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Use of Doubling Number as an Arithmetic Measure of Plant Cell Growth and Metal-Induced Cell Growth Inhibition

Mert METIN*, Tomonori KAWANO

Graduate School of Environmental Engineering, The University of Kitakyushu, 1-1 Hibikino, Wakamatsu, Kitakyushu, Fukuoka 808-0135, Japan

Highlights

- In this study, methods of the calculation of cell growth and growth inhibition were discussed.
- The topic is not a new issue but this article is a good update for studying cell/tissue growth.
- Differently from other studies, here, we also calculated day by day growth performance of cells.
- Based on that, we suggested that Cd metal disrupts the cell cycle of tobacco BY-2 cells.

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Abstract

Cell growth inhibition is generally handled as a measure of toxicity. Shortly, more toxicity implies more growth inhibition. Then, the question arises; How to calculate & evaluate cell growth inhibition in a universal manner? Actually, the method for calculating growth inhibition is not considered to be a central issue, in general. There are various approaches (subtractive, divisionary, and logarithmic) for calculating cell growth. Among these approaches, two of them are highly easy and popular, subtraction-based and division-based calculations. However, these two methods for the calculation of cell growth do not strongly reflect the nature of cell growth. Alternatively, the use of a doubling number-based formulation can provide a better approach and performance in the evaluation of cell growth and cell growth inhibition unless the culture attains the confluent status. Here, we discussed different methods of growth calculation which we applied to the study of "growth inhibition of BY-2 cells under Cd exposure".

1. INTRODUCTION

Cell growth usually refers to cell proliferation. Cell proliferation is the process in which each cell grows in size and then divides to produce two daughter cells. The repetition of these processes leads to an exponential increase in cell number (equivalent to the Malthus curve in population dynamics). Cell growth requires both an increase in cell size and cell division to occur in the same period, therefore the average size of cells remains constant as the population increases. There are two popular approaches employed to calculate growth. One of the approaches is "subtraction-based calculation" [1-3] while the other one is "division-based calculation" [1,4,5]. These two approaches are very easy to account and popular. Here, we provided the formulation of both approaches for the evaluation of the growth of plant cells using tobacco cell suspension culture, as a model.

1.1. Theoretical Background for Arithmetic Evaluation of Cell Growth Inhibition

1) Subtraction-based calculation simply defines the growth as follows:
Growth = Final Cell Density - Initial Cell Density; or $G_{sub} = D_t - D_0$

2) Division-based calculation emphasizes the ratio of growth as follows:
Growth = Final Cell Density / Initial Cell Density; or $G_{div} = D_t / D_0$

*Corresponding author, e-mail: mertmetin3335@gmail.com

In growth inhibition kinetics, these two approaches are handled to score the impact of growth limiting factors. Then, the growth of each sample is often evaluated relative to the growth of the control (as a ratio). In this way, the relative growth of samples compared to the growth of the control is worked out through simple division, thus, it can be expressed as the growth relative to the control where D_0 is the "Initial Density" of the cell culture and D_t is the "Final Density". Or the changes in growth can be expressed as the percentage of control, i.e. G (%).

1) Subtraction-based calculation defines the G (%) as follows:

G (%) = 100 x Growth of Sample / Growth of Control; or Grelative = 100 x (G_i / G_{cont})
= 100 x (D_t of Sample – D_0 of Sample) / (D_t of Control – D_0 of Control);
or 100 x ($Dt_{.i}$ – $D_{0.i}$) / ($D_{t.cont}$ – $D_{0.cont}$)

2) Division-based calculation elucidates the G (%) as follows:

G (%) = 100 x (Growth of Sample / Growth of Control); or 100 x (G_i / G_{cont})
= (D_t of Sample / D_0 of Sample) / (D_t of Control / D_0 of Control);
or 100 x ($D_{t.i}$ / $D_{0.i}$) / ($D_{t.cont}$ / $D_{0.cont}$)

Cellular growth is driven by cell division which is actually a doubling event. An immature cell grows firstly in size and then divides into two smaller cells. This process continues as long as the cellular environment meets "good" conditions. As a result of cell growth, cellular fresh mass increases in parallel, so it is basically doubled, too. In the case of cell cultures, the change in cell density is a measure of cell growth. In cultures of suspension cells, cell growth depends on a variety of factors, including initial cell density, type of cells, the ratio of live cells over total cellular population in initial culture, composition and size of the growth medium, the abundance of medium nutrients, and so on [6]. As the cells continue to grow in a cell culture medium, cell density exponentially increases. However, this exponential growth slows down in the late phase mainly due to a decrease in medium nutrient content. The phase where cells show rapid growth is called as "exponential phase" whereas the late phase of cell growth with a heavily slowed growth rate is referred to "stationary phase". In general, cells from the exponential phase are preferred for most biological experiments [7-11].

We have handled the kinetics of the plot of algal cell growth based on the logistic kinetics; dN / dt = r x N x [(K – N) / K] with and without the Allee effect [12-14]. In addition, the impacts of growth inhibition metals were assessed by applying the above logistic models. However, for drastic simplification, we can mathematically simplify and summarize the cell growth in terms of its doubling characteristics, specifically in a time period corresponding to the exponential phase, by focusing on the number of doubling events (N) and D_0 with high living/nonliving cell ratio as follows:

D_0 x 2 x 2 x 2 x 2 x 2 x 2 (N times) …. = D_t
D_0 x 2N = Dt
$2N = D_t / D_0$
$\log_2 (2N) = \log_2 (D_t / D_0)$
$N = \log_2 (D_t / D_0)$.

Here N = 1 means D_0 is doubled whereas N = 0 means no change, and N < 0 signs that D_t is lower than D_0. This formulation better reflects the reality when the living/nonliving cell ratio of D_0 is very high, thus, D_0 is almost identical to the density of living cells. If nonliving cells in D_0 increase, the apparent division capacity of cell culture looks lowered. So, a lower living/nonliving cell ratio refers to a lower N and it is also true vice-versa. Thus, one can talk about the division capacity of a cell culture via the elucidation of N. When N is divided by the time period of investigation (for instance 7 days), the average doubling speed (ADS, doubling per day) can be obtained. If the time period investigated is divided by N, the average doubling time (ADT, how many days are needed to double the density) can be worked out. ADS and N are directly proportional while ADT and N are reversely proportional. Here are the formulations for ADS and ADT:

$ADS = N / t,$
$ADT = t / N .$

In this binary logarithmic calculation, N represents the capability of cell division. Therefore, ADT and ADS can be reliable measures of cell growth. The binary logarithmic calculation for G (%) can be defined as below:

$G\ (\%) = 100 * (N_i / N_{cont})$.

Alternatively, G (%) can also be calculated with the help of ADT and ADS:

$G\ (\%) = 100 * (ADT_{cont} / ADT_i)$,
$= 100 * (ADS_i / ADS_{cont})$

In fact, the binary logarithmic calculation is merely a simple estimation of the number of doublings. So, it is not a novel suggestion. Similar to the binary logarithmic approach shown here, the OECD guideline [15] proposes the use of the calculation of specific growth rate (SGR) as a measure of growth. According to OECD, $SGR = [\ln (D_t / D_0)] / t$, where t is the time period of the investigation. So, the only difference between OECD-suggested SGR and N is the base of the logarithm. However, the difference disappears when G (%) is calculated. In the case of the calculation of G (%), both approaches end up with the same result. Similar to the OECD-suggested approach, the binary logarithmic calculation can be applied only if there is exponential growth in the controls (or growth is close to an exponential pattern), no significant periods of lag (nor stagnancy) is observed, and the course of the growth curve is monotonous [15].

In contrast to the binary logarithmic calculation, conventional subtraction-based and division-based calculations designed to evaluate the extent of growth largely remain insufficient in some aspects. In the case of the subtraction-based calculation, the role of initial density (D_0) seems to be underestimated. Just oppositely to this, the role of initial density appears to be over-estimated in the division-based calculation. Actually, these approaches are not scoring the actual cell division which is the driving force for an exponential increase in cell density. The exponential increases cannot be sufficiently described by subtraction and division-based calculations. Distinctly, a logarithmic approach can provide a better insight into exponentially dynamic issues [15]. We think that the best way among the aforementioned methods to express cell growth is the binary logarithmic calculation ($N = \log_2 (D_t / D_0)$, emphasizing the doubling nature of cell growth.

There seem some other advantages of the binary logarithmic calculation over the other two (subtraction-based and division-based calculation). For example, samples which are started with different cell densities can be compared by this type of calculation. In addition, it also lets us compare the experiments carried out with the cell cultures whose division characteristics differ from each other. However, such comparison is limited only to superficial conclusions if there are significant disparities among the division characteristics of compared samples.

As one of the soft heavy metals, cadmium (Cd) is toxic to living. This metal can act on various cellular and physiological events [16,17]. Chlorosis and growth blockage are easily observable outcomes of Cd phytotoxicity [18]. Reduction in rate of photosynthesis and stomatal density, decreases in the mineral uptake and enzymatic activities, reduced seed germination, and low yield and low protein content in grain are among other results of Cd actions in plants [18]. Cytotoxicity of Cd is well-known to relate with the cellular thiols. Cd can strongly react with S-S (found in proteins) and S-H (found in antioxidants) groups to create proteotoxicity and ROS imbalance. These two factors are suggested to be prominent actors driving Cd-exposed cells to be driven into cell death [19-22]. In addition, Cd is also referred as a genotoxic element [23].

Here, we investigated the impact of cadmium on the growth of tobacco (Nicotiana tabacum) BY-2 cells. Specifically, we provided a sight for the calculation of the cell growth with & without Cd.

2. MATERIAL METHOD

Shortly, BY-2 cells were propagated weekly in the dark at 23°C. We used three different sources of cell cultures which were 3 days old, 7 days old and 9 days old. Experiments done with different sources of cell cultures are labelled according to the age of cells, 3DOE, 7DOE, and 9DOE. Cells were cultured in Murashige - Skoog (MS) medium [24] which was sterilized by autoclaving (121°C). 2 mL of 3 days old cells were added to 28 mL of MS liquid medium while 1 mL of 7 and 9 days old cells were added to 29 mL of MS liquid medium following the addition of Cd to the medium. 300 mM and 240 mM stock solutions of $CdSO_4$ (2.5 H_2O) were sterilized via micro-filters. Stock solutions were diluted with sterile water to produce lower molarities. Small amounts of Cd solutions (max. 100 µL) were added to cell culture media to produce final metal concentrations of 800, 100, 80, 50, 40, 20, 16, 10, 8, 4 and 1.6 µM (final culture volume is nearly 30 mL). By this way, we covered a wide range of Cd doses. 100, 80, 50, 40, 20, 16, and 10 µM doses were handled for 3DOE while 800, 80, 40, 16, 8, 4, and 1.6 µM doses were handled for 7DOE. Lastly, 800, 80, 40, 16, and 4 µM doses were used for 9DOE.

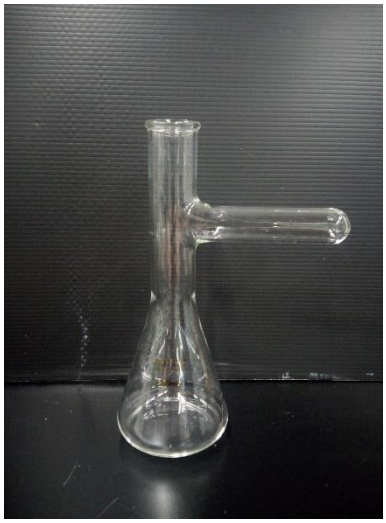

Figure 1. *Specially designed side-arm flask*

Special flasks, each with a side-arm (Figure 1), are designed for non-invasive optical monitoring of cell density (turbidity). In addition to facile optical measurement, these flasks permitted us to do experiments without opening the flasks and, thus, without disrupting the experimental system. Therefore, the experimental system was kept closed throughout the experimentation. Experiments were carried out at 28°C in the dark. The changes in cell density were monitored with McFarland Den-1B Densitometer.

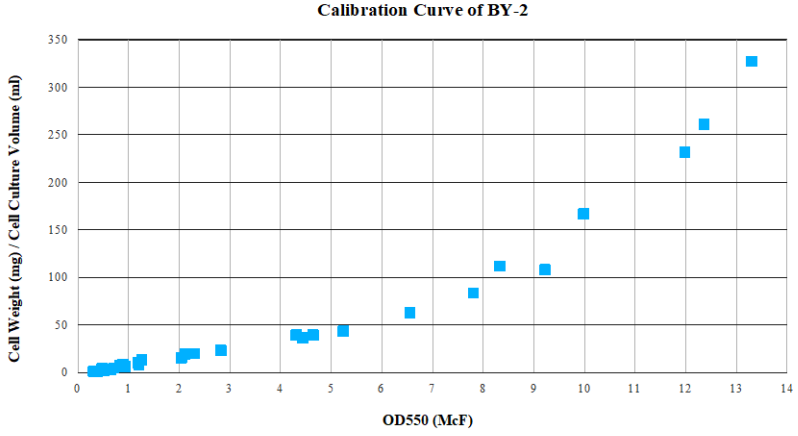

Figure 2a. *Calibration curve*

To convert the "McF" unit of the densitometer to the unit of cellular density, namely, "mg of cells per mL of cell culture", a calibration curve needed to be constructed. In the procedure of calibration construction, BY-2 cell suspension cultures were vacuum-filtrated to collect the cells. Then, known amounts of cellular mass were re-suspended in MS liquid media to reproduce 10 mL of cell suspensions. Following that, the densities of these model cell suspensions were measured with the densitometer. At the end of the process, we obtained a data set big enough to create a calibration curve (Figure 2a). It was decided to evaluate the data set in two parts, below 5.24 McF (Figure 2b) and above 5.24 McF (Figure 2c). After this dissection of the range, we obtained a pair of linear & exponential formulae as follows; $y = 8.477x$ for McF ≤ 5.24 (Figure 2b) and $y = 3.403x^2 - 29.371x + 107.308$ for McF ≥ 5.24 (Figure 2c).

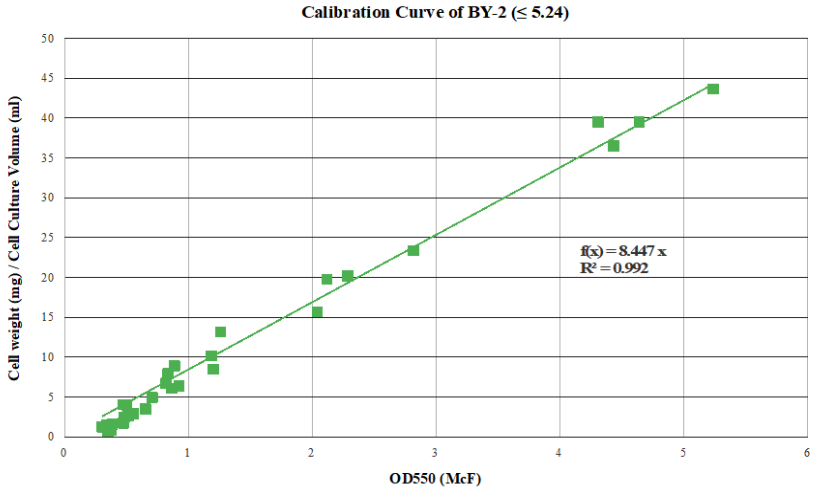

Figure 2b. *Calibration curve for McF ≤ 5.24*

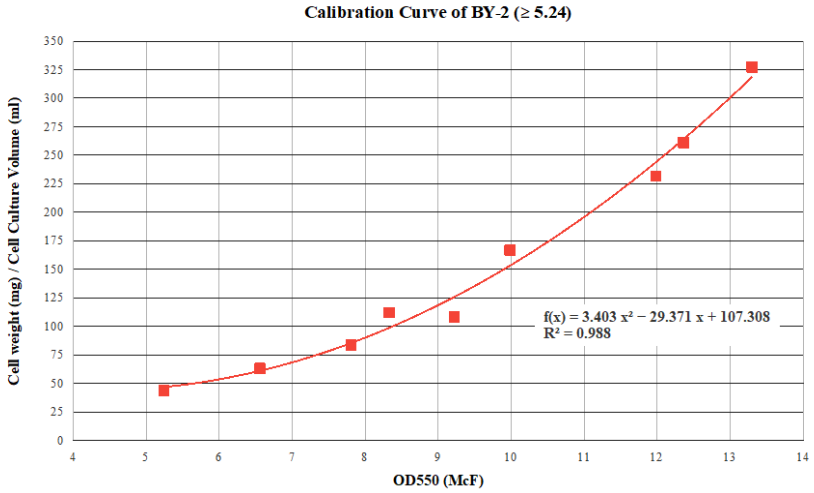

Figure 2c. *Calibration curve for McF ≥ 5.24*

To evaluate G (%), we mainly focused on the binary logarithmic calculation (Table 1). In addition, results obtained through the other two calculations are also provided in Figures 4a, 4b, and 4c for comparison of calculation approaches. We calculated G (%) for all three experiments for 0-7 days intervals. Furthermore, the daily doubling performance of samples of 3DOE (the only experiment which we continuously observed) is evaluated with the account of N for subsequent days (Figure 5).

3. RESULTS AND DISCUSSION

Formulae gained in Figures 2b and 2c are applied to the optically measured data which were in McF (Supplementary Data). Based on the converted data; "Cell Density vs Time" graphs (Figure 3a, 3b and 3c) could be created. What we clearly see from these three graphs is a dose-dependent growth inhibition by Cd. Moreover, in 3DOE, there seems a threshold dose for Cd to lower the carrying capacity (defined in the logistic model) of the BY-2 cell population. If there is a such threshold, it locates between 20 and 40 µM.

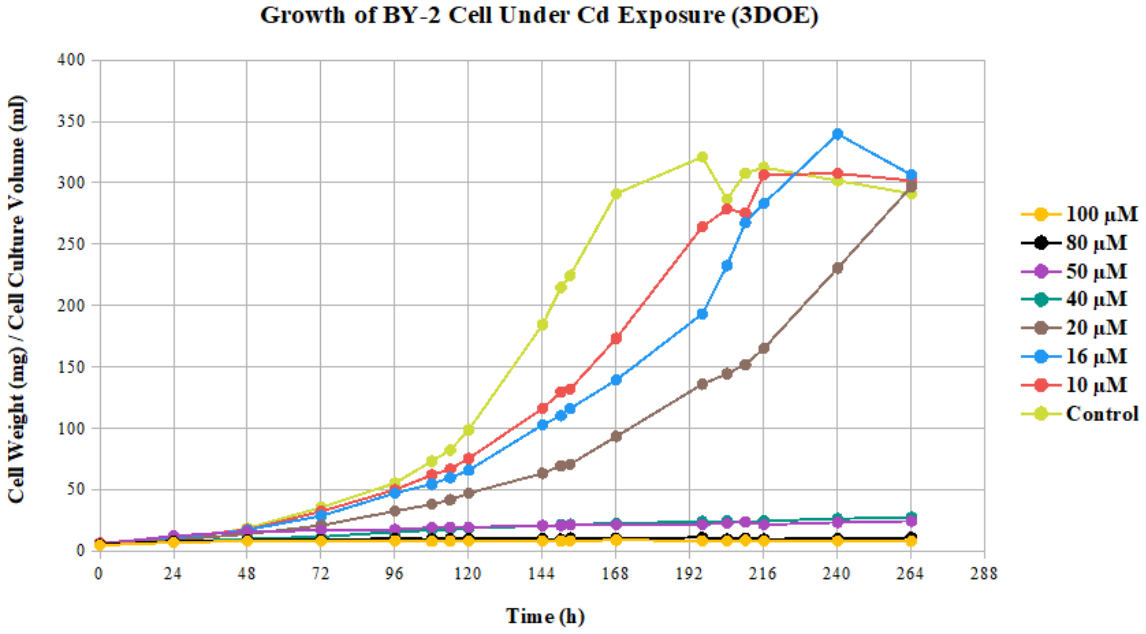

Figure 3a. *"Cell Density vs Time" graph belonging to 3DOE*

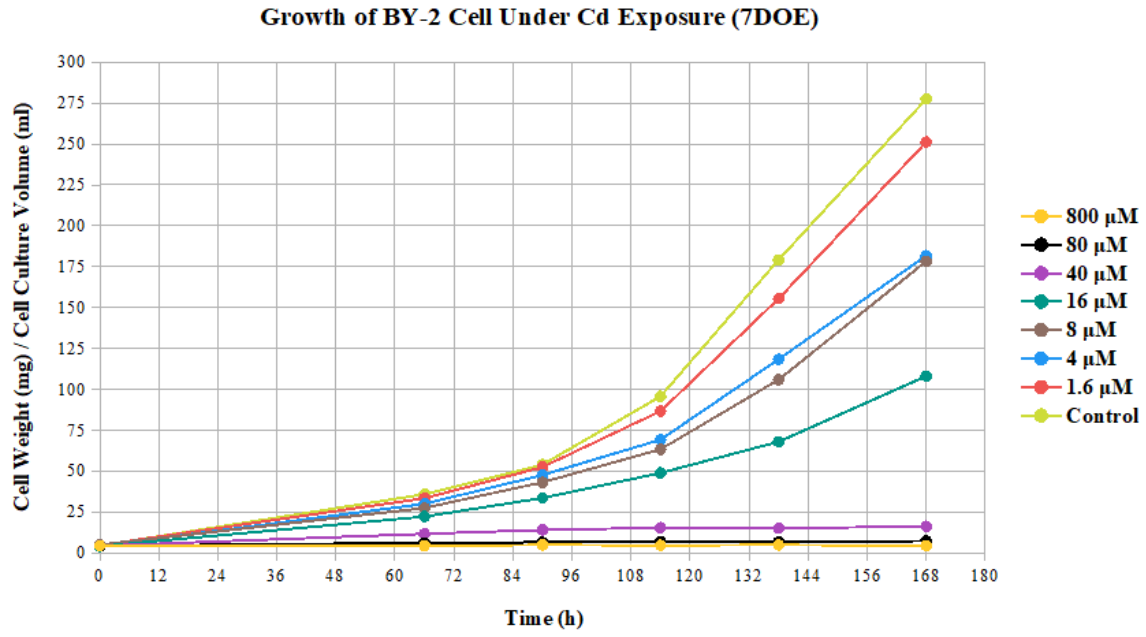

Figure 3b. *"Cell Density vs Time" graph belonging to 7DOE*

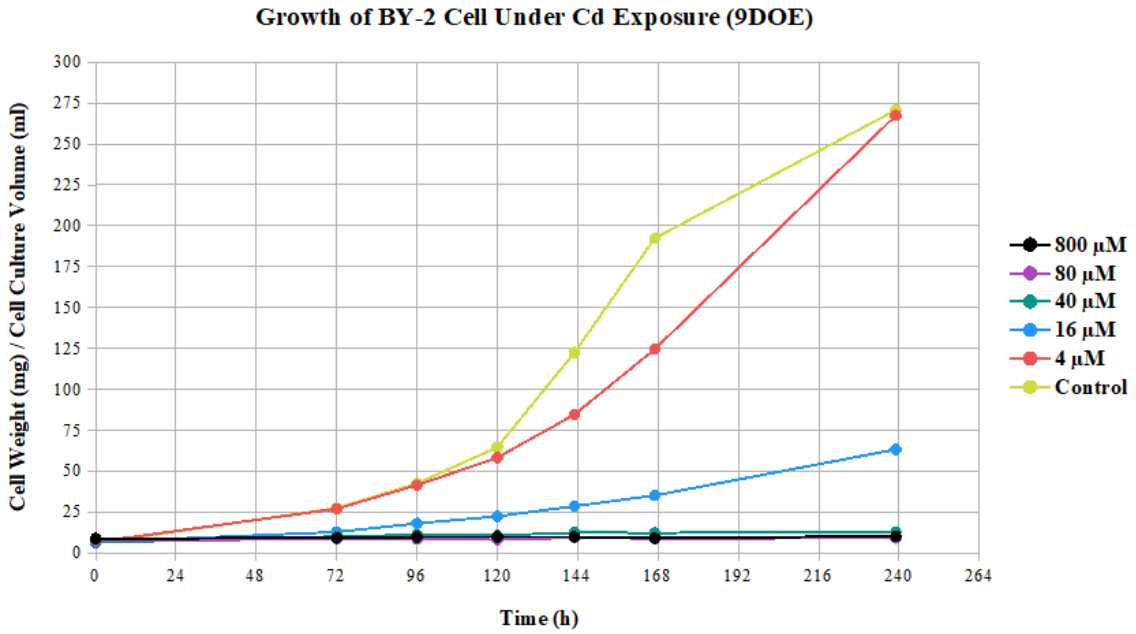

Figure 3c. *"Cell Density vs Time" graph belonging to 9DOE*

According to Table 1, N of 3DOE control and N of 7DOE control is somehow close to each other. However, the N of 9DOE control is the lowest among controls. This signs that the division capacities of well-aged 9DOE cells were lower than those of 7DOE and 3DOE. Therefore, it is assumed that 3DOE and 7DOE are directly comparable with each other while 9DOE is not directly comparable with others. In the case of the comparison of 3DOE with 7DOE, it can be observed that G (%) values are very close. If an example is given according to Table 1; While the 80 µM Cd Dose G (%) value was 17.88 in 3DOE, it is seen as 13.58 in 7DOE at the same dose. There appears only 4 % difference which could be even observed among the replicates of the same experiment. In three experiments, samples with 800, 100 and 80 µM Cd did not grow. 40 and 50 µM doses of Cd also significantly blocked the growth. Doses ≤ 20 µM slowed down the growth in a concentration-dependent manner. Growth inhibition was positively associated with Cd dose. As 9DOE superficially compared with others (3DOE and 7DOE), it is seen that the same doses of Cd more strongly acted on 9DOE cells. Conclusively, a cell population with lower division capacity is affected more strongly by the action of the metal.

Table 1. *Growth Inhibition Calculations by Binary Logarithmic Approaches*

Experiments	Cd Dose (µM)	D_0 (mg of cells per mL of cell culture)	D_t (mg of cells per mL of cell culture)	D_t / D_0	N (Doubling per 7 Days)	ADT (Days)	G (%)
3DOE	100	4.60	8.80	1.92	0.94	7.47	15.90
	80	5.05	10.49	2.08	1.05	6.64	17.88
	50	5.51	21.49	3.90	1.96	3.56	33.30
	40	4.70	22.32	4.75	2.25	3.11	38.12
	20	5.00	93.24	18.65	4.22	1.66	71.56
	16	5.73	139.33	24.33	4.60	1.52	78.06
	10	6.35	173.25	27.27	4.77	1.47	80.86
	0	4.88	291.22	59.65	5.90	1.19	100
7DOE	800	4.43	4.46	1.01	0.01	638.04	0.18
	80	4.16	7.30	1.76	0.81	8.62	13.58
	40	4.29	16.24	3.78	1.92	3.65	32.10
	16	4.02	108.11	26.89	4.75	1.47	79.39
	8	4.98	178.34	35.78	5.16	1.36	86.29
	4	4.22	181.78	43.04	5.43	1.29	90.74

	1.6	4.68	251.21	53.68	5.75	1.22	96.07
	0	4.39	277.49	63.18	5.98	1.17	100
9DOE	800	8.77	9.00	1.03	0.04	182.29	0.76
	80	7.32	8.80	1.20	0.27	26.23	5.32
	40	6.35	12.23	1.93	0.95	7.41	18.83
	16	6.57	35.16	5.35	2.42	2.89	48.20
	4	6.62	124.53	18.80	4.23	1.65	84.33
	0	5.93	192.37	32.44	5.02	1.39	100

3.1. Calculation Disparities

As shown in Figures 4a, 4b, and 4c, there is a tendency that the G (%) values gained through the binary logarithmic calculation (48.20 % in 16 µM dose (Supplementary Data)) to become greater than the G (%) values gained through conventional two types of calculations, namely, the values worked out through the division-based (16.49 % in 16 µM dose (Supplementary Data)) and subtraction-based (15.33 % in 16 µM dose (Supplementary Data)) calculations which somehow appeared to be highly similar. This could be a clue for the question "Why there are two popular methods to calculate growth?" It is probably because their results did not remarkably contradict in general.

As shown in Figures 4a, 4b, and 4c, EC_{50} (concertation of the chemical of interest lowering the growth by 50%) originating from binary logarithmic calculations seemed higher than the EC_{50} values derived from division and subtraction-based calculations. Interestingly, EC_{50} values obtained through division- and subtraction-based calculations appear to be very close to each other (even the same). Here, we evaluated EC_{50} for Cd-induced growth inhibition from 3DOE data via binary logarithmic calculations. As a calibration curve from the results of binary logarithmic calculation of 3DOE was drawn (Figure 4a), the formula of "$y = 0.01x^2 - 1.878x + 101.393$" emerged where y is G (%) and x is the dose of Cd (in µM). As the equation solved for y = 50, EC_{50} appeared to be 33.25 µM.

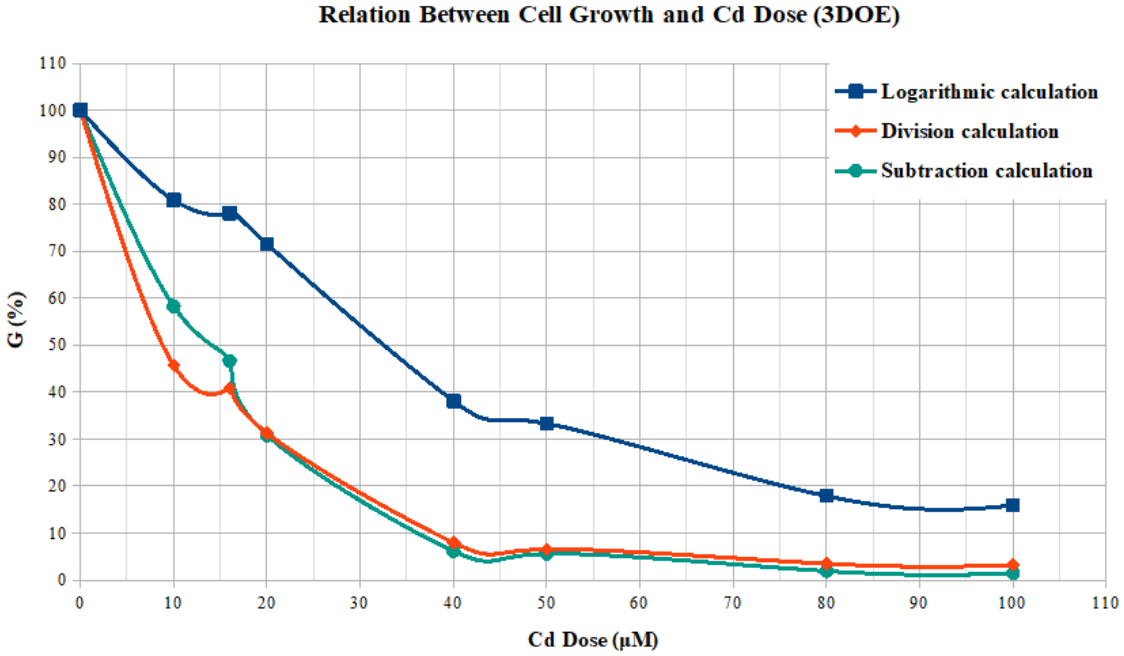

Figure 4a. *Relation Between Cell Growth and Cd Dose (3DOE)*

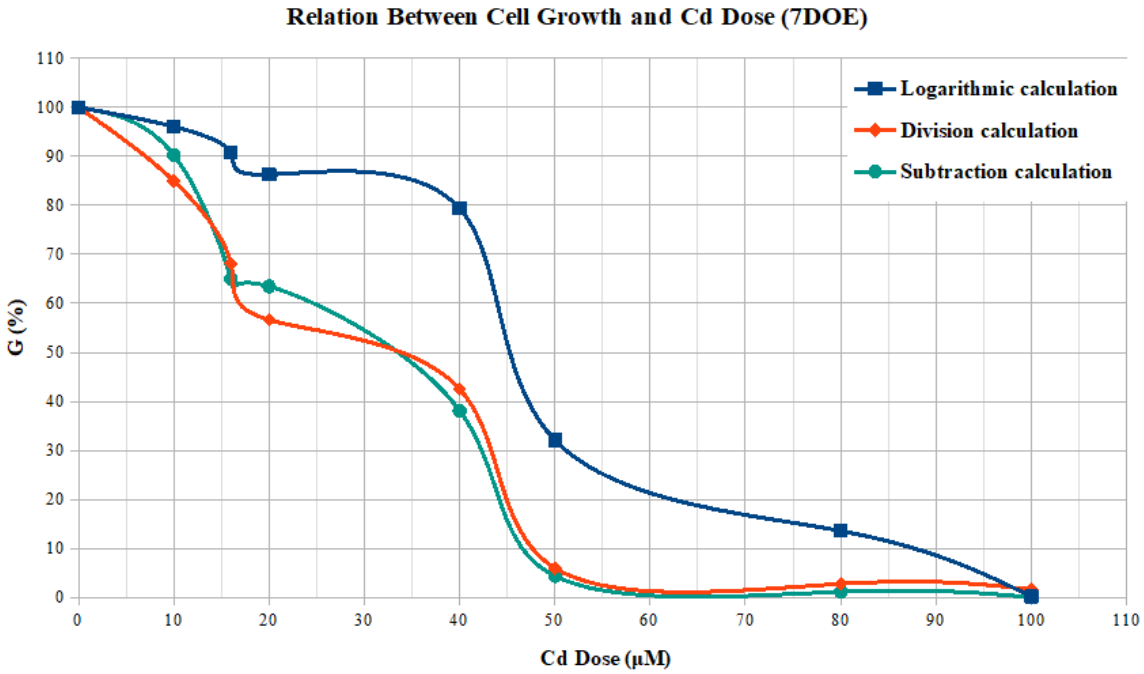

Figure 4b. *Relation Between Cell Growth and Cd Dose (7DOE). For a clearer view and easy evaluation, data regarding 800 µM are excluded from the graph*

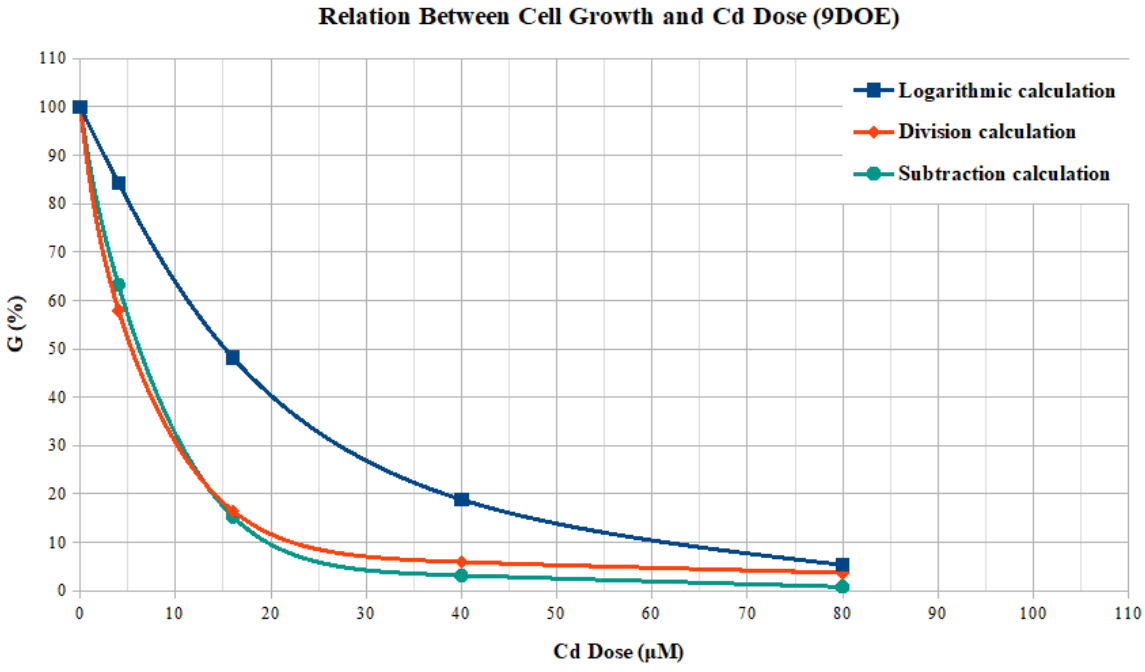

Figure 4c. *Relation Between Cell Growth and Cd Dose (9DOE). For a clearer view and easy evaluation, data regarding 800 µM are excluded from the graph*

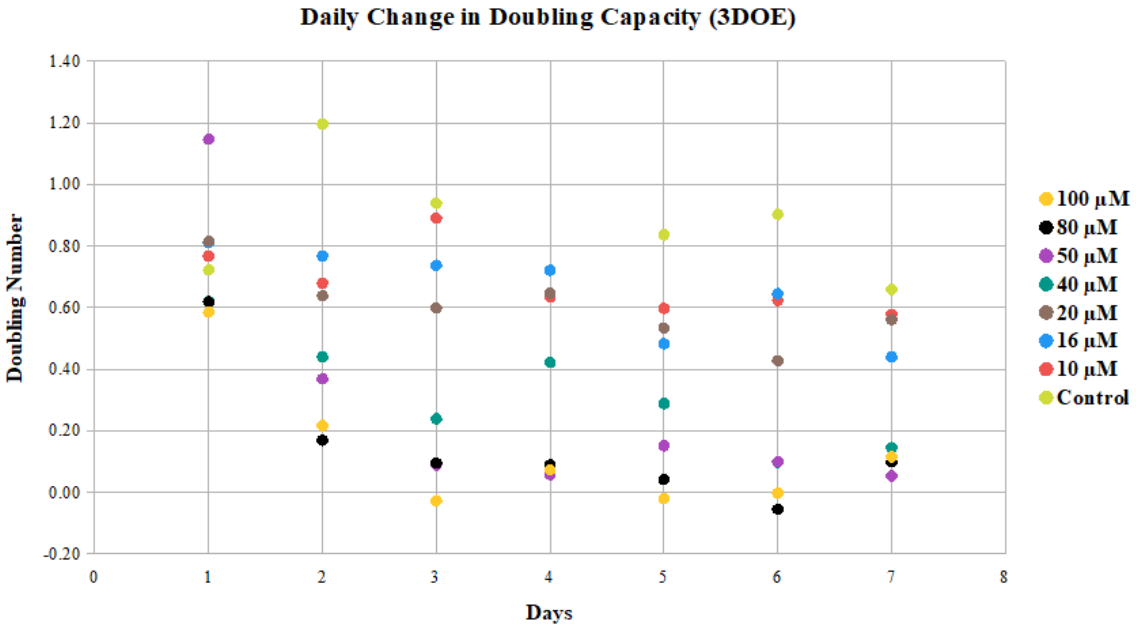

Figure 5. *"Number of Doubling vs Time" graph belonging to 3DOE*

3.2. Daily Change in Doubling Performance

Based on Figure 5, it can be stated that, on the 1st day of the week, cells grow regardless of the dose applied within the range of 0-100 µM. We started to observe a decrease in the growth of metal-supplemented samples as compared to the control on the 2nd day of the week. Furthermore, it is recognized that N of samples with low doses Cd (≤ 20 µM) and of control get closer on the 4th day. While the control experiences a lowered doubling performance in the passage from day 3 to day 4, samples with Cd do not follow this decrease. The situation becomes outstanding with samples supplemented by low doses of Cd (≤ 20 µM). Moreover, samples with high doses of Cd behave similarly to the samples supplemented by low doses of Cd (≤ 20µM). So "What happens on the 4th day?", "Is a defense mechanism (such as an anti-oxidative system) activated against metal ions?" These questions remained to be answered. However, one point is obvious; the cell cycle of the control is not followed by Cd-exposed samples which means Cd disrupts the cell cycle.

4. CONCLUSION

Here, we listed our conclusions:

* Cell growth is an exponential issue. Subtraction and division-based calculations do not sufficiently report the nature of an exponential curve. Thus, cell growth can be better described with the binary logarithmic calculation. Via the help of the binary logarithmic approach, it is possible to compare the results of different growth inhibition experiments which are carried out by different teams and/or with different species/varieties.

* In general, the culture age of the inoculating source cells, where the cells used for the experiments originate, is stated in research articles. For some experiments, it is sufficient. In most of the experiments, the ages of cells are provided to inform readers about the use of young cells with high-level division capacity. However, for some experiments such as the ones investigating cell growth, it could be important to provide more data. Generally, 7 days old cells are accepted as "young". However, this may not be a sign that they have high division capacity. If subculturing of these cells is done with a lot of cells, on the 7th day, the cells will be at the stationary phase. This population of cells will have a lower living / nonliving cell ratio which means lower division capacity. Although this is not the case most of the time, it is better to provide some data regarding the division capacity of the initial cells. We suggest scientists, working on cell

growth, provide N or ADT with the investigated period of time in addition to the age of the source cell culture. In this study, we see that 2 mL of 3 days old cells and 1 mL of 7 days old cells have a similar pattern of cell growth regardless of the presence/absence of Cd (apparent by G (%)). Via the help of N, we could better recognize that cells of 9DOE control differ from controls of 3DOE and 7DOE.

* 9DOE control had a lower division capacity (N=5.02) as compared to controls of 3DOE (N=5.90) and 7DOE (N=5.98). We also observed that 9DOE is more harshly affected by Cd toxicity in terms of cell growth. This phenomenon suggests that a cell population with lower division capacity is more strongly influenced by Cd toxicity. The reason behind this stronger impact could be the higher amounts of Cd ions per living cell.

* In these conditions, EC_{50} for Cd-induced growth inhibition of BY-2 cells (3DOE) is 33.25 μM. Readers should note that even the cell lines derived from the same source can show different characteristics. So, the EC_{50} value presented here cannot be used for generalized conclusions on tobacco.

* Cd disrupts the cell cycle of BY-2 cells.

* Our questions regarding day 4 remain to be investigated.

* The calculation styles we mentioned here are just a small and easy part of the issue of calculation of cell growth. Actually, there are many methods to evaluate cell growth and growth inhibition and they are discussed in a variety of articles. For more please visit the following studies: [15,25-28].

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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