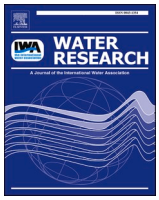
Water Research 245 (2023) 120585

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres



Self-repair and resuscitation of viable injured bacteria in chlorinated drinking water: *Achromobacter* as an example

Zengyi Hu, Xiaohui Bai *

State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

ARTICLE INFO

Keywords:
Drinking water
Chlorine disinfection
Intermediate injured state
Cellular repair
Resuscitation

ABSTRACT

Chlorine disinfection for the treatment of drinking water can cause injury to the membrane and DNA of bacterial cells and may induce the surviving injured bacteria into a viable but non-culturable (VBNC) state. It is difficult to monitor viable injured bacteria by heterotrophic plate counting (HPC), and their presence is also easily miscalculated in flow cytometry intact cell counting (FCM-ICC). Viable injured bacteria have a potential risk of resuscitation in drinking water distribution systems (DWDSs) and pose a threat to public health when drinking from faucets. In this study, bacteria with injured membranes were isolated from chlorinated drinking water by FCM cell sorting. The culture rate of injured bacteria varied from 0.08% to 2.6% on agar plates and 0.39% to 6.5% in 96-well plates. As the dominant genus among the five identified genera, as well as an opportunistic pathogen with multiple antibiotic resistance, *Achromobacter* was selected and further studied. After treatment with chlorine at a concentration of 1.2 mg/L, *Achromobacter* entered into the intermediate injured state on the FCM plot, and the injury on the bacterial surface was observed by electron microscopy. However, the CTC respiratory activity assay showed that 75.0% of the bacteria were still physiologically active, and they entered into a VBNC state. The injured VBNC *Achromobacter* in sterile drinking water were resuscitated after approximately 25 h. The cellular repair behavior of injured bacteria was studied by Fourier transform infrared attenuated total reflectance (FTIR-ATR) and comet assays. It was found that DNA injury rather than membrane injury was repaired first. The expression of *Ku* and *ligD* increased significantly during the DNA repair period, indicating that non-homologous end-joining (NHEJ) played an important role in repairing DNA double-strand breaks. This study deepened the understanding of the effect of chlorine disinfection on bacterial viability in drinking water and will provide support for the improvement of the chlorine disinfection process for the treatment of drinking water.

1. Introduction

Access to clean and safe drinking water is important for health and prosperous communities. Monitoring microbial contamination is a necessary measure to safeguard drinking water quality and an essential task for water utilities (Favere et al., 2021). Heterotrophic plate counting (HPC) is one of the most common microbial monitoring methods used by water utilities (Sartory, 2004; Chowdhury, 2012), and its basic principle is that the number of viable bacteria in the drinking water is reflected by the culturable bacterial counts on the plate (Chen et al., 2018). However, chlorine, as a widely used disinfectant for the treatment of drinking water (Deborde and von Gunten, 2008; Ma and Bibby, 2017), can cause cellular injury to bacteria and easily induce them into a viable but nonculturable (VBNC) state given that chlorine is a strong oxidative stressor (Turetgen, 2008; Chen et al., 2018; Qi et al., 2022; Zhu et al., 2022). Notably, most bacteria in the environment are naturally in the VBNC state and have never been cultured on the medium (Achtman and Wagner, 2008). The presence of disinfectant-induced injured VBNC bacteria and natural VBNC bacteria led to a significant underestimation of viable bacterial counts in the drinking water when HPC was applied for quantification.

To more accurately assess the drinking water disinfection efficiency and the microbiological quality, another bacterial quantification method, flow cytometry (FCM), has been used as an alternative to HPC by water utilities and researchers in the last two decades (Hoefel et al., 2003; Prest et al., 2014; Van Nevel et al., 2017). FCM intact cell counting

* Corresponding author.
E-mail address: xhbai@sjtu.edu.cn (X. Bai).

https://doi.org/10.1016/j.watres.2023.120585
Received 5 July 2023; Received in revised form 24 August 2023; Accepted 4 September 2023
Available online 5 September 2023

(ICC) discriminates between live and dead bacteria based on the integrity of the cell membrane, rather than the culturability, providing a fast, accurate, and reproducible technique for microbial monitoring (Kennedy et al., 2011; Van Nevel et al., 2017). However, the potential viability of bacteria with injured membranes in chlorinated drinking water has not yet been fully understood in FCM-ICC. As a strong oxidizing agent, chlorine acts on the cell wall and membrane, attacking the double bonds of the lipid bilayer and the amino acids of the membrane proteins, leading to the loss of membrane integrity and the leakage of inner cell components (Arnhold et al., 2001; Cho et al., 2010). Moreover, due to its small molecular size and lack of electrical charge, hypochlorous acid (HClO) easily diffuses into cells and rapidly reacts with the nucleophilic structure of proteins and DNA, resulting in misfolding of proteins and breakage of DNA chains (Hawkins and Davies, 2002; Winter et al., 2008; da Cruz Nizer et al., 2020). Injured but viable bacteria are generally not included in FCM-ICC because injury, especially cell membrane injury, is considered by most researchers as a reliable indicator of bacterial viability loss (Vives-Rego et al., 2000; Ramseier et al., 2011; Kirschner, 2016; Brown et al., 2019).

However, it may be unreasonable to dismiss all injured bacteria as dead. Whether bacteria are completely inactivated should depend on the degree of injury. Water utilities have to limit the chlorine dosage due to the formation of chlorinated disinfection byproducts (Kali et al., 2021), and some injured bacteria may survive a nonlethal disinfectant dose in an intermediate state. This intermediate state between the “live” and “dead” populations can be observed on the FCM plot when bacterial cells are stained with SYBR Green I and propidium iodide. The occurrence of intermediate states corresponds to a specific degree of membrane injury, which is higher than that of the “live” population but lower than that of the “dead” population. However, the above hypothesis must be based on the fact that these injured bacteria in the intermediate state are actually viable. In our study, intermediate-state bacteria were considered potentially viable injured bacteria in chlorinated drinking water and should be further studied.

The study of viable injured bacteria can facilitate a better evaluation of the chlorine disinfection efficiency of drinking water and provide support for its improvement. Moreover, viable injured bacteria in the water distribution system are closely related to the phenomenon of microbial regrowth and its control. In drinking water distribution systems (DWDSs), viable injured bacteria may attach to the pipe wall as biofilms (van der Kooij et al., 2003) and thus have a sufficient amount of time for the repair of injured cellular structures, such as the cell membrane and DNA (Cox, 1999; Cooper and McNeil, 2015; Dupuy et al., 2019; Endutkin and Zharkov, 2021), and then regain the multiplication ability. Similarly, secondary water supply facilities also provide long retention times for the resuscitation of injured bacteria (Zhao et al., 2020), which finally results in excessive microbial detection in the users’ tap water. However, little attention has been given to the presence of surviving injured bacteria after chlorine disinfection in the microbial quality evaluation of drinking water, and their potential contribution to microbial regrowth in DWDSs has been greatly overlooked. It is essential to answer the following questions: (1) Are the intermediate state bacteria actually viable injured bacteria in chlorinated drinking water? (2) Can injured bacteria complete cellular repair and then be resuscitated in DWDSs? (3) What are the core biological mechanisms involved in the repair and resuscitation of injured bacteria?

To answer the above questions, FCM cell sorting was used to isolate the injured bacteria from chlorinated drinking water samples. FCM cell integrity analysis, transmission electron microscopy (TEM), scanning electron microscopy (SEM) and CTC respiratory activity assays were used to study the characteristics of chlorine-treated *Achromobacter*, a representative species of culturable injured bacteria sorted from drinking water. Sterile drinking water was used as the resuscitation condition for injured *Achromobacter* to simulate the environment in DWDSs. Fourier transform infrared attenuated total reflectance (FTIR-ATR), comet assay and RT−qPCR were used to study the cellular repair behavior in injured *Achromobacter* and the expression of related genes. Our study demonstrated the viability of injured bacteria in chlorinated drinking water, in which their presence results in an overestimation of disinfection efficiency and microbiological quality by HPC or FCM-ICC. Viable injured bacteria have a potential risk of resuscitation in DWDSs after completing DNA repair, and opportunistic pathogens may pose health risks.

2. Materials and methods

2.1. Water samples

Water sampling was conducted in a drinking water treatment plant (DWTP) and its drinking water distribution systems (DWDSs) in Shanghai, China (Fig. 1). A detailed description of the water quality is displayed in Table S1. The drinking water treatment process includes sedimentation, sand filtration, ozone oxidation, biological activated carbon filtration, and chlorine disinfection. Samples were collected in October 2022 and May 2023, kept on ice until transport to the laboratory and processed within 6 h.

2.2. Flow cytometry (FCM) cell integrity analysis and injured bacteria sorting

Moreover, 7-Aminoactinomycin D (7-AAD), an ideal substitute for propidium iodide (PI), was used to stain bacteria in the drinking water for cellular integrity analysis due to the smaller emission spectra overlap between 7-AAD and SYBR Green I (SGI) (Schmid et al., 1992). SGI working solution was made by diluting SGI (Solabio, China) 100-fold with dimethyl sulfoxide (Hammes et al., 2008). For each water sample, 1 mL was added to 10 μL SGI working solution (Lee et al., 2016) and 1 μL 7-AAD (Sangon Biotech, China). The samples were incubated in the dark for 15 min before analysis (Lee et al., 2016). FCM analysis of injured bacteria and cell sorting were performed on CytoFLEX (Beckman Coulter, USA) and BD FACSAria II (BD Biosciences, USA), respectively, with 488 nm excitation from a blue solid-state laser. Red fluorescence was measured at 690 nm (PC5.5-A channel), and green fluorescence was measured at 525 nm (FITC-A channel). Data were acquired and analyzed using CytExpert Software and BD FACSDiva Software. Manual gating of cell populations was performed according to the previously described strategy (Berney et al., 2007), and cells in the region of the “intermediate state” (Fig. S1 and Fig. S2) were conceived as potentially viable injured bacteria. A single injured bacteria was loaded into each well of the 96-well plate containing R2A broth, and a total of 10,000 injured bacteria were loaded into 1 mL sterile PBS solution to be spread on R2A agar plates.

2.3. Cultivation and identification of injured bacteria (genus level)

The 96-well plates were incubated at 28 ℃ for 10 days with shaking at 120 r/min. Agar plates were incubated at 28 ℃ for 10 days. The culture rate of injured bacteria was calculated based on the turbidity of the liquid medium in the 96-well plate and colony formation on the agar plate (Fig. S3). The cultured strains were purified three times for DNA extraction. Universal primers 27F and 1492R were used to amplify the 16S rRNA gene (Johnson et al., 2019), and the amplified sequences were used for NCBI BLAST (http://blast.ncbi. nlm.nih.gov/Blast.cgi) (Miao et al., 2022).

2.4. Species identification of Achromobacter and culture conditions

According to the previously described method (Spilker et al., 2013), *Achromobacter* isolates were analyzed by the *nrdA* gene sequence cluster to differentiate species. A pure culture of *Achromobacter* was incubated in LB broth at 37 ℃ for 12 h with shaking at 180 r/min to obtain cells in the early stationary phase. The bacterial culture was centrifuged at

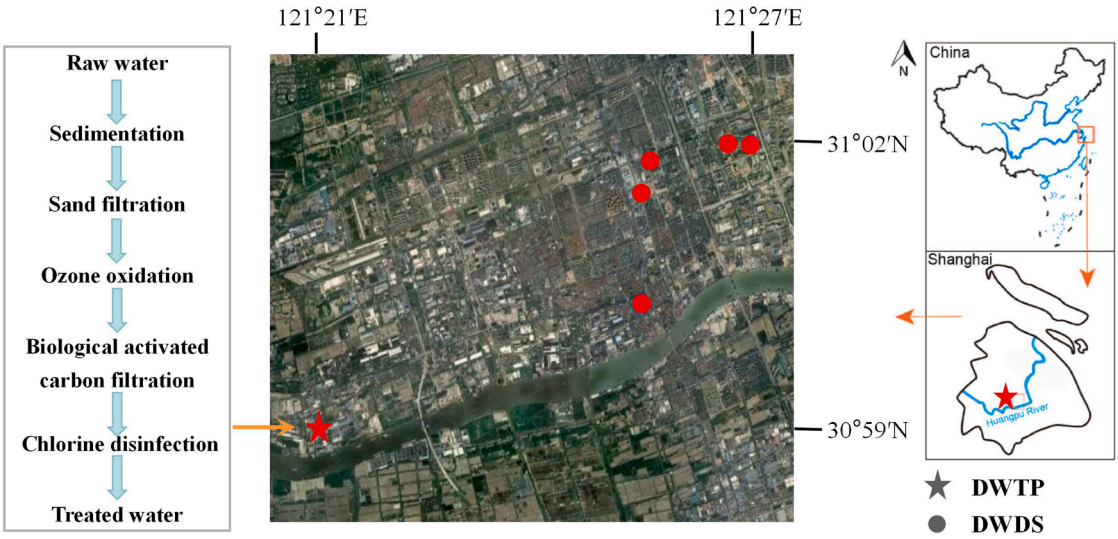


Fig. 1. Geographic locations of the sampling sites. (Two treated water samples were collected from the DWTP, and five tap water samples were collected from its DWDSs).

5000 × g for 5 min, and the supernatant was discarded and washed twice with sterile PBS solution.

2.5. Chlorine disinfection

The cells described above were resuspended and diluted in sterile PBS solution to approximately 10^5 CFU/mL. Sodium hypochlorite (NaClO) aqueous solution was made by diluting a NaClO commercial solution 100 times, and the residual chlorine was measured by a Pocket Colorimeter II (HACH, USA). A certain amount of NaClO aqueous solution was added to the bacterial suspension to achieve the target initial disinfectant concentrations (0.4, 0.8, 1.2, 1.6, 2.0, 3.0, 4.0 and 5.0 mg/L). Samples were taken at 1 h for analysis of bacterial characteristics (e.g., membrane integrity, morphology, respiratory activity), and excess sodium thiosulfate solution (1.5%, 1:1000) was added to quench the chlorine in the sample.

2.6. Culturable cell counting

A 200 μL appropriately diluted bacterial suspension was plated on an LB agar plate and incubated at 37 ℃ for 48 h to obtain the quantity of culturable cells.

2.7. Resuscitation assay

An *Achromobacter* suspension of approximately 10^5 CFU/mL was treated with chlorine at a dose of 2 mg/L for 1 h to ensure that all bacteria entered into the intermediate state, indicating membrane injury, and culturable cell counts were 0 CFU/mL. The 200 μL sample described above was added to 20 mL sterile drinking water (filtered through a 0.22 μm polyethersulfone membrane and sterilized under UV light) in a 50 mL centrifuge tube and incubated at 28 ℃ for 32 h. One hundred microliter samples were taken each hour and plated on LB agar plates to determine the time required for the appearance of colonies. Three independent experiments were performed, and colony counts were performed in triplicate. Resuscitation of *Achromobacter* in the VBNC state was considered to have occurred if the generation time of cells was far longer than that of control *Achromobacter* (Chen et al., 2018).

2.8. Characteristics of injured bacteria

2.8.1. Morphological observations

TEM observation: Control *Achromobacter* and injured *Achromobacter* were fixed with 2.5% glutaraldehyde for 6 h at 4 ℃. Cells were washed four times with PB solution and then stained with osmic acid for 1.5 h. After gradually dehydrating using ethanol and acetone, cells were infiltrated and embedded with resin. The prepared samples were cut using an EM UC7 Ultra-Microtome (Leica, Germany), and the sliced samples were stained with uranyl acetate and lead citrate. Finally, the prepared grids were observed using a HT7800 (HITACHI, Japan).

SEM observation: The samples were fixed with 2.5% glutaraldehyde for 6 h at 4 ℃. Cells were washed with PBS and then sequentially dehydrated for 10 min in ethanol (50%, 70%, 90%, and 100%). The cells then underwent vacuum freeze drying and were coated with gold. Morphological observations were performed using an S3400II (HITACHI, Japan).

2.8.2. Respiratory activity

5-cyano-2,3-dimethylphenyl tetrazolium chloride (CTC) can be reduced to red fluorescent CTC formazan by the electron transport system of cells, which is a direct indicator of oxidative metabolism and viability (Creach et al., 2003). A 20 μL CTC solution (Dojindo, Japan) was added to 1 mL of bacterial suspension to a final concentration of 1 mM. The samples were incubated at 37 ℃ for 30 min, and the fluorescence intensity at 585 nm (Sieracki et al., 1999) was measured by CytoFLEX (Beckman Coulter, USA). The gating strategy for CTC-stained viable cells is shown in Fig. S4.

2.8.3. Determination of the peroxidation degree of membrane lipids

To investigate the cell membrane repair behavior of chlorine-injured *Achromobacter*, Fourier transform infrared attenuated total reflectance (FTIR-ATR) spectroscopy was used to assess the peroxidation extent of bacterial membrane lipids. According to the method described previously (Minnikin et al., 1984), membrane lipids of untreated *Achromobacter*, injured *Achromobacter* immediately after chlorine disinfection, and injured *Achromobacter* incubated in sterile drinking water for 10 h and 20 h were extracted. The extracted lipid solution was placed on a crystal of the ATR sampling accessory and allowed to form a film.

A Nicolet 6700 FTIR spectrophotometer (Thermo Fisher Scientific, USA) was used to record spectra in the 4000–400 cm^{-1} range with a 4 cm^{-1} resolution. The extent of lipid peroxidation was characterized by the following metrics: the ratio of the intensity of bands ν(C = O) and

ν(C = C), the ratio of the intensity of bands $\nu_{as}(CH_3)$ and $\nu_{as}(CH_2)$, and the ratio of the intensity of bands ν_s(PO- 2) and ν(R-O-P-O-R').

2.8.4. Detection of DNA damage

A Bacterial Comet Assay Kit (GENMED, USA) was used to detect DNA strand breaks and lesions of the injured *Achromobacter*. In the comet assay, the damaged denatured DNA migrates out of the cell by electrophoresis, forming the "tail" of the "comet", while the intact superhelical DNA remains in the nucleus (Dunkenberger et al., 2022). The images of 100 random comets from each sample were captured on an Eclipse 50i fluorescence microscope (Nikon, Japan), and the percentage of tail DNA (relative fluorescence intensity of the tail) was calculated using ImageJ Software.

2.8.5. Gene expression levels analyzed by RT–qPCR

Total RNA was extracted using RNAisoPlus (TaKaRa, Japan) and transcribed to cDNA with Hifair III 1st Strand cDNA Synthesis SuperMix for qPCR (Yeasen, China) according to the manufacturer's instructions. Specific primers were designed by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) according to the corresponding sequences in the reference genome of *Achromobacter pulmonis* and then evaluated by Oligo 7 Software. The qPCRs were performed in triplicate with Universal Blue qPCR SYBR Green Master Mix (Yeasen, China) using a CFX96 Connect Real-Time PCR Detection System (Bio-Rad, USA). The normalized fold changes in target gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method (Ginzinger, 2002).

2.9. Statistical analysis

Intact, viable cell counts and colony-forming units were log10 transformed. Percentage, mean, and standard deviation (SD) were calculated in Microsoft Excel. Analysis of variances (ANOVA) was performed using RStudio (Version 4.2.2), and all data analyzed in this study were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. FCM sorting and cultivation of intermediate injured bacteria

The unique pattern of intermediate state bacteria was observed by FCM analysis of a tap water sample with a residual chlorine concentration of 0.58 mg/L (Fig. 2a), as well as other chlorinated drinking water samples (Fig. S1). They were suspected to contain viable injured bacteria. Intermediate injured bacteria from seven water samples were sorted by FCM (Fig. 2b) and then cultured in 96-well plates containing R2A broth and on R2A agar plates. The culture rate of injured bacteria varied from 0.39% to 6.5% in 96-well plates and 0.08% to 2.6% on agar plates (Fig. 2c). A strong negative correlation ($R^2 > 0.8$) was found between the culture rate and the total chlorine concentration in the drinking water, and the culture rate of injured bacteria in tap water was much higher than that in treated water. This may be because the originally unculturable injured bacteria recovered their culturability with the decrease in chlorine concentration during the drinking water distribution process. The sorting and culture mode of single cells provided a better niche for injured bacteria and thus achieved a higher culture rate than that on plates, which implied an underestimation of the number of viable injured bacteria in drinking water when using heterotrophic plate counting (HPC).

A total of five genera were observed in the culture results: *Achromobacter, Stenotrophomonas, Pannonibacter, Pseudomonas* and

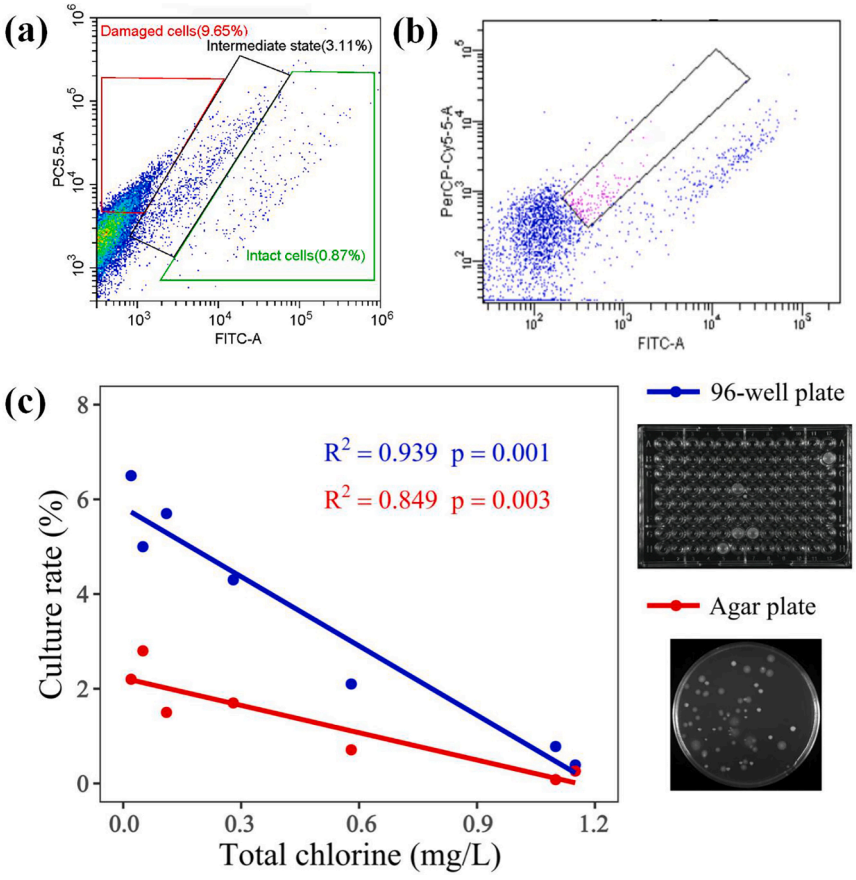


Fig. 2. FCM cell sorting and cultivation of injured bacteria. (a) The gating strategy for intermediate-state bacteria in FCM analysis. (b) The target area in FCM cell sorting. (c) Correlation between the culture rate of injured bacteria and total chlorine in the water samples.

Microbacterium (Table 1). Among these culturable injured bacteria, *Achromobacter* was the most abundant genus, an emerging opportunistic pathogen of environmental origin that is generally recovered from persons with cystic fibrosis (CF) (Isler et al., 2020). Moreover, *Achromobacter* can cause difficulties in clinical treatment due to its resistance to multiple antibiotics, e.g., aminoglycosides, tetracyclines and β-lactams. Therefore, *Achromobacter* was selected as the representative species of culturable injured bacteria in the drinking water and was further studied.

Culturability is sufficient unnecessary proof of bacterial viability. To obtain bacterial strains for the subsequent study, we used a culture-based strategy to demonstrate the viability of intermediate injured bacteria sorted by FCM. This is a high-speed and high-throughput method for the identification and acquisition of viable injured bacteria and is applicable to different types of water samples. However, it should be noted that oxidative stress can easily induce bacteria into a viable but nonculturable (VBNC) state, which means that the number of culturable injured bacteria in chlorinated drinking water is far lower than that of actually viable injured bacteria. A more appropriate research strategy should be developed for their quantification.

In addition, the membrane injury caused by chlorine disinfection may not be the only reason why the intermediate state bacteria on FCM dot plots were stained with 7-AAD. Some bacterial cells that are actively dividing may also be incorrectly stained due to altered permeability (Shi et al., 2007). To exclude this possibility, we further investigated the occurrence of injury and the survival ability of *Achromobacter* with chlorine treatment.

3.2. Cell membrane injury of Achromobacter with chlorine treatment

Chlorine-treated *Achromobacter* were stained with SGI and 7-AAD to provide information on cell membrane injury. The bacterial population showed a clear and distinctive staining pattern on the flow dot plots (Fig. 3): intact cell population (green gate area), damaged cell population (red gate area) and intermediate state cell population between them (black gate area). Before chlorine treatment, the vast majority of *Achromobacter* cells appeared in the intact and damaged cell populations (Fig. 3a), the so-called “live” and “dead” populations. As the chlorine doses increased, the fluorescence intensity of 7-AAD increased, while the fluorescence intensity of SYBR Green I diminished, resulting in a shift in the cell population from the intact cell area toward the intermediate state area. When the chlorine doses reached 1.2 mg/L (Fig. 3d), over 99% of the *Achromobacter* entered into the intermediate state area, marking the loss of cell membrane integrity and altered permeability. Such a disinfectant dose was close to that used in actual situations, indicating that the reason why *Achromobacter* were in the intermediate state cell population in FCM sorting might indeed be the injury by chlorine disinfection. With a further increase in chlorine doses to 1.6 mg/L (Fig. 3e), the concentration of bacterial cells in the intermediate state decreased, which can be attributed to the aggravation of membrane damage.

To provide direct morphological evidence for the occurrence of cell membrane injury, SEM and TEM were used to observe the injured *Achromobacter* in the intermediate state (Fig. 4). Before chlorine treatment, the boundary of the cell membrane appeared clear and smooth in SEM observations; however, the treated cells exhibited a crumpled appearance, and the surfaces were no longer smooth. TEM images also found that chlorine disinfection blurred the cell boundary and made it look rough, indicating injury to the cell membrane and cell wall. The cytoplasmic material condensed in some regions, but no leakage of the cytoplasm was observed, which remained a separate entity from the environment. In addition, the chlorine-treated bacteria maintained the basic morphology of a cell, suggesting the potential viability of injured *Achromobacter*.

Table 1
Composition of culturable injured bacteria isolated from the drinking water samples.

Order	Family	Genus	Proportion
Burkholderiales	Alcaligenaceae	*Achromobacter*	45.1%
Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	26.4%
Hyphomicrobiales	Stappiaceae	*Pannonibacter*	16.2%
Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	7.8%
Micrococcales	Microbacteriaceae	*Microbacterium*	4.5%

In previous studies, it was generally acknowledged that the occurrence of cell membrane injury implies the loss of bacterial viability. Injured cells cannot maintain any electrochemical gradient (Vives-Rego et al., 2000). Their cellular structures are exposed to the environment and will eventually degrade. However, such a view does not take into account the extent of membrane injury and the time node at which the bacteria are eventually inactivated. Although unbalanced transmembrane transport is toxic to the bacterial cell (Espiritu, 2021), this may not mean that the injured bacteria will die immediately. Moreover, as long as the enzymes indispensable for bacterial life are still active and the DNA template remains intact, the cell is likely to have metabolic capacity and proliferation potential (Burby and Simmons, 2020). The intermediate-state bacteria on FCM dot plots may suffer nonlethal membrane injury, and further studies are needed to investigate their actual viability.

3.3. Respiratory activity of injured Achromobacter

Chlorine disinfection can cause injury to the cell membrane of *Achromobacter* and effectively decrease the FCM intact cell counts, as shown in Fig. 3. However, the decrease in physiologically viable cell counts was not noticeable in comparison. As shown in Fig. 5a, after 1 h of exposure to 1.2 mg/L free chlorine, the intact cell counts of *Achromobacter* decreased from 10^5 cells/mL to 10^3 cells/mL, indicating that the disinfectant caused membrane injury of 99% of the bacteria. However, the viable cell counts were still close to 10^5 cells/mL at this time, and 75% of the *Achromobacter* maintained a certain level of respiratory activity. The significant difference between the intact cell counts and viable cell counts strongly suggested the existence of viable injured *Achromobacter* in the intermediate state (Fig. 3d). To truly inactivate *Achromobacter*, the concentration of chlorine disinfectant was increased to 5 mg/L, and a 97.7% decrease in viable cell counts was observed. Fig. 5b shows the respiratory activity levels of the injured viable *Achromobacter* treated with various doses of chlorine. Bacterial respiratory activity significantly decreased ($P < 0.05$) after chlorine treatment and plateaued at a low level (approximately 49.2% of the control) after 3 mg/L. Lower respiratory activity means less energy and substance metabolism in the injured cells, and therefore fewer substrates are taken up from the environment, as well as chlorine (Chen et al., 2018). Notably, low metabolic activity has been found in many studies to be closely related to the transition of bacteria to a viable but nonculturable (VBNC) state (Lin et al., 2017).

As a cultivation-independent method, FCM is currently widely used to monitor microbiological drinking water quality and evaluate disinfection efficiency. However, our results suggested that for some bacteria, cell membrane injury may not be a conservative indicator of cell death. It is well known that the envelope of gram-negative bacteria is composed of both an inner and an outer membrane. The outer membrane provides the bacterial cell with a remarkable permeability barrier, and the inner membrane is where life-sustaining activities such as electron transport and ATP synthesis occur (Doerrler, 2006). The presence of the intermediate state bacteria on the flow dot plot corresponds to the first step of membrane injury, the outer membrane injury (Berney et al., 2007), while the inner membrane of the bacteria may still be fully functional. This may explain why the injured *Achromobacter* in the intermediate state maintained their respiratory activity. When treated with 5 mg/L chlorine disinfectant, more injured *Achromobacter* further shifted from

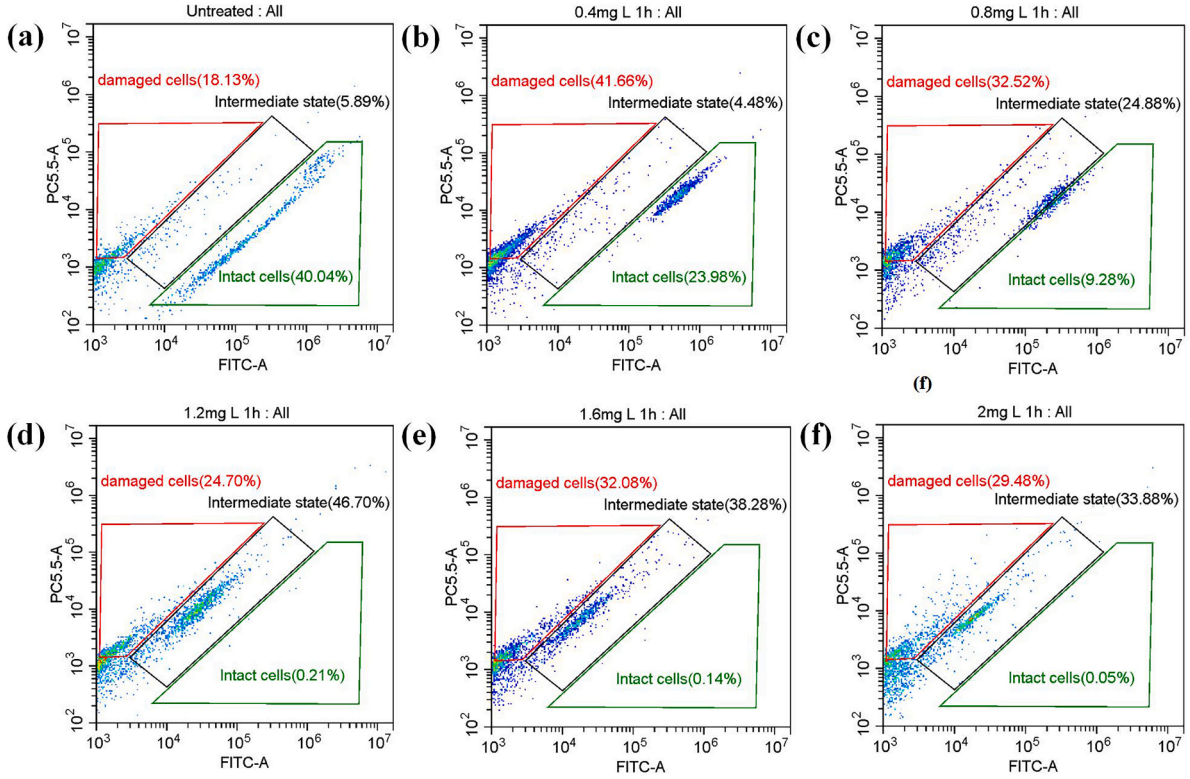


Fig. 3. FCM cell integrity analysis of *Achromobacter* treated with various doses of chlorine. (a) 0 mg/L. (b) 0.4 mg/L. (c) 0.8 mg/L. (d) 1.2 mg/L. (e) 1.6 mg/L. (f) 2.0 mg/L.

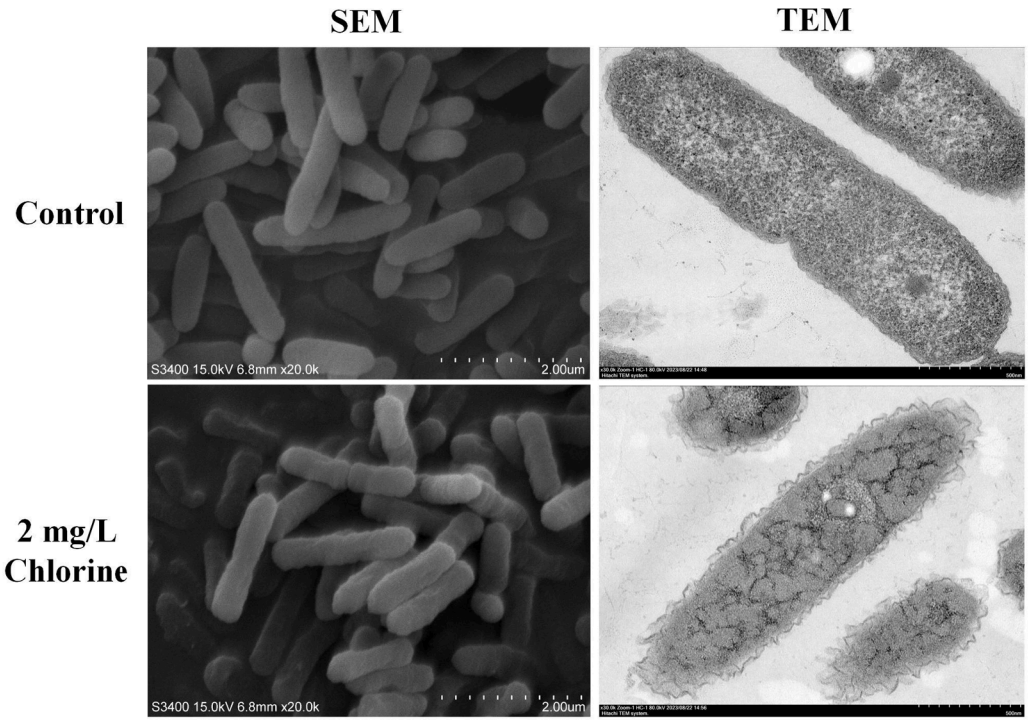


Fig. 4. Morphological characteristics of chlorine-injured *Achromobacter* under SEM (scanning electron microscopy) and TEM (transmission electron microscopy).

the intermediate state area to the damaged cell area on the flow dot plot (Fig. S5), indicating the occurrence of inner membrane injury and true bacterial inactivation.

Viable injured bacteria in chlorinated drinking water have long been neglected in FCM intact cell counting because they are in the intermediate state. To obtain a more realistic assessment of the chlorine disinfection efficiency of drinking water by using FCM, as well as the true inactivation of certain opportunistic pathogens, it is necessary to include

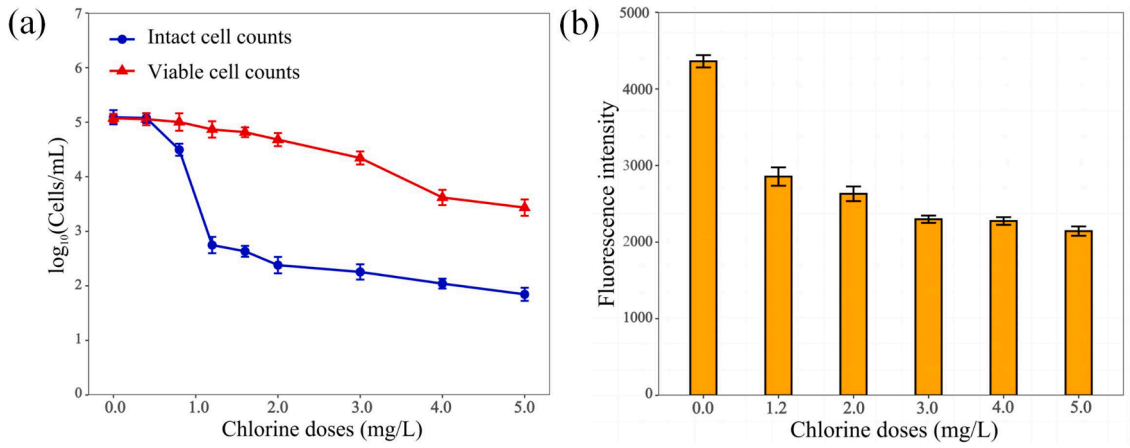


Fig. 5. CTC respiratory activity assay of *Achromobacter*. (a) Intact and viable *Achromobacter* cell counts after treatment with various doses of chlorine. (b) Levels of respiratory activity (calculated by the average florescence intensity per cell).

the intermediate injured bacteria in the investigation and injure them further. The different microbial counting methods described in this study should also facilitate a more accurate characterization of the efficiency of other oxidative disinfection processes (e.g., ozone), as well as the improvement of drinking water disinfection techniques.

3.4. Resuscitation of injured VBNC Achromobacter

The injured *Achromobacter* with 2 mg/L of chlorine treatment were immediately spread on LB agar plates, but no colony-forming unit was observed, indicating that the injured bacteria had entered into a viable but nonculturable (VBNC) state. To simulate the real living conditions of bacteria in DWDSs, sterile drinking water was used as the resuscitation environment for injured VBNC *Achromobacter* (Fig. 6). After the addition of injured VBNC *Achromobacter* into sterile drinking water, the culturable cell counts were 0 CFU/mL for up to 24 h and then rapidly reached approximately 10^3 CFU/mL at 25 h, 28 h, and 29 h in three different batches. However, the control *Achromobacter* only took approximately 9 h to enter into the exponential growth phase. The different generation times of the control *Achromobacter* and that of the injured *Achromobacter* suggested that VBNC cells were successfully resuscitated. In our study, the appearance of culturable cells was the result of resuscitation of injured bacteria rather than a small amount of intact bacteria in the sample, since the intact cell concentration was diluted to less than 1 cell/mL.

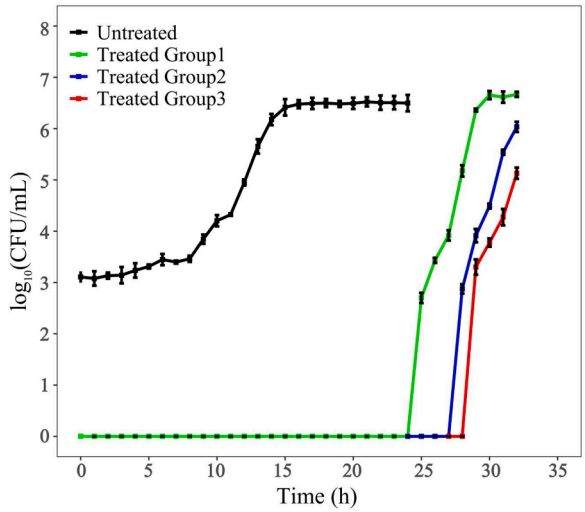


Fig. 6. Resuscitation of injured VBNC *Achromobacter* in sterile drinking water.

In previous studies, favorable resuscitation conditions such as LB broth (Zhang et al., 2015) were controlled for VBNC bacteria, but drinking water is in fact an oligotrophic environment. Resuscitation of VBNC bacteria in the medium does not mean that the same result will occur in DWDSs. In this study, the nutritional conditions of bacteria in DWDSs were well simulated, and resuscitation was also observed. However, there may still be some limitations in our study because the shear forces of water flow in pipelines were not taken into account, which may be detrimental to the survival of fragile injured cells. However, this is the first report that chlorine-induced VBNC bacteria can be resuscitated in drinking water and that they are membrane injured and not detected when using HPC or FCM-ICC. This study on *Achromobacter* provides valuable insight into the resuscitation behavior of other injured VBNC pathogens in drinking water. In the future, more studies are needed to focus on the actual viability of chlorine-injured bacteria in treated drinking water and their fate in the water distribution system.

3.5. Cellular repair behavior of injured VBNC Achromobacter before resuscitation

To some extent, the resuscitation of VBNC bacteria is a reshaping process involving cellular repair. The cell membrane and DNA are the permeability barrier and genetic material of the cell. We investigated the repair behavior of injured VBNC *Achromobacter* on these two important cellular structures.

3.5.1. Repair behavior of injured VBNC Achromobacter on the cell membrane

FCM analysis was used to track the changes in cell membrane integrity of injured *Achromobacter* after chlorine disinfection (Fig. 7a). No significant decrease was observed in the number of intermediate injured bacteria up to 25 h after disinfection (Fig. 7b), which may imply that the new daughter cells were generated by the division of bacterial cells with injured membranes, and membrane repair was not a necessary preparation for resuscitation. In addition, the daughter cells will inherit a part of the injured membrane from the mother cells and synthesize new membranes at the equatorial plane at the same time. Therefore, the new cell population that occurred at 30 h after disinfection retained a certain membrane permeability, and their clustering on the FCM dot plot was similar to that in Fig. 3b.

Fourier transform infrared attenuated total reflectance (FTIR-ATR) spectroscopy was used to provide information on the peroxidation of the phospholipid bilayer structure (Fig. 7c), which is directly related to the alteration of membrane permeability in chlorine-treated bacteria. A new strong band and a new weak band occurred at 3674 cm^{-1} and 3380 cm^{-1} in the FTIR-ATR spectra of all three chlorine-treated samples,

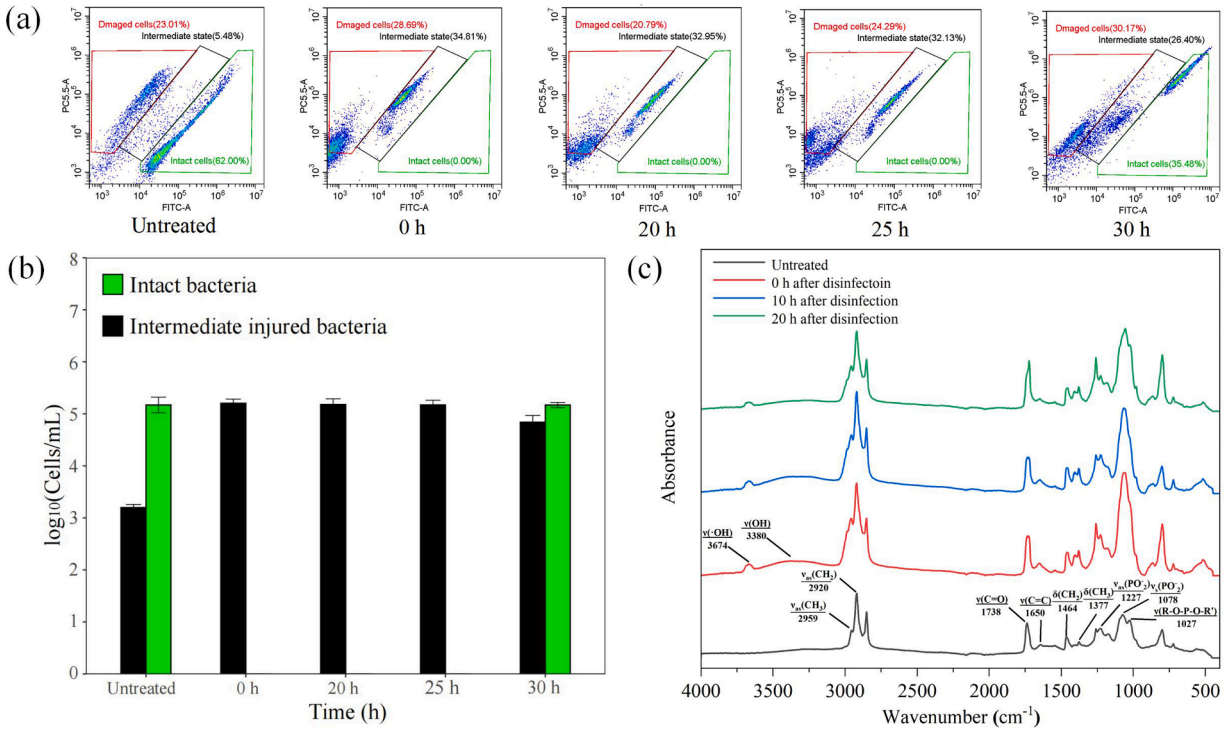


Fig. 7. Exploring the cell membrane repair behavior of injured *Achromobacter*. (a) FCM cell integrity analysis of *Achromobacter* at different times after chlorine disinfection. (b) Bar graph showing bacterial number in FCM analysis. (c) FTIR-ATR spectra of the membrane lipids extracted from *Achromobacter* at different times after chlorine disinfection.

which are attributed to the stretching vibration of hydroxyl radicals (•OH) and associative hydroxyl groups (-OH), respectively. •OH is a main reactive oxygen species formed by chlorine in the cell, which initiates the chain reaction of radical peroxidation of fatty acids in phospholipids, and lipid hydroxide (LOH) is one of the products of the termination stage. These two newly appearing bands strongly indicated the oxidative state of membrane lipids of injured *Achromobacter*. Moreover, in unsaturated fatty acyl chains, the hydrogen atoms on methylene groups adjacent to double bonds are more easily abstracted by reactive radicals, forming lipid oxides ($L = O$). The ratio of the absorbance of the ν(C = O) band at 1738 cm^{-1} to the absorbance of the ν(C = C) band at 1650 cm^{-1} in the chlorine-treated samples (3.2-, 2.7- and 3.0-fold) increased compared with that in the untreated sample (2.3-fold). Cleavage of fatty acyl chains caused by rearrangement of alkoxyl radicals is another result of peroxidation of phospholipids. The ratio of the asymmetric stretching vibration intensity of the methyl group ($\nu_{as}(CH_3)$) at 2959 cm^{-1} to that of the methylene group ($\nu_{as}(CH_2)$) at 2920 cm^{-1} increased in the chlorine-treated samples (from 0.43-fold to 0.60-, 0.58- and 0.57-fold). Alkanes, alkenes, hydroxides and aldehydes of different carbon chain lengths are released in the oxidation process of phospholipids (Reis and Spickett, 2012). The phosphate group of phospholipids is responsible for three main vibration bands at 1227, 1078 and 1027 cm^{-1}, corresponding to an R-O-P-O-R' vibration and to the symmetric and asymmetric PO- 2 stretching vibrations, respectively. The decrease in the intensity ratio of the ν(R-O-P-O-R') band to the ν_s(PO- 2) band (from 0.87-fold to 0.68, 0.65 and 0.70-fold) in chlorine-treated samples also indicated the cleavage of fatty acyl chains.

The FTIR-ATR spectra showed that the cell membrane lipids of the injured *Achromobacter* incubated in sterile drinking water for 10 h and 20 h still had typical oxidative structures, and the extent of lipid peroxidation was similar to that of the samples immediately treated with chlorine. Chlorine-injured *Achromobacter* may not undergo cell membrane repair or synthesize new cell membranes before resuscitation, which was consistent with the results of FCM. In fact, prokaryotes are rarely reported to possess the ability to repair cell membrane injury, and they are naturally protected by a hardened and impermeable cell wall. However, eukaryotic cells have always faced potentially lethal plasma membrane injury, and they have evolved powerful repair mechanisms such as vesicle fusion and contractile rings (Cooper and McNeil, 2015).

3.5.2. DNA repair behavior of injured VBNC Achromobacter

Genome stability is critical for the survival of cells and their division. The comet assay is a microelectrophoretic technique that can be used to visualize DNA damage in individual bacterial cells (Fig. 8a). Severe DNA damage was observed in *Achromobacter* immediately after 1 h of exposure to 2 mg/L free chlorine. The breakage of the DNA strand resulted in the formation of tailing in the electric field in the presence of the dye. However, comet images of the treated *Achromobacter* incubated in sterile drinking water showed that the tail became shorter at 10 h after disinfection and almost disappeared at 20 h after disinfection. This indicated that DNA damage was gradually repaired during this period, and the bacterial genome was restored to its original integrity. Furthermore, we quantified the DNA damage degree of the bacterial populations by calculating the percentage of tail DNA (Fig. 8b) and similarly determined DNA repair behavior.

Oxidative DNA injury may cause cell mutations or death and impede the progression of DNA replication along chromosomes unless repaired. The DNA repair of injured *Achromobacter* can be completed at 20 h after disinfection, which may be essential for subsequent resuscitation (Fig. 6). The first goal of cell division is to pass the intact and unchanged genetic material to the next generation (Verni, 2022), and cells must detect and repair DNA damage before replicating. Many studies have reported that the rejoining of DNA strand breaks in most cells is a rapid process with a half-time of only a few minutes (Frankenberg-Schwager, 1989). However, in our study, DNA repair of injured *Achromobacter* appeared to be very slow. This may be because they sustained oxidative damage from residual chlorine in drinking water during their repair incubation.

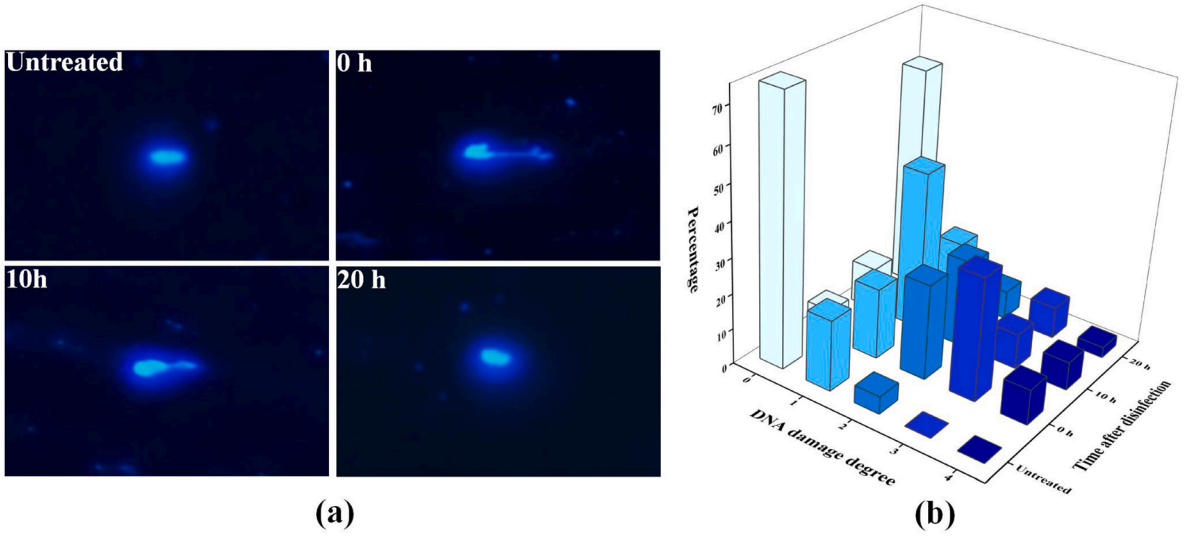


Fig. 8. Comet assay of *Achromobacter* at different times after chlorine disinfection. (a) Representative comet images of untreated cells and injured cells at 0 h, 10 h and 20 h after treatment. (b) Quantitative evaluation of DNA damage degree (Level zero: tail DNA% < 5, Level one: 5 ≤ tail DNA% < 20, Level two: 20 ≤ tail DNA% < 40, Level three: 40 ≤ tail DNA% < 95, Level five: tail DNA% ≥ 95).

3.5.3. *The pathway of DNA damage repair in chlorine-injured Achromobacter*

Modified bases and DNA double-strand breaks (DSBs) are common forms of oxidative DNA damage (Dizdaroglu, 2005). The expression levels of related genes were measured to explore the molecular mechanism of DNA damage repair in chlorine-treated *Achromobacter* (Fig. 9). *MutM* and *nth* are DNA glycosylases that recognize and release the modified base, generating an apurinic/apyrimidinic (AP) site; *Xth* is an AP endonuclease that generates a 5′-deoxyribosephosphate (5′-dRP) group and a nicked strand; *PolA* and *ligA* are a DNA polymerase and ligase, respectively, incorporating a new nucleotide and resealing the DNA backbone. These five genes described above constitute the base-excision repair (BER) pathway in bacteria. However, their expression levels either did not change significantly ($P > 0.05$) or decrease significantly ($P < 0.05$) after chlorine treatment (0 h, 10 h and 20 h), indicating that BER is not the critical repair mechanism involving the DNA damage repair of *Achromobacter*. Similarly, no significant upregulation was measured in *recA*, a key recombinase gene in the homologous recombination (HR) pathway of DSB repair. However, *Ku*, which encodes the recruiting protein, and *ligD*, which encodes the multifunctional enzyme carrying out nuclease, polymerase and ligase activities in the non-homologous end-joining (NHEJ) pathway of DSB repair, were both significantly upregulated ($P < 0.05$) after chlorine treatment (10 h and 20 h). In particular, the fold change of *ligD* expression was up to 7.63-fold at 20 h after chlorine treatment, implying that DNA damage was being effectively repaired at this time, which was consistent with the results in Fig. 8.

In contrast to previous research strategies (Bodet et al., 2012; Tong

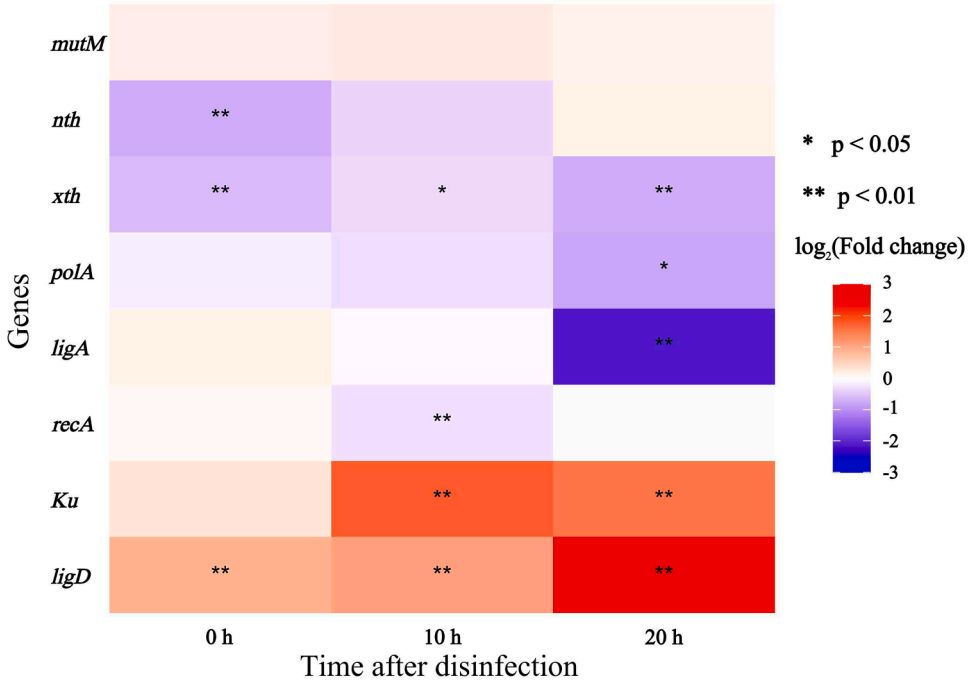


Fig. 9. Gene expression of *Achromobacter* after chlorine disinfection.

et al., 2021), we focused not only on the immediate stress response of bacteria against chlorine but also on the dynamic changes in gene transcript levels throughout the bacterial repair process. Based on the results of RT−qPCR, it was speculated that the main form of DNA damage in chlorine-disinfected *Achromobacter* might be the breakage of DNA double-strands, rather than the base pairs being affected, which is consistent with Chen's findings (Chen et al., 2018). Moreover, the injured *Achromobacter* used the low-fidelity NHEJ pathway independent of DNA templates to repair DSBs, rather than the traditional HR pathway. This phenomenon can be explained by the VBNC state of injured bacteria, in which DNA is not replicating and cannot be used as a template for HR repair. Notably, the NHEJ pathway is not ubiquitous in bacteria; it is found in approximately 20–25% of the kingdom (Bowater and Doherty, 2006; Amare et al., 2021). Some bacterial species possessing this repair apparatus, such as *Bacillus subtilis, Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*, are opportunistic pathogens, and their presence is frequently reported in DWDSs (Luo et al., 2021). This is an alarming phenomenon; injured pathogenic bacteria with the NHEJ pathway may be endowed with stronger DNA repair capacity and thus have resuscitation potential in DWDSs after the completion of DNA damage repair.

4. Conclusions

Chlorine disinfection for the treatment of drinking water can cause bacteria to lose cell integrity and enter an intermediate injured but viable state. Their low culture rate and injured cell membrane made it easy for them to escape the monitoring of HPC and FCM-ICC.

Achromobacter was one of the main viable injured bacteria identified and acquired by FCM cell sorting and cultivation. Most of the injured *Achromobacter* still had a certain level of respiratory activity and were not truly inactivated after different doses of chlorine disinfection.

Injured *Achromobacter* can enter a VBNC state and be resuscitated in drinking water. This report provided evidence for the resuscitation of injured VBNC bacteria in a real drinking water environment.

Completion of DNA damage repair was essential for the resuscitation of injured *Achromobacter* in drinking water. Time regulation and maintaining a certain level of residual chlorine concentration in the water distribution system can be helpful in avoiding the completion of bacterial DNA repair and thus controlling microbial regrowth in DWDSs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (NSFC) (51878406).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120585.

References

Achtman, M., Wagner, M., 2008. Microbial diversity and the genetic nature of microbial species. Nat. Rev. Microbiol. 6 (6), 431–440. https://doi.org/10.1038/nrmicro1872.

Amare, B., Mo, A., Khan, N., Sowa, D.J., Warner, M.M., Tetenych, A., Andres, S.N., 2021. LigD: a structural guide to the multi-tool of bacterial non-homologous end joining. Front Mol. Biosci. 8, 787709 https://doi.org/10.3389/fmolb.2021.787709.

Arnhold, J., Osipov, A.N., Spalteholz, H., Panasenko, O.M., Schiller, J., 2001. Effects of hypochlorous acid on unsaturated phosphatidylcholines. Free Radic. Biol. Med. 31 (9), 1111–1119. https://doi.org/10.1016/s0891-5849(01)00695-5.

Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.U., Egli, T., 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. Appl. Environ. Microbiol. 73 (10), 3283–3290. https://doi.org/10.1128/aem.02750-06.

Bodet, C., Sahr, T., Dupuy, M., Buchrieser, C., Hechard, Y., 2012. Legionella pneumophila transcriptional response to chlorine treatment. Water Res. 46 (3), 808–816. https://doi.org/10.1016/j.watres.2011.11.059.

Bowater, R., Doherty, A.J., 2006. Making ends meet: repairing breaks in bacterial DNA by non-homologous end-joining. PLoS Genet. 2 (2), e8. https://doi.org/10.1371/journal.pgen.0020008.

Brown, M.R., Hands, C.L., Coello-Garcia, T., Sani, B.S., Ott, A.I.G., Smith, S.J., Davenport, R.J., 2019. A flow cytometry method for bacterial quantification and biomass estimates in activated sludge. J. Microbiol. Methods 160, 73–83. https://doi.org/10.1016/j.mimet.2019.03.022.

Burby, P.E., Simmons, L.A., 2020. Regulation of cell division in bacteria by monitoring genome integrity and DNA replication status. J. Bacteriol. 202 (2), e00408–e00419. https://doi.org/10.1128/jb.00408-19.

Chen, S., Li, X., Wang, Y., Zeng, J., Ye, C., Li, X., Guo, L., Zhang, S., Yu, X., 2018. Induction of Escherichia coli into a VBNC state through chlorination/chloramination and differences in characteristics of the bacterium between states. Water Res. 142, 279–288. https://doi.org/10.1016/j.watres.2018.05.055.

Cho, M., Kim, J., Kim, J.Y., Yoon, J., Kim, J.H., 2010. Mechanisms of Escherichia coli inactivation by several disinfectants. Water Res. 44 (11), 3410–3418. https://doi.org/10.1016/j.watres.2010.03.017.

Chowdhury, S., 2012. Heterotrophic bacteria in drinking water distribution system: a review. Environ. Monit. Assess. 184 (10), 6087–6137. https://doi.org/10.1007/s10661-011-2407-x.

Cooper, S.T., McNeil, P.L., 2015. Membrane Repair: mechanisms and Pathophysiology. Physiol. Rev. 95 (4), 1205–1240. https://doi.org/10.1152/physrev.00037.2014.

Cox, M.M., 1999. Recombinational DNA repair in bacteria and the RecA protein. Prog. Nucleic Acid Res. Mol. Biol. 63, 311–366. https://doi.org/10.1016/s0079-6603(08)60726-6.

Creach, V., Baudoux, A.C., Bertru, G., Rouzic, B.L., 2003. Direct estimate of active bacteria: CTC use and limitations. J. Microbiol. Methods 52 (1), 19–28. https://doi.org/10.1016/s0167-7012(02)00128-8.

da Cruz Nizer, W.S., Inkovskiy, V., Overhage, J., 2020. Surviving reactive chlorine stress: responses of gram-negative bacteria to hypochlorous acid. Microorganisms 8 (8), 1220. https://doi.org/10.3390/microorganisms8081220.

Deborde, M., von Gunten, U., 2008. Reactions of chlorine with inorganic and organic compounds during water treatment-Kinetics and mechanisms: a critical review. Water Res. 42 (1–2), 13–51. https://doi.org/10.1016/j.watres.2007.07.025.

Dizdaroglu, M., 2005. Base-excision repair of oxidative DNA damage by DNA glycosylases. Mutat. Res. 591 (1–2), 45–59. https://doi.org/10.1016/j.mrfmmm.2005.01.033.

Doerrler, W.T., 2006. Lipid trafficking to the outer membrane of Gram-negative bacteria. Mol. Microbiol. 60 (3), 542–552. https://doi.org/10.1111/j.1365-2958.2006.05130.x.

Dunkenberger, L., Reiss, K., Del Valle, L., 2022. Comet assay for the detection of single and double-strand DNA breaks. Methods Mol. Biol. 2422, 263–269. https://doi.org/10.1007/978-1-0716-1948-3_18.

Dupuy, P., Sauviac, L., Bruand, C., 2019. Stress-inducible NHEJ in bacteria: function in DNA repair and acquisition of heterologous DNA. Nucleic. Acids. Res. 47 (3), 1335–1349. https://doi.org/10.1093/nar/gky1212.

Endutkin, A.V., Zharkov, D.O., 2021. [GO system, a DNA repair pathway to cope with oxidative damage]. Mol. Biol. (Mosk) 55 (2), 223–242. https://doi.org/10.31857/s0026898421020063.

Espiritu, R.A., 2021. Repairing plasma membrane damage in regulated necrotic cell death. Mol. Biol. Rep. 48 (3), 2751–2759. https://doi.org/10.1007/s11033-021-06252-w.

Favere, J., Waegenaar, F., Boon, N., De Gusseme, B., 2021. Online microbial monitoring of drinking water: how do different techniques respond to contaminations in practice? Water Res. 202, 117387 https://doi.org/10.1016/j.watres.2021.117387.

Frankenberg-Schwager, M., 1989. Review of repair kinetics for DNA damage induced in eukaryotic cells in vitro by ionizing radiation. Radiother. Oncol. 14 (4), 307–320. https://doi.org/10.1016/0167-8140(89)90143-6.

Ginzinger, D.G., 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp. Hematol. 30 (6), 503–512. https://doi.org/10.1016/s0301-472x(02)00806-8.

Hammes, F., Berney, M., Wang, Y., Vital, M., Koster, O., Egli, T., 2008. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Res. 42 (1–2), 269–277. https://doi.org/10.1016/j.watres.2007.07.009.

Hawkins, C.L., Davies, M.J., 2002. Hypochlorite-induced damage to DNA, RNA, and polynucleotides: formation of chloramines and nitrogen-centered radicals. Chem. Res. Toxicol. 15 (1), 83–92. https://doi.org/10.1021/tx015548d.

Hoefel, D., Grooby, W.L., Monis, P.T., Andrews, S., Saint, C.P., 2003. Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. J. Microbiol. Methods 55 (3), 585–597. https://doi.org/10.1016/s0167-7012(03)00201-x.

Isler, B., Kidd, T.J., Stewart, A.G., Harris, P., Paterson, D.L., 2020. Achromobacter infections and treatment options. Antimicrob. Agents Chemother. 64 (11) https://doi.org/10.1128/aac.01025-20 e01025-01020.

Johnson, J.S., Spakowicz, D.J., Hong, B.Y., Petersen, L.M., Demkowicz, P., Chen, L., Leopold, S.R., Hanson, B.M., Agresta, H.O., Gerstein, M., Sodergren, E., Weinstock, G.M., 2019. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat. Commun. 10 (1), 5029. https://doi.org/10.1038/s41467-019-13036-1.

Kali, S., Khan, M., Ghaffar, M.S., Rasheed, S., Waseem, A., Iqbal, M.M., Bilal Khan Niazi, M., Zafar, M.I., 2021. Occurrence, influencing factors, toxicity, regulations, and abatement approaches for disinfection by-products in chlorinated drinking water: a comprehensive review. Environ. Pollut. 281, 116950 https://doi.org/10.1016/j.envpol.2021.116950.

Kennedy, D., Cronin, U.P., Wilkinson, M.G., 2011. Responses of *Escherichia coli, Listeria monocyt*ogenes, and Staph*ylococcus aureus* to simulated food processing treatments, determined using fluorescence-activated cell sorting and plate counting. Appl. Environ. Microbiol. 77 (13), 4657–4668. https://doi.org/10.1128/aem.00323-11.

Kirschner, A.K.T., 2016. Determination of viable legionellae in engineered water systems: do we find what we are looking for? Water Res. 93, 276–288. https://doi.org/10.1016/j.watres.2016.02.016.

Lee, Y., Imminger, S., Czekalski, N., von Gunten, U., Hammes, F., 2016. Inactivation efficiency of *Escherichia coli* and autochthonous bacteria during ozonation of municipal wastewater effluents quantified with flow cytometry and adenosine triphosphate analyses. Water Res. 101, 617–627. https://doi.org/10.1016/j.watres.2016.05.089.

Lin, H., Ye, C., Chen, S., Zhang, S., Yu, X., 2017. Viable but non-culturable E. coli induced by low level chlorination have higher persistence to antibiotics than their culturable counterparts. Environ. Pollut. 230, 242–249. https://doi.org/10.1016/j.envpol.2017.06.047.

Luo, L.W., Wu, Y.H., Yu, T., Wang, Y.H., Chen, G.Q., Tong, X., Bai, Y., Xu, C., Wang, H.B., Ikuno, N., Hu, H.Y., 2021. Evaluating method and potential risks of chlorine-resistant bacteria (CRB): a review. Water Res. 188, 116474 https://doi.org/10.1016/j.watres.2020.116474.

Ma, X., Bibby, K., 2017. Free chlorine and monochloramine inactivation kinetics of Aspergillus and Penicillium in drinking water. Water Res. 120, 265–271. https://doi.org/10.1016/j.watres.2017.04.064.

Miao, X., Han, X., Liu, C., Bai, X., 2022. Intrinsic chlorine resistance of bacteria modulated by glutaminyl-tRNA biosynthesis in drinking water supply systems. Chemosphere 308 (Pt 2), 136322. https://doi.org/10.1016/j.chemosphere.2022.136322.

Minnikin, D.E., O'Donnell, A.G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A., Parlett, J.H., 1984. An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. J. Microbiol. Methods 2 (5), 233–241. https://doi.org/10.1016/0167-7012(84)90018-6.

Prest, E.I., El-Chakhtoura, J., Hammes, F., Saikaly, P.E., van Loosdrecht, M.C., Vrouwenvelder, J.S., 2014. Combining flow cytometry and 16S rRNA gene pyrosequencing: a promising approach for drinking water monitoring and characterization. Water Res. 63, 179–189. https://doi.org/10.1016/j.watres.2014.06.020.

Qi, Z., Huang, Z., Liu, C., 2022. Metabolism differences of biofilm and planktonic Pseudomonas aeruginosa in viable but nonculturable state induced by chlorine stress. Sci. Total Environ. 821, 153374 https://doi.org/10.1016/j.scitotenv.2022.153374.

Ramseier, M.K., von Gunten, U., Freihofer, P., Hammes, F., 2011. Kinetics of membrane damage to high (HNA) and low (LNA) nucleic acid bacterial clusters in drinking water by ozone, chlorine, chlorine dioxide, monochloramine, ferrate(VI), and permanganate. Water Res. 45 (3), 1490–1500. https://doi.org/10.1016/j.watres.2010.11.016.

Reis, A., Spickett, C.M., 2012. Chemistry of phospholipid oxidation. Biochimica et Biophysica Acta (BBA) - Biomembranes 1818 (10), 2374–2387. https://doi.org/10.1016/j.bbamem.2012.02.002.

Sartory, D.P., 2004. Heterotrophic plate count monitoring of treated drinking water in the UK: a useful operational tool. Int. J. Food Microbiol. 92 (3), 297–306. https://doi.org/10.1016/j.ijfoodmicro.2003.08.006.

Schmid, I., Krall, W.J., Uittenbogaart, C.H., Braun, J., Giorgi, J.V., 1992. Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry 13 (2), 204–208. https://doi.org/10.1002/cyto.990130216.

Shi, L., Gunther, S., Hubschmann, T., Wick, L.Y., Harms, H., Muller, S., 2007. Limits of propidium iodide as a cell viability indicator for environmental bacteria. Cytometry A 71 (8), 592–598. https://doi.org/10.1002/cyto.a.20402.

Sieracki, M.E., Cucci, T.L., Nicinski, J., 1999. Flow cytometric analysis of 5-cyano-2,3-ditolyl tetrazolium chloride activity of marine bacterioplankton in dilution cultures. Appl. Environ. Microbiol. 65 (6), 2409–2417. https://doi.org/10.1128/aem.65.6.2409-2417.1999.

Spilker, T., Vandamme, P., Lipuma, J.J., 2013. Identification and distribution of Achromobacter species in cystic fibrosis. J. Cyst. Fibros. 12 (3), 298–301. https://doi.org/10.1016/j.jcf.2012.10.002.

Tong, C., Hu, H., Chen, G., Li, Z., Li, A., Zhang, J., 2021. Chlorine disinfectants promote microbial resistance in Pseudomonas sp. Environ. Res. 199, 111296 https://doi.org/10.1016/j.envres.2021.111296.

Turetgen, I., 2008. Induction of Viable but nonculturable (VBNC) state and the effect of multiple subculturing on the survival of *Legionella pneumophila* strains in the presence of monochloramine. Ann. Microbiol. 58 (1), 153–156. https://doi.org/10.1007/BF03179460.

van der Kooij, D., Vrouwenvelder, J.S., Veenendaal, H.R., 2003. Elucidation and control of biofilm formation processes in water treatment and distribution using the unified biofilm approach. Water Sci. Technol. 47 (5), 83–90. https://doi.org/10.2166/wst.2003.0287.

Van Nevel, S., Koetzsch, S., Proctor, C.R., Besmer, M.D., Prest, E.I., Vrouwenvelder, J.S., Knezev, A., Boon, N., Hammes, F., 2017. Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring. Water Res. 113, 191–206. https://doi.org/10.1016/j.watres.2017.01.065.

Verni, F., 2022. DNA damage response (DDR) and DNA repair. Int. J. Mol. Sci. 23 (13), 7204. https://doi.org/10.3390/ijms23137204.

Vives-Rego, J., Lebaron, P., Nebe-von Caron, G., 2000. Current and future applications of flow cytometry in aquatic microbiology. FEMS Microbiol. Rev. 24 (4), 429–448. https://doi.org/10.1111/j.1574-6976.2000.tb00549.x.

Winter, J., Ilbert, M., Graf, P.C., Ozcelik, D., Jakob, U., 2008. Bleach activates a redox-regulated chaperone by oxidative protein unfolding. Cell 135 (4), 691–701. https://doi.org/10.1016/j.cell.2008.09.024.

Zhang, S., Ye, C., Lin, H., Lv, L., Yu, X., 2015. UV disinfection induces a VBNC state in *Escherichia coli* and *Pseudomonas aeruginosa*. Environ. Sci. Technol. 49 (3), 1721–1728. https://doi.org/10.1021/es505211e.

Zhao, L., Liu, Y.W., Li, N., Fan, X.Y., Li, X., 2020. Response of bacterial regrowth, abundant and rare bacteria and potential pathogens to secondary chlorination in secondary water supply system. Sci. Total Environ. 719, 137499 https://doi.org/10.1016/j.scitotenv.2020.137499.

Zhu, L., Shuai, X., Xu, L., Sun, Y., Lin, Z., Zhou, Z., Meng, L., Chen, H., 2022. Mechanisms underlying the effect of chlorination and UV disinfection on VBNC state Escherichia coli isolated from hospital wastewater. J. Hazard. Mater. 423 (Pt B), 127228 https://doi.org/10.1016/j.jhazmat.2021.127228.