Experimental validation of stability and applicability of Start Growth Time method for high-throughput bacterial ecotoxicity assessment

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Abstract
Ecotoxicity assessments based on bacteria as model organisms are widely used for routine toxicity screening because it has the 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 requirements, but traditional viable cell count methods are relatively labor- and time-intensive. The Start Growth Time 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 in 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 h to finish the assessment and obtain 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.

Keywords Ecotoxicity assessment · Start Growth Time method · Bacterial growth · High-throughput · Effective concentration · Flow cytometry

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 synergistic 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 and Simon 2006, Wang et al. 2015; Yao et al. 2018). In contrast to multi-cellular eukaryotic organisms, bacteria have rapid rates of growth. Ecotoxicity assessment using bacteria as model organisms is easily applicable for widely and routinely toxicity screening because it has the advantages of relatively short assay time, high sensitivity and 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. The bioluminescent signal is subsequently determined after the bacterial cells are exposed to the target chemicals for 15 or 30 min. The amount of the target chemicals to cause a 50% luminescence inhibition is called the median effect concentration (EC$_{50}$) (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. 2016), 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 the toxicity response of regulation concerning chemicals to prospective bioremediation bacteria (Kang and 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 most straightforward method and is applicable for these purposes (Baek and An 2011, Giri and 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. Although the colony counting method can be improved by preparing the agar layer on a specialized complementary metal–oxide–semiconductor (CMOS) sensor, the number of colonies can be determined at a very early growth stage via automatically microscopic monitoring (Jung and Lee 2016). However, the microscopy device can only monitor one sample at a time. On the other hand, few novel techniques to use varied pH or electrical conductivity signals as bacterial cell growth changes have been established (Chandra and Singh 2018; Zhang et al. 2018). These methods have the advantages of high sensitivity and accuracy than traditional optical signals. However, preparation of the detection platform requires a more complicated but low throughput analysis capacity. Alternatively, a Start Growth Time (SGT) method for high throughput determination of viable bacterial cell counts in 96-well plates has been established previously (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 h 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 h. 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 still 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). Only two studies applied 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 SGT method’s applicability and stability when applied to gram-positive and gram-negative bacteria. Besides, the applicability of the SGT method as an alternative ecotoxicity assessment method was also detailed and 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, the flow cytometry measurement prepared various live and dead cell mixture ratios were prepared and verified. 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.

**Materials and methods**

**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 30 °C. *S. xylosus, E. faecalis,* and *E. coli* were grown at 37 °C 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 its growth temperature overnight with agitation at 120 rpm. We transferred the precultured cells to 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.

**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 tenfold 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.\(_{600}\)). 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.\(_{600}\) 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 s\(^{-1}\). 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 \(10^{-8}\).

**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 8000 \(\times\) g for 10 min. The cell pellet was resuspended in 70% isopropanol for 1 h (Kaprelyants and Kell 1992, Pascaud et al. 2009) to kill the cell. After removing the 70% isopropanol, the dead cells were washed twice with 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 \(2 \times 10^{7}\) 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 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 $10^{-4}$.

**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 $2 \times 10^7$ 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$_4$·5H$_2$O 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$_{600}$ nm threshold of 0.15.

**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 a non-linear exponential curve. One-way ANOVA compared means between each condition with a post hoc Tukey test. Stars shown in figures denote 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).

**Results and discussion**

**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., *B. megaterium* and *E. 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 are shown in Fig. 1. We could readily monitor their growth curves using the 96-well microplate reader and establish their SGT correlations (subplots in Fig. 1) for all the examined gram-positive and gram-negative bacteria. For the gram-negative bacteria, *E. coli*, *P. aeruginosa*, and *S. oneidensis* (Fig. 1b, d, and e), their calibration curve between the SGT values (O.D$_{600}$ nm threshold of 0.15, the horizontal line placed in Fig. 1) and the serial dilution factors were highly linear with a correlation coefficient $R^2$ above 0.998 (listed in each subplot). 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*, *E. faecalis*, and *S. xylosus* (Fig. 1a, c, and f), 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 factors 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. Our results indicate that the SGT correlation is bacteria-dependent. Most of their SGT slope values were around $-0.5$ ($-0.43$ to $-0.55$) (Fig. 2a), but only a more negative SGT slope ($-0.7$) was obtained for the *E. faecalis*. The result speculated that the SGT slope is associated with the bacterial specific growth rate ($\mu_{max}$). We observed that the *E. faecalis* had a $\mu_{max}$ that was twice higher than those of the other bacteria in our study (data not shown). The relatively high $\mu_{max}$ of *E. faecalis* has also been observed previously, compared with those of *E. coli* and *Lactobacillus plantarum*, *S. aureus*, *Streptococcus mutans*, *Serratia marcescens*, and *Klebsiella oxytoca* (Konopacki and Rakoczy 2019; Wilson et al. 2013). The intercept values of the SGT correlation (Fig. 2b) are supposed to represent the cell density or dilution factor where the SGT value was 0, however, it was hard to find any correlation against the SGT intercept.

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 (Fig. 3a). Only the *S. oneidensis* showed a slight increase in 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. *Shewanella* sp. has been recognized as a 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 rapidly activating gene expression by the σ^5_5 factor at 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 (Fig. 3b) of *E. 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. From the above results, the SGT method is applicable to both gram-positive and gram-negative bacteria. However, a consistent procedure, including the preculture duration of inoculum harvesting and constant dilution factors, would
guarantee the stability of the established SGT correlation. The SGT stability was established on a single bacterium at a time. SGT application for assessing multiplexed and mixed culture still may not be possible to determine the correlation. Methods by applying fluorescent nanosensors would be an alternative method for mixed culture detection (Yin et al. 2019).

The effect of live-to-dead cell ratio

As our previous examination, the SGT method used to be established by a tenfold 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 a live and dead cell mixed culture, which is not intuitional to be defined through the standard SGT method. Therefore, we evaluated the effect of different live-to-dead cell ratios on establishing the SGT correlation to the six bacteria. The viability confirmation was presented in Fig. S2, which suggests a complete deactivation where no cell growth was observed for the prepared dead cells. We 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 showed verification of their exact mixing ratios analyzed 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 treated bacterial cells with isopropanol (Chen and Li 2005).

Fig. 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

Fig. 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 Fig. S1
However, the 100% live or 100% dead positive samples still had cells (from 0.9 to 21.1%) (i.e., Fig. S7) in the opposite region. Therefore, evaluation of the following SGT results would be correlated with the exact ratios determined via the flow cytometry.

The six bacterial cell cultures with a total cell density of approximately $2 \times 10^7$ cells mL$^{-1}$ and various live-to-dead cell ratios were used for their SGT correlation establishment. Figure 4 elucidates the slope and intercept results of their SGT correlations determined by the same data analyzing procedure as shown in Fig. 1 (data not shown). With different live and dead cell mixing ratios, slopes of the established SGT correlation (Fig. 4a) have no significant difference between each live-to-dead cell mixing ratio. The slope values of *E. faecalis* ranged from $-0.65$ to $-0.71$ and were still much more 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 against the dead cell percentage in *E. coli*, *E. 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).

$$y = a + b \cdot e^{-kx}$$  \hspace{1cm} (1)

Table 1 lists 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 (six bacterial strains) or the SGT set-up.
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 \( (y = a + b \times e^{kt}) \) |
|-------------------|---------------------|---------------------------------------------------------------|
|                   | 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                                  |
| E. 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                                  |
|                   | 10^{-2}             | 0.0604 ± 0.0140                                             | 0.9795                                  |
|                   | 10^{-3}             | 0.0760 ± 0.0208                                             | 0.9768                                  |
|                   | 10^{-4}             | 0.0843 ± 0.0237                                             | 0.9791                                  |

dilution factor (from 10^{-1} to 10^{-4}). Almost all 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 around 0.97. The imperfect distinction to define live and dead cells may be the major reason to 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 using the non-linear exponential equation provides an alternative calculation option to facilitate using the SGT method for ecotoxicity assessment. By applying the alternative calculation with the SGT method for ecotoxicity assessment, interesting toxic chemicals can be readily examined for their toxicity by adding them to the culture medium the evaluation concentrations. Starting with a relatively low dilution factor, such as 10^{-1}, can reduce the assessment duration to less than 2 to 4 h. The improvement makes the SGT method a moderate time-saving but high cost-effective ecotoxicity assessment method than others (Dalzell et al. 2002; Ngoc et al. 2021).

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 are 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 EC_{50} by the Cu^{2+} ions at 2.27 ± 0.04 mM. Various ecotoxicity assessment methods were applied to determine the EC_{50} of Cu^{2+} ions to E. coli, including electrochemical biosensor, resazurin reduction bioactivity, β-galactosidase activity, and reporter gene luminescence, and cell growth. The EC_{50} defined by the different methods has distinct results, whereas metabolism-based methods have a sensitive EC_{50} of less than 1.0 mM. For instance, the Cu^{2+} ecotoxicity to E. coli, determined by the electrochemical biosensor, has a predicted EC_{50} 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 EC_{50} 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 EC_{50} 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 EC_{50} was about 0.2 mM. Our predicted EC50 was similar to those reported EC_{50} by applying the growth- or cell-count-based methods for determination. Jo et al. (2004) obtained an estimated acute EC_{50}, ranging from 2.0 to 2.5 mM by culturing the E. coli in a Luria Bertani medium and another EC_{50} of 2.2 mM after exposing the E. coli cells with Cu^{2+} ions for 12 h. 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 $10^{-2}$), the determination of the bacterial ecotoxicity requires less than 6 to 7 h to finish the test. Except by adding the interesting toxic chemicals directly to the culture medium, the effect of diluting culture medium for assessing the toxicity of real aqueous samples on the bacterial cell growth and stability of SGT method is still unclear. More investigations for understanding the impact of mixing the actual environmental or food samples for ecotoxicity assessment are required in the future.

Conclusions

A high-throughput and alternative method, called Start Growth Time (SGT) 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.
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Chang-Chun Shih: Investigation, Data Curation.

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Declarations

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