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Research Paper

Efficacy of Residual Ozone on Surrogate Microorganisms for Waterborne Pathogens in Bottled Water

William Ryan Schwaner [1,2], Sanjay Kumar [2,*], Harshavardhan Thippareddi [3]

[1] Department of Food Science and Technology, University of Nebraska, Lincoln, NE 68588, United States
[2] Quality Assurance and Food Safety, Niagara Bottling LLC, Diamond Bar, CA 91765, United States
[3] Department of Poultry Science, University of Georgia, Athens, GA 30602, United States

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ABSTRACT

Ozone is a powerful disinfectant that is widely used in the bottled water (BW) industry. Primary ozone disinfection of water for bottling occurs in a reaction tank with a specific contact time. Residual ozone in the bottled water may still possess disinfection activity. The purpose of this study was to evaluate the efficacy of residual ozone in BW in reducing the populations of surrogate microorganisms for waterborne pathogens (*Escherichia coli* [BAA-1427], *Enterococcus faecalis* [ATCC 19433] and *Burkholderia cepacia* [ATCC 25416]). The effect of water pH and total dissolved solids (TDS) on the disinfection process was also evaluated. A pilot scale ozone delivery system and filler were assembled to allow filling of 0.5 L polyethylene terephthalate (PET) plastic water bottles with ozonated (0.1, 0.2, 0.3, and 0.4 mg/L) water. Ozonated water was inoculated with microorganisms to attain ca. 6.0 log and 4.0 log CFU/mL, and microbial populations were determined after 5, 30, 60, and 180 min at 25 °C. Samples (100 mL) were filtered through Neogen NEO-GRID membrane filters and placed on tryptic soy agar, incubated for 48 h at 37 °C, and enumerated. Ozone dissipation in BW was measured with and without biological load (6.0 log CFU/mL) at 21 and 38°C for 6 h. Greater reductions ($P \leq 0.05$) in *E. faecalis* (4.61 and 3.68 log CFU/mL) and *B. cepacia* (5.24 and 4.12 log CFU/mL) were observed at 0.4 and 0.1 mg/L ozone in BW, respectively. Longer contact time (>5 min) did not result in greater reduction ($P > 0.05$) in microbial populations. Faster ozone dissipation ($P \leq 0.05$) was observed at 38 °C and the dissipation rate increased with biological load. Except at higher pH (9.0) and TDS (50 and 300 mg/L) concentrations, the residual ozone in BW (≥0.1 mg/L) can provide ≥4.0 log reductions in pathogen surrogates *E. coli*, *E. faecalis,* and *B. cepacia*, providing an additional measure of microbiological safety in BW.

A clean and safe drinking water supply is a fundamental need for all human populations. Waterborne disease has been a problem throughout history and has been responsible for numerous deaths and widespread epidemics. However, the threat to public safety due to waterborne illness has been significantly reduced. This reduction in waterborne illness can be attributed to several factors including general education on the importance of water handling, water storage, disinfection and distribution systems, and the biological and chemical agents that cause disease. Bottled water has grown over popularity in the last half century as an alternative to municipal water supplies for those who prefer a packaged product that meets their taste, convenience, storage, and/or source water preferences. BW recalls are not a common occurrence due to the several layers of safeguards that manufacturers implement including source water management and testing, in-plant quality assurance testing, and a multibarrier approach to disinfection. One of the key factors in this multibarrier approach for BW production is the use of ozone for water disinfection.

Ozone has a long history of use in water disinfection. In the early 1900s, ozone equipment was developed for use in municipal drinking water treatment facilities (Rice, 1999). A key factor for the continued use of ozone has been its high disinfection capability on a multitude of microorganisms including bacteria, viruses, and protozoa. The mode of action for ozone disinfection is cell lysis through membrane disruption leading to leakage of cellular contents and cell death (Pascual et al., 2007). Due to the high oxidation capability, ozone is very effective at mitigating taste and odor issues with particularly troublesome water sources. The fact that ozone does not impart a residual taste or odor in the finished package while providing a robust disinfection capability has led to its extensive use in the BW industry (Ahmad and Azam, 2019).

* Corresponding author.
E-mail address: skumar@niagarawater.com (S. Kumar).

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In the United States, municipal drinking water treatment facilities are regulated by the U.S. Environmental Protection Agency (USEPA) while the bottled water industry is regulated by the US Food and Drug Administration (FDA). Bottled water manufacturers must follow the water treatment guidelines outlined in 21 CFR §129.80(a) and meet the bottled water quality standards in 21 CFR §165.110(b). The regulation specifies that all finished product water must be treated by distillation, ion-exchange, filtration, ultraviolet light (UV), reverse osmosis (RO), carbonation, mineral addition, or other processes effective in accomplishing the proposed objective to reduce bacterial and chemical contaminants. In the United States, some states have imposed additional regulations requiring frequent sampling and monthly reporting to local/state regulatory bodies. In addition to the State and Federal regulatory requirements, many bottled water manufacturers adhere to the International Bottled Water Association (IBWA) code of practice by treating and monitoring the source of the water supply and finished products in accordance with that code as an additional level of security (IBWA, 2021). The bottled water industry was among the food industry leaders in implementing HACCP and science-based preventive controls. This approach has served both the manufacturers and consumers well in providing consistently safe products.

The USEPA mandates microbiological standards for water supplies produced by the municipal water treatment facilities. The municipal corporations use various methods of disinfection (including chlorine, chloramines, UV, and ozone) to process the water to meet these drinking water standards. Chlorine and/or chloramines remain the primary method of disinfection for municipal water treatment, and they provide a residual level of disinfectant to achieve adequate microbial destruction throughout the distribution system, something that is not possible with ozone as it dissipates quickly. Contact time (CT) values are used to define the amount of disinfectants applied to a water source. CT is defined as a known concentration (C) of a disinfectant (chlorine, ozone) multiplied by a known exposure or contact time (T) and is typically expressed in units of mg-min/L. The required CT values vary based on temperature and the microorganism of interest. These guidelines for CT values have assured consistency in determining the appropriate levels of ozone and other disinfection agents in the design of regulations surrounding municipal water treatment plants, and these concepts have been carried over for designing bottled water disinfection processes. The CT value concept from the EPA and municipal water treatment is a good foundation for the science behind ozone treatment in the bottled water industry, but there remain some differences in the needs of the product distribution systems that should be addressed.

Escherichia coli has long been considered an indicator microorganism for fecal contamination of water supply. *E. coli* is not normally found in natural water supplies, but its presence, along with other coliforms, can result from contamination of water supplies with either animal or human waste. The EPA enforces monitoring of all water sources used for human consumption to provide protection against enteric pathogens, and *E. coli* is the indicator organism of choice. The FDA, IBWA, and all state and local regulations for bottled water mandate that there is no detectable *E. coli* in the finished product. Likewise, source waters that are found to have confirmed *E. coli* present are not allowed to be used for BW production until corrective actions have been implemented and the source of the *E. coli* has been eliminated.

Enterococcus faecalis is a predominant streptococcus found in the colon of virtually all mammals. While not as abundant in the gut flora as *E. coli*, this microorganism has been proposed as a secondary indicator of pathogen contamination for water supplies (Edberg et al., 2000). One benefit of *E. faecalis* is that it is generally more salt tolerant than *E. coli,* providing a good indicator for marsh or salt-water sources. Additionally, *E. faecalis* indicates both recent and historical contamination, as it persists longer in the environment (week to months) and survives harsh conditions like, chlorination, UV exposure, and antibiotics. Enterococci are resilient due to their thick cell walls, biofilm formation, and intrinsic resistance genes (e.g., *van* genes conferring vancomycin resistance). While *E. coli* is gut-specific, *Enterococcus spp*. inhabits diverse niches, including soil and water, complicating contamination source tracking but broadening their utility as persistent indicators (Byappanahalli et al., 2012; Fisher & Phillips, 2009; Lata et al., 2009). Therefore, it is a good alternative to *E. coli* for evaluating the efficacy of ozone in bottled water.

Burkholderia cepacia is found naturally throughout the environment in soil, roots, and water. It also can occur in purified water. Although incoming potable water did not show *B. cepacia* as the primary bacteria species, it was the second-highest primary bacteria species found in purified water and water for injection (Sandle, 2015). These microorganisms have contaminated many pharmaceutical products and can pose public safety issues. Drugs that are contaminated with *B. cepacia* may cause serious health consequences in immunocompromised patients and young children (Torbeck et al., 2011). This microorganism is considered a potential threat to the bottled water industry primarily due to the use of these products for the preparation of infant formulas.

The objectives of this research were to: (1) evaluate the impact of temperature and time on ozone dissipation in bottled water and develop a predictive model for ozone dissipation in bottled water and (2) evaluate the destruction of foodborne pathogen surrogates by ozone in BW. This research will provide information on additional reductions in foodborne pathogen surrogates that can be achieved beyond the ozone treatment (contact tank) typically referenced for BW disinfection processes.

Materials and methods

Bacterial strains. *Escherichia coli* (*E. coli*; EC; BAA 1427), *Enterococcus faecalis* (*E. faecalis*; EF; ATCC 19433), and *Burkholderia cepacia* (*B. cepacia*; BC; ATCC 25416) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The *E. coli* was streaked onto Trypticase Soy Agar (TSA, Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 100 mg/L of rifampicin (GoldBio; St. Louis, MO) while *E. faecalis* was streaked onto Brain Heart Infusion Agar (BHI, Becton, Dickinson and Company, Franklin Lakes, NJ) and *B. cepacia* culture was streaked onto Nutrient Agar (NA, Remel, Waltham, MA). Stock cultures of *E. coli* (EC), *E. faecalis* (EF), and *B. cepacia* (BC) were prepared in MicroBank™ (ProLab Diagnostics, ON, Canada) and maintained at −80 °C till further use. The EC and EF were grown in their respective liquid medium (50 mL) and incubated for 24 h at 35 °C. The BC strain was grown in nutrient broth for 48 h at 30 °C. Freshly grown cultures were centrifuged at 7,850*g* for 10 min, and cell pellets were washed twice with Butterfield Phosphate Buffer (BPB, Neogen Corporation, Lansing, MI) and suspended in 2.5 mL of BPB to obtain optical density of 2.6 at 600 nm.

Ozone generation. A pilot scale ozonation system and filler (Steelhead Inc, San Antonio, TX) were assembled to allow filling of 0.5 L polyethylene terephthalate (PET) plastic water bottles with ozone at 0.1, 0.2, 0.3, and 0.4 mg/L concentrations (Fig. 1). For this, tap water was connected to Reverse Osmosis Unit (Model AT-500, Axeon Water Technologies, CA, USA) to obtain purified water which was later ozonated using a custom-designed ozone generator and filler combination. The ozone concentration (499 Series Ozone Sensors, Rosemount Analytical, Atlanta, GA), pH (3900 pH/ORP sensor, Rosemount Analytical) and total dissolved solids (TDS; 400/400VP Endurance Conductivity Sensor, Rosemount Analytical) were measured using the probes attached to the 1057 multiparameter analyzer (Fig. 2, Rosemount Analytical) and further verified using ozone, pH, and TDS handheld meters (Hach Company, Loveland, CO).

Experiment procedures. To measure bacterial inactivation by residual ozone, 0.5 L of polyethylene terephthalate (PET) plastic water bottles was filled with ozonated water (0.1, 0.2, 0.3, and 0.4 mg/L).

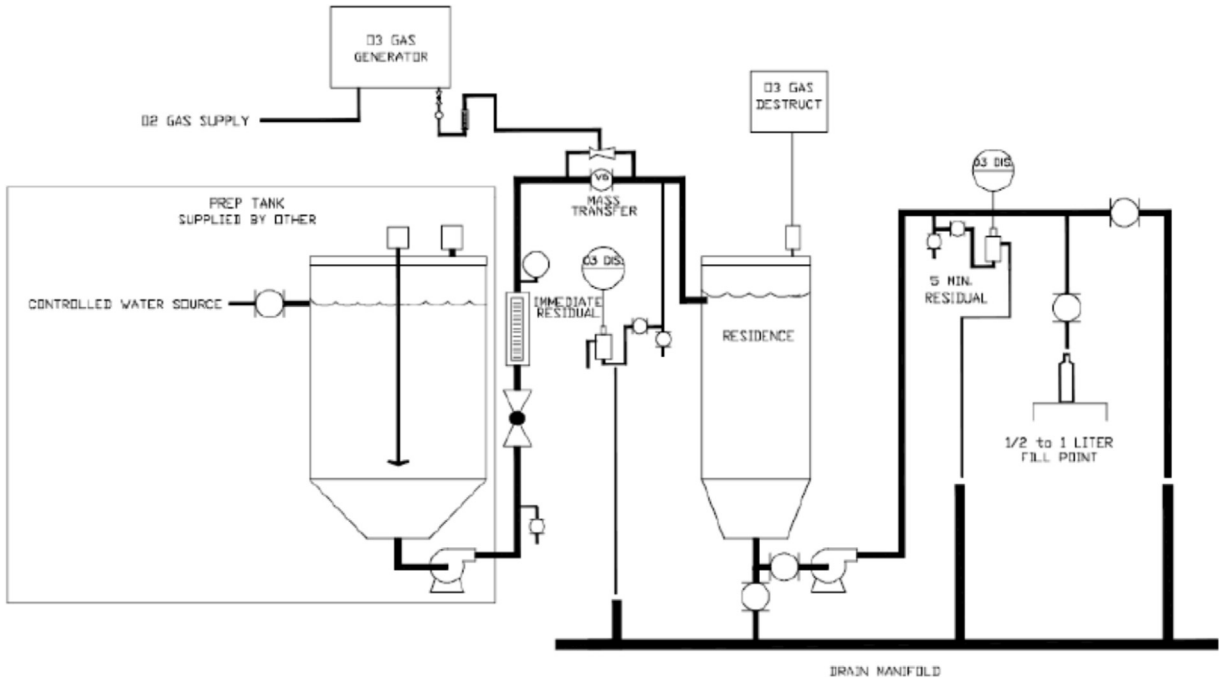

Figure 1. Ozone Pilot System Design (Steelhead Manufacturing, San Antonio, TX).

Figure 2. Ozone filling and monitoring equipment.

Each bottle was immediately inoculated with an individual microorganism, *E. coli* (EC), *Enterococcus faecalis* (EF), or *Burkholderia cepacia* (BC) to attain ca. 6.0 log and 4.0 log CFU/mL population. The inoculated bottles were maintained at 25 °C for predefined contact times (5, 30, 60, and 180 min) to simulate postbottling ozone exposure. To terminate ozone activity at each timepoint, 1.0 mL of 50% of sodium thiosulfate (Thermo Fisher Scientific, Waltham, MA) was added in 500 mL bottled water to neutralize residual ozone after specified exposure time a practice aligned with industry. The 180-min maximum duration was selected based on preliminary data showing complete ozone dissipation in PET bottles by this timeframe and industry prioritization of rapid disinfection (≤5 min) for operational efficiency and expedited warehouse shipping timelines.

To measure the efficacy of residual ozone in water with varying pH and TDS concentrations, purified water was adjusted to pH 5.0, 7.0, or 9.0 using 0.1 N HCl or 0.1 N NaOH. Similarly, three tanks filled with purified water were adjusted to TDS of 5, 50, and 300 mg/L using a mineral salts blend (potassium bicarbonate, sodium bicarbonate, magnesium oxide, sodium chloride; ProTab Laboratories, Foothill Ranch, CA) used in the BW industry for assessing the effect of TDS. Each tank was ozonated (0.1 mg/L), and 0.5 L PET bottles were filled and subsequently inoculated with each microorganism to attain ca. 4.0 $\log_{10}$ CFU/mL. Each bottle was stored for 30 min at 25 °C. After ozone treatment, bottled water was neutralized using 1 mL of 50% of sodium thiosulfate (Thermo Fisher Scientific).

Microbial enumeration. For evaluation of the efficacy of the residual ozone concentrations (0.1, 0.2, 0.3, and 0.4 mg/L) for different treatment times (5, 30, 60, and 180 min), the water sample was serially diluted in BPB and 100 mL of each dilution was filtered through Neogen NEO-GRID hydrophobic membrane filter using a manifold system (Neogen, Lansing, MI). The NEO-GRID hydrophobic filters were placed onto TSA, BHI, and NA supplemented with Fast Green FCF (FCF: for coloring food; 0.25 g/L; Sigma Aldrich, St. Louis, MO) for enumeration of *E.* coli, *E. faecalis,* and *B. cepacia,* respectively. The Fast Green FCF was added to enhance the visibility of the colonies on the NEO-GRID filter showing bluish green or green color colonies. The plates were incubated as mentioned earlier, and the number of positive squares (colonies) were counted. The number of positive squares was multiplied by the appropriate dilution factor and converted to the corresponding most probable number using NEO-GRID Manual Methods (Neogen, Lansing, MI), and reported as log CFU/mL. The log reduction in microbial population was calculated to determine the efficacy of residual ozone.

Ozone dissipation. Dissipation of ozone in bottled water (0.5 L PET bottle) under static conditions was measured using the colorimetric method. PET bottles containing ozonated water (500 mL, 0.4 mg/L ozone) were incubated in a water bath maintained at 21 and 38 °C for 6 h. To assess the effect of biological load on ozone dissipation, each bottle was inoculated with 20 μL of bacterial strain (*E. coli*) to attain a final concentration of ca. 6.0 log CFU/mL. Ozone concentration was measured using an ozone handheld colorimeter (Indigo Method 8311, Hach Company, Loveland, CO) after every 30 min (separate bottle analyzed for each sampling time) until 6 h.

Ozone dissipation model. The ozone dissipation model was also generated using temperatures 38 °C and 4 °C. Simple linear model was used to describe ozone dissipation in bottled water with and without microbial load at 38 °C, and a quadratic model was used, with and without microbial load, at temperature 4 °C. Eq. (1) is a simple linear model, and Eq. (2) is a quadratic model.

$$Ozone = \beta_0 + \beta_1 * Time + \varepsilon \quad (1)$$

$$Ozone = \beta_0 + \beta_1 * Time + \beta_2 * Time^2 + \varepsilon \quad (2)$$

R^2 was used to determine the goodness of fit and statistical analyses were performed in R (Version 4.1.3; R Foundation for Statistical Computing, Vienna, Austria) to build the ozone dissipation model.

Statistical analysis. Three independent replications were performed for each treatment, using fresh inoculum. Prior to analysis, bacterial counts were transformed into log counts (CFU/mL) to stabilize variance and meet normality assumptions (McDonald, 2014). Analysis of variance (ANOVA) using the general linear model procedure of the Statistical Analysis System (Release 8.01, SAS Institute, Inc., Cary, N.C.) was conducted. Tukey's test was used to separate means ($\alpha = 0.05$) of *E. coli, E. faecalis, and B. cepacia* (log CFU/mL) with different ozone concentrations and time of contact.

Results and discussion

A key benefit of using ozone for disinfection of water for bottling applications is its dissipation within a short duration after the bottling process (Nwaiwu and Ibekwe, 2019). The water temperature, pH, and the amount of organic and inorganic materials affect the ozone decomposition rate. An immediate (within 1 min) decrease in ozone concentration from 0.40 to 0.28 mg/L was observed upon the addition of microbial load (dead bacterial cells) to the ozonated water (Fig. 3). Complete dissipation of ozone in bottled water required 1.5 h and 6.0 h when stored at 21 °C, and 38 °C, respectively (Fig. 3). Santos et al. (2021) reported a higher ozone dissipation rate in water stored at higher temperatures (25 °C vs. 4 °C) and higher pH values (7.0 vs. 5.0). This confirms that lower water temperature and pH can result in a slower ozone dissipation. The solubility of ozone (concentration of gas that dissolves in a liquid) at a constant temperature is linearly proportional to the partial pressure of the gas in equilibrium with that liquid. The lower the water temperature, the greater the solubility of the gas in water (Khadre et al., 2001) and consequently, lower solubility in water and higher ozone dissipation rate (Di Bernardo and Dantas, 2006).

An immediate reduction in the ozone concentration was observed when a biological load was introduced in the ozonated bottled water compared to the nonsupplemented samples (0.1 vs 0.2 at 38 °C and 0.2 vs 0.3 at 21 °C), and the ozone decomposition was greater with longer exposure times (90 min vs 180 min at 38 °C; Fig. 3). Okafor (2011) reported that the same pH, natural organic matter, and dissolved solids can interact to affect the oxidation efficiency of ozone. The organic matter present in the water directly and indirectly reacts with ozone and consumes available hydroxyl radicals. The reaction between hydroxyl radicals and the organic matter produces a superoxide, which quickly reacts with ozone to reconstitute hydroxyl radicals. The chain reaction of superoxide generation ends in the presence of inhibitors or compounds that interfere with superoxide release after reaction with hydroxyl radicals. Kwon et al. (2017) and Galdeano et al. (2018) reported that higher temperature, higher pH, and lower initial water quality (higher TDS, etc.) can all accelerate ozone decay and therefore, lower available ozone for disinfection. Despite the immediate reduction in the ozone concentration at 21 °C and 38 °C with and without biological load, there was adequate ozone (>0.1 mg/L) to provide comparable disinfection performance to other methods used for water treatment.

The coefficients of simple linear model and quadratic model are shown in Table 1 at temperature 38 °C, for both with and without added cell (microbial load) models. Ozone concentration decreased with time and was depleted by 100 min (Fig. 3). The ozone depletion was linear at 4 °C (Fig. 4), and similar coefficients were observed for water with and without microbial load at both temperatures (4 and 38 °C; Fig. 4). Nghi et al. (2018) reported that samples after different duration of ozone disinfection duration showed a similar rate ozone decomposition. However, in water, ozone interacts with dissolved solids, and therefore, ozone decomposition is affected by the amount of TDS. We can calculate the half-life time of the ozone reaction with the matters. The Pearson correlation coefficient (R^2) of the models indicated a good fit except for water stored at 4 °C containing the biological load, with an R^2 of 0.588 (Table 1).

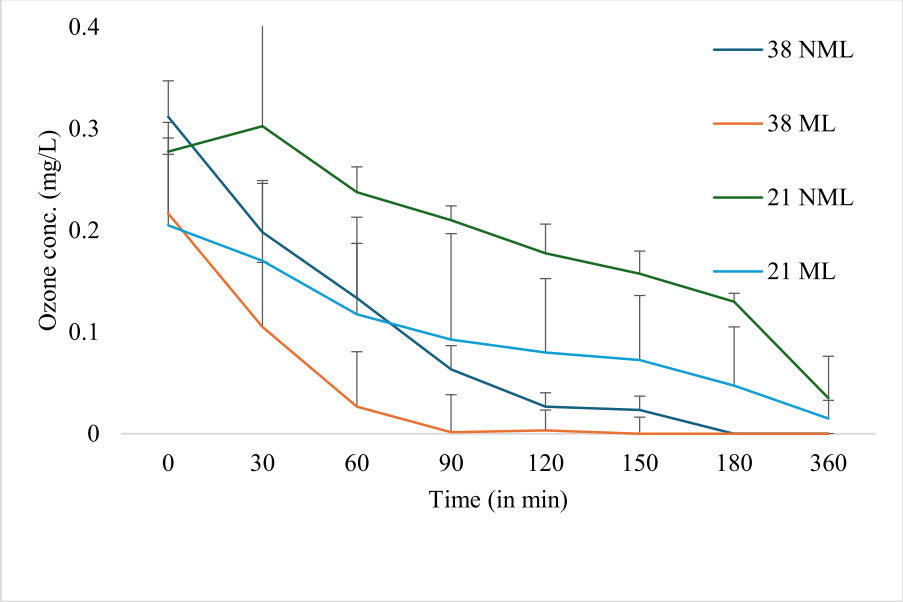

Figure 3. Dissipation of ozone in bottled water during storage at different temperatures (38 °C and 21 °C) with microbial load (ML; ca. 6.0 log) and no microbial load (NML) at different time points (in min).

Table 1
Coefficients and R^2 of simple linear model and quadratic model used for ozone dissipation. Simple linear model was used to describe ozone dissipation in bottled water with and without microbial load at 38 °C and quadratic model was used with and without microbial load at temperature 4 °C

Coefficients	β_0	β_1	β_2	R^2
38 °C with microbial load	0.04	−0.316	0.338	0.7418
38 °C without microbial load	0.095	−0.552	0.454	0.7699
4 °C with microbial load	0.216	−0.00069		0.588
4 °C without microbial load	0.03	−0.00074		0.772

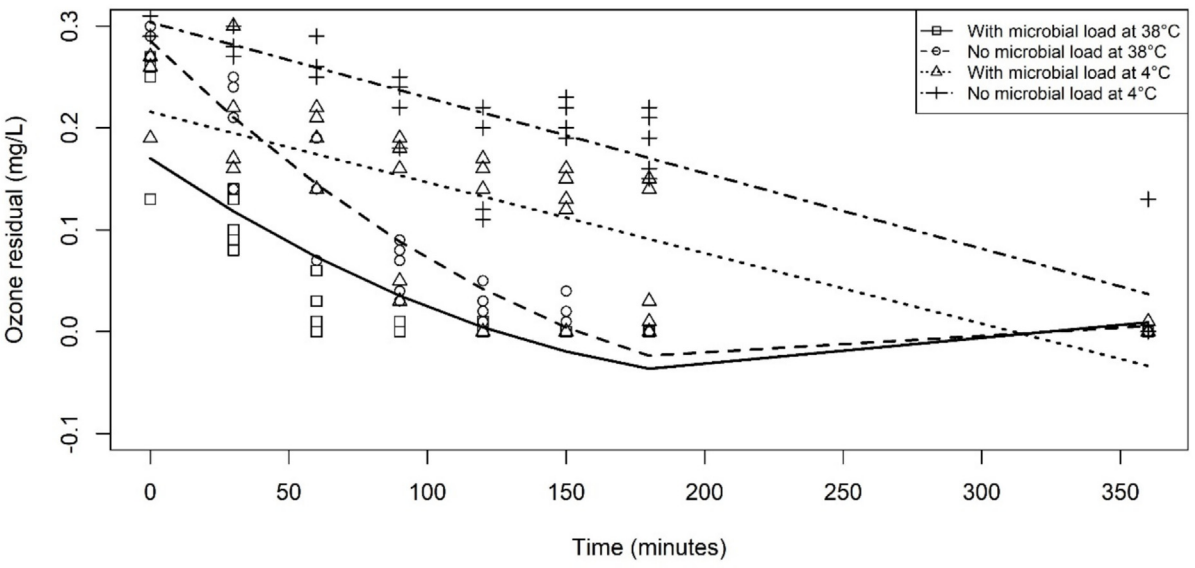

Figure 4. Ozone dissipation model at temperatures 38 °C and 4 °C. Simple linear model was used to describe ozone dissipation in bottled water with microbial load ((ML; ca. 6.0 log) and no microbial load (NML) at different time points (in min) at 38 °C. Quadratic model was used to describe ozone dissipation in bottled water with microbial load ((ML; ca. 6.0 log) and no microbial load (NML) at different time points (in min) at 4 °C.

Several factors contribute to the ozone dissipation rate and consequently, the available ozone for microbial disinfection. Farooq et al. (1977) evaluated reduction in *Mycobacterium fortuitum* and *Candida parapsilosis* population in a constant flow reactor system simulating municipal treatment processes with ozone and reported that water pH affected the microbial destruction, with higher pH (>8.5) in the system resulting in greater survival. The authors hypothesized that the lower reductions in microbial population observed were due to faster ozone dissipation at higher pH, and not necessarily a direct effect of pH on the disinfection activity of ozone. Similar results were observed in this study, with increasing temperature and pH resulting in increased ozone dissipation rates in bottled water. This should be considered in the water bottling process, especially as it relates to alkaline water products (generally produced with minerals or electrolysis resulting under a pH > 8.5) that may use the same treatment systems as other bottled water products.

Reduction in pathogen surrogate (*E. coli*; *B. cepacia,* and *E. faecalis*) populations of ≥4.0 log CFU/mL was observed in ozonated bottled water regardless of the initial population (either 6.0 or 4.0 log CFU/mL) of the microorganisms (Tables 2–4). A slightly greater reduction ($P \leq 0.05$) was observed at higher ozone concentrations of 0.3 and 0.4 mg/L. Longer contact times (30, 60, and 180 min) for ozone disinfection resulted in minimal overall reduction ($P \leq 0.05$) in microbial population beyond 5 min. Majority of the ozone disinfection occurs within a very short time (>5 min) with minimal but significant reductions (>4.0 log) observed subsequently. Jamil et al. (2017) reported similar findings in controlled experiments using a batch ozone contact setup evaluating the disinfection capability on *E. coli* and *Salmonella*. The authors reported that most of the ozone disinfection occurred within 1 min of contact time. Similarly, Santos et al. (2021) reported that 1 min of ozone contact time was adequate to reduce *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida albicans* population by >2.23 log CFU/mL (29). Similar effects of ozone (1.3–0.5 mg/L) were reported by Bialoszewski et al. (2011) on *E. coli* and *Staphylococcus aureus* with 1 min of contact time.

The susceptibility of microorganisms to ozone is affected by the dissolved solids concentration. Microorganisms present in the natural sources of water are protected by the conversion of ozone to less reactive species having weak or no disinfection activity by the organic matter that may be present in naturally sourced water (Bancroft et al., 1984; Elford and van den Ende, 1942; Ingram and Barnes, 1954; Ingram and Haines, 1949; Prabha et al., 2015). Williams et al. (2004) reported that ascorbic acid and organic matter in orange juice reduced the efficacy of ozone in reducing the *E. coli* population. Among the dissolved solids, carbonates and bicarbonates act as strong scavengers of ozone and thus, reduce the ozone efficacy. Adding minerals in the water as is practiced in the bottled water industry increases the total dissolved solids content in the water, therefore, it is necessary to evaluate the effect of mineral supplementation of BW on ozone disinfection efficacy. In this study, a mineral blend (potassium bicarbonate, sodium bicarbonate, magnesium oxide, and sodium chloride) was supplemented at varying concentrations of 5.0, 50, and 300 mg/L and the efficacy of ozone in reducing microbial population was evaluated (Table 5). Increasing mineral concentration (5 to 300 mg/L) reduced the ozone disinfection activity. Both *E. coli* and *B. cepacia* reductions were lower (<4.0 CFU/mL) at TDS concentration of >50 mg/L, similar to the findings of Williams et al. (2004). Galdeano et al. (2018) and Kwon et al. (2017) reported that higher TDS, higher temperature, and higher pH decrease ozone disinfection efficacy.

Increases in pH and temperature affect ozone decomposition differently in various media, thus affecting ozone disinfection efficacy (Chittrakorn et al., 2014; Galdeano et al., 2018). Considering the expanding market of alkaline water, it is necessary to evaluate the efficacy of ozone in reducing microbial population in BW with varying pH values. Greater decomposition of ozone in BW at high pH (~9.0) was observed resulting in lower efficacy of ozone. Higher pH values (>8.0) of BW lead to rapid ozone decomposition due to the presence of hydroxyl ions which reduce the half-life of the ozone (Jamil et al., 2017). Nevertheless, it has been reported that the oxidizing potential of hydroxyl radicals has higher disinfecting attributes (Di Bernardo and Dantas, 2006). On the contrary, at pH below 6.0, the disinfection efficiency is related to molecular ozone and has a direct effect on bacterial cells (Galdeano et al., 2018; Jamil et al., 2017). Therefore, faster ozone decomposition causes a reduction in the CT value required for the effective killing of ozone less effective (Jamil et al., 2017). Reductions of ≥4 log CFU/mL were observed in the *E. coli*, *B. cepacia,* and *E. faecalis* populations in the BW, regardless of the pH (5 or 7). However, higher pH (Elford and van den Ende, 1942) resulted in a lower reduction (<4.0 log CFU/mL) for *E. coli*, *B. cepacia,* and *E. faecalis* (Table 5). The results indicate reduced ozone efficacy at higher pH in the BW. Rapid ozone breakdown in alkaline solutions has been associated with the catalytic effects of hydroxyl ions in the medium (Alder and Hill, 1950; Hewes and Davison, 1973). Leiguarda et al. (1949) observed marginally superior bactericidal action of ozone against *E. coli* and *C. perfringens* at pH 6.0 compared to pH 8.0. Farooq et al. (1977) reported a lower reduction in *Mycobacterium fortuitum* counts with higher pH during the ozone disinfection process. The authors reported that greater survival was due to lower ozone residual in alkaline water. Furthermore, Foegeding (1985) investigated the effect of ozone on *Bacillus* and *Clostridium* spores at various pH values (Bancroft et al., 1984; Bialoszewski et al., 2011; Bouland et al., 2004) and concluded

Table 2
Reduction of *E. faecalis* (mean ± S.D.; log CFU/mL) in bottled water by ozone (0.1–0.4 mg/L) for various periods of time at 25 °C in the presence of biological load (ca. 6.0 or 4.0 log CFU/mL) subsequent to bottling

Target population (log CFU/mL)[c]	Exposure time (min)	Ozone concentration (mg/L)			
		0.1	0.2	0.3	0.4
4.0	5	3.23 ± 0.05^{aX}	2.57 ± 1.13^{aX}	3.24 ± 0.05^{aX}	3.24 ± 0.05^{aX}
4.0	30	1.89 ± 0.86^{bX}	2.90 ± 0.43^{aX}	3.24 ± 0.05^{aX}	3.24 ± 0.05^{aX}
4.0	60	3.01 ± 0.45^{aX}	3.24 ± 0.05^{aX}	3.24 ± 0.05^{aX}	3.24 ± 0.05^{aX}
4.0	180	2.11 ± 1.04^{bX}	3.13 ± 0.24^{aX}	3.17 ± 0.10^{aX}	3.16 ± 0.11^{aX}
6.0	5	3.68 ± 0.82^{aX}	3.75 ± 0.95^{aX}	3.94 ± 1.28^{aX}	4.61 ± 1.37^{aY}
6.0	30	4.38 ± 1.22^{bX}	3.94 ± 1.28^{aX}	4.61 ± 1.37^{aX}	4.61 ± 1.37^{aX}
6.0	60	4.20 ± 1.06^{bX}	4.97 ± 0.45^{bY}	4.20 ± 1.19^{aX}	4.19 ± 1.19^{aX}
6.0	180	3.65 ± 0.43^{aX}	4.91 ± 1.37^{bX}	5.27 ± 0.22^{bY}	4.29 ± 1.20^{aX}

[a] Within columns (Same ozone concentration), means with different superscripts (^{ab}) differ significantly ($P < 0.05$) between the time points.
[b] Within rows (Same exposure time), means with different uppercase superscripts (^{XY}) differ significantly ($P < 0.05$) between the ozone concentrations.
[c] Initial *E. faecalis* populations subsequent to inoculation (0 min) were 6.0 and 4.0 log CFU/mL; and the calculated populations (based on the inoculum) were 5.27 and 3.24 log CFU/mL.

Table 3
Reduction of *B. cepacia* (mean ± S.D.; log CFU/mL) in bottled water by ozone (0.1–0.4 mg/L) for various periods of time at 25 °C in the presence of biological load (ca. 6.0 or 4.0 log CFU/mL) subsequent to bottling

Target population (log CFU/mL)[c]	Exposure time (min)	Ozone concentration (mg/L)			
		0.1	0.2	0.3	0.4
4.0	5	3.28 ± 0.48^{aX}	3.55 ± 0.06^{aX}	3.42 ± 0.24^{aX}	3.24 ± 0.24^{aX}
4.0	30	3.19 ± 0.69^{aX}	3.57 ± 0.02^{aX}	3.10 ± 0.83^{aX}	3.57 ± 0.02^{aX}
4.0	60	3.37 ± 0.34^{aX}	3.56 ± 0.02^{aX}	3.32 ± 0.45^{aX}	3.57 ± 0.02^{aX}
4.0	180	3.57 ± 0.03^{aX}	3.57 ± 0.02^{aX}	3.56 ± 0.02^{aX}	3.57 ± 0.02^{aX}
6.0	5	4.12 ± 1.12^{aX}	4.97 ± 0.22^{aY}	4.82 ± 0.57^{aY}	5.24 ± 0.36^{aY}
6.0	30	4.19 ± 0.62^{aX}	5.31 ± 0.17^{aY}	5.30 ± 0.26^{aY}	4.38 ± 0.89^{bX}
6.0	60	5.28 ± 0.21^{bXY}	4.93 ± 0.57^{aXY}	4.77 ± 1.12^{aX}	5.44 ± 0.03^{aY}
6.0	180	4.83 ± 0.61^{aY}	5.17 ± 0.48^{aXY}	5.44 ± 0.03^{bX}	5.42 ± 0.01^{aX}

[a] Within columns (Same ozone concentration), means with different superscripts (^{ab}) differ significantly ($P < 0.05$) between the time points.
[b] Within rows (Same exposure time), means with different uppercase superscripts (^{XY}) differ significantly ($P < 0.05$) between the ozone concentrations.
[c] Initial *B. cepacia* populations subsequent to inoculation (0 min) were 6.0 and 4.0 log CFU/mL; and the calculated populations (based on the inoculum) were 5.44 and 3.57 log CFU/mL.

Table 4
Reduction of *E. coli* (mean ± S.D.; log CFU/mL) in bottled water by ozone (0.1–0.4 mg/L) for various periods of time at 25 °C in the presence of biological load (ca. 6.0 or 4.0 log CFU/mL) subsequent to bottling

Target population (log CFU/mL)[c]	Exposure time (min)	Ozone concentration (mg/L)			
		0.1	0.2	0.3	0.4
4.0	5	3.77 ± 0.40^{aX}	3.25 ± 0.06^{bY}	3.96 ± 0.24^{aX}	3.93 ± 0.15^{aX}
4.0	30	3.96 ± 0.12^{aX}	3.96 ± 0.12^{aX}	3.96 ± 0.83^{aX}	3.96 ± 0.12^{aX}
4.0	60	3.96 ± 0.12^{aX}	3.96 ± 0.12^{aX}	3.96 ± 0.45^{aX}	3.96 ± 0.12^{aX}
4.0	180	3.96 ± 0.12^{aX}	3.96 ± 0.12^{aX}	3.96 ± 0.02^{aX}	3.83 ± 0.29^{aX}
6.0	5	5.61 ± 0.55^{aX}	5.31 ± 1.07^{abX}	5.97 ± 0.08^{aX}	5.48 ± 0.89^{aX}
6.0	30	4.96 ± 0.84^{bX}	5.32 ± 0.18^{abXY}	5.44 ± 0.84^{aXY}	5.97 ± 0.08^{aY}
6.0	60	5.88 ± 0.04^{aY}	5.17 ± 0.97^{aX}	5.11 ± 0.94^{bX}	5.44 ± 0.83^{aXY}
6.0	180	5.70 ± 0.37^{aX}	5.97 ± 0.08^{bX}	5.97 ± 0.08^{aX}	5.97 ± 0.08^{aX}

[a] Within columns (Same ozone concentration), means with different superscripts (^{ab}) differ significantly ($P < 0.05$) between the time points.
[b] Within rows (Same exposure time), means with different uppercase superscripts (^{XY}) differ significantly ($P < 0.05$) between the ozone concentrations.
[c] Initial *E. coli* populations subsequent to inoculation (0 min) were 6.0 and 4.0 log CFU/mL; and the calculated populations (based on the inoculum) were 5.97 and 3.96 log CFU/mL.

Table 5
Reduction of *E. coli, B. cepacia, E. faecalis* (mean ± S.D.; log CFU/mL) in bottled water by ozone (0.1 mg/L) at three TDS concentrations (5, 50, and 300 mg/L) and pH concentrations (5, 7, and 9)

pH/TDS	*E. coli*	*B. cepacia*	*E. faecalis*
TDS[b]			
5	4.53 ± 0.22^{a}	4.18 ± 0.24^{a}	5.02 ± 0.26^{a}
50	3.62 ± 0.19^{b}	2.70 ± 0.23^{b}	4.78 ± 0.26^{ab}
300	3.05 ± 0.19^{b}	2.14 ± 0.20^{b}	4.24 ± 0.26^{b}
pH[c]			
5.0	4.72 ± 0.40^{a}	5.34 ± 0.27^{a}	5.67 ± 0.30^{a}
7.0	4.20 ± 0.40^{b}	4.15 ± 0.27^{b}	3.96 ± 0.29^{b}
9.0	3.71 ± 0.35^{c}	3.28 ± 0.27^{c}	3.86 ± 0.27^{b}

[a] Within columns, means with different superscripts (^{ab}) differ significantly ($P < 0.05$) between the TDS and pH values.
[b] Initial *E. coli*, *B. cepacia,* and *E. faecalis* populations subsequent to inoculation (0 min) were 4.53, 4.18, and 5.02 log CFU/mL, respectively, for TDS concentrations.
[c] Initial *E. coli*, *B. cepacia,* and *E. faecalis* populations subsequent to inoculation (0 min) were 4.72, 5.34, and 5.67 log CFU/mL, respectively, for pH concentrations.

that greater reductions were observed at lower pH (Bancroft et al., 1984) by ozone.

It is assumed that higher concentrations of ozone in BW will result in greater microbial reduction and thus, the greatest protection for the finished product. While this may be true from a microbiological safety perspective, the formation of disinfection by-product by ozone in BW is a concern. Ozone oxidizes bromide (a common component in spring waters) to bromate, depending on the initial bromide concentration, ozone concentration, water temperature, and pH. Bottled water facilities that produce bottled spring water need to design a system that will disinfect the product to assure microbiological safety while not exceeding the regulatory bromate concentration. This is usually a non-issue for purified bottled water products that remove most of the bromide through reverse osmosis or other purification strategies. Bouland et al. (2004) provides a thorough review of controlling the conversion of bromide into bromate in water by ozone. It is important that manufacturers of bottled water optimize ozone concentration to achieve microbial reduction while minimizing the risk of bromate formation.

Several studies have evaluated the disinfection capability of ozone in drinking water using contact tanks where the ozone is injected into the water in a tank with a continuous flow of water, providing a specific contact time for disinfection. However, literature on the ability of the residual ozone in bottled water in reducing microbial population is lacking. This research will be useful to understand the role of time and temperature on the design of ozone disinfection strategies for the bottled water industry as a supplement to the microbial reduction achieved during processing (contact tanks). Bottled water manufacturers utilize multiple technologies such as micron filtration, carbon filtration, UV treatment, and ozone treatment to significantly reduce the risk of waterborne illness. Further, the concentrations of microorganisms used in these experiments would not likely be encountered under normal operating conditions, yet it is reassuring that low con-

centrations of residual ozone in the bottled water can afford significant microbial reduction (>4.0 log CFU/mL), assuring the safety of bottled water. Based on our study we suggest that for optimal microbial safety (≥4 log reduction) in bottled water, apply ozone at 0.3–0.4 mg/L with minimum 5 min contact time, prioritizing pH 5.0–7.0 and ambient temperatures (25 °C) to slow ozone decay. Minimize dissolved solids (<50 mg/L) and adjust dosing for alkaline/mineral waters (pH > 8.5 or TDS > 50 mg/L) to counteract reduced efficacy, while limiting bromate risks in bromide-containing sources via lower ozone (≤0.3 mg/L) and pretreatment.

CRediT authorship contribution statement

William Ryan Schwaner: Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Sanjay Kumar:** Writing – review & editing, Validation, Methodology, Formal analysis. **Harshavardhan Thippareddi:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

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.

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