OZONE: SCIENCE & ENGINEERING
2017, VOL. 39, NO. 6, 407–416
https://doi.org/10.1080/01919512.2017.1322489

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Ozone Disinfection Efficiency for Indicator Microorganisms at Different pH Values and Temperatures

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ABSTRACT

Disinfection efficiency of ozone was determined in various types of water at different pH (6, 7 and 8) values and temperatures (15, 25 and 35 °C) for *E. coli* and *Salmonella*. Three different applied ozone concentrations (1.5, 1.7, and 2 mg/L) in the gas phase were applied, and samples were taken at different time intervals to determine microbial survival using spread plate count (SPC) and ozone residual. Highest microbial inactivation was observed in distilled water with applied ozone concentration of 2 mg/L in the gas phase. Survival of *E. coli* was higher at pH 8 and 15 °C as compared to lower pH values and temperatures as depicted by the inactivation kinetics of the test microbes used in the study. *Salmonella* showed 5 and 6 log removal after contact time of 45 and 60 sec, respectively, at 2 mg/L. Disinfection of mixed culture showed relatively more survival of *E. coli*; as 3 and 4 log removal of *E. coli* and 4 and 5 log removal of *Salmonella* was observed after 45 and 60 sec.

ARTICLE HISTORY
Received 24 January 2017
Accepted 27 March 2017

KEYWORDS
Ozone; Disinfection; Drinking Water; Indicator Microorganisms; Ozone Residual; pH; Temperature

Introduction

Microbial contamination of water is one of the major problems throughout the world as water can carry a number of different organisms to a large number of consumers over wide geographic areas. Microbiological contamination is the primary cause of disease outbreaks in drinking water. Different diseases such as cholera, typhoid, dysentery, and hepatitis have been linked to drinking water, mainly contaminated by human waste. Pakistan is one of those countries facing serious problems related to water quality and the supply of clean and safe water to different parts of the country (Hashmi, Qaiser, and Farooq 2012). Disinfection of water and protection of water sources from sewage contamination have proven effective in preventing transmission of these diseases by water (Ahmed et al. 2004). Microbes of *Enterobacteriaceae* have been used as indicators of food and water quality and to assess the efficacy of disinfection process.

Among the commonly used chemical disinfectants, ozone can be rated as a promising disinfectant for microbial contaminants in water. Ozone is an unstable gas that can be broken down to oxygen gas (O_2) and oxygen atoms (O). Oxygen atoms, being strong oxidizers, appear to be a good agent for sterilization. This property therefore becomes very useful for the inactivation of microorganisms in water (Larocque 1999; Rice 1999). Ozone has been tested against *Pseudomonas fluorescens, Escherichia coli* O157:H7, *Leuconostoc mesenteroides*, and *Listeria monocytogenes* in various studies (Al-Hashimi, Mason, and Joyce 2015; Edward-Brandt et al. 2007; Kim and Yousef 2008). They demonstrated that the treatment with ozone in concentration of 1 mg/L resulted in a significant reduction of 93% in the number of live cells. Exposure of bacteria to ozone at 2.5 mg/L for 40 sec caused a 5- to 6-log decrease in count. A descending order for resistance of bacteria to ozone was reported as: *E. coli* O157:H7, *P. fluorescens, L. mesenteroides*, and *L. monocytogene* (Kim and Yousef 2008).

A study reported the effect of ozonation on rate of disinfection of *E. coli* and found the rate of disinfection as a function of ozone concentration, ozonation duration, and flowrates (Zuma, Lin, and Jonnalagadda 2009). The influence of pH and temperature in aqueous systems on the rate of ozone-initiated disinfection of microbes was stronger at lower pHs than at basic pH. Further, molecular ozone was found more effective in disinfection than hydroxyl radicals (Zuma, Lin, and Jonnalagadda 2009).

Disinfection is a rate process and is dependent upon the rates of reaction between the disinfectant and the microorganisms in water. Disinfection efficiency of ozone depends upon multiple factors such as ozone concentration, contact time, pH, and

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temperature. We hypothesized that pH has an effect on the kinetics of ozone inactivation of microorganisms. The ozone decomposition rate has been reported to increase with temperature under the same conditions of mass transfer. Ozone residual with a given applied concentration decreases with increasing temperature due to an increased rate of ozone decomposition and a decreased solubility of ozone (Li, Gyurek, and Finch 2001). This study was specifically designed to study the effect of pH and temperature on ozone disinfection against indicator microbes of public health importance and the effect of water quality in different water samples. Inactivation of pure and mixed cultures of *E. coli* and *Salmonella* was carried out by applying various ozone concentrations and contact times to study the inactivation behavior of these indicator microbes in water samples of various origins. The study is important to the deteriorating water quality situation in Pakistan in identifying and studying the disinfection process against common indicator microbes of great public health importance.

Materials and methods

Pure cultures of *E. coli* and *Salmonella* were isolated from wastewater of Nullah Lai using standard protocols (APHA 2005). Different biochemical tests were performed for their identification and characterization. To carry out disinfection with ozone using these cultures, the following steps were taken.

Preparation of phosphate buffer

The required buffer was prepared by adding potassium dihydrogen phosphate 0.34 g and sodium hydroxide 0.06 g/L in 1,000 mL of distilled water, giving strength of 0.0025 M. Neutral pH was maintained. The buffer was autoclaved and sterilized at 121 °C and 15 psi pressure for 15 min before use. Similarly, phosphate buffers of pH 6 and 8 were prepared according to standard methods (APHA 2005).

Preparation of microbial culture suspension

Slants of Merck KGA nutrient agar were prepared and inoculated using a sterile inoculating loop by picking pure *E. coli* colonies from EMB agar plates and *Salmonella* colonies from bismuth sulphite agar plates. The slants were incubated at 37°C for overnight growth of culture (Bergey and Holt 1994). The slants were washed with phosphate buffer and a thick suspension was obtained. It was centrifuged for 10 min at 4,000 rpm. The supernatant was thrown and the resulting pellet was dissolved on a gyro mixer (TKA 0300–100). It was again centrifuged and washed with phosphate buffer. This procedure was repeated (Larson and Marinas 2003). A thick suspension of microbial cultures was obtained as in Hunt and Marinas (Hunt and Marinas 1997). Optical density (OD) was measured using an OD meter at 400 nm wavelength to determine the cell density. An aliquot of 2 mL of the original culture suspension was added in 1,000 mL of phosphate buffer and stirred for 5 min for complete mixing. The temperature of this sample was maintained at 15, 25, and 35 °C in a water bath (Memmert, Type WB-7). In the case of tap water and well water analyses, the phosphate buffer was replaced by these samples while making the bacterial suspension for ozonation.

Ozonation of samples

The ozone contactor consisted of a Pyrex gas washing bottle autoclaved and sterilized at 171 °C(Ahmed and Farooq 1984). Ozone was generated from a commercial ozone generator (XeTin, Ozone Air and Water purifier) Air Zone XT-301, using atmospheric air as the oxygen source. The concentration of ozone in the gas phase was determined as described in the standard methods (Ahmed and Farooq 1984). Ozonation was carried out in a batch system; 600 mL of the phosphate buffer inoculated with *E. coli* culture was poured into a sterile ozone gas washing bottle. Ozone was bubbled into the solution and the inlet gas ozone concentration was 1.5, 1.7 and 2 mg/L, and applied for 3 min. Samples were taken out at different time intervals, *i.e.*, 5, 10, 20, 30, 45, 60 sec and 3 min during ozonation to determine the ozone residual and SPC.

Ozonated samples were collected in test tubes containing sodium thiosulfate to instantly reduce free ozone residual prior to enumeration (Farooq and Akhlaque 1983). Both non-treated control (without ozone) and ozone-treated samples, 0.1 mL each, were collected at different time intervals. The samples were diluted up to 1/10, 1/100, 1/1000, and so on depending on the initial viable number of microbes (Choi et al. 2007). Then the samples and dilutions were spread onto nutrient agar and incubated at 37 °C. The colonies appearing on plates were counted with a colony counter (Stuart-SC 6, Bibby Sterlin LTD USA) and calculated as CFU/mL (Thanomsub et al. 2002).

Standard plate count (SPC)

Standard plate counts (SPC) was determined as per standard methods (APHA 2005). It is a procedure for estimating the number of live heterotrophic bacteria in water. Serial dilutions of the samples were prepared (using phosphate buffer) so that following incubation, one of the dilutions will yield growth of 30–300 colonies (the ideal counting range) on the agar plate. For SPC, 0.1 mL of the sample or dilution was transferred to a sterile, media containing petri dish. The sample was spread onto the agar with the help of a sterile L-shaped bent rod, while the petri dish is spun on a turntable.

Determination of ozone residual concentration

A Merck Spectroquant colorimeter picco kit (SN 059008) was used for determining ozone residual concentration in the gas phase present in water samples.

Results and discussion

Disinfection studies of E. coli in distilled, tap, and well water samples

A comparison of percent survival of *E. coli* in distilled, tap, and well water is shown in Figure (1a). The well water sample showed the highest percentage of survival as compared to tap and distilled water samples. This shows that the disinfection efficiency of ozone is found to be less in the case of well water samples due to their chemical characteristics. In the distilled water sample, percent survival of *E. coli* is shown to be least, compared to the other two water samples. Ozone disinfection was greater in distilled water sample causing inactivation of *E. coli* at applied ozone concentration of 2 mg/L in gas phase for the same contact time of 60 sec. These results reveal a variation in disinfection efficiency of ozone in lab and field conditions, i.e., in distilled water and drinking water samples. When the applied ozone concentration was 2 mg/L in gas phase, a significant decrease in bacterial

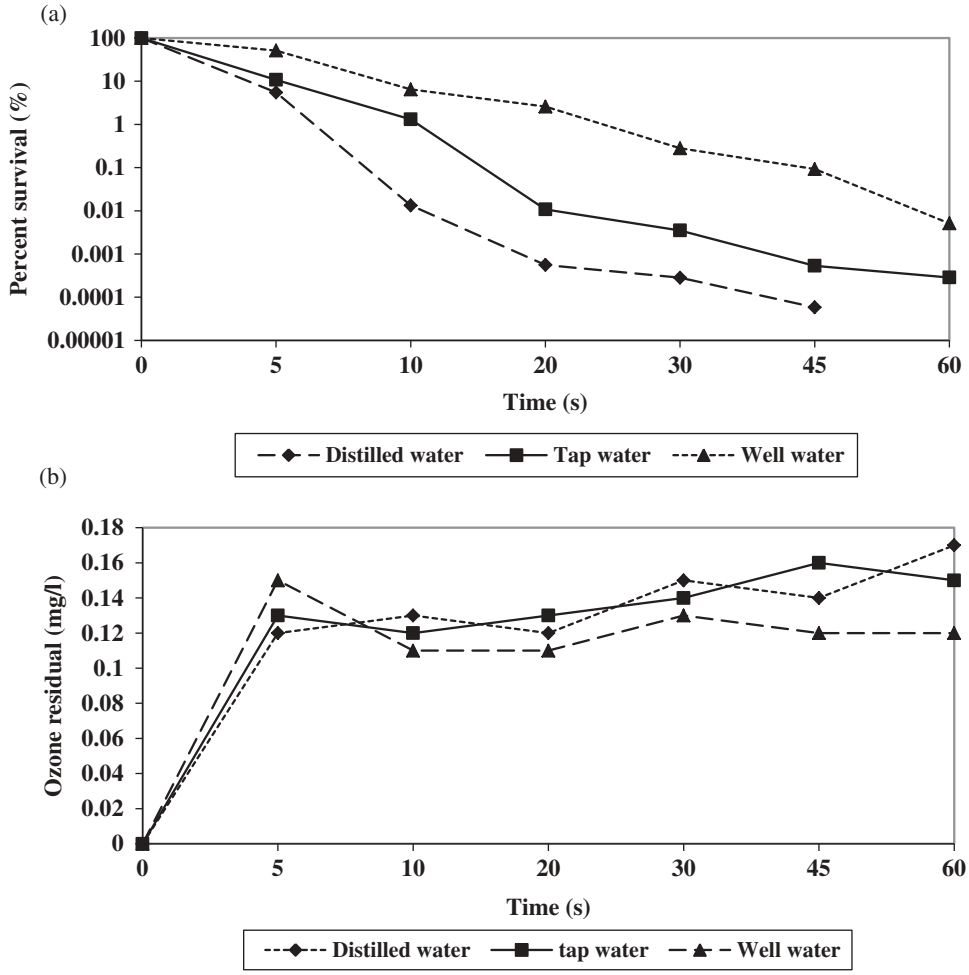

Figure 1. (a) Comparison of percent survival of *E. coli* in different water samples at applied ozone concentration of 2 mg/L in gas phase. (b) Comparison of ozone residual concentration with time at applied ozone concentration of 2 mg/L in various water samples.

counts (1.6×10^3) and 4 log removals was revealed just after contact time of 10 seconds in distilled water, which led to further decrease in the subsequent time intervals. So, after 20, 30, 45, and 60 sec, the *E. coli* counts were, 6.7×10^1, 3.4×10^1, 7, and 0 CFU/mL, respectively. Further, in the case of the tap water sample, *E. coli* counts reduced to 2.20×10^2 after the same contact time with ozone. Data up to 60 sec of disinfection is shown in figures due to the major inactivation of microbes during the initial period of ozonation.

Chemical analyses of the initial tap water and well water are given in Table 1. The samples from tap water and well water were given the similar ozone treatment to observe the effects of the water parameters and quality on disinfection process of ozone. With an applied ozone concentration of 2 mg/L in the gas phase in a well water sample, *E. coli* counts of 1.64×10^6 and 1.04×10^6 CFU/mL were observed after 5 and 10 sec of ozonation. A similar trend was shown by Macauley, Qiang, and Adams (2006), who reported that at high ozone concentration bacterial inactivation efficiency may be increased due to more ozone molecules contacting with microbes to carry out the inactivation process. The bacterial counts were 1.36×10^4, 1.72×10^3, 2.90×10^2, and 2.10×10^1 after contact time of 20, 30, 45, and 60 sec, respectively in this study. The water quality parameters were found to affect the disinfection efficiency of ozone in this case.

It was reported by few studies that the organic matter, turbidity, hardness, and TOC present in drinking water exerts a high ozone demand as compared to distilled water as a medium (Cho, Chung, and Yoon 2003; Filho 2010). This would, in turn, result in lower ozone residual for a given ozone concentration and, consequently lead to poor inactivation. The applied ozone concentration of 2 to 3 mg/L and contact time of 5 to 10 min are required for the disinfection of good quality surface water, while this demand is 3 to 4 mg/L of ozone concentration with same contact time in the case of poor quality surface water (Farooq, Chian, and Engelbrecht 1977). The present results depict the ozone disinfection process of various water samples collected from surface water and groundwater against the native microbes isolated from the same environment. The results may be helpful to optimize the disinfection in the ambient environment of the study area with respect to specific indicator microbes of public health importance.

Table 1. Chemical analysis of initial tap and well water samples.

S. No	Parameters	Tap water	Well water
1	pH	6.71	7.17
2	Temperature	18 °C	17.6 °C
3	Total Dissolved Solids	198 mg/L	688 mg/L
4	Conductivity	412 µS/cm	1387 µS/cm
5	Turbidity	0.83 NTU	0.62 NTU
6	Chemical Oxygen Demand	00	00
7	Alkalinity	55 mg/L ($CaCO_3$)	175 mg/L ($CaCO_3$)
8	Hardness	212 mg/L	500 mg/L

Figure (1b) shows a comparison of ozone residual concentration in different water samples when 2 mg/L ozone concentration in gas phase was applied for inactivation of *E. coli*. The data show a trend of high ozone residual concentration in distilled water compared to tap and well water samples. The low ozone residual in the well water sample is primarily due to its increased ozone demand for carrying out disinfection compared to distilled water. The well water sample has a high alkalinity value compared to tap water. Alkalinity may inhibit decomposition of ozone as carbonate and bicarbonate radical react with •OH will result carbonate radical that will not react with ozone (von Gunten 2003). This also depends upon the chemical nature of water samples being used as disinfection media. As the presence of acidity, alkalinity, hardness and other water qualities results into high ozone demand to carry out the disinfection process.

Disinfection studies of E. coli *at different pH values*

Figure (2a) shows the survival *of E. coli* at pHs of 6, 7 and 8 when the applied ozone concentration was 1.7 mg/L in gas phase and temperature was maintained at 25 °C in distilled water. When the ozonation of *E. coli* culture inoculated in distilled water was carried out at the pH of 7 and temperature of 25 °C, the bacterial count reduced from an initial count of 5.50×10^6 to 1.36×10^5 and 6.90×10^4 CFU/mL, showing one log removal after 5 and 10 sec of ozonation time. Similarly after 20, 30, 45 and 60 sec, the *E. coli* counts reduced to 4.5×10^3, 2.0×10^3, 1.34×10^2 and 5.80×10^1 CFU/mL revealed 2-log, 3-log and 4-log removal of *E. coli* counts, respectively. The applied ozone concentration of 1.7 mg/L resulted in ozone residual concentration ranged between 0.1 to 0.11 during the contact time of 5 and 60 sec. Although 2.0 mg/L ozone concentration in gas phase in distilled water resulted in ozone concentration of 0.15 and 0.12 mg/L. According to a study, there has to be a surplus of residual ozone in the solution to assure that every microorganism has been contacted (Rosen, Lowther, and Clark 1974).

Disinfection study of *E. coli*, with the same initial count of 5.50×10^5 at pH of 8, resulted in reduction in *E. coli* counts of 3.4×10^5 and 2×10^5 after 5 and 10 sec, respectively. Similarly 2 log and 1 log removal of *E. coli* was observed after 45 and 60 sec of contact time, respectively. Variation in ozone residual concentration is given in Figure (2b). It is evident from the results that the survival of *E. coli* was high at pH value of 8, than survival of *E. coli* observed at pHs of 7 and 6 as after ozonation of

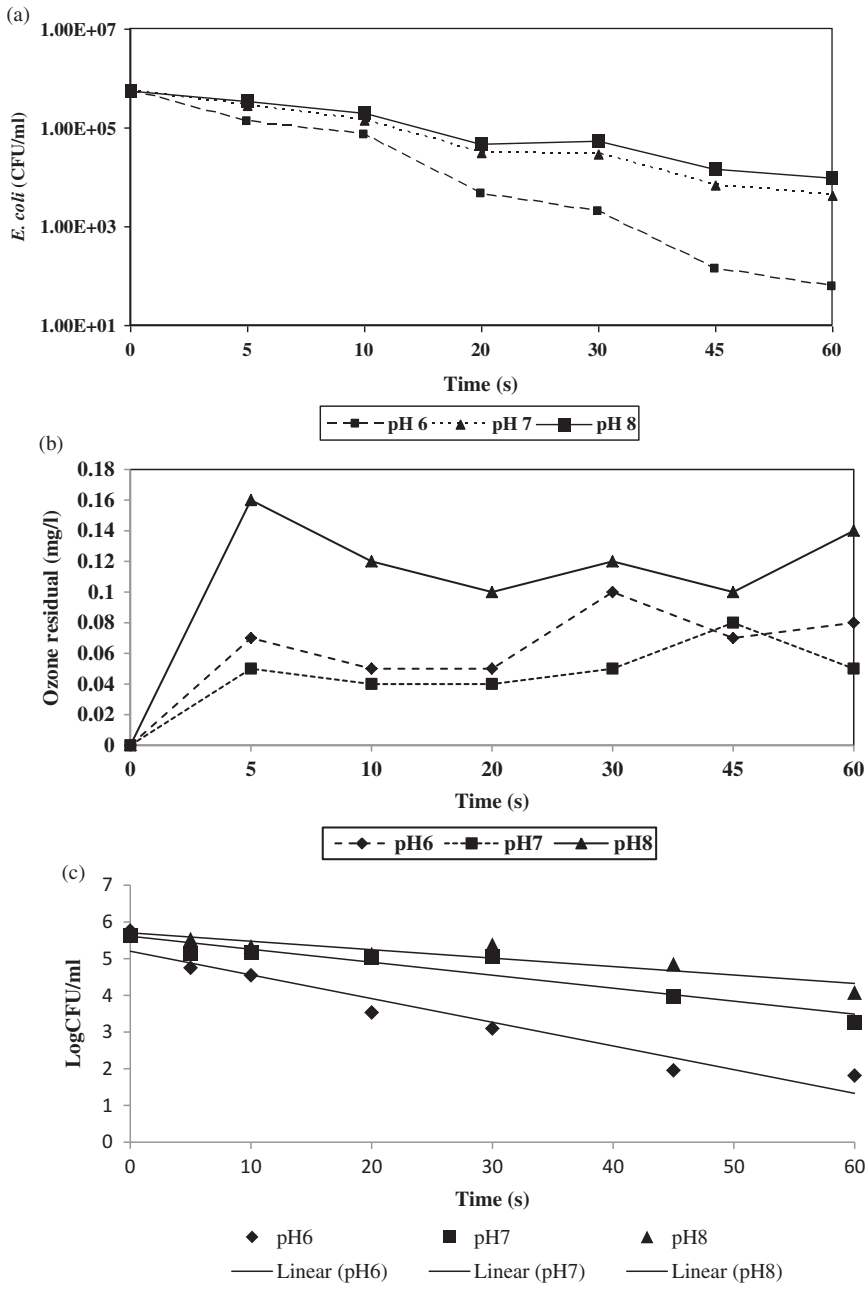

Figure 2. (a) Survival of *E. coli* at various pH and 25 °C with applied ozone concentration of 1.7 mg/L in gas phase in distilled water. (b) Variation in ozone residual concentration with time at various pH with applied ozone concentration of 1.7 mg/L in gas phase in distilled water. (c) Effect of various pH on *E. coli* inactivation rate; plot of log CFU/ml vs. time.

60 sec, the *E. coli* counts were 5.80×10^1 at pH of 6, while it was 4.21×10^3 and 9.50×10^3 at pH of 7 and 8, respectively. The results of this study are according to the findings that the inactivation of microorganisms is mostly through reaction with molecular ozone when the pH is low as ozone decomposes at high pH values, and the resulting radicals contribute to its efficacy (Khadre, Yousef, and Kim (2001). The kinetics of inactivation of microbes were found faster at low pH than in basic medium, as depicted by rate constant K′ values at pHs 6, 7 and 8 were calculated as 0.06, 0.035 and 0.02/s, respectively, in our experiments (Table 2). Figure (2c) shows the plot of logCFU/mL vs. time, which yields a fairly straight line showing the order of microbial inactivation as one line, and the rate constants indicated the effect of different pH values on the disinfection process.

Table 2. Effect of pH and temperature on disinfection kinetics of ozone.

pH	K′/s	Temperature °C	K′/s
6	0.064	15	0.024
7	0.035	25	0.047
8	0.023	35	0.058

Disinfection studies of E. coli *at different temperatures*

Figure (3a) shows the survival trend of *E. coli* at an applied ozone concentration of 1.7 mg/L when the disinfection was carried out at three different temperature conditions i.e., 15, 25 and 35 °C. At 15 °C, with an initial count of 1.32×10^9, only 2-log and 3-log removal was observed after 30 sec and 60 sec of contact time. Similarly, at 25 °C, 2- and 3-log removal of *E. coli* was observed after the contact time of 5 and 10 sec, respectively. However, the contact times of 45 and 60 sec resulted in 4-log removal of *E. coli* under the same temperature and pH conditions. These results are in accordance with previously reported findings of Hunt and Marinas (1997), as they conducted experiments at temperatures of 5, 10, 15 and 20 °C and reported that ozone disinfection is dependent on temperature.

Disinfection experiments of the same *E. coli* counts at 35 °C showed a great reduction in *E. coli* survival with time.

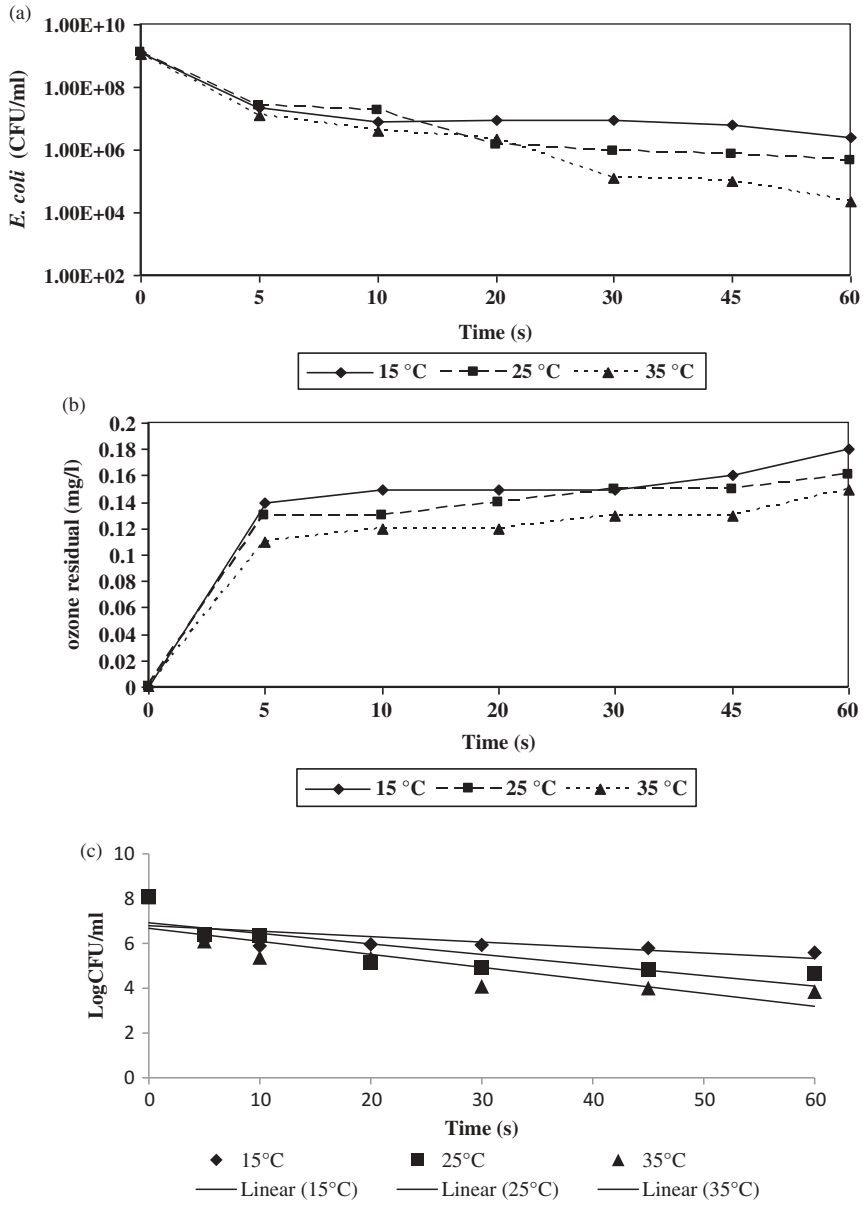

Figure 3. (a) Survival of *E. coli* at different temperatures and pH 7 with applied ozone concentration of 1.7 mg/L in gas phase in distilled water. (b) Variation in ozone residual concentration with time at different temperatures and pH 7 with applied ozone concentration of 1.7 mg/L in gas phase in distilled water. (c) Effect of various temperatures on *E. coli* inactivation rate; plot of log CFU/ml vs. time.

As the counts were 1.27×10^7 and 4.20×10^6 CFU/mL thus showing 2- and 3-log removal of *E. coli* under the same conditions, although 4- and 5-log removal of *E. coli* was observed after 45 and 60 sec, respectively. So, at this temperature, less survival and more *E. coli* inactivation were observed. The experiments to investigate the effect of temperature on the disinfection rates were conducted at 8 °C and 25 °C using *E. coli*, 10^8 CFU/mL, flow rate of 2 L/min and ozone, 0.906 mg/L (Zuma, Lin, and Jonnalagadda 2009). Temperature was observed to have a marginal effect on the rate of disinfection of the test microorganism, *E. coli*. Ozone residual concentration ranged between 0.14 and 0.18 mg/L at 5 and 60 sec of contact time at 15 °C. At 25 °C, the ozone residual was 0.13 and 0.16 mg/L after 5 and 60 sec, respectively. In the temperature case of 35 °C, the ozone residual was 0.11 mg/L after 5 sec, 0.12 and 0.13 mg/L after 10 and 30 sec, respectively. After 60 sec the ozone residual was found to be 0.15 mg/L with an applied ozone concentration of 1.7 mg/L ozone concentration in gas phase as shown in Figure (3b).

Table 2 shows the K′ values at temperatures of 15, 25 and 35 °C with applied ozone concentration of 1.7 mg/L in distilled water. It showed marginal effects on the disinfection rate of the test microorganisms as K′ values were found to be 0.02, 0.04 and 0.05 s^{-1}, respectively (Figure 3c). It may be due to very little variation in temperature range applied in this study. Previous studies have reported that although increasing the temperature can significantly reduce the solubility of ozone and increase the decomposition rate, temperature change has no significant effect on the inactivation of bacteria (Zuma, Lin, and Jonnalagadda 2009).

Disinfection studies of Salmonella

Survival of *Salmonella* at various applied concentrations of ozone in gas phase is shown in Figure 4(a). Initial count of 2.86×10^7 CFU/mL was inoculated in distilled water at pH 7 and 25 °C. The reduction in *Salmonella* counts after 5 and 10 sec of ozonation was 1.36×10^6 and 1.04×10^6 CFU/mL, respectively, showing 1-log removal when the applied ozone concentration was 2 mg/L in gas phase in distilled water. Similarly, after 20 and 30 sec of contact time, 3- and 4-log removal of *Salmonella* counts were observed. After contact times of 45 and 60 sec, the counts were 2.90×10^2 and 1.10×10^1 CFU/mL, respectively showing 5- and 6-log removal in the *Salmonella* counts. A study showed that treatments with ozone (1.6 and

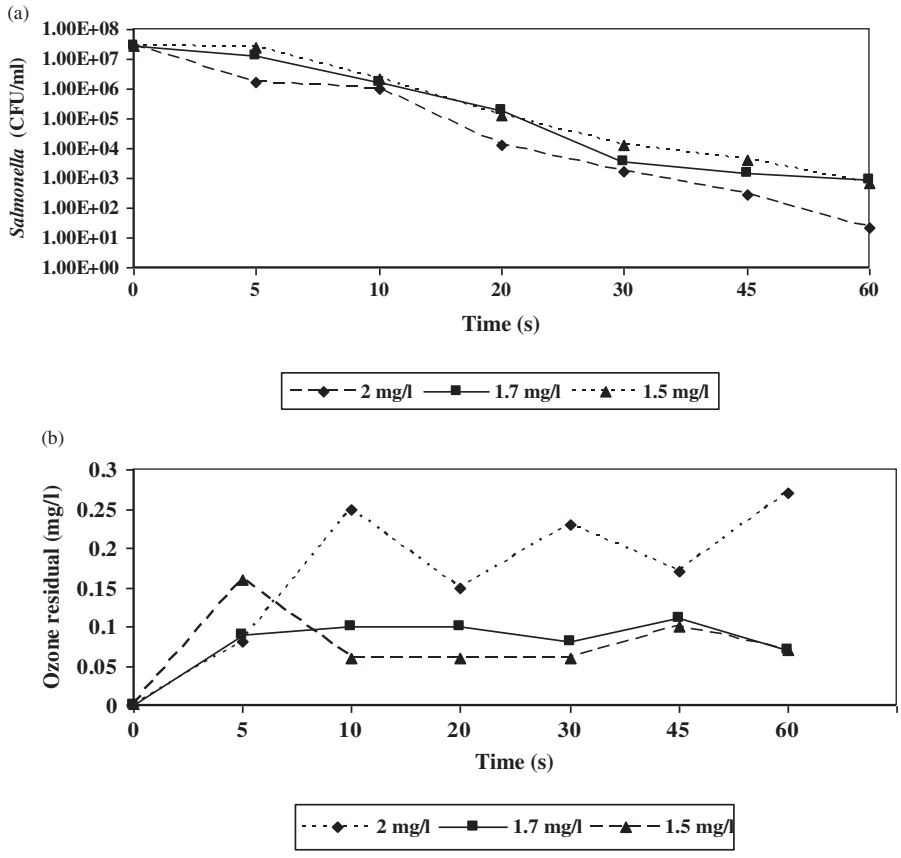

Figure 4. (a) Survival of *Salmonella* at various applied ozone concentrations in gas phase at pH 7 and 25 °C in distilled water. (b) Variation in ozone residual concentration with time at various applied ozone concentrations in gas phase at pH 7 and 25 °C in distilled water.

2.2 mg/L) for 60 sec decreased the *S. sonnei* population in water by 3.7 and 5.6 log CFU/mL, respectively (Selma et al. 2007). When the applied ozone concentration was 1.7 mg/L in the gas phase in distilled water, *Salmonella* counts reduced to 1.27×10^7 and 1.60×10^6 CFU/mL after contact time of 5 and 10 sec. The applied ozone concentration of 1.5 mg/L in gas phase resulted in 2-log removal after contact time of 20 sec. Although the contact time of 45 and 60 sec at this applied ozone concentration resulted in 4.30×10^3 and 7.20×10^2 CFU/mL showing 4- and 5-log removal of *salmonella*. The variation in ozone residual concentration is shown in Figure 4(b).

Disinfection studies of mixed culture

When mixed culture of *E. coli* and *Salmonella* were inoculated in distilled water and disinfected with ozone, a reduction in the counts of mixed culture was observed. The initial count of *E. coli* was 10^8 in the mixed culture, which reduced to 1.13×10^7 and 3.2×10^6 CFU/mL just after the contact time of 5 and 10 sec at an applied ozone concentration of 1.5 mg/L in gas phase. Although the contact time of 45 and 60 sec reduced the *E. coli* counts to 3×10^5 and 2.9×10^4, respectively. *Salmonella* counts reduced to 3.2×10^6 and 3×10^5 CFU/mL after 10 and 20 sec of contact time. Four-log and 5-log removal was observed after 45 and 60 sec of contact time and 6-log removal was obtained after 3 min of ozonation. (Thanomsub et al. 2002) reported that after ozone exposure, the number of bacteria in cultures at 10^3, 10^4 and 10^5 CFU/mL decreased in a time-dependent manner and bacterial growth was no longer detectable when they ozonated a mixed culture of *E. coli, Salmonella* and *Staphylococcus aureus* at 28 °C.

The applied ozone concentration of 1.7 mg/L in gas phase resulted in 2-log removal of *E. coli* in mixed culture after 10 sec of ozonation. After 45 and 60 sec of contact time, 3- and 4-log removal was observed. Although 6-log removal was observed in contact time of 3 min in mixed culture. The same initial count of *E. coli* when subjected to 2 mg/L ozone concentration, 2-log and 3-log removal of *E. coli* was observed after contact time of 5 and 10 sec, respectively. Two-log and 4-log removal of *Salmonella* was observed after 10 and 20 sec, respectively, although the removal was 6-log just after contact time of 45 and

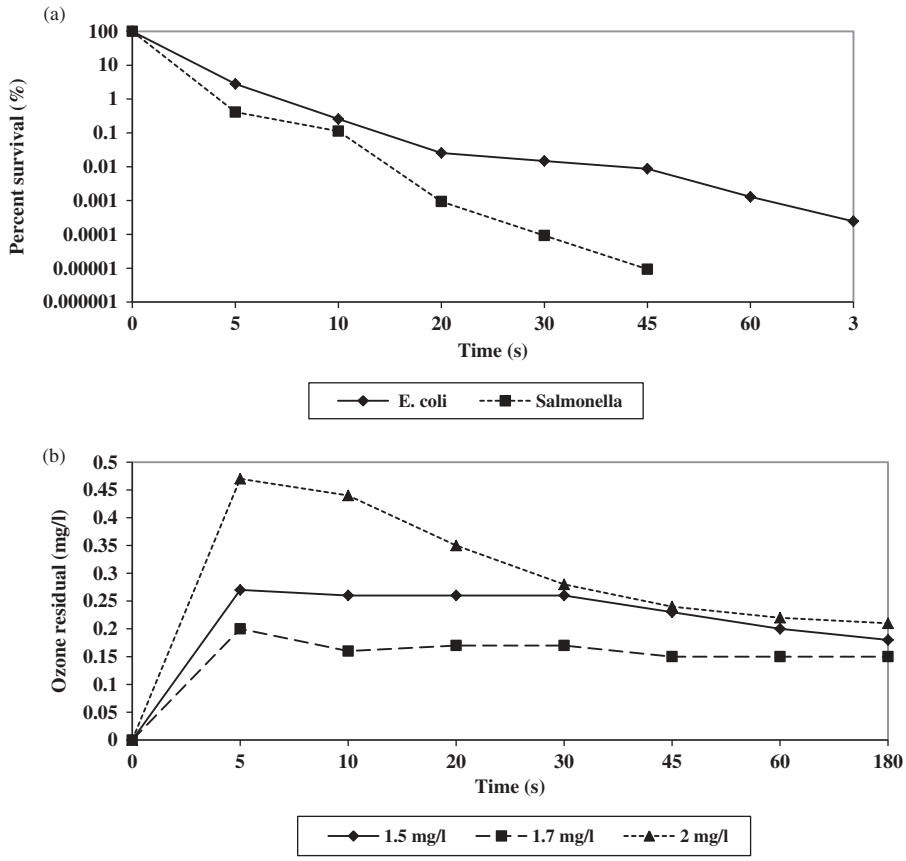

Figure 5. (a) Comparison of percent survival of *E. coli* and *Salmonella* in mixed culture at applied ozone concentration of 2 mg/L in disitilled water. (b) Variation in ozone residual concentration with time at pH 7 and 25 °C in mixed culture and distilled water.

60 sec, respectively. Thus, *E. coli* was found to be more resistant to ozonation as compared to *Salmonella* in the experiments with mixed culture. A comparison of percent survival of *E. coli* and *Salmonella* is shown in Figure 5(a) for applied ozone concentration of 2 mg/L in gas phase. Variation in ozone residual concentration is shown in Figure 5(b). The percent survival for *E. coli* was shown to be high as compared to *Salmonella* for the same applied ozone concentration and contact time in a mixed culture of both organisms. The results are in line with a previous study that *Salmonella* was found to be least resistant among the five different microbes (Farooq and Akhlaque 1983). This justifies that *E. coli* is known to be an indicator micro-organism as it is more resistant to disinfection than known bacterial pathogens for a given dosage and contact time of ozone.

Conclusions

This study depicts the inactivation process of indicator microorganisms of public health importance; *E. coli and Salmonella* in drinking water collected from various sources using ozone treatment. A range of different pH and temperatures were studied and effects of water quality have been demonstrated using different types of water for the disinfection process. The well water sample showed relatively less inactivation of *E. coli* during given contact time and ozone concentrations due to its high ozone demand and water characteristics. Although the disinfection study at various pH values showed comparatively less inactivation of microbes at pH 8 as compared to lower pH values also depicted by inactivation kinetics. At the temperature of 35 °C, relatively more inactivation and marginal effect on inactivation kinetics were observed. During the disinfection studies of mixed culture of *E. coli* and *Salmonella, E. coli* was found to be comparatively more resistant to ozone disinfection among the two microbes tested for their inactivatioin behavior.

Acknowledgments

We are thankful for laboratory support from IESE, NUST in carrying out these experiments.

Funding

The research work was supported by a grant from Higher Education Commission of Pakistan (Project No. 20-874/HEC/R&D/07/379).

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