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Chlorine disinfection of drinking water assessed by flow cytometry: new insights

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Abstract

The efficacy of chlorine disinfection was assessed for the first time over a range of disinfection conditions using flow cytometry (FCM) to provide new insights into disinfection processes. Inactivation was assessed for pure culture bacteria (*Escherichia coli)* and micro-organisms in real treated water from operational water treatment works (WTWs). A dose dependent increase in inactivation rate (*k*) was observed for both test matrices, with values of 0.03 to 0.26 and 0.32 to 3.14 L/mg.min for the WTW bacteria and *E. coli,* respectively. After 2 minutes, *E. coli* was reduced by 2 log for all chlorine doses (0.12 to 1.00 mg/L). In the case of the WTW filtrate bacteria, after 2 minutes log reductions were between 0.54 and 1.14 with increasing chlorine concentration, reaching between 1.32 and 2.33 after 30 minutes. A decrease in disinfection efficacy was observed as temperature decreased from 19 to 5°C for both microbial populations. With respect to chlorination at different pH (pH 6, 7, 8), membrane damage was more pronounced at higher pH. This was not consistent with the higher disinfection efficacy seen at lower pH. when culture based methods are

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used to assess bacterial reductions. This provides evidence that more understanding into the fundamental mechanisms of chlorine disinfection are required and that methodological alterations may be required (e.g. pH standardisation) to fully utilise FCM over the entire range of chlorination conditions observed in operational environments.

Keywords

Drinking water; flow cytometry; bacteria; chlorination; disinfection; *Escherichia coli*

1. Introduction

Microbiologically safe drinking water is a priority in any water treatment and distribution system, and disinfection using chlorine is commonplace for the inactivation of microorganisms, including pathogens, and suppression of regrowth during periods of water storage (Farrell et al. 2018). The mechanism of action of free chlorine on bacterial cells relates to oxidative damage to membranes, nucleic acids, proteins, amino acids, cell walls and other lipids causing a loss of viability (Camper et al. 1979; Haas and Engelbrecht, 1980). Chlorine exhibits different reactivity towards some of these functional groups (Deborde and von Gunten, 2008) compounded by intrinsic differences in bacterial chlorine susceptibility (phenotypic chlorine resistance) within the drinking water microbial community (Gray et al. 2013; Ridgway and Olson, 1982). For example, previous studies have demonstrated that the exposure required for a specified inactivation of 3 log (99.9%) varies significantly, with *Staphylococcus epidermis* being almost 10-fold more resistant to chlorine than *Escherichia coli* (*E. coli*), and *Mycobacterium aurum* 1,000 times more resistant than the latter (Helbling and VanBriesen, 2007). Consequently, the operating conditions need to be set appropriately to ensure effective disinfection of the microbial community present within the water at any given moment.

There are four important abiotic factors affecting the efficiency of disinfection: the free chlorine concentration, exposure time, temperature and pH. Operationally, this is delivered by the ‘Ct’ concept, whereby disinfection is achieved by allowing sufficient contact time (residence time, t) between the water and chlorine disinfectant (concentration, C) at a fixed pH. The World Health Organisation (WHO) recommends a minimum Ct of 15 mg.min/L, where the concentration of free chlorine is 0.5 mg/L,

the residence time is 30 minutes, and water pH is less than 8 at a turbidity ≤1 NTU (WHO, 2011).

Recent research has shown that culture-based methods are not always reliable for determining disinfection performance (Chen et al., 2018). For example, the shortcomings of using heterotrophic bacteria (HPC) for assessing microbial water quality are well understood. Most notably, because typically less than 1% of bacteria are culturable on standard growth media due to poor recovery and the prevalence of viable but non-culturable (VBNC) organisms (Van Nevel et al., 2017). Mild chlorination has been reported to induce such loss of culturability although bacteria are still deemed viable (Health Canada, 2012). The resulting limitations of cultivation methods has driven the need for alternative diagnostic methods to help explain and diagnose disinfection processes. Flow cytometry (FCM) has gained popularity as an alternative to traditional microbial monitoring approaches (Hammes *et al*., 2008; Prest *et al*., 2013; Nevel *et al*., 2013; Gillespie *et al*., 2014). FCM has been shown to have a wide variety of applications such as monitoring bacterial numbers in treatment and distribution (Nescerecka *et al*., 2018), monitoring natural water sources (Besmer *et al*., 2016) and evaluating biological process performance (Ziglio *et al*., 2002). Based on staining cells with fluorescent dyes, FCM enables both the assessment of total (TCC) and intact cells counts (ICC) (Cheswick et al., 2019). The latter are typically assessed when aiming at quantification of disinfection efficacy, as most chemical disinfectants lead to damage of the bacterial cell envelope (Virto *et al*., 2005; Xu *et al.,* 2018). Alternative methods could therefore provide additional insight into these processes. As an example, in an assessment of 213 WTWs, the FCM data enabled true quantification of microbial log removal rates across different treatment stages (Cheswick et al., 2019). This was impossible with HPCs due to the infrequent occurrence of positive

detections of cultured bacteria. FCM can also measure the intensity of fluorescence from each detected event. Different intensities originate from bacteria with different nucleic acid contents. Typically two distinct clusters can be distinguished, often termed high nucleic acid (HNA) or low nucleic acid (LNA) content bacteria based on high or low green fluorescence, respectively (Lebaron et al., 2001). It has been proposed that these two sub-populations of bacteria have different characteristics that may influence their response to water treatment disinfectants (Ramseier et al., 2011).

While applied across a range of application areas, to date no work has reported utilising FCM to assess inactivation by chlorination over a range of typical disinfection operational conditions. The aim of this work was to use FCM to critically assess the impact of dose, pH and temperature on the kinetics and efficacy of microbe disinfection with free chlorine. Results were obtained for water containing pure cultures of *E. coli*, as well as for microbes present in real environmental water samples to enable disinfection variables to be explored for organisms with known differences in chlorine susceptibility.

2. Materials and Methods

All glassware was prepared to remove any chlorine demand. Organic free glassware was prepared according to the method described in APHA-AWWA-WEF (2012). In brief, borosilicate glassware was machine-washed and rinsed three times with ultrapure (UP) water (Purelab Option – S7/15, 18.2 Ω-cm and TOC <3 ppb). Glassware was incubated overnight in 0.2 M hydrochloric acid and rinsed with UP water. Glassware was air dried, covered with aluminum foil and incubated at 550 °C for 6 h. Teflon coated screw caps were washed with HCl as above and immersed into hot (60 °C) sodium persulphate solution (10%) for 1 h, rinsed three times with UP water and air dried. Glassware was stored in a dry place until use. Vessels were left to stand overnight in a 1% sodium hypochlorite solution before being left to air dry and capping with foil prior to use.

2.1 Cultivation of E. coli

Experiments were performed with *E. coli* (ATCC 25922) as a routinely measured compliance indicator organism for assessment of the microbial risk posed from drinking waters. *E. coli* from a glycerol cryo-stock at -80 ˚C were streaked onto a tryptic soy agar (TSA) plate at 37 ˚C for 24 hours. Colonies were then inoculated into 10% tryptic soy broth (TSB) for 15 h at 30 ˚C and stirred to reach the stationary growth phase (approx. 10^9 cells/mL). The *E. coli* were harvested by centrifugation at room temperature (5000 × g, 5 min). The supernatant was carefully removed using a pipette and filter tip. Bacteria were aspirated and resuspended in an equal volume of 0.1 µm filtered phosphate buffered saline (10 mM, pH 7) (Sigma Aldrich). The wash step was repeated three times. A final experimental concentration of approximately 1 x 10^5 ICC/mL was used, representative of typical cell counts of water from conventional WTW at the stage prior to disinfection.

2.2 Water samples containing natural drinking water bacteria

Water samples were taken from two conventional surface water drinking WTWs in Scotland. The treatment train was: coagulation, flocculation, clarification, rapid gravity filtration, disinfection, storage and distribution and samples were obtained from the outlet of the rapid gravity filter for the two sites (termed 'WTW-A Filtrate' (pH 6.8); 'WTW-B Filtrate' (pH 6.4)). The samples were therefore representative of treated water prior to it being subjected to chlorine disinfection. The microbial population in the environmental samples was used to understand the difference in chlorine susceptibility between natural microbes and laboratory reference indicator organisms as measured by FCM.

2.3 Disinfection by chlorine assay

Experiments were carried out in 250 mL borosilicate glass Erlenmeyer flasks prepared as described above to ensure minimal chlorine demand. Phosphate buffer (100 mM) was added to a 0.5% NaCl solution for *E. coli* and directly into the natural environmental water to provide a final buffer concentration of 10 mM at the required pH. To this, the washed *E. coli* were added and prior to chlorine addition, the vessel was left for 30 minutes to equilibrate to test conditions. Chlorine concentration experiments were conducted at pH 7 using a range of free chlorine concentrations (0.12, 0.25, 0.50, 0.75, 1.00 mg/L). Free and total chlorine was measured a N,N–diethyl-p-phenyldiamine (DPD) colorimetric method and a Hach pocket colorimeter (Hach-Lange, Salford, UK). The pH tests were carried out at pH 6, 7 and 8 at room temperature (measured at 21 °C) and using chlorine doses of 0.12 and 0.25 mg/L. Each experiment was stirred continuously using a magnetic stirrer (150 rpm). At time points following chlorination (0, 0.5, 1.0, 1.5, 2, 5, 10, 20, 30 minutes), a 500 µL sample was taken and added to a microcentrifuge tube containing 5 µL of 0.1 M sodium

thiosulphate to quench any residual chlorine. For the long exposure tests the same procedure was followed but sampling times were increased to 0, 20, 40, 60, 80 and 120 minutes at free chlorine concentration of 1.0 and 2.0 mg/L. In these experiments controls were undertaken which were not chlorine treated but were still treated with a stoichiometric ratio of sodium thiosulphate to show that the quenching agent had no effect on the viability of these organisms. Each experiment was performed in triplicate. Inactivation of bacteria followed second order kinetics: $\log\left(\frac{N}{N_0}\right) = -kC_0t$, where N_0 is the initial number of bacteria, N is the number of surviving bacteria after time, t, and C_0 is the free chlorine concentration (Haas and Karra, 1984).

2.4 Flow cytometry analysis

FCM analysis was undertaken according to Gillespie et al. (2014) and Cheswick et al. (2019). To quantify total cell concentrations (TCC), a 10,000× stock of SYBR Green I (SG; cat. S-7567; Life Technologies Ltd., Paisley, UK) was diluted with 0.22 µm filtered dimethyl sulphoxide (DMSO) (Fisher Scientific, Fair Law, NJ). Aliquots of this 100× SYBR Green I working solution were added to samples to achieve a 1× final concentration. For the quantification of ICC, a dye mixture was made of SYBR Green I and propidium iodide (PI) (1 mg ml^{-1}, corresponding to 1.5 mM; cat. P3566; Life Technologies Ltd, Paisley, UK) at a ratio of 5:1 respectively. This mixture was added to samples to give final concentrations of 1× SYBR Green I and 3 µM of PI. Prior to staining, a 10% volume of phosphate buffer (100 mM, pH 7) was added to each sample to normalise the pH value and to avoid pH effects on analysis. Once the dyes and sample had been mixed, they were incubated in the dark for 10 min at 37 °C in a plate incubator (PHMP, Grant Instruments, UK), consistent with our previous validated approach for environmental samples (Cheswick et al., 2019). For the analysis, a BD Accuri C6 flow cytometer with a 488nm solid-state laser (Becton Dickinson UK. Ltd,

Oxford, UK) was used. Green fluorescence was recorded in the FL1 channel at 530 nm and red florescence in the FL3 channel at 675 nm. A sample volume of 25 µL was analysed at a flow rate of 66 µL min^{-1}. For the analysis of TCC and ICC a fixed gate described previously was used (Gatza *et al*., 2013). The gate was designed to include both autochthonous bacteria from natural waters and laboratory-grown bacteria and no adjustments was necessary. An example set of data from the FCM showing bacteria in the gated region can be seen in Supporting Information (SI) Figure S1. High and low nucleic acid content (HNA/LNA) analysis was carried out in the FL1/FL3 fluorescence plot (Lebaron et al., 2001; Berney et al., 2008; Nocker et al., 2017). As populations of bacteria can shift, the distinction point between the HNA and LNA bacteria was always placed between the two clear peaks on the sample prior to disinfectant addition at t = 0. Data processing was undertaken on the CSampler software (BD, Belgium) and using Microsoft Excel.

2.5 The impact of water pH on the fluorescence intensity of propidium iodide

To understand whether emission of DNA-intercalated propidium iodide changes with pH, propidium iodide was added to 1 µg of calf thymus DNA (Life Technologies Ltd, Paisley, UK) dissolved in filter sterilized UP water to a final dye concentration of 3 µM. Samples were incubated at 37°C for 10 minutes in the dark. After incubation, samples were transferred to a 96 well flat-bottomed black plate, where an equal volume of phosphate buffer (10 mM) was added. The pH of the phosphate buffer was adjusted using 1 M NaOH and 1 M HCl to achieve the desired pH (3, 4, 5, 6, 7, 8 and 9). Fluorescence was then measured using a microplate reader (TecS2an Infinite 200 Pro, Männedorf, Switzerland). Instrument settings were as follows: excitation wavelength of 485 nm (excitation bandwidth = 9 nm), emission recorded at both 620 nm and 670 nm (Emission bandwidth = 20 nm) and gain = 120.

3. Results and Discussion

3.1 Impact of chlorine dose and inactivation measured using flow cytometry

To assess the effect of chlorine exposure on membrane integrity, incremental increases in chlorine concentrations were applied to either pure culture *E. coli* (N_0 = 1.18 x 10^5 ± 1.5 x 10^4 ICC/mL) or the WTW-A filtrate bacteria (N_0 = 1.45 x 10^5 ± 2.5 x 10^4 ICC/mL) (Figure 1a and b). *E. coli* had a 1.8 log reduction at the lowest chlorine concentration of 0.12 mg/L after 1.5 minutes contact time (Figure 1a). Increasing the chlorine concentration had minimal impact on disinfection of *E. coli* (Figure 1a). Similarly, increasing contact time above 2 minutes did not result in increased log removal of *E. coli* ICC (data not shown). However, within a minute of chlorine addition there was a chlorine concentration dependent reduction in the ICC for *E. coli*. For example, at t = 1 min, log reductions of *E. coli* ICC went from 0.29 to 1.8 with increasing chlorine dose from 0.12 to 1 mg/L.

WTW-A filtrate bacteria required substantially higher chlorine exposure time for equivalent log reduction to those seen for *E. coli* (Figure 1a and b). As observed for *E. coli*, there was no change in the WTW-A filtrate bacteria ICC over the duration of the 30 minute test period in the control test, when no chlorine was dosed into the water (Figure 1b). At t = 0.5 minutes, log reduction of between 0.1 and 0.25 was observed at doses between 0.25 and 1 mg/L chlorine. Dose dependent chlorine inactivation was evident, with much greater difference in the cell reductions between the chlorine doses than seen for *E. coli*, albeit over much longer exposure times. For example, for the filtrate bacteria after 10 minutes, there was a 1 log reduction for a chlorine dose of 0.25 mg/L, while this was 1.7 for a chlorine dose of 1 mg/L (Figure 1b). Similar reductions were seen after only 1 minute for *E. coli*.

The inactivation rate constant (*k*) increased in magnitude as chlorine dose increased for both the *E. coli* and the natural water bacteria (Figure 1c). A small rate constant equates to a slow reaction of chlorine with cellular components which inactivate bacteria. In this case, the flow cytometry method relies on membrane integrity as a viability parameter, therefore inactivation rate values represent chlorine's impact on membrane integrity/permeability (Hassard et al. 2016; Nocker et al. 2017). The inactivation rate constant decreased from 0.03 to 0.26 L/mg.min as dose increased for the WTW A filtrate bacteria. In contrast, the *E. coli* inactivation rate constants were higher due to the rapid decline in cell counts over the first minutes of chlorine exposure. The *k* decreased by an order of magnitude as chlorine dose went from low to high, with values between 0.32 and 3.14 L/mg.min for 0.12 and 1 mg/L chlorine concentrations, respectively. This was a similar range to the 4.71 L/mg.min seen when *E. coli* was chlorinated using concentrations between 0-1 mg/L and assessed by FCM (Cunningham et al., 2008). Similar data obtained from culture based methods have resulted in higher values. For example, Lee *et al.,* (2010) reported inactivation rate constants of 31.29 L/mg.min for *E coli* at an initial chlorine concentration of 1 mg/L. Our attempts to directly compare FCM with data obtained from culture based methodologies has been hampered by the low or zero cell counts that are observed using the latter (Cheswick et al., 2019; Leziart et al., 2019). This results in poor quality data with low resolution. The difference owes itself to the fact that using the staining approach, viability assessment by FCM is based on membrane integrity and not on the ability to form colonies. More chlorine is necessary to induce membrane damage than to prevent colony formation (Virto et al., 2005). The higher sensitivity afforded by FCM enables detection and tracking of intact cells that may not be culturable following disinfection, even in the case of an organism such as *E. coli* that generally has a high

sensitivity to chlorine. In this case, approximately 10^3 ICC cells remained following disinfection.

The difference in the inactivation rates obtained for *E. coli* and the real water sample demonstrates that this bacterial indicator is not suitable for monitoring disinfection efficacy of the total microbial community, which was reduced at a much slower rate. It was also noted that the inactivation curves for *E. coli* and the natural water had different profiles. The natural water bacteria had a long tail at higher contact times, while the *E. coli* had a more sudden drop, or shoulder, very shortly after the addition of chlorine. These observations are consistent with the presence of a heterogeneous bacterial population present in the natural water that have a wider range of chlorine susceptibility and tolerance (Cerf, 1977). As a consequence, no single specific bacterial strain would be likely to reflect the chlorine susceptibility of the diverse bacterial population contained in natural water. This would also apply to heterotrophic plate counts (HPC) that reflect only a small percentage of the total bacterial population. As a consequence, HPC bacteria in drinking water only form a few (if any) colonies (Cheswick et al., 2019), such that their cultivation is not suitable either for monitoring chlorine disinfection.

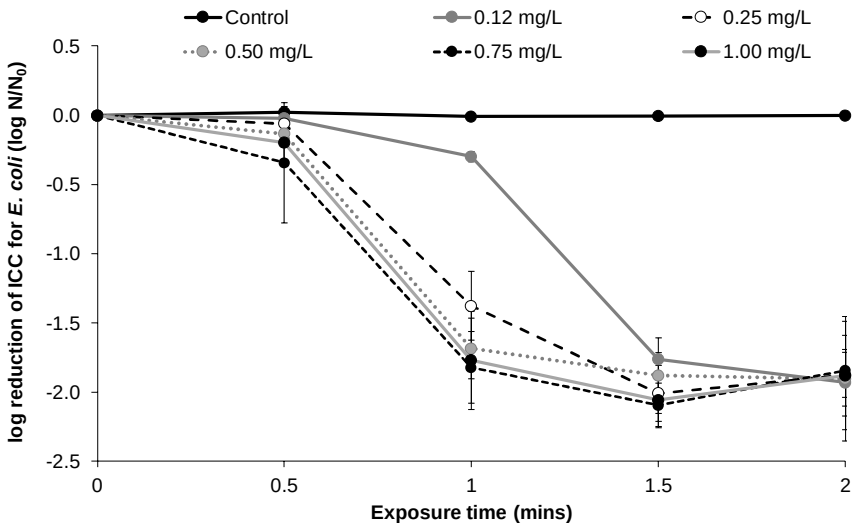
In this research, changes in ICC have been used to evaluate disinfection by chlorine to account for damage to cellular membranes. This is because TCC typically show little change during the initial exposure to chlorine. However, after long-term chlorine exposure, smaller reductions in TCCs have been observed (Nocker et al. 2017), but this often gets little attention as in the context of disinfection. It is therefore of interest to determine what happens to TCCs with increasing contact time.

Although not as prominent as was seen with the ICC, the TCCs decreased slightly with increasing exposure time for all chlorine concentrations. In the case of *E. coli*, there was a dose-response relationship between chlorine concentration and cell reduction up to exposure times of 20 minutes, with increasing disinfectant concentration having a bigger impact (Figure 2). At t = 30 min the differences in the TCC reduction for chlorine concentrations ≤0.75 mg/L became much less, grouping around log reductions of 0.1. However at the highest chlorine concentration applied (1 mg/L), the log reduction was much greater at 0.24.

In the case of WTW-A filtrate, longer exposure and higher chlorine doses had to be applied to see differences in TCC (Figure 2 and SI Figure S2). After 40 minutes of exposure, TCC began to decrease more rapidly for both of the chlorine concentrations applied. After 120 minutes a maximum log reduction of 1.8 was recorded at both 1 and 2 mg/L chlorine concentration. At 2 mg/L chlorine concentration, the loss of TCC was more rapid, with the maximum log reduction seen after 60 minutes, while the reduction slowed above 80 minutes at the 1 mg/L concentration. This decrease in total cells has been reported previously when chlorine exposures exceeded 0.3 mg/L (Song *et al*., 2019) and is further supported by Phe *et al*. (2005) who suggested that after 80 minutes of chlorine exposure, nucleic acids become sufficiently damaged such that the binding of the stain is reduced. As bacteria suffer nucleic acid damage, the binding of SG is also affected due to the destruction of binding sites. As a consequence, FCM signals can migrate left on the green fluorescence axis and leave the gated area. This in turn leads to a reduction of TCC. The effect varies significantly between different samples. As such, better understanding is required as to whether the TCC population is susceptible to nucleic acid damage as a result of increased penetration of chlorine into the cells.

However, these results show that for both *E. coli* and environmental bacteria, TCC were reduced in a similar way and show that nucleic acids are subject to continued damage resulting in a decline of SYBR Green signals due to loss of binding sites. Phe et al. (2009) hypothesize that nucleic acid damage is necessary to achieve the actual killing of microorganisms. As ICC measurement only detects membrane damage, assessing the extent of nucleic acid damage might be of value. Further research is therefore needed to assess in more detail how far the reduction of TCC reflects nucleic acid damage.

a



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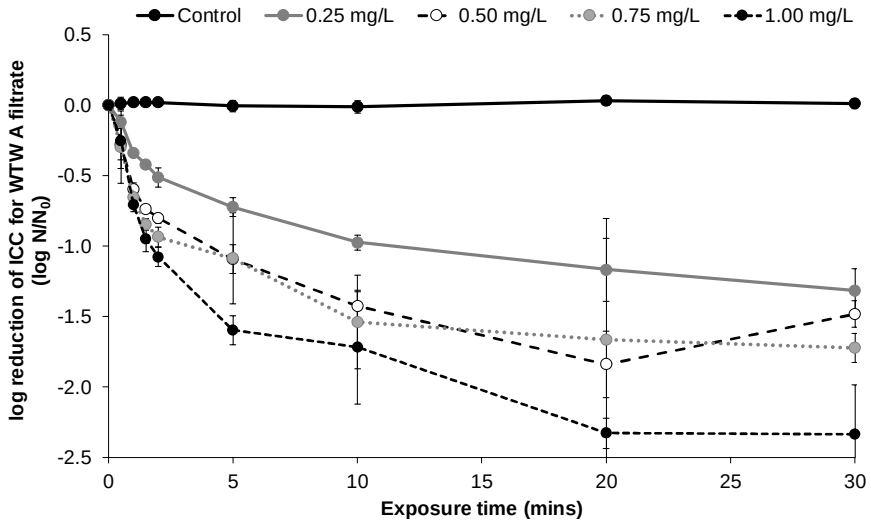

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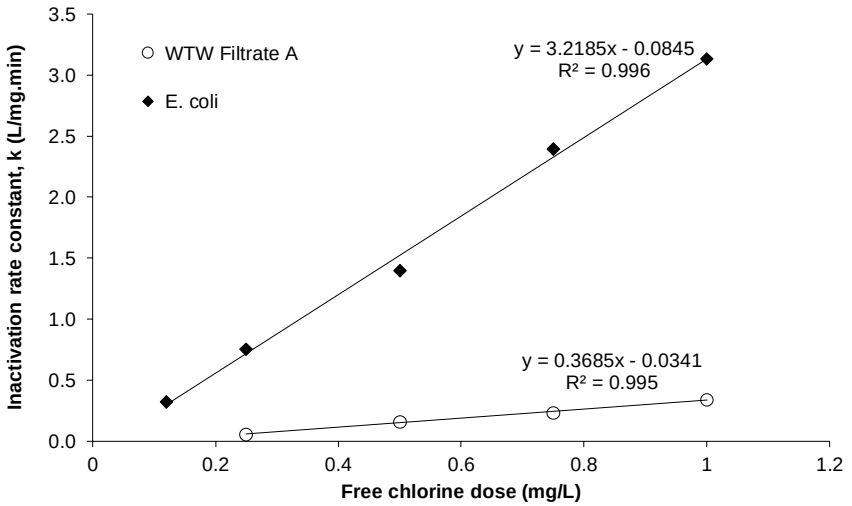


Figure 1. a) Kinetics of cell reduction for ICC *E. coli* (10^5 ICC/mL) exposed to increasing chlorine doses; b) Kinetics of cell reduction for ICC in WTW-A filtrate exposed to increasing chlorine doses; c) Rate constants of cell reduction. All tests were carried out at pH 7.

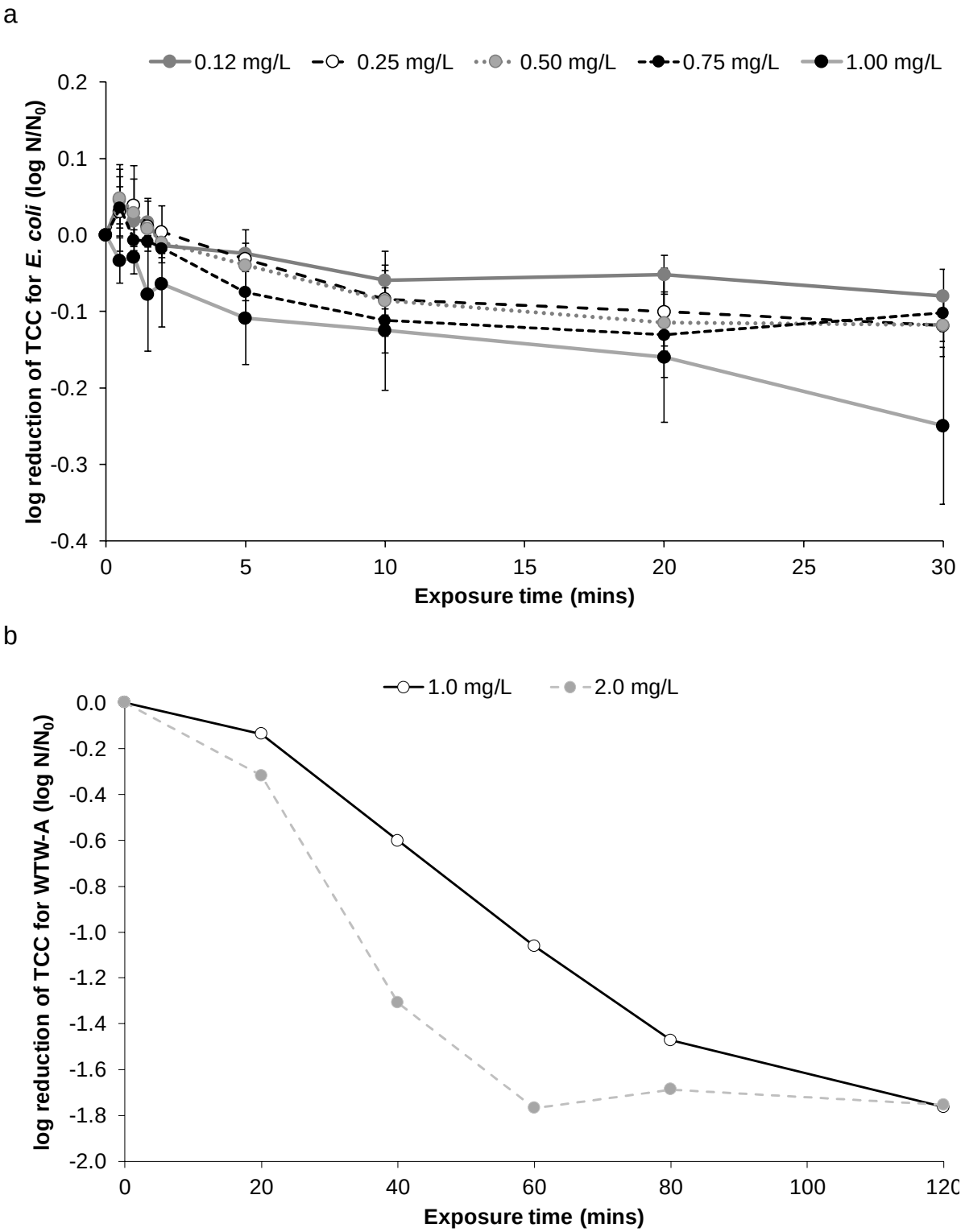


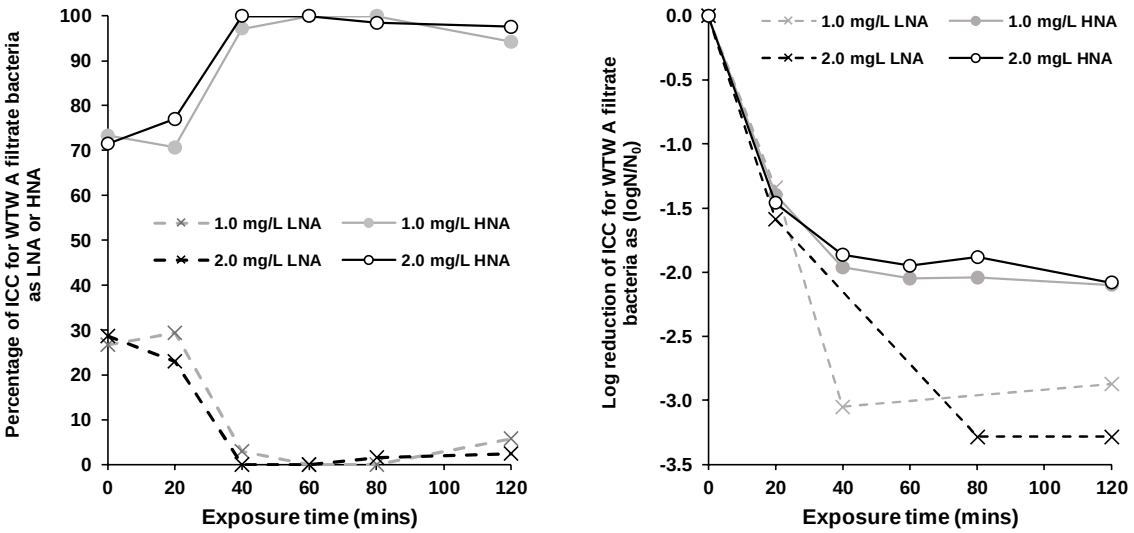
Figure 2. a) Log reduction of TCC for *E. coli* exposed to chlorine concentrations of 0.12, 0.25, 0.50, 0.75 and 1.0 mg/L. b) Log reduction of TCC for bacteria in WTW-A filtrate water exposed to chlorine concentrations of 1.0 and 2.0 mg/L. Experiments were conducted at pH 7 and room temperature.

3.2 HNA and LNA bacteria

The effect of different chlorine concentrations on HNA and LNA clusters was assessed as a function of exposure time for the ICC (Figure 5). The author's acknowledge that there is some debate about the interpretation of bacteria associated with being either HNA and LNA. For example, whether these bacteria represent different types of organism or organisms that are more or less active/healthy (Prest et al., 2013; Liu et al., 2017). However, despite this, the relative proportions between LNA and HNA changed, suggesting that there was a greater susceptibility of LNA bacteria to chlorine than for the HNA organisms. Log reductions of 2.87 and 3.28 were seen for LNA bacteria and 2.10 and 2.08 for HNA bacteria at 1.00 and 2.00 mg/L chlorine doses, respectively. Similar observations were seen for the TCC (SI Figure S3).

In the current study LNA bacteria were shown to be the most sensitive to inactivation from chlorination. In contrast, Ramseier *et al*. (2011) found HNA bacteria in drinking water were slightly more susceptible to free chlorine at Ct of less than 15 mg.min/L but at 20 mg.min/L the inactivation rate of each group was almost equal. These results shows that the susceptibilities of different populations can be distinct for different waters. In the present case, there was a common cluster of HNA cells with a green fluorescence value between 4-6 x 10^4 that were able to persist even after 120 mins of exposure suggesting these are chlorine tolerant organisms. This cluster of organisms represented around 2 x 10^3 TCC/mL and 5 x 10^2 ICC/mL (1.7% and 0.6% of the original count respectively). Such a finding aligns with the fact that some organisms are highly tolerant to chlorine. Considering the natural water in this study was of an unknown heterogeneous environmental composition it is plausible that bacteria whose fluorescent signals remain stable even at high Ct values show high chlorine tolerance. Examples of chlorine tolerant bacteria includes *Flavobacterium* and *Mycobacterium*

avium (Wolfe *et al.*, 1985; Luh and Mariñas, 2007). Therefore, this work has highlighted that specific differences in the HNA and LNA populations could reflect susceptibility to free chlorine and that the stability of certain clusters might therefore be indicative of bacteria with high chlorine resistance.



354 Figure 5. HNA and LNA from ICC of WTW-A filtrate bacteria at chlorine exposures
355 of 1.0 and 2.0 mg/L: a) Percentage of HNA/LNA at each time step; b) log reduction
356 rates for HNA/LNA.
357

3.3 New insights into the interaction between pH, chlorination and bacterial membrane integrity using flow cytometry

The impact of pH on disinfection was assessed for both the pure culture and environmental bacteria. Following disinfection, but prior to FCM analysis, the pH of all samples was adjusted to 7 to avoid any pH effects on fluorescent signals. In the case of the environmental bacteria in WTW-A filtrate, log reductions in intact cells of 0.69, 1.32 and 1.46 were seen after 30 minutes at pH of 6, 7 and 8 respectively (Figure 6a). To confirm these observations, a second real water was assessed (WTW-B filtrate). In this case, the same order was observed, with log reductions of 0.56, 1.31 and 1.63 at pH 6, 7 and 8 (Figure 6b). When the pure culture *E. coli* was considered, there was little discrimination between the inactivation at the different pH, with log reductions around 1.6 after 30 minutes of chlorine exposure (Figure 7).

The observed results were unexpected in that disinfection efficacy as determined by FCM was apparently enhanced under increasingly alkaline conditions for the environmental bacteria, which is the inverse of traditional disinfection theory established from culture based assessment of bacteria. It was likely that the high sensitivity of *E. coli* to chlorine explained why less distinction was seen for the pure culture bacteria. Further investigation of a potential pH effect was carried out by investigating the intercalation of the PI dye with nucleic acids by using a calf thymus DNA solution with pH adjusted to different values. Staining was performed under the same conditions as applied for the FCM analysis (SI Figure S4). Emission at both 670 nm (which replicates the FL3 670/LP bandpass filter of the Accuri C6 FC) and 620 nm (which is the optimum emission point for PI) revealed no direct impact as a function of pH across the range of interest. Similar results have previously been reported for SYBR Green I (Baldock et al. 2013), indicating that the intensity of fluorescence of

FCM dyes is not impacted by the pH range observed in drinking water analysis. The bacterial inactivation with pH relationship was therefore unlikely to be a methodological artefact.

While it is known that chlorine disinfection efficacy increases with decreasing pH (as shown by traditional culture based enumeration of bacteria), the results obtained here suggest that more damage to bacterial membranes is inflicted by chlorine at higher pH. Membrane damage is the parameter that is measured by FCM when using propidium iodide as a vitality dye. The effect on membrane integrity therefore appears to be different from the effect on overall bacterial viability. Other studies that have used FCM to assess disinfection over a pH range have shown similar observations. For example, Ramseier *et al.* (2010) showed that more cells with damaged membranes were observed at pH 8.5 (55%) compared to pH 7 (32%) following chlorine disinfection of a real drinking water, a result consistent with that seen here. Howard and Inglis (2003) found that *Burkholderia pseudomallei* inactivation as assessed by FCM occurred at a greater rate at pH 7 and 8, compared to lower pH values of 4, 5 and 6. In this case, the authors proposed that *B. pseudomallei* employs survival strategies at low pH or is able to exclude chlorine under these conditions, rather than any change in membrane integrity. However, these observations may have been in-line with those seen here. The established view of chlorine disinfection is that HOCl is able to penetrate through the membrane cell wall more effectively than the charged OCl^- species, a mechanism known as the permeability theory (Haas and Engelbrecht, 1980). Accordingly, established practice is to operate disinfection under lower pH environments to maximise inactivation rates (White, 2010). In cases where FCM has been applied in the assessment of chlorine disinfection efficacy over a pH range, the results have not been conclusive, or disagree with the established permeability theory

of increased disinfection efficacy at low pH. This then raises the question as to whether the pH effect of chlorine on membrane integrity is different from the effect on overall cell viability. Further investigations therefore hold much promise in being able to refine the method of action of chlorine. As FCM is gaining more popularity as a public health tool to evaluate the microbial quality of water, understanding the implications of these observations is requried. Consequently, this may limit the application of FCM in assessing bacterial reductions over a pH range.

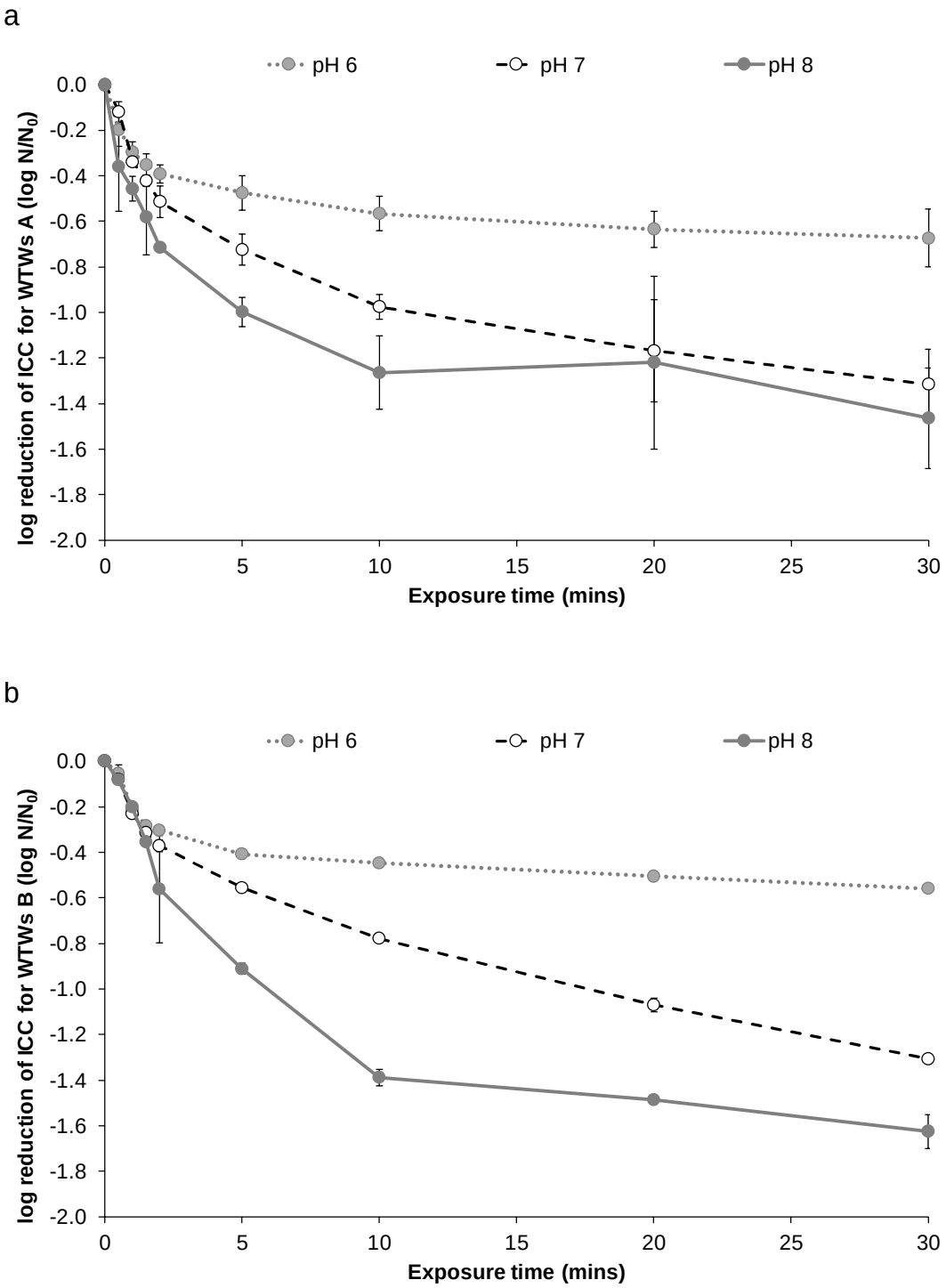


Figure 6. Log reduction of WTW-A (a) and WTW-B (b) filtrate bacteria exposed to a chlorine dose of 0.25 mg/L at varying pH values (pH 6, 7, 8) at room temperature.

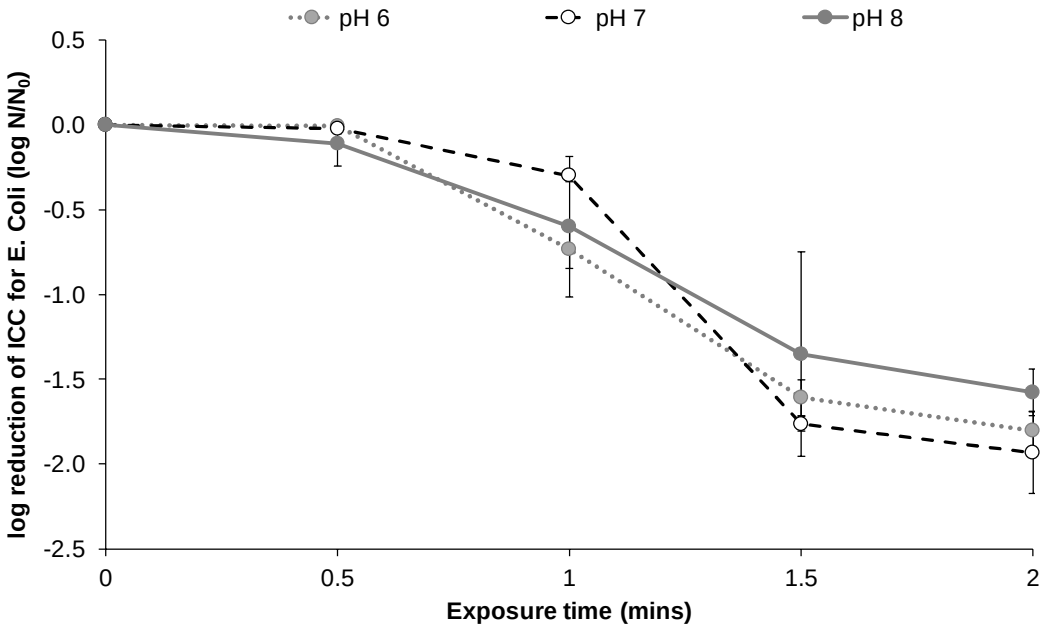


Figure 7. Log inactivation of *E. coli* cells exposed to a chlorine dose of 0.12 mg/L at varying pH values (pH 6, 7, 8) at room temperature.

3.4 The effect of temperature on bacterial inactivation using flow cytometry

Chlorine inactivation was tested at 5, 12 and 19 °C (room temperature) for *E. coli* and WTW-A filtrate bacteria (Figure 8a, b). At 5 °C, log reductions of ICC did not exceed 1.6 and 0.73 for *E. coli* and WTW-A filtrate bacteria, after 5 and 30 minutes respectively. In comparison, the efficacy of chlorine inactivation improved as temperature was increased to 12°C for the *E. coli* ICC following two minutes contact time (Figure 8a). Increased temperatures yielded better log removals. For example, the maximum *E. coli* ICC log reduction of 2.13 was seen after 5 minutes at 19°C. As expected, the real water bacteria were less easily removed and had very similar profiles with increasing temperature and exposure time, with log reduction for ICC at 12°C and 19°C reaching 1.26 after 30 minutes, respectively (Figure 8b). Reduced inactivation occurred at the lower temperatures, as a result of three different mechanisms i) reduced collision between the free chlorine and bacterial membranes

at sufficient activation energy (LeChevallier et al. 1988; Spalding 1962); ii) lower diffusion rate constants for free chlorine therefore poorer penetration of free chlorine into cells (Jolley and Carpenter, 1982) and iii) reduced microbial activity caused by the lower temperatures (Harakeh et al., 1985). While the results presented here are in line with previous observations of bacterial inactivation with temperature, such sensitive monitoring by FCM allows for the rapid development of accurate water specific temperature correction factors that will help water utilities account for differences in disinfection efficacy.

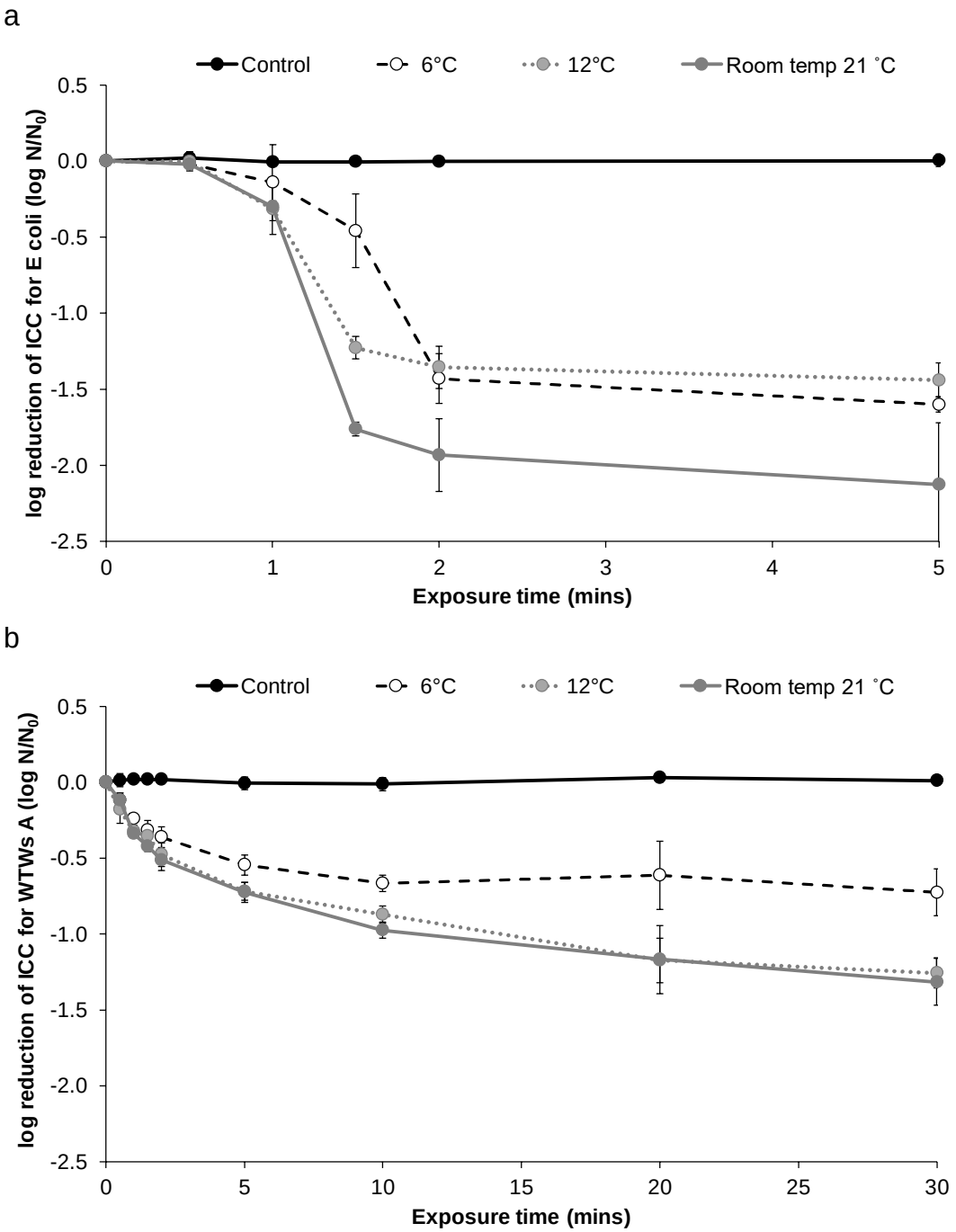


Figure 8. Impact of temperature on chlorine inactivation efficiency: (a) Log inactivation of *E. coli* ICC exposed to a chlorine dose of 0.12 mg/L across a temperature range (6, 12 and 19 °C (room temperature)); (b) Log inactivation of ICC from WTW-A filtrate bacteria exposed to a chlorine dose of 0.25 mg/L at varying temperatures (6, 12 and 19 °C (room temperature)). All tests were carried out at pH 7. Error bars show standard deviation of three independent experimental repeats.

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4. Conclusions

FCM in combination with membrane integrity staining was shown to be suitable at assessing cell damage caused by chlorine over a range of dose and exposure times and this was found to be repeatable in both controlled (pure culture) and practical (WTW filtrate bacteria) scenarios. Likewise, the effects of lower temperature can be seen by the reduced effectiveness of chlorine. Chlorine, however, not only leads to a reduction in ICC, but prolonged exposure caused both a reduction in the TCC and fluorescence, indicating DNA was becoming damaged and the cells were undergoing complete lysis. We also identified a chlorine tolerant population in the filtrate sample that profiled as ‘HNA’, highlighting that HNA bacteria are not necessarily more susceptible to chlorine damage. As a result, the relative proportion of LNA decreased after 40-60 minutes of exposure to chlorine at 1-2 mg/L in our study. An unexpected observation was that the higher pH led to an increased loss of membrane integrity when compared to low pH. This might be due to pH-dependent differences in the mechanistic action of chlorine on the bacterial cell membrane, a finding that warrants further investigation.

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