Evidently diverse effects of silver nanoparticles on *Vibrio parahaemolyticus* across different estuarine water samples

Qianqian Yang[a,b]†, Xiangyi Hou[a]†, Feng Lu[a], Dahai Zhang[b*], Wentao Lin[c], Nick Schlensky[c], Zhixiang Chen[b], Yan Zhang[a], Xuzhi Zhang[a*]

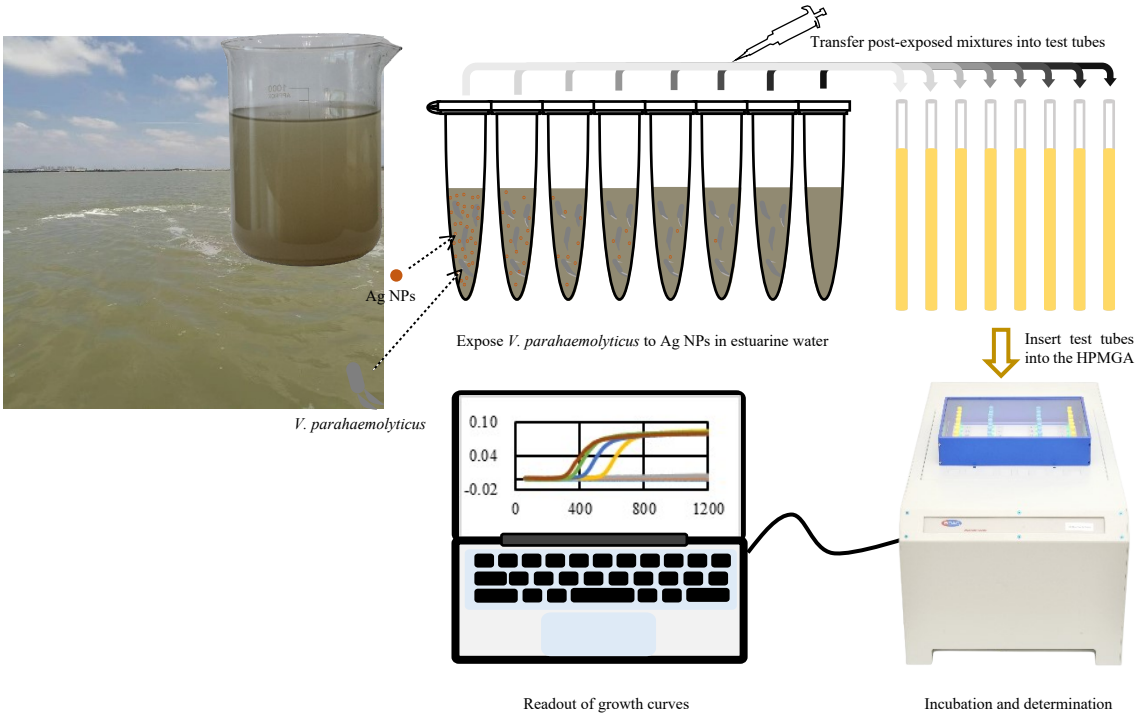
[a] *State Key Laboratory of Mariculture Biobreeding and Sustainable Goods, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, Shandong 266071, China*

[b] *Key Laboratory of Marine Chemistry Theory and Technology, Ministry of Education, Ocean University of China, Qingdao 266100, China*

[c] *eDAQ Pty Ltd, 6 Doig Ave, Denistone East NSW 2112, Australia*

Corresponding authors: E-mail: zhangxz@ysfri.ac.cn (X. Zhang); dahaizhang@ouc.edu.cn (D. Zhang)

† These authors contributed equally to this work.



Schematic S1 The working steps of determining MICs of Ag NPs against *V. parahaemolyticus* in estuarine water with the electronic microbial growth analyzer (EMGA) method.

Validation of the electronic microbial growth analyzer-based phenotypic method

Determination of V. parahaemolyticus growth curves with the EMGA and a Bioscreen C microbiology reader

The determination of *V. parahaemolyticus* growth and growth rate curves with the EMGA was conducted with a method we reported previously.[1] A starter LB broth, which held approximately 10^8 CFU/mL *V. parahaemolyticus*, was used to inoculate the tested LB broths. Five consecutive 10-fold dilutions of the tested LB broths were separately loaded into test tubes (5 mm NMR tube, NORELL, USA) in triplicate, and each of the test tubes was covered with a 0.22-μm/13-mm Millex syringe filters (Merck-Millipore, Germany) to guarantee sterile gas exchange. Then, these test tubes and the negative controls (LB broth) were inserted into the working channels of the HPEMGA. Unless otherwise stated, trials were performed in triplicate. The culture temperature, sampling interval, excitation frequency, excitation level, reference channel, and sampling count were 36°C, 1 min, 200 kHz, 90%, channel No.1 and 1200, respectively. After the determination of growth curves, namely, the ΔC^4–t curves, the polynomial regression algorithm listed in the "Curve Fitting" function was used to smooth curves with a polynomial order of 15. Then, the growth rate curves were decomposed using the data analysis tool implemented in CellStatz software. We denoted the incubation time of the beginning of a decline in the growth rate as the time of maximum growth rate (T_{mgr}).[1] ΔC^4 and T_{mgr} values on the growth rate curves were obtained by clicking the "Peak Display" button.

The determination of *V. parahaemolyticus* growth curves using the automated Bioscreen C microbiology reader (Helsinki, Finland) was performed according to the method reported by Aalto-Araneda *et al*.[2] Briefly, a starter LB broth containing 10^8 CFU/mL of *V. parahaemolyticus* was used to inoculate tested LB broths and prepared 5 consecutive 10-fold dilutions (10^2, 10^3, 10^4, 10^5 and 10^6 CFU/mL). A 200 μL volume of the resulting suspension was pipetted into a well of the 100-well honeycomb plate. LB broth was used as the negative control. A cover was used on the plate to prevent evaporation. The honeycomb plate was incubated in the Bioscreen C microbiology reader at 36°C, and OD_{600} values were automatically collected for each well every 10 min during a 20 h period. The suspensions were homogenized with a slight orbital shake for 10 s prior to each OD reading.

Figure S1a (Column I) shows the triplicate growth curves of 10^2, 10^3, 10^4, 10^5 and 10^6 CFU/mL of inoculum *V. parahaemolyticus* determined using the EMGA method. In the exponential phase, the slopes of the lines were almost identical for all samples, despite the variation in inoculum concentrations. The final C^4 outputs reached similar levels, suggesting that for *V. parahaemolyticus*, the increase in incubation amount accelerated the detectable onset of growth, rather than increasing the maximum growth rate and maximum growth. For negative controls, there were no sigmoidal growth curves over the same period, suggesting that without viable bacteria, there was no conductivity change during the incubation(Zhang et al., 2018).[3] Figure S1b (Column I) shows the growth rate curves. There was a linear relationship between the logarithmic value of the initial inoculum concentration of *V. parahaemolyticus* and T_{mgr} (Figure S1c (Column I)). The values of the trials in triplicate indicated a standard deviation (SD) of $T_{mgr} \leq 4$ min. Further studies showed that the EMGA method exhibited good precision, accuracy and repeatability (Figure S2 and Table S1).

As a counterpart to these results, sigmoidal OD-t curves obtained with the automated Bioscreen C microbiology reader are shown in Figure S1a (Column II). The corresponding growth rate curves are shown in Figure S1b (Column II). A linear relationship was observed between the logarithmic value of the initial inoculum concentration of *V. parahaemolyticus* and the average $C_{t,}$ with a correlation coefficient *r* of 0.9926 (Figure S1c (Column II)). The values of the trials in triplicate indicated an SD of $C_t \leq 5$ min.

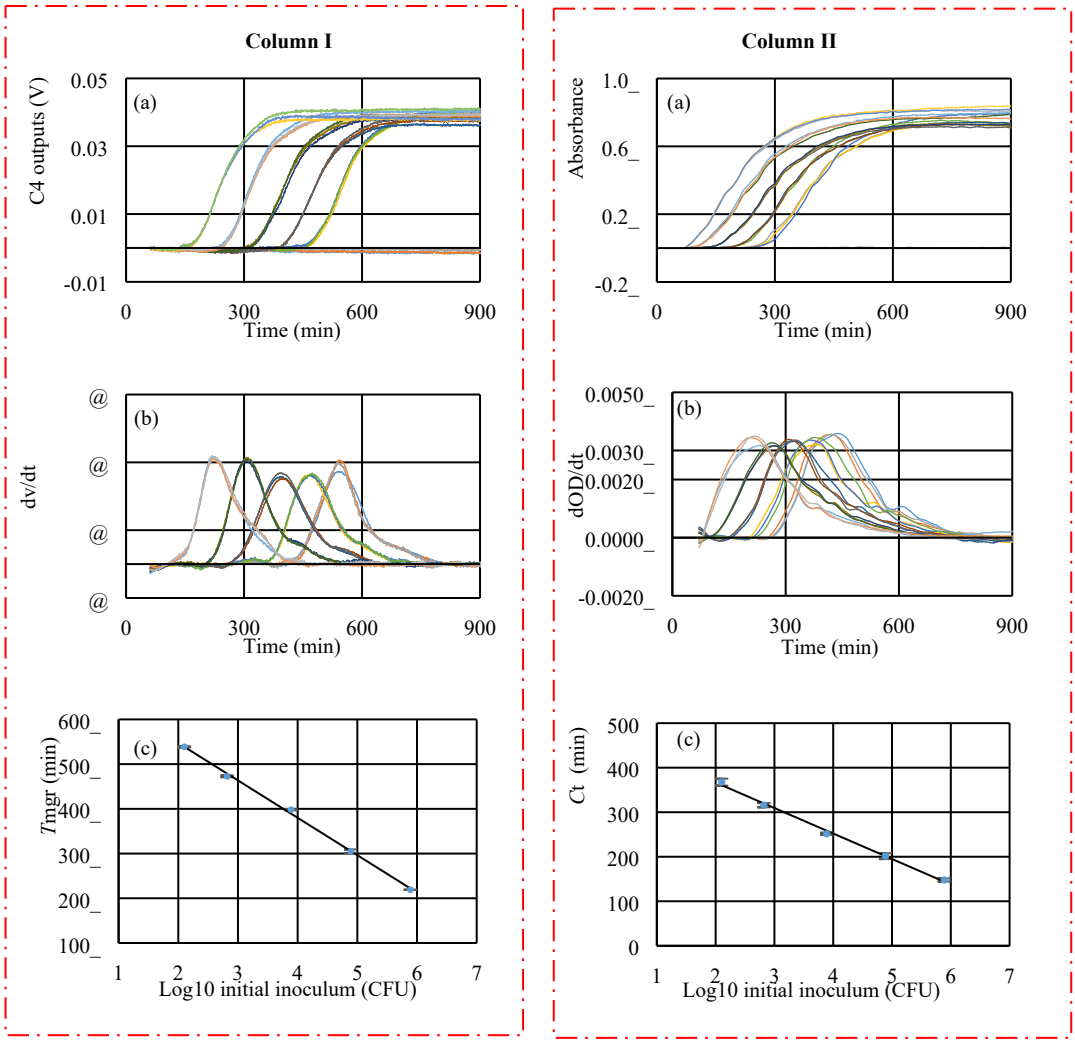


Figure S1 Column I: Triplicate growth (a) and growth rate (b) curves of *V. parahaemolyticus* determined using the EMGA method. The linear relationship between the logarithmic values of the initial inoculum and T_{mgr} (c). Column II: Triplicate growth (a) and growth rate (b) curves of *V. parahaemolyticus* determined using the OD method. The linear relationship between the logarithmic values of the initial inoculum and C_t (c). In (a) and (b), from left to right, the initial inoculums were 10^6, 10^5, 10^4, 10^3 and 10^2 CFU/mL, respectively. Error bars represent the SD of three measurements.

In homogeneous simple laboratory media, the shapes of the growth curves were similar to those obtained by the OD method, which clearly indicated the lag phase, acceleration phase, exponential phase, deceleration phase, and stationary phase. Moreover, over the range of 10^2 – 10^6 CFU/mL, the linear

regression equation was T_{mgr} (min) = −83.601log [*V. parahaemolyticus* (CFU/mL)] + 714.33 with a correlation coefficient *r* of 0.9981. This suggests that there is a linear relationship between the logarithmic value of the initial (surviving) *V. parahaemolyticus* and T_{mgr}. The correlation and accuracy were as good as or even better than those obtained with the automated OD method. These outcomes showed that the EMGA was capable of showing the growth curves of *V. parahaemolyticus* online. Moreover, the EMGA method exhibited good precision, accuracy and repeatability (Figure S2 and Table S1).

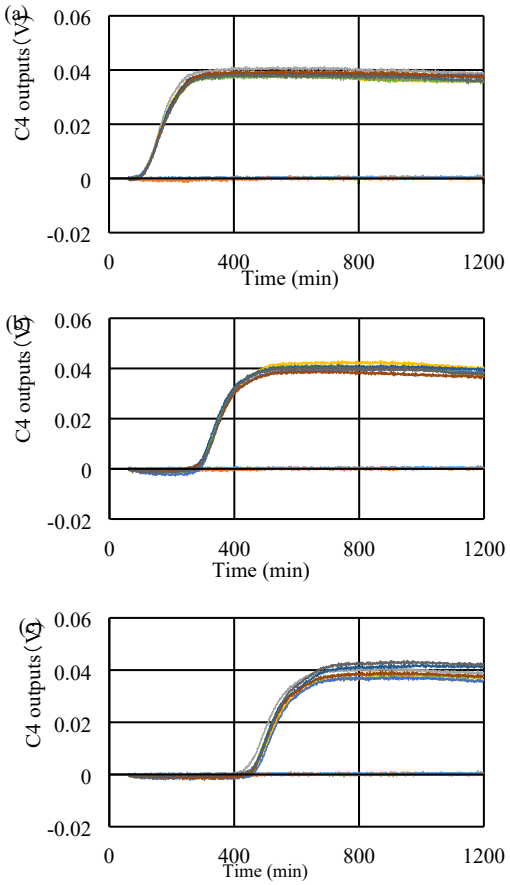


Figure S2 Growth curves of *V. parahaemolyticus* in LB broth determined using the EMGA method in septuplicate. The initial inoculum was 10^7 CFU/mL (a), 10^5 CFU/mL (b) and 10^3 CFU/mL (c). The culture temperature, sampling interval, excitation frequency, excitation level, reference channel and sampling count were 36°C, 1 min, 2 MHz, 90%, channel 1 and 1200, respectively.

Table S1: SD and RSD of T_{mgr} values for different initial inoculum.

	10^3	10^5	10^7
SD	9.01	3.58	1.96
RSD	1.78%	1.06%	1.22%

Determination of MICs of Ag NPs against V. parahaemolyticus in physiological saline

The determination of MICs with the EMGA method consisted of two steps, i.e., the transfer of post-exposed mixtures to test tubes and the determination of surviving *V. parahaemolyticus* growth curves. In brief, the re-suspended *V. parahaemolyticus* cells in physiological saline with a final concentration of approximately 10^5 CFU/mL were exposed to 0.75, 1.5, 3.0, 6.0 and 12.0 mg/L Ag NPs at 25°C for 30 min under gentle shaking. Then, a 200 μL volume of each resulting mixed suspension was transferred into a test tube, in which 1.8 mL LB broth was pre-loaded. We covered the test tubes with Millex syringe filters and then inserted them into the HPEMGA to determine the growth curves of the surviving bacteria. LB broths with and without bacterial cells served as positive and negative controls, respectively. The culture temperature, sampling interval, excitation frequency, excitation level, reference channel, and sampling count were the same as above. The MIC was defined as the lowest antibiotic concentration that inhibited the growth of bacteria (Theophel et al., 2014),[4] as assessed from the absence of the sigmoidal curve.

The determination of MICs with the OD method in physiological saline also involved two steps, namely, the transferring of post-exposed mixtures and the determination of the surviving bacterial growth curves (Theophel et al., 2014).[4] In summary, re-suspended *V. parahaemolyticus* cells (10^5 CFU/mL) in physiological saline were incubated in an incubator at 25°C for 30 min under gentle shaking. A 200-μL volume of each post-exposed mixture was pipetted into a well of the 100-well honeycomb plate. In the wells, serial 2-fold dilutions of Ag NPs were added to produce the expected final concentrations. Two hundred microliters of physiological saline with and without 10^5 CFU/mL *V. parahaemolyticus* were used as the positive and the negative control, respectively. The honeycomb plate was incubated in the Bioscreen C microbiology reader, and the remaining procedures were performed as stated above. The MIC was harvested based on the absence of the sigmoidal curve.

The determination of MICs with the plate counting method in physiological saline was performed according to the report by Feng *et al*.[5] with minor modifications. A 10-mL aliquot of physiological saline containing 10^5 CFU/mL *V. parahaemolyticus* was separately loaded into five 50-mL centrifuge tubes. Different amounts of Ag NPs were added to the centrifuge tubes to produce the expected final concentrations. Samples with and without 10^5 CFU/mL bacterial cells were used as the positive and negative controls. Then, the centrifuge tubes were incubated in an incubator (RADOBIO Scientific CO., Ltd., Shanghai, China) at 25°C for 30 min under gentle shaking. Three 100-μL aliquots of each physiological saline matrix were directly plated on three LB agar plates. Then, three 100-μL aliquots of each suspension containing surviving bacterial cells were plated on three LB agar plates. All plates were incubated in the incubator at 36°C for 20 h. Then, images of typical desired culture results were taken using a handheld 90D Canon camera.

The determination of MICs with the broth microdilution (BMD) method in physiological saline was performed according to the CLSI guidelines (Ceriotti et al., 2012).[6] In summary, re-suspended *V. parahaemolyticus* cells (10^5 CFU/mL) in physiological saline were incubated at 25°C for 30 min under gentle shaking. Then, a 200-μL volume of each post-exposed mixture was pipetted into a well of a Corning 96-well plate (Corning Incorporated, USA). The wells contained serial 2-fold concentrations of Ag NPs. Samples with and without 10^5 CFU/mL *V. parahaemolyticus* were used as the positive and

negative controls, respectively. The plates were sealed with parafilm and incubated in an incubator at 36°C for 20 h. The MIC was defined as the lowest concentration at which the growth was inhibited completely, as determined visually.

Figure S3a shows typical growth curves of surviving *V. parahaemolyticus* in LB broths obtained with the EMGA. Sigmoidal curves were obtained as expected for the positive controls (with inoculum concentration of 10^5 CFU/mL in the liquid broth) due to normal bacterial growth. Versus the positive controls, the lag phase durations of *V. parahaemolyticus* regularly extended with an increasing concentration of Ag NPs. The delayed onset of bacterial growth suggests that the higher the concentration of the Ag NPs is, the less surviving *V. parahaemolyticus*. With concentrations of Ag NPs ≥6.0 mg/L, there were no sigmoidal growth curves over the incubation time. Thus, an MIC of 6.0 mg/L was directly obtained.

Figure S3b shows typical triplicate OD-t growth curves of surviving *V. parahaemolyticus* in LB broths. Sigmoidal curves were obtained as expected for the positive controls. Versus the positive controls, the lag phase durations extended with increasing concentration of the Ag NPs. An MIC of 6.0 mg/L was also directly obtained according to the presence of a straight line.

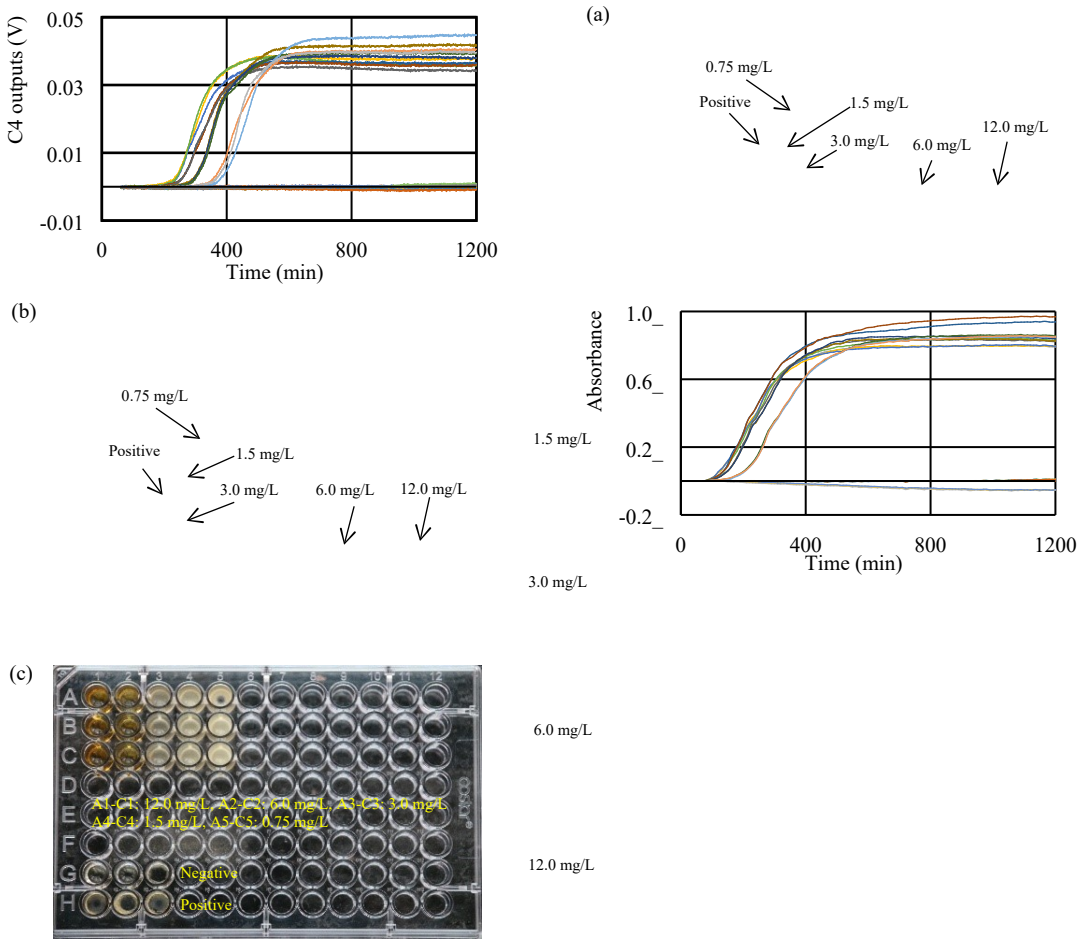


Figure S3 Effects of Ag NPs on *V. parahaemolyticus* in physiological saline. Growth curves of surviving *V. parahaemolyticus* obtained using the EMGA (a) and OD (b) methods in triplicate. Images of BMD (c) and plate counting (d) results. *V. parahaemolyticus* (~10^5 CFU/mL) in physiological saline was

exposed to 0.75, 1.5, 3.0, 6.0 and 12.0 mg/L Ag NPs at 25°C for 30 min under gentle shaking. Then, surviving *V. parahaemolyticus* were cultured in LB broth/agar for 20 h.

Figure S3c and Figure S3d show typical images of BMD and plate counting results for determining the effects of Ag NPs on *V. parahaemolyticus*. The MICs obtained using these endpoint methods were in good agreement with those obtained using automated methods, namely, the EMGA and Bioscreen C microbiology reader.

Determination of MICs of Ag NPs against V. parahaemolyticus in estuarine water samples

In estuarine water, the MICs of Ag NPs against *V. parahaemolyticus* was also determined with the EMGA method. The re-suspended *V. parahaemolyticus* cells in S1 sample with a final concentration of approximately 10^5 CFU/mL were exposed to 1.5, 3.0, 6.0, 12.0, 24.0 and 48.0 mg/L Ag NPs. Then, post-exposed mixtures were transferred into test tubes. Test tubes were inserted into the EMGA to determine the growth curves of surviving bacteria. The operation parameters and the readout of MICs were the same as stated above. When plate counting and BMD methods were employed, of note, post-exposed estuarine water matrices were treated with centrifugation at 5, 000 g for 10 min using a Beckman OPTIMA XL-90 centrifuge to remove indigenous suspended solids (Nayak et al., 2005).[7]

Figure S4a shows typical growth curves of surviving *V. parahaemolyticus* in LB broths. Sigmoidal curves were obtained as expected for the positive controls. Versus the positive controls, the lag phase durations of *V. parahaemolyticus* extended with increasing concentrations of Ag NPs from 3.0 mg/L to 12.0 mg/L. In cases where the concentrations of pulse-exposed Ag NPs were 24.0 mg/L and 48.0 mg/L, there were no sigmoidal growth curves. This indicates that in the S1 sample, the MIC of pulse-exposed Ag NPs against *V. parahaemolyticus* is 24.0 mg/L. This MIC was also validated using the BMD (Figure S4b) and plate counting (Figure S4c) methods. In other words, the MIC of Ag NPs against *V. parahaemolyticus* in estuarine water determined using the EMGA method was consistent with those obtained using the BMD and plate counting methods. Thus, this efficient method was reliable for determine the adverse effects of nanomaterials on bacteria.

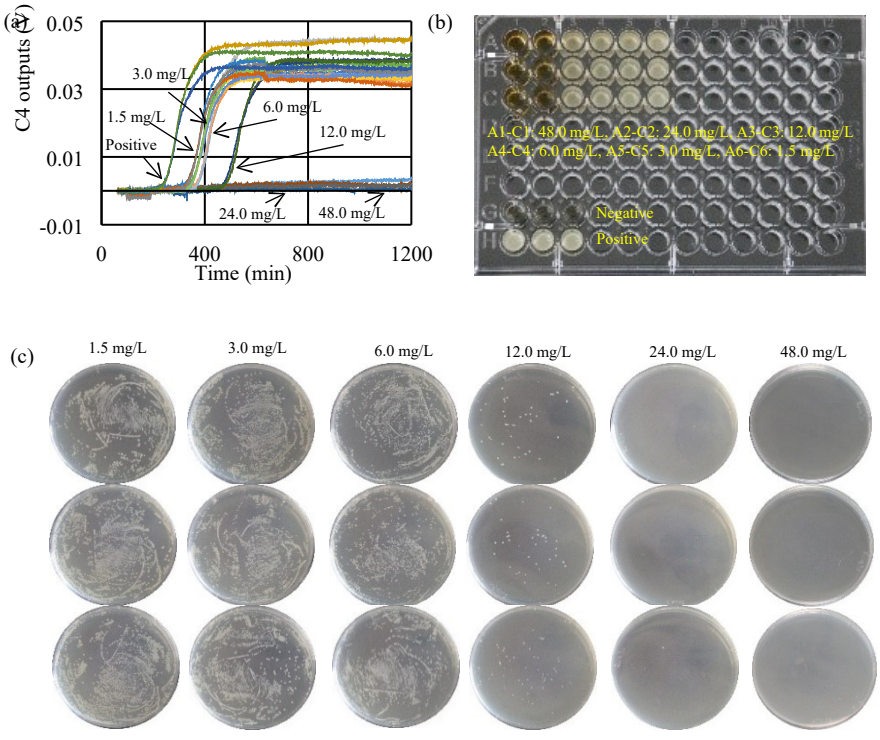


Figure S4 Effects of Ag NPs on *V. parahaemolyticus* in estuarine water. Triplicate growth curves of surviving *V. parahaemolyticus* obtained with the EMGA (a). Images of BMD (b) and plate counting (c) results. Approximately 10^5 CFU/mL *V. parahaemolyticus* in the S1 sample was exposed to 1.5, 3.0, 6.0, 12.0, 24.0 and 48.0 mg/L Ag NPs at 25°C for 30 min under gentle shaking. Surviving *V. parahaemolyticus* were cultured in LB broth/agar for 20 h.

In conclusion, the EMGA-based thenotypic method was reliable for harvesting MICs of Ag NPs against *V. parahaemolyticus* in both homogeneous transparent laboratory mediums and heterogeneous complex matrices.

References

1. Zhang X, Yang Q, Ma L, Zhang D, Lin W, Schlensky N, Cheng H, Zheng Y, Luo X, Ding C, Zhang Y, Hou X, Lu F, Yan H, Wang R, Li C, Qu K. Automatically showing microbial growth kinetics with a high-performance microbial growth analyzer. Biosens. Bioelectron. 2023, 239, 115626.
2. Aalto-Araneda M, Pöntinen A, Pesonen M, Corander J, Markkula A, Tasara T, Stephan R, Korkeala H. Strain variability of Listeria monocytogenes under NaCl stress elucidated by a high-throughput microbial growth data assembly and analysis protocol. Appl Environ Microb. 2020, 86, e02378-19.
3. Zhang X, Jiang X, Yang Q, Wang X, Zhang Y, Zhao J, Qu K, Zhao C. Online Monitoring of Bacterial Growth with an Electrical Sensor. Anal. Chem. 2018, 90, 6006-6011.
4. Theophel K, Schacht VJ, Schluter M, Schnell S, Stingu CS, Schaumann R, Bunge M. The importance of growth kinetic analysis in determining bacterial susceptibility against antibiotics and silver nanoparticles. Front Microbiol. 2014, 5, 544.
5. Feng J, Guo X, Chen Y, Lu D, Niu Z, Tou F, Hou L, Xu J, Liu M, Yang Y. Time-dependent effects of ZnO nanoparticles on bacteria in an estuarine aquatic environment. Sci Total Environ. 2020, 698,134298.
6. Ceriotti F, Zakowski J, Sine H, Altaie S, Horowitz G, Pesce AJ, Boyd J, Horn P, Gard U, Horowitz G. CLSI 2012; M07-A10.
7. Nayak BB, Kamiya E, Nishino T, Wada M, Nishimura M, Kogure K. Separation of active and inactive fractions from starved culture of Vibrio parahaemolyticus by density dependent cell sorting. Fems Microbiol Ecol. 2005, 51,179-186.