Using ANOVA to Evaluate the Effects of Swine Slaughterhouse Wastewater Conditions on Algae Growth

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***Abstract* - Wastewater is a major environmental problem. Swine slaughterhouses generate a large volume of wastewater with high an organic load and nutrients. It therefore has the potential to cause environmental problems. The effects of swine slaughterhouse wastewater conditions on algae growth are evaluated. Microalgae, Chlorella vulgaris TIST8580 in water mixtures Tris acetate phosphate medium (control) and in diluted sewage from a slaughter house with the ratio of 25:75 and 50:50 (sewage: water) were experimentally studied. We used One-way ANalysis Of Variance (One-way ANOVA) and Two-ways analysis of variance (Two-way ANOVA) techniques for testing the differences between the 3 cases of the culture conditions used. It was found that from the One-Way ANOVA, the average number of cells of algae in wastewater in the 25% and 50% groups is not significantly different but both of these groups are significantly different from that of the control group. In addition, from the Two-Way ANOVA, we found that unlike the control group, the same kind of data in both 25% and 50% groups had no significant difference. This implies that the characteristic growth of algae in these two group do not significantly change over the culture period. We believe that our finding could benefit the researchers to properly design experiments especially for the case of resource limitation.**

Keywords- statistical analysis; ANOVA; algae growth; wastewater; Swine Slaughterhouse

I. INTRODUCTION

Wastewater is a major environmental. Wastewater contains high amount of nutrients such as nitrogen and phosphorus which lead to ecological problems [1]. This problem can lead to a high chemical oxygen demand (COD) [2] and it is harmful to aquatic life as the quality of water drops and there is an increasing of the plant population [3,4]. Specifically, swine wastewater is highly toxic to the environment because it is rich in nitrogen, phosphorus, heavy metals, and other compounds which can lead to pollution. In many countries swine wastewater is treated before being released [5-7]. Recently, Kitrungloadjanaporn et. al. studied nutrient removal from the effluent of swine slaughterhouse wastewater by *Chlorella vulgaris.* This sytem was considered to be a very challenging system to study due to the wastewater conditions. Swine slaughterhouse generates a large volume of wastewater with a high organic load and nutrients [8,9]. Nitrogen and phosphorus without treatment may cause eutrophication in

rivers, lakes, sea, and the upset the balance of the ecosystem and they have other negative effects such as algal bloom, low dissolved oxygen concentration, undesirable pH shifts and cyanotoxin production. Nutrient removal by chemical and physical treatment can be costly in terms of energy and chemical consumption [10,11]. Cultivation of algae in wastewater is considered a potential option for the sustainable production of algal biomass because of reduction in the production cost and the wastewater cleaning by an environmentally friendly method [12]. Microalgae could grow in different types of wastewater, such as sediment in septic tank, effluent, cattle slaughterhouse, poultry manure and primary piggery wastewater [13-18].

II. Literature Review and limitations of Current Techniques

There are many microalgae that are capable of growing in wastewater and treating wastewater. Microalgae require nitrogen, phosphorus, CO2, and light to grow. Hence, microalgae can be utilized to remove these nutrients from wastewater[19,20]. One of them is *Chlorella vulgaris*, a unicellular green alga. *Chlorella vulgaris* uses nitrogen and phosphorus from wastewater for food. It can tolerate a harsh environment, is easy to grow and reproduce, and is effective at removing nutrients in water [21]. There are many other ways to remove the nutrient from wastewater. However, they are more expensive and require complicated technology [1]. Microalgae is a better way to treat wastewater not only because it is cheap but also because it has a valuable by-product. The benefits of growing algae in swine slaughterhouse wastewater are absorbing nutrients and transforming it into biomass that can eventually be processed to biodiesel by transesterification process. The remains can be used in animal food industries, soil conditioners, supplements or pharmaceuticals [22-25].

In this work, we study the system of algae in the swine slaughterhouse wastewater. The main objective is to investigate the effects of wastewater conditions on the growth of algae using the one-way and two-way analysis of variance (ANOVA) methods. To the best of our knowledge this is the first study to use ANOVA to analyze this particular kind of problem. ANOVA is the extension of the t- and the z-tests. The one-way ANOVA is used to determine whether there are any statistically significant differences between the means of three or more independent groups. A two-way ANOVA is an extension of the one-way ANOVA. With a one-way ANOVA, you have one independent variable affecting a dependent variable. With a two-way ANOVA, there are two independent variables. It tests the effect of two factors at the same time. Here we shall use this ANOVA to investigate the algae growth in the swine slaughterhouse wastewater system.

III. A New Evaluation Technique

Here the microalgal strain of *Chlorella vulgaris* TISTR 8580 used in this study was purchased from the Thailand Institute of Scientific and Technological Research. Then, it was streaked on agar plates, and kept under fluorescent light to be purified. After that, the algae was transferred to TAP agar slant, and kept under a 3,000 lux daylight fluorescent lamp with the photoperiod of 12 hours light and 12 hours dark. The atmosphere was controlled at 28±2ºC. Then, the algae was prepared for the next experiments.

The Swine Slaughterhouse wastewater was collected from Samphran slaughterhouse Limited company in Nakhon Pathom, Thailand after the anaerobic digestion. In order to be realistic, no filtration was done on the wastewater unlike in another study which filtered the wastewater before conducting the experiment [26]. The nutrients concentration found in wastewater are shown in Table I. However, we have diluted wastewater with distilled water into two concentrations, 25% and 50% for further experiments.

The total nitrogen, phosphorus, and COD concentrations were analyzed using electrode, persulfate, and dichromate methods respectively, which were done by the Environmental Clinic, Faculty of Environmental and Resource Studies of Mahidol University, Thailand. The *C. vulgaris* starter was constructed by moving several colonies of purified *C. vulgaris* from the TAP agar slant into a 1000 ml sterilized glass bottle containing 800 ml TAP medium. Then, *C. vulgaris* in the TAP medium was grown under the conditions of 3,000 lux fluorescent light (12 hrs light : 12 hours dark), 28±2ºC, and 0.4 vvm (volume of air per volume of culture per minute) filtered air. The cell concentration were observed daily using a haemocytometer until the cell density of *C. vulgaris* reached 3±0.3x105 cell/ml. Then, the experimental groups were prepared by diluting swine slaughterhouse wastewater with distilled water into 25% swine slaughterhouse wastewater and 50% swine slaughterhouse wastewater concentrations in sterilized glass bottles (3 repeats for each concentration), and adding 1 ml of starter of *C. vulgaris* grown into each bottle. *C. vulgaris* were also grown in TAP medium (3 repeats) as a control group. Each trial was set under the same conditions which are 0.4 vvm filtered air, 28±2ºC, and 3,000 lux red LED light (12 hrs day: 12 hrs night). Cell density was observed and recorded daily by taking 1 ml of each trial and counting the cells under microscope using the haemocytometer method for 20 days [27]. This is because the nutrients analysis requires at least 500 ml of swine slaughterhouse wastewater, so the experiment needed to be stopped as the amount of water left in the bottles were about to be under 500 ml as some of them were taken as samples for cell density observation and some had evaporated. After 20 days, the bottles that were used in the experiment were delivered to the Environmental Clinic, Faculty of Environment and Resource Studies of Mahidol University to analyze the amount of nutrients left in the solution.

TABLE I. NUTRIENT CONCENTRATIONS OF THE EFFLUENT OF SWINE SLAUGHTERHOUSE WASTEWATER AFTER DILUTION USED IN EXPERIMENTS

	Total Nitrogen (mg/L)	Total Phosphorus (mg/L)	COD (mg/L)
1. 25% concentration	77.73	4.76	73.67
2. 50% concentration	129.50	8.90	75.67

Consider the growth of *C. vulgaris* TISTR 8580. The algae were raised in 3 different media which are TAP media, 25% swine slaughterhouse wastewater, and 50% swine slaughterhouse wastewater. The experiment was conducted for 20 days.

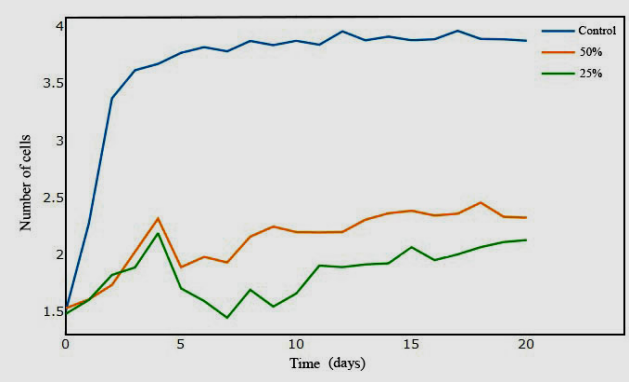

Figure 1. Cells density of *C. vulgaris* TISTR 8580 cultivated in the effluent of swine slaughterhouse wastewater at the concentration of 25% (Green line) and 50% (Red line) and the control TAP (Blue line).

From Fig.1, the algae can grow in every media but *C. vulgaris* can grow the best in TAP media. Algae is able to grow more in TAP media than in swine slaughterhouse wastewater because the nutrients in TAP media are more suitable for algae to grow than swine slaughterhouse wastewater. Moreover, swine slaughterhouse wastewater are contaminated with other bacteria and protozoa that can inhibit algal growth [28]. From Fig. 1, the algae grown in TAP media stay in the lag phrase for 2 days and start the log phase during day 2 to day 8. *C. Vulgaris* in TAP media starts the stationary phase in day 8 until the end of the experiment at day 20. *C. vulgaris* starts from 32.3 $\times 10^4$ cell/ml at day 0 and reaches 7.3×10^4 cell/ml at day 20. The growth of *C. vulgaris* in TAP media follows the normal growth curve. The highest algae cell density in TAP media is 9.08 $\times 10^7$ cell/ml in day 17. The highest cell density of TAP media is approximately 32 times more than the highest cell density of 50% swine slaughterhouse wastewater. From Fig.1, the algae can grow in swine slaughterhouse wastewater in both 25% and 50% concentration as stated in the study that the suitable N:P ratio for microalgae to grow is between 10 to 30 [29]. In this experiment, the N:P ratio of 25% swine slaughterhouse wastewater and 50% swine slaughterhouse wastewater are 16.32 and 14.55 respectively which are suitable for algae to grow. The algae in 50% swine slaughterhouse wastewater has higher growth rate than the algae in 25% swine slaughterhouse wastewater due to the higher amount of nutrient in the media. The result of this experiment supports the result in the study of [30] which showed that the higher the concentration of swine wastewater, the higher the growth rate of the microalgae. The result reveals that the higher the COD in the wastewater, the better the growth of the algae [31]. However, it cannot be determine the timing of the lag phase, log phase and stationary phase in the algae growth in swine slaughterhouse wastewater. The growth rate during first 4 days of 25% and 50% swine slaughterhouse wastewater is higher than the growth rate the day after. The highest cell density for algae in 25% swine slaughterhouse wastewater is 1.54 $\times 10^6$ cells/ml. The highest cell density for algae grown in 50% swine slaughterhouse wastewater is 2.85×10^6 cells/ml. During day 5 the cell density of algae in swine slaughterhouse wastewater declined and the media turned yellow - green. It started to turn green again in day 10.

Moreover, this study found that the number of algae cells cultured in a control case will get the highest average (mean = 6050) next, in mixed water with 50% of wastewater case (mean = 116) lastly, in algae in water mixed 25% of wastewater (mean = 81.9), respectively.

From Fig.1, we can see that the number of algae cells in the control water case is greater than in the case of water mixed with both wastewater samples. It can be seen that the number of algae cells in the mixed wastewater 50% is close to that for 25%.

IV. METHODOLOGY TO OBTAIN RESULTS

In this research, ANalysis Of VAriance (ANOVA) was used to analyze the difference between the number of cells in wastewater 25% and 50%, respectively. Hereafter we will refer 25%, 50% and control TAP as C1, C2 and C3, respectively.

IV. Statistical Analysis Method

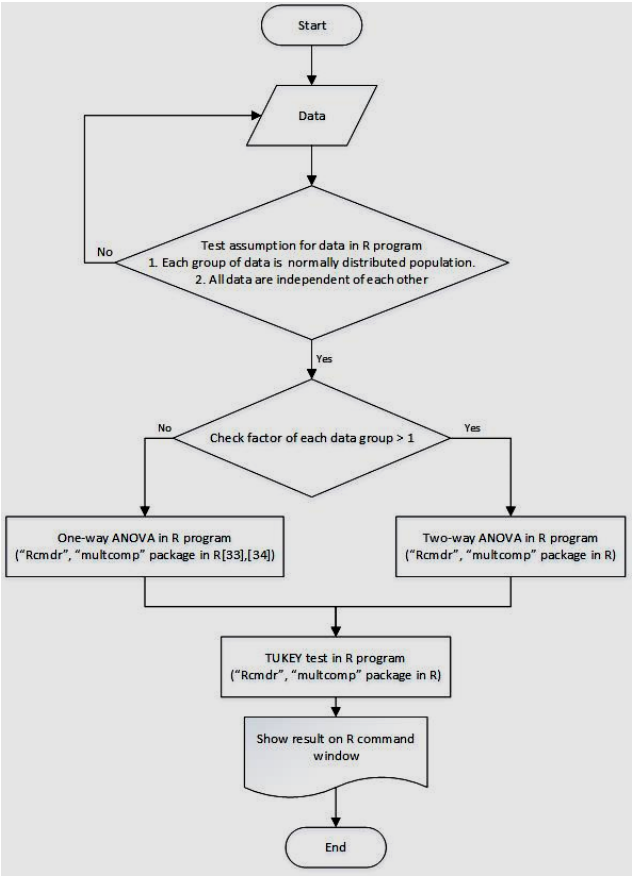

Figure 2. Flow chart for algorithm of statistical analysis of algae growth.

V. Results and Discussion

A. Normal distribution requirement

To implement the ANOVA approach, each group sample is drawn from a normally distributed population. Using the significant value, it can be determined whether the correlation is a significant one or not. The null hypothesis (H0) and alternate hypothesis (H1) are two types of hypothesis. If the null hypothesis gives a p-value zero or nearly zero, then we will reject this at the 5% level if the significance is less than 0.05. Here calculations are shown in Table II.

TABLE II. P-VALUES OF EACH CASE.

Type	p-value
25% (C1)	0.2449
50% (C2)	0.2923
control (C3)	0.05329

The P-value of 25% (C1) is 0.2449, of 50% (C2) is 0.2923 and of the control (C3) is 0.05329. They are greater than the level of significance 0.05. So, we do not reject the null hypothesis that the number of algae cells of water 3 cases are normally distributed populations. The Fig. 3 shows the data.

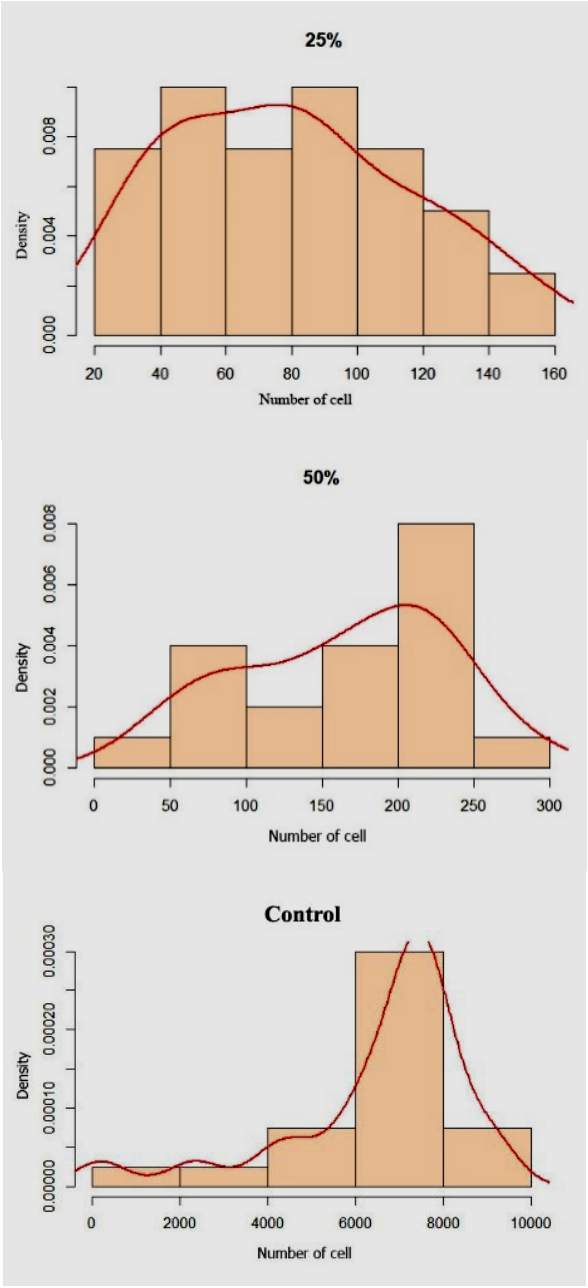

Figure 3. Histogram graph of number of cells of water 3 cases.

B. Independency of Population

Next, we need to check if all samples are drawn independently from each other. We test the hypothesis that the number of cells for each type of algae in the water is independent at 0.05 significance level. As the p-value 0.236 is greater than the 0.05 significance level, we do not reject the null hypothesis that the number of cells.

C. One-way analysis of variance (one-way ANOVA)

A one-way ANOVA is used to compare two means from two independent (unrelated) groups using the F-distribution. The null hypothesis for the test is that the two means are equal. Therefore, a significant result means that the two means are unequal. A positive one-way ANOVA result will show that at least two groups are different from each other. But it will not say what groups are different. If the test returns a significant f-statistic, we may need to run an ad hoc test (like the honestly significant difference test) to tell that exactly which groups had a difference in means [35]. Here calculations are shown for a one-way ANOVA in Table III where P (>F) is <2e-16 which is less than the level of significance 0.05. So the ANOVA test rejects the null hypothesis.

TABLE III. ONE-WAY ANOVA FOR THE NUMBER OF ALGAE CELLS.

Description	Value
df	2
F value	**117.7**
Pr(>F)	**<2e-16**

Tukey's HSD (honest significant difference) test, also known as Tukey's test is a single-step multiple comparison procedure and statistical test. It can be used on original data or in conjunction with an ANOVA (post-hoc analysis) to find means that are significantly different from each other. It compares all possible pairs of means, and is based on a standardized range distribution. Using the Tukey test in Table IV where Pr(>|t|) of C1-C2 is 0.983 which is greater than the level of significance 0.05.

TABLE IV. THE TUKEY TEST OF THE ONE-WAY ANOVA FOR COMPARISON BETWEEN THE CASES OF WATER.

Case of water	t value	Pr(>|t|)	Conclusion
C1-C2	0.177	**0.983**	not different
C1-C3	13.376	**<0.00001**	different
C2-C3	13.199	**<0.00001**	different

The ANOVA tests the null hypothesis that means for C1 and C2 are not different. Pr(>|t|) of C1-C3 and C2-C3 are <0.00001 which is less than the level of significance 0.05. So the ANOVA test rejects the null hypothesis these cases.

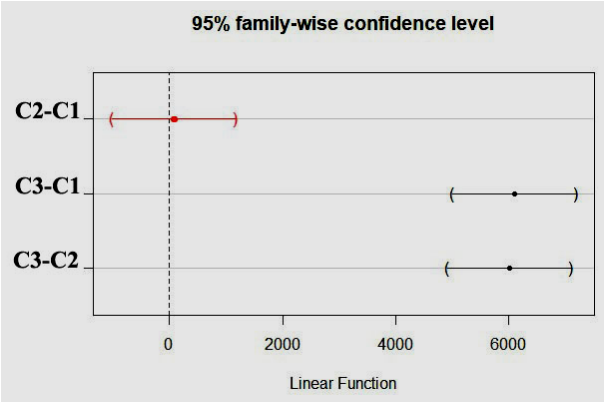

Figure 4. The graph of the confidence interval of TUKEY test of One-way ANOVA.

Fig.4 shows the 95% confidence intervals for each pair difference. From the average number of cells in each curve which has passed the x-axis can be concluded that the difference between the average values in each pair is not different. The top row shows the value of the average difference between C1 and C2 with a line drawn through the x-axis. So, the number of cells of C1 is not different from C2. So the number of algae cells of C1 and C2 are not different with C3.

D. Two-Way Analysis Of Variance (Two-Way ANOVA)

In other words, if the experiment has a quantitative outcome and there are two categorical explanatory variables.

D1. [Day 1 to day 10] and [day 11 to day 20]

Here calculations are shown for a two-way ANOVA for day1 to day10 (P1) and day11 to day20 (P2) in Table5 where Pr(>F) is <2e-16 which is less than the level of significance 0.05. ANOVA test the null hypothesis that there are at least 2 parts with the mean difference. Pr(>F) of type is < 2.2e-16 which is less than the level of significance 0.05. The ANOVA test rejects the null hypothesis that there are at least 2 cases with means different.

TABLE V. TWO-WAY ANOVA FOR 2 PARTS.

Source of Variation	df	F value	Pr (>F)	Conclusion
P	1	13.538	0.0005403	different
C	2	268.080	< 2.2e-16	different
P: C	2	11.592	0.00006477	

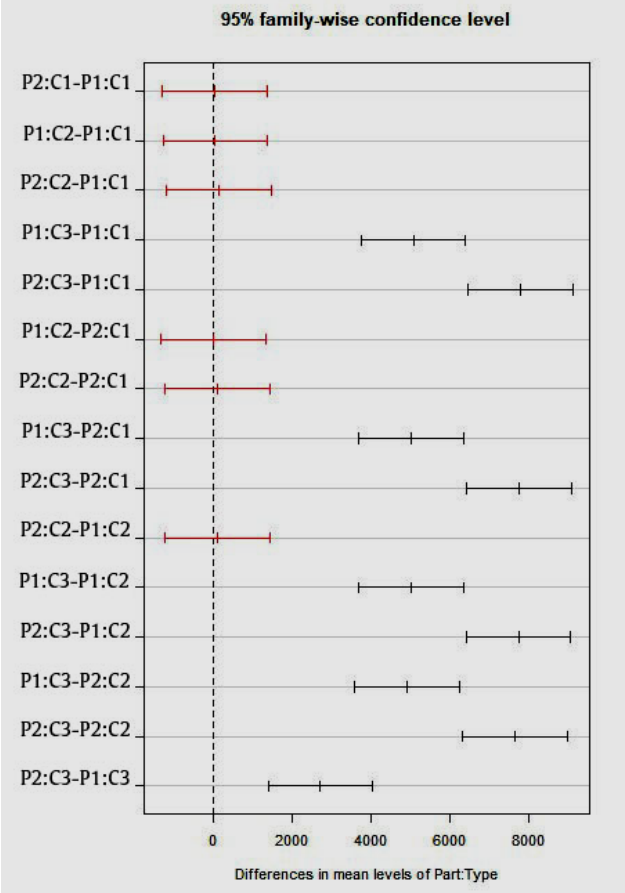

Figure 5. The Tukey test of the confidence interval of 2 parts.

In Fig. 5, the Tukey test results are shown. From the average pair of curves, if there is line passing x or zero in the confidence interval, it can be concluded that the difference between the average value pairs is not different. In the top row, it shows the value of the average difference between P2: C1 and P1: C1 with a line drawn though the zero and we can see that each P of C1 and C2 will have a line drawn thought the zero so the average value between C1 and C2 is not different. Clearly P1 and P2 of C3 is no line drawn through the x-axis so there it is different. It shows that algae growth phase of control water has 2 phases. And P1 and P2 of C1 have a line drawn through the x-axis so the algae growth of C1 has 1 phase. Similarly, the algae growth of C2 has 1 phase.

D2. [Day 1 to day 5], [day 6 to day 10], [day11 to day 15] and [day 16 to day 20]

Here calculations are shown for a two-way ANOVA for day1 to day5 (P1), day6 to day10 (P2), day11 to day15 (P3) and day16 to day20 (P4) in Table 5 where Pr(>F) of P is 0.0000006 which is less than the level of significance 0.05. The ANOVA tests the null hypothesis that there are at least 2 parts with the mean difference. Pr(>F) of C is < 2.2e-16 which is less than the level of significance 0.05. The ANOVA test rejects null hypothesis that there are at least 2 cases with the mean difference.

TABLE VI. TWO-WAY ANOVA FOR 4 PARTS.

Source of Variation	df	F value	Pr (>F)	Conclusion
P	3	13.538	0.000000588776	different
C	2	268.080	< 2.2e-16	different
P: C	6	11.592	0.000000006171	

From Fig. 6, the average pair of curves pasiing x-axis (with zero) can be concluded that the difference between the average value pairs is not different. In the top row, it shows the value of the average difference between P2:C1 and P1:C1 with a line drawn through the x-axis so the average value pairs is not different. For the other pair with C3, there are no lines drawn through the x-axis so there is a difference. Clearly, line P1:C3 compared with P2-P4:C3 does not pass the x-axis or zero meaning the average of cell density of algae of P1 is different from other P pf C3.
have a line drawn thought the x-axis so the average value pairs for P2, P3 and P4 of C3 is not different. This shows that algae growth of the control water has 2 phases. And P1, P2, P3 and P4 of C1 have a line drawn through the x-axis so the algae growth of C1 has 1 phase. Similarly, algae growth of C2 has 1 phase.

VI. SUMMARY AND CONCULSIONS

The effects of swine slaughterhouse wastewater conditions on algae growth were experimentally studied with three groups of samples namely, control, 25%, and 50% diluted wastewater. Rigorous systematic statistical analyses via ANOVA methods was used to analyze the data. It was found that from the one-way ANOVA, the average numbers of cells of algae in the wastewater 25% and 50% groups is not significantly different but both of these groups are significantly different from that of the control group. In addition, from the two-way ANOVA, we found that unlike the control group, the same kind of data in both 25% and 50% group has no significant difference. This implies that the characteristic growth of algae in these two group do not significantly change over the culture period. We believe that our finding could benefit the researchers to properly design experiments especially for the case of resource limitation.

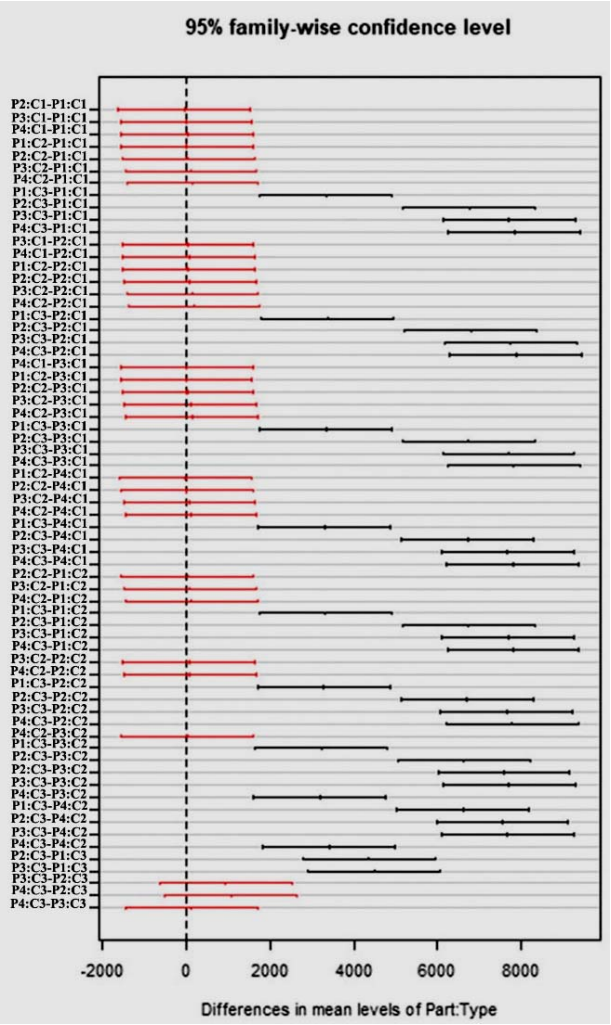

Figure 6. The Tukey test of the confidence interval of [Day 1 to day 5], [day 6 to day 10], [day11 to day 15] and [day 16 to day 20].

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