Supplementary materials

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

Manna Wang[1], Mohamed Ateia[2,*], Yuta Hatano[1], Chihiro Yoshimura[1,*]

[1]*Department of Civil and Environmental Engineering, Tokyo Institute of Technology, Tokyo 152-8552, Japan*

[2]*United States Environmental Protection Agency, Center for Environmental Solutions & Emergency Response, Cincinnati, OH, USA*

*Corresponding authors: [M.A.] Ibrahim.mohamed@epa.gov; [C.Y.] yoshimura.c.aa@m.titech.ac.jp

Submitted to: *Environmental Science: Water Research & Technology*

Type of submission: *Research article*

Supplementary material

Text S1 Water sampling and characterization

After sampling, the water was filtered with a 0.22 μm membrane to ensure no bacteria in the filtered water. Concentration of dissolved organic carbon (DOC) was analyzed by TOC analyzer (TOC-CHP, Shimadzu V-series, Japan). Concentration of anions (e.g., PO_4^{3-}, Cl^-, NO_3^- and SO_4^{2-}) and cations (e.g., Na^+, K^+, Mg^{2+}, Ca^{2+} and NH_4^+) were analyzed by ion chromatography (LC-20ADSP-100V, Shimadzu). The suspended solids (SS) was not characterized, but the data was obtained from the water quality report for the same sampling locations by the local government. The water characteristics are summarized in **Table S1**.

Text S2 Fluorescence-based viability test (Wang et al., 2022)

First, a combined reagent mixture of the same volumes of SYTO 9 and PI is prepared. Then, 3 μL of the dye mixture is added for each mL of the sample solution. The stained samples are then mixed in a lightproof microtube and incubated at room temperature in the dark for 15 minutes. The subsequent analytical procedures followed our previous work (Wang et al., 2022). Using the established linear models for each water matrix in our previous work (Wang et al., 2022) (**Table S1**), the concentration of viable cells was calculated. The fluorescence emission spectrum of each sample was measured in the range of 480-700 nm by fixing excitation wavelength at 470 nm using a fluorescence spectrophotometer (RF-5300 PC, Shimadzu, Japan). Triplicate measurement of the fluorescence spectrum of each sample was conducted. The background spectrum of each water matrix was measured and subtracted from the corresponding spectrum of the sample. Preprocessed spectra were used to calculate the integrated fluorescence intensity of SYTO 9 in GraphPad Prism (version 9.1.2).

Text S3 Modeling of the concentration ratio of culturable to viable *E. coli*

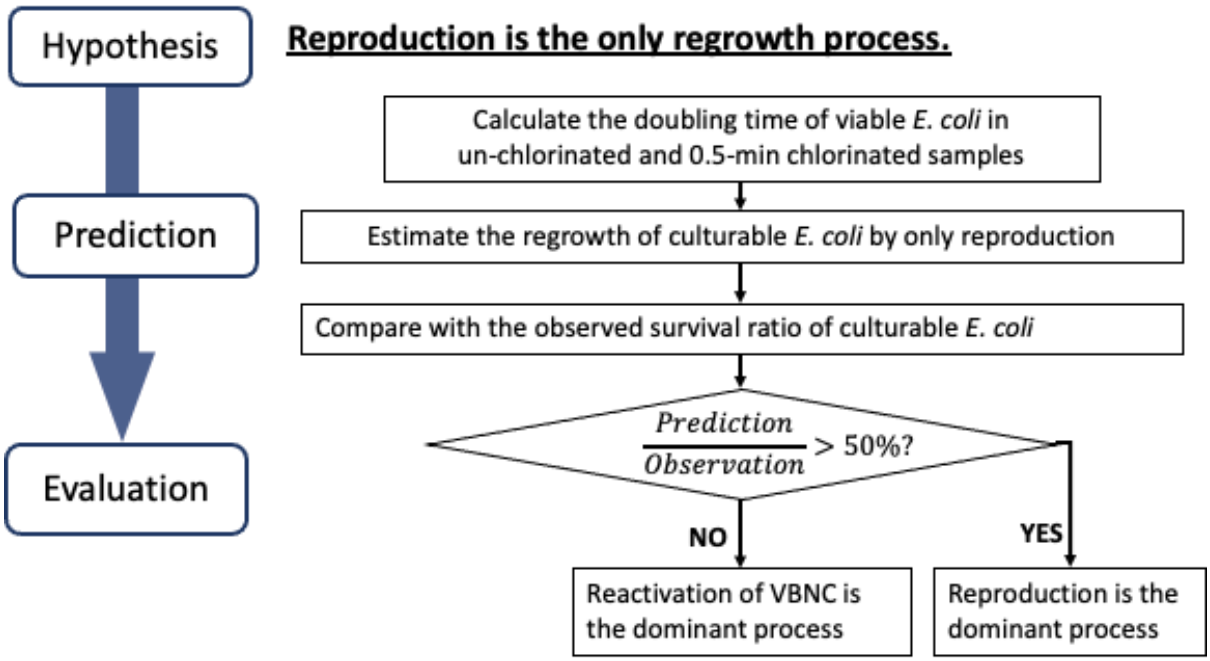
The second-order regrowth model (Equation (6) in the main text) was modified to describe the concentration ratio of culturable *E. coli* to viable *E. coli* during the regrowth phase, as Equation S1. In this model application, we assumed that the viable *E. coli* concentration (N_v) was constant during the regrowth phase, based on the observations shown in **Fig. 4** in the main text. Then, the model parameters, S_m and k_R, were adjusted in Excel Solver to get the minimum sum of squared error between the experimental and modeled values of the ratio of culturable *E. coli*.

$$\text{Ratio of culturable } E.coli = \frac{N_R}{N_V} = \frac{100 \times N_0 \cdot S_m/N_V}{1 + [S_m/S_0 - 1] \cdot e^{-k_r \cdot S_m \cdot t_r}} \qquad \text{Equation S1}$$

Text S4 The hypothetico-deductive strategy to identify the dominant regrowth process.

To identify the dominant regrowth process, we adopted the hypothetico-deductive method (**Scheme S1Error! Reference source not found.**), described in step 1) to 4) below.

Scheme S1



1) We hypothesized that reproduction is the only regrowth process in chlorinated sample during the 3-day storage. The following steps include predictions based on this hypothesis and hypothesis evaluation by comparing the prediction with observation.
2) The doubling time of un-chlorinated samples or 0.5-min chlorinated samples was calculated, as shown below.

	Doubling time (day) $Doubling\ time = \frac{time \times \ln(2)}{\ln(Nt) - \ln(N0)}$			
Water	**A** Original sample	**B** A + 0.85% NaCl	**C** A + Tama River water	**D** A + Nomi River water
Un-chlorinated	3.06	2.70	8.23	3.50
0.5-min chlorinated	6.67	4.10	16.57	6.35

3) The increase of culturable *E. coli* via reproduction during the regrowth phase was estimated using the calculated doubling time for each water condition. The initial state of this calculation was the beginning of the regrowth phase, which was the end of chlorination treatment. In addition, the prediction of culturable *E. coli* after 0.5-min chlorination used the doubling time of viable cells in 0.5-min chlorinated samples, while the others used the doubling time in un-chlorinated samples.
4) The ratio of predicted regrowth to the observed regrowth in terms of survival ratio of culturable *E. coli* was calculated and compared with the criterion of 0.5. If the ratio is less

than 0.5, it could be concluded that the reactivation of VBNC is the dominant regrowth process. Otherwise, the reproduction could be considered as the dominant regrowth process.

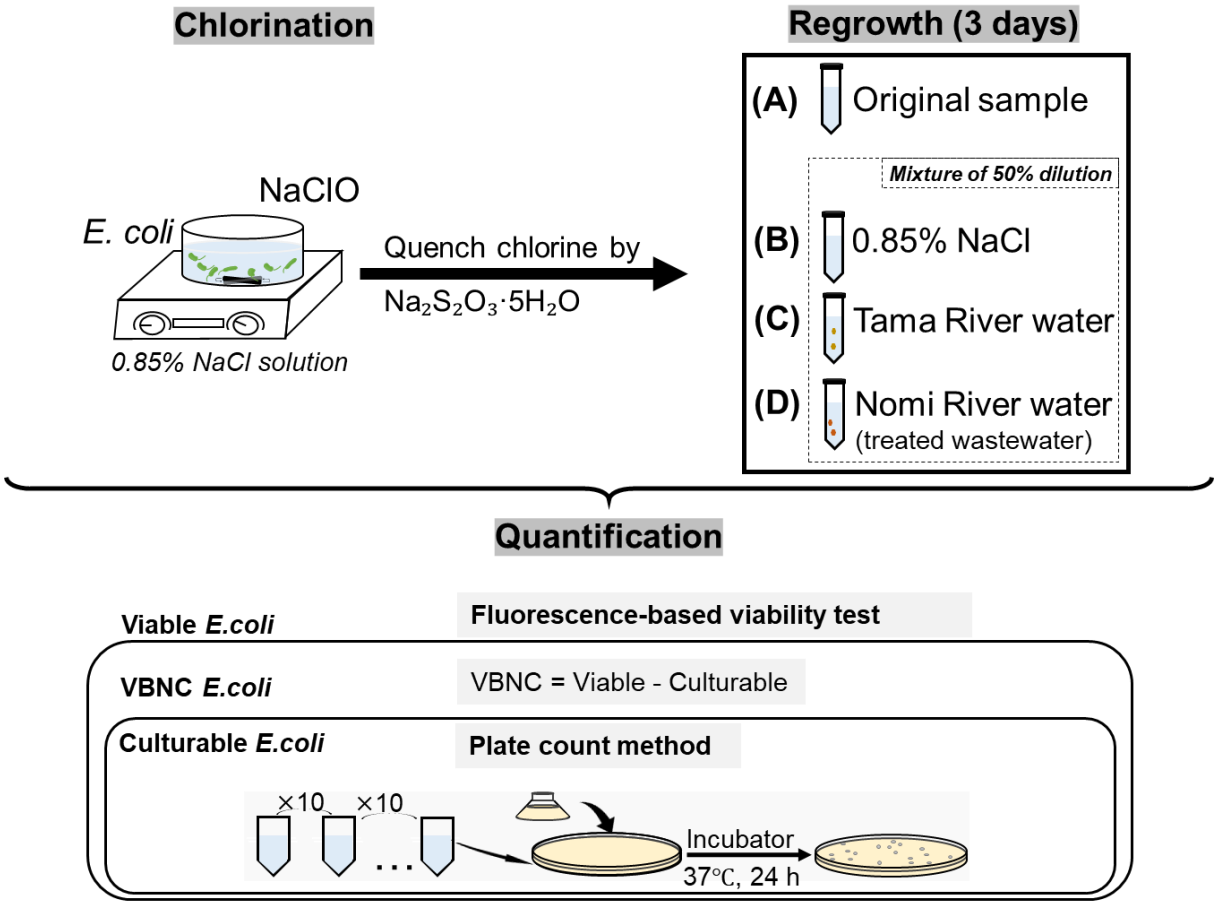


Fig. S1 Experimental scheme of chlorination and post-chlorination regrowth test.

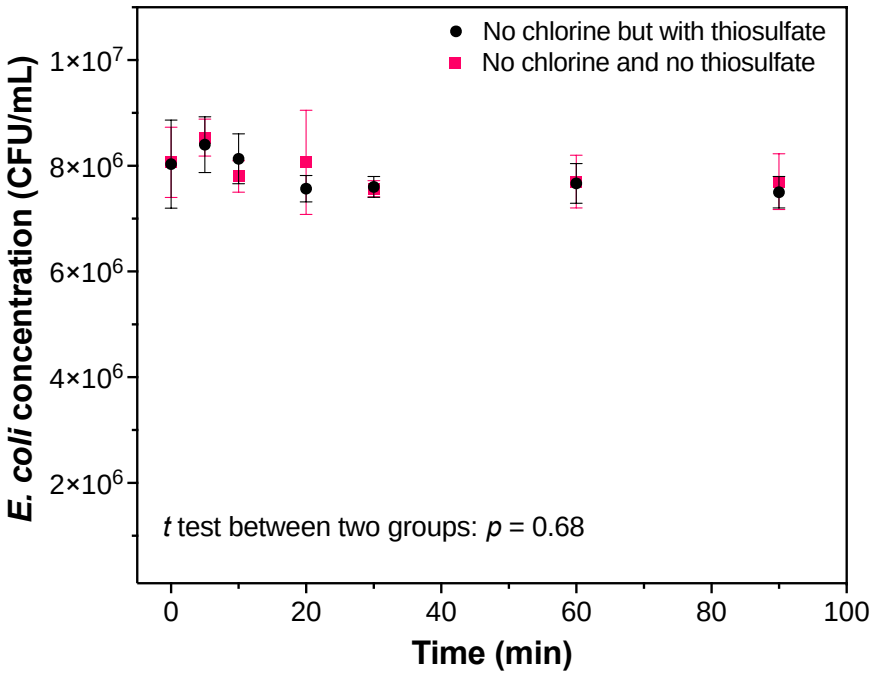


Fig. S2 The concentration of culturable *E. coli* (CFU/mL) in the control experiments (black, no chlorine but with thiosulfate; red, no chlorine and no thiosulfate).

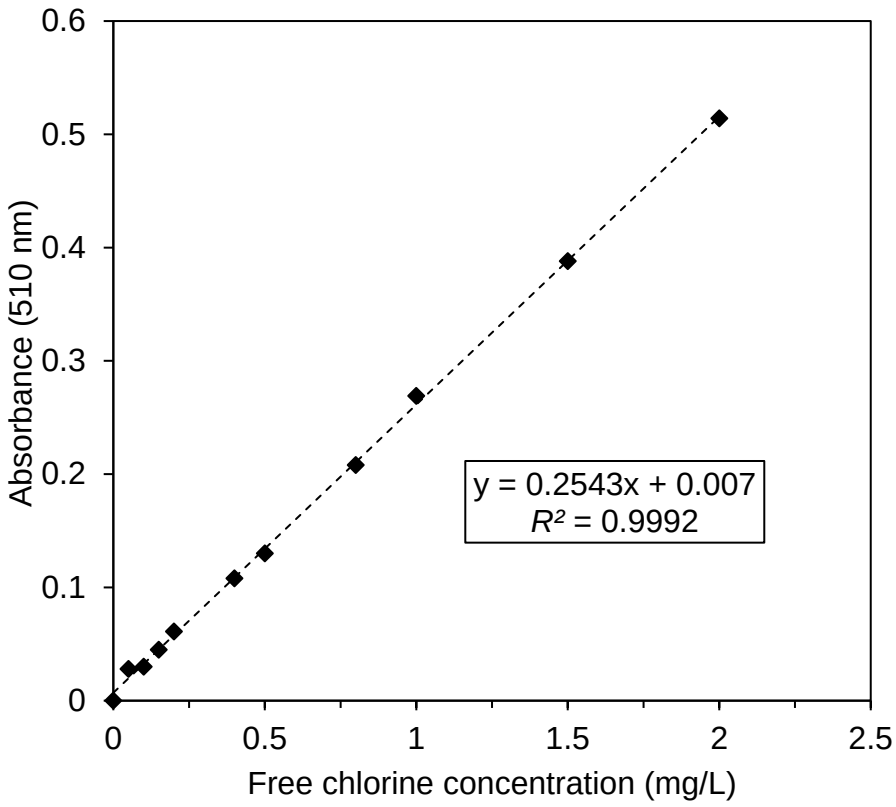


Fig. S3 Calibration curve for free chlorine measurement using DPD reagent and UV-vis spectrophotometer

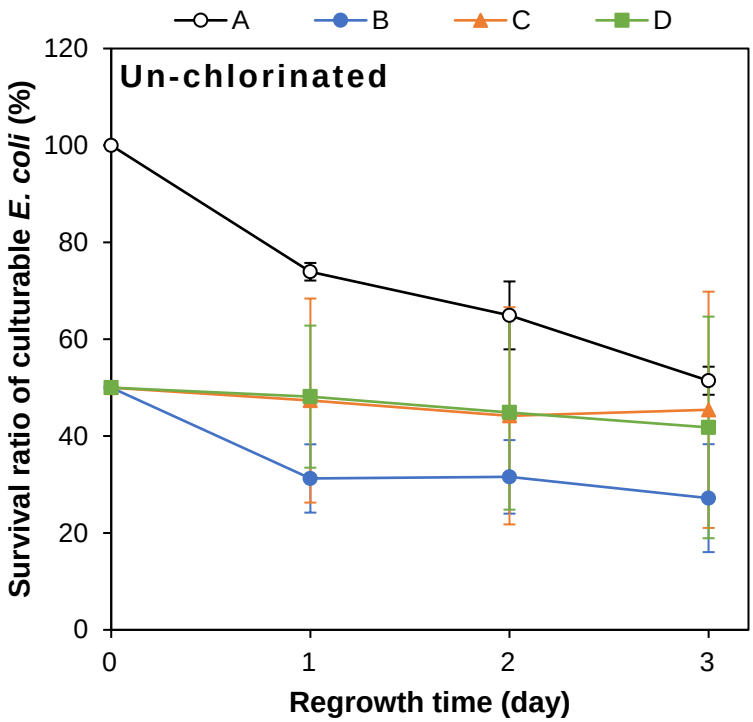


Fig. S4 The survival ratio of culturable *E. coli* in four types of water without chlorine treatment. The errors bars represent the standard deviation from replicate experiments. **A**: original sample; **B**: the mixture of original sample with 0.85% NaCl solution; **C**: the mixture of original sample with Tama River water; **D**: the mixture of original sample with Nomi River water.

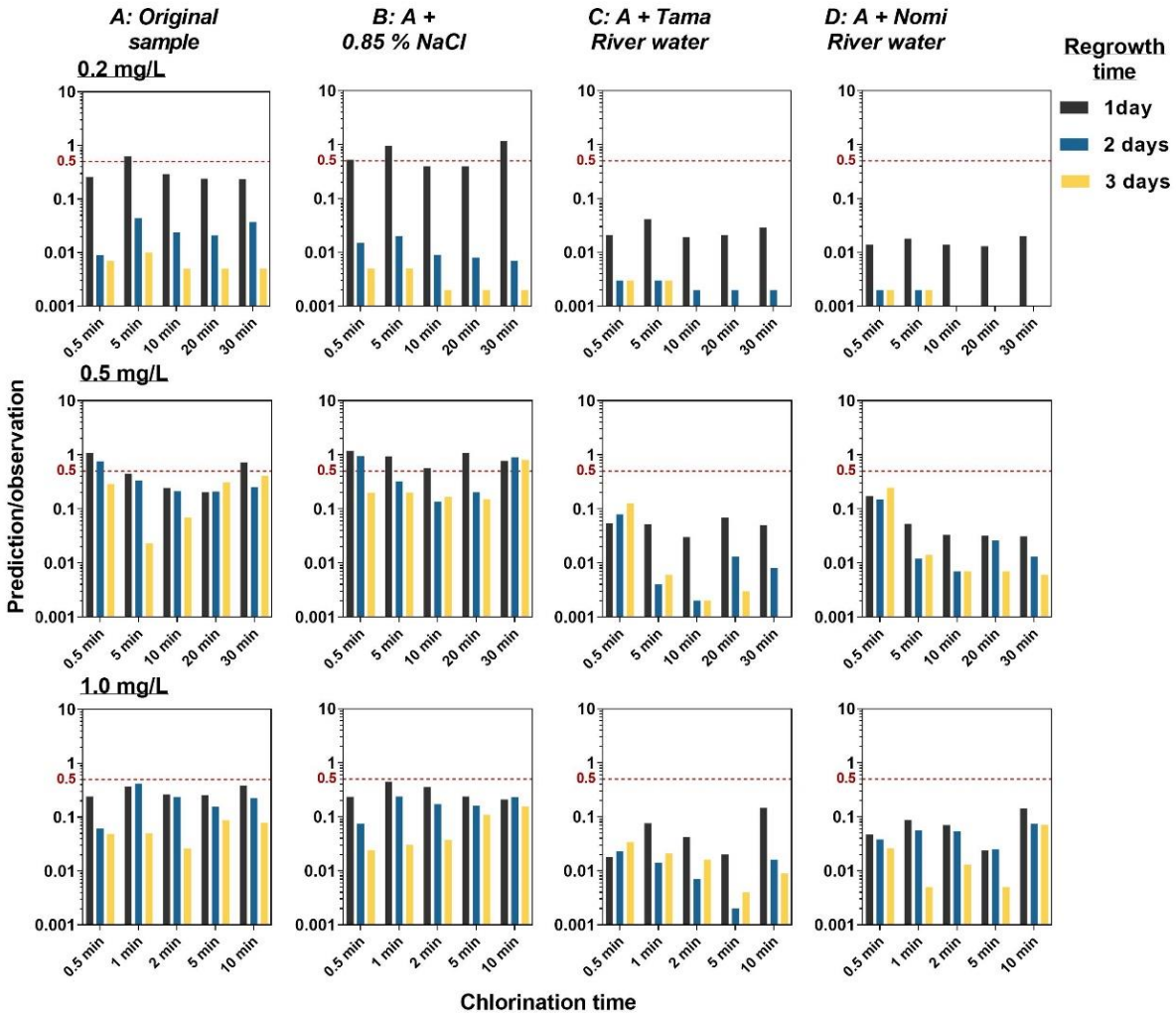


Fig. S5 The ratio of hypothetical estimation to experimental observation of the regrowth in three days. The red dotted line indicates the ratio of 0.5, which is the criterion to evaluate the hypothesis in the hypothetico-deductive analysis described in Text S4.

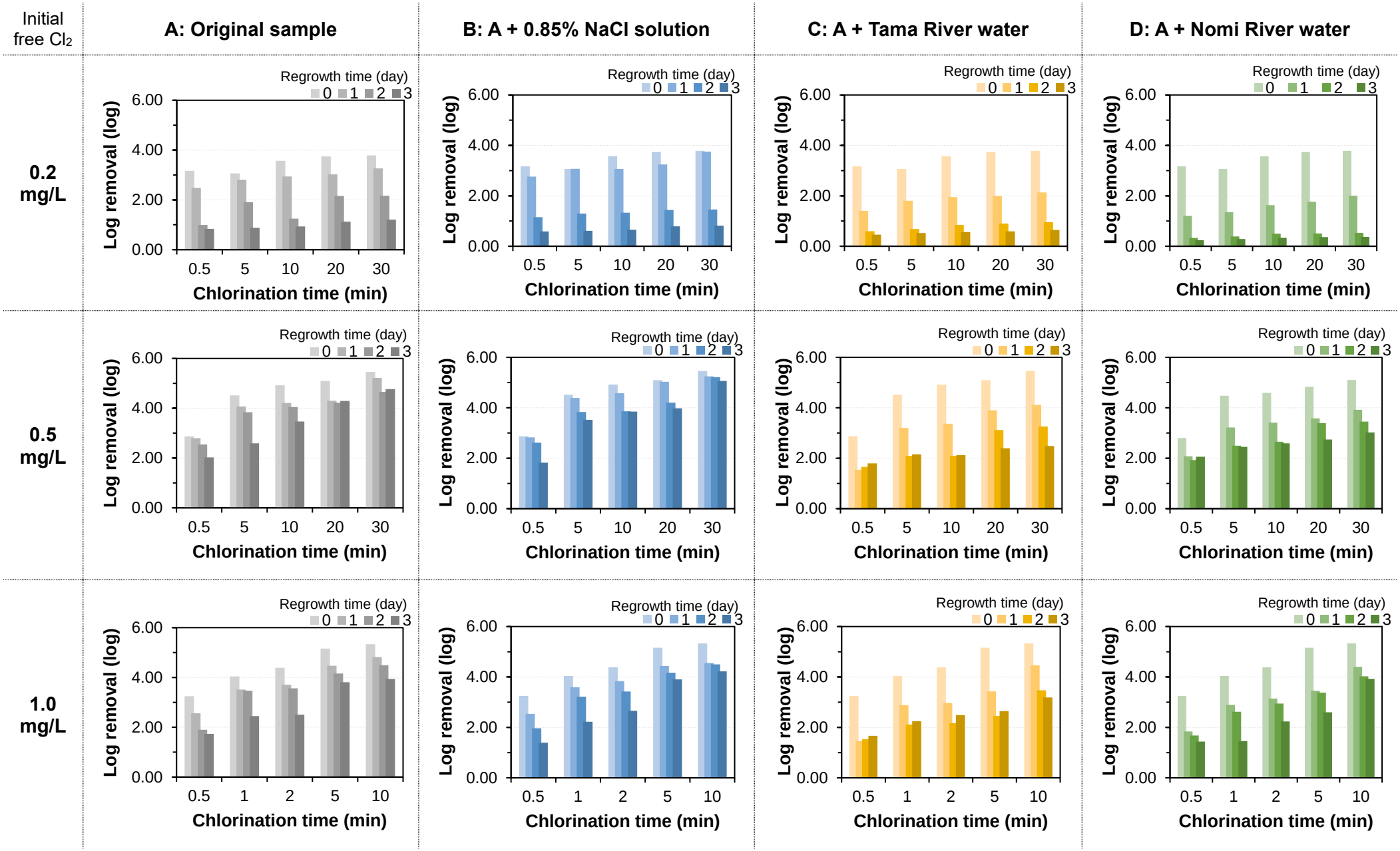


Fig. S6 The log removal of culturable *E. coli* considering regrowth in four types of water after treatment with various initial doses of chlorine (0.2, 0.5, and 1 mg/L). The log removal was calculated using the average concentrations from duplicate experiments with triplicate measurements.

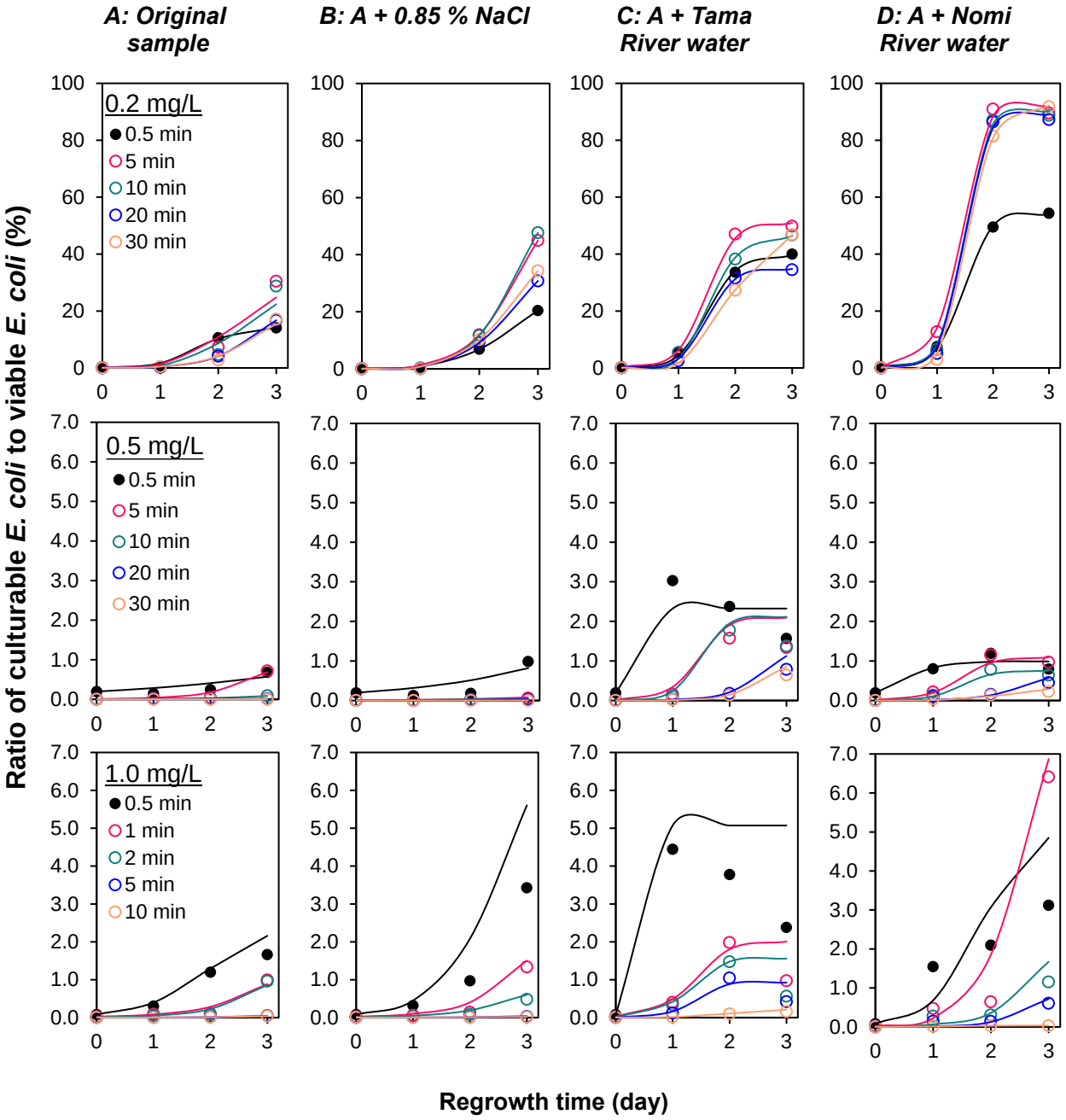


Fig. S7 The concentration ratio of culturable *E. coli* to viable *E. coli* during regrowth in four types of water after treatment with various initial chlorine concentrations (0.2, 0.5, and 1 mg/L) and contact time (0.5 min to 10 min or 30 min). The curves represent the modeling output (**Equation S1**, **Text S2**).

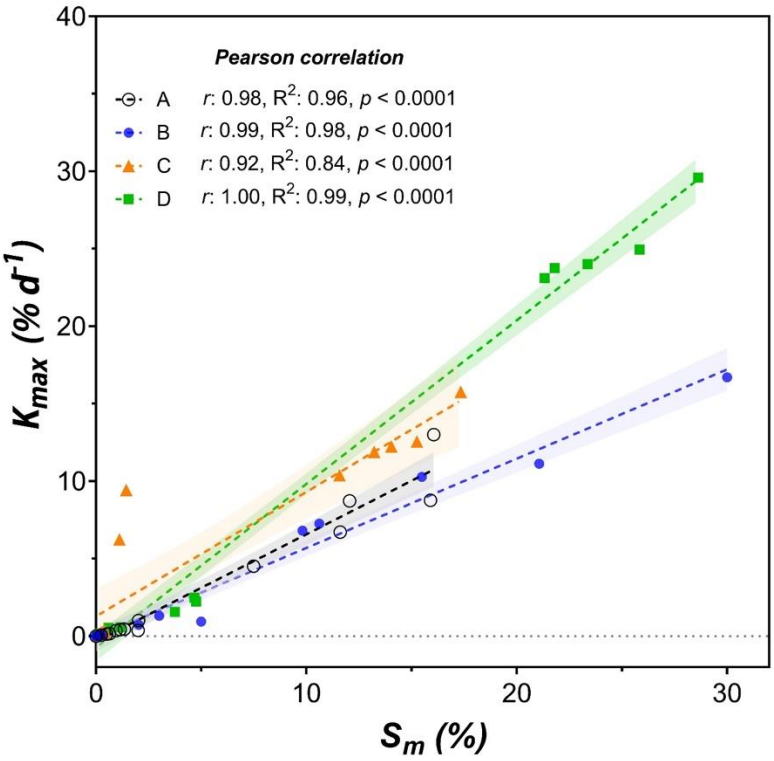


Fig. S8 Correlation analysis between the maximum real regrowth rate and maximum survival ratio. **A**: original sample; **B**: the mixture of original sample with 0.85% NaCl solution; **C**: the mixture of original sample with Tama River water; **D**: the mixture of original sample with Nomi River water.

Table S1 Characteristics of Tama River water, Nomi River water, and saline solution.

Water characterization		Tama River water	Nomi River water	0.85% NaCl solution
pH		7.50	7.10	6.20
Conductivity (mS/m)		17.77	22.3	79.1
SS (mg/L)		3 (Feburary, 2021) [a]	1 (annual average in 2020) [b]	n.a.
DOC (mg C/L)		2.198	9.725	n.a.
Ions (mg/L)	Na^+	38.86 ± 0.12	49.68 ± 0.08	n.a.
	NH_4^+	0.08 ± 0.00	n.d.	n.a.
	K^+	7.8 ± 0.07	13.06 ± 0.52	n.a.
	Mg^{2+}	4.81 ± 0.04	5.09 ± 0.1	n.a.
	Ca^{2+}	24.17 ± 0.47	19.43 ± 0.27	n.a.
	PO_4^{3-}	1.23 ±0.03	2.73 ±0.06	n.a.
	Cl^-	38.13 ± 0.03	48.88 ± 0.10	n.a.
	NO^{3-}	20.83 ± 0.02	60.08 ± 0.15	n.a.
	SO_4^{2-}	35.78 ± 0.45	36.89 ±0.52	n.a.

- n.a.: not available; n.d.: not detected.
- [a]: River water quality survey in fiscal year 2020, Setagaya city, Tokyo, Japan. https://www.city.setagaya.lg.jp/mokuji/sumai/011/006/003/d00124121_d/fil/R2_suishitu.pdf (in Japanese)
- [b]: Nomi River pollution survey in 2020, Ota city, Tokyo, Japan. https://www.city.ota.tokyo.jp/seikatsu/sumaimachinami/kankyou/shiryo/kankyouchousa_houkokusho/kankyochosa_r02/R2_mizu-kankyou_houkoku.files/303_mizu-nomikawa.pdf (in Japanese)

Table S2 Summary of linear regression models used in the fluorescence-based method (Wang et al., 2022).

Water type	Linear regression model [a, b]	R^2
0.85 % saline solution	$y = 3.72 \times 10^{-5}x - 1.67$	0.95
The mixture of 0.85 % saline solution and Tama River water	$y = 4.46 \times 10^{-5}x + 3.95$	1.00
The mixture of 0.85 % saline solution and Nomi River water	$y = 5.00 \times 10^{-5}x + 7.14$	0.99

[a.] Here, *y* is the SYTO 9 peak area in 500-510 nm, and *x* is the viable cell plate count.
[b.] The *p*-value of all the linear regression models was less than 0.0001.

Table S3 Summary of parameters of second-order regrowth model applied to the survival ratio of culturable *E. coli* (Equation (6)).

	0.2 mg/L					0.5 mg/L					1 mg/L				
	k	***S_m***	***K_{max}***	***R^2***	***p value***	***k***	***S_m***	***K_{max}***	***R^2***	***p value***	***k***	***S_m***	***K_{max}***	***R^2***	***p value***
A	0.202	16.056	13.014	0.99	**	0.377	2.000	0.377	0.88	ns	0.988	2.023	1.011	1.00	**
	0.139	15.899	8.777	0.97	*	1.655	0.944	0.369	0.96	*	0.990	1.350	0.451	0.94	*
	0.241	12.055	8.741	0.90	*	4.498	0.257	0.074	0.98	*	1.320	1.150	0.436	0.96	*
	0.200	11.611	6.725	1.00	***	711.593	0.006	0.006	0.97	*	1.646	0.641	0.169	0.99	**
	0.322	7.489	4.520	0.97	*	672.641	0.002	0.001	0.79	ns	2.120	0.500	0.133	0.99	**
B	0.074	30.000	16.704	0.99	**	0.151	5.000	0.943	0.88	ns	0.584	3.000	1.314	0.99	**
	0.100	21.066	11.129	1.00	***	3.748	0.209	0.041	0.99	**	0.705	2.033	0.729	0.97	*
	0.171	15.490	10.279	1.00	***	226.855	0.008	0.004	0.93	*	4.872	0.298	0.108	0.98	*
	0.258	10.614	7.269	1.00	***	177.512	0.007	0.002	0.97	*	9.132	0.109	0.027	0.96	*
	0.283	9.818	6.813	1.00	***	23.096	0.005	0.000	0.94	*	392.690	0.004	0.001	0.90	ns
C	0.210	17.333	15.769	1.00	**	20.115	1.114	6.243	0.80	ns	18.335	1.435	9.432	0.87	ns
	0.216	15.258	12.564	1.00	**	10.332	0.390	0.393	0.97	*	10.178	0.341	0.296	0.91	*
	0.249	14.036	12.244	1.00	***	11.365	0.402	0.458	0.99	**	16.288	0.250	0.255	0.73	ns
	0.272	13.218	11.899	1.00	***	6.086	0.383	0.223	1.00	***	32.614	0.145	0.172	0.88	ns
	0.310	11.581	10.392	1.00	***	9.520	0.272	0.175	1.00	***	35.415	0.058	0.030	0.95	*
D	0.144	28.628	29.596	1.00	***	6.195	0.586	0.531	0.92	*	0.395	4.756	2.231	0.86	ns
	0.149	25.843	24.957	1.00	**	16.656	0.205	0.175	1.00	***	0.454	4.681	2.485	0.99	**
	0.176	23.368	24.016	1.00	***	24.982	0.147	0.136	1.00	***	0.448	3.745	1.571	0.99	**
	0.200	21.802	23.751	1.00	***	11.488	0.185	0.098	0.99	**	1.728	1.160	0.581	0.99	**
	0.203	21.332	23.119	1.00	***	41.224	0.063	0.040	0.99	**	29.407	0.038	0.010	0.79	ns

- **A**: original sample; **B**: the mixture of original sample with 0.85% NaCl solution; **C**: the mixture of original sample with Tama River water; **D**: the mixture of original sample with Nomi River water.
- ns: $p \geq 0.05$, not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.