The synergistic effect of *Escherichia coli* inactivation by sequential disinfection with low level chlorine dioxide followed by free chlorine

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ABSTRACT

To the best of our knowledge, there was little information available on pathogen removal using low level disinfectant followed by free chlorine in sequential disinfection (SD). This study investigated *Escherichia coli* inactivation by four types of disinfection: single step disinfection (SSD), SD, traditional sequential disinfection (TSD) and mixed disinfectant disinfection (MDD). Results indicated that SD had higher ability to inactivate *E. coli* than the others, indicating there was a positive synergistic effect on chlorine disinfection by prior dosing with a low level of chlorine dioxide (ClO_2). The ONPG assay suggested that the permeability of cell wall rather than the viability of *E. coli* were changed under 0.02 mg/l ClO_2 treatment. The coexistence of residual ClO_2 and free chlorine also plays an active synergistic effect. Additionally, temperature had a positive effect on *E. coli* inactivation in SD, while inactivation was reduced in alkaline compared to neutral and acidic conditions.

Key words | chlorine dioxide, *Escherichia coli*, free chlorine, low level prior dosing, sequential disinfection

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INTRODUCTION

Elimination of pathogenic microorganisms (e.g. bacteria, viruses, and protozoa) is of great concern in water treatment systems (White 1992). Free chlorine is the most widely used disinfectant to remove these pathogens (Sawyer *et al.* 1994). However, because of the emergence of hazardous microorganisms as well as formation of chlorinated disinfection by-products, alternative disinfectants such as ozone (Bashtan *et al.* 1999), chlorine dioxide (ClO_2) (Hoehn 1992), and UV irradiation (Chizuko *et al.* 2000) have been proposed. In addition, traditional sequential disinfection (TSD) with alternative disinfectants to get 1 log inactivation followed by free chlorine to produce further inactivation has been proven to be an effective approach to remove these pathogens (Driedger *et al.* 2000; Corona-vasquez *et al.* 2002; Ballester & Malley 2004; Leggett *et al.* 2008; Murphy *et al.* 2008). Sequential treatments are based on the use of a stronger oxidant, such as ozone or ClO_2 as the primary disinfectant, followed by a weaker oxidant, such as free chlorine, as the secondary disinfectant. The synergistic effect in sequential treatments is likely caused by the activity of disinfection agents reacting with specific chemical groups in the bacterial cell wall (Son *et al.* 2005). It appears that the secondary disinfectant is allowed to permeate through partially reacted cell wall layers at a faster rate because of the damage brought about by the primary disinfectant (Corona-vasquez *et al.* 2002). The synergistic effects are beneficial in reducing the disinfectant dose and retention time required for the same level of inactivation and, thereby leading to reduction in both operating costs and formation of disinfection by-products (Cho *et al.* 2006). However, in previous sequential disinfection experiments which we named traditional sequential disinfection, the primary disinfectant

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was used to inactive 90% of the microorganisms (1 log inactivation), while only 10% were inactivated by the secondary disinfectant (Liyanage *et al.* 1997; Driedger *et al.* 2000; Corona-vasquez *et al.* 2002; Cho *et al.* 2006). This procedure does not seem to be promising for practical applications because of possible elimination of the primary disinfectant in the secondary disinfection system.

A low level of disinfectant has been demonstrated to be able to effectively inactivate *Escherichia coli* phage MS2 (Hornstra *et al.* 2010). Son *et al.* (2005) observed a synergistic effect when a small amount of ClO_2 was mixed with free chlorine (concentration ratio 1.8/200) to inactive *Bacillus subtilis* spores. However, to the best of our knowledge, no information was available about pathogen removal using low level disinfectant followed by free chlorine in sequential disinfection (SD). *E. coli* is very sensitive to chemical disinfectants. To obtain 1 log inactivation of *E. coli,* previous studies showed that CT (concentration of disinfectant × time of contact) value was about 0.08 mg/l min for free chlorine and 0.03 mg/l min for ClO_2 respectively (Kouame & Haas 1991; Cho *et al.* 2010). Cho *et al.* (2010) investigated inactivation mechanisms of *E. coli* by several disinfectants based on 1 log inactivation. They found that free chlorine had a negligible effect on cell permeability, while ClO_2 partially damaged both cell surface and inner components. Huang *et al.* (2000) established a relationship between stepwise cellular damage and its impact on the overall process in the photo-catalytic inactivation of *E. coli* by titanium dioxide, and the results indicated that the cell permeability should be changed before intracellular materials reacted. Davis *et al.* (1973) demonstrated that there was no loss in viability of *E. coli* when the cell wall alone was damaged. Moreover, the damaged cell wall could be repaired during subculture onto agar plates for the viability study. Additionally, as a small amount of residual disinfectant is left in the secondary water supply system, there could have an unplanned SD when a disinfectant is dosed to ensure enough disinfection before distributing water to users.

In this paper, SD was adopted and defined as a procedure in which two disinfectants were used and sequentially applied in the same system. The objectives of this study were: (a) to determine whether low level ClO_2 could enhance free chlorine disinfection, (b) to elucidate the synergistic effect of low level ClO_2 with free chlorine on *E. coli* inactivation, and (c) to evaluate the role of pH and temperature in the SD. Four disinfection techniques were investigated: single step disinfection (SSD), SD, TSD, and mixed disinfectant disinfection (MDD).

MATERIALS AND METHODS

Preparation and viability assessment of *E. coli*

E. coli (ATCC 29522) samples were prepared according to the procedure described by Cho *et al.* (2010). In brief, a freeze-dried pellet of *E. coli* was first rehydrated aseptically using tryptic soy broth and grown at 37 °C for 18 h. Bacterial cells were harvested from the broth by performing the following procedure twice: the suspension was centrifuged at 4,000 *g* and 4 °C for 10 min and the pellets were washed with 0.15 M phosphate buffered saline (PBS) (pH 7.1). Then the stock suspension of *E. coli* was prepared by suspending final pellets in 10 ml PBS and stored at 4 °C for usage in 24 h.

E. coli cells were enumerated using a membrane filter procedure (Ayyildiz *et al.* 2009). One or ten milliliter (diluted) samples were filtered directly using sterile 0.45 μm membrane filters (Beihua, China) with a sanitized metal vacuum apparatus. Filters were then incubated in Endo medium (BD CO., USA) at 37 °C for 24 h. *E. coli* was counted using an optical counter. The number of *E. coli* was expressed as colony-forming units (CFU)/ml.

Preparation and analysis of disinfectants

The ClO_2 stock solution (100 mg/l) was prepared by oxidizing sodium chlorite with hydrogen sulfate (Son *et al.* 2005; Chauret *et al.* 2001) and stored at 4 °C in the dark (Hornstra *et al.* 2010). In disinfection experiments, the stock solution was diluted to a working solution of about 1.0 mg/l using deionized water. The concentration of ClO_2 in the working solution was determined by measuring the absorbance at 359 nm in a 1 cm quartz cuvette using a UV 2550 spectrophotometer (Shimazu, Japan). The working solution with ClO_2 concentrations below 0.2 mg/l was obtained by diluting 1 mg/l ClO_2 solution directly.

The free chlorine stock solution (100 mg/l) was produced by oxidizing concentrated hydrochloric acid with manganese

dioxide. In disinfection experiments 0.5 mg/l free chlorine was used, based on the inactivation rate and efficiency; according to Vicuna-Reyes *et al.* (2008), disinfectant concentration has no effects on the disinfection efficiency at certain CT values. Concentration of free chlorine was determined by the DPD (*N*, *N*-diethyl-*p*-phenylelenediamine) method at 515 nm.

ONPG assay for cell wall permeability

As described by Huang *et al.* (2000), the ONPG assay was used to determine whether a small amount of ClO_2 could change the cell wall permeability of *E. coli*. ONPG is a synthetic chromogenic substrate for the intracellular β-D-galactosidase of *E. coli* that can be used as a permeability probe. ONPG is hydrolysized to ONP when it reacts with β-D-galactosidase, which gives rise to a yellow color under alkaline conditions (pH 10.0).

In this study, a procedure described by Huang *et al.* (2000) and Cho *et al.* (2010) was used to enhance the synthesis of β-D-galactosidase. In brief, after being grown in Luria-Bertani broth (LB broth) at 37 °C for 18 h, *E. coli* was further spiked into a flask containing 1 mM isopropyl β-D-thiogalactopyranoside and 100 ml LB broth, and incubated at 32 °C for 4 h in a shaking incubator. After that, the suspension was centrifuged at 4,000 *g* and 4 °C for 10 min and then washed twice with PBS. After dosing with low level ClO_2, 9 ml *E. coli* samples were taken at each time point and mixed with 1 ml of 5 mM ONPG. To measure maximum absorbance at 420 nm, a 1.8 ml sample was then mixed with 0.2 ml of 1 M bicarbonate buffer (pH 10.0) in the cuvette at 4-min intervals for 16 min to stop the reaction between enzyme and substrate. Hydrolysis kinetics were evaluated by recording variation of absorbance at 420 nm with reaction time. The extinction coefficient of ONP was determined at pH 10.0 by measuring its absorbance at 420 nm. The hydrolysis rate of ONPG was expressed as moles of released chromophore per mg cell dry weight per min at 20 °C (Huang *et al.* 2000). The estimated mass of *E. coli* used in the calculation was 6.65×10^{-13} g/cell (Ilic *et al.* 2004; Cho *et al.* 2010).

Disinfection experiments

Disinfection experiments were performed in amber flask reactors. All glassware was soaked in 5 mg/l ClO_2 or free chlorine solution overnight, and then rinsed thoroughly with ultrapure water before use. Other materials in disinfection experiments were autoclaved at 121 °C for 15 min. All experiments were repeated three times.

In SSD experiments, an initial microbial population of 3×10^6 to 1×10^7 CFU/ml was added to 490 ml PBS and stabilized for 20 min, and then a 1 ml sample was withdrawn at the initial point (N_0) to assay the viability. Ten milliliters ClO_2 or Cl_2 stock solution was dosed into the reactor for disinfection. During disinfection, two 1 ml samples were taken at appropriate time points; one was immediately quenched with 0.02 M sodium thiosulfate to assay the viability (N_t) and the other used to determine the residual disinfectant concentration.

In SD experiments, the microbial population was added and stabilized following the same procedure as SSD, then ClO_2 at the concentration based on ONPG hydrolysis experiments was added. After the desired reaction time, a 1 ml sample was taken to assay the viability (N_0). After that, 10 ml Cl_2 stock solution was dosed to obtain a final Cl_2 concentration of 0.5 mg/l. Similarly, two 1 ml samples (N_t) were taken to assay the viability and determine the residual concentration.

Both TSD and MDD experiments were conducted by following a similar procedure to SD. In TSD experiments, sodium thiosulfate was added to quench ClO_2 after the desired reaction time determined by ONPG hydrolysis experiments, and the solution in the reactor was centrifuged at 4,000 *g* and 4 °C for 10 min and resuspended in PBS in the same conditions as used for the ClO_2 disinfection experiment. After that, Cl_2 solution was dosed for SD. In MDD experiments, the mixture of Cl_2 and ClO_2 was dosed.

The different procedures of the four technique mentioned above are summarized in Table 1.

Additionally, the effect of both pH (5.6, 7.1, 8.2) and temperature (4, 20, 35 °C) on the inactivation kinetics of *E. coli* was investigated in SD experiments.

Calculation

The disinfection level was expressed as CT value versus log inactivation, where CT value is defined as disinfectant concentration (C_t, mg/l) multiplied by contact time (t, min) and can be calculated from $CT = \int_0^t C_t dt$, and log inactivation is the logarithm of N_t/N_0. In order to get an

Table 1 | Procedures used in the experimental disinfection techniques

	Steps				
Disinfection technique	**Preparation of *E. coli* in PBS suspension**	**ClO_2 dosing**	**Quenching ClO_2 after a certain reaction time and centrifuging the suspension**	**Re-suspending *E. coli* in PBS solution**	**Free chlorine dosing**
SSD	√	√	×	×	×
SD	√	√	×	×	√
TSD	√	√	√	√	√
MDD	√	√[a]	×	×	√[a]

[a]Chlorine dioxide and chlorine were added as a mixed disinfectant.

accurate CT value, a fitting curve between chlorine concentration and contact time was initially obtained using Origin 8.0 software, and then the CT value was estimated based on this fitting curve using Wolfram Mathematica 7 software.

All data were calculated from triplicate experiments.

RESULTS AND DISCUSSION

Inactivation of *E. coli* with low level ClO_2

Cho *et al.* (2010) investigated the mechanisms of *E. coli* inactivation by several disinfectants, and found ClO_2 caused some surface damage and inner component degradation as inactivation progressed. On the other hand, no loss in *E. coli* viability was detected when only the cell wall was damaged (Davis *et al.* 1973). Based on these findings, the process of *E. coli* inactivation by ClO_2 can be described as a sequence of reactions of ClO_2 with cell wall components, before it reaches the inner cell, followed by increase in cell wall permeability and subsequent ClO_2 penetration into cytoplasm, and ultimately cell death (Arana *et al.* 1999; Cho *et al.* 2011). Thus, there could be a particular level of ClO_2 that just damages the cell wall, and when ClO_2 exceeds this level cell death would occur.

In order to test the procedure, four different concentration of ClO_2 (0.005, 0.01, 0.02, and 0.05 mg/l) were tested in SSD experiments. As shown in Figure 1, the viability of *E. coli* was changed slightly with ClO_2 concentration below 0.02 mg/l, while 3.75 log inactivation was observed at 0.05 mg/l ClO_2 for 30 min treatment. Therefore, it could conclude that ClO_2 at below 0.02 mg/l is only able to damage cell walls, and thus change cell permeability,

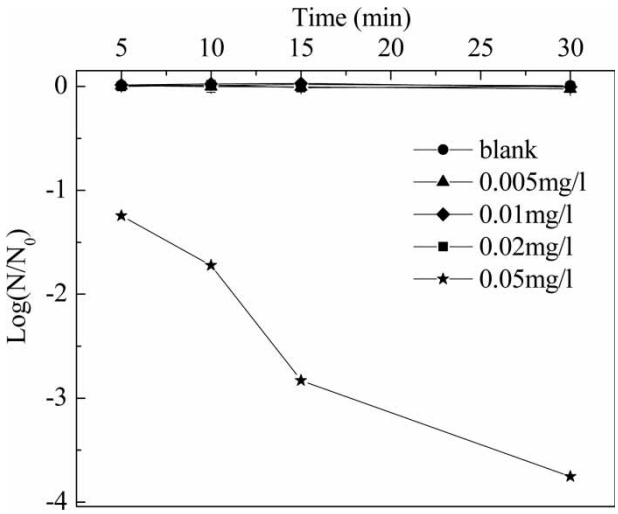

Figure 1 | *E. coli* inactivation at various ClO_2 concentrations (pH 7.1, 20 °C).

while having a negligible effect on inner cell components. However, when its concentration is above 0.05 mg/l, ClO_2 can penetrate into the cell after damaging the cell wall and thus reacts with inner cell components to decrease viability of *E. coli*. Based on these results, 0.02 mg/l ClO_2 solution was used in the following experiments. Coincidentally, Chinese Standards for Drinking Water Quality (GB5749-2006) rules 0.02 mg/l as the limit of residual disinfectant concentration in the endpoint of a supply network with ClO_2 as the disinfectant.

Permeability variation of cell wall by low level ClO_2

As described by Huang *et al.* (2000), the intact *E. coli* cells have very low permeability to ONPG. As shown in Figure 2, the limited accessibility of ONPG to β-D-galactosidase in

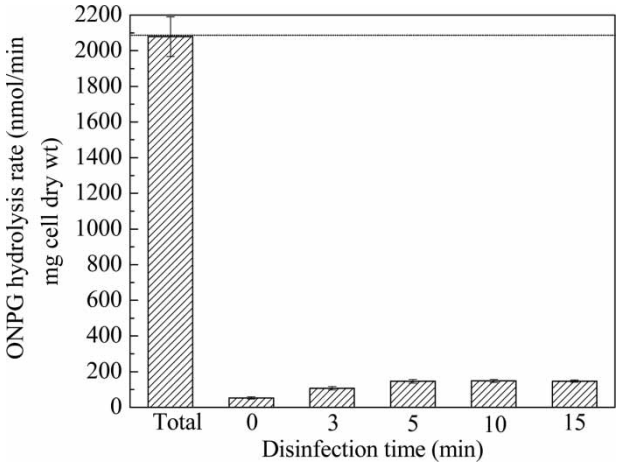

Figure 2 | Variation of ONPG hydrolysis rate with time for *E. coli* (1×10^7 to 3×10^7 CFU/ml) by dosing 0.02 mg/l ClO_2 (pH 7.1, 20 °C).

intact cells was reflected by its very low hydrolysis rate, which was less than 2.6% (52.9 ± 6.5 n mol·(min mg cell dry w)$^{-1}$) of that in lysed cells (2,078.9 ± 111.6 n mol·(min mg cell dry w)$^{-1}$). This is consistent with values obtained in previous studies (less than 2 and 2.3%) (Huang *et al.* 2000; Cho *et al.* 2010). After dosing 0.02 mg/l ClO_2 and reacting 5 min, the hydrolysis rate was increased to 148.0 ± 8.9 n mol·(min mg cell dry w) $^{-1}$, indicating the damage to the cell wall by ClO_2 (Huang *et al.* 2000; Cho *et al.* 2010). However, the ONPG hydrolysis rate kept constant with the further increase of reaction time, suggesting (a) ClO_2 could damage cell wall within a few minutes, and (b) the low level ClO_2 was consumed in 5 min. Therefore, a 5 min interval was chosen in the subsequent SD experiments.

Kinetics of disinfectant decay

The first-order kinetic ($C_t = C_0 \exp^{-kt}$) was found acceptable for predicting chemical disinfectant decay when ClO_2 or O_3 was used (Cho *et al.* 2003; Hornstra *et al.* 2010). However, the power function pow2p2 ($C_t = C_0 \times (1 + t)^{-k}$) rather than the first-order kinetics appeared a better regression in this study. A higher decay rate (k value) was determined using pow2p2 compared to the first-order decay model; this may be because the PBS was prepared without using chlorine-demand-free water, so the PBS could have consumed a certain amount of chlorine that leads to a pseudomorph of a higher decay rate according to DPD determination. This explanation was further supported by

Table 2 | The fitting parameters between C_t and time fitted by Origin 8.0

C_0 (mg/l)	Experimental condition	Data points	k	r^2
0.5	pH 7.1, $T = 4$ °C	9	0.5586	0.9923
	pH 7.1, $T = 20$ °C	9	0.9026	0.9920
	pH 7.1, $T = 35$ °C	9	1.2826	0.9927
	pH 5.6, $T = 20$ °C	9	1.3539	0.9829
	pH 8.2, $T = 20$ °C	9	0.7897	0.9742
0.2	pH 7.1, $T = 20$ °C	7	1.3178	0.9593
1.0	pH 7.1, $T = 20$ °C	8	0.4162	0.9513
1.5	pH 7.1, $T = 20$ °C	8	0.3041	0.9387

the fact that k value was reduced with the increase of initial disinfectant concentration (Table 2). As initial chlorine concentration increased from 0.2 to 1.5 mg/l, k value decreased from 1.3178 to 0.3041. Additionally, higher temperature or lower pH resulted in a higher k value (Table 2).

Dosing 0.02 mg/l ClO_2 in SD, MDD, and TSD experiments can result in coexistence of residual ClO_2 and Cl_2. To determine the impact of low level ClO_2 on the decay of Cl_2, additional experiments were conducted by dosing 0.02 mg/l ClO_2 into PBS solution under certain conditions before dosing with different concentrations of Cl_2. A negligible difference for Cl_2 concentration was found with and without ClO_2 dosage (data not shown) by the DPD method. Furthermore, 0.02 mg/l ClO_2 was beyond the detection limit of the DPD method. Thus, we did not determine ClO_2 concentration nor calculate its CT value. The CT values below were estimated only from Cl_2 concentration.

Disinfection enhancement in SD

As shown in Figure 3, in the first 0.2 mg/l min (30 s) 4.4 log inactivation was obtained in SD but only 2.7 log inactivation in SSD. Moreover, the CT value was only 1.0 mg/l min for SD to achieve a 6.0 log inactivation (by 29.6% reduction) compared to SSD (1.4 mg/l min) indicating that existence of low level ClO_2 did have a synergistic effect on the subsequent chlorine disinfection. This could be explained in the following way: (a) the permeability of the cell wall was changed by a low level of ClO_2, which accelerated the penetration rate of chlorine into cell inside and resulted in a greater inactivation efficiency; (b) there may be a synergistic effect of the oxidant with its intermediates. In order to find

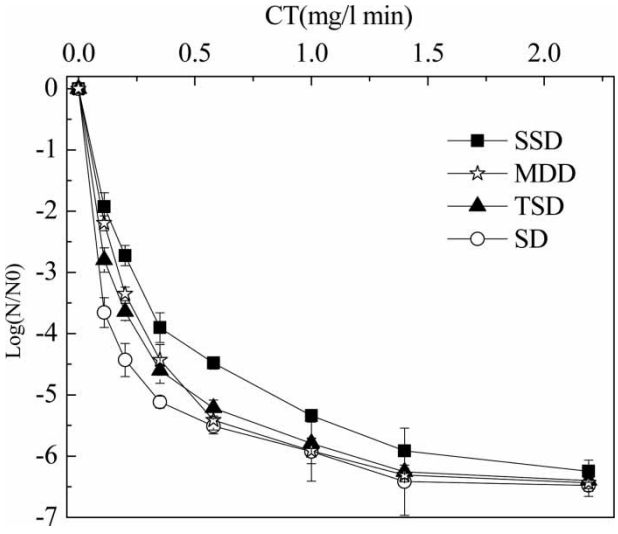

Figure 3 | Comparison of *E. coli* inactivation in four types of disinfection experiments (pH 7.1, 20 °C).

the key reason, both TSD and MDD experiments were conducted, and the results are shown in Figure 3. The inactivation efficiency in both MDD and TSD was higher compared to SSD, while lower than that in SD within 1.0 mg/l min. Within 0.46 mg/l min (~1.6 min), a higher inactivation efficiency was observed in TSD compared to MDD, but their difference gradually reduced with the increase of CT, for example, the difference between them was about 0.6 log at 0.11 mg/l min but almost negligible at 0.46 mg/l min. In CT range of 0.46–1.4 mg/l min, there was a higher inactivation efficiency in MDD compared to TSD. Nevertheless, no difference of disinfection efficiency was observed among MDD, TSD, and SD when CT was higher than 1.4 mg/l min.

Additionally, the above results give us a hint that the existence of low level residual disinfectant may play an active role in the disinfection system, e.g. by shortening disinfection time or reducing disinfectant dose. Therefore, dosage of extra disinfectant before traditional disinfection could potentially be useful in practical operations.

Possible synergism mechanism

As previously mentioned, when only the cell wall was damaged, no loss in viability of *E. coli* was detected. However, this is remarkably different with other types of pathogens encountered in water treatment, such as *Cryptosporidium parvum* and viruses, since damage to the oocyst wall and virus coat may result in their inability to reproduce. In the case of *E. coli*, chemical disinfectants may undergo reactive transport through cell wall and damage inner cell components such as β-D-galactosidase (Huang *et al.* 2000). The enhanced inactivation efficiency in both SD and TSD compared to SSD demonstrated that there was a synergistic function of low level ClO_2 for free chlorine disinfection by changing cell wall permeability and facilitating subsequent transport of free chlorine.

On the other hand, despite no loss in viability of *E. coli* being detected under 0.02 mg/l ClO_2 treatment, the inactivation efficiency in SD was found to be higher than that in TSD, implying the synergistic effect of residual ClO_2 and/or its formed intermediates on the disinfection of free chlorine. Son *et al.* (2005) investigated the variation of UV absorbance with time for the mixture of Cl_2 and ClO_2. Results indicated that ClO_2 was stable in concentrated chlorine solution under acidic conditions. Therefore, the enhanced disinfection efficiency may be only due to the synergistic effect of ClO_2 itself with free chlorine.

In summary, the mechanism of synergy for low level ClO_2 on *E. coli* inactivation by chlorine can be described as a sequential procedure. First, the low level ClO_2 reacts with *E. coli* cell wall components and result in cell wall permeability increase. Second, the increased cell wall permeability accelerate chlorine and residual ClO_2 penetration into cytoplasm, and then disinfectant reacts with cytoplasm and cause cell death. Additionally, coexistence of residual ClO_2 and free chlorine also plays an active synergistic role.

The mechanism of synergy gives a clear explanation of why the inactivation efficiency in both TSD and SD was higher than that in SSD. On the other hand, the higher inactivation in SD compared to TSD can be attributed to the synergistic effect of coexisting residual ClO_2 and free chlorine. Although both the cell wall permeability increase and coexistence of disinfectant occur in MDD, a lower efficiency in MDD was observed in the first 0.46 mg/l min (~1.6 min) than in TSD, which may be due to the length of time required to change the permeability by ClO_2. With prolonged reaction time, the difference of inactivation efficiency was reduced between MDD and TSD, and finally

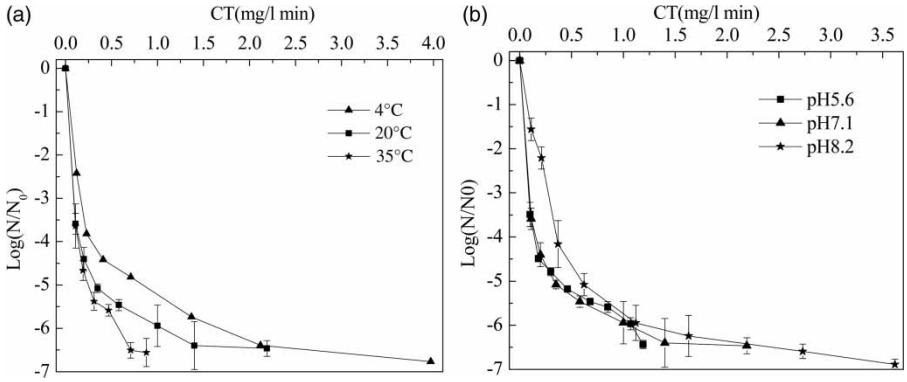

Figure 4 | Effect of (a) temperature (pH 7.1) and (b) pH (20 °C) on *E. coli* inactivation in SD.

led to a higher inactivation efficiency in MDD after 0.46 mg/l min.

Role of pH and temperature in SD

Both pH and temperature are key factors for pathogen inactivation by free chlorine. As shown in Figure 4(a), temperature had a positive effect on *E. coli* inactivation in SD, which is similar to those with traditional chemical disinfectants (USEPA 1999). A significant reduction of inactivation efficiency was observed when pH was increased from 7.1 to 8.2, but a negligible difference between neutral and acidic conditions (Figure 4(b)).

CONCLUSIONS

This study demonstrated that inactivation of *E. coli* could be enhanced by prior dosing with low level of ClO_2 in free chlorine disinfection, due to permeability variation of the cell wall caused by ClO_2 to facilitate free chlorine transport and the synergistic effect of coexisting residual ClO_2 and free chlorine. Additionally, the inactivation ability increased with the increase of temperature in the SD, while lower disinfection efficiency was found in alkaline conditions than in acid and neutral conditions. This study also implied that dosage of low level disinfectant before traditional disinfection could potentially be a useful strategy in practical disinfection operations.

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