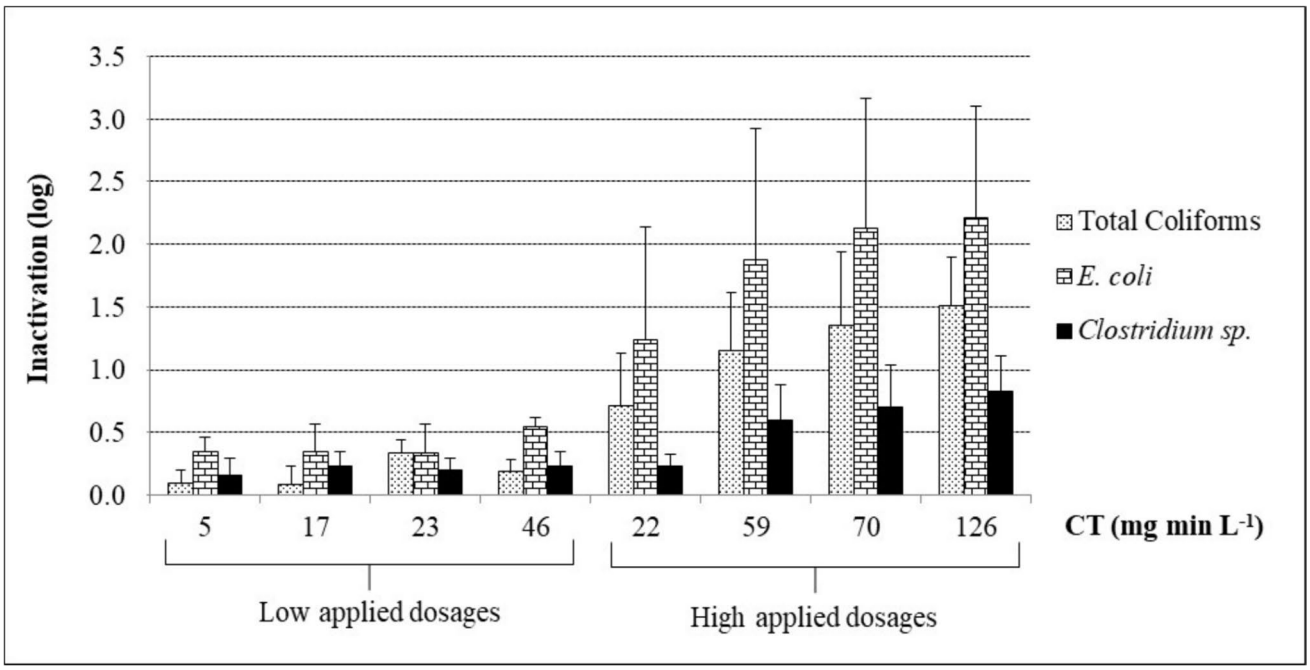

Fig. 4 Microorganism inactivation obtained by individual ozonation at low and high applied dosages (bars refer to standard deviation)

Table 3 Chick, Chick-Watson, Hom and Collins inactivation kinetics modeling parameters for ozonation

Target organism	Model	Constants/ coefficients	Values	R^2
Total coliforms	Chick	*k* (low)	0.0582	0.7921
		k (high)	0.3966	**0.9842**
	Chick-Watson	*k*'	0.0204	0.6307
		N	1.1304	
	Hom	*K*	0.2079	0.9001
		N	1.6567	
		m	−0.6568	
	Collins	*n* (low)	0.1399	0.5258*
		b (low)	1.0043	
		n (high)	0.7132	0.9632
		b (high)	1.2197	
E. coli	Chick	*k* (low)	0.1721	0.9506
		k (high)	0.6557	0.9661
	Chick-Watson	*k*'	0.1272	0.4322*
		n	0.6638	
	Hom	*K*	1.0489	0.9384
		n	1.0510	
		m	−0.5046	
	Collins	*n* (low)	0.3585	0.8545
		b (low)	0.6397	
		n (high)	1.1560	**0.9857**
		b (high)	1.0705	
Clostridium sp.	Chick	*k* (low)	0.0729	0.9407
		k (high)	0.2133	**0.9876**
	Chick-Watson	*k*'	0.0593	0.4524*
		n	0.4886	
	Hom	*K*	0.1964	0.8218
		n	0.7082	
		m	0.1463	
	Collins	*n* (low)	0.1540	0.8929
		b (low)	0.6495	
		n (high)	0.3977	0.8774
		b (high)	1.4961	

*not significant. Low = voltage of 40%; high = voltage of 60%, data in bold indicate the best fit

dose of 10 Wh m^{-3}, a result comparable to that reported by Gehr et al. [48]. Li et al. [49] and Gehr et al. [48] observed the inactivation of total coliforms and *E. coli*, respectively, within a UV dose range similar to that used in the present study (~ 10.8 mJ cm^{-2} to 108 mJ cm^{-2}). However, Wang et al. [6] reported similar results for *E. coli* and total coliforms at lower doses.

Regarding UV inactivation kinetics, both the Chick and Collins models strongly fit the disinfection results, with the Collins model providing the best fit (Table 4).

These findings on microbial decay rates in relation to intrinsic resistance to the disinfectants studied are highly important for the future design, operation, and monitoring of disinfection units. However, it is important to note that the studied models have limitations when applied to real-world conditions because of variations in the flow rate, effluent quality, and other factors.

3.3 Sequential disinfection (Chlorine–UV)

The results for the physicochemical variables following the application of chlorine as the primary disinfectant, followed by ultraviolet radiation as the secondary disinfectant, are shown in Table S5.

For the chlorine–UV sequential disinfection, the resistance of microorganisms to treatment followed the following order: *E. coli* = total coliforms < *C. perfringens* spores (*t*-test, $p < 0.05$). This trend was observed for both chlorine doses of 5 mg L^{-1} for 5 min and 10 mg L^{-1} with a dose of 10 mg L^{-1} for 10 min. The main inactivation results are shown in Table 5.

Inactivation results obtained for the three microorganisms using standalone chlorine at 10 mg L^{-1} for 10 min were statistically similar to those achieved with sequential disinfection, where the lowest chlorine dosage was followed by UV radiation (*t*-test, $p > 0.05$). Thus, this reduction in chlorine requirement suggests a lower disinfection byproduct formation while maintaining the same potential for inactivation of microorganisms, which is consistent with the findings of Wang et al. [6], who also reported synergistic effects on heterotrophic bacterial counts, total bacterial counts, and total coliforms in effluents treated with a sequential disinfection process involving ultraviolet radiation followed by chlorination.

According to Table 6, synergistic effects were more pronounced for the most resistant microorganism, *C. perfringens*, which presented averages of 0.26 and 0.43 $\log_{10}$ when treated with 5 and 10 mg L^{-1} of chlorine, respectively, followed by UV radiation.

Neither standalone nor sequential treatments involving chlorine resulted in measurable reduction in the concentration of *Giardia* spp. cysts or *Cryptosporidium* spp. oocysts. *Cryptosporidium* oocysts were detected in only 25% of the samples (4/16), which hindered the analysis of viability or fluorescence changes. Among the oocysts detected, 60% were viable. Rennecker et al. [50] also highlighted the challenges in inactivating *Cryptosporidium* oocysts with chlorine, which require CT values greater than 1000 mg min L^{-1} for 90% inactivation. Similarly, Driedger et al. [51] reported that a CT of 3700 mg min L^{-1} was needed to achieve a 2-log inactivation of *Cryptosporidium*.

Giardia cysts were assessed for morphological damage through cyst blooming and for predictive inference via the vital dye propidium iodide, as shown in Fig. 6.

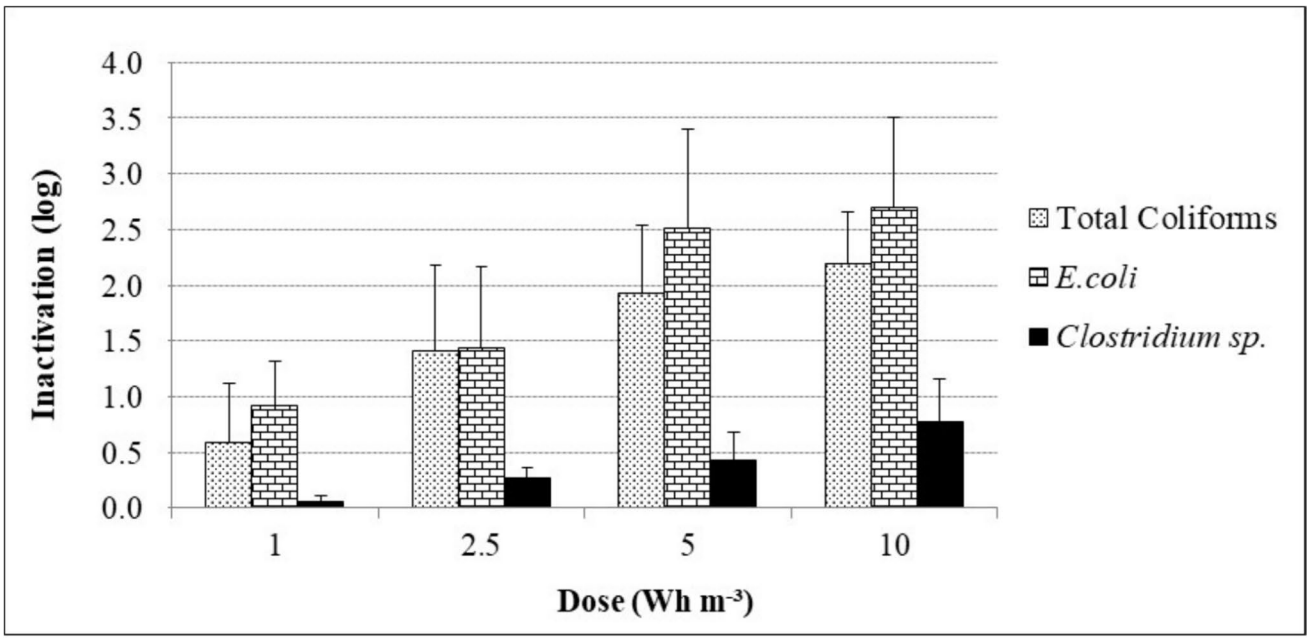

Fig. 5 Inactivation of indicator bacteria by different doses of UV radiation

Table 4 Chick and Collins inactivation kinetics modeling parameters for UV irradiation

Target organism	Model	Constants/ coefficients	Values	R^2
Total coliforms	Chick	*k*	0.6196	0.8878
	Collins	*n*	1.6870	**0.9660**
		b	0.4232	
E. coli	Chick	*k*	0.7660	0.8903
	Collins	*n*	1.9387	**0.9373**
		b	0.3555	
Clostridium sp.	Chick	*k*	0.1845	0.9927
	Collins	*n*	0.6885	**0.9554**
		b	0.9318	

Data in bold indicate the best fit.

Large standard deviations were observed in the test results, making it challenging to perform repeated disinfection trials with sewage. The variability in sample quality, which is influenced by numerous factors, affects both the chemical oxidation process and the physical action of ultraviolet radiation [6]. Despite this variability, a reduction in the percentage of *Giardia* cysts exhibiting standard fluorescence was noted after disinfection with ultraviolet radiation and varying chlorine doses. Ultraviolet radiation primarily targets cellular DNA, leading to observable changes in cyst viability [52]. In contrast, chlorine affects the cyst cell wall [53, 54], as evidenced by an increase in the percentage of cysts showing altered fluorescence. Sequential disinfection appears to amplify fluorescence alterations and enhance cyst inactivation, suggesting a synergistic effect between the two disinfection methods.

3.4 Sequential disinfection (Ozone-UV)

The results for physical and chemical parameters of ozone applied as a primary disinfectant followed by UV radiation are presented in Table S6. The temperature remained unchanged after ozonation and subsequent ultraviolet

Table 5 Inactivation, in logarithmic units, of the indicator microorganisms (mean ± standard deviation) subjected to chlorination followed by ultraviolet radiation

Treatment	Inactivation (log_{10})		
	E. coli	Total coliforms	*C. perfringens*
UV disinfection [2.5 Wh m^{-3}]	1.70 ± 0.33	1.47 ± 0.32	0.12 ± 0.04
Chlorination [5 mg L^{-1}; 5 min]	2.46 ± 0.77	1.94 ± 0.97	0.77 ± 0.48
Chlorine [5 mg L^{-1}; 5 min] + UV [2.5 Wh m^{-3}]	3.62 ± 0.61	2.78 ± 0.32	1.15 ± 0.76
Chlorination [10 mg L^{-1}; 10 min]	3.59 ± 0.39	3.15 ± 0.38	1.06 ± 0.57
Chlorine [10 mg L^{-1}; 10 min] + UV [2.5 Wh m^{-3}]	4.04 ± 0.43	3.50 ± 0.45	1.61 ± 0.77

Table 6 Synergistic effects in sequential disinfection chlorine–UV disinfection

Microorganism	Assay	Σ Individual inactivation (Ii)	Sequential inactivation (Si)	Synergism
E. coli	[5;5]-UV^4	3.38	3.56	0.18
Total coliforms	[5;5]-UV^4	2.54	2.67	0.13
Clostridium perfringens	[5;5]-UV^1	1.33	1.71	0.38
	[10;10]-UV^1	1.54	1.80	0.26
	[5;5]-UV^2	1.48	2.08	0.60
	[10;10]-UV^2	1.96	2.78	0.82
	[10;10]-UV^3	0.86	1.06	0.20
	[5;5]-UV^4	0.33	0.40	0.07
	[10;10]-UV^4	0.36	0.78	0.42

*Synergism = Observed Si–(Σ Ii) (USEPA, 1999). The inactivation values and synergism values are given as log values.

[5;5]: chlorination with 5 mg L^{-1} and 5 min; [10;10]: chlorination with mg L^{-1} and 10 min; UV of 2.5 Wh m^{-3}. $^{1;2;3;4}$: assay numbers.

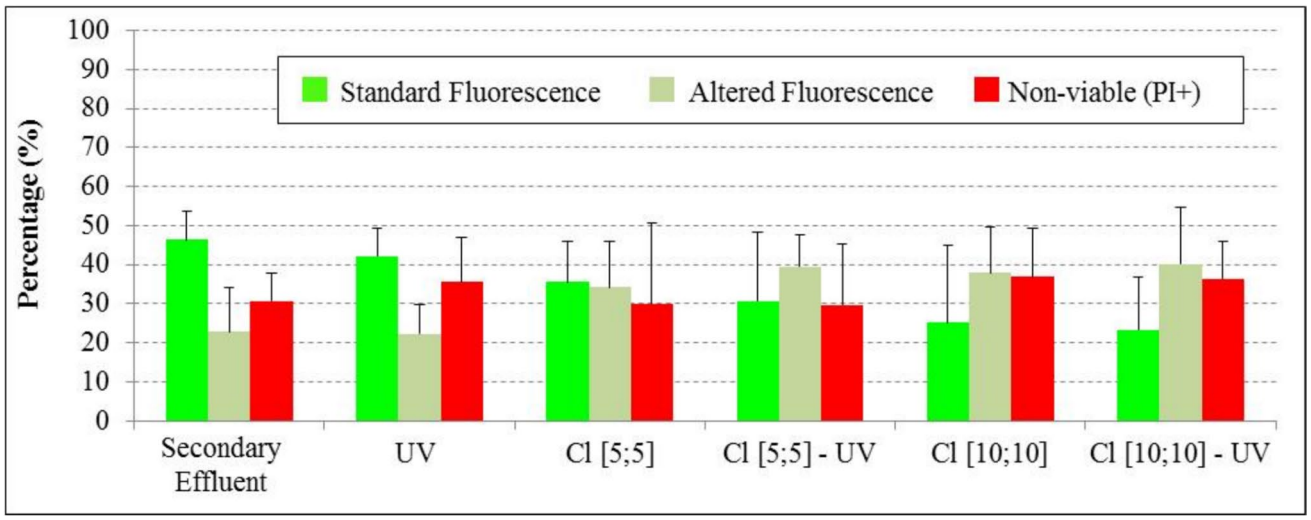

Fig. 6 Assessment of morphological damage to *Giardia* spp. cysts following individual and sequential disinfection treatments with chlorine and UV radiation. The central bars indicate the standard deviation. Chlorine doses applied: 5 mg L^{-1} for 5 min, and 5 mg L^{-1} for 10 min. UV dose: 2.5 Wh m^{-3}

radiation treatment, while pH increased significantly (*t*-test, $p < 0.05$) following both ozonation and sequential disinfection. At a CT of 23 mg min L^{-1}, average removals of 20.2% for turbidity and 10.6% for COD were achieved. When the CT was increased to 83 mg min L^{-1}, the removal rates improved to 51% for turbidity and 22.4% for COD. The UV radiation dose following ozone did not influence turbidity or COD removal. The results for the inactivation of indicator microorganisms are shown in Table 7.

No statistical differences were observed in the resistance of microorganisms—*E. coli*, total coliforms, and *Clostridium perfringens*—to individual ozonation. However, in the sequential tests involving ozone followed by UV radiation, the resistance order for both ozone CT values was as follows: *E. coli* = total coliforms < *Clostridium perfringens* (*t*-test, $p < 0.05$), which was consistent with the results from the sequential chlorine and UV radiation treatments.

Inactivation results for the three microorganisms, when ozonation was applied alone at 11 mg L^{-1} for 7 min, were statistically equivalent to those achieved with sequential disinfection (3.3 mg L^{-1} for 5 min of ozone followed by UV radiation). The application of ozone before UV radiation can reduce the cost of the UV disinfection system by decreasing the effluent's absorbance in the spectrum range of 200–700 nm, as shown in Supplementary Fig. S1 (Supplementary Material), particularly at 254 nm. This reduction allows for fewer UV lamps and smaller channels, in accordance with White [44]. Therefore, both the costs and benefits of sequential disinfection should be carefully considered. One potential explanation for this cost-effectiveness

Table 7 Synergistic effect of sequential disinfection chlorine–UV disinfection

Treatment	Inactivation (log_{10})		
	E. coli	Total coliforms	*C. perfringens*
UV disinfection [2.5 Wh m^{-3}]	1.70 ± 0.33	1.47 ± 0.32	0.12 ± 0.04
Ozonation [3.3 mg L^{-1}; 7 min]	0.04 ± 0.05	0.29 ± 0.35	0.21 ± 0.18
Ozone [3.3 mg L^{-1}; 7 min] + UV [2.5 Wh m^{-3}]	1.92 ± 0.26	1.78 ± 0.29	0.31 ± 0.19
Ozonation [11.9 mg L^{-1}; 7 min]	1.40 ± 0.90	1.24 ± 0.48	0.68 ± 0.34
Ozone [11.9 mg L^{-1}; 7 min] + UV [2.5 Wh m^{-3}]	2.44 ± 0.39	2.02 ± 0.38	0.84 ± 0.31

is that ozone pretreatment reduces bacterial agglomeration and microbial attachment to particulate matter surfaces [55]. The synergistic effects of sequential disinfection (ozone-UV) are shown in Table 8.

Synergistic effects of ozone and UV disinfection were observed, particularly in the inactivation of total coliforms, with reductions of up to 1.28 log. For *E. coli*, synergism was more pronounced with ozone as the primary disinfectant than with chlorine, resulting in up to 0.60 log inactivation. For *C. perfringens*, the sequential application of ozone at CTs of 23 mg min L^{-1} and 83 mg min L^{-1}, followed by UV radiation, resulted in average log inactivation values of 0.06 and 0.10, respectively. These values were lower than those achieved with sequential chlorine and UV disinfection.

Jung et al. [56] also reported synergistic effects with the sequential application of ozone and UV radiation, reporting an average of 0.5 log inactivation for *Bacillus subtilis* spores. However, no synergy was observed when UV radiation was applied before ozone, indicating that ozone applied first disrupts the spore wall, facilitating UV penetration and allowing the radiation to more effectively target cellular DNA and RNA.

Unlike sodium hypochlorite, ozone effectively reduced the concentration of *Giardia* cysts and *Cryptosporidium* oocysts in both individual and sequential disinfection tests. With a CT of 23 mg min L^{-1}, an average inactivation of 0.24 log (± 33%) of *Giardia* cysts was observed, similar to the inactivation rates for *E. coli*, total coliforms, and *Clostridium perfringens* (*t*-test, $p > 0.05$). Increasing the CT to 83 mg min L^{-1} significantly enhanced *Giardia* cysts inactivation, with an average of 1.65 log (± 0.68), which was also comparable to the inactivation of the three fecal indicator bacteria (*t*-test, $p > 0.05$). However, the addition of UV radiation in sequential treatments did not further improve *Giardia* cyst inactivation.

Cryptosporidium oocysts were detected in only 31% of the ozonated samples (5/16), with 80% of them being viable on the basis of viability assessment via the vital dye propidium iodide. In the remaining samples, *Cryptosporidium* oocysts were below the detection limit of the method. Morphological damage to *Giardia* cysts was evaluated by assessing cyst blooming, whereas viability was inferred via the use of propidium iodide, as shown in Fig. 7.

High standard deviation values were once again observed in the results, which made statistical comparisons challenging. Nevertheless, a reduction in the percentage of *Giardia* cysts with standard fluorescence was noted after ozonation, suggesting that oxidative damage rendered the cysts nonviable, as indicated by propidium iodide staining.

Ozone treatment alone resulted in *Giardia* cyst inactivation ranging from 0.72 log (at the lowest ozone dose) to 1.80 log (at the highest dose); whereas, sequential treatments with ozone achieved up to 1.50 log reduction. Interestingly, a more pronounced correlation was observed

Table 8 Synergistic effects in sequential disinfection ozone–UV disinfection

Microorganism	Assay	Σ Individual inactivation (Ii)	Sequential inactivation (Si)	Synergism
E. coli	[23]-UV^2	1.23	1.54	0.31
	[83]-UV^2	1.53	1.94	0.41
	[23]-UV^3	1.64	2.24	0.60
	[23]-UV^4	1.78	2.04	0.26
Total coliforms	[23]-UV^2	0.98	2.26	1.28
Clostridium perfringens	[23]-UV^1	0.36	0.46	0.10
	[83]-UV^1	0.39	0.51	0.12
	[83]-UV^3	1.22	1.30	0.08
	[23]-UV^4	0.06	0.08	0.02
	[83]-UV^4	0.54	0.63	0.09

[23]: CT of 23 mg min L^{-1}; [83]: CT of 83 mg min L^{-1}; UV of 2.5 Wh m^{-3}; $^{1;2;3;4}$: assay number.

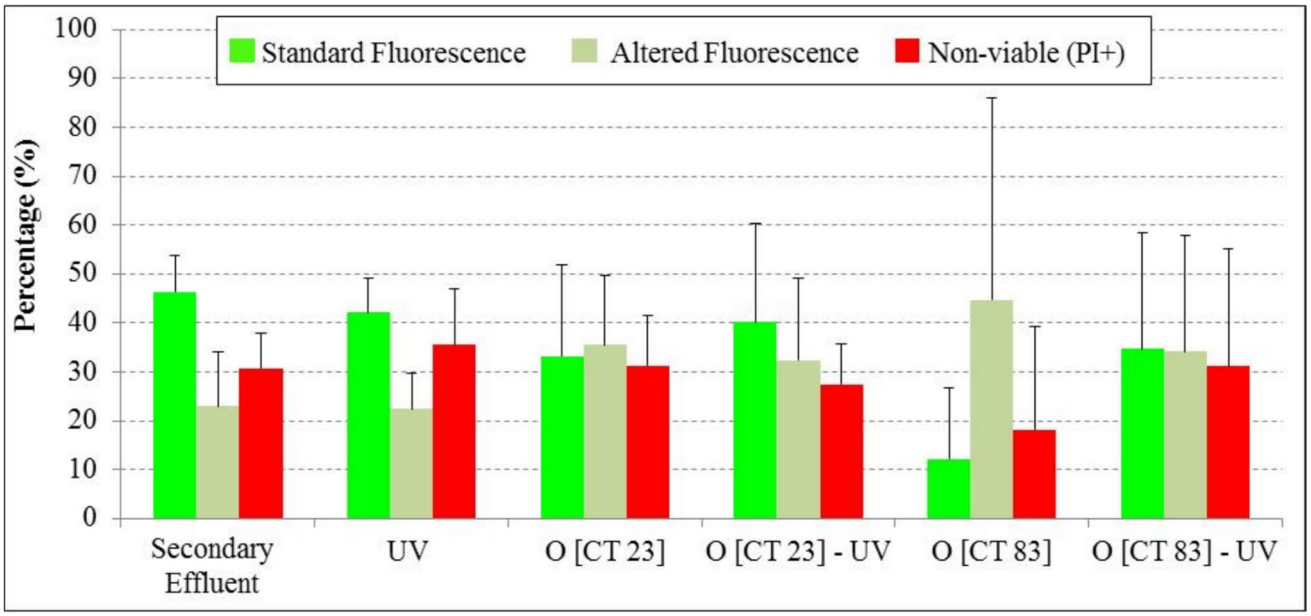

Fig. 7 Assessment of morphological damage to *Giardia spp.* cysts following individual and sequential disinfection treatments with ozone and UV radiation. The central bars represent the standard deviation. Ozone CT was applied at 23 mg.min.L^{-1} and 83 mg.min.L^{-1}. UV dose: 2.5 Wh.$m.^{-3}$

between the mass of ozone consumed and cysts inactivation than between the mass of ozone consumed and the applied CT. Ozone disinfection not only reduced *Giardia* cysts concentrations but also decreased cysts fluorescence in sequential treatments (ozone–UV), likely due to oxidative damage to the cyst walls.

Cho et al. [57] highlighted that ozone, as a strong oxidant, reacts with multiple components of the cell wall when it penetrates the cell, subsequently targeting the cytoplasm. The synergistic effects of ozone and UV radiation are particularly advantageous for addressing resistant pathogens and emerging challenges, such as the treatment of endocrine-disrupting compounds. These compounds, which are commonly found in personal hygiene products and pharmaceuticals, have been shown to reduce the estrogenic activity of municipal effluents [44].

Koivunen and Heinonen-Tanski [34] further explained the mechanism behind synergistic disinfection, noting that the use of two distinct disinfectants can inflict different types of damage on microorganisms. This multifaceted approach leads to more effective inactivation by targeting multiple vulnerabilities within pathogens.

Ozonation improved the physicochemical quality of the effluent and enhanced the effectiveness of subsequent ultraviolet disinfection. As a result, sequential disinfection significantly reduces the microbiological risk posed by resistant agents, protozoan (oo)cysts, and *Clostridium* sp., potentially driving the growing adoption of water reuse projects.

4 Conclusion

This study details the disinfection potential of chlorine, ozone, and UV radiation, both individually and in sequential applications, for secondary effluent contaminated with various microorganisms. The findings highlight the benefits and limitations of these methods, focusing on indicator bacteria, protozoan cysts, and implications for effluent quality.

The resistance of microorganisms followed the order *E. coli* = total coliforms < *Clostridium perfringens*, which was consistent across all disinfection methods, including chlorination, ozonation, UV disinfection, and sequential disinfection. The greater resistance of *C. perfringens* than that of *E. coli* or total coliforms raises concerns about the suitability of using low-resistance indicators in regulations and microbiological risk assessments. These indicators may not accurately reflect the effectiveness of disinfection processes against more resistant pathogens, potentially underestimating risks.

Synergistic effects were observed for sequential disinfection using chlorine or ozone as primary disinfectants followed by UV radiation. These effects were significant across all studied microorganisms, with the most pronounced results for *Clostridium* sp. For ozone-UV treatments, synergistic effects ranged from 0.02 to 1.28 log inactivation, whereas the chlorine-UV combinations ranged from 0.07 to 0.82 log inactivation. This suggests that lower ozone CT (concentration vs. contact time)

values for primary disinfectants may suffice, reducing the potential for DBP formation while maintaining high disinfection efficiency.

Standalone chlorine-based treatments, including sequential chlorine–UV, did not significantly reduce *Giardia* cysts. In contrast, ozone alone achieved up to 1.80 log inactivation, and ozone–UV sequential treatments resulted in up to 1.50 log. Both ozone and sequential treatments reduced cyst fluorescence, indicating oxidative damage to cyst walls and increased disinfection potential. However, *Cryptosporidium* oocysts remained highly viable after disinfection tests, posing a public health concern.

The application of ozone before UV treatment also improved effluent quality by reducing the absorbance at 254 nm, which in turn allowed for a shorter UV detention time. This improvement in oxidative activity prior to UV exposure is particularly advantageous, as it reduces the oxidation demand and can minimize the required size and number of UV lamps in the system. However, the cost and benefit of installing an additional disinfection unit, such as ozone, should be carefully evaluated.

Kinetic models, particularly those proposed by Chick and Collins, provide valuable parameters for the design and optimization of disinfection units. These models demonstrate the best fit for the various microorganisms and disinfectants applied to the studied effluent, further supporting the development of efficient and effective disinfection strategies.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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