Experimental Validation of Stability and Applicability of Start Growth Time Method For High-Throughput Bacterial Ecotoxicity Assessment.

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Research Article

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Abstract

Ecotoxicity assessments based on bacteria as model organisms is widely used for routine toxicity screening because it has advantages of time-saving, high sensitivity, cost-effectiveness, and less ethical responsibility. Determination of ecotoxicity effect via bacterial growth can avoid the restriction of model bacteria selection and unique equipment requirement, but traditional viable cell count methods are relatively labor- and time-intensive. The Start Growth Method (SGT) is a high-throughput and time-conserving method to determine the amount of viable bacterial cells. However, its usability and stability for ecotoxicity assessment are rarely studied. This study confirmed its applicability in terms of bacterial types (gram-positive and gram-negative), growth phases (middle exponential and early stationary phases), and simultaneous existence of dead cells (adjustment by flow cytometry). Our results verified that the stability of establishing SGT correlation is independent of the bacterial type and dead-cell portion. Moreover, we only observed the effect of growth phases on the slope value of established SGT correlation in *Shewanella oneidensis*, which suggests that preparing inoculum for the SGT method should be consistent in keeping its stability. Our results also elucidate that the SGT values and the live cell percentages meet the non-linear exponential correlation with high correlation coefficients from 0.97 to 0.99 for all the examined bacteria. The non-linear exponential correlation facilitates the application of the SGT method on the ecotoxicity assessment. Finally, applying the exponential SGT correlation to evaluate the ecotoxicity effect of copper ions on *E. coli* was experimentally validated. The SGT-based method would require about 6 to 7 hours to finish the assessment and obtained an estimated EC50 at 2.27 ± 0.04 mM. This study demonstrates that the exponential SGT correlation can be a high-throughput, time-conversing, and wide-applicable method for bacterial ecotoxicity assessment.

1 Introduction

Environmental protection has become a global issue of concern. To properly establish legislation regulating wastewater effluent discharge and monitoring water quality, novel risk-based approaches and test methods adopted to assess water body status, including ecological safety, chemical quality, and biological impact, become essential (Bodini et al. 2018). As the representative index of biological impact, water toxicity is determined to assess the hazardous effects of pollutants, chemicals, or heavy metals on ecosystems or environments. Ecotoxicity tests are the most frequently used tools for assessing water toxicity by detecting the biological response produced by microorganisms or higher organisms affected by the toxic chemicals (García-Gómez et al. 2015, Petric et al. 2016). Compared with physiochemical analyses to understand the water quality, ecotoxicity tests can overcome the limits of physiochemical analyses in demonstrating the biochemical influence of toxic chemicals on living organisms, bioavailability toward toxic chemicals, and the antagonistic and synergetic interactions (Bolan et al. 2015, Rosado et al. 2016, Rosal et al. 2010). In ecotoxicity tests, organisms' trophic levels, including mammalian cells, algae, plants, fish, zooplankton, phytoplankton, and bacteria, are examined as model and target organisms. (Hund-Rinke &Simon 2006, Wang et al. 2015, Yao et al. 2018). In contrast to multi-cellular eukaryotic organisms, bacteria have rapid rates of growth. Ecotoxicity test based on bacteria as

model organisms are easily applicable for widely and routinely toxicity screening because it has advantages of relatively short assay time, high sensitivity, cost-effectiveness, and less ethical responsibility (Muneeswaran et al. 2021, Parvez et al. 2006, Wang et al. 2010).

Various kinds of biochemical responses have established the bacteria-based toxicity assessment method. For instance, luminescent bacteria, such as *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum*) (Venancio et al. 2021) or *Photobacterium leiognathid* (Muneeswaran et al. 2021, Neale et al. 2017) are the most widely used method for evaluating and monitoring ecotoxicity. They have been applied to assess the ecotoxicity of petroleum hydrocarbon, pesticide-contaminated soils, contaminated river sediments, nanoparticles, and industrially processed wastewater (Jarque et al. 2016, Moi et al. 2017, Zhang et al. 2020). The luminescent bacteria can naturally produce bioluminescence by expressing their luciferase gene and the bioluminescent signals can be monitored using a luminometer. After the bacterial cells are exposed to the target chemicals for 15 or 30 min, the bioluminescent signal is subsequently determined. The amount of the target chemicals to cause a 50% luminescence inhibition is called the median effect concentration (EC50) (Froehner et al. 2000). Alike the principle of monitoring the bioluminescent signal, other biochemical indicators, such as nitrification, electron transfer, respiration, or unique enzyme expression, have also been applied to ecotoxicity tests, which are related to nitrifying bacteria (Gernaey et al. 1997), iron-oxidizing bacteria (Yang et al. 2017), sulfur-oxidizing bacteria (Eom et al. 2019), electroactive bacteria (Chu et al. 2021), and fermentative bacteria (Eom et al. 2020). However, detecting or monitoring all the above biochemical responses requires their specific equipment; thus, extending the generalization of those ecotoxicity methods is restricted. Besides, the primary purpose of those ecotoxicity assays is to collect and establish the toxicity profile and database of interesting chemicals as much as possible based on one reliable microbiological system. Those methods are unable to adapt the requirement for an opposite purpose, for instance, to evaluate toxicity response of regulation concerning chemicals to prospective bioremediation bacteria (Kang &Park 2010, Ruggiero et al. 2005), specific plant growth-promoting bacteria (Mubeen et al. 2006, Verma et al. 2016), and sewage bacteria (Strotmann et al. 1994). Therefore, ecotoxicity assessment determined based on the inhibition of bacterial growth is still the simplest method and applicable for these purposes (Baek &An 2011, Giri &Golder 2015).

For quantifying bacterial growth and inhibition after toxic chemical exposure, the viable-count or spread plate method is one of the most commonly used techniques by counting the number of forming colonies on an agar plate and evaluating the difference between samples with or without adding chemicals. However, the main disadvantage of the spread plate method is that it takes a relatively long time (at least overnight) for incubation before the results are obtained. Alternatively, a Start Growth Time (SGT) method for high throughput determination of viable bacterial cell counts in 96-well plates has been established (Hazan et al. 2012). The SGT method prepares a series dilution of the bacterial liquid culture, monitors the growth curve of each diluted culture, and sets up an optical density (O.D.) threshold around 0.15 to 0.20 to point as the SGT value. Then, it establishes a linear correlation between the SGT values and the cell density to quantify the viable bacterial cell counts for the other samples. Because the SGT value represents the bacterial growth during the early exponential phase, the duration of the overall monitoring

process is relatively short. For instance, the time required to establish the SGT correlation for Pseudomonas aeruginosa strain PA14 only took 11.5 hours to reach the SGT for the most diluted culture (Hazan et al. 2012). Xia et al. (2020) applied the SGT analysis for four common pathogenic aquaculture bacteria, e.g., *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio alginolyticus*, and *Vibrio harveyi*, and their highest SGT values were from 4 to 10 hr. Although using the SGT method to quantify the viable bacterial cell count has the advantages of high throughput and short test duration, its applicability and stability to gram-negative and gram-positive bacteria are unclear. The target bacteria in the previous studies, which have applied the SGT method, were almost gram-negative bacteria, such as the genera of *Pseudomonas*, *Aeromonas*, *Edwardsiella*, *Vibrio*, *Burkholderia*, and *Coxiella* (Ahn et al. 2017, Khan et al. 2019, Maura et al. 2016, Xia et al. 2020). There were only two studies applying the SGT method to investigate the gram-positive bacteria, *Bacillus megaterium* and *Enterococcus faecalis*, but no detailed SGT correlation was established in their study (Li et al. 2018, Oyama et al. 2017). Besides, the SGT method has only been applied in rare studies for investigating the ecotoxic effects on bacteria, e.g. chlorhexidine gluconate and benzalkonium chloride toward *Burkholderia cenocepacia* (Ahn et al. 2017) and ruminal and antimicrobial peptide toward *E. faecalis* (Oyama et al. 2017). Its applicability for ecotoxicity assessment and the effect of existence of dead/injured bacterial cells is still unclear.

Therefore, this study aims to evaluate the applicability and stability of the SGT method when it is applying to gram-positive and gram-negative bacteria. Besides, the applicability of the SGT method as an alternative ecotoxicity assessment method was also detailed verified in this study. This study established the SGT correlations of three gram-positive and three gram-negative bacteria with their cells collected from the middle exponential and the early stationary phases. Besides, various live and dead cell mixture ratios were prepared and verified by the flow cytometry measurement. The effects of the existence of dead bacterial cells on the SGT correlation establishment were examined. Finally, the ecotoxicity and inhibitory impact of copper ions on *Escherichia coli* cells was evaluated using its SGT correlation established with the different live-to-dead cell ratios.

2 Materials And Methods

2.1 Bacteria and growth conditions

This study assessed three gram-positive and three gram-negative bacteria, including *Bacillus subtilis* ATCC 6633^T, *Staphylococcus xylosus* ATCC 29971^T, *Enterococcus faecalis* ATCC 29212^T, *Escherichia coli* ATCC 23716^T, *Pseudomonas aeruginosa* BCRC 11078^T, and *Shewanella oneidensis* ATCC 700550^T. We obtained these bacterial type strains from the Bioresource Collection and Research Center, the Food Industry Research and Development Institute (BCRC, FIRDI, Hsinchu, Taiwan). *B. subtilis*, *P. aeruginosa*, and *S. oneidensis* were grown in the tryptic soy broth (TSB) medium at 30ºC. *S. xylosus*, *Ent. faecalis*, and *E. coli* were grown at 37ºC in the medium of TSB, brain heart infusion (BHI), and nutrient broth (NB), respectively. We purchased the dehydrated culture media mentioned above from BD Biosciences (Franklin Lakes, NJ, USA). Before starting any experiment, we plated out the bacterial cells on their growth medium

to obtain a fresh stock of bacteria ready to inoculate for the following preculture procedure. Subsequently, we inoculated a single colony on the fresh stock solid medium in the corresponding liquid broth of 25 mL in flasks. Then, it was cultivated at their growth temperature overnight with agitation at 120 rpm. We transferred the precultured cells for a second liquid culture by controlling the inoculum (1:10 dilution factor) and the growing duration. Subsequently, we harvested cells grown to the middle exponential or the initial stationary phases for the SGT experiments.

2.2 Determination of SGT correlations

We established the SGT correlations by following the method in the previous report (Hazan et al. 2012) with minor modifications. Cells from the second liquid culture with growth to the middle exponential phase were used directly as the inoculum for the general SGT determination. The above-collected cells were 10-fold serially diluted in a factor range of 10^{-2} to 10^{-7}. We monitored their growth curves using an automated 96-well microplate reader (AccuReader M965, Metertech, Taiwan) with an optical density of 600 nm ($O.D._{600nm}$). The microplate reader was set at the growth temperature depending on the examined bacteria and with 3 s of circular shaking every 30 min. The SGT value of each diluted culture was defined as the time required to reach an $O.D._{600nm}$ threshold of 0.15. Then we established the SGT correlation between the required SGT time and the dilution factor by the linear regression. Besides, we measured the initial cell density (cell mL^{-1}) with proper dilution using a CytoFlex S flow cytometer (Beckman Coulter, Fullerton, CA, USA) at a fixed flow rate of 20 µL min^{-1} and below its upper measuring limitation of 30,000 events sec^{-1}. We also determined the SGT correlation of the cells from the second liquid culture with growth to the initial stationary phase for comparison. Because the cells in the stationary phase were relatively dense, we increased the factors of the serial dilution for SGT correlation to the range of 10^{-3} to 10^{-8}.

2.3 Effect of live-to-dead cell ratio

We prepared the dead bacterial cell as the following procedures. Cells from the second liquid culture during the middle exponential phase were collected by centrifugation at 8,000 × g for 10 min. The cell pellet was resuspended in 70% isopropanol for 1 hr (Kaprelyants &Kell 1992, Pascaud et al. 2009) to kill the cell. After removing the 70% isopropanol, the dead cells were washed twice by phosphate buffered saline (PBS) solution to be the dead cell stock. For preparing cell suspensions of live and dead bacterial cells with different ratios, we used cells freshly grown to the middle exponential phase as the live-cell stock. Cell densities of both the live and dead cell stock were measured in advance by the flow cytometry and adjusted to the same cell density of 2×10^7 cells mL^{-1}. We mixed suspensions of live and dead cell stocks at the same concentration to give different live-to-dead cell ratios of 100:0, 75:25, 50:50, 25:75, and 1:99. We used the flow cytometer to verify the exact live-to-dead cell ratio again. Quadrant gating of the flow cytometry plots was applied to separate the live and dead bacterial cells and verify their exact mixed ratio depending on their contour plot diagram with their distinctly forward scatter (FSC) and side scatter (SSC) signals (Fig. S3 to S8) by using the Kaluza Analysis Software version 2.1 (Beckman Coulter, USA). Subsequently, we determined their SGT correlation for live and dead cells under various mixed

ratios by following the previous procedures. Factors of the serial dilution during examining the effects of live and dead cell ratio on the SGT correlations were controlled in the range of 10^{-1} to 10^{-4}.

2.4 Ecotoxicity assessment of copper ions to *E. coli*

We assessed the ecotoxicity of copper (Cu^{2+}) against *E. coli* by applying the established correlation between SGT values and different live and dead cell ratios. For the growth inhibition toxicity test, *E. coli* cells grown to the middle exponential phase were first mixed with fresh NB medium to adjust the cell density to 2×10^7 cells mL^{-1}. The cell density was verified by flow cytometry and defined as the *E. coli* cell stock. Subsequently, we performed a similar 1:10 serial dilution of the *E. coli* cell stock into a factor range from 10^{-1} to 10^{-4} to establish the SGT correlation. Each diluted *E. coli* culture was dosed with 1.0, 2.0, 2.5, 3.0, and 4.0 mM of Cu^{2+} by adding a 100-fold concentrated Cu^{2+} stock solution ($CuSO_4 \cdot 5H_2O$ prepared with DI water). Then, we monitored the growth curves of the *E. coli* culture with different Cu^{2+} amounts and determined the SGT values of each dilution by the same $O.D._{600nm}$ threshold of 0.15.

2.5 Data and statistical analysis

The correlation between the culture dilution factor and its corresponding SGT was established by linear regression. We fitted the correlation between the live-to-dead cell ratio and their corresponding SGT by an non-linear exponential curve. One-way ANOVA compared means between each condition with a post-hoc Tukey test. Stars shown in figures denotes the degree of significance, one star (*) indicates a p-value < 0.05; two stars (**) indicate a p-value < 0.01; three stars (***) indicate a p-value < 0.001. We performed all the above data and statistical analysis with OriginPro 2018 SR1 (OriginLab, USA).

3 Results And Discussion

3.1 The effect of bacterial discrimination and growth phases

In the past, studies that applied the SGT method to study their bacteria mainly focused on gram-negative bacteria; only two gram-positive bacteria, e.g., *Bacillus megaterium* and *Enterococcus faecalis*, have been studied (Li et al. 2018, Oyama et al. 2017), but no clear SGT correlation was shown. Thus this study established the SGT correlation for three gram-negative and three gram-positive bacteria, and their results were shown in Fig. 1. We could readily monitor their growth curves using the 96-well microplate reader and establish their SGT correlations for all the gram-positive and gram-negative bacteria. For the gram-negative bacteria, *E. coli*, *P. aeruginosa*, and *S. oneidensis* (Fig. 1b, 1d, and 1e), their calibration curve between the SGT values ($O.D._{600nm}$ threshold of 0.15) and the serial dilution factors were highly linear with a correlation coefficient R^2 above 0.998. Although our study used different strains of *E. coli* (strain K12) and *P. aeruginosa* (strain BCRC 11078), the results of high linearity were consistent with the reported linearity (R^2 above 0.994) in the previous study (Hazan et al. 2012).

Similarly, the gram-positive bacteria, including *B. subtilis*, *Ent. Faecalis*, and *S. xylosus* (Fig. 1a, 1c, and 1f), also presented the correlation of high linearity (R^2 above 0.996) between their serial dilution factors and the SGT values. Our results demonstrated that the SGT correlation is readily established for both gram-negative and gram-positive bacteria between the range of dilution factor between 10^{-2} and $10^{-7,}$ and the initial cell density was around 10^8 to 10^9 cell mL-1. Slopes of the SGT correlations showed high stability within the six bacteria (Fig. 2a). Moreover, our results indicate that the SGT correlation is bacteria-dependent. Most of their SGT slope values were around -0.5 (-0.43 to -0.55), but only a more negative SGT slope was obtained for the *Ent. faecalis*. The result speculated that the SGT slope is associated with the specific growth rate (μ_{max}). Although linearizing their SGT slope values and μ_{max} had a low correlation coefficient, we observed that the *Ent. faecalis* had a μ_{max} that was twice higher than those of the other bacteria in our study (data not shown). The relatively high μ_{max} of *Ent. faecalis* have also been observed previously, compared with those of *E. coli* and *Lactobacillus plantarum*, S. aureus, *Streptococcus mutans*, *Serratia marcescens*, and *Klebsiella oxytoca* (Konopacki &Rakoczy 2019, Wilson et al. 2013). The intercept values of the SGT correlation are supposed to represent the cell density or dilution factor where the SGT value was zero, which was less meaningful for evaluation.

The same SGT correlation established with bacterial cells collected from the early stationary phase was shown in Fig. S1. We can successfully establish the SGT correlations with high correlation coefficients (all R^2 above 0.979). However, we only had to increase the dilution factor one order higher (10^{-3} to 10^{-8}) because the cell inoculum at the stationary phase was denser than that at the middle exponential phase. The effect of inoculation with cells from different growth phases on the SGT correlation establishment was shown in Fig. 3. Our results reveal that, for most of the examined bacteria, the slope value of the SGT correlations has no significant difference. Only the *S. oneidensis* showed a slight increase of its SGT correlation slope value from -0.49 to -0.39 (p-value < 0.001) while using cells from the stationary phase as the inoculum for the SGT method. The *Shewanella* sp. has been recognized as the skillful bacteria in stress tolerance (Le Laz et al. 2016, Tseng et al. 2018, Wang et al. 2016). Beg et al. (2012) have demonstrated that *S. oneidensis* strain MR-1 has a noticeable shift of transcriptional profiles from cells at the exponential phase to the early stationary phase. Most of the changing gene expression is involved in growth-dependent activities, such as ATP biosynthesis, aminoacyl-tRNA biosynthesis, and amino acid metabolic process. Bouillet et al. (2017) also elucidated that *S. oneidensis* strain MR-1 has a specialized mechanism that allows bacterial adaptation in versatile environments by protecting its σ^S factor from proteolysis, which enables rapid gene activation by the σ^S factor as the cells enter the early stationary phase. The sensitive characteristics of *S. oneidensis* to sensing the growth environment may vary its subsequent growth rate, thus affecting the corresponding SGT correlation establishment. Again, although the intercept values of *Ent. faecalis*, *S. oneidensis*, and *S. xylosus* were significantly decreased with cells at the stationary phase as the inocula, the intercept values showed no strong correlation and tendency between the six bacteria. Consequently, the SGT method is applicable and capable of applying to both gram-positive and gram-negative bacteria. However, a consistent procedure, including the preculture

duration of inoculum harvesting and constant dilution factors, can guarantee the stability of the established SGT correlation.

3.2 The effect of live-to-dead cell ratio

As our previous examination, it used to establish the SGT method by a 10-fold serial dilution of fresh bacterial liquid culture for simulating the same principle of quantitative PCR (Hazan et al. 2012). However, assessing ecotoxicity mainly focuses on the definition of EC50 or half-maximal inhibitory concentration (IC50) in mixed culture in the company of live and dead cells, which is not intuitional to be defined through the current SGT method. Therefore, we examined the effect of different live-to-dead cell ratios on establishing the SGT correlation to the six bacteria. We prepared the dead cell by mixing with the 70% isopropanol for 1 hr. The viability results were presented in Fig. S2, which suggests a complete deactivation where no cell growth was observed. Our experiments set up the expected live-to-dead cell ratios by 100:0, 75:25, 50:50, 25:75, and 1:99, and Fig.S3 to Fig. S8 present verification of their exact mixing ratios by flow cytometry. By detecting the height signals of FSC and SSC and presenting them in the contour plot, the high-density regions among the contour plot were easily distinguishable for live and dead cells, and only very slight points outside of the contour region were overlapped. The other study also observed a clear shift of the SSC signals after bacterial cells were treated with isopropanol (Chen &Li 2005). However, the live positive and dead negative samples still had cells (from 0.9–21.1%) in the opposite region. Therefore, evaluation of the following results that are involved in the live-to-dead cell ratios would be applied by the exact ratios determined via the flow cytometry.

The six bacterial cell cultures with a total cell density of approximately 2×10^7 cells mL^{-1} and various live-to-dead cell ratios were used for their SGT correlation establishment. Fig. 4 elucidates the slope and intercept results of their SGT correlations. With different amounts of dead cells, slopes of the established SGT correlation (Fig. 4a) have no significant difference between each live-to-dead cell ratio. The slope values of *Ent. faecalis* ranged from -0.65 to -0.71 and were still much negative than the other bacteria (-0.42 to -0.59). The intercept values were gradually increased along with the dead cell percentage. However, they could not get a good positive correlation between each other in *E. coli*, *Ent. faecalis*, and *S. xylosus* (data not shown). Interestingly, using the following non-linear exponential equation (Eq 1) can adequately stimulate the correlation of each exact live-cell ratio (*x variable*) and its corresponding SGT value (*y variable*) for every diluted culture (dilution factor of 10^{-1} to 10^{-4}) (Fig S9).

$$y = a + b * e^{-kx} \text{ (Eq 1)}$$

Table 1 listed their fitting constants and correlation coefficients in detail. From the results, successful fitting by using the non-linear exponential curve was irrelevant to either the type of bacteria or the SGT set-up dilution factor. Most curve fitting can reach an extremely high correlation coefficient (R^2) above 0.99, but only the R^2 of *S. oneidensis* and *S. xylosus* were relatively low to around 0.97. The imperfect distinction between the live and dead cells, defined from the quadrant gating regions, may cause a decrease in their correlation coefficient. However, their correlation of the SGT values and the live cell ratios still followed the same trend of the exponential equation. The non-linear curve of the exponential

equation has also been used to present the growth condition of *Clostridium difficile* in the previous study (Courson et al. 2019). Similarly, the equation helps model the growth condition of Salmonella enterica serovar Typhimurium (Chong et al. 2019). Consequently, the results demonstrate that establishing the correlation between the SGT value and the live cell percentage by using the non-linear exponential equation provides an alternative calculation option to facilitate using the SGT method for ecotoxicity assessment.

Table 1
Non-linear exponential curve fitting results between the SGT values and the exact live cell ratios for different diluted cultures.

Bacteria	SGT dilution factor	non-linear exponential correlation ($y = a + b * e^{-kx}$)	
		Constant (k)	Correlation coefficient (R^2)
B. subtilis	10^{-1}	0.0393 ± 0.0023	0.9986
	10^{-2}	0.0399 ± 0.0023	0.9986
	10^{-3}	0.0330 ± 0.0030	0.9969
	10^{-4}	0.0378 ± 0.0032	0.9970
E. coli	10^{-1}	0.0366 ± 0.0047	0.9942
	10^{-2}	0.0401 ± 0.0009	0.9998
	10^{-3}	0.0385 ± 0.0058	0.9917
	10^{-4}	0.0192 ± 0.0042	0.9919
Ent. faecalis	10^{-1}	0.0473 ± 0.0060	0.9925
	10^{-2}	0.0483 ± 0.0015	0.9996
	10^{-3}	0.0489 ± 0.0034	0.9978
	10^{-4}	0.0415 ± 0.0051	0.9932
P. aeruginosa	10^{-1}	0.0381 ± 0.0034	0.9971
	10^{-2}	0.0361 ± 0.0036	0.9967
	10^{-3}	0.0313 ± 0.0030	0.9972
	10^{-4}	0.0313 ± 0.0033	0.9965
S. oneidensis	10^{-1}	0.1178 ± 0.0197	0.9868
	10^{-2}	0.1096 ± 0.0251	0.9745
	10^{-3}	0.0896 ± 0.0055	0.9980
	10^{-4}	0.1179 ± 0.0179	0.9891
S. xylosus	10^{-1}	0.0414 ± 0.0083	0.9851

Bacteria	SGT dilution factor	non-linear exponential correlation ($y = a + b * e^{-kx}$)	
		Constant (k)	Correlation coefficient (R^2)
	10^{-2}	0.0604 ± 0.0140	0.9795
	10^{-3}	0.0760 ± 0.0208	0.9768
	10^{-4}	0.0843 ± 0.0237	0.9791

3.3 Experimental validation of copper ion ecotoxicity to *E. coli*

To validate the application of the SGT method in non-linear exponential correlation for ecotoxicity assessment, we selected Cu^{2+} ion as the toxicant to evaluate its acute toxicity to *E. coli* at the dosage concentration of 1.0, 2.0, 2.5, 3.0, and 4.0 mM. Again, four dilution factors from 10^{-1} to 10^{-4} were applied to measure their SGT. Then we substituted the measured SGT values into the exponential equations we established in Fig. S9b to get the corresponding live cell percentage. The assessment results were shown in Fig. 5. For the four dilution factors, adding Cu^{2+} ions to the concentration above 1.0 mM started to have a toxic effect on *E. coli*. The linear interpolation to the live-to-dead cell ratio of 50:50 reflects a predicted EC50 by the Cu^{2+} ions at 2.27 ± 0.04 mM. Various ecotoxicity assessment methods were applied to determine the EC50 of Cu^{2+} ions to *E. coli*, including electrochemical biosensor, resazurin reduction bioactivity, β-galactosidase activity, reporter gene luminescence, and cell growth. The EC50 defined by the different methods has distinct results, where metabolism-based methods have a sensitive EC50 less than 1.0 mM. For instance, the Cu^{2+} ecotoxicity to *E. coli*, determined by the electrochemical biosensor, has a predicted EC50 at 0.1 mM to 0.3 mM (Fang et al. 2016, Wang et al. 2008). Moreover, determination by the *E. coli* whole cell-based electrochemical biosensor would obtain a higher EC50 concentration for Cu^{2+} where a dose of 0.15 mM only had an inhibitory effect of less than 20% (Gao et al. 2016). The Cu^{2+} ecotoxicity determined via the *E. coli* β-galactosidase activity depends on the composition of the liquid medium, which would change the estimated EC50 ranging from 0.3 to 1.9 mM (Choate et al. 2008). By applying the resazurin reduction of *E. coli* for the Cu^{2+} ecotoxicity, its predicted EC50 was about 0.2 mM. Our predicted EC50 was similar to those reported EC50 by applying the growth- or cell-count-based methods for determination. Jo et al. (2004) obtained an estimated acute EC50, ranging from 2.0 to 2.5 mM by culturing the *E. coli* in Luria Bertani medium and another EC50 of 2.2 mM after exposing the *E. coli* cells with Cu^{2+} ions for 12 hr. Deryabin et al. (2013) used *pSoxS::lux* and *pRecA::lux* reporter gene system for Cu^{2+} ecotoxicity determination and observed the 50% luminescence reduction at 1.2 mM and 2.3 mM, respectively. Using the SGT method accompanied by the non-linear exponential correlation and selecting an appropriate dilution factor (such as 10^{-1} or 10^{-2}), determination of the bacterial ecotoxicity requires less than 6 to 7 hr to finish the test.

4 Conclusions

A high-throughput and alternative method, called Start Growth (SGT) Time method, was investigated for bacterial ecotoxicity assessment based on simply monitoring bacterial growth. Our results demonstrate that the SGT correlation can be readily established for gram-positive and gram-negative bacteria, including *Bacillus subtilis*, *Staphylococcus xylosus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Shewanella oneidensis*. However, a consistent procedure, like controlling the growth phase, to prepare inoculum for the SGT method can improve correlation establishment stability. The existence of dead cells together would not affect the reproducibility and stability of establishing the SGT correlations, thus increasing its applicability for ecotoxicity assessment. Besides, the SGT value and the corresponding live cell percentage fit the non-linear exponential correlation. Experimental validation in this study demonstrates that the SGT exponential correlation is helpful for bacterial ecotoxicity assessment with advantages of broad applicability, time-conserving capability, and high stability.

Declarations

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Ethical Approval and Consent to Participate

Not applicable

Consent to Publish

All authors mutually agreed to publish the work in this journal.

Authors Contributions

Siang Chen Wu: Conceptualization, Methodology, Formal analysis, Validation, Data Curation, Writing, Visualization, Supervision, Project administration

Chang-Chun Shih: Investigation, Data Curation.

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Competing Interests

The authors declare that they have no conflict of interest

Availability of data and materials

Not applicable

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Figures

Figure 1

The linearity of SGT correlations was established in the six bacterial strains while their cells were collected at the middle exponential phase for **(a)** *B. subtilis*; **(b)** *E. coli*; **(c)** *Ent. faecalis*; **(d)** *P. aeruginosa*; **(e)** *S. oneidensis*; **(f)** *S. xylosus*. The main figure represents growth curves of bacterium in different diluted cultures, the time when the growth curves crossed the threshold of $O.D._{600nm}$ in 0.15 was defined as their SGT. The subplot within the main plot expresses the linear regression and correlation coefficient (R^2) of the SGT values (hr) and cell density (cell mL^{-1}) in a logarithmic scale.

Figure 2

Comparison in the **(a)** SGT slope and the **(b)** SGT intercept of the SGT correlations from the six bacteria established from the results of Fig. 1.

Figure 3

Comparison in the **(a)** SGT slope and the **(b)** SGT intercept of the SGT correlations from the six bacteria established from cells collected from the middle exponential (bacteria_expo) and the early stationary (bacteria_stat) phases. The SGT correlations from cells of the early stationary phase were listed in the Fig. S1.

Figure 4

Comparison in the **(a)** SGT slope and the **(b)** SGT intercept of the SGT correlations from the six bacteria established from various expected live-to-dead cell ratios as 100:0; 75:25; 50:50; 25:75; and 1:99.

Figure 5

Observed SGT values of various *E. coli* diluted cultures **(a)** 10^{-1}; **(b)** 10^{-2}; **(c)** 10^{-3}; and **(d)** 10^{-4}; with the addition of Cu^{2+} ions at the concentration of 1.0, 2.0, 2.5, 3.0, and 4.0 mM. The red vertical lines indicate the live-to-dead cell ratio of 50:50 with annotations of linear interpolation for the corresponding Cu^{2+} ionic concentrations.

Supplementary Files

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