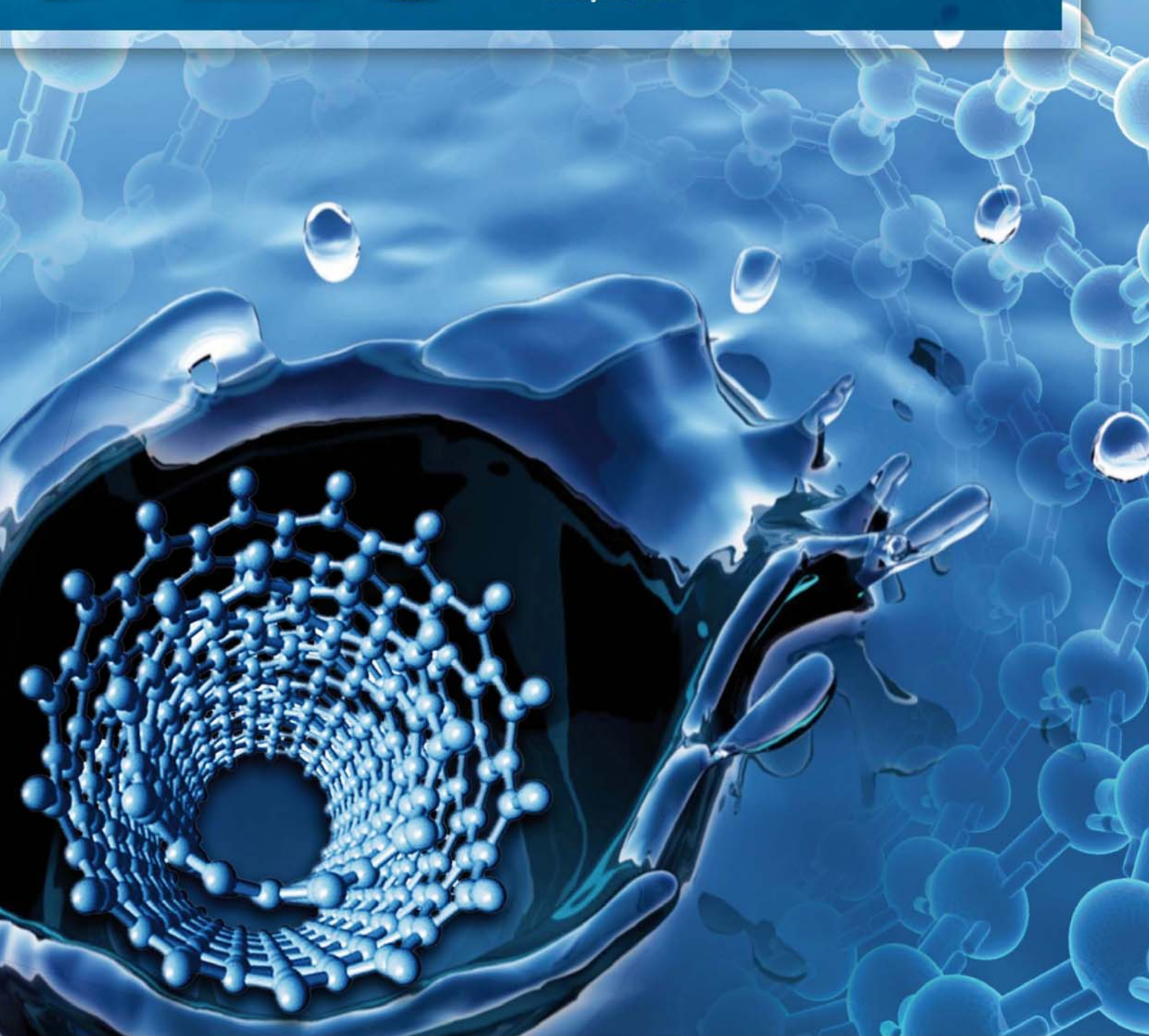


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## Inactivation, reactivation and regrowth of indigenous bacteria in reclaimed water after chlorine disinfection of a municipal wastewater treatment plant

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### Abstract

Disinfection of reclaimed water prior to reuse is important to prevent the transmission of pathogens. Chlorine is a widely utilized disinfectant and as such is a leading contender for disinfection of reclaimed water. To understand the risks of chlorination resulting from the potential selection of pathogenic bacteria, the inactivation, reactivation and regrowth rates of indigenous bacteria were investigated in reclaimed water after chlorine disinfection. Inactivation of total coliforms, *Enterococcus* and *Salmonella* showed linear correlations, with constants of 0.1384, 0.1624 and 0.057 L/(mg·min) and  $R^2$  of 0.7617, 0.8316 and 0.845, respectively. However, inactivation of total viable cells by measurement of metabolic activity typically showed a linear correlation at lower chlorine dose (0–22 (mg·min)/L), and a trailing region with chlorine dose increasing from 22 to 69 (mg·min)/L. Reactivation and regrowth of bacteria were most likely to occur after exposure to lower chlorine doses, and extents of reactivation decreased gradually with increasing chlorine dose. In contrast to total coliforms and *Enterococcus*, *Salmonella* had a high level of regrowth and reactivation, and still had 2% regrowth even after chlorination of 69 (mg·min)/L and 24 hr storage. The bacterial compositions were also significantly altered by chlorination and storage of reclaimed water, and the ratio of *Salmonella* was significantly increased from 0.001% to 0.045% after chlorination of 69 (mg·min)/L and 24 hr storage. These trends indicated that chlorination contributes to the selection of chlorine-resistant pathogenic bacteria, and regrowth of pathogenic bacteria after chlorination in reclaimed water with a long retention time could threaten public health security during wastewater reuse.

**Key words:** inactivation; regrowth; reactivation; indigenous bacteria; reclaimed water

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### Introduction

Available water resources in cities are becoming increasingly scarce because of increasing population, changing precipitation patterns, and/or degradation of existing sources of water (US EPA, 2004; WHO, 2006). Treated wastewater has been used to alleviate some of these water shortages (Hoffbuhr et al., 2007). As wastewater contains a range of pathogens, one of the important goals is the inactivation of pathogens during treatments to minimize the public health risks associated with reclaimed water (Costán-Longares et al., 2008). Disinfection is an essential treatment that safeguards consumers against pathogenic microorganisms in reclaimed water, and chlorine-based disinfectants are widely utilized disinfectants for drinking

water and reclaimed water worldwide (US EPA, 2004).

Reclaimed water contains a high level of organic matter, which potentially reacts with the disinfectants but also provides nutrition for microorganisms (Weinrich et al., 2009; Jjemba et al., 2010). Additionally, a number of pathogenic bacteria have been shown to enter a viable but nonculturable (VBNC) state during wastewater treatments, such as *E. coli*, *Salmonella* sp., *Enterococcus* sp., *Shigella dysenteriae*, *Vibrio cholerae*, and *Helicobacter pylori* (Reissbrodt et al., 2002; Adams et al., 2003; Gupte et al., 2003). The VBNC bacteria can be alive and show very low levels of metabolic activity, and can become reactive in certain conditions (Oliver, 2005). Consequently, the bacteria in reclaimed water have the potential to reactivate and grow during storage and distribution before consumption (Jjemba et al., 2010).

Nowadays, the microbial quality of reclaimed water is regulated by guidelines and specific regulations based

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on determining levels of coliform bacteria, *E. coli* and *Enterococcus* (US EPA, 2004; WHO, 2006). However, the removal of coliform bacteria does not adequately reflect the removal of pathogenic bacteria achieved in removal and disinfection procedures, and most of these indicator bacteria are nonpathogenic. In addition, their presence does not clearly correlate with the presence of actual opportunistic pathogens, such as *Salmonella* and *Mycobacterium* (Harwood et al., 2005; Narasimhan, 2007). *Salmonella* exist widely in water environments and can cause fever and diarrhea. A study indicated that 80% of wastewater samples used as an irrigation source tested positive for *Salmonella* and the presence of *Salmonella* in reclaimed water can pose a threat to public health during exposures (Nutt et al., 2003). To address concerns about the risk of reclaimed water, these pathogens, in addition to the indicator bacteria, should be studied.

Previous studies on disinfection efficacy have used predominantly pure cultures of indicators or pathogens (Cho et al., 2010). However, bacteria cultivated under realistic low nutrient conditions exhibit considerably diverse physiology and different susceptibilities towards disinfectants compared to cells cultivated in a rich medium under laboratory conditions (Berney et al., 2006). The treated wastewater has a complicated indigenous microbial community, and the composition and physiological state of the bacterial community can influence the efficiency of bacterial disinfection (Luh and Mariñas, 2007; Ramseier et al., 2011). Even though disinfection of reclaimed water targets the entire indigenous bacterial community in the water, such communities are not commonly used in disinfection studies. It is still not clear whether regrowth or reactivation of total viable cells is more common than that of indicator and pathogenic bacteria in waters.

The use of the tetrazolium salts, such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 2-(4-iodophenyl)-3-(4-nitro-phenyl)-5-phenyl tetrazolium chloride (INT), for evaluating the metabolic activity of aerobic, facultative, and anaerobic bacteria has gained wide application (Blenkinsopp and Lock, 1990). The CTC and INT are colorless, membrane-permeable compounds that produce a red-fluorescing precipitate in the cell when it is reduced by the electron transport system of a bacterial cell (Blenkinsopp and Lock, 1990). This approach for estimating active bacterial cells has been used widely and is comparable in simplicity and reliability to any epifluorescence technique commonly used for the enumeration of total bacterial density (Dufour and Colon, 1992). Fluorescent staining in combination with flow cytometry (FCM) is a rapid, cultivation-independent approach for analysis of bacteria in water on the single-cell level (Berney et al., 2008; Hammes et al., 2008). FCM does not provide information on the type of species that are measured (e.g., *E. coli*, *Legionella*, *Vibrio*, non-pathogenic unknown bacteria) unless species-specific labeling is applied (Hammes and Egli,

2010). However, it can provide information on the physiological state of the entire community that is analyzed. The CTC staining combined with FCM was applied to detect and qualify viable cells in water samples in the present study.

The objective of this study was to investigate the effect of chlorine disinfection on indigenous bacteria in the reclaimed water of a wastewater treatment plant (WWTP). The inactivation, reactivation and regrowth rates of indicators (total coliforms and *Enterococci*), pathogenic bacteria (*Salmonella*), and total viable cells were evaluated and compared in reclaimed water after a series of doses of chlorine disinfection. The proportions of indicator and pathogenic bacteria in the microbial communities were also analyzed to indicate survival and resistant bacteria in reclaimed water after disinfection.

## 1 Materials and methods

### 1.1 Water samples

The reclaimed water samples were collected from effluents of a wastewater treatment plant in Beijing, China. The source water was the secondary-treated municipal wastewater, and the treatment processes included coagulation sedimentation and sand filtration. The reclaimed effluent was used for irrigation and car washing inside of the plant. All the samples were aseptically collected in sterile containers and transported to the lab on ice for immediate processing. The water parameters of the samples are as follows: temperature 15°C–18°C, pH 6.8, TOC 8–10 mg/L, total coliforms  $3.23 \pm 0.18 \log_{10}$ CFU/100 mL, *Enterococci*  $1.97 \pm 1.08 \log_{10}$ CFU/100 mL, *Salmonella*  $3.16 \pm 0.51 \log_{10}$ CFU/100 mL, total viable cells detected by CTC-FCM  $6.71 \pm 0.49 \log_{10}$ cells/100 mL.

### 1.2 Chlorine disinfection of reclaimed water samples

The chlorine disinfection of reclaimed water samples was carried out in 1000-mL glass beakers at room temperature (20°C). The samples were exposed to sodium hypochlorite to establish different initial concentrations of chlorine (0.2–3.0 mg Cl<sub>2</sub>/L) following the *N,N*-diethyl-*p*-phenylenediamine method. After 30 min exposure, the chlorine disinfection was terminated by addition of a sodium thiosulfate solution (2%). The initial and residual chlorine concentrations after 30 min exposing are shown in **Table 1**. The final exposure time of 30 min was chosen according to the standards for reclaimed water quality of China (SL368-2006).

The dosage of chlorination was calculated using Eq. (1).

$$\text{Chlorine dose} = \int_0^t C_t \times t \quad (1)$$

where,  $C_t$  (mg/L) is the residual chlorine concentration at exposure time  $t$  (min).

**Table 1** Initial and residual chlorine concentrations after 30 min exposure in reclaimed water

Initial chlorine (mg Cl <sub>2</sub> /L)	Residual chlorine (mg Cl <sub>2</sub> /L)
0.25	0.07
0.53	0.09
0.75	0.14
1.20	0.26
1.45	0.34
2.04	0.92
3.05	1.62

To evaluate chlorination effects on reclaimed water, the inactivation efficiency of cells was quantified by Eq. (2).

$$\text{Inactivation of cells} = \log_{10} \frac{N_{CT}}{N_0} \quad (2)$$

where, the cells included total coliforms, *Enterococci*, *Salmonella*, and total viable cells detected by CTC staining in reclaimed water.  $N_0$  (CFU/L or cells/L) and  $N_{CT}$  (CFU/L or cells/L) are the concentrations of the cells before and after chlorine disinfection, respectively.

### 1.3 Regrowth and reactivation experiments

The regrowth experiments were carried out in the dark to simulate storage conditions of reclaimed water in a tank before use by the customers. The raw and chlorinated reclaimed water samples after neutralization by sodium thiosulfate were retained in beakers at room temperature (20°C) for 24 hr. Then total coliforms, *Enterococcus*, *Salmonella* and total viable cells were detected in the retained reclaimed waters.

### 1.4 Bacteria detection methods

Concentrations of total coliforms, *Enterococcus* and *Salmonella* were determined using the membrane filtration method (with a 0.45- $\mu$ m-pore-size cellulose nitrate filter) and 0.1, 1, 10, and/or 100-mL aliquots. Total coliform contents were determined using m-Endo LES Agar (BR, Beijing, China) incubated at 37°C for 24 hr, and presumptive positive results were confirmed based on formation of glittering with gold colonies. *Enterococcus* was detected using mEI Agar (Cat. No. 256620, BD, USA) incubated at 37°C for 24 hr, and presumptive positive results were confirmed based on forming dark blue colonies. *Salmonella* was detected using Bismuth Sulfite Agar (Cat. No. 273300, BD, USA) incubated at 37°C for 24 hr, and presumptive positive results were confirmed based on forming black and/or brown colonies.

The total viable cells in water samples were detected by CTC staining and followed by flow cytometry. Stock solutions of CTC (50 mmol/L) were prepared as described previously for water sample measurements and stored at -20°C until use (Berney et al., 2008). The total actively respiring cells in the sample aliquots were stained separately with 2 mmol/L CTC final concentration and incubated at 37°C in the dark for 3 hr prior to analysis. Then the stained

samples were measured on a FCM (Beckman Coulter, USA). The FCM was equipped with a 22 mW solid-state laser, emitting at a fixed wavelength of 488 nm, and volumetric counting hardware that measures the number of particles in 100  $\mu$ L of each sample. Green and red fluorescence were collected on the FL1 (520  $\pm$  20 nm) and FL3 (> 615 nm) channels, respectively. The specific instrumental gain settings were: FL1 = 520 nm, FL3 = 650 nm, event rate < 1000 events/sec, no compensation was used and the data collection trigger was set on the red fluorescence channel (FL3). The limit of quantification is 1000 cells/mL and the instrumental error is below 5%. All data were collected as logarithmic (3 decades) signals and processed with the QuantaCollection software (Beckman Coulter, USA). The negative control was ddH<sub>2</sub>O stained with CTC.

### 1.5 Evaluation of reactivation or decay of bacterial cells

To evaluate the reactivation of bacterial cells after 24 hr retention in the dark, the degree ( $D$ , %) of reactivation or decay was quantified by Eq. (3).

$$D = \frac{N_{CTr} - N_{CT}}{N_0 - N_{CT}} \times 100\% \quad (3)$$

where,  $N_{CTr}$  (CFU/L or cells/L) is the concentration of the bacteria or cells in reclaimed water treated by chlorine and after 24 hr storage in the dark. Equation (3) shows the percentage of repaired bacteria among bacteria inactivated by chlorine disinfection. The degree of reactivation could be considered the degree of decay if the value is negative. All the data were subjected to statistical analysis.

### 1.6 Evaluation of the ratio of indicator and pathogenic bacteria in total active microorganism community

The percentages of indicator and pathogenic bacteria in total viable cells immediately after chlorine disinfection ( $P$ , %) and after 24 hr incubation in the dark ( $P_r$ , %) were evaluated by Eqs. (4) and (5), respectively.

$$P = \frac{N_P}{N_T} \times 100\% \quad (4)$$

$$P_r = \frac{N_{Pr}}{N_{Tr}} \times 100\% \quad (5)$$

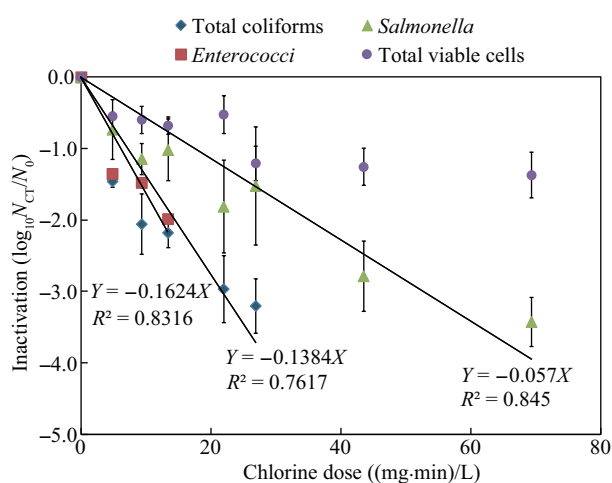
where,  $N_T$  (cells/L) and  $N_P$  (CFU/L) are the concentrations of the total viable cells and pathogenic bacteria after chlorine disinfection, respectively.  $N_{Tr}$  (cells/L) and  $N_{Pr}$  (CFU/L) are the concentrations of the total viable cells and pathogenic bacteria after chlorine disinfection and 24 hr storage in dark.

## 2 Results and discussion

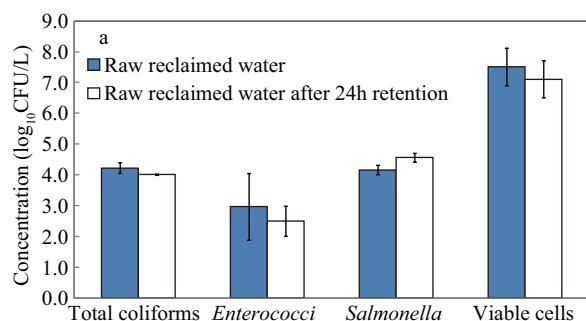
### 2.1 Inactivation of bacteria in reclaimed water by chlorine disinfection

Inactivation curves of native indicator bacteria (total coliforms and *Enterococcus*) and pathogenic bacteria (*Salmonella*) by chlorine disinfection in reclaimed water were compared with that of actively respiring cells. The inactivation curves of native total coliforms, *Enterococcus* and *Salmonella* (Fig. 1) indicated that the reductions were first order with an apparent inactivation rate constant of 0.1384, 0.1624 and 0.057 L/(mg·min) and  $R^2$  of 0.7617, 0.8316 and 0.845, respectively. The results estimated that 3 log<sub>10</sub> reductions of total coliforms, *Enterococcus*, and *Salmonella* required chlorine doses of approximately 21.7, 18.5, and 52.6 (mg·min)/L, respectively, which were interpolated from the linear-regression lines in Fig. 1. Clearly, the *Salmonella* was more resistant to chlorine than both total coliforms and *Enterococcus*.

As shown in Fig. 1, the inactivation curves of total viable cells detected by CTC staining and FCM typically



**Fig. 1** Inactivation curves of indigenous total coliforms, *Enterococcus*, *Salmonella*, and total viable cells in reclaimed water. No total coliforms were detected when chlorine dose was higher than 27 (mg·min)/L and no *Enterococcus* were detected when chlorine dose was higher than 14 (mg·min)/L.

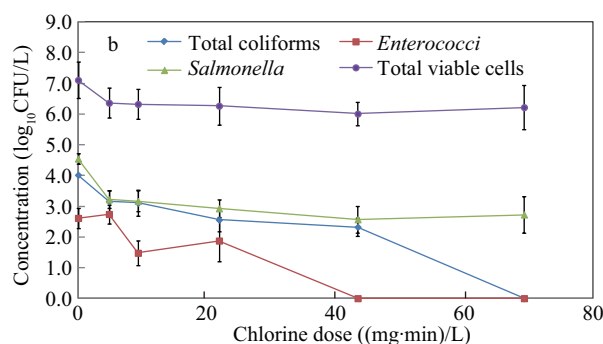


showed first-order inactivation at lower chlorine dosages and a trailing region with increasing chlorine dose. The maximum reduction of total actively respiring cells was approximately 1.3 log<sub>10</sub> when the chlorine dose ranged from 30 to 69 (mg·min)/L. A similar phenomenon was reported by Ramseier et al. (2011), whose results showed that the total indigenous intact cells in drinking water had a two-phase inactivation curve. The results demonstrated the chlorine-resistance phenomena of indigenous microorganisms in reclaimed water. Tailing may be attributed to the presence of chlorine-resistant sub-populations of the microorganisms and the presence of particulate-associated and clumped microorganisms (US EPA, 2004). However, the mechanism of the resistance effect is still not clear and it has been related to microorganisms' aggregation or population heterogeneity (Simonet and Gantzer, 2006; Ramseier et al., 2011).

### 2.2 Regrowth of bacteria after chlorine disinfection

The consumption of reclaimed water is usually irregular and it may take several days or even a week for reclaimed water to reach consumers (US EPA, 2004). To simulate the storage conditions, the raw and chlorinated reclaimed waters were stored for 24 hr in the dark at room temperature. As shown in Fig. 2a, total coliforms, *Enterococcus*, and total viable cells decreased about 0.2, 0.5 and 0.4 log<sub>10</sub> respectively, while, *Salmonella* increased approximately 0.4 log<sub>10</sub> in raw reclaimed water after 24 hr incubation in the dark. Both the regrowth and decay was less than 0.2 log<sub>10</sub> for the studied bacteria. The *t*-test results also showed there was no significant regrowth after 24 hr storage ( $p > 0.05$ ). Previous studies also found that there was no significant regrowth of total heterotrophic bacteria in un-chlorinated secondary effluents after 24 or 48 hr (Huang et al., 2011).

The regrowth of indigenous bacteria in reclaimed water disinfected with a serial dose of chlorine was also investigated after 24 hr storage in dark at room temperature. As shown in Fig. 2b, the chlorine disinfection can clearly reduce regrowth of bacteria after 24 hr retention. There was no *Enterococcus* or total coliforms regrowth with chlorination of more than 44 and 69 (mg·min)/L, respec-



**Fig. 2** (a) Regrowth of indigenous total coliforms, *Enterococcus*, *Salmonella*, and viable cells in reclaimed water before and after chlorine disinfection. (b) Regrowth of indigenous bacteria as a function of chlorine dose. The reclaimed water samples were incubated in the dark for 24 hr at room temperature.

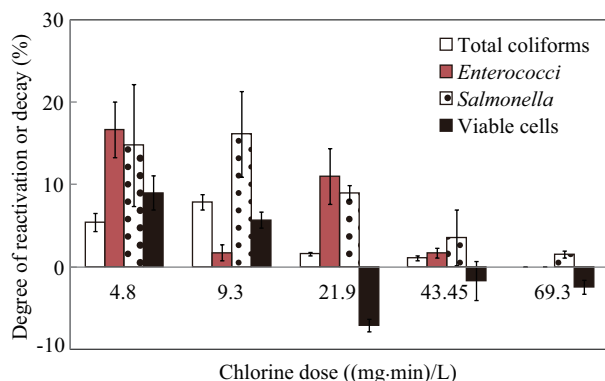
tively. However, there were still *Salmonella* and actively respiring cells after 24 hr storage even with chlorination of 69 (mg·min)/L, which indicated *Salmonella* has higher regrowth potential than those of the indicators (total coliforms and *Enterococcus*).

Previous studies indicated that many bacteria can enter into a VBNC state after disinfection, including *E. coli*, *Enterococcus* and *Salmonella* (Gupte et al., 2003; Oliver, 2005). The VBNC bacteria cannot grow in conventional bacteriological medium; however, they can be reactivated or resuscitated in certain conditions, rendering them culturable again (Higgins et al., 2007). As a result, the regrowth of bacteria after chlorination derives from the regrowth of living bacteria, reactivation of VBNC bacteria and regrowth of reactivated ones in reclaimed water (Oliver, 2005).

### 2.3 Reactivation or decay of bacteria and total actively respiring cells after chlorine disinfection in reclaimed water

Percentages of reactivation/decay of native total coliforms, *Enterococcus*, *Salmonella*, and total viable cells were calculated (Fig. 3). Reactivation/decay of the detected bacteria and cells were observed when the chlorination was lower than 69.3 (mg·min)/L. Reactivation of bacteria was most likely to occur after exposure to a low dose of chlorination, and the extent of reactivation decreased gradually as the dose of chlorination increased.

As shown in Fig. 3, the reactivation percentages of *Salmonella* were higher than that of total coliforms, for example, the reactivation percentages were 4.3% for *Salmonella* and 1.8% for the total coliforms at 43.45. As the initial concentration of *Enterococcus* in reclaimed water was relatively low, *Enterococcus* could not be detected after 24 hr storage when the chlorination was more than 43.45 (mg·min)/L. The reactivation of total actively respiring cells decreased with chlorine dose less than 10 (mg·min)/L, while the decay increased with chlorine dose

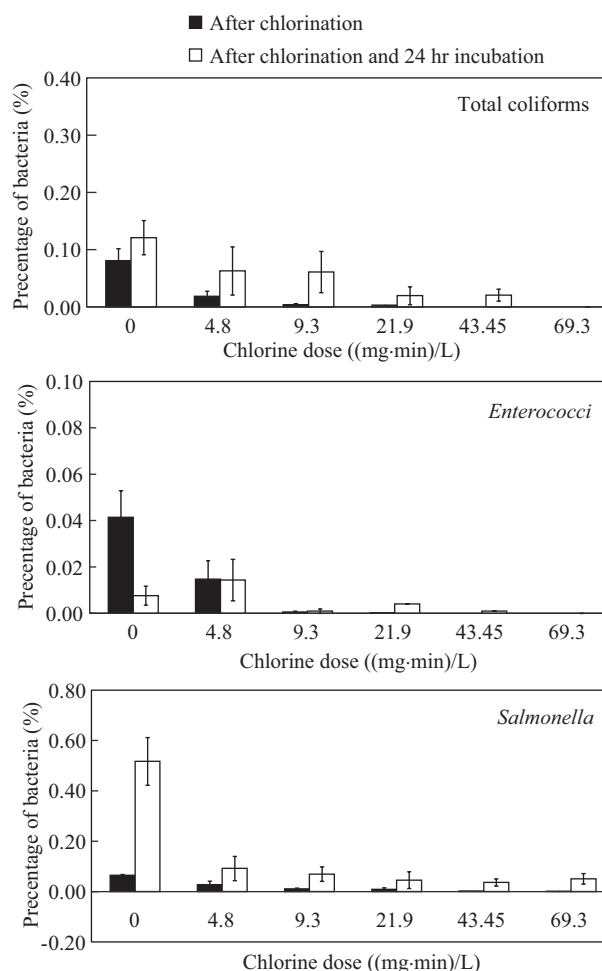


**Fig. 3** Degree of reactivation or decay of indigenous total coliforms, *Enterococcus*, *Salmonella* and total viable cells in reclaimed water after chlorine disinfection. The reclaimed water was disinfected by chlorine and then incubated in the dark for 24 hr at room temperature.

increasing from 21.9 to 69.3 (mg·min)/L. A previous study reported that total heterotrophic bacteria decayed under an initial concentration of 0.5 and 1.0 mg Cl<sub>2</sub>/L, but reactivation occurred after 2.0 mg Cl<sub>2</sub>/L exposure for 10 min and then incubation in the dark for 24 hr (Huang et al., 2011). According to the different reactivation/decay of total coliforms, *Enterococcus*, *Salmonella*, and total viable cells, the proportion of indicator and pathogenic bacteria in the reclaimed water would significantly change after incubation for 24 hr in the dark.

### 2.4 Effect of chlorine disinfection on the change of microbial community in reclaimed water

Since the proportion of pathogenic bacteria in a microbial community is very important for the potential public health risk, the ratios of total coliforms, *Enterococcus* and *Salmonella* in the reclaimed water were evaluated. As shown in Fig. 4, the proportions of total coliforms, *Enterococcus*, and *Salmonella* after chlorination decreased with the increasing chlorination, and chlorine disinfection



**Fig. 4** Proportions of the total coliforms, *Enterococcus* and *Salmonella* in total viable cells of the reclaimed water after chlorine disinfection and after further 24 hr storage in dark at room temperature.



could significantly decrease the proportions of *Salmonella* and total coliforms after 24 hr retention in the dark. As for the reactivation and regrowth, when the chlorine dose was more than 21.9 (mg·min)/L, the percentages of total coliforms, *Enterococcus* and *Salmonella* were lower than 0.001% just after chlorination, however, the percentages of total coliforms and *Salmonella* were remarkably increased to 0.02% and 0.045% after 24 hr storage in the dark, respectively (Fig. 4). The proportion of *Enterococcus* after 24 hr retention was still very low, as there was little regrowth. The present results indicated that it is not sufficient to only use indicators (total coliforms and *Enterococcus*) to evaluate the inactivation, regrowth and microbe quality of reclaimed water, and more chlorine-resistant pathogens should be evaluated, such as *Salmonella*. It can also be inferred that the proportions of indicator and pathogenic bacteria in the microbial community of the reclaimed water will be changed by chlorine disinfection and following retention. Furthermore, chlorine disinfection may lead to chlorine-resistant bacteria becoming a dominant species in the reclaimed water.

### 3 Conclusions

This study investigated the inactivation, reactivation and regrowth of the indigenous indicators and pathogenic bacteria in reclaimed water after chlorine disinfection. The results revealed that potential risk of bacterial prevalence and regrowth in reclaimed water still exists after chlorine disinfection. *Salmonella* was more resistant to chlorine than indicator bacteria, including total coliforms and *Enterococcus*, and the inactivation of total viable cells demonstrated the chlorine-resistance of indigenous microorganisms in reclaimed water. Although chlorine disinfection can reduce rates of regrowth and reactivation of bacteria, *Salmonella* still clearly re-grew even with chlorination of 69 (mg·min)/L, and proportions of *Salmonella* significantly increased compared to those of both total coliforms and *Enterococcus* after 24 hr storage in the dark. Clearly, chlorine disinfection brought about selection of chlorine-resistant pathogenic bacteria in reclaimed water after storage. In order to protect against public health risk caused by pathogens, not only indicator bacteria but also chlorine-resistant pathogenic bacteria should be evaluated during production, storage and distribution of reclaimed water.

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