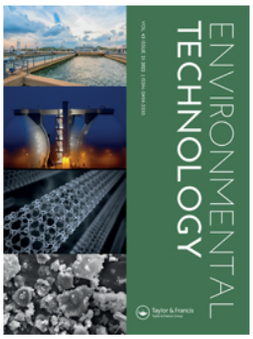

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Flow cytometric assessment of the chlorine/chloramine efficacy of particle-associated bacteria in drinking water

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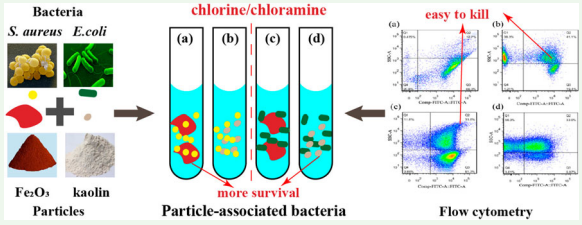
ABSTRACT

Chlorine and chloramine are widely used to maintain the microbial safety after drinking water treatment plants. Particles existing in the treated water may react with these chemical disinfectants, and impact the efficacy of disinfection. However, the protective effects of particles without-disinfectant demand on bacteria in the chlorination/chloramination are not well known. In this study, two laboratory-derived bacteria (*Staphylococcus aureus* and *Escherichia coli*) and two no-disinfectant demand particles (Fe_2O_3 and kaolin) in drinking water were selected to build particle-associated bacteria (PAB) systems, and their resistance to chlorine/chloramine was further assessed. Flow cytometry (FCM) was employed to image PAB systems and assess the removal rate of bacteria. The results were that particles showed protective effects on bacteria in half of chlorine experiments and 90% of chloramination. The protection was related to the combination form of particles and bacteria tied to neither particle species nor size, and there was no positive relationship between the protection effect and water turbidity. *S. aureus* attached to Fe_2O_3 had stronger resistance than kaolin, and kaolin protected *E. coli* better than Fe_2O_3. The same trend was observed in both chemical disinfectants, and more significant resistance had been shown in chloramination than chlorination. FCM images which gave a qualitative description on the combination states of different PAB systems may be a clue to explain the strength of the resistance. Environmental bacterial strains and particles are recommended in the future to explore practical applications.



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Disinfection; Particle-associated bacteria; Drinking water; Flow cytometry; Turbidity

1. Introduction

Chlorine and chloramine are the two widely used disinfectants to maintain the microbial safety of water in the process of long-distance transportation [1]. Particulate matter existing in treated water such as humic substances from water sources and powder activated carbon leached from water treatment [2] may consume chemical disinfectants, and thus impact the efficacy of disinfection [3,4]. However, particles in treated water have a range of diverse sources, and not all of them react with chlorine or chloramine. For example, suspended particles (e.g. clay) from water sources and chemical precipitates (e.g. iron oxide [5]) from distribution systems are typical inert particles in drinking water. The effects of these no-disinfectant demand particles on chemical disinfection are not well known.

Several studies have revealed that particulate matter without chlorine demand may not provide significant protective effects for bacteria in the chemical disinfection process, and the organic content of particles was determined as an important predictable parameter on chlorine efficacy [6,7]. But these studies did not deny that the inorganic particles may protect bacteria against chemical disinfectants by covering bacteria in

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their aggregates [4,8,9]. Winward et al. [10] reported that the inactivation rate of total coliform increased from 4 logs to 6 logs with the mean particle size decreasing from approximately 500 to 200 μm in the chlorine disinfection of grey water, which demonstrated that particles could affect the resistance of bacteria to chlorine disinfection through preventing chlorine from permeating into particles [10]. In addition to the complex background, single type of particles without chlorine demand such as goethite and soil particles collected from the environment has been demonstrated to protect bacteria from chlorine disinfection [9]. The survival rate of *Serratia marcescens* after short-term association with goethite was 30% higher than that of pure bacteria treated with 0.1 mg/L chlorine [9]. Soil particles gained more bacterial survival rate up to 44% in the experiments of all three tested bacteria [9]. Thus, different types of particle-associated bacteria (PAB) systems are likely to behave differently in chlorine disinfection. In addition, chloramine has attracted considerable attention since it has been proven to improve the organoleptic properties of drinking water, reduce the formation of disinfection by-products and control biofilms better compared with chlorine [11]. Although chloramine has been the most common alternative disinfectant to chlorine, there is less research on its inactivation effects on PAB systems.

Another important factor influencing the inactivation of bacteria that have to discuss is turbidity, which is a more intuitive way to describe the distribution of particles in water. Worldwide regulations have recommended that the turbidity of treated water should be reduced to a certain level before disinfection to ensure sufficient bacteria inactivation [12]. Therefore, although turbidity has no definite mathematical relationship with the concentration of particles, the former is generally used as a parameter to represent the abundance of particles in the exploration of the chlorine efficacy of PAB [6,7,10].

The oxidative damage of chlorine/chloramine disinfection on bacteria causes culturability, membrane permeability, metabolism, and genetic injuries [13], but not all microorganisms can be killed in treated water [14]. For example, some microorganisms may be induced into a viable but nonculturable (VBNC) state through chlorine/chloramine disinfection process [15]. Bacteria in VBNC state cannot be detected by the conventional plate culture method, but the resuscitation and regrowth of these bacteria in distribution systems would bring microbiologically risk to the final tap water [16]. Flow cytometry (FCM) has been developed as a rapid and accurate quantitation of bacteria in drinking water recently [17], and it can detect bacteria with intact cell membrane which including bacteria in VBNC state. Thus, FCM was employed in this study to detect the bacterial survival rate in different systems treated with disinfection. In addition, the apparatus can also be applied to give a qualitative description on combination states of different PAB systems [18].

The aim of this study was to determine if there were protective effects when particles without-disinfectant demand co-exist with bacteria in chlorine/chloramine disinfection. Two inorganic substances iron trioxide (Fe_2O_3) and kaolin clay were selected as representative no-disinfectant demand particles from water sources and distribution systems. *Staphylococcus aureus* (*S. aureus*, ATCC 6538) and *Escherichia coli* (*E. coli*, ATCC 25922) were chosen as routinely measured fecal indicator organism and opportunistic pathogen related to microbial safety of drinking water. The two bacteria and two particles were built into four PAB systems to explore the effects of particles impacting chlorine/chloramine inactivation. Further, the factors impacting disinfection including particle types, particle size, turbidity and the combination states of different PAB systems were also discussed to offer knowledge of the resistance of PAB for the related practitioners.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and characterisation methods

S. aureus and *E. coli* (Table 1) were purchased from China Center of Industrial Culture Collection (CICC), resuscitated in accordance with conventional methods, and stocked in glycerol solution at −80°C. Bacterial cultures were streaked from glycerol stocks on Nutrition Agar plates and grown at 37°C for 48 h. A single colony was loop inoculated aseptically into lysogeny broth in Erlenmeyer flask followed by incubation overnight at 37°C in shaking incubator (120 rpm) to early stationary phase (OD600 = 1.0–1.5). The bacteria were harvested by centrifugation for 10 min at 5000 rpm, 4°C. After discarding the supernatant, the cells were washed two times with sterilising phosphate buffered solution (PBS) and resuspended in sterilising PBS solution for the following analyses. All operations were carried out in line with biosafety and aseptic conditions.

Microbial adhesion to hydrocarbons (MATH) method [19,20] with N-hexadecane (purity > 98%; Sinopharm

Table 1. Characteristics of tested bacteria.

Bacteria	Phylum/class	Gram staining	Shape
S. aureus	Firmicutes/Bacilli	G+	Spherical
E. coli	Proteobacteria/Gamma	G−	Rod-shaped

Chemical Reagent Co., Ltd, China) was used to measure the hydrophobicity on the surface of bacteria cells. The bacteria suspensions were adjusted to the resulting optical density (OD600 = 0.5 ± 0.05) by 10-mM KH2PO4 buffer at pH = 7. Turbid washed suspension of microbial cells (3.0 mL) mixed with 1.0 mL liquid test hydrocarbon and vortexed in 15 mL sealable plastic test. Vortex (Vortex-Genie2, MO BIO, USA) was performed with the maximum speed of 30 s and was repeated for 30 s after 1 min interval. After 15 min standing, the hydrophilicity can be determined by measuring OD600 of bottom aqueous phase in disposable polystyrene cuvettes with an effective volume of 1.5 mL. The control and blank groups were mixed without N-hexadecane or bacteria, respectively. The hydrophobic partitioning of the bacterial suspension was calculated by using Equation (1):

$$\mathrm{CSH} = (\mathrm{OD}_c - \mathrm{OD}_s)/(\mathrm{OD}_c - \mathrm{OD}_b), \quad (1)$$

where CSH is hydrophobicity of cell surface, OD_c, OD_s, OD_b are the OD_{600} value of control, sample and blank group, respectively. The analysis was carried out in triplicate at room temperature (24 ± 1°C).

Zeta potential measurements were conducted at room temperature (24 ± 1°C) using Zetasizer Nano ZS 90 (Malvern, UK) equipped with a gas laser emitting at a fixed wavelength of 633 nm. The bacterial suspensions with OD600 = 0.1 ± 0.01 were measured three times to obtain their zeta potential at pH = 7 and different IS (0, 1, 20 mM KCl).

2.2. Particles selection and characterisation methods

Fe_2O_3 and kaolin particles were rinsed three times by sterile ultrapure water to remove water-soluble impurities before use. The colloidal fraction of the particles was separated by sedimentation for 24 h and the supernatant was discarded. Then, particles were harvested from drying in the oven for 24 h and compounded according to the following concentration unless were specifically explained. The processed kaolin particles were spread into PBS buffer solution and reached 0.1% wt. Fe_2O_3 particles were weighed right before use to guarantee effective particle concentration in consideration of its large size and high settlement rate. Particle size distribution was characterised by a focused beam reflectance measurement (EyeTech Particle Size and Shape Analyser, Ankersmid, Netherlands). The zeta potential was measured at room temperature using a Zetasizer Nano ZS 90 (Malvern, UK).

2.3. PAB systems building and methods

PAB systems were built with the above two bacteria and two particles. Each PAB system contains 1 mL bacteria solution (OD600 = 0.3, ~107 cells/mL), 1 mL 0.1 wt% particle solution and 8 mL PBS buffer. Fe_2O_3 powder (0.05 g) was added directly to PAB systems to ensure the particle concentration. The above mixing operations were finished in decarbonised and sterilised glass test tubes. The tubes were turned upside down for 20 times to mix the particles and bacteria and applied to the following experiments and characterisation.

For FCM (BD FACSVerse, BD Bioscience) qualitative analysis, PAB systems and the related particle and bacteria samples were stained by SYBR Green I (Life Technologies, Inc., USA), which was diluted with dimethyl sulphoxide (DMSO) to a working stock concentration of 100×. Then, the nucleic acid stain was filtered through a 0.22 μm filter to remove particles.

2.4. Disinfection experiments

Disinfection on pure bacteria and particles was implemented to study their disinfection characteristics, respectively. The results were used to determine the disinfection exposed time with 0.8 mg/L chlorine and 1.0 mg/L chloramine solution which were adopted usually in actual operation [21]. Working particle solutions were diluted to 20, 10, 5, 2 and 0.5 NTU with the 100 NTU particle stock solution. Total 10 mL PAB system contained 1 mL bacteria (OD600 = 0.3) and different turbid solutions or the control group (0 NTU). Each PAB system was prepared according to Section 2.3 (Table S1). The disinfection termination time was 20 s for 0.8 mg/L chlorine and 4 h for 1.0 mg/L chloramine and sodium thiosulfate solution (0.08%) was utilised to neutralise residue disinfectant at the end point. The contact time for each disinfectant was determined by the actual percentage of live bacteria in chlorine/chloramine disinfection inactivation tests, respectively (Figure S1). Prior to quantification, sonication (40 kHz in 4°C for 5 min) was used to separate particles and bacteria. The determination of the optimum ultrasonic time was shown in Figure S2. The disinfecting properties of experimental particles were also conducted compared with powdered activated carbon (Sinopharm Chemical Reagent Co., Ltd, China) which was a typical chlorine-consuming substance.

Qualitative analyses of the dead and live bacteria before and after disinfection were performed by the double staining method and detected by FCM referring

to the previous method [22]. Briefly, 5 µL treated SYBR Green I and Propidium Iodide (Life Technologies, Inc., USA) were added to 500 µL sample and the reaction was incubated in the dark for 15 min, 37°C prior to be evaluated by FCM (BD FACSVerse, BD Bioscience). The proportion of viable bacteria was calculated by green fluorescence for each sample. The percentage of viable bacteria ratio before and after disinfection was used to evaluate the resistance of PAB systems to chlorine/chloramine.

2.5. Statistical analysis

The effects of particle type and turbidity on the chlorination or chloramination of different bacteria were evaluated using a three-way ANOVA (Analysis of Variance). A two-way ANOVA was used when particle type, turbidity, or bacteria had significant interactive effects on the disinfection. Meantime, Dunnett's test was selected as post hoc tests to determine whether the bacterial survival rates of different turbidity groups were greater than the control group (0 NTU). For other levels, Duncan's test was run to compare the means of different turbidity groups and assess statistical significance accordingly. The assumptions of ANOVA were met and the difference in the ANOVA was deemed significant at $p < 0.05$.

3. Results

3.1. Bacterial strains and particle characteristics

MATH was investigated to evaluate the hydrophobic property of bacteria strains. *S. aureus* (93.12%±2.86%) was observed to have higher hydrophobicity than *E. coli* (38.27% ± 12.87%). Particle size distribution of two particles is shown in Table 2. Kaolin particles had over 80% smaller aspect ratio than Fe_2O_3 particles in volume average diameter. On average, Fe_2O_3, the particle size larger than 8.66 µm constituted 90% of the total Fe_2O_3 particles, and kaolin particles larger than 8.20 µm made up 10% of the total volume. In terms of particle size, two selected particles were totally different from each other.

The zeta potential of tested bacteria and particles were all negative and became less negative with ion concentration increased (Figure 1). It is elucidated that high ion concentration by adding potassium chloride would influence counter-ions with compressing the double electron layer by DLVO theory. The amounts of negative charge in *S. aureus* are greater than those in *E. coli*. Generally, the isoelectric point (pl) of Gram-positive bacteria and Gram-negative bacteria are 2–3 and 4–5, respectively [23]. Therefore, the bacteria have negative surface charges when cultivated at physiological pH values (7.4) [24], and Gram-positive bacteria usually have a lower surface charge than Gram-negative bacteria. The zeta potential of *E. coli* as Gram-negative was obviously higher than *S. aureus* belonging to Gram-positive bacteria, especially with ionic strength increased. Similar phenomena were observed in previous studies using electrophoretic mobility [25]. Compared with bacteria, kaolin particles are more negatively charged than Fe_2O_3. Bacteria would be poor at adsorption to cells because both of them are negatively charged in consideration of overcoming electrostatic repulsion. Moreover, the attachment was even harder than cell aggregation in view of the absolute difference of zeta potential between different bacteria and particles simply.

Table 2. Particle size distribution of three particles.

Particle	Fe_2O_3	Kaolin
Volume average diameter (µm)[a]	36.38 ± 21.65	5.20 ± 2.17
D10 (µm)	8.66	2.21
D90 (µm)	68.59	8.20

[a]Values are given as mean concentration ± standard deviation; $n = 5$.

3.2. PAB systems and characterisation

The side scatter and fluorescence intensity of particles were detected to partition and quantify particles and bacteria in PAB systems using FCM. In Figure 2, the vertical axis shows information about the internal complexity or granularity of particles through side scatter channel (SSC). The horizontal axis is fluorescence intensity channel which detects particles binding with a fluorescent dye. Generally, particles usually have a high side scatter signal and low fluorescence intensity because of no binding with dye. Bacteria can emit fluorescence signal after dying and

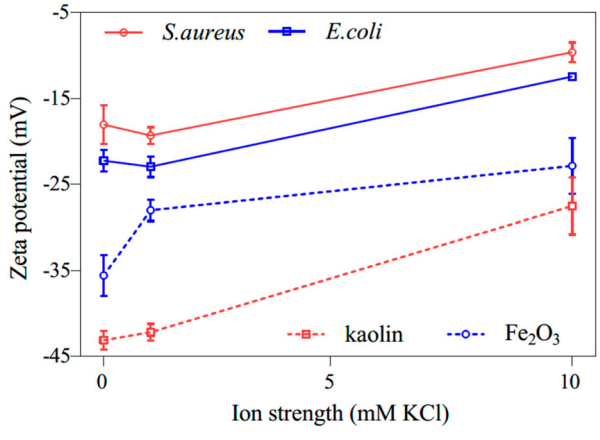

Figure 1. The zeta potential of bacterial strains and particles.

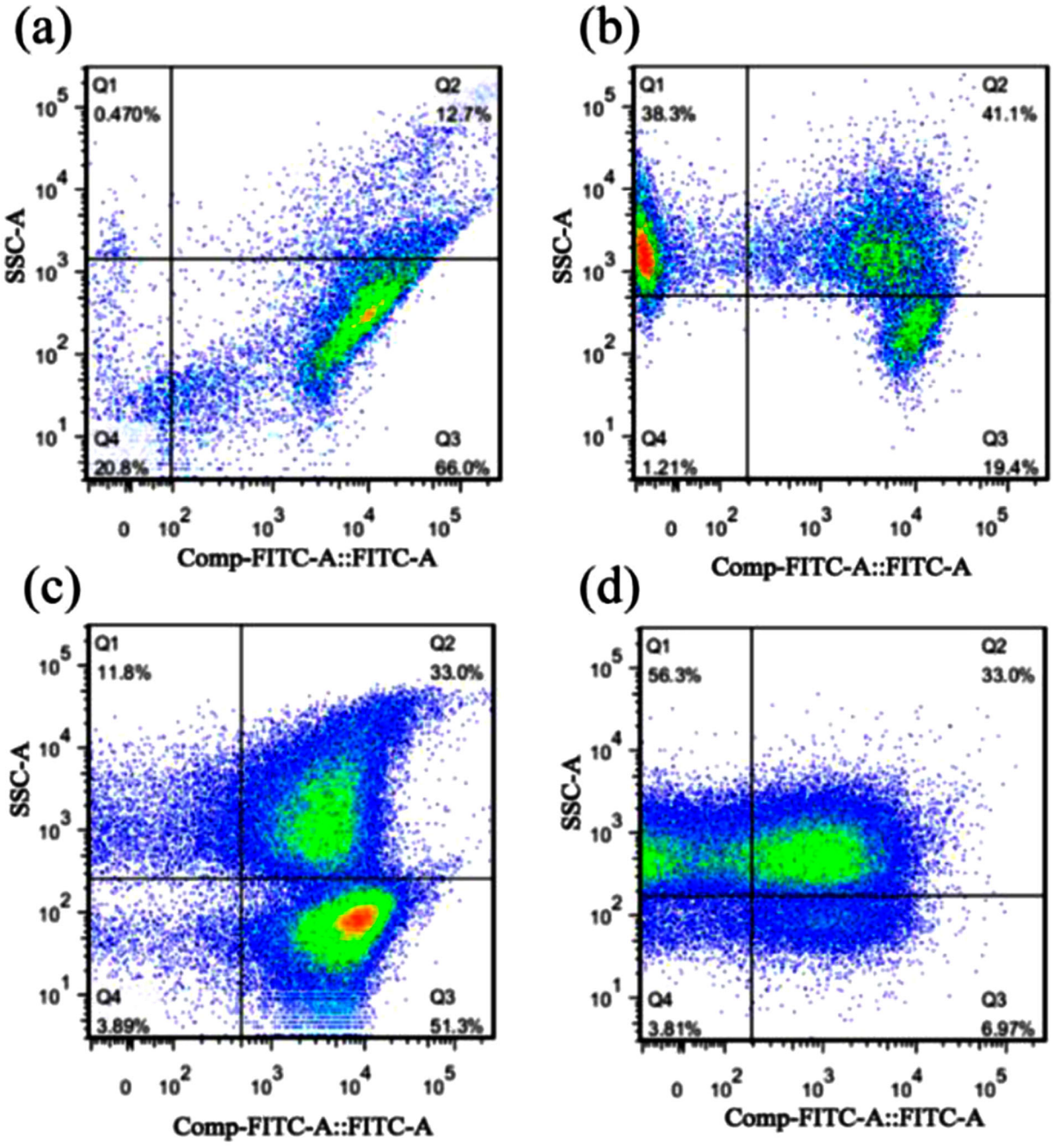

Figure 2. The attachment of particles and bacteria showed by FCM. (a) *S. aureus* + Fe_2O_3; (b) *S. aureus* + kaolin; (c) *E. coli* + Fe_2O_3; (d) *E. coli* + kaolin.

have a low side scatter signal. Both high signals in two channels would be acquired when particles attached with bacteria. Different particles distribute in partition Q1 like Fe_2O_3 and kaolin in Figure S3. Bacteria like *S. aureus* and *E. coli* are mainly distributed in Q3 in Figure S3. Above all, the two bacteria are seen to be combined with particles in Q2 from the qualitative analysis of FCM. The more noticeable partition appeared in *E. coli* attached with Fe_2O_3 (Figure 2(c)) and *S. aureus* with kaolin (Figure 2(b)).

3.3. Disinfectant resistance of PAB systems

Bacteria (*S. aureus* and *E. coli*) and particles (Fe_2O_3 and kaolin) were mixed to be PAB systems for disinfection tests. The disinfectant consumption of two experimental particles themselves approached zero compared with powdered activated carbon (Figure S4). The results showed that *S. aureus* had significant higher disinfectant resistance than *E. coli* (chlorine: $F(1,70) = 1238.67$, $p = 3.01 \times 10–46$, chloramine: $F(1,70) = 787.05$, $p = 8.30 \times$

10 – 40) regardless of disinfectant types or other factors. The different disinfection efficacy of chlorine and chloramine has also been observed in the experiments. The inactivation ratio in the chlorination was higher than in the chloramination although the latter was applied for a longer time and at a higher dose. In addition, the protective effect of particles was less obvious in the chlorination. In post-chlorination, *S. aureus* only demonstrated remarkable increased resistance in higher turbidity (10 and 20 NTU) (Figure 3(a)). *E. coli* showed stronger activity than control groups both in 0.5 and 20 NTU after chlorination, and particle-associated advantage was obtained additionally with kaolin in 5 and 10 NTU than that with Fe_2O_3 (Figure 3(b)). By contrast, although the removal rates of *S. aureus* associated with kaolin in 5 and 10 NTU had no significant decrease when treated with chloramine, the protective effects were obvious under all other conditions with chloramination for all PAB systems (Figure 4). The two kinds of bacteria all showed resistance in the highest turbidity (20 NTU) regardless of a particular type. Remarkably, microbial resistance to inactivation could also exist in low turbidity. Second to the highest turbidity (20 NTU), the lowest turbidity (0.5 NTU) have the highest live cell ratio in the chlorination of *E. coli*, which was closer to the turbidity level in the pre-disinfection water from the majority of drinking water treatment plants. Two highest bacteria survival probability were detected with the turbidity of 0.5 and 2 NTU in the chloramination of particle attached *S. aureus* systems. The highest survival rate of *E. coli* in the chloramine disinfection occurred with the turbidity of 5 NTU. The above information was reached both in Fe_2O_3 and kaolin systems. In summary, the protective effects were attained in all combination types of particles and bacteria under two chemical disinfectants.

Particle type had no significant influence on the bacteria survival rate of PAB systems on the whole. However, particles have a more complicated impact on protection behaviour depended on bacteria types with the changes of turbidity. That was that *S. aureus* attached with Fe_2O_3 had stronger resistance than kaolin in some circumstances (chlorine, 10 NTU: $p = 0.045$; chloramine, 5 NTU: $p = 0.005$; chloramine, 10 NTU: $p = 0.004$). As mentioned above, microbial resistance to inactivation was observed when *S. aureus* attached with Fe_2O_3 in 5 and 10 NTU with chloramine but kaolin did not show any significant protective effects under the same conditions. Correspondingly, the reverse had taken place with *E. coli*, which attached with kaolin had greater resistance than that with Fe_2O_3 (chlorine, 5 NTU: $p = 0.021$; chloramine, 5 NTU: $p = 0.007$, 20 NTU: $p = 0.031$).

Results obtained in this investigation showed that there were large differences in chlorine/chloramine-resistance depending on the microorganisms investigated and the composition of disinfectants. *S. aureus* was found to have higher resistance to chemical disinfectants than *E. coli*. These phenomena may be ascribed to that the higher susceptibility of Gram-negative bacteria to multi-target antimicrobials comparatively to that of Gram-positive bacteria [26,27]. Chlorine as a non-selective oxidant has been proved to react with cellular components and influences metabolic processes [26]. The cell wall of *S. aureus* (~20–40 nm [28]) as gram-positive bacteria consists of a thick layer of peptidoglycan interlaced with much teichoic or teichuronic acids (TAs). TAs form a continuum of negative charge and can bind cationic groups, which in turn endows the cell envelope with good permeability, toughness and electrostatic properties [29]. *E. coli* as Gram-negative organisms, in contrast, lack of TAs and have outer membrane functions along with a thin layer of peptidoglycan and a distinct periplasm between them. The cell wall may be related to the accessibility of chlorine to targets within the cell and influence the resistance [26]. The germicidal efficacy of chloramination was weaker than that of chlorination in this study, which may magnify the protective effects of particles when chloramine was used for disinfection. In addition, the resistance of PAB systems was not significantly influenced by particle type. As mentioned in the literature [6], turbidity has also no significant impact on the protection of bacteria.

Results have demonstrated that different combination of particles and bacteria had different disinfectant resistance. significantly different protective effects were demonstrated in their disinfection experiments. Kaolin provided fewer protective effects to *S. aureus* than Fe_2O_3, and the reverse happened in the experiments with *E. coli*. Images attained from FCM may provide some evidence to explain the above result. There was a clear dividing line between *S. aureus* and kaolin (Figure 2(a)) to distinguish PAB (Q2) and free-living bacteria (Q3). A similar situation appeared in *E. coli* attached with Fe_2O_3 (Figure 2(c)). However, little distinction was observed between Q2 and Q3 partition when *S. aureus* was attached with Fe_2O_3 (Figure 2(b)) and *E. coli* attached with kaolin (Figure 2(d)). Then, the two pairs at the front had a lower inactivation ratio than the two other couples. Linear model has shown that approximately 65% of the protective effects could be predicted by adhesive behaviour [10]. Free-living bacteria are easier to be killed by disinfectants than PAB, which lie in Q2. The clear partitions offered specific aims for disinfectants, whilst the universal protection provided by the

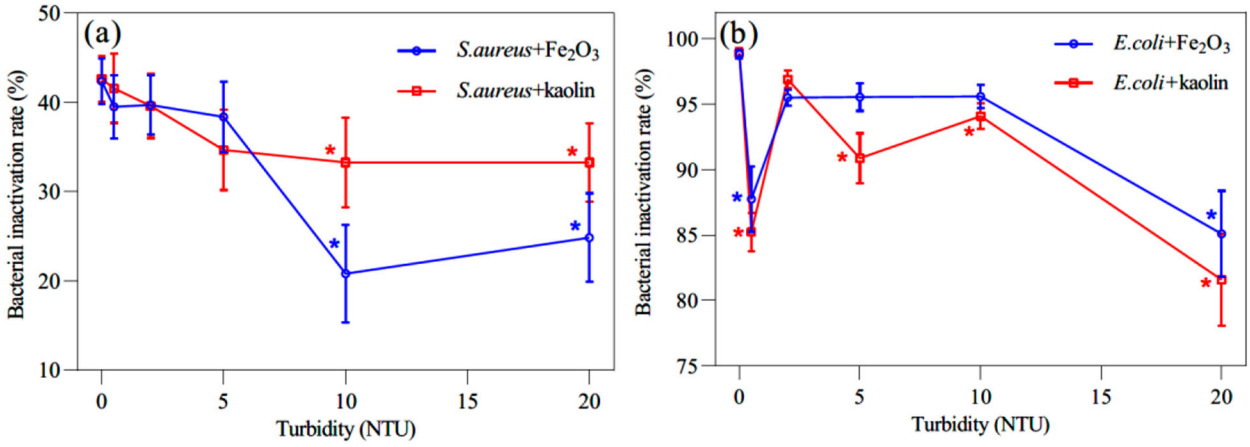

Figure 3. The bacterial inactivation rate of (a) *S. aureus* or (b) *E. coli* after chlorine disinfection in the presence of particles (Fe_2O_3 or kaolin) at different turbidity. * indicates the current group has significant difference with the control group (0 NTU).

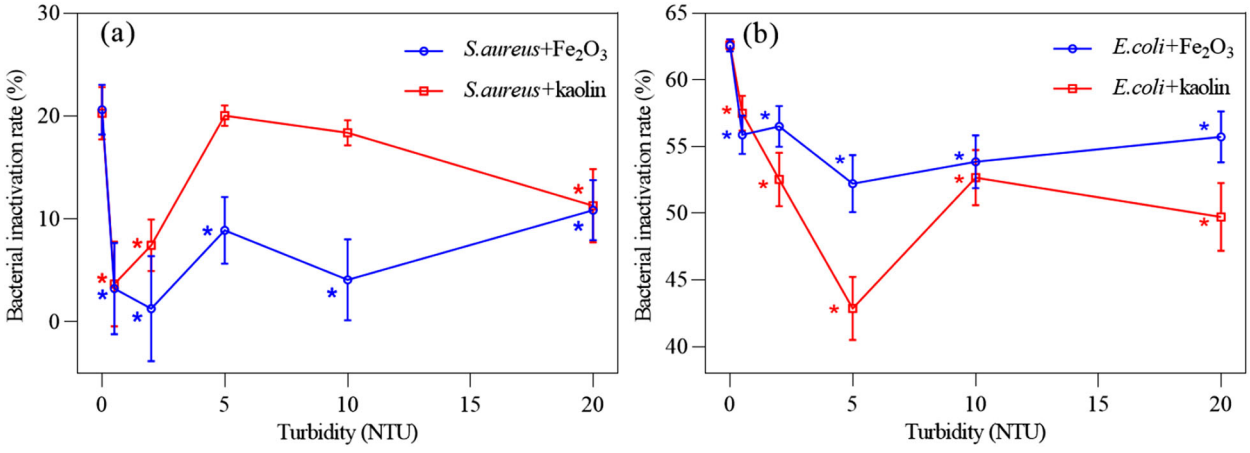

Figure 4. The bacterial inactivation rate of (a) *S. aureus* or (b) *E. coli* after chloramine disinfection in the presence of particles (Fe_2O_3 or kaolin) at different turbidity. * indicates the current group has significant difference with the control group (0 NTU).

attachment of particles and bacteria. Microbe existed in four main forms according to different niches: free-living bacteria, PAB, bacteria in loose deposits and in biofilms on the pipe walls in the drinking water distribution systems [30]. The first two kinds of bacteria co-exist and relate directly to the microbial safety of tap water [30]. The PAB was not selected deliberately from the whole system, and thus the prediction results would fit the actual situation more precisely. On the other hand, these results showed that settling methods for isolating cells attached to particles [31] may not be suitable for quantifying PAB resistant to disinfectant. The percent of PAB obtained from settling methods was higher than FCM [18]. The crossing protective of bacteria and particles are underestimated when only PAB systems were considered. There is extensive evidence in the environmental monitoring that PAB usually has pronounced taxonomic differences with free-living bacteria in water [32]. Further studies are required to verify the above findings with environmental bacterial strains and particles.

4. Conclusions

This study with laboratory-derived bacteria and particles expanded on knowledge relating to the protective effects which particles in drinking water provided to bacteria. The protection was not significantly influenced by particle characteristics or turbidity. Instead, different PAB systems had shown different disinfectant resistance but the same trend in both chemical disinfections; that is, *E. coli* attached with kaolin and *S. aureus* with Fe_2O_3 had stronger disinfectant resistance than the corresponding bacteria with another particle. The image performed by FCM may express explicitly the combination of bacteria and particles and provide a clue to the phenomena. The stronger resistance to chemical disinfectants of one PAB system was consistent with the

less noticeable partition represented in FCM images, and the latter means the particles in the system bind more tightly to the bacteria. In addition, the protective effect was more obvious with a weaker disinfectant chloramine compared with chlorine. For future studies, monitoring is needed to further verify the conclusions with environmental bacterial strains and particles.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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