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Changes in groundwater and surface water bacterial communities under disinfection processes: Chlorination, ozonization, photo-fenton and ultraviolet radiation

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ABSTRACT

Pathogenic bacteria, introduced in water sources through faecal contamination, have traditionally been investigated as individual species, leading to the establishment of microbial, sanitary, and environmental quality indicators. Recent advancements in our understanding of the microbiome and its intricate interactions within the human-microbiome-environment network advocate for a broader evaluation of the impact of disinfection on the entire microbial community. In this study, we conducted a comprehensive screening experiment involving four disinfection processes; ozone, ultraviolet radiation with wavelengths between 200 – 280 nm (UV-C), photo-Fenton, and chlorination, applied to two distinct water sources; surface (SW) and groundwater (GW). The cells that remained viable after treatment were recovered using Brain Heart Infusion (BHI) broth, and 16S rRNA gene sequencing was used for their identification. Our findings confirmed the presence of faecal contamination in the water sources and revealed distinct effects of each treatment on the recovered bacterial populations. The chlorination of groundwater samples likely had a greater impact on bacteria in a vegetative state than on spores. Consequently, this led to a higher abundance in the BHI cultures of sporulating bacteria such as *Bacillus* (increasing from 0.36 to 93.62 %), while ozonation led to an elevated recovery of *Pseudomonas* (increasing from 45.2 to 69.9 %). Conversely, in surface water, calcium hypochlorite and ozone treatments favored the selection of *Staphylococcus* and *Bacillus*, whose relative abundance in the cultures increased from 0 to 39.22 % and from 0.35 to 96.6 %, respectively. In groundwater, *Pseudomonas* was resistant to UV-C radiation and their relative abundance increased from 45.2 % to 93.56 %, while photo-Fenton was effective against this bacterial group decreasing its relative abundance to 0.46 %. However, other genera such as *Bacteroides, Aeromonas*, and *Citrobacter* seemed to be less injured by this disinfection process. BHI broth was successful in recovering various bacterial groups that exhibited resistance to sublethal water disinfection.

Introduction

Only 2.5 % of the water on Earth is freshwater, and 69.0 % is frozen in polar ice caps, glaciers and other ice (NASA, 2020). Drinking water comes mainly from rivers and groundwater (0.49 % and 30.1 %, respectively). This water can be consumed after purification in water treatment plants or after simple treatments such as boiling, chlorination, exposure to solar radiation, filtration or a combination of them in rural settings (Chu et al., 2019). Inadequate investment policies for water and sanitation, long distances from urban centres, and lack of utilities such as electricity or alternative energy sources cause approximately 122 million people to drink water directly from rivers, lakes, and other groundwater sources (WHO and UNICEF, 2021). Contaminated water and poor sanitation are linked to the transmission of diseases such as cholera, diarrhea, dysentery, hepatitis A, typhoid fever and polio (Ramírez-Castillo et al., 2015). Chlorination has long been a standard method for disinfection of drinking water and wastewater. It effectively eliminates a wide range of harmful microorganisms, ensuring the safety of water for human consumption and reducing the risk of waterborne diseases. Nevertheless, several concerns have arisen overtime, leading to its replacement or supplementation with other disinfection methods (Chu et al., 2019; Alvear-Daza et al., 2021; Bodzek, 2022; Juvakoski

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Table 1
Experimental conditions for disinfection processes.

Treatment	Sample Volumen (mL)		Time of exposure (min)		Disinfectant concentration		Irradiation intensity (μW/cm^{2}\)		H_2O_2:Fe (mg/L)	
	SW	GW	SW	GW	SW	GW	SW	GW	SW	GW
$Ca(ClO)_2$	500	500	360	120	9 mg/L*	1 mg/L*		–		–
O_3	500	500	50	25	101 g/m^{3}*	101 g/m^{3}*		–		–
UV-C	100	100	720	360	–	–	1500	1500		–
PhF	80	80	18	30	–	–	30,000	30,000	10:06	10:06

SW: Surface water, GW: Groundwater, $Ca(ClO)_2$: Calcium hypochlorite, O_3: Ozone, UV-C: Ultraviolet radiation, PhF: Photo-Fenton,. * Chlorine and Ozone concentrations were established based on oxidant demand assays previously performed with each water sample (data not shown), following the standard methods for water analysis (APHA 2018bAPHA 2018b).

et al., 2022). One of the main concerns is the formation of disinfection by-products (DBPs), which are a consequence of the reaction of chlorine with the natural organic matter present in water. These reactions can lead to the formation of potentially harmful by-products such as trihalomethanes (THMs) and haloacetic acids (HAAs), which have been linked to an increasing risks of health problems, including cancer, and miscarriages (Kumari and Gupta, 2022). Moreover, this disinfectant may not be effective against certain microorganisms such as *Cryptosporidium* and certain viruses. Depending on the microbial and organic load in the water, higher doses and contact times are required for disinfection, which can lead to other drawbacks such as taste and odour issues, generating consumer complaints.

Advancements in DNA sequencing technologies have significantly enhanced our understanding of the complex interactions and coevolution between the human microbiome and various environments (Gilbert et al., 2018; Proctor et al., 2019). Although there is compelling evidence challenging the reliability of microbial indicators in assessing water quality (McClary-Gutierrez et al., 2021; Ramírez-Castillo et al., 2015; Wen et al., 2020), some water disinfection studies remain focused solely on monitoring a small number of pathogen microorganisms (Morrison et al., 2022; Wang et al., 2022). The evolution of novel disinfection methods, considering the relationship among the human host, its microbiome, and the environment, remains in preliminary stages. Therefore, exploring the impact of water disinfection on the broader microbial community is crucial. Drawing from our expertise in disinfection processes, we conducted a thorough screening experiment using ozone, ultraviolet radiation with wavelengths between 200 – 280 nm (UV-C), photo-Fenton, and chlorination, using two water sources, surface and groundwater. We explored the use of BHI broth to recover injured bacteria and 16S rRNA gene sequencing to monitor the shifts in the recovered bacterial community. This broth is a nutrient-rich medium derived from calf brains and beefs hearts, with a great potential in recovering viable bacteria post-disinfection. Previous experiments showed that the protein components in a culture medium can benefit the recovery of injured bacteria (Sanabria et al., 2011). Moreover, this medium has been used for culturing waterborne bacterial pathogens; for the pre-enrichment of water samples, aiding in the recovery of low-concentration bacteria, and for antibiotic susceptibility testing (Ersoy Omeroglu et al., 2021; Jorgensen and Ferraro, 2000). This research offers valuable perspectives on employing an enrichment culture media and DNA sequencing to track the microbial changes in raw water samples, emphasizing its potential to detect pathogenic bacteria that remain viable after sub-lethal disinfection.

Methods

Water sampling

A total of 18 L of raw surface water (SW) and groundwater (GW) were collected from a contaminated river (*La vieja* river, Cartago, Colombia, 4°45′18.45″ N, 75°53′28.72″ W), and a sub-superficial well (La Regina, Candelaria, Colombia, 3°22′16.11″ N,76°19′52.81″ W) in 20 L sterilized vessels. The samples were transported to the laboratory and stored overnight at room temperature (24 °C ± 2) in the dark. Overnight storage allowed sedimentation of the debris, reducing suspended particles that could interfere with the disinfection process. Sedimentation before water chlorination is a common practice in rural communities in Colombia (Betancur, 2020). The experimental conditions for each treatment are shown in Table 1. The physicochemical parameters monitored during disinfection are summarized in Table 2.

Bacteria recovery and molecular analysis

Aliquouts of 1 mL were taken from all treated water samples and controls, and then transferred to new tubes containing 100 μL of 0.1 N sodium thiosulfate (Sigma Aldrich, USA) to halt the oxidation process. This process was carried out at different exposure times, as detailed in Supplementary material – Table S1. Subsequently, each quenched sample and all corresponding controls were transferred to new tubes containing 9 mL of Brain Heart Infusion (BHI) broth (Sigma Aldrich, USA) to facilitate the recovery of viable bacteria. The inoculated tubes were promptly placed in an incubator at 37 ± 2 °C for 48 h. During this incubation period, microbial growth was tracked by measuring the optical density (OD) of the cultures at 600 nm every 24 h. All cultures

Table 2
Physicochemical parameters, upper and lower ranges monitored during treatment.

Disinfection treatment	Groundwater			Surface water		
	pH (± SE)	Temperature (°C) (± SE)	Turbidity (NTU) (± SE)	pH (± SE)	Temperature (°C) (± SE)	Turbidity (NTU) (± SE)
$Ca(ClO)_2$	7,13–7,45 (± 0,04)	27–30 (± 0,4)	0,26–0,31 (± 0,02)	7,88–8,16 (± 0,03)	28–31 (± 0,7)	8,98–9,22 (± 1,22)
O_3	7,7–8,45 (± 0,31)	28–28 (± 0,0)	0,44–0,36 (± 0,04)	7,7–7,73 (± 0,38)	28–27 (± 0,2)	5,21–23,4 (± 0,51)
UV-C	7,48–8,49 (± 0,19)	27–30 (± 0,5)	0,44–1,89 (± 0,08)	7,3–8,0 (± 0,12)	27–33 (±0,8)	8,98–3,41 (± 0,23)
PhF	7,58–8,74 (± 0,29)	25–37 (± 0,5)	0,18–0,15 (± 0,02)	7,76–8,57 (± 0,05)	25–37 (±0,43)	5,83–5,18 (± 0,22)

All measurements were performed using the standard methods for water analysis (APHA 2018a, 2018c). NTU: Nephelometric Turbidity Unit, SE: Standard Error, $Ca(ClO)_2$: Calcium hypochlorite, O_3: Ozone, PhF: Photo-Fenton, UV-C: Ultraviolet irradiation.

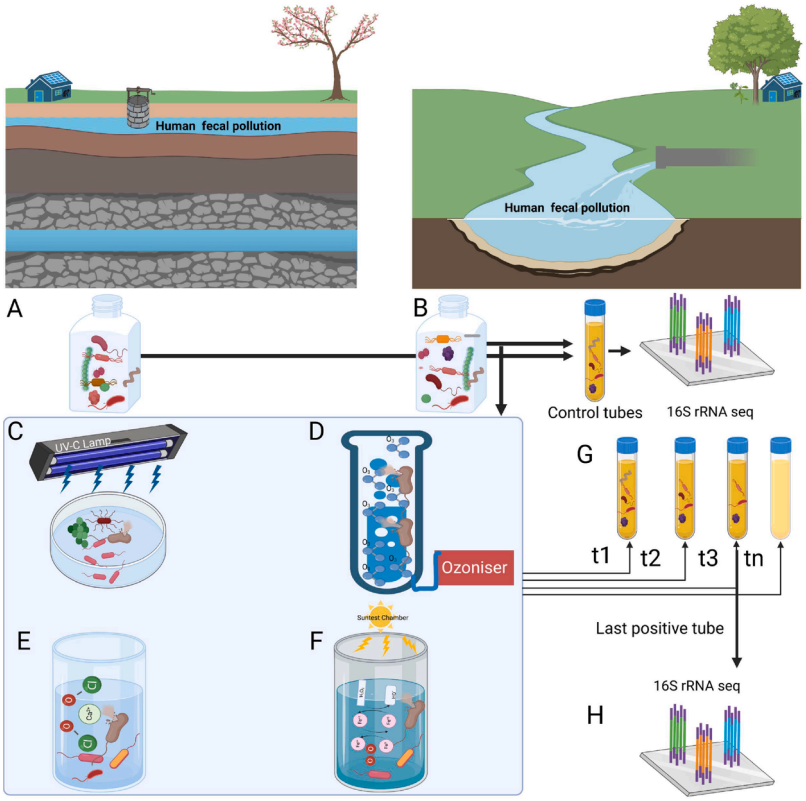

Fig. 1. Experimental setup. A. Sample from groundwater. B. Sample from surface water. C. UV irradiation. D. Ozone treatment. E. Calcium chloride treatment. F. Photo-Fenton process. G. Recovery of viable bacteria in BHI broth at different exposure times. H. DNA extraction and sequencing of the 16S rRNA genes V1–2 fragment.

showing an OD > 0.01 were considered to contain viable cells. After incubation, the cultures were homogenized by using a vortex mixer at 1000 rpm for 1 min, and 1 mL was extracted from each final positive tube and all controls for DNA extraction. The extraction of genomic DNA was performed using the GeneJET Genomic DNA purification kit (Thermo Scientific, USA). The experimental setup is depicted in Fig. 1.

DNA extraction and informatic analysis

Ten DNA samples were sequenced on an Illumina MiSeq platform (Mr DNA commercial facility, USA) targeting the V1–2 hypervariable regions of bacterial 16S rRNA genes. High-throughput sequence data were analysed using *dada2* (Callahan et al., 2016) and visualised using *phyloseq* (McMurdie and Holmes 2013) in the RStudio v4.1.2 environment. Sequences were quality filtered (maxN = 0) and length was truncated to 280 base pairs. Error rates were calculated and then inferred from 105,458,640 bases in 376,638 reads. Sequences were de-replicated and high-resolution amplicon sequence variants (ASVs) were produced using the dada algorithm, followed by removal of chimaeras. Taxonomy was assigned to ASVs using two approaches (i) assignTaxonomy function within dada2, which uses the RDP classifier algorithm with reference to the SILVA 16S bacteria v132 data; and (ii) BLASTN algorithm using the NCBI RefSeq 16S rRNA database (downloaded June 2022), taxonomic lineage was retrieved from top hits using the taxize package.

Reproducible code and data analysis files are available at [https://github.com/siobhon-egan/waterTreatments], and all data were uploaded to the European Nucleotide Archive under project accession number PRJEB54073 (ERA16057005) (BioSample accession numbers: SAMEA110284847 - SAMEA110284856). In addition, to evaluate the overlap of taxonomy with that associated with the human gut microbiome, the genera identified in the samples were compared with a list of bacteria taxa downloaded from GMrepo (https://gmrepo.humangut.info/home) [downloaded June 2022], which is a curated database of consistently annotated human gut metagenomes.

Results and discussion

Recovery of bacteria from non-treated water in BHI broth

A total of 554,760 raw reads were retrieved from high-throughput sequencing (an average of 53,014 reads per sample). After denoising and chimaera removal, 468,421 reads remained. Analysis against the NCBI RefSeq 16S database identified 84.5 % (466,236/554,760) as bacteria (Supplementary material – Table S2). In addition, the sequencing depth was enough to fully characterize the bacterial community in the samples, as supported by the plateau reached by all the samples in the rarefaction curves (Supplementary material – Fig. S2).

Previous studies have evaluated the pre-enrichment of water samples with BHI broth to facilitate the recovery of injured or difficult-to-grow bacteria, and enrich bacteria present at such a low concentration that it cannot be detected with conventional nutrient media or molecular techniques. (Ersoy Omeroglu et al., 2021). Moreover, this broad-spectrum culture medium has been successfully used for growing several waterborne bacterial pathogens (Adzitey et al., 2012; Chin et al., 2015; Kaur et al., 2018; Paixão et al., 2013). Based on these studies, we explored the use of this culture medium to track the changes in the microbial community of raw water samples throughout different disinfection treatments. Also, our main goal was to enrich and detect multiple pathogenic bacteria that could remain viable when sub-lethal disinfection conditions are applied.

The sequencing results of the non-treated samples that were cultured in BHI showed 52 bacterial genera in surface water, and 33 in groundwater. It is worth noting that the initial microbial composition of the raw

Table 3
Overlap of taxonomy at genera level with that associated with the human gut microbiome.

	Genera in the sample (n)	Change in the number of genera after treatment (*)	Human gut-microbiome related genera (n)	Change in the number of Human gut-microbiome-related genera after treatment (**)
Surface water				
NT	52	–	41	–
$Ca(ClO)_2$	23	↓56 %[a]	18	↓56 %
O_3	30	↓42 %	27	↓34 %
PhF	28	↓46 %	22	↓46 %
UV-C	31	↓40 %	26	↓37 %
Groundwater				
NT	33	–	24	–
$Ca(ClO)_2$	29	↓12 %	26	↑8 %
O_3	24	↓27 %	20	↓17 %
PhF	39	↑18 %	31	↑29 %
UV-C	35	↑6 %	30	↑25 %

NT: non-treated water, $Ca(ClO)_2$: Calcium hypochlorite, O_3: Ozone, PF: Photo-Fenton, UV-C: Ultraviolet irradiation. (*) These percentages represent the change in the number of genera detected after each treatment compared to the control sample. [a]Example of the calculation: (1 – 23/52).100 ≅ 56 %. (**) The arrows show the increase or decrease in the proportion of these genera after the treatment compared to the control. Error estimates are not shown because only one sample per treatment was sequenced.

water samples could be affected by the overnight storage and sedimentation steps that were applied before performing the disinfection experiments. Nevertheless, these samples were used as a baseline to determine the changes in the abundance of bacteria after each treatment. Although the use of BHI enrichment followed by 16S rRNA gene sequencing does not provide data on the original microbial abundance of the water sources, it allowed us to identify several pathogenic bacteria which could outcompete other naturally occurring bacteria. As shown in Table 3, bacteria genera, which are common in the gut microbiome, were found in both water samples. Multiple markers of *Lachnospiraceae* have been previously reported in surface waters using Microbial Source Tracking methods and were linked with host-specific faecal pollution (Feng, 2019). Moreover, recent studies suggest this family as an alternative indicator of human faecal pollution (McLellan et al., 2015; Feng et al., 2018). The recovery of *Lachnospiraceae* using BHI enrichment of non-treated water samples showed differences between both water sources, with a relative abundance of 12.25% in surface water and only 0.4% in groundwater. The low abundance of this family would indicate that the faecal pollution in the groundwater reservoir was low (Supplementary material – Fig. S3). Nevertheless, the high relative abundance of the families *Enterobacteriaceae* (15.99 %) and *Bacteroidaceae* (20.49 %), which are also associated with faecal contamination, suggest the contrary (Supplementary material – Fig. S3). It is very likely that the surrounding septic tanks, which are not properly built and lack maintenance, are a source of faecal contamination by lixiviation of filtration into the supply wells in *La regina*. These results highlight the importance of discussing how the criteria for selecting microbial quality indicators are established.

In this study, we examined the impact of disinfection on bacterial cultivability, and multiple factors must be considered when interpreting the results: (i) the likelihood of survival of a given species increases if its initial abundance is higher, which could lead to a higher number of this bacteria in the culture media after the treatment, (ii) there are inter- and intra-species variations in the survival of cells due to inherent differences in resistance and repair mechanisms, which are activated in response to the disinfectant, (iii) the culture medium will favour the growth of bacteria with a strong affinity for available nutrients, and for other critical factors such as pH, temperature, and oxygen concentration. In future experiments, it is essential to investigate these factors individually to understand their impact on the observed microbial community structure.

The results of the BHI culture of the non-treated groundwater indicated that *Pseudomonas* and *Bacteroides* were the most abundant genera, with 45.2 % and 20.4 %, respectively. In contrast, the cultures inoculated with non-treated surface water were dominated by the genera *Bacteroides* (13.8 %), *Comamonas* (12.5 %), and *Aeromonas* (12.2 %) (Fig. 2). As expected, the disinfection processes caused a marked decrease in the alpha diversity of the recovered microbial community (Supplementary material – Fig. S1). While ozone caused the highest drop in the diversity of the samples from both water sources, UV did not affect the diversity in surface water (Supplementary material – Fig. S1). Overall, the results show that all treatments affected the bacterial communities initially present in raw water differently. For instance, in groundwater, chlorine caused a sharp increase in the relative abundance of *Bacillus* (from 0.36 to 93.62 %), and ozone in *Pseudomonas* (from 45.2 to 69.9 %) in the BHI cultures. In contrast, the same disinfectants in surface water increased the abundance of *Staphylococcus* (from 0 to 39.22 %) and *Bacillus* (from 0.35 to 96.6 %), respectively (Fig. 2). The treatment with UV radiation of groundwater led to a dominance of *Pseudomonas* in the culture, which accounted for up to 93.56 % of the reads obtained during sequencing. On the contrary, photo-Fenton was effective in the removal of this genera, decreasing its abundance to 0.46 %. Moreover, the relative abundance of *Bacteroides, Aeromonas*, and *Citrobacter* didn't change after treatment, which suggests that these bacteria might not be affected by this disinfection method. Notably, if the same analysis is performed for these treatments in surface water, the

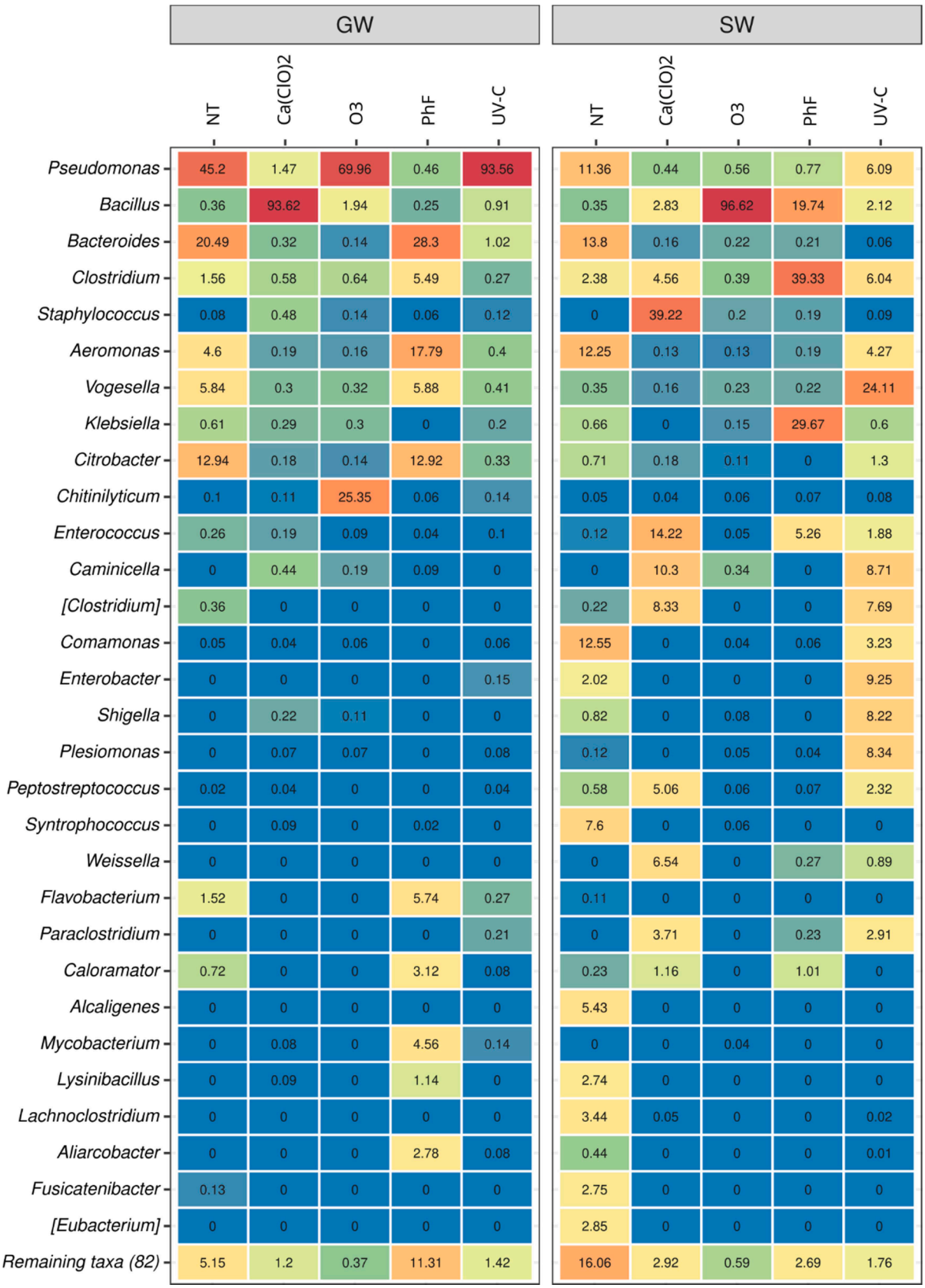

Fig. 2. Relative abundance heatmap at genus level before and after treatments. GW: groundwater, SW: surface water, NT: no treatment (control), $Ca(ClO)_2$: Calcium hypochlorite, O_3: Ozone, PF: Photo-Fenton, UV-C: Ultraviolet irradiation.

Table 4
List of genera that were recovered in BHI after sublethal disinfection and examples of potentially pathogenic species.

Genera	Water source	Treatment	Examples of potentially pathogenic species
Pseudomonas	GW	O_3 UV-C	*Pseudomonas aeruginosa*
Bacillus	GW SW	$Ca(ClO)_2$ O_3	*Bacillus cereus*
Bacteroides	SW	PhF	*B. thetaiotaomicron* *B. fragilis*
Clostridium	SW	PhF	*C. perfringens* *C. difficile*
Staphylococcus	SW	$Ca(ClO)_2$	*S. aureus*
Aeromonas	GW	PhF	*A. caviae* *A. veronii* *A. dhakensis*
Klebsiella	SW	PhF	*K. pneumoniae*

SW: Surface water, GW: groundwater, O_3: Ozone, UV-C: Ultraviolet irradiation, $Ca(ClO)_2$: Calcium hypochlorite, PhF: Photo-Fenton.

results are completely different. For instance, photo-Fenton seemed to favor the growth of *Clostridium* and *Klebsiella*, whose relative abundance went from 2.38 % to 39.33 % and from 0.66 % to 29.67 %, respectively. Altogether, these results highlight the profound impact of the water source and their microbial community composition on the effect of these disinfection methods.

The continuous exposure of bacteria to disinfectants develops disinfectant adaptability and tolerance, with horizontal gene transfer (HGT) of disinfectant resistance genes facilitating the survival of some species (Tong et al., 2021). Additionally, the growth rate of disinfectant-resistant bacteria is shocking (Zhu et al., 2021). This difference could explain how the bacterial groups selected as resistant in BHI after the disinfectant treatments show a relative abundance that exceeds that of the cultures of the untreated samples. As expected, sporulated species, such as the genus *Bacillus,* showed a sharp increase in their relative abundance. For instance, this genus increased above 90 % after passing through chlorination in GW and ozone in SW. It is well known that this bacterial group exhibit a wide range of physiologic abilities that allow them to live in multiple natural environments. In addition, their spores are known to survive heat, cold, radiation, desiccation, and disinfectants (Silva et al., 2018). The genera *Staphylococcus*, which also belongs to the class *Bacilli*, also showed a significant increase in its relative abundance after SW chlorination from 0 to 39.22 %. The high tolerance of various *Staphylococcus* strains to chlorine-containing disinfectants has been documented by Yixiao et al. (2023).

Last, we have summarized in Table 4 the list of potentially pathogenic genera recovered in BHI after sublethal disinfection and provided examples of species of clinical concern. Nevertheless, further experiments are required to isolate and confirm the taxonomy at the genus level of these bacteria, as well as characterize their antibiotic resistance profiles, virulence and pathogenicity.

Conclusions

This study provides evidence of potentially pathogenic bacteria that might be able to recover in a nutritive environment when water disinfection processes such as chlorination, ozonation, UV-C radiation and photo-Fenton are applied in sub-lethal conditions. It also shows the differential response of the same bacterial groups across different treatments and water sources, which highlights the importance of considering the whole microbial community in disinfection studies. The alternative use of BHI as a pre-enrichment medium to track the faith of viable bacterial pathogens throughout the treatment has a great potential for application and could be a useful method for detecting resistant bacteria with increased tolerance to disinfectants. With these results, we will now explore the use of different culture media and other cultivation parameters on the recovered microbial community.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

DNA sequences were deposited in a public repository. Link to DNA analysis code is provided in the manuscript.

Credit author statement

Castaño-Henao L., and Garcia Mendez D.F., performed all the experiments, participated in the data analysis and curation, and writing the original draft. Egan S. conducted the analysis of the sequencing data and contributed to the original draft. Sanabria J., contributed to conceptualization and experiment desing, review and editing of the manuscript, supervision, project administration, and funding acquisition.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2024.100244.

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