Supplementary information

Novel Fluorescence-Based Method for Rapid Quantification of Live Bacteria in River Water and Treated Wastewater

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Type of submission: *Communication*

Content

Text 1: Absence of VBNC bacteria in the prepared samples in this study

Previous studies reported that free soluble copper (0.05 mM or higher, equivalent to 3.2 mg/L or higher) could reduce bacterial culturability (within 1 day or 1 hour) [10,11]. However, surveys on the Tama River and treated wastewater along Tama River showed that the concentrations of heavy metals (Cd, Co, Cu, Mn, Mo, Ni, Pb, V, Zn, and Al) were at the level of μg/L or not detectable [12,13], much lower than the concentrations used to induce VBNC bacteria in previous studies. Liu *et al.*[14] designed experiments to understand the induction of *E. coli* O157:H7 into VBNC state in environmental waters (e.g., river water), and showed that *E. coli* O157:H7 could survive in a culturable state for weeks in river water at 25 °C. In this study, the sample preparation and analysis were completed within one day at 25 °C. Therefore, it is reasonable to assume no induction of VBNC bacteria within the experimental time and condition in our study.

Table S1 Comparison of current bacterial detection methods with the current study

Method	Time to detection	LOD (bacteria/mL)	Disadvantages	References
Plate count	days	1-10	▪Only detect culturable bacteria ▪Time-consuming	1,2
ATP measurements	Minutes	1-10	▪Overestimation due to extracellular ATP ▪Low reproducibility	3,4
Quantitative PCR	Hours	1-10	▪Difficulty in selection of indicator gene ▪Complex procedures and high cost	5,6
Fluorescence microscopy	Hours	$< 10^4$	▪Time-consuming ▪Inability to deal with complex water	1
Flow cytometry	Minutes	10^4	▪High facility cost and technical requirement ▪Sophisticated data analysis	1,7
Fluorescence microplate reader	Minutes	10^6	▪High detection limit ▪Can only measure at specific wavelengths	8,9
Fluorescence spectroscopy	Minutes	10^4-10^5	▪Requirement of calibration for each water matrix ▪Currently only verified with *E. coli*	This study

Table S2 Comparison of this study and previous studies on cell quantification using BacLight Kit and fluorometer.

Target water	Target bacteria	Analytical equipment	Excitation wavelength	Emission range used for peak integration	Model type	Model variables		R^2	LOD (bacteria/mL)	Reference
						Independent variable	Dependent variable			
▪ Minimal A salts medium with 0.2% glucose ▪ 0.85% saline solution	*E. coli*	Optrode system	NA	505-515 nm 600-610 nm	Linear regression	Percentage of live cells measured by flow cytometry (%)	Ratio [a] or adjusted ratio [b] of fluorescence peaks	▪ 0.96, 0.99 ▪ 0.52, 0.62	▪ 10^6 ▪ 10^7	[11]
0.85% saline solution	*E. coli*	Optrode system	473 nm	509-529 nm 609-629 nm	▪ Principle component regression ▪ Partial least square regression ▪ Support vector regression	Flow cytometry counts of live cells (cells/mL)	Integrated SYTO 9 intensity	▪ 0.77 ▪ 0.87 ▪ 0.84	10^6	[6]
Saline solution	*E. coli*	Optrode system	473 nm	The full spectral data was used	▪ Single-spectrum support vector regression ▪ Multi-spectra support vector regression	Percentage of live bacteria measured by flow cytometry (%)	Full-spectrum	▪ 0.85 and 0.91 for 10^8 and 10^7 bacteria/ml samples. ▪ 0.87 and 0.91 for 10^8 and 10^7 bacteria/ml samples.	10^6	[12]
Peptone water (0.1% peptone, 0.85% sodium chloride, pH 7)	▪ *E. coli* ▪ *S.* Typhimurium ▪ *S. aureus* ▪ *B. cereus*	Optrode system	473 nm	490-590 nm [c]	Linear regression	Logarithm of bacterial cell (Log (CFU/mL))	Logarithm of fluorescence intensity (490-590 nm)	NA	▪ 10^5 ▪ 10^5 ▪ 10^5 ▪ 10^3	[13]
0.85% saline solution	▪ *E. coli* ▪ *S. aureus*	Fluorescence spectroscopy	470 nm	510-540 nm 620-650 nm	Linear regression	Percentage of live cells	Ratio [a] of fluorescence peaks	NA	NA	The Live/Dead BacLight kit manual
▪ 0.85% saline solution (SA) ▪ Tama River water (TM) ▪ Nomi River water (NM) ▪ Mixture of SA and TM ▪ Mixture of SA and NM	*E. coli*	Fluorescence spectroscopy	470 nm	500-510 nm 595-605 nm	Linear regression	Viable cell plate count (CFU/mL)	Integrated SYTO 9 intensity	▪ 0.96 ▪ 0.99 ▪ 0.99 ▪ 1.00 ▪ 0.99	▪ 10^4 ▪ 10^4 ▪ 10^4 ▪ 10^5 ▪ 10^4	This study

[a] The ratio of the integrated intensity of SYTO 9 peak to PI peak. $Ratio = \frac{SYTO\ 9}{PI}$.

[b] The adjusted ratio was proposed as Adjusted ratio $= \frac{100 \times Ratio}{1+Ratio}$ [10].

[c] Only SYTO 9 was applied to stain samples.

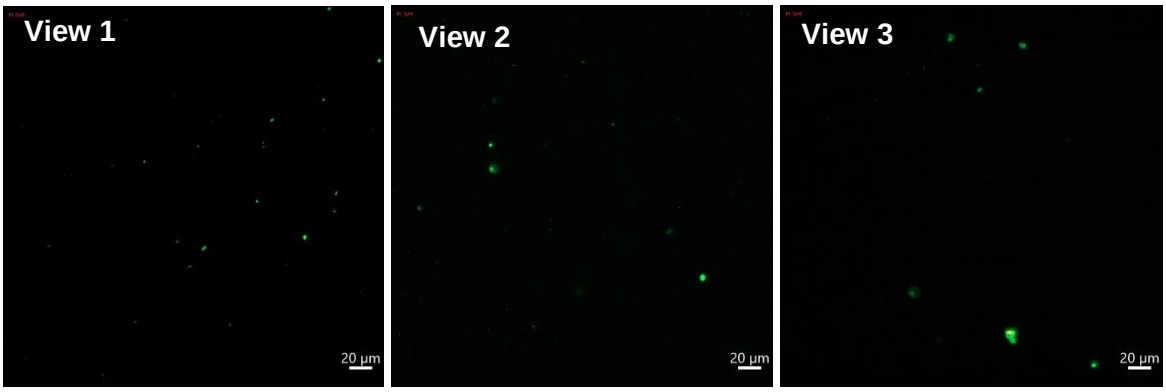


Fig. S1 Fluorescence microscopy images of SYTO 9 and PI-stained 0.22 µm-membrane filtered Tama River water. The three panels are different views of one sample. The stained sample (5 µL) was placed on an agarose gel-coated glass slide and covered by a square coverslip. The images were acquired under an Inverted Phase Contrast Fluorescence Microscope (Axio Observer Z1, ZEISS^{TM}, Germany) equipped with fluorescence filter cubes GFP (Excitation at 450-490 nm; Emission at 500-550 nm) and DsRed (Excitation at 538-562 nm; Emission at 570-640 nm), at a total magnification up to 400× (objective lens of 40× and eyepiece of 10×).

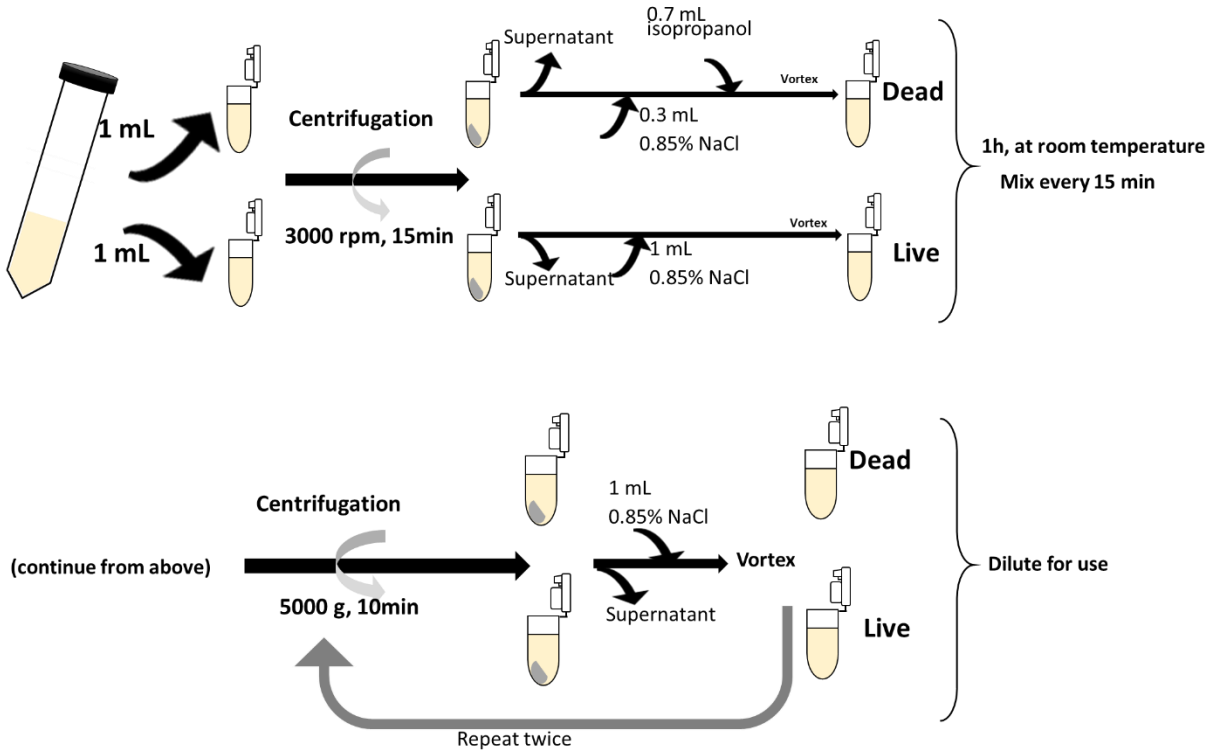


Fig. S2 Procedures for preparing living and dead cell suspensions (BacLight Kit manual with modifications).

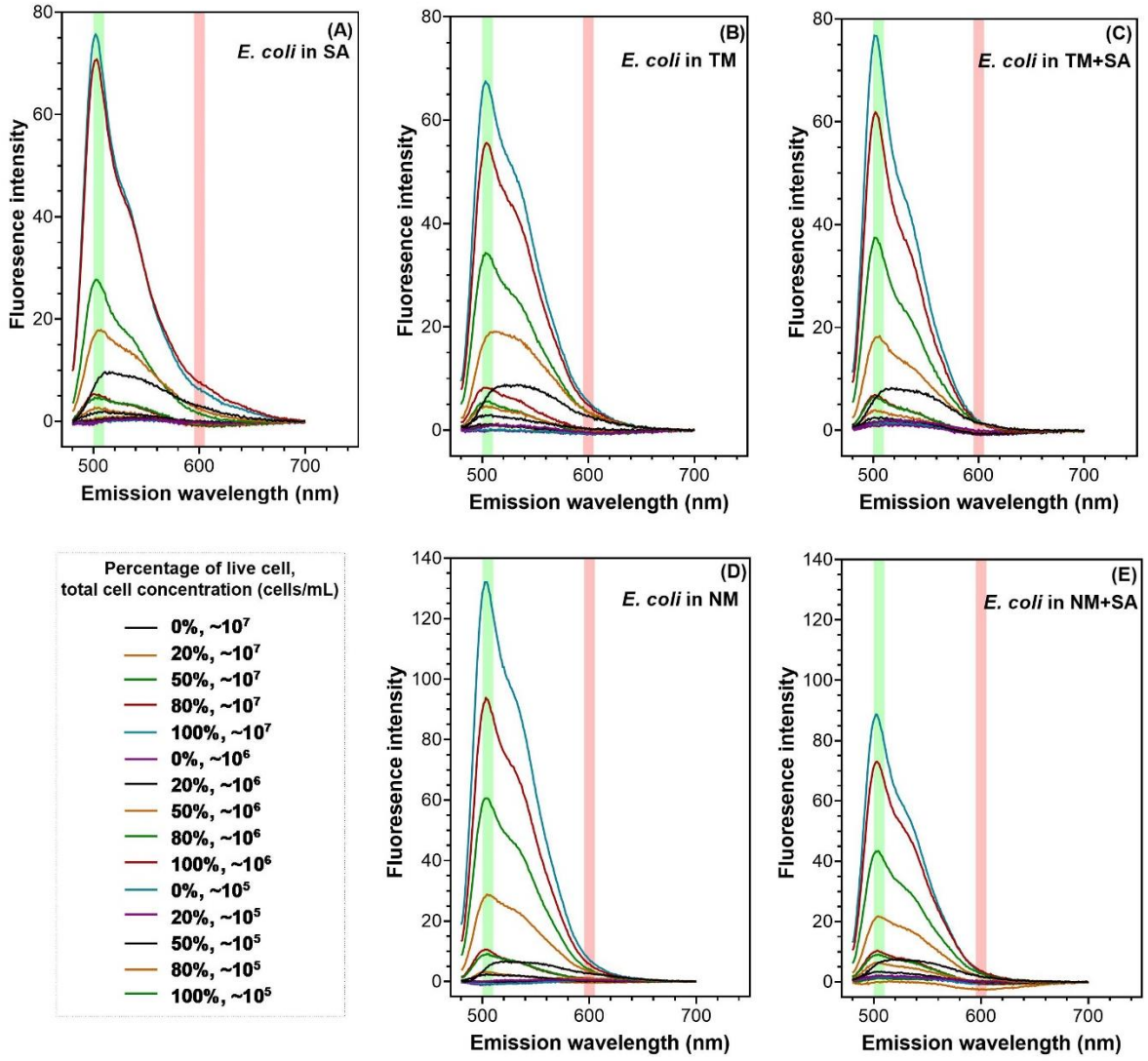
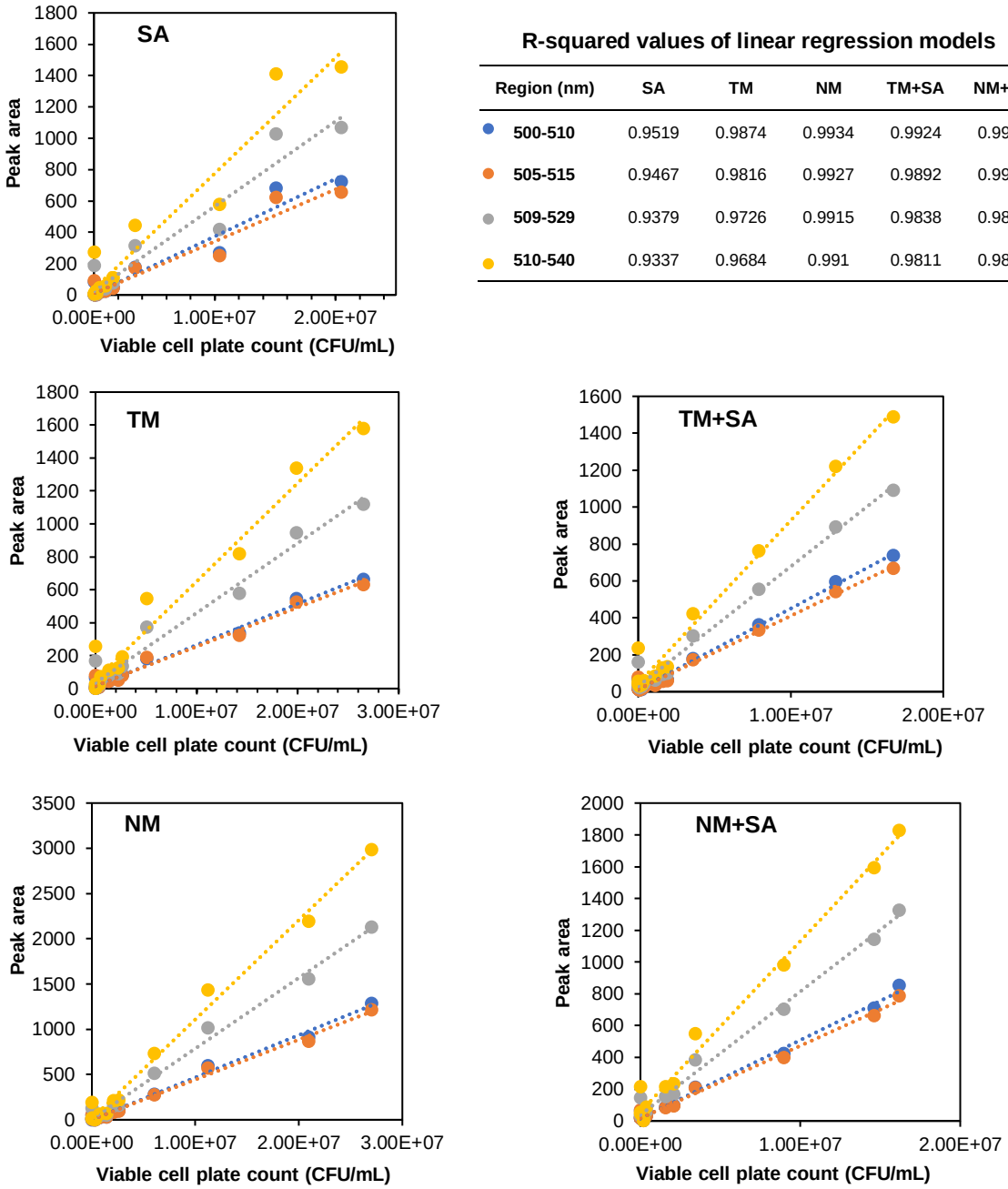


Fig. S3 Preprocessed spectra of *E. coli* suspended in those water matrices. The green and red ribbons indicate the fluorescence emission peaks of SYTO 9 and PI, respectively. The spectra were acquired by fixing the excitation wavelength at 470 nm. The fluorescence emission spectrum of *E. coli* suspension was the average of triplicate measurements, and the background spectrum of the water matrix was subtracted.



R-squared values of linear regression models

Region (nm)	SA	TM	NM	TM+SA	NM+SA
500-510	0.9519	0.9874	0.9934	0.9924	0.9923
505-515	0.9467	0.9816	0.9927	0.9892	0.9916
509-529	0.9379	0.9726	0.9915	0.9838	0.9896
510-540	0.9337	0.9684	0.991	0.9811	0.9885

Fig. S4 Linear regression models of SYTO 9 peak areas in different regions against cell count in five types of water and their R-squared values. The peak area was calculated using the preprocessed spectra (i.e. the background spectrum was removed). SA: 0.85% saline solution; TM: Tama River water; NM: Nomi River water; TM+SA: the mixture of Tama River water and 0.85% saline solution; NM+SA: the mixture of Nomi River water and 0.85% saline solution.

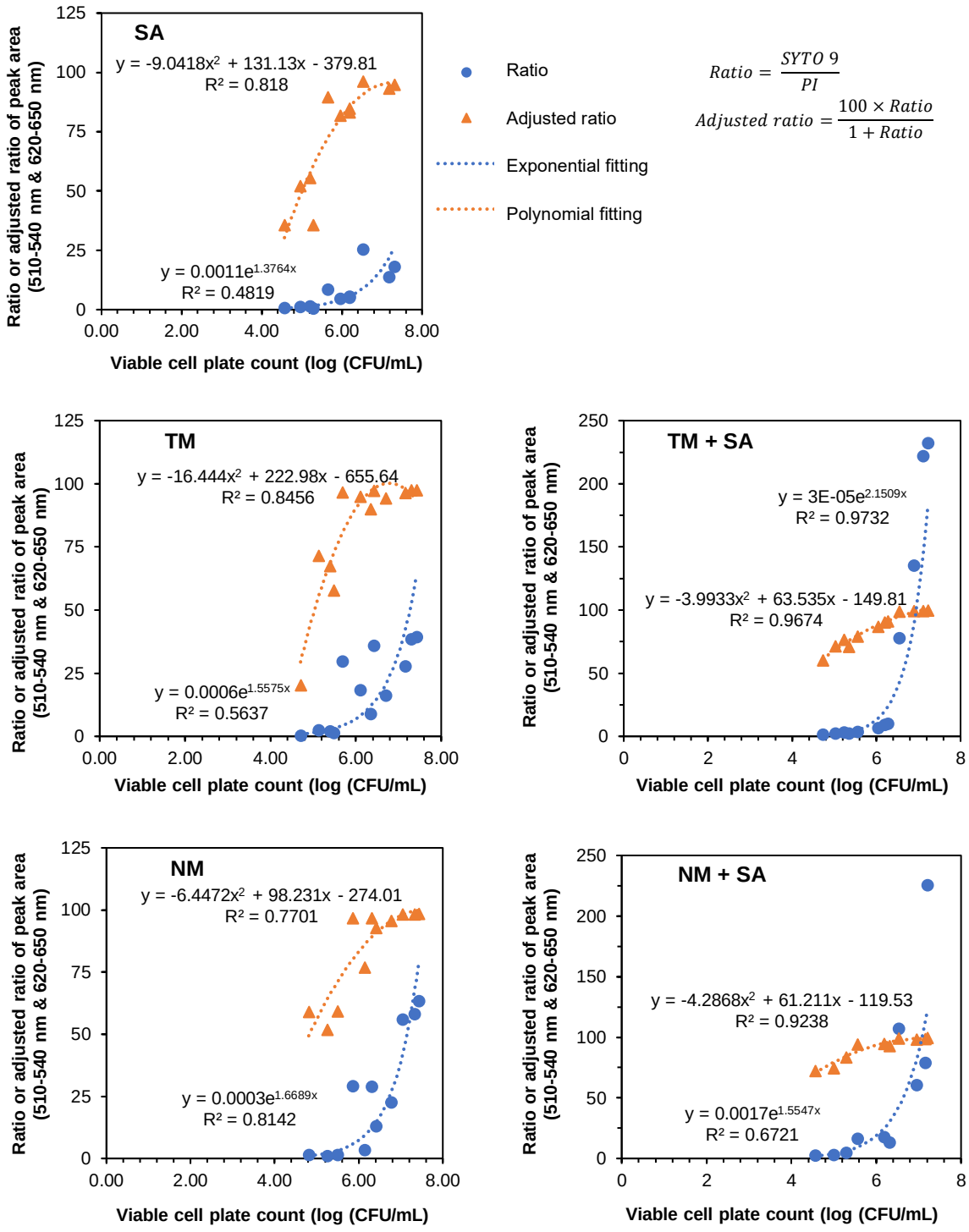


Fig. S5 Nonlinear regression models of ratio or adjusted ratios between SYTO 9 and PI peak areas across 510-540 nm and 620-650 nm, respectively, against logarithmic cell count in five types of water. The peak regions and ratio calculation were based on the BacLight Kit instructions, while the adjusted ratio was proposed by a previous study [10]. SA: 0.85% saline solution; TM: Tama River water; NM: Nomi River water; TM+SA: the mixture of Tama River water and 0.85% saline solution; NM+SA: the mixture of Nomi River water and 0.85% saline solution.

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