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Regrowth of *Escherichia coli* in environmental waters after chlorine disinfection: shifts in viability and culturability†

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Bacterial regrowth after water/wastewater disinfection poses severe risks to public health. However, regrowth studies under realistic water conditions that might critically affect bacterial regrowth are scarce. This study aimed to assess for the first time the regrowth of *Escherichia coli* (*E. coli*) in terms of its viability and culturability in environmental waters after chlorine disinfection, which is the most widely used disinfection method. Post-chlorination regrowth tests were conducted in 1) standard 0.85% NaCl solution, 2) river water receiving domestic wastewater effluents, and 3) river water that is fully recharged by domestic wastewater effluents. The multiplex detection of plate count and fluorescence-based viability test was adopted to quantify the culturable and viable *E. coli* to monitor the regrowth process. The results confirmed that chlorine treatment (0.2, 0.5 and 1.0 mg L^{-1} initial free chlorine) induced more than 99.95% of *E. coli* to enter a viable but non-culturable (VBNC) state and the reactivation of VBNC *E. coli* is presumably the major process of the regrowth. A second-order regrowth model well described the temporal shift of the survival ratio of culturable *E. coli* after the chlorination (R^2: 0.73–1.00). The model application also revealed that the increase in initial chlorine concentration and chlorine dose limited the maximum regrowth rate and the maximum survival ratio, and the regrowth rate and percentage also changed with the water type. This study gives a better understanding of the potential regrowth after chlorine disinfection and highlights the need for investigating the detailed relation of the regrowth to environmental conditions such as major components of water matrices.

Water impact

We demonstrated multiplex detection as an effective tool to understand and assess the regrowth process after chlorine disinfection. The gained in-depth knowledge of post-chlorination regrowth in environmental water provides a scientific basis for controlling bacterial regrowth in the context of wastewater treatment and reuse.

1. Introduction

Domestic wastewater is a major point source of waterborne pathogens and disseminating antibiotic resistance through discharge or reuse.[1] Recently, the growing water stress caused by water scarcity and quality deterioration has necessitated and promoted wastewater reuse, including agricultural irrigation, urban reuse, and natural water body recharge.[2] For disinfection to be successful, microorganisms must be sufficiently removed during the treatment while ensuring low microbial risk to public health and the ecosystem after the disinfection process. However, recent studies have reported an undesirable increase in bacterial population after wastewater disinfection, revealing a hidden microbial health risk.[3–5] This issue becomes particularly serious when the regrowth of pathogenic and/or antibiotic-resistant bacteria happens after disinfection.[6,7]

Under environmental stress, bacteria can enter the viable but non-culturable (VBNC) state, an adaptive survival strategy that can possibly continue for years (up to 11 years as has been reported).[8,9] Bacteria in the VBNC state are not culturable but are actually live and metabolically active.[10] Disinfection treatment can act as environmental stress to bacteria and induce bacteria to enter the VBNC state.[11] For instance, Chen *et al.* (2018)[11] have reported that both chlorination and chloramination (0.5–4 mg L^{-1}) in 0.9% NaCl

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solution could induce *Escherichia coli* (*E. coli*) into the VBNC state, although the maximum disinfectant dose that allows VBNC reactivation varied with the disinfectant and water matrix.

While losing culturability, VBNC *Legionella pneumophila* induced by monochloramine and VBNC *Pseudomonas aeruginosa* induced by UV-C irradiation are able to synthesize virulence-related proteins.[12,13] Moreover, pathogenic bacteria in the VBNC state may not be infectious but can regain culturability and virulence when the environment is favorable.[6,14] For example, VBNC *Vibrio cholerae* O1 was reported to revert to a culturable and virulent state in the human intestine.[15] While bacterial culturability is determined by a conventional culture-based method (*i.e.*, plate count), the assessment of bacterial viability requires a culture-independent method as viable bacteria include the VBNC fraction that cannot be cultured. Therefore, multiplex detection methods that combine culture-based and culture-independent approaches should be applied in order to monitor viable cells and evaluate the regrowth.[5] All available evidence supports that VBNC bacteria are a potential risk to public health and the ecosystem, and thus it is essential to assess and control viable bacteria, including culturable and VBNC cells, during and after disinfection. To the best of our knowledge, no study has monitored the changes of bacterial viability with time after disinfection treatment, limiting our understanding of the actual health risk associated with the viable bacteria in disinfected water.

Among various wastewater disinfection processes, chlorination is the most widely used because of its simple application and low cost. As mentioned above, however, chlorination is challenged by the potential risk of bacterial regrowth. The process of post-chlorination regrowth may be influenced by various factors such as temperature,[16–18] availability of nutrients,[19] residence time,[19,20] water matrix,[19,21] and oxidant residual.[22] These influential factors indicate the need for regrowth tests under a number of possible conditions. Moreover, regrowth tests after chlorination have been performed in a few studies.[11,23–25] In these reports, no systematic test was run under realistic water conditions after chlorination, which remains a critical knowledge gap regarding the safe reuse of treated water.[5] Similarly, regrowth kinetic models have been developed to simulate the photoreactivation and/or dark-repair process after UV disinfection,[16,17,26,27] but no research effort has been put on modeling the post-chlorination process. Therefore, it is worth modeling the regrowth process after chlorine disinfection to understand the regrowth process better and enhance our capacity against relevant microbial risk.

In this study, we address the gaps in the literature and target for the first time investigating the regrowth process of a model bacterium *E. coli* after chlorine treatment under environment-relevant conditions. This study constitutes the first report to assess the post-chlorination regrowth in environmental water by adopting multiplex detection of plate count and fluorescence-based viability tests. We also adopted a modeling approach to evaluate the regrowth process and relate it to the chlorination and post-chlorination process conditions.

2. Materials and methods

2.1 Water sampling and characterization

To represent the conditions relevant to wastewater reuse or discharge scenarios, river water and treated wastewater were also used in this study. The river water was sampled in the downstream section of the Tama River (Tokyo, Japan) on March 4th, 2021, which receives treated domestic wastewater from several treatment plants in its upstream basin. Treated wastewater was sampled from the Nomi River (an urban channel in Tokyo, Japan) on May 12th, 2021, which is fully fed by treated domestic wastewater. After the sampling, the water was filtered with a 0.22 μm membrane to ensure no bacteria present in the filtered water. The concentrations of dissolved organic carbon (DOC) and ions in the filtered water were analyzed. The chemical analysis and the results are summarized in Text S1 and Table S1,† respectively.

2.2 Chlorination and regrowth tests

A pure culture of *E. coli* K-12 (NBRC3301) provided by NITE Biological Resource Center, Japan, was used. A fresh liquid culture of *E. coli* was prepared by inoculation on LB broth (L3022, Sigma-Aldrich) and incubation at 37 °C for 12–18 hours in a shaker incubator to reach the stationary phase. To prepare test suspensions, 1.0 mL of the liquid culture was centrifuged at 3000 rpm for 15 min at 4 °C and resuspended in 1 mL sterile 0.85% NaCl solution. The centrifugation and resuspension were repeated twice to wash off the LB nutrient medium. Finally, the prepared *E. coli* suspension achieved a concentration of $\sim 10^9$ cells per mL. In the chlorination treatment, 1 mL of the prepared *E. coli* suspension was diluted in 100 mL of 0.85% NaCl solution, which resulted in an initial concentration of $\sim 10^7$ cells per mL for the chlorination.

The experimental setup for chlorination and regrowth tests is shown in Fig. S1.† Chlorine disinfection was conducted in 100 mL batch tests in a sterile 200 mL glass beaker while being mildly stirred with a magnetic stirrer at 25 °C. First, the prepared *E. coli* suspension was added into a sterile 200 mL glass beaker containing sterile 0.85% NaCl solution. Solutions of sodium hypochlorite (NaClO) (20, 50, and 100 mg L^{-1} free chlorine) were prepared by diluting a stock NaClO solution (>5%, Kanto Chemical). Then, sodium hypochlorite at different concentrations was added to the reactor to reach 0.2, 0.5, and 1 mg L^{-1} initial free chlorine. The applied chlorine concentrations in this study were designed to be lower compared to those in practical wastewater treatment (typically, 5–20 mg L^{-1} (ref. 28)) because the subjected water to *E. coli* in our experiment does not contain chlorine-demanding inorganic and organic components. The treatment continued up to 30 minutes for the 0.2 and 0.5 mg L^{-1} groups and up to 10 minutes for the 1

mg L^{-1} group. At each sampling time (0.5, 5, 10, 20 and 30 minutes for 0.2 and 0.5 mg L^{-1}, and 0.5, 1, 2, 5 and 10 minutes for 1.0 mg L^{-1}), 0.5 mL of sodium thiosulfate solution was added into the reactor and mixed for 5 minutes to terminate the chlorination process (the final sodium thiosulfate concentration was 0.6% (w/v)). Control experiments (*i.e.*, no chlorine but with thiosulfate, no chlorine and no thiosulfate) were also conducted to observe the fate of non-disinfected *E. coli* and assure no effect of sodium thiosulfate on bacterial viability (Fig. S2†). In addition, the free chlorine residual was measured at each contact time using the *N*,*N*-diethyl-*p*-phenylenediamine (DPD) reagent for free chlorine (Hanna instruments) and a UV-vis spectrophotometer (Shimadzu UV-2600, Japan) (calibration curve shown in Fig. S3†). Specifically, a 10 mL sample was filtered with a 0.45 μm membrane and mixed with the DPD reagent for 20 seconds. Then, the absorbance was measured at a wavelength of 510 nm.[29] The chlorine dose was calculated by integrating the area under the chlorine decay curve within each contact time and used to represent the dosage of chlorination (mg min L^{-1}).

In the subsequent regrowth test, the chlorinated samples were kept in the dark at 25 °C in an incubator. To represent the conditions relevant to wastewater reuse or discharge scenarios, chlorinated *E. coli* were kept in four types of water after quenching the residual chlorine at each contact time (Fig. S1†).

The original chlorinated samples (A) could mimic the conditions of treated wastewater stored in a tank before being reused or discharged. The mixtures with 0.85% NaCl solution (B), Tama River water (C), and Nomi River water (D), respectively, simulated the conditions of treated wastewater introduced to the environment. For the mixed samples, specifically, each 15 mL chlorinated sample was mixed with 15 mL of 0.85% NaCl solution, filtered Tama River water, and filtered Nomi River water, respectively, in a 50 mL sterilized polypropylene centrifuge tube and kept while the cap was loosely closed. The viable and culturable *E. coli* concentrations were monitored every day for 3 days after the chlorination.

2.3 Quantification of culturable and viable *E. coli*

The plate count method was used to quantify culturable *E. coli*. After quenching chlorination, 1 mL of each appropriately diluted sample was mixed with LB agar medium and incubated for 24 h at 37 °C. For each dilution rate of samples, plates were prepared in triplicate. A LIVE/DEAD BacLight bacterial viability kit (L7012) was used to quantify viable *E. coli*, following our previous work[30] (details in Text S2†). The viable and culturable *E. coli* counts in the control experiment showed a significant correlation (Pearson correlation coefficient r = 0.86, $p < 0.05$). All samples were measured in triplicate. VBNC cells were calculated as the difference between the viable and culturable cell counts.

The percentage of regrowth was calculated by eqn (1)[31] to evaluate the regrowth of *E. coli* after chlorination. It indicates how much the inactivated cells could regrow.

$$\text{Percentage of regrowth (\%)} = \frac{N_R - N}{N_0 - N} \times 100 \quad (1)$$

Here, N_0 is the culturable cell count before chlorination treatment (CFU mL^{-1}), N is the culturable cell count immediately after chlorination treatment (*i.e.*, the beginning of regrowth test) (CFU mL^{-1}), and N_R is the culturable cell count for a certain time during the regrowth phase (CFU mL^{-1}).

The log removal considering regrowth was calculated by eqn (2) to quantify the effect of regrowth on the actual disinfection efficiency.

$$\text{log removal} = \log_{10}(N_0) - \log_{10}(N_R) \quad (2)$$

2.4 Modeling of inactivation and regrowth kinetics

Hom's model (eqn (3))[32] describes the non-linear disinfection kinetics with initial lag or tailing off frequently observed in practice[33,34] and in this study.

$$\ln\frac{N_t}{N_0} = -kD^n t^m \quad (3)$$

Here, k is the pseudo-first-order inactivation rate constant, D is the disinfectant concentration, and n and m are empirical constants. This model was applied to obtain the inactivation rate constants of culturable and viable *E. coli*, k_c and k_v (eqn (4) and (5)).

$$\text{Viable cells (cells per mL) } N_V = N_{V(0)} \cdot e^{-k_v \cdot D^{n_v} \cdot t^{m_v}} \quad (4)$$

$$\text{Culturable cells (CFU per mL) } N_C = N_{C(0)} \cdot e^{-k_c \cdot D^{n_c} \cdot t^{m_c}} \quad (5)$$

Several empirical models have been proposed to represent bacterial regrowth after UV disinfection. The models describe the process as a saturation-type first-order or second-order reaction with/without a decay phase.[17,26] For the regrowth process after chlorination, we selected the second-order regrowth model (eqn (6)) because it depicts a logistic curve that was observed in our experimental results. The model fitness was evaluated from the *R*-squared value (R^2) between the observed and predicted survival ratios of culturable *E. coli*.

$$\text{Second-order regrowth model}: S_R = \frac{N_R}{N_0} \times 100 = \frac{S_m}{1 + [S_m/S_0 - 1] \cdot e^{-k_R \cdot S_m \cdot R_t}} \quad (6)$$

Here, S_R is the survival ratio of culturable *E. coli* (%) during the regrowth phase; S_0 is the survival ratio of culturable *E. coli* immediately after chlorination (%); S_m is the maximum

survival ratio of culturable *E. coli* (%); R_t is the regrowth time (day); k_R is the regrowth rate constant (% per day). The survival ratio of viable *E. coli* was calculated as well to compare with that of culturable *E. coli* and to understand the behavior of viable cells in different types of water after chlorine disinfection. In addition to modeling the survival ratio of culturable *E. coli*, a modified model of the second-order regrowth model was applied to simulate the concentration ratio of culturable *E. coli* to viable *E. coli* during the regrowth phase (details in Text S3†).

In the second-order regrowth model (eqn (6)), k_R is not the actual reaction rate and the actual regrowth rate K (% per day) is expressed by eqn (7). The actual regrowth rate reaches its maximum (K_{max}) when S_R is half of S_m. Thus, K_{max} is calculated by eqn (8) (Li *et al.*, 2017).[16]

$$\text{Actual regrowth rate} \quad K = k_R (S_m - S_R) S_R \tag{7}$$

$$\text{Actual maximum regrowth rate} \quad K_{max} = \frac{k_R {S_m}^2}{4} \tag{8}$$

In addition, multiple linear regression was applied to examine the relationship between the regrowth model parameters (*i.e.*, k_R, S_m and K_{max} as dependent variables) and the chlorination conditions (*i.e.*, the initial chlorine concentration and the chlorine dose as independent variables). The model fitness was evaluated from R^2 between the observed and predicted values of the model parameters.

3. Results and discussion

3.1 Impact of chlorination on the culturability and viability of *E. coli*

Chlorine treatment reduced the culturability and viability of *E. coli* to different extents. Given the initial free chlorine concentrations of 0.2, 0.5 and 1 mg L^{-1}, the culturable cell counts of *E. coli* decreased from 6.9 log CFU mL^{-1} to 3.1 log CFU mL^{-1} in 30 minutes, to 1.4 log CFU mL^{-1} in 30 minutes, and to 1.6 log CFU mL^{-1} in 10 minutes, respectively (Fig. 1(1)). However, the viable cell counts were reduced by less than 10% and were still approximately 10^6 cells per mL at the end of chlorine treatment. By chlorination, the concentration ratio of culturable to viable *E. coli* decreased

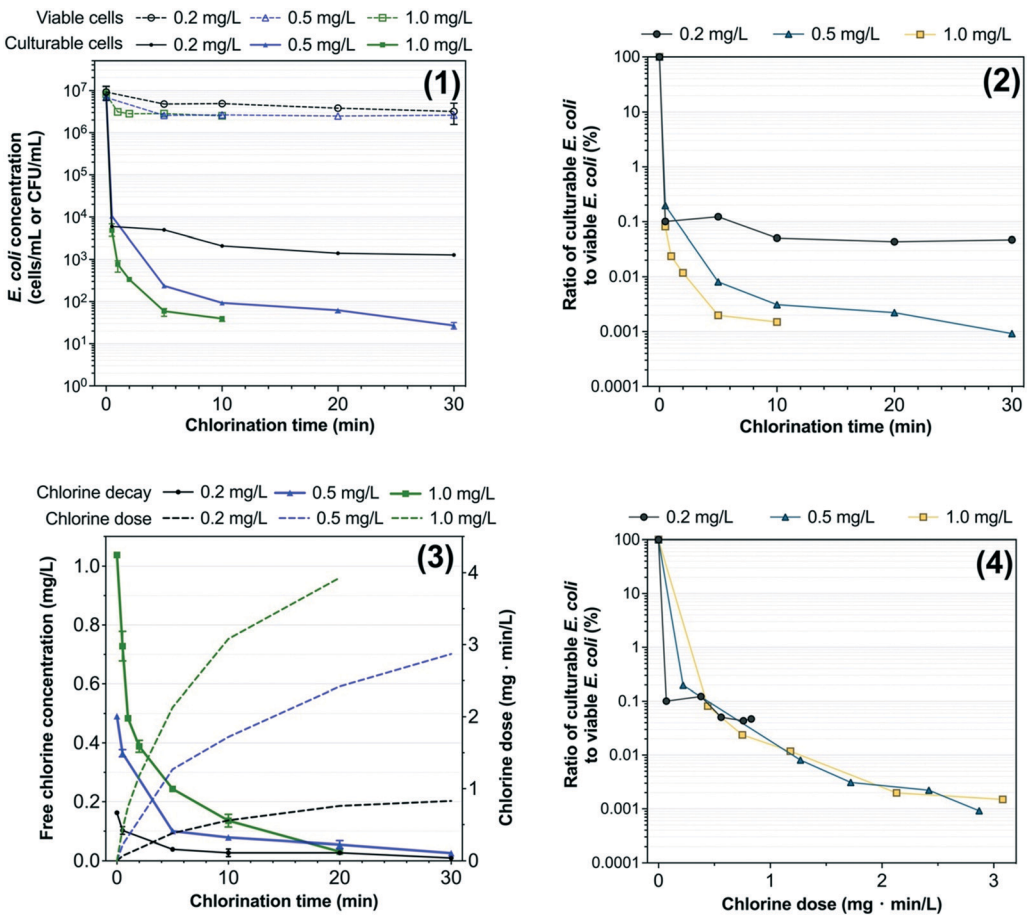

Fig. 1 (1) Viable and culturable *E. coli* cell counts, (2) the concentration ratio of culturable *E. coli* to viable *E. coli*, (3) chlorine decay and dose as a function of time, and (4) the concentration ratio of culturable *E. coli* to viable *E. coli* as a function of chlorine dose, during chlorine disinfection (initial free chlorine of 0.2, 0.5, and 1 mg L^{-1}). The error bars represent the standard deviation from duplicates with measurements conducted in triplicate. The error bar is not visible when it is shorter than the size of the symbol.

from 100% to 0.047%, 0.00092% and 0.0015% after chlorine treatment with initial free chlorine of 0.2 mg L^{-1} for 30 minutes, 0.5 mg L^{-1} for 30 minutes, and 1.0 mg L^{-1} for 10 minutes, respectively (Fig. 1(2)). The culturable and viable *E. coli* concentrations were reduced rapidly within the first 5 minutes, followed by a slow decline, showing a similar trend to that of the chlorine decay with the increase of contact time (Fig. 1(3)). Despite the initial free chlorine concentration, the concentration ratio of culturable to viable *E. coli* showed a similar decreasing rate with the increase of chlorine dose (Fig. 1(4)). The difference in the concentrations of viable and culturable *E. coli* indicates the cell concentration of the VBNC state. As a result, the VBNC *E. coli* accounted for over 99.95% of the viable *E. coli* at the end of chlorine treatment. Previous studies also showed that low-level chlorination (0.3–1.5 mg L^{-1}) could effectively reduce the culturability of *E. coli* and induce *E. coli* to enter the VBNC state.[36,37] In addition to chlorination, other disinfection treatments such as chloramination, ozonation and UV irradiation were also reported to induce bacteria to enter into a VBNC state.[38–41]

Regardless of the initial free chlorine concentration, the inactivation of viable and culturable *E. coli* showed a tailing phase (Fig. 1(1)). The tailing phase may appear due to the aggregation of microorganisms, the appearance of resistant subpopulations or the decline of disinfectant concentration over time.[42,43] As the result of model application, the inactivation kinetics of culturable and viable *E. coli* were well described by Hom's model (Table 1). The inactivation rate constants of culturable *E. coli* (k_c) were much higher than those of the viable *E. coli* (k_v) (Table 1). Meanwhile, the rate constants increased with the initial chlorine concentration. However, k_v increased more slowly than k_c with the initial chlorine concentration, indicating that a higher initial chlorine concentration induced the VBNC state more rapidly than a lower initial chlorine concentration. The existence of VBNC *E. coli* after chlorine disinfection indicated the substantial potential of their regrowth.

3.2 Regrowth process of *E. coli* after chlorine disinfection

Adopting the multiplex detection (*i.e.*, the combination of the plate count method and fluorescence-based viability test), we presumed that the reactivation of VBNC *E. coli* was the major regrowth process after chlorine disinfection. During the regrowth phase, the viable *E. coli* concentration showed diverse patterns depending on the contact time of chlorine treatment. In the un-chlorinated samples, the viable *E. coli* increased up to 0.3 log cells per mL in four types of water (Fig. 2). Consequently, the survival ratio of viable *E. coli* significantly increased (Pearson correlation between survival ratio and regrowth time: $r > 0$, p value of 0.028, 0.0065, 0.17, and 0.13 in water (A), (B), (C) and (D), respectively) (Fig. 3). In 0.5 min chlorinated samples, we observed a similar increase in the viable *E. coli* concentration (Fig. 2) and survival ratio (Fig. 3). In contrast, the viable cell concentration and survival ratio fluctuated in other chlorinated samples with longer contact times and showed no significant differences (Fig. 2 and 3). It was unexpected to observe the increase of viable *E. coli* in the saline solution ((A) and (B)) that contains no energy source. However, one study reported that a 0.9% saline solution can support significant growth of *Enterobacteriaceae* strains, including the pure strain of *E. coli*, while the mechanism was not elaborated.[44] In natural aquatic environments, enteric bacteria (*e.g.*, *E. coli*) could survive but are not able to multiply[45] or could multiply at a very low specific growth rate.[46] The estimated doubling time of viable *E. coli* in the un-chlorinated and 0.5 min chlorinated samples in this study was at least three days, much longer than the reported doubling time of 15 hours in a wild environment.[47] Therefore, we concluded that *E. coli* reproduction was not a significant process involved in the chlorinated samples under our experimental conditions.

On the other hand, in the un-chlorinated samples, the concentration and survival ratio of culturable *E. coli* decreased over time (Pearson correlation between survival ratio and regrowth time: $r < 0$, p value of 0.025, 0.14, 0.14, and 0.13 in water (A), (B), (C) and (D), respectively) (Fig. 2 and S6†). The loss of culturability in saline solution was possibly due to nutrient starvation.[48] However, in all the chlorinated samples, culturable *E. coli* showed a notable increasing trend and eventually reached a much higher concentration and survival ratio than those at the beginning of the regrowth phase (Fig. 2 and 4). Nevertheless, *E. coli* reproduction in the chlorinated samples is still a possible process because the remaining active cells after chlorination were much less than those in the un-chlorinated samples. To identify the dominant regrowth process, reproduction or reactivation of VBNC, we adopted the hypothetico-deductive analysis (Text S4, Scheme S1†). In this analysis, we hypothesized that reproduction is the only regrowth process

Table 1 Kinetic parameters of Hom's model applied to the inactivation of viable and culturable *E. coli* by chlorination with initial free chlorine of 0.2, 0.5, and 1 mg L^{-1}. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Initial chlorine concentration	Viable				Culturable			
	k_v	n_v	m_v	R^2	k_c	n_c	m_c	R^2
0.2 mg L^{-1}	0.35	0.00	0.32	0.98**	7.22	0.00	0.05	0.99***
0.5 mg L^{-1}	0.96	0.02	0.03	1.00***	7.81	0.00	0.15	1.00***
1.0 mg L^{-1}	0.97	0.00	0.07	1.00***	10.64	0.27	0.30	1.00***

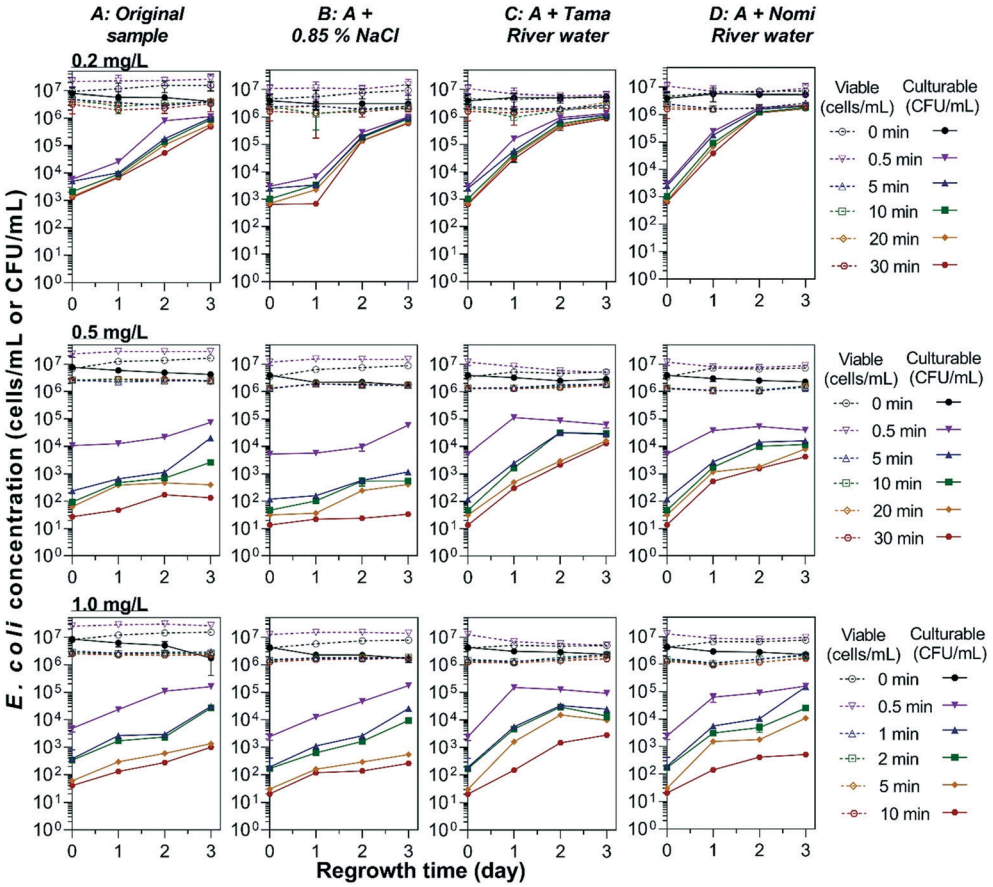

Fig. 2 The regrowth of *E. coli* in four types of water after treatment with various initial doses of chlorine (0.2, 0.5, and 1 mg L^{-1}). The original sample was chlorinated *E. coli* in 0.85% NaCl solution without dilution or mixing treatment. In the legend, the chlorination time was noted, 0 to 30 min for 0.2 and 0.5 mg L^{-1} and 0 to 10 min for 1.0 mg L^{-1} treatment. The error bars represent the standard deviation from duplicate experiments where cell plate count and fluorescence measurement were conducted in triplicate. The error bar is not visible when it is shorter than the size of the symbol.

and tested it by comparing the hypothetical calculation with the experimental results. The calculation was based on the doubling time of un-chlorinated or 0.5 min chlorinated viable *E. coli*, which was considered shorter than that of chlorinated culturable *E. coli*. Thus, this calculation provided an estimate of the maximum concentration resulting only from reproduction (no reactivation). As a result (Fig. S5†), the ratio of the hypothetical estimation to the experimental observation of the regrowth was less than 0.5 under most of the conditions. Particularly, in the environmental waters (Tama River and Nomi River), the ratio was always less than 0.5 regardless of the chlorination time and the regrowth time after chlorination. Therefore, we concluded that the reactivation from the VBNC state is likely the dominant regrowth process.

Direct evidence of reproduction and reactivation in the experiment and quantitative analysis of different processes remains a challenge for understanding the regrowth process. To address this challenge, future studies should (1) explore the reproduction ability of damaged and un-damaged bacteria in environmental water, to understand the contribution of reproduction to the regrowth; (2) investigate the regrowth process in environmental water after achieving the complete removal of culturable cells, to provide direct evidence of the VBNC reactivation; and (3) implement frequent monitoring of the viable and culturable cells (*e.g.*, hourly), to catch the dynamic changes in the regrowth phase.

Destiani and Templeton (2019)[25] reported the increase of antibiotic-resistant *E. coli* and *Pseudomonas aeruginosa* in phosphate-buffered saline solution by the plate count method after chlorination. However, their study did not determine the dominant regrowth process, which might be the reactivation of VBNC cells or the reproduction of culturable cells.[5] Chen *et al.* (2018) applied a high dose of disinfectant (4 mg L^{-1} chlorine and chloramine) to eliminate culturable *E. coli* and confirmed their reactivation from the VBNC state in LB broth (rich medium) by calculating the generation time.[11] In this study, we confirmed the existence of VBNC *E. coli* in the chlorination process (Fig. 1) and the non-significant reproduction in chlorinated samples (Fig. 3). Therefore, the reactivation of VBNC *E. coli* was concluded as the dominant regrowth process in our experiment. Environmental stresses (*e.g.*, low temperature or high salinity) trigger the VBNC state of bacteria, and elimination

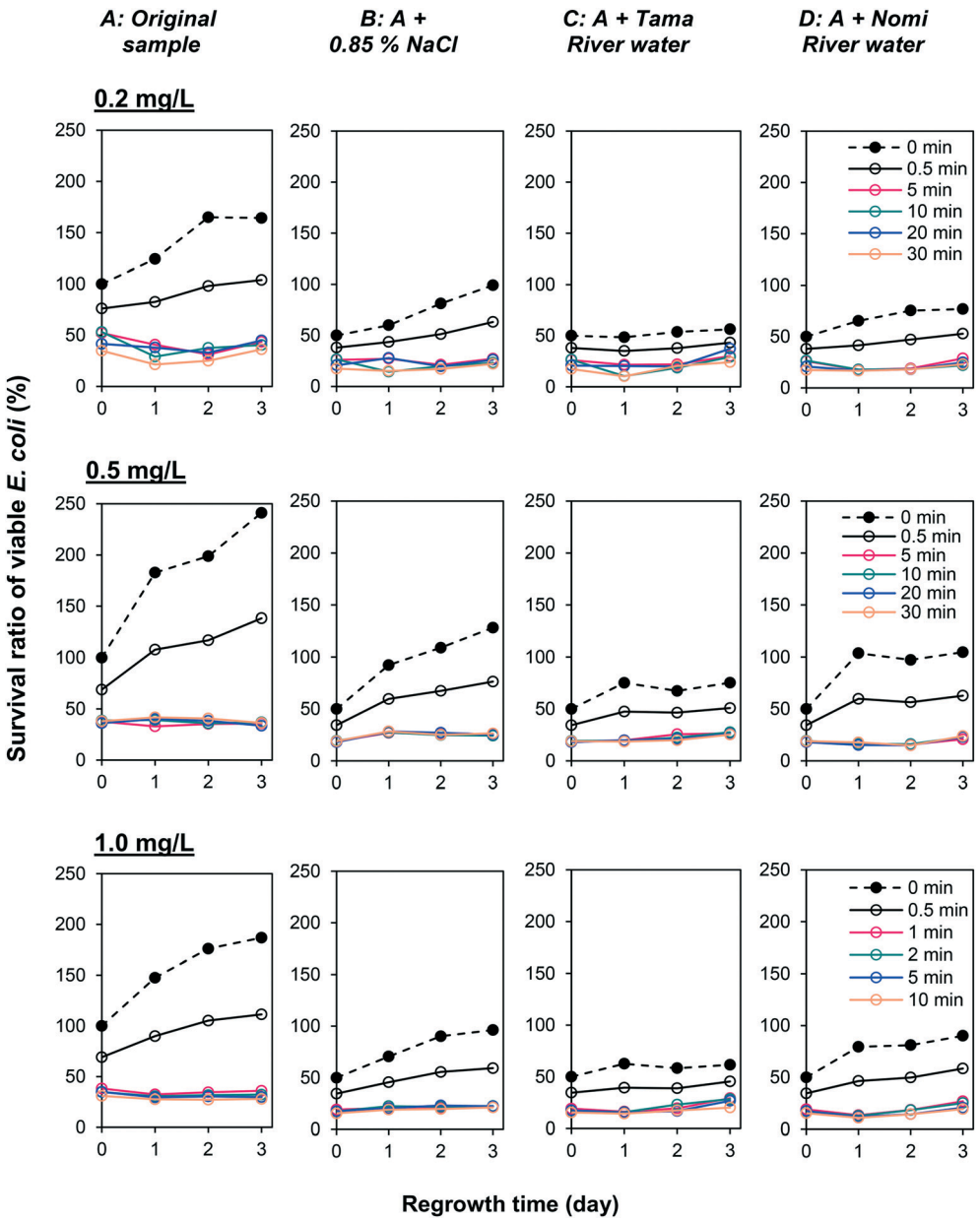

Fig. 3 The survival ratio of viable *E. coli* in four types of water after treatment with various initial chlorine concentrations (0.2, 0.5, and 1 mg L^{-1}) and contact times (0.5 min to 10 min or 30 min). A: Original sample; B: the mixture of the original sample with 0.85% NaCl solution; C: the mixture of the original sample with Tama River water; D: the mixture of the original sample with Nomi River water.

of these stresses (*e.g.*, temperature upshift or relief from saline stress) could make bacteria regain culturability.[10,49–51] In the presented results, the removal of environmental stress (*i.e.*, oxidative stress from chlorine) allowed *E. coli* to regrow, which was attributed to the reactivation from the VBNC state, leading to the increase of culturable cell counts.

3.3 Effect of disinfection conditions on the regrowth of *E. coli*

Disinfection conditions (*i.e.*, initial chlorine concentration and chlorine dose) played an important role in determining the speed and degree of regrowth. The percentage of regrowth and log removal considering regrowth were calculated to quantify the degree of regrowth (Fig. 5) and the impact of regrowth on the chlorination efficiency (Fig. S6†). After chlorine disinfection at 0.2 mg L^{-1}, the percentage regrowth in all water matrices increased over time after disinfection, reaching an eventual level of 6.2% (30 min chlorinated sample) to 28.7% (0.5 min chlorinated sample introduced into Nomi River water) after three days (Fig. 5). Consequently, the log removal considering regrowth after 0.2 mg L^{-1} chlorination showed a substantial reduction of approximately 1 log per day, resulting in a 2.2 log to 3.4 log reduction in the log removal after three days (Fig. S6†). When higher initial chlorine concentrations (0.5 and 1.0 mg L^{-1}) were applied, the percentage of regrowth ranged from

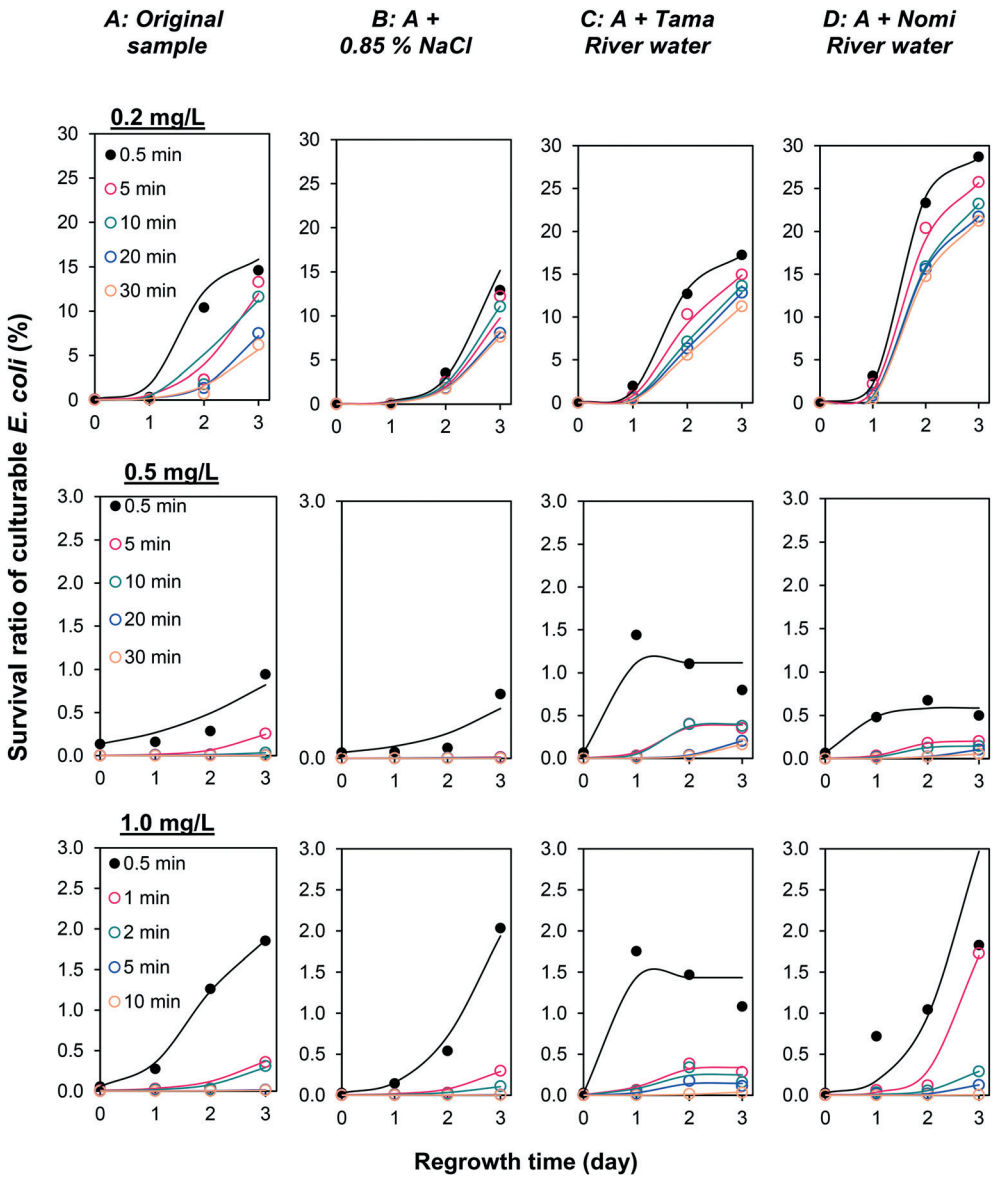

Fig. 4 The survival ratio of culturable *E. coli* during regrowth in four types of water after treatment with various initial chlorine concentrations (0.2, 0.5, and 1 mg L^{-1}) and contact times (0.5 min to 10 min or 30 min). The lines represent the modeling output of eqn (6).

0.0003–1.80% during the 3 day storage, meaning that only less than 1.80% of the VBNC *E. coli* regained culturability (Fig. 5), leading to a 0.39 to 2.97 log reduction in the log removal in three days (Fig. S6†).

Meanwhile, even though a longer treatment time by 0.2 mg L^{-1} chlorination did not alter the trend or reduce the degree of regrowth, the contact time of chlorination did affect the regrowth pattern and level under the four water conditions when higher initial chlorine concentrations (0.5 and 1.0 mg L^{-1}) were applied (Fig. 2). Similarly, Huang *et al.* (2011) reported that a higher chlorine concentration could lower the extent of regrowth of antibiotic-resistant bacteria and total heterotrophic bacteria.[23] Thus, an increase of the initial chlorine concentration could limit the regrowth of *E. coli* to some extent.

The second-order regrowth model (eqn (6)) was applied to simulate the survival ratio of culturable *E. coli versus* the regrowth time in four types of water after chlorine disinfection (Fig. 4). This model showed a good fitness with the experimental data, mostly giving R^2 values over 0.90 (Table S3†). The modified model (eqn (S1)†) could also well describe the temporal shift of the concentration ratio of culturable to viable *E. coli* (R^2: 0.75–1.00) (Fig. S7†), and the fitted values of model parameters (k_R and S_m) were close to those fitted with the second-order regrowth model (eqn (6)). These model applications suggested that the post-chlorination regrowth followed typical regrowth kinetics that was initially identified for the photoreactivation kinetics after UV disinfection,[17] including a lag phase, an exponential increase phase, and a stabilization phase, within the 3 day

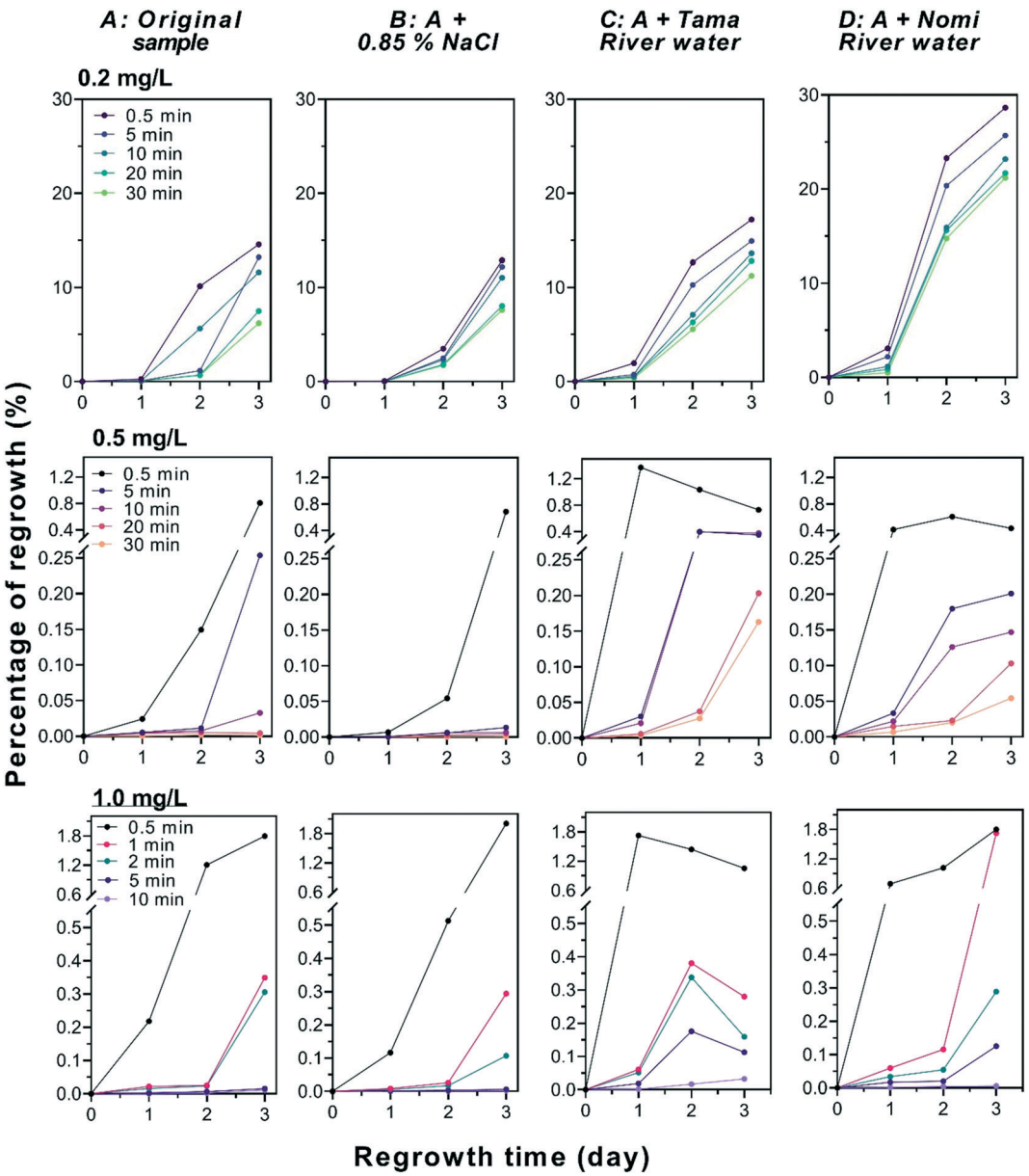

Fig. 5 The percentage of regrowth (%) of culturable *E. coli* in four types of water after treatment with various initial chlorine concentrations (0.2, 0.5, and 1 mg L^{-1}) and contact times (0.5 min to 10 min or 30 min).

storage. The initial free chlorine concentration showed an influence on the regrowth parameters (k_R, S_m, and K_{max}) (Fig. 6(A)–(C)). Generally, for the higher initial chlorine concentration, k_R increased but S_m and K_{max} decreased, even though the difference between initial chlorine concentrations of 0.5 and 1.0 mg L^{-1} was quite minor.

The regrowth rate constant k_R (% per day) generally showed an increasing tendency with the increase of chlorine dose (mg min L^{-1}) (Fig. 6(D)) while it decreased with the initial concentration ratio of culturable to viable *E. coli* (%) (Fig. 6(G)). On the other hand, the maximum survival ratio S_m and maximum actual regrowth rate K_{max} decreased with increasing chlorine doses (Fig. 6(E) and (F)) and increased with the concentration ratio of culturable to viable *E. coli* at the beginning of the regrowth phase (Fig. 6(H) and (I)). At an initial chlorine concentration of 0.2 mg L^{-1}, S_m decreased from 16.1% to 7.5%, and K_{max} decreased from 13.0% per day to 4.5% per day in the original sample when the chlorine dose increased from 0.07 to 0.83 mg min L^{-1} (Fig. 6(E) and (F), and Table S3†). Notably, at an initial chlorine concentration of 0.5 mg L^{-1}, S_m and K_{max} were reduced over 100 times from 2.00% and 0.377% per day while increasing the chlorine dose from 0.22 to 2.87 mg min L^{-1} (Table S3†). This suggested that a longer treatment time with chlorine effectively limited the extent and rate of *E. coli* regrowth under the tested conditions. Although the values of S_m and K_{max} were low, for an initial *E. coli* concentration of 10^6 cells per mL, an S_m value of 1% indicates the maximum

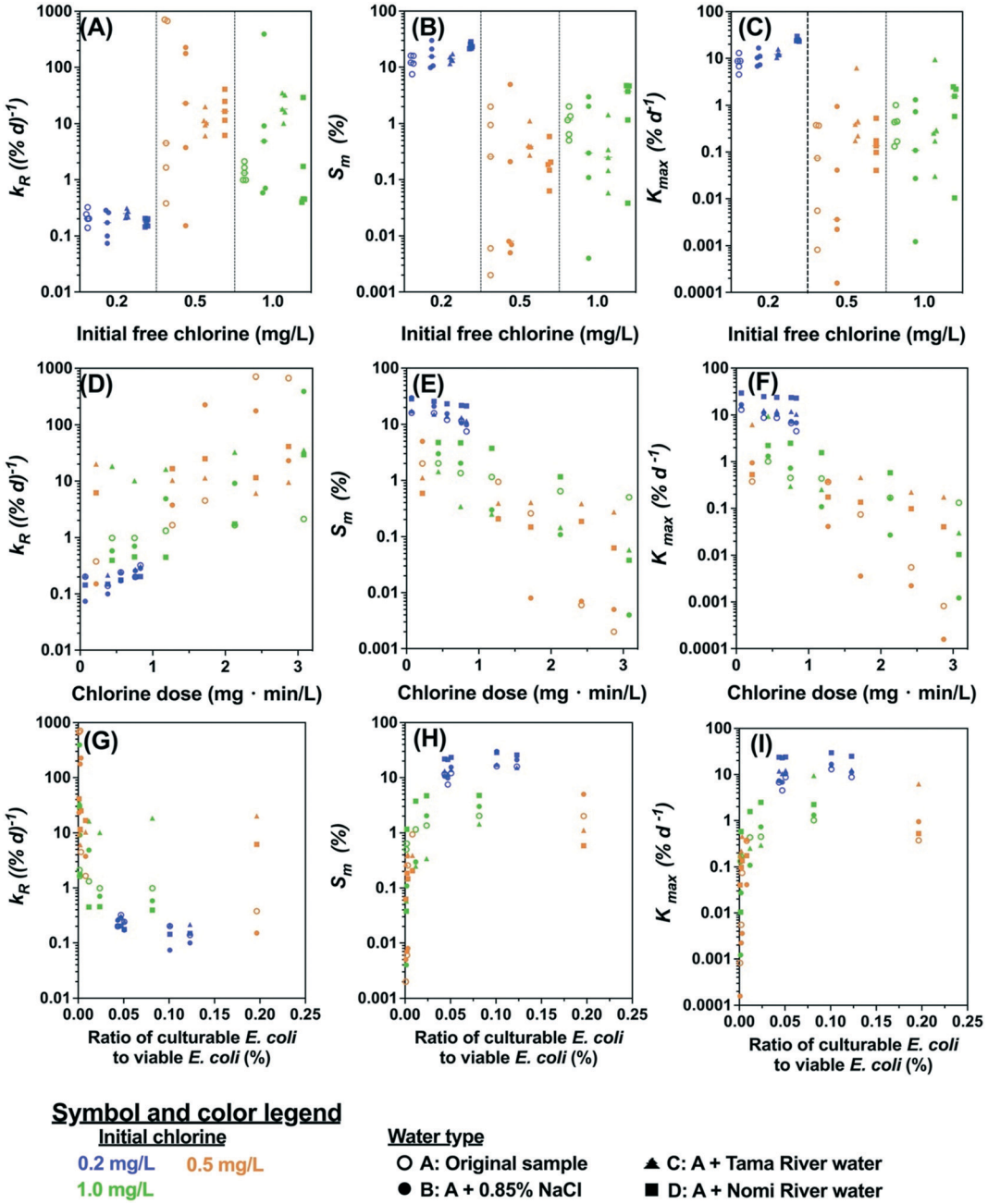

Fig. 6 The regrowth rate k_R (% per d), maximum survival ratio S_m (%) and maximum actual regrowth rate K_{max} (% per d) as a function of the initial chlorine concentration ((A)–(C)), the chlorine dose (mg·min L^{-1}) ((D)–(F)), and the ratio of culturable *E. coli* to viable *E. coli* (%) at the beginning of the regrowth phase ((G)–(I)). The *y*-axes in (A)–(I) are shown in logarithmic scale to break up data clusters. The values of k_R and S_m were obtained by applying eqn (6) to the experimental data, and the values of K_{max} were calculated by eqn (8) using the model output. The displayed values were calculated from the average of triplicate analysis in duplicate experiments.

reactivation of 10^4 VBNC cells, and a K_{max} value of 1% per day means the fastest reactivation rate of 10^4 cells per day, equivalent to reactivation of 416 VBNC cells per mL per hour. Therefore, even a minor reduction in S_m and K_{max} is of great significance in controlling microbial regrowth.

As a result from multiple regression analysis, R^2 of the regression was 0.58 ($p < 0.001$) for ln k_R, 0.68 ($p < 0.001$) for ln S_m, and 0.69 ($p < 0.001$) for ln K_{max} (Table 2). The initial chlorine concentration and chlorine dose were significant in the multiple regression model of all regrowth parameters ($p < 0.05$), except that the initial chlorine concentration was not significant in the regression model for ln k_R ($p = 0.09$) (Table 2). The absolute values of the model coefficient of chlorine dose were larger than that of the initial chlorine concentration, suggesting that a per unit change in chlorine dose is more impactful than a per unit change in initial chlorine concentration in the regrowth parameters (Table 2). In addition, a positive correlation was observed between the

Table 2 Multiple linear regression analysis for the regrowth model parameters (k_R, S_m, and K_{max}). Natural log transformation was applied to the dependent variables. The multicollinearity of the independent variables was evaluated from the variance inflation factors (VIFs) and we confirmed that all the applied variables showed a VIF value less than five. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Independent variable	R^2	Dependent variable	Coefficient
ln k_R	0.58***	*(intercept)*	−2.07***
		Initial chlorine concentration	1.17
		Chlorine dose	1.76***
ln S_m	0.68***	*(intercept)*	3.36***
		Initial chlorine concentration	−1.36*
		Chlorine dose	−2.04***
ln K_{max}	0.69***	*(intercept)*	3.26***
		Initial chlorine concentration	−1.55*
		Chlorine dose	−2.31***

maximum survival ratio S_m and maximum actual regrowth rate K_{max} in all types of water (Pearson's $r > 0.92$, $p < 0.0001$) (Fig. S8†). Nevertheless, the multiple regression based on chlorination settings was not good enough (R^2 less than 0.70) to accurately estimate the regrowth parameters. When multiple regression was applied to each water type separately, the regression performance improved (R^2 increased up to 0.88, data not shown), which infers that the addition of variables indicating the water properties might better predict the regrowth parameters.

Previous studies reported that DNA repair after UV disinfection reached its maximum level faster (less than 12 hours)[16,17,26] than the reactivation of VBNC *E. coli* after chlorination observed in this study. Li *et al.* (2017)[35] reported that after achieving a 4.5 log removal of culturable *E. coli* in 0.9% saline solution with low-pressure UV, the maximum survival ratio (S_m) and the maximum actual regrowth rate (K_{max}) were 8.04% and 2.46% per h under light, while the survival ratio under dark conditions fluctuated within 0.002–0.006% and could not be modeled.[16] In contrast, in this study, after achieving a 4.5 log removal in 0.85% saline solution by chlorination (0.5 mg L^{-1} initial chlorine, 5 minutes), S_m and K_{max} were 0.257% and 0.074% per day in the dark. Chen *et al.* (2018) observed the regrowth of *E. coli* in LB broth after treatment with 0.5, 1, 2, and 3 mg L^{-1} chlorine for up to 2 hours and 4 mg L^{-1} chlorine for 0.5 hours. However, no cell regrowth occurred in LB broth after 4 mg L^{-1} chlorination for 2 hours,[11] probably due to the lethal damage caused by the high disinfectant concentration and longer treatment time. In addition, a sunlight/H_2O_2 process (H_2O_2 50 mg L^{-1}, 90 min) was reported to inhibit the regrowth of multidrug-resistant *E. coli*, while chlorination (1 mg L^{-1}, 15 min) led to an increase in the multidrug-resistant *E. coli* in 48 hours after chlorination, even though both processes achieved complete inactivation (*i.e.*, culturable *E. coli* below the detection limit).[52] It was stated that the formation of hydroxyl radicals during the sunlight/H_2O_2 process contributed to bacterial inactivation and controlled the regrowth.[52] Therefore, the disinfection mechanism and the treatment conditions (*e.g.*, dose) play essential roles in determining the extent and rate of bacterial regrowth.

3.4 Effect of the receiving water body on the regrowth of *E. coli*

A comparison among the samples mixed with 0.85% NaCl solution, Tama River water, and Nomi River water allows us to assess the impact of the receiving water body on the regrowth of *E. coli* after chlorination. During the three day storage, the culturable *E. coli* in Tama River and Nomi River water showed a more notable increase in its concentration than in saline solution (Fig. 2 and 5). For instance, after 0.5 mg L^{-1} chlorine treatment for 30 minutes, the percentage of regrowth in saline solution was only 0.00014% after three days, two to three orders of magnitude lower than those in Tama River water (0.16%) and Nomi River water (0.054%) (Fig. 5). Moreover, when applying a higher chlorine dose, the difference between the saline solution and the river waters tended to increase in terms of the percentage of regrowth (Fig. 5), the maximum survival ratio (S_m), and the maximum regrowth rate (K_{max}) (Table S3†).

However, the difference between Tama River and Nomi River water was difficult to address regarding the promoting effect on *E. coli* regrowth. The regrowth of *E. coli* water was higher in Nomi River water than in Tama River water after 0.2 mg L^{-1} and 1.0 mg L^{-1} chlorine treatment, but slightly higher in Tama River water than in Nomi River water in 0.5 mg L^{-1} chlorine-treated samples (Fig. 5 and Table S3†). A previous study on solar disinfection and post-irradiation *E. coli* survival found that the *E. coli* population showed a noticeable increase within one day in wastewater but not in lake water and Milli-Q water.[19] One day after 1 mg L^{-1} chlorination, antibiotic-resistant *E. coli* showed a percentage regrowth of less than 20% in phosphate buffer solution[25] and up to 100% in secondary wastewater effluent.[23] The more significant regrowth in the wastewater could be attributed to the high concentration of organic matter and the increased assimilability after wastewater treatment.[53]

Studies on the bacterial regrowth in drinking water of several regions revealed that the substrate availability determined the potential of bacterial regrowth, including assimilable organic carbon (AOC) and microbially available phosphorous (MAP).[54–56] As seen in Table S1,† the sampled

Nomi River water has a higher DOC concentration (9.7 mg C per L) than Tama River water (2.2 mg C per L). Although AOC was not measured in the present study, AOC typically accounts for less than 4% of DOC in surface water,[57] and thus the AOC in the sampled Tama River and Nomi River water was estimated to be less than 88 μg C per L and 380 μg C per L. Several studies proposed 50–100 μg AOC per L as the minimum requirement for maintaining microbial stability in chlorinated drinking water.[37,58,59] According to this range for AOC, Tama River water is considered to have microbe-stable conditions while Nomi River water promotes bacterial growth, consistent with the changes of viable *E. coli* concentrations shown in Fig. 3. In addition, this study showed that they regrew in all types of water (Fig. 2 and 5), indicating that AOC is not the only influential component in determining regrowth.

Besides AOC, inorganic nutrients such as MAP can be essential for bacteria. For example, dissolved inorganic phosphorus (*i.e.*, phosphate) regulates carbon mineralization, and it is the most frequently reported limiting nutrient for bacterial production.[60–62] The sampled Nomi River water contains a higher phosphate concentration (2.73 mg L^{-1}) than Tama River water (1.23 mg L^{-1}). Considering that Nomi River water is more nutritious than Tama River water in terms of AOC and MAP, it was reasonable to expect more regrowth in Nomi River water under all conditions, consistent with our observation (Fig. 5) and modeling output (Table S3†).

Concerning the reactivation of VBNC *E. coli*, the dominant regrowth process under the experimental conditions, the carbon availability and phosphate availability are not the only influential factors. Previous studies have proved that the promoting factors for the reactivation from their VBNC state are sodium pyruvate,[63] amino acids,[49] vitamins,[64] quorum-sensing molecules (autoinducer),[65] active proteins such as the protein YeaZ,[66] and catalase.[67] Researchers also pointed out that some currently unidentified signaling molecules in water environments could increase the culturability of bacteria.[68,69] Treated wastewater is discharged into freshwater or coastal water environments, containing components that can interfere and complicate the regrowth of disinfected bacteria. For instance, in receiving water, the dissolved organic compounds would provide carbon sources for bacteria and react with the residual disinfectant (*e.g.*, chlorine),[55,56] and the suspended solids could shield bacteria from the residual disinfectant,[70] thus promoting the bacterial regrowth. Meanwhile, residual antibiotics and high salinity in receiving water could disrupt the metabolic activities of bacteria[71] or act as an environmental stress to induce active bacteria to enter the VBNC state,[72] inhibiting the bacterial regrowth. Therefore, the bacterial regrowth in natural water and treated wastewater is a complex process and should be carefully evaluated. The presented data here were not sufficient to model how much bacterial regrowth could happen in complicated natural water and treated wastewater. Nevertheless, this problem could be overcome when collecting more data step by step, if (1) a greater range of environmental water samples (*e.g.*, more than ten water sources) is included in one regrowth study, (2) comprehensive water characterization is conducted, and (3) frequent and continuous monitoring (*e.g.*, daily for 7 days) is implemented.[73] For further scientific understanding of the regrowth phenomenon after chlorination, it is inevitable to design systematic and multi-factor experiments under controlled conditions of chlorination and regrowth processes. Such studies could help understand the role of each factor individually, their interactive effect in the regrowth, and build solid relationships between influential factors and regrowth parameters. In addition, the multiplex approach for bacterial detection/quantification, such as the combination of culture-based methods and molecular tools (*e.g.*, polymerase chain reaction and fluorescence viability test), is a powerful tool for future investigations.

4. Conclusions

This study demonstrated the effect of chlorine treatment on different *E. coli* subpopulations (*i.e.*, viable and culturable fractions) and the regrowth in environmental water. The major findings were concluded as follows:

- Chlorine treatment with 0.2, 0.5 and 1.0 mg L^{-1} initial chlorine induced *E. coli* to enter the VBNC state, and a higher chlorine concentration could speed up the transition from the culturable state to the VBNC state.
- Multiplex detection (*i.e.*, the combination of the plate count method and fluorescence-based viability test) allowed the understanding of the regrowth process. The reactivation of VBNC *E. coli* was presumably the dominant regrowth process in the tested waters after chlorine treatment.
- Removing environmental stress (*i.e.*, oxidative stress from chlorine) was adequate to reactivate the VBNC subpopulation of *E. coli*.
- The second-order regrowth model could simulate the survival ratio of culturable *E. coli* during regrowth (R^2: 0.75–1.00), suggesting that post-chlorination regrowth follows typical regrowth kinetics including lag, exponential and stabilization phases.
- The application of the regrowth model estimated regrowth parameters (*i.e.*, regrowth rate constant (k_R), maximum survival ratio (S_m) and maximum actual regrowth rate (K_{max})). This paves the way for establishing the relationship between regrowth parameters and influential factors in chlorination and regrowth phases.
- Multiple linear regression analysis revealed that the initial chlorine concentration and chlorine dose significantly determined the regrowth rate and maximum regrowth level.
- Environmental waters (*i.e.*, Tama River water and Nomi River water) allowed a higher percentage of regrowth than a simple saline solution, although the specific promoting components in the water are to be identified.

Overall, the observed notable regrowth of *E. coli* in environmental water highlighted the importance of

monitoring the total viable bacteria during wastewater disinfection and after disinfection. This study enhanced our understanding of the environmental adaptability of *E. coli* after being stressed by chlorine treatment. The approach and the findings in this investigation provide significant insights for developing countermeasures to effectively control bacterial regrowth. When available and affordable, the quantification of viable bacteria should be adopted in wastewater treatment facilities to ensure the removal of viable bacteria and limit the potential of regrowth. Considering that detection of viable bacteria is not commonly adopted, disinfection may be targeted to achieve a higher removal of viable bacteria and suppress their potential regrowth, especially when the treated wastewater is discharged into environments with high organic and nutrient content. In addition, more research efforts are required to assess the regrowth in a large variety of well-characterized water and bacterial species or communities, which would assist in establishing a predictive model of regrowth and eventually facilitate the design of an effective disinfection treatment to prevent post-disinfection regrowth.

Disclaimer

The research presented was not performed or funded by the EPA and was not subject to EPA's quality system requirements. The views expressed in this article are those of the author(s) and do not necessarily represent the views or the policies of the U.S. Environmental Protection Agency.

Conflicts of 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.

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