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Bacterial regrowth in water reclamation and distribution systems revealed by viable bacterial detection assays

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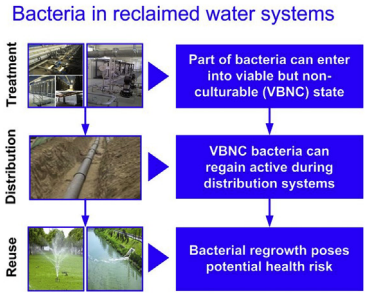
HIGHLIGHTS

- Reclaimed water treatment and disinfections can induce bacteria into a VBNC state.
- The regrowth of *Salmonella* was higher than that of *Escherichia coli* in the distribution system.
- The regrowth of *E. coli* mainly depended on the depletion of total chlorine.
- PMA-qPCR is recommended to predict bacterial regrowth in distribution systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Microbial regrowth needs to be managed during water reclamation and distribution. The aim of present study was to investigate the removal and regrowth of *Escherichia coli* (*E. coli*) and *Salmonella* in water reclamation and distribution system by using membrane integrity assay (PMA-qPCR), reverse transcriptional activity assay (Q-RT-PCR) and culture-based assay, and also to evaluate the relationships among bacterial regrowth, and environmental factors in the distribution system. The results showed that most of the water reclamation processes potentially induced bacteria into VBNC state. The culturable *E. coli* and *Salmonella* regrew 1.8 and 0.7 $\log_{10}$ in distribution system, which included reactivation of bacteria in the viable but non-culturable (VBNC) state and reproduction of culturable bacteria. The regrowth of culturable *E. coli* and *Salmonella* in the distribution system mainly depended on the residual chlorine levels, with correlations (R^2) of −0.598 and −0.660. The abundances of membrane integrity and reverse transcriptional activity bacteria in reclamation effluents had significant correlations with the culturable bacteria at the end point of the distribution system, demonstrating that PMA-qPCR and Q-RT-PCR are sensitive and accurate tools to determine and predict bacterial regrowth in water distribution systems. This study has improved our understanding of microbial removal and regrowth in reclaimed water treatment and distribution systems. And the results also recommended that more processes should be equipped to remove viable bacteria in water reclamation plants for the sake of inhibition microbial regrowth during water distribution and usages.

1. Introduction

Reclaimed water provides an opportunity to supplement water resources and alleviate environmental loads. Wastewater reuse is practiced worldwide, especially in arid zones and urban areas

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(Chen et al., 2012; Shi et al., 2008). For example, in Beijing, more than 50% of treated wastewater is reclaimed and reused, which accounts for approximately 19% of total water consumption (Chang and Ma, 2012). Reclaimed water has been used for several purposes in Beijing, including agricultural irrigation, toilet flushing, recreational and environmental supply, and groundwater recharge (Chang and Ma, 2012). Therefore, close attentions need to be paid to the quality of reclaimed water.

When considering reclaimed water quality, bacterial regrowth is one of the issues that needs to be managed in order to prevent infectious diseases that would lead to the occurrence of pathogenic bacteria, the deterioration of reclaimed water quality, and corrosion and biofouling in distribution systems (Jjemba et al., 2010; Vital et al., 2010; Li et al., 2011; Thayanukul et al., 2013). Currently, evaluation of the microbial quality of reclaimed water mainly focuses on indicator organisms in the treated effluents, such as total and fecal coliforms, *Escherichia coli* (*E. coli*), and *Enterococci* (Costán-Longares et al., 2008). However, most of these indicator organisms are non-pathogenic, and their presence do not correlate with the presence of certain pathogens during wastewater reuse, such as *Salmonella*, *Vibrio parahemolyticus*, and *Mycobacteria* (Harwood et al., 2005; Ishii et al., 2007; Lee et al., 2011). Thus, in order to address the microbial risks of reclaimed water, pathogenic bacteria, in addition to the indicator bacteria, also need to be investigated.

Recently, an increasing number of studies have reported the removal of bacteria during water the reclamation processes and the regrowth of bacteria in the distribution systems (De Luca et al., 2013; Ryu et al., 2005; Thayanukul et al., 2013). Generally, microbial occurrences and regrowth in reclaimed water distribution systems are only investigated by culture-based assay (Jjemba et al., 2010; Thayanukul et al., 2013; Derry and Attwater, 2014). However, a number of bacteria, such as *E. coli*, *Enterococcus* and *Salmonella*, can enter into a viable, but non-culturable (VBNC) state during the wastewater treatment and disinfection processes (e.g., chlorine, ozone, and UV disinfections) (Bjergbaek and Roslev, 2005; Lleo et al., 2011; Trevors, 2011; Moreno et al., 2007). VBNC bacteria do not grow in conventional bacteriological media, but are still alive and have low levels of metabolic activity. More importantly, VBNC bacteria may restart active growth when optimal conditions are restored (Oliver, 2005; Alam et al., 2007; Coutard et al., 2007). These phenomena suggest that VBNC bacteria in treated effluents can enter reclaimed water distribution systems and may serve as "seeds" for regrowth, which pose potential health risks when reclaimed water is used. Therefore, it is necessary to monitor not only culturable, but also VBNC bacteria in treated effluents and at the point of use for reclaimed water.

As conventional culture-based assays cannot detect VBNC bacteria, innovative, cultivation-independent techniques have been developed using the distinguishing physiological characteristics of viable bacteria, such as the integrity of cell membranes, transcriptional activity, and metabolic activity.

The integrity of cell membranes can be determined by using some DNA-intercalating dyes, which can only intercalate into DNA from non-viable bacteria with permeable membranes or extracellular DNA (Thayanukul et al., 2013; Alleron et al., 2013). Recent improvements in the quantitative PCR technique, such as the pretreatment of samples with the DNA-intercalating dyes (e.g. propidium monoazide (PMA) and ethidium monoazide (EMA)) prior to DNA extraction have been reported to only detect DNA from viable microorganisms (Nocker and Camper, 2006; Nocker et al., 2007). PMA/EMA-qPCR methods are based on the ability of bacteria with intact cellular membranes to resist penetration of DNA-intercalating dyes, whereas DNA in non-viable bacteria with permeable membranes or extracellular DNA does interact with the dyes and cannot be amplified by PCR following light-induced cross-linking (Nocker et al., 2007). It has been reported that PMA-qPCR can evaluate viable bacteria, including both culturable and VBNC bacteria, after chemical disinfection processes (e.g. chlorine and ozone disinfections) (Tong et al., 2011; Nocker et al., 2007; Li et al., 2014).

As the half-life of bacterial RNA ranges from 0.5 to 50 min, RNA has been used as the target for the transcriptional activity of viable cells (Takayama and Kjelleberg, 2000). Quantitative reverse transcription PCR (Q-RT-PCR) has been developed, which can target bacterial RNA and has been used to detect viable bacteria in environmental samples. Previous studies reported that the use of DNA standards, including PCR products, and plasmid and genomic DNA, produces in an overestimation of the RNA target molecules, largely due to different reaction efficiencies between RT-PCR and PCR (Fey et al., 2004). Therefore, RNA standards are needed for accurate quantification of RNA. Several studies have shown that Q-RT-PCR can effectively and sensitively detect viable *E. coli* and *Salmonella* in environmental samples (Fey et al., 2004; González-Escalona et al., 2009).

In this study, the removal and regrowth of two culturable and viable bacteria, including an indicator organism (*E. coli*) and a pathogenic bacteria (*Salmonella*), in two reclaimed water treatment and distribution systems were comprehensively investigated over one year using a number of viable bacteria detection methods. These were culture-based, PMA-qPCR, and Q-RT-PCR assays, which target culturability, membrane integrity, and the transcriptional activity of bacteria, respectively. The environmental factors that affect the regrowth of bacteria in distribution systems were also evaluated. This study demonstrated the feasibility and sensitivity of Q-RT-PCR and PMA-qPCR assays. They are able to detect viable bacteria in reclaimed water, which should reduce the implicated public health risks associated with bacterial regrowth in reclaimed water systems.

2. Materials and methods

2.1. Sampling sites and sample collection

The study was performed in two (Q and W) water reclamation plants (WRP) in Beijing, China. Table 1 shows the process configurations and the sampling points used in this study. These two WRPs (Q and W) receive secondary effluents from the activated sludge process and are located in domestic wastewater treatment facilities. The water samples were collected once a month from June 2012 to May 2013 (12 sampling occasions and 108 samples in total) using sterile 5 L plastic containers. The samples were transferred to the laboratory within 4 h of sampling and placed on ice before analysis (<24 h).

The samples collected from the Q WRP and distribution system were 1) secondary effluent (Q1); 2) after ultrafiltration treatment (Q2); 3) after ozonation and granular activated carbon filtration (Q3); 4) after chlorine disinfection (WRP effluent, Q4) and 5) at the end of the distribution pipe (Q5) (Table 1). Samples collected from W WRP and distribution system were: 1) secondary effluent (W1); 2) after chlorination, coagulation and sand filtration (W2); 3) after UV and chlorination disinfection (WRP effluent, W3); and 4) at the end of the distribution pipe (W4) (Table 1).

2.2. Physicochemical analyses

The total organic carbon (TOC) was quantified using a TOC analyzer (TOC-V CPH, Shimadzu, Japan). Total nitrogen (TN), total phosphorus (TP), ammonia nitrogen (NH_4–N) and nitrate nitrogen

Table 1
Process configurations of the two wastewater reclamation plants (WRPs) and the sampling points used in this study.

WRPs	Treatment processes	Treatment unit	Sample points
Q	Secondary	Aeration tank	–
		2nd sedimentation	Q1
	Tertiary	Ultrafiltration	Q2
		Ozonation	–
		Granular activated carbon	Q3
	Disinfection	Post-chlorination	Q4
	Distribution	End of pipes (8 km)	Q5
W	Secondary	Aeration tank	–
		2nd sedimentation	W1
	Tertiary	Chlorination	–
		Coagulation and sand filtration	W2
	Disinfection	UV disinfection	–
		Chlorination	W3
	Distribution	End of pipes (2 km)	W4

(NO_3–N) analyses were performed using standard procedures (Wei et al., 2002). The concentrations of total chlorine in the plant effluent and samples taken from the end of the distribution pipe were measured by the N, N-diethyl-p-phenylendiamin (DPD) colorimetric method using a colorimeter (46700, Hach, USA). The water temperature were also determined, and the average temperature form June 2012 to May 2013 were 25.1 °C, 27.7 °C, 26.6 °C, 21.0 °C, 14.3 °C, 6.2 °C, 5.1 °C, 4.8 °C, 4.1 °C, 6.6 °C, 14.2 °C, and 21.6 °C in each sampling occasion.

2.3. Quantification of culturable bacteria using culture-based assays

The *E. coli* and *Salmonella* were determined using the membrane filtration method. Briefly, 100 mL water samples were serially diluted and then filtered with a 0.45-μm-pore-size cellulose nitrate filter. Then the filters were placed on selective agar medium for *E. coli* or *Salmonella*.

For *E. coli*, the filters were placed on m-TEC agar (USEPA, 2002), incubated for 2 h at 35 ± 0.5 °C, and then incubated at 44 ± 0.5 °C for 22 h. The *E. coli* colonies were confirmed with a urea substrate (2 g urea with 10 mg phenol red/100 mL water) after incubation for 15 min and subsequent enumeration of the yellow or yellowish brown colonies under a UV lamp (Jjemba et al., 2010).

For *Salmonella*, the filters were placed on bismuth sulfite agar plates and then incubated overnight at 35 °C. Distinct black or surrounded by a black or brownish-black zone colonies were considered to be *Salmonella* (William, 2000; Murray et al., 1999).

2.4. Quantification of bacteria membrane integrity using the PMA-qPCR assay

The PMA-qPCR assay had been previously developed, optimized, and then used to analyze the bacterial inactivation efficacies of chlorine and monochloramine in our laboratory, which proved that PMA treatment removed more than 99% of DNA from non-viable cells and that the PMA-qPCR assay could differentiate viable bacteria from the non-viable ones in treated wastewater (Tong et al., 2011; Li et al., 2014).

In this study, the bacteria in reclaimed water samples were concentrated using a cellulose nitrate filter according to our previous study (Tong et al., 2011). Briefly, 1 L water samples were filtered using a 0.45-μm-pore-size cellulose nitrate filter (Xingya, Shanghai, China). Then the filter was transferred to a sterile 10 mL centrifuge tube. Exactly 8 mL of sterile PBS was added to the tube and then vortexed for 5 min to release the bacteria into the eluent solution. After centrifuging at 13,000 rpm for 15 min, the supernatant was removed, and the remaining pellets were resuspended in 500 μL PBS and moved to a 2 mL tube and then centrifuged at 13,000 rpm for 5 min. The remaining pellets were re-suspended in 500 μL PBS.

Then the concentrated samples were treated with PMA according to our previous research (Li et al., 2014; Tong et al., 2011). Briefly, PMA (Code: 40013, Biotium, USA) was dissolved in 20% dimethyl sulfoxide (DMSO) to obtain a 20 mM stock concentration and then stored at −20 °C in the dark. Around 2.5 μL of PMA solution (20 mM) was added to 500 μL re-suspended samples in a 1.5 mL tube to obtain a final PMA concentration of 100 mM. The tube was incubated in the dark with occasional mixing for 5 min, followed by light exposure for 4 min using a 2500 W halogen light source (sealed beam lamp, FCW 120 V, 3200 K, GE Lighting, USA). In order to avoid excessive heating during light exposure and to maximize light exposure, the tubes containing the concentrated samples and PMA were placed horizontally on ice at a distance of approximately 20 cm from the light source and were periodically shacked.

After PMA treatment, the genomic DNA was extracted using the FastDNA® Spin Kit for Soil (Code: 6560200, MP Biomedicals, USA) according to the instructions, and the DNA was immediately subjected to qPCR or stored at −20 °C until needed.

Primers specific to *E. coli* were used to target the *uidA* gene (uidA-F (5′–3′): ATGGAATTTCGCCGATTTTGC; uidA-R (5′–3′): ATTGTTTGCCTCCCTGCTGC) (Heijnen and Medema, 2006), and the primers and a specific probe for *Salmonella* targeted the *invA* gene (invA-F (5′–3′): CATTTCTATGTTCGTCATTCCATTACC, invA-R (5′–3′): AGGAAACGTTGAAAAACTGAGGATTCT; invA-P (5′–3′): 6FAM-TCTGGTTGATTTCCTGATCGCGCTGAATATC-TAMRA) (Pusterla et al., 2010). In order to quantify the *E. coli* and *Salmonella* genes, a plasmid containing the target gene was constructed and used as an external calibration standard (Li et al., 2009; Hu et al., 2008). Briefly, the DNA was extracted from *E. coli* and *Salmonella* and then subjected to PCR analysis. The PCR product was then separated on a 1% agarose gel and purified using a gel extraction kit (Code: 9762, TaKaRa, Japan). Then the products were linked with a pMD®18-T vector cloning kit (Code: D101A, TaKaRa, Japan) and transferred into competent *E. coli* DH5α. The plasmids were extracted and purified using a Plasmid Maxprep Kit (Code: N001, Vigorous Biotechnology, China), and then sequenced and analyzed to confirm the target genes. Quantification of the plasmid DNA was carried out by UV spectrophotometry (NanoDrop 2000, Thermo, Japan) at 260 nm. The qPCR protocols for *E. coli* and *Salmonella* were developed in our laboratory (Tong et al., 2011). The qPCR was performed in 20 μL reaction mixtures in an iQ5 icycler (Bio-Rad, CA, USA) following the manufacturer's instructions.

For *E. coli*, the mixtures consisted of 10 μL 2 × SYBR Premix Ex Taq™ (TaKaRa, Japan), 0.2 μL of each primer (10 μM final concentrations), 2 μL of DNA template, and 7.6 μL of dH_2O. The thermo cycling profile for the *E. coli uidA* gene was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, 72 °C for 20 s, melting curve analysis at 95 °C for 15 s, and finally, annealing at 60 °C for 1 min.

For *Salmonella*, the mixtures consisted of 10 μL Premix Ex Taq™ (Code: DRR041A, TaKaRa, Japan), 0.2 μL of each primer (10 μM final concentrations), 0.2 μL of Taqman®Probe (20 μM final concentration) (Code: DRR039A, TaKaRa, Japan), 2 μL of DNA template, and 6.6 μL of dH_2O. For the *Salmonella invA* gene, the thermo cycling profile was 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

A serially diluted plasmid DNA that contained the target gene was constructed and used to establish the standard curve, and double distilled water (ddH_2O) was used as a negative control. The

plasmids were extracted, purified, and then analyzed by DNA sequencing to confirm the target genes.

2.5. Quantification of bacteria by reverse transcriptional activity using Q-RT-PCR assay

The RNA standards were recombined according to previous studies (Fey et al., 2004) in order to accurately quantify the bacterial RNA targets. The Q-RT-PCR assay was established and optimized using data from a previous study (Lin et al., 2012). Briefly, two primer sets were designed for construction of the RNA standards for *E. coli* (uidA-T7-F (5′–3′): TAATACGACTCACTATAGGGGCGTTACAAGAAAGCC; uidA-T7-R (5′–3′): GCATCTCTTCAGCGTAAGGGTAATGCGA) and *Salmonella* (invA-T7-F (5′–3′): TAATACGACTCACTATAGGGAACAGTGCTCGTTTACG; invA-T7-R (5′–3′): GCAGAGTTCCCATTGAAATGGTC) containing the sequences for the T7 promoter. The PCR products amplified by these primers (*uidA*-T7 primers and *invA*-T7 primers) contained the PCR products amplified by the primers for qPCR. These primers were used to amplify the genomic DNA of *E. coli* and *Salmonella* by PCR, according to the following protocol: initial denaturation, 4 min at 94 °C; 30 cycles of 1 min at 94 °C; 1 min at 58 °C (*Salmonella*) or 56 °C (*E. coli*); 1 min at 72 °C; and then final elongation at 72 °C for 10 min. The PCR mixtures (50 μL per sample) contained 25 μL of Premix *Taq*® (Code: D331A, TaKaRa, Japan), 1 μL of each relevant primer (400 nM final concentration), 5 μL of genomic DNA and 18-μL of dH_2O. To obtain the RNA standards, the PCR products were purified with a PCR purification kit (Code: A7170, Promega, USA) and subsequently transcribed in vitro by the T7 RiboMAX™ Express LargeScale RNA Production System (Code: P1320, Promega, USA). The products were immediately followed by digestion with DNase I (15 min, 37 °C) and subsequently purified. The transcripts were quantified by Nanodrop 2000C (Thermo Scientific, USA). The standards were diluted in nuclease free water and stored at −80 °C. The Q-RT-PCR assay consisted of two steps: first the RT reactions were performed in 20 μL reaction mixtures, and secondly the quantitative PCR assays were carried out according to Section 2.4.

A number of 1 L samples were collected and concentrated as described in Section 2.4. The total RNA was extracted using the FastRNA® Pro Soil-Direct Kit, and the RNA was subjected to Q-RT-PCR using the PrimeScript® RT reagent Kit perfect Real Time (Code: RR014A, TaKaRa, Dalian, China). The RT reaction was performed in 20 μL reaction mixtures that contained 8 μL RNA, 4 μL of 5 × PrimeScript® Buffer (for Real Time analysis), 1 μL of PrimeScript® RT Enzyme Mix I, 1 μL of Oligo dT (50 μM) and 1 μL Random 6 mers (100 μM), and 5 μL of ddH_2O (Code: RR014A, TaKaRa, Dalian, China). The RT reaction protocol included incluation at 37 °C for 15 min and 85 °C for 5 s. The cDNA products were subjected to qPCR, which was carried out as described in Section 2.4, or stored at −20 °C until needed.

2.6. Data analysis

The detection limits for the culture-based assays were 1 CFU/100-mL for *E. coli* and *Salmonella*. The detection limits for the PMA-qPCR assays were about 50 and 20 copies/100 mL for *E. coli* and *Salmonella*, respectively (Murray et al., 1999). The detection limits for the Q-RT-PCR assays were about 200 copies/100 mL for both *E. coli* and *Salmonella* (Lin et al., 2012).

The Log_{10} removal was quantified by Eq. (1):

$$\text{Log}_{10}\ \text{Removal of a bacteria} = \text{Log}_{10}\frac{N_i}{N_o} \quad (1)$$

where N_i is the bacteria concentration after a treatment, and N_0 is the bacteria concentration before a treatment. They are based on three assays,

Regrowth was quantified by Eq. (2):

$$\text{Log}_{10}\ \text{Regrowth of a bacteria} = \text{Log}_{10}\frac{N_{end}}{N_{eff}} \quad (2)$$

where N_{end} is the bacteria concentration at the end point and N_{eff}: is the bacteria concentration in effluent samples detected by three assays.

The degree of reactivation can be considered to be the degree of decay if the value is negative. All statistical analyses were carried out using OriginPro 8.1 (OriginLab, USA) and the Graphpad Prism 5.0 (GraphPad Software, USA). The statistical analyses were performed by SPSS 18.0 (SPSS Ltd, Hong Kong).

3. Results

3.1. Occurrences of E. coli and Salmonella by viable bacteria detection assays at different points in the reclaimed water treatment and disinfection processes

The occurrences of *E. coli* and *Salmonella* after using a number of viability detection methods at two WRPs over 1 year are shown in Fig. 1. It is notable that the occurrences of viable bacteria with membrane integrity (detected by PMA-qPCR assay) and transcriptional activity (detected by Q-RT-PCR assay) were relatively higher than the culturability detected by the culture-based assay (Fig. 1).

In the Q system, the concentrations of culturable *E. coli* were gradually reduced from secondary effluent (Q1) to after ultrafiltration (Q2), ozonation and granular activated carbon filtration (Q3) and chlorination (Q4), while, significantly increased at the end point of the distribution pipe (Q5), respectively (Fig. 1). The geometrical average concentrations of *E. coli* with membrane integrity detected by PMA-qPCR were more than 10^5 in Q1 and Q2, after disinfection the values reduced to about 10^4 copies/L in Q3 and Q4. After distribution, however, the value increased to about 10^5 in Q5. The geometrical average abundances of *E. coli* with transcriptional activity detected by Q-RT-PCR were similar with the results of PMA-qPCR.

In the W system, the abundance of culturable *E. coli* and *Salmonella* in secondary effluents were similar to that in the Q system, which were about 10^5 CFU/L. The concentrations of culturable *E. coli* and *Salmonella* were reduced after chlorination, coagulation-sand filtration (W2), and disinfection (W3), and ranged from 10^5 to about 10^1 CFU/L, but increased to about 10^4 CFU/L after the distribution system. However, the geometrical average concentrations of *E. coli* and *Salmonella* with membrane integrity and transcriptional activity varied little along the treatment and disinfection processes, and ranged from 10^5 copies/L to 10^6 copies/L (Fig. 1). Besides, the concentrations of culturable *Salmonella* in effluents of two plants were higher, indicating that *Salmonella* was more resistant to chlorine disinfection than *E. coli* (Mahmoud et al., 2007; Mahmoud and Linton, 2008).

Statistic analysis indicated that there were significant differences between culturable bacteria and viable bacteria detected by PMA-qPCR and Q-RT-PCR in the same sampling sites ($^*p < 0.05$ and $^{**}p < 0.01$) (Fig. 1). And relatively higher concentrations of viable bacteria than the culturable bacteria in the two reclaimed water systems indicated that VBNC bacteria existed in all the reclamation processes. For example, disinfection treatment can induce VBNC bacteria. Previous research found that *Legionella pneumophila* can enter into a VBNC state after monochloramine treatment. In this state, *L. pneumophila* was unable to form colonies on standard media, but was still able to synthesize proteins, some of which are involved in virulence (Alleron et al., 2013). Furthermore, the VBNC

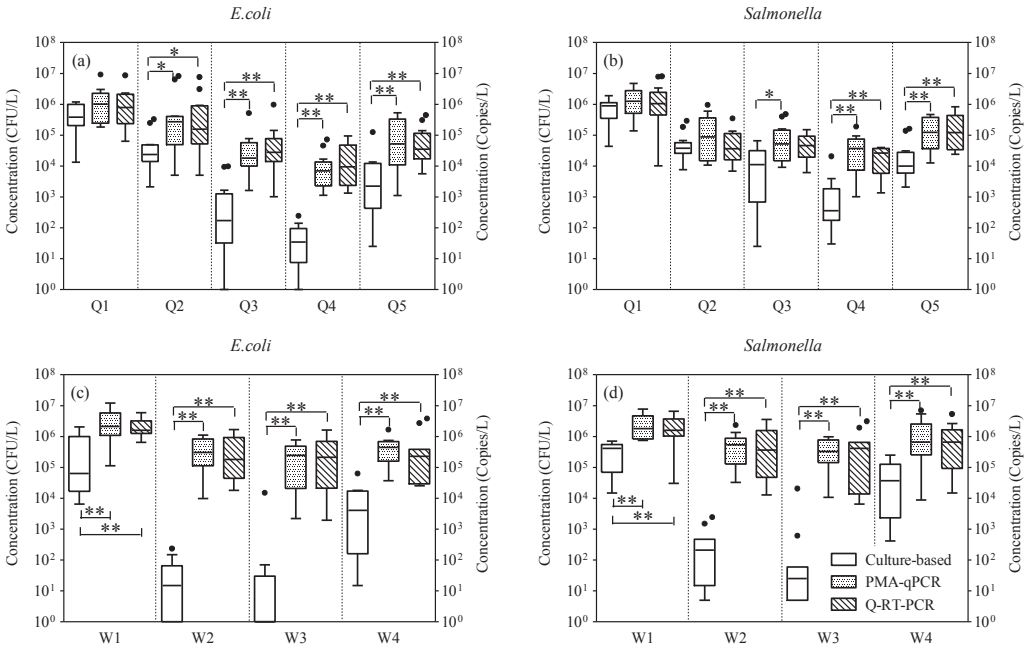

Fig. 1. Concentrations of the viable indicator (*E. coli*) and pathogen (*Salmonella*) bacteria in the Q (a and b) and W (c and d) reclaimed water treatment and distribution systems at different process points. The whiskers of each box indicate the minimum and maximum values, excluding the abnormal values (shown as dots). The lines in the middle of each box indicate the median value of the whole year's data. Detection limits were used as concentrations for the samples where bacteria were not detected. Significant differences between different detection assays in the same sampling sites were tested with the S–N–K test (*, $p < 0.05$ and **, $p < 0.01$). Detailed information about the sampling points are shown in Table 1.

bacteria were able to retain their viability for much longer periods than suggested by the culture-based method (Gin and Goh, 2013).

3.2. Removal and inactivation of E. coli and Salmonella by the different reclaimed water treatment and disinfection processes

Removal and inactivation of *E. coli* and *Salmonella* by the different reclaimed water treatment and disinfection processes are shown in Fig. 2. Both *E. coli* and *Salmonella* were continuously removed along the reclamation processes, with each process providing different removal efficiencies. The *E. coli* and *Salmonella* removal efficiencies, based on culture-based assay, were slightly higher than by PMA-qPCR and Q-RT-PCR assays, mainly because some bacteria entered into a VBNC state under treatment and disinfection conditions and were not detected by the culture-based assay.

The removal efficiencies of culturable *E. coli* were about 1, 2.2, and 1 $\log_{10}$ by ultrafiltration, ozonation and granular activated carbon filtration, and chlorine disinfection in the Q system, respectively (Fig. 2). The removal efficiencies of *E. coli* with membrane integrity and transcriptional activity were similar, at approximately 0.8, 1.1, and 0.3 $\log_{10}$ along the ultrafiltration, ozone and chlorination treatments, respectively (Fig. 2a). For *Salmonella*, the ultrafiltration removal efficiencies evaluated by these three assays were similar, and ranged from 1 to 1.3 $\log_{10}$ (Fig. 2b). However, ozone and chlorine disinfections removed more culturable *Salmonella* than bacteria with membrane integrity and transcriptional activity. The culture-based assay indicated that ozone and chlorine disinfections removed 1.8 and 1.2 $\log_{10}$ culturable *Salmonella*, respectively. However, the PMA-qPCR and Q-RT-PCR assays showed less than 0.4 $\log_{10}$ *Salmonella* when those with membrane integrity and transcriptional activity were removed (Fig. 2a and b).

In the W system, the integrated processes of chlorination, coagulation, and sand filtration removed 3.2–3.7, 0.8–1.0, and 0.7–1.0 $\log_{10}$ of viable bacteria with culturability, membrane integrity, and transcriptional activity, respectively (Fig. 2c and d). The UV and chlorination disinfection treatments inactivated 0.4–0.6, 0.1–0.2 and 0.1–0.2 $\log_{10}$ of the viable bacteria with culturability, membrane integrity and transcriptional activity, respectively (Fig. 2c and d). The removal of culturable bacteria with culturability, were significantly different with viable bacteria detected by PMA-qPCR and Q-RT-PCR in W system ($p < 0.05$) (Fig. 2c and d).

The advanced treatment process in this WRT is ultrafiltration using 0.02 μm hollow-fiber membranes. Theoretically, all the bacteria that are larger than 0.02 μm would be removed. However, comparing with the new membrane, previous study has showed that the removal efficiencies are reduced due to membrane fouling and membrane damage (Xiao et al., 2011). A previous study reported that removal efficiencies were as high as 5 $\log_{10}$ for *E. coli* and 4.5 $\log_{10}$ for *Enterococci* (Ottoson et al., 2006). However, in this study, the removal efficiencies of culturable and viable *E. coli* and *Salmonella* were similar at about 0.8–1.4 $\log_{10}$ (Fig. 2), which were much lower than previous studies. This was probably due to membrane fouling and damage at the treatment plant, which has been running since 2006 (Xie, 2008).

Ozone treatment is mainly used to remove chromaticity in this WRP, but it can also attack numerous cellular constituents, including proteins, unsaturated lipids, and respiratory enzymes in cell membranes, and enzymes and nucleic acids in the cytoplasm (Khadre et al., 2001). The inactivation efficiency of ozone is governed by the organic matrix in water samples and the ozone dose (C_t), which is the product of the dissolved ozone concentration (mg/L) and ozone exposure time (min). As shown in Fig. 2, for both *E. coli* and *Salmonella*, it is notable that the removal efficiencies by ozone treatment of culturable bacteria were much higher than for bacteria with membrane integrity and transcriptional activity. This indicated that ozone treatment may make some bacteria enter into VBNC states.

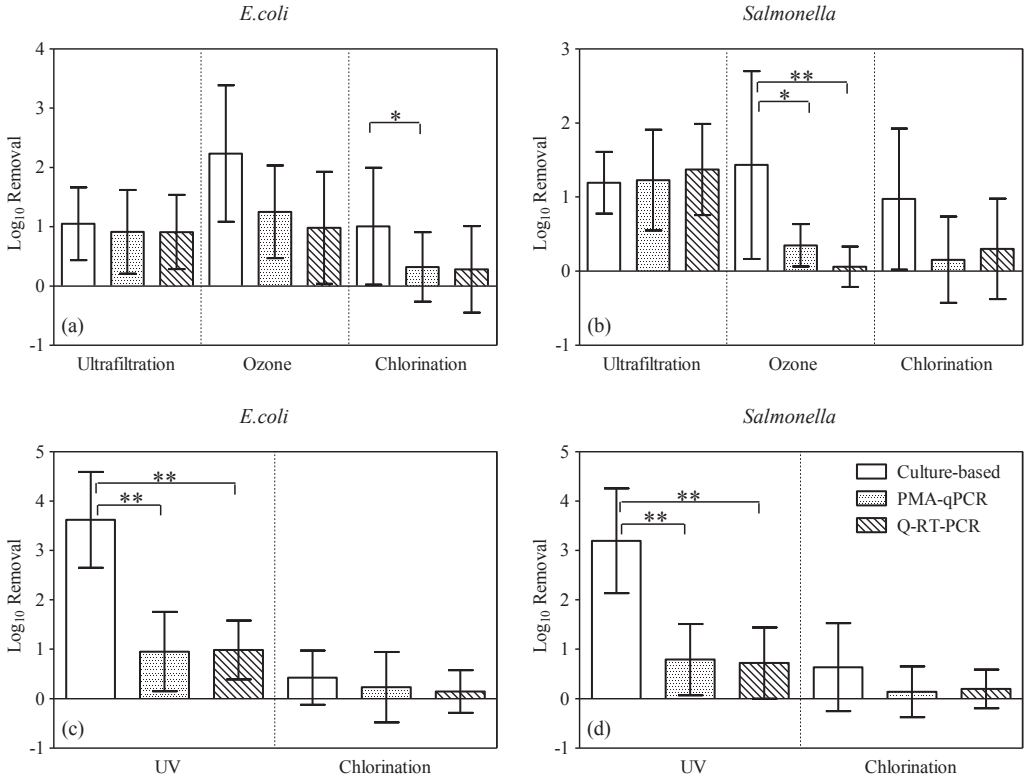

Fig. 2. Removal values for viable *E. coli* and *Salmonella* in the Q (a and b) and W (c and d) systems using culture-based, PMA-qPCR, and Q-RT-PCR assays. Significant differences between different detection assays in the same sampling sites were tested with the S–N–K test (*, $p < 0.05$ and **, $p < 0.01$). Detection limits were used as concentrations for the samples where no bacteria were detected.

Chlorine is added to the effluents to prevent microbial growth during reclaimed water distribution. Similar to ozone, chlorine is generally considered to be a non-selective oxidant that can react with a wide variety of cellular components and affects metabolic processes (Virto et al., 2005). The chlorine disinfection efficiency is affected by chorine dose (chlorine concentration × exposure time), organic compounds in the water matrix, and especially temperature (Virto et al., 2005). Temperature is an important factor that affects chlorine residuals in the water (Thayanukul et al., 2013). As shown in Fig. 2, the removal efficiencies of culturable *E. coli* and *Salmonella* were about 1 $\log_{10}$, and the removal efficiency for viable *E. coli* was slightly higher than *Salmonella*. These results indicated that *Salmonella* were more resistant to chlorine than *E. coli*, which were similar to other studies (Lezcano et al., 1999; Kim and Yousef, 2000). For example, one study reported that the removal efficiencies were 1–4.5 $\log_{10}$, based on a chlorine dose ranging from 0.15 to 1.2 mg/L (Tong et al., 2011).

3.3. Evaluation the regrowth of E. coli and Salmonella in the reclaimed water distribution system

Bacterial regrowth in the distribution system includes reactivation from the VBNC state, and the reproduction by culturable and reactivated VBNC bacteria, which can be detected by culture-based, PMA-qPCR, and Q-RT-PCR assays (Fig. 3). As shown in Fig. 3, the *E. coli* and *Salmonella* with culturability, membrane integrity, and reverse transcriptional activity regrew in the distribution systems. In the Q distribution system, the regrowth efficiencies were lowest in summer and highest in winter, and were about 1.0 and 2.4 $\log_{10}$ for *E. coli*, and 0.5 and 2.3 $\log_{10}$ for *Salmonella*, respectively. However, in the W system, the regrowth efficiencies in summer and autumn were higher than in winter and spring, at 2.7–2.8 and 0.7 $\log_{10}$ for *E. coli*, and 3.3 and 1.4 $\log_{10}$ for *Salmonella*, respectively.

When the regrowth efficiencies evaluated by culture-based, PMA-qPCR and Q-RT-PCR assays are compared, the regrowths of *E. coli* and *Salmonella* detected by the culturability assay were higher than for the membrane integrity and reverse transcriptional activity assays. For example, the regrowth efficiencies of *E. coli* ranged from 1.0 to 2.4 $\log_{10}$ (culturability), from 0.3 to 1.4 $\log_{10}$ (PMA-qPCR assay), and from 0.2 to 1.1 $\log_{10}$ (Q-RT-PCR assay) in the Q distribution system. These results indicated that a number of the *E. coli* and *Salmonella* with membrane integrity and reverse transcriptional activity can become reculturable during distribution and reuse.

Statistically significant correlations were found between the concentrations of viable bacteria in the Q reclamation plant effluents (detected by PMA-qPCR and Q-RT-PCR assays) and concentrations of culturable bacteria at the end point of Q distribution system (detected by culture-based assay) (Table S1 and S2), with the correlation coefficients (R^2) about 0.8 ($p < 0.01$) for *E. coli* and 0.6 ($p < 0.05$) for *Salmonella*, respectively. In the W reclamation system, however, the correlations were not significant because of the less regrowth due to the higher concentration of chlorine in the end point which. Those results indicated that PMA-qPCR and Q-RT-PCR assay are useful and efficient tools for evaluating and predicting the regrowth of VBNC bacteria in the distribution system and can provide more information than culture-based assay for microbial risk assessment.

This study is the first to use PMA-qPCR, Q-RT-PCR and culture-based assays together to monitor viable bacteria in reclaimed water systems, which enabled this study to evaluate and quantify the reactivation of VBNC bacteria in the distribution system. Our study

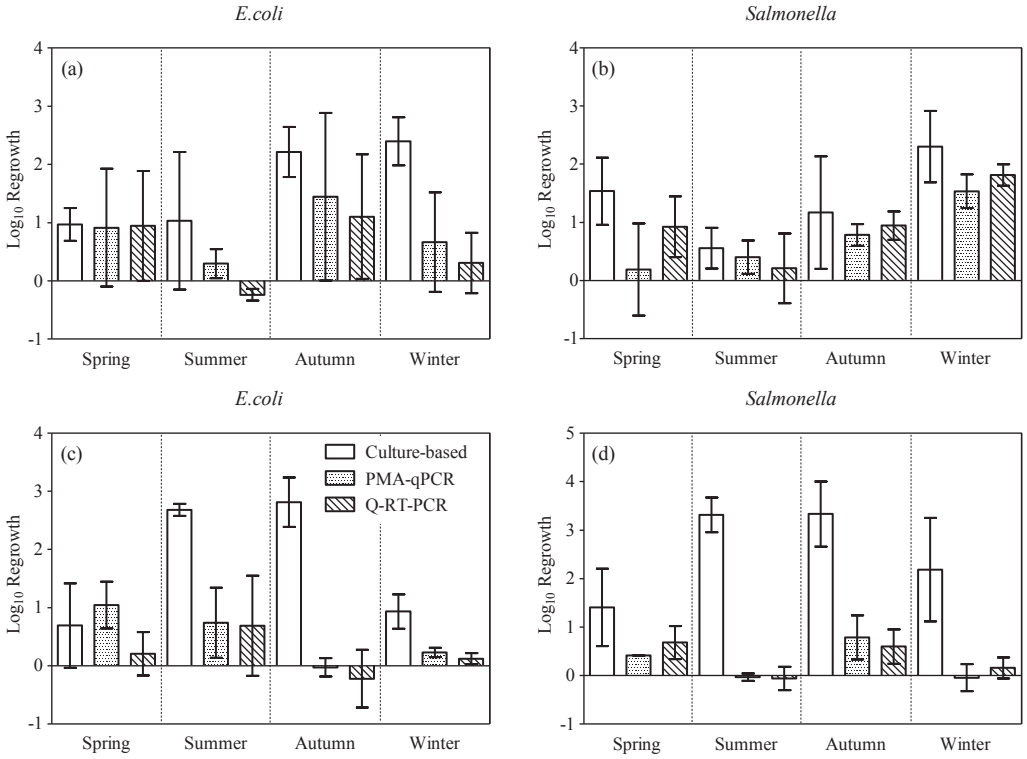

Fig. 3. Regrowth of *E. coli* and *Salmonella* in the Q (a and b) and W (c and d) distribution systems by using culture-based, PMA-qPCR, and Q-RT-PCR assays in different seasons.

proved that a considerable proportion of the VBNC bacteria, which appeared during the reclaimed water treatment and disinfection processes, can become culturable again in the distribution system and pose significant health risks to reclaimed water users. For this reason, we have begun monitoring viable bacteria as well as culturable ones in the effluents from WRP.

3.4. Relationships among bacterial regrowth, storage periods, nutrients, and total chlorine in distribution systems

3.4.1. Effects of storage period on bacterial regrowth

The reclaimed water consumption rate is usually irregular, so it may take several days or even a week for reclaimed water to reach consumers (Huang et al., 2011). In order to evaluate the bacterial regrowth under these conditions, the concentrations of viable bacteria in effluents from the Q and W systems were investigated using culture-based, PMA-qPCR, and Q-RT-PCR assays after a retention time of up to 6 d. The reclaimed water samples were collected from Q and W plants with total chlorine levels of 0.2 and 0.52 mg/L, and were stored at 15 °C in the dark.

Fig. 4a and b show that the culturable *E. coli* and *Salmonella* in reclaimed water from the Q system increased considerably during storage, and the regrowth of *Salmonella* occurred earlier than that of *E. coli*. The regrowth of *E. coli* was non-significant during the first 3 d of storage, but it regrew rapidly from 3 to 5 d storage, and increased from 7 to 4.4 × 10^2 CFU/100 mL (Fig. 4a). In contrast, *Salmonella* regrew considerably from 1 to 3 d storage, and the rise was 3.9 × 10^2 to 1.8 × 10^4 CFU/100 mL (Fig. 4b). However, the *Salmonella* regrowth rate fell from 3 to 6 d storage with a final concentration of 4.6 × 10^4 CFU/100 mL (Fig. 4). In reclaimed water collected from the W system, the culturable *E. coli* regrew to 28 CFU/100 mL after the first day's storage, increased slowly between 1 and 4 d storage, and only increased to 5.5 × 10^2 CFU/100 mL between 4 and 6 d storage (Fig. 4c). The regrowth of culturable *Salmonella* was 1.5 × 10^3 CFU/100 mL during the first 3 d of storage, and remained the same during the last 3 d of storage (Fig. 4d).

The viable *E. coli* with membrane integrity and reverse transcriptional activity in reclaimed water from the two systems slightly decreased during 6 d of storage. However, the viable *Salmonella* with membrane integrity and reverse transcriptional activity slightly increased (Fig. 4). The regrowth of the bacteria investigated by this study includes reactivation of VBNC bacteria and the reproduction of culturable and reactivated ones. Previous results also showed that there was no significant regrowth of culturable bacteria in the unchlorinated secondary effluents after retention in the dark at room temperature for 22–48 h, which indicated that culturable bacteria didn't reproduce significantly (Huang et al., 2011). The results of this study indicated that a proportion of the VBNC bacteria can recover to a culturable state during storage and distribution, which will pose a public risk during wastewater reuse.

3.4.2. Effects of total chlorine and nutrients on bacterial regrowth

Physical and chemical factors that have been reported to affect the regrowth of pathogens in reclaimed water were also detected, including nutrients (TN, TP, NO_3–N, NH_3–N, and TOC) and total chlorine, in the distribution system (Table S3 and S4). The Pearson correlations (r) for the culturable and viable *E. coli* and *Salmonella* abundances in effluents (Q4 and W3) and end points (Q5 and W4) with various physical and chemical factors in reclaimed water are summarized in Table 2. Only total chlorine was significantly related to culturable *E. coli* and *Salmonella*, with correlation coefficients of −0.598 ($p < 0.001$) and −0.660 ($p < 0.001$), respectively (Table 2). The concentration of culturable *E. coli* and *Salmonella* in reclaimed water after distribution mainly depended on the total chlorine, with correlations (R^2) of −0.598 and −0.660 (Table 2). Previous research also indicated that an average chlorine concentration of 1.63 mg/L in the effluents was needed to control coliform

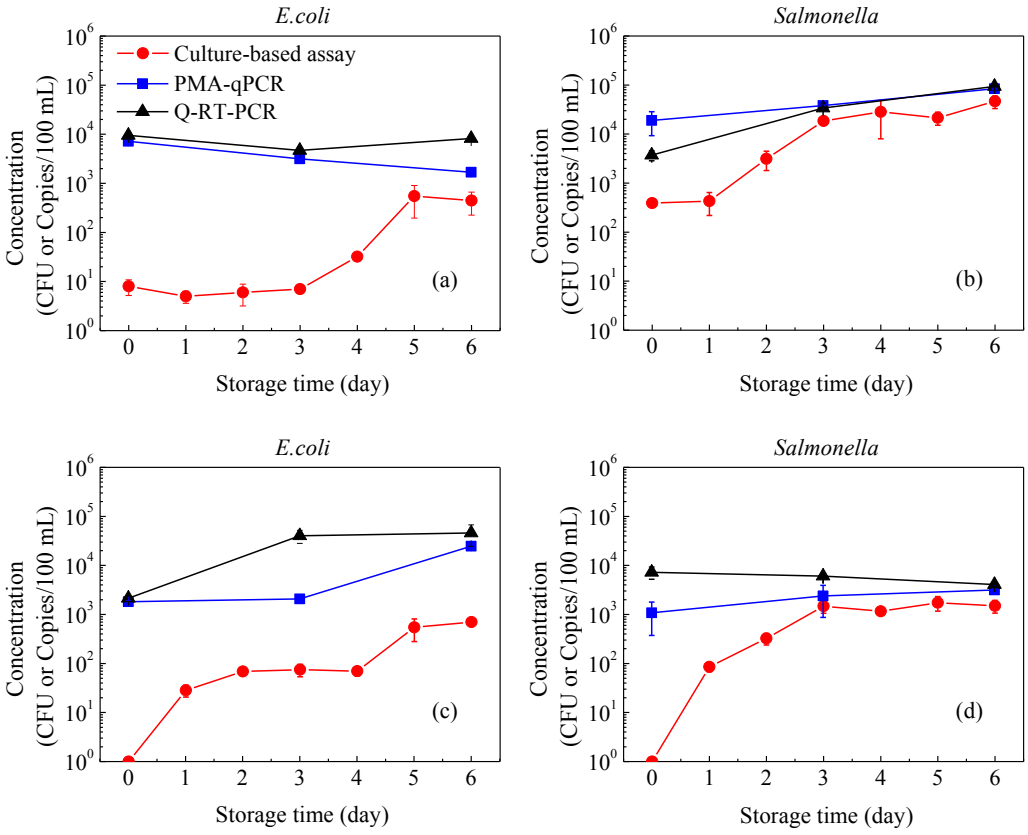

Fig. 4. Effects of storage period on the regrowth of *E. coli* and *Salmonella* in the Q (a and b) and W (c and d) distribution systems.

bacteria (Jjemba et al., 2009). In another study of a chlorined distribution system, the same range of heterotrophic bacteria was detected with corresponding levels of residual chlorine (Zhang and DiGiano, 2002). The strong negative relationship between the total chlorine and *E. coli* suggested that total disinfectant was an important factor influencing the growth of *E. coli* in the distribution system.

The nutrients showed little correlations with viable *E. coli* and *Salmonella*, only nitrate and TOC showed significant correlations. However, the nutrients levels showed that the reclaimed water was copiotrophic and therefore was suitable for many bacteria, such as coliforms, which require high nutrient concentrations (1 mg/L TN for 1.0×10^3 MPN coliform/100 mL) (Jjemba et al., 2009). The concentrations of most nutrients (TN, NO_3–N, NH_3–N, and TOC) and total chlorine (Table S3 and S4) fell after distribution, which was similar to other studies (Zhang and DiGiano, 2002). The correlation between TOC and the bacteria concentrations was statistically insignificant, which indicated that bacteria can grow in TOC concentrations ranging from 3.4 to 18.4 mg/L. A previous study indicated the concentration of *E. coli* significantly increased when the TOC concentration was above 2.4 mg/L (LeChevallier et al., 1991). Zhang also suggested that bacterial regrowth is a potential problem because water entering the distribution system usually contains biodegradable organic matter and nutrients, albeit in low concentrations (Zhang and DiGiano, 2002).

In general, reducing nutrient levels and increasing the total chlorine will inhibit bacterial regrowth in reclaimed water systems (Lin et al., 2012). However, higher total chlorine levels could introduce other risk issues, such as disinfection by-products, which have various toxic effects and carry significant health risks (Richardson et al., 2007). Additionally, it's crucial to minimize viable bacteria in treated effluents by controlling bacterial regrowth in the distribution system. Therefore, innovative and efficient bacteria detection methods, e.g. PMA-qPCR assay, are recommended for monitoring and predicting bacterial regrowth in reused wastewater.

Table 2
Correlation coefficients (r) for *E. coli* and *Salmonella* concentrations and various water quality parameters in the reclaimed water samples.

Microbe	Detection methods	*n*	TP	NH_3–N	NO_3–N	COD	TOC	Chlorine
E. coli	Culture	48	−0.148	0.165	−0.166	−0.072	−0.073	−0.598**
	PMA-qPCR	48	0.249	−0.033	0.489**	0.379*	−0.147	−0.029
	Q-RT-PCR	48	0.250	−0.185	0.428**	0.295	−0.414**	0.055
Salmonella	Culture	48	−0.241	0.177	−0.305	−0.249	−0.089	−0.660**
	PMA-qPCR	48	0.244	0.002	0.458**	0.486**	−0.220	−0.048
	Q-RT-PCR	48	0.302	−0.013	0.327*	0.360	0.022	−0.129

n = number of samples.
An asterisk indicates significance at the 0.05 (*) level.
An asterisk indicates significance at the 0.01 (**) level.

4. Conclusion

The results of this study have provided a comprehensive and long-term evaluation of the removal and regrowth of viable bacteria throughout the water reclamation treatment process and their subsequent distribution systems. All the reclamation treatment and disinfection processes have the potential to induce bacteria into the VBNC state, and both *E. coli* and *Salmonella* can regrow during the storage and distribution processes. This includes bacteria derived from reactivation from the VBNC state and reproduction by viable bacteria. The growth of *E. coli* and *Salmonella* mainly in reclaimed water in distribution system depended on the total chlorine, with correlations (R^2) of −0.598 and −0.660 for the culture-based assay. The significant correlations between bacteria with membrane integrity in the reclamation effluents shown by the PMA-qPCR, Q-RT-PCR and culturable bacteria assays of the samples taken at the end of distribution systems suggested that PMA-qPCR and Q-RT-PCR assay are efficient ways of measuring bacterial regrowth and can be used to predict bacterial regrowth during wastewater reclamation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2015.10.071.

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