GENETIC CHARACTERIZATION OF ANAEROBIC MICRO-ORGANISMS APPLIED TO WASTEWATER TREATMENT: AN ALTERNATIVE IN AREAS OF HEIGHT

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ABSTRACT
The degradation of organic matter in wastewater from livestock stables is analyzed with the use of anaerobic micro-organisms obtained from wastewater samples from the study area. The research is divided into three phases. In the first, wastewater samples are taken from three farms in the area; in the second phase the samples are taken to the laboratory and three replicates of each collected sample are prepared to be analyzed and identified and the methanogenic bacteria that are responsible for degrading organic matter in anaerobic processes are isolated. In the last phase, two experiments are carried out with three repetitions each; in the first one, the contaminated water is placed without a previous dilution process. In the second experiment, a 75% dilution is carried out to recreate the real conditions in the treatment plants where the micro-organisms are going to be applied, which suffer dilutions due to the presence of constant precipitations in the analysis area. The results show a high elimination of organic matter and fecal coliforms in the wastewater treated with the identified micro-organisms, producing degradation kinetics of order 1 (exponential) with particular constants for each process.
Keywords: anaerobic micro-organisms, wastewater treatment, genetic characterization.

1 INTRODUCTION

The livestock sector has increased significantly during the last decades, according to the United Nations Food and Agriculture Organization (FAO) livestock contributes 40% of world agricultural production [1]. This intensification in exploitation brings, as a consequence, an incorrect environmental management. Waste from livestock farms have organic matter, nitrogen compounds, phosphorus, potassium and heavy metals [2]; biological systems are involved in processes of purification of organic matter by anaerobic decomposition [3] so this technology produces low amounts of sludge which does not require the administration of oxygen during the process, therefore generates lower energy demand and low installation cost, consequently a low treatment cost [4].

Anaerobic digestion is reduced to four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis; within the methanogenic bacteria, four genera stand out: Methanococcus, Methanebacterium, Methanosacosin, and Methanospirillum which transform the organic material present in wastewater by catabolizing acetate and monocarbon compounds to produce methane gas and generate biogas [5], [6]. The specific methanogenic activity (SMA) is carried out to evaluate the microbial activity of converting the organic substrate into methane [7]; this activity establishes the maximum capacity of chemical oxygen demand (COD) removal that allows to establish the minimum and the maximum organic concentration load to be applied in a reactor to ensure the reduction of organic matter into biogas [8]; the determination of biochemical oxygen demand (BOD), COD and suspended solids, both sedimentable and non-sedimentable (SS), are sufficient to assess the organic material removal. In addition, to assess fecal contamination and the effectiveness of the treatment processes, indicator organisms such as coliform bacteria, (which are normally

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found in the intestine of warm-blooded animals and excreted in faeces) are used. Coliform bacteria are found in proportional densities to the amount of fecal contamination, on the other hand its absence indicates that water is suitable for human consumption [9] hence, there are several methods of removal or inactivate these indicator bacteria: filtration materials, ultraviolet radiation, activated sludge with membrane bioreactor, with anaerobic reactor ascending flow, through chlorine–gas disinfection and biological systems, among others [10]. Within the processes of purification of wastewater by biological systems there are some limitations, such as: the temperature between 30–35°C is an optimal range, however, there are bacterial populations with a high yield in the production of biogas at 70°C, on the other hand, biogas producing bacteria cannot be discarded at low temperatures, therefore a sufficient conditioning time must be managed [11], [12]; the hydraulic retention time (HRT) is required for bacteria to degrade organic matter, the dry matter content must not exceed 10% of the water mixture – manure. HRT varies according to temperature, the higher temperature the lower HRT in obtaining biogas, thus it varies from 30 to 90 days from tropical to cold climate [13]; pH plays an important role in the degradation processes due to acidification influences negatively, studies have shown that from pH 8.2 to 8.4 is a satisfactory anaerobic process [14]; finally, process inhibitors, high concentrations of mainly toxic compounds such as: heavy, alkaline, alkaline earth metals, ammonium and sulphates, and because of the fact that anaerobic biological systems are highly vulnerable, the production of biogas stops completely [15], [16].

Considering these limitations, the objective of this study was to analyze the degradation of organic matter in wastewater from livestock stables, throughout the use of an anaerobic biological system with methanogenic bacteria obtained from wastewater samples from the study area.

2 METHODS

2.1 Collecting and obtaining of samples

The sampling of effluents discharges from stables was performed in triplicate with sterile 60 ml syringes, which were introduced approximately 40 cm under the water mirror [17], according to the anaerobic treatment to be carried out, the sampling was separated into three areas: (1) wetland of Tarqui river sub-basin; (2) stagnant water near the experimental farms; and (3) sewage running of the investigated farms. The samples were kept under anaerobic conditions and below 10°C for transportation to the laboratory [18].

2.2 Isolation and obtaining micro-organisms

To obtain the individual micro-organisms for wastewater treatment with high loads of organic matter, selective means were used for their isolation: Barker–Taha for Methanobacterium (MB) and Stadtman–Barker for Methanococcus (MC) [19]. Pre-reduction of the liquid means was carried out by thermal shock with the following conditions: 10 minutes at 100°C, so as to the oxygen in the tube could escape, cold water jet cooled; the solid means were left 2 hours in the anaerobic jar and the atmosphere generators were used (AnaeroGen OXOID) [20]. For the inoculation of the sample, 100 μL of each liquid means was taken and incubated for 15 days at 37°C in an anaerobic atmosphere (AnaeroGen OXOID), afterwards the bacterial growth was verified by the presence of turbidity in the liquid means. In solid means was planted MC and MB pre-reduced, they were incubated with the same conditions for liquid means previously described, and finally the presence of bacilli

and cocci growth obtained in the liquid and solid means was confirmed by Gram staining [17], [21].

2.3 Phenotypic identification and production of methane gas

The phenotypic identification was carried out based on traditional schemes such as the observable characteristics in morphology, development, biochemistry and metabolism [22] In addition to the Gram staining, the metabolic capacity of the bacteria to produce methane gas was evaluated, by means of: 16 x 150 mm with lateral detachment, rubber stoppers, latex hoses of 20 cm and tweezers; the system was assembled in a biosafety cabinet, 6 ml of MC and MB liquid culture means were supplied, providing the space for gas storage that bacteria will generate; the colonies obtained from the solid media of MC and MB were inoculated before sealing the system subsequently, the qualitative measurement was made by the water displacement technique, demonstrated by the presence of bubbles that were generated by the gas production and the increasing of water volume [17], [21].

2.4 Design of wastewater treatment

The anaerobic digestion test for the treatment of wastewater was carried out in six reactors at a laboratory scale with a capacity of 15 litres each, the reactors are arranged in two groups with different concentrations of waste water coming from the stable's washing of the farms in study, this allows to simulate dilutions by presence of precipitation in the study area and with three different dosages of bacteria to evaluate the performance of the treatment. The contaminants reduction behavior, the microbiological behavior and its efficiency during 30 days of analysis is evaluated [23].

Group 1: R1 (white) wash water from farm stable without dosage of anaerobic bacteria, R2 (100% bacteria), R3 (75% bacteria); 52 ml solution with presence of anaerobic bacteria per cubic meter of wastewater in R1, 0.77 ml in R2 and 0.58 ml in R3 were used for the dosage. Group 2: in this group of reactors the sample is kept diluted to 25% in order to represent the environmental conditions of the area due to the variant climate presenting constant rainfall, R4 (white), R5 (100% bacteria), R6 (75% of bacteria); 52 ml of the solution per cubic meter of wastewater, 0.77 ml in R5 and 0.58 ml in R6 were used for the dosage.

The control parameters established to evaluate the reduction of contamination by anaerobic digestion during days 0, 10, 20 and 30 are: COD, BOD, fecal coliforms, conductivity, pH, salinity, temperature, suspended solids, being able to assess the degree of pollution reduction and the system behavior [16].

3 RESULTS

3.1 Statistical analysis of the data obtained from the study of anaerobic biodegradation

Fig. 1 shows a considerable decreasing in the load of wastewater in all the reactors. BOD2 reactor contains 100% concentration of pollutant and bacteria dose, it started with a maximum amount of 840 mg/l to a minimum of 80 mg/l. BOD3 reactor has a concentration of 100% and bacteria dose of 75% and started with a maximum amount of 840 mg/l to a minimum of 81 mg/l. BOD2 reactor had a greater reduction in the BOD, both reactors BOD5 and BOD6 are diluted at 25%, where bacteria dose of 100% and 75% was applied, respectively. The reactor with the higher degradation is BOD5, which started 100% with a maximum of 640 mg/l to a minimum of 76 mg/l.

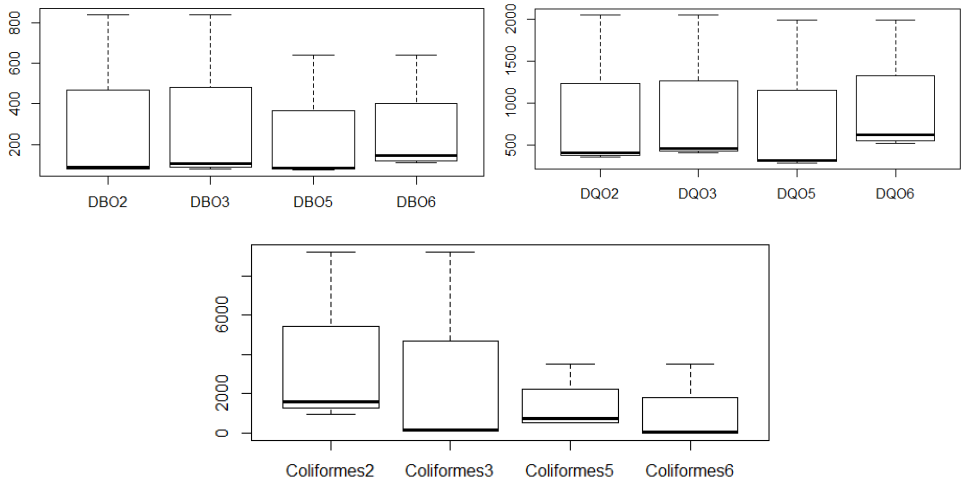

Figure 1: Statistical analysis of BOD, COD and fecal coliforms.

Regarding COD analysis, both reactors COD2 and COD3 showed similar behavior in the reduction, the difference is because of the amount of bacteria dose. COD2 reactor started with a maximum of 2,045 mg/l to a minimum of 360 mg/l; COD3 reactor reached a minimum of 410 mg/l which means that the most effective dose of bacteria was 100%. COD5 and COD6 reactors that were diluted at 25% showed a lower concentration, both reactors started with a maximum of 1,982 mg/l. the most effective degradation occurred in COD5 reactor with a minimum of 294 mg/l. COD6 reactor only reached a minimum of 521 mg/l which also illustrates that effectiveness in treatment depends on the bacteria dose.

In the coliform analysis, Coliform2 and Coliform3 behaved different in their reduction of contamination levels. Both reactors started with a maximum concentration of 9,200 NMP/100 ml, where Coliform2 reached a minimum of 920 NMP/100 ml and Coliform3 reached 79 NMP/100 ml. The same behavior was found in reactors 5 and 6 starting with a maximum concentration of 3,500 NMP/100 ml and reaching a minimum of 540 NMP/100 ml and 2 NMP/100 ml respectively. This particular parameter behaves in a different way than BOD and COD because coliform tend to be represented with most likely number of the sample therefore the data obtained is an approximation than the total existing coliforms.

3.2 Degradation curve of residual water

3.2.1 BOD degradation curve

Polynomic lines of grade 3 were used to analyze the behavior curve of the organic material with the anaerobic treatment of the BOD getting an exponential model through a mathematical analysis which describes, in a real way, the behavior of the pollutant degradation (Fig. 2).

For the analysis of the reactors BOD2, BOD3, BOD5 and BOD6 a polynomic line of grade 3 was used to get equation 1 as result. Where “x” data axis represents the days where the biggest reduction of BOD occurs.

Data from Table 3 was replaced to solve the equations in Table 1. This amount is in mg/l of BOD.

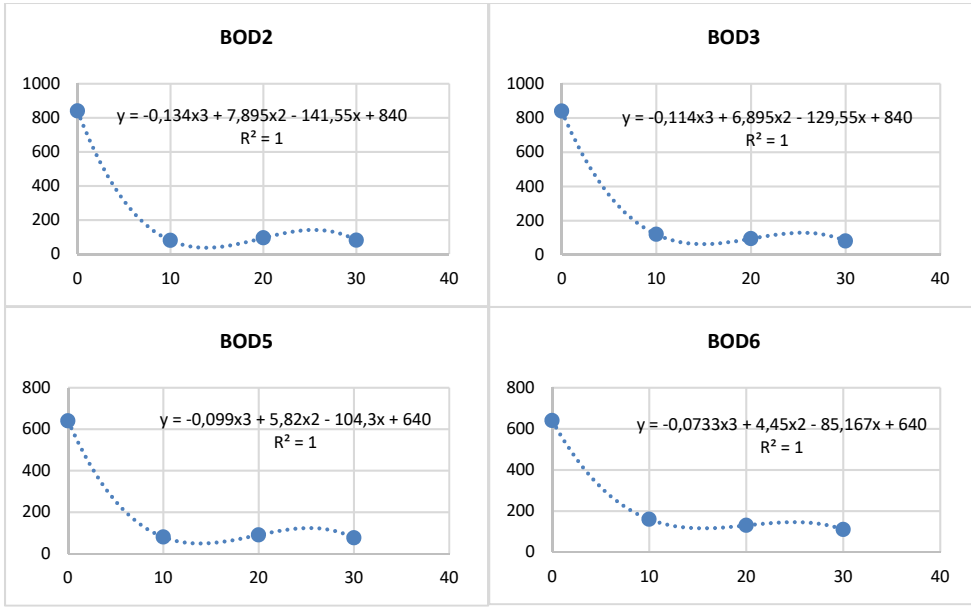

Figure 2: Polynomial degradation curves in reactors BOD2, BOD3, BOD5 and BOD6.

Table 1: Resultant equations for each reactor.

BOD2	y= -0.134x^3+7.895x^2-141.55x+840
BOD3	y= -0.114x^3+6.895x^2-129.55x+840
BOD5	y= -0.099x^3+5.82x^2-104.3x+640
BOD6	y= -0.0733x^3+4.45x^2-85.167x+640

Table 2: Equation 2 of mathematical derivation in the 4 reactors.

BOD2	-0.402x^2+15.79x-141.55=0
BOD3	-0.342x^2+13.79x-129.55=0
BOD5	-0.297x^2+11.64x-104.3=0
BOD6	-0.21x^2+8.9x-85.167=0

Table 3: Day with the highest degradation for BOD in the 4 reactors.

BOD2	13.89
BOD3	17.46
BOD5	13.87
BOD6	14.61

Table 4: Equation 1 with Table 3 values in the 4 reactors.

BOD2	y= -0.134(13.89)^3+7.895(13.89)^2-141.55(13.89)+840
BOD3	y= -0.114(17.46)^3+6.895(17.46)^2-129.55(17.46)+840
BOD5	y= -0.099(13.87)^3+5.82(13.87)^2-104.3(13.87)+640
BOD6	y= -0.0733(14.61)^3+4.45(14.61)^2-85.167(14.61)+640

Table 5: BOD degradation of the four reactors in mg/l.

BOD2	38.03 mg/l
BOD3	73.22 mg/l
BOD5	48.84 mg/l
BOD6	117.15 mg/l

Fig. 3 illustrates the results after the points of degradation model were found.

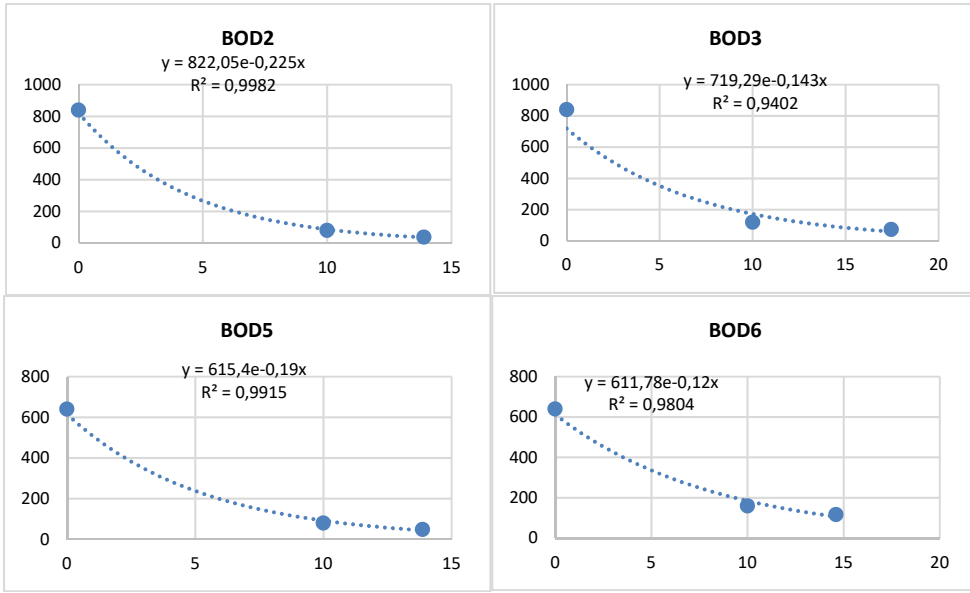

Figure 3: Exponential degradation curves in reactors BOD2, BOD3, BOD5 and BOD6.

Degradation curve of BOD2 reactor was decreasing in the BOD concentration after 10 days with an amount lower than 100 mg/l, accomplishing the dumping limits required to fresh water sources, even reaching lower values than 38.03 mg/l after 14 days. BOD3 reactor showed a variation in its adjustment in comparison with the BOD2 reactor model, the results obtained from this model are very efficient because they reached the environmental policy and also improves hydraulic retention time, between 20 days in cold weather and reaching 14 days with the greatest degradation of the pollutant. These variations are because of the dosage that generates that degradation variation levels in BOD, in this specific research

reached 17 days moreover, it degraded less amount of pollutant with an amount of 73.22 mg/l. BOD5 reactor showed a good fit in the organic material behavior curve, due to it has less concentration of the pollutant, 25% of dilution and 100% of the bacteria dosage applied, as it is shown in the degradation curve at day 10 the outcomes are less than 100 mg/l started with an initial concentration of 640 mg/dl of BOD therefore, it obeys the environmental policy and reached a maximum value of degradation of 48.84 mg/l at day 14. This also optimized the hydraulic retention values in cold weather. The reactor BOD6 has also a 25% concentration, the results are not encouraging, they only reached a value of 160 mg/l at day 10 and a maximum degradation at day 15 with 117.15 mg/l, these values failed to fulfill the environmental policy because this reactor had only 75% of the dosage. Consequently, this treatment is unsuitable.

3.2.2 COD curve degradation

For COD degradation parameter model, a polynomic line of grade 3 was performed to present the measurement results, also a mathematical analysis using deriving equations to achieve the exponential model that describes, in a real way, the behavior of the pollutant degradation (Fig. 4).

For the analysis of the reactors COD2, COD3, COD5 and COD6, a polynomic line of grade 3 was used. Equation 1 in Table 6 resulted of this analysis.

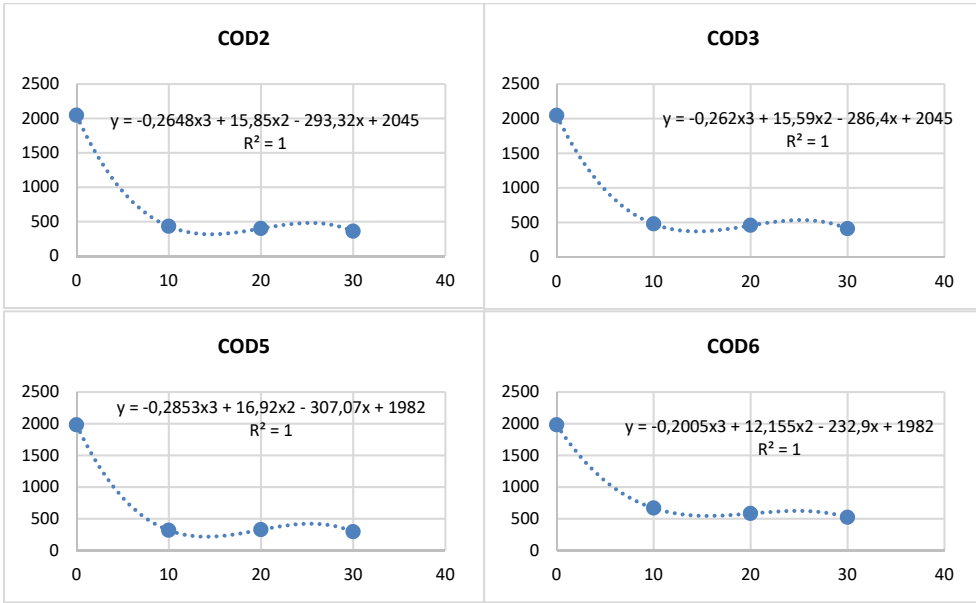

Figure 4: Polynomial degradation curves in s reactor COD2, COD3, COD5 and COD6.

Table 6: Equation 1 cleared in the 4 reactors.

COD2	y= -0.2648x^3+15.85x^2-93.32x+2045
COD3	y= -0.262x^3+15.59x^2-286.4x+2045
COD5	y= -0.2853x^3+16.92x^2-07.07x+1982
COD6	y= -0.2005x^3+12.155x^2-32.9x+1982

Table 7: Equation 2 of mathematical derivation in the 4 reactors.

COD2	-0.794x^2+31.7x-293.32=0
COD3	-0.78x^2+31.18x-286.4=0
COD5	-0.855x^2+33.84x-307.07=0
COD6	-0.6015x^2+24.3x-232.9=0

Table 8: Day with the highest degradation for COD in the 4 reactors.

COD2	14.64
COD3	14.30
COD5	14.09
COD6	15.68

Data from Table 8 is used in Table 6 to find the "y" value which is the amount in mg/l of COD.

Table 9: Equation 1 with values of Table 8 in the 4 reactors.

COD2	y= -0.2648(14.64)^3+15.85(14.64)^2-293.32(14.64)+2045
COD3	y= -0.262(14.30)^3+15.59(14.30)^2-286.4(14.30)+2045
COD5	y= -0.262(14.30)^3+15.59(14.09)^2-286.4(14.09)+2045
COD6	y= -0.2005(15.68)^3+12.155(15.68)^2-232.9(15.68)+1982

Table 10: COD degradation of the four reactors in mg/l.

COD2	319.55 mg/l
COD3	371.33 mg/l
COD5	216.43 mg/l
COD6	544.44 mg/l

Once the necessary points were found to fit the degradation model, Fig. 5 illustrates the outcomes of the laboratory test.

By using the polynomic line of grade 3, an exponential model is created for the reactor COD2 which started with a value of 2,045 mg/l and 100% of bacteria dosage. In the first 10 days, a decreasing was observed and reached a value of 432 mg/l and a maximum limit of degradation of 319.55 mg/l at day 15, so it failed to fulfill the environmental policy in spite of the degradation was good, since there was a significant reduction of the pollutant concentration because of an excessive concentration of organic material (cattle waste). For the reactor COD3 with 75% of bacteria dosage, outcomes showed that after 10 days of COD concentration reached a value of 478 mg/l and a maximum degradation of 371.33 mg/l at day 14. This result also failed to fulfill the environmental policy, however the levels of concentration are easier to handle. The reactor COD5 presented a dilution of 25% in its pollutant's initial concentration with an initial value of 1,982 mg/l of COD, a 100% of

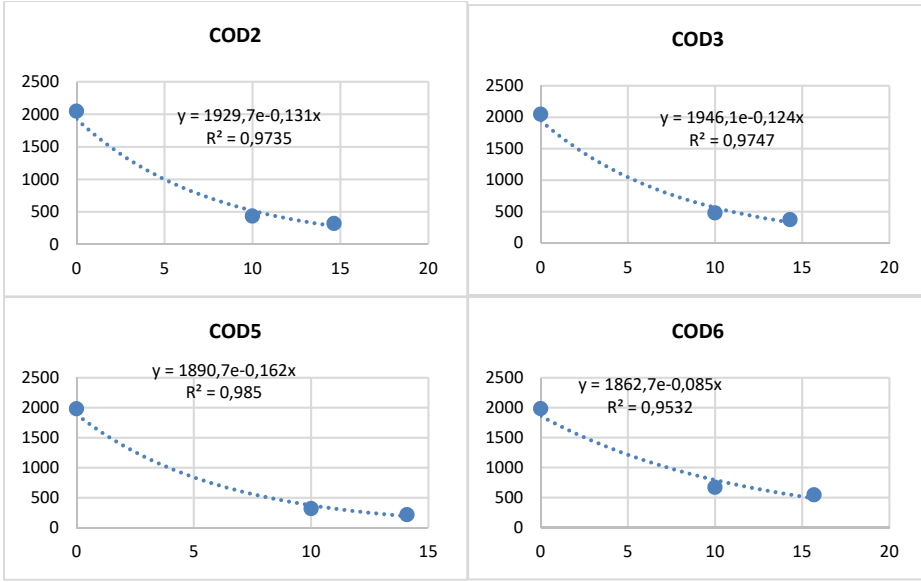

Figure 5: Exponential degradation curves in reactors COD2, COD3, COD5 and COD6.

anaerobic bacteria dosage was applied; at day 10, showed a reduction of 318 mg/l and, at day 14 displayed a value of 216.43 mg/l, the reduction is favorable almost reaching the environmental policy for dumping limits. For the reactor COD6 with a 25% of dilution in its initial concentration, 75% of anaerobic bacteria dosage was applied. At day 10 a decreasing of 668 mg/l of COD value was observed, at day 15 it reached a maximum degradation of 544.4 mg/l. These results are unfavorable since they only reached less than the half of degradation of COD in a longer time than the other reactors.

3.2.3 Fecal coliform degradation curve

A polynomic line of grade 3 was used to understand the degradation curve. Then, a mathematical analysis was performed to create and exponential model to describe, in a real way, the behavior of the pollutant degradation (Fig. 6).

A polynomic line of grade 3 was used to obtain the equation 1 in the Table 11 for the reactors Coliform2, Coliform3, Coliform5 and Coliform6.

Data from Table 11 was used to calculate the initial equation and to find the missing value in the "y" axis and it represents the biggest degradation in NMP/100 ml.

Once the necessary points were found to fit the degradation model, the outcomes of the laboratory test are illustrated in Fig. 7.

Reactor Coliform2 had an initial concentration of 9,200 NMP/100 ml, 100% of bacteria dosage was applied. At day 10 a reduction of 920 NMP/100 ml, at day 13 a value of 621.5 NMP/100 ml was observed. Results from this reactor fits with the environmental policy of dumping limits. On the other hand, it is possible to keep smaller hydraulic times to reach a bigger amount of Coliforms degradation. Reactor Coliform3, the degradation at day 10 had a value of 79 NMP/100 ml after this period the records showed zero value. In the adjusted exponential model, the behavior of the experiment at day 5 was considered and showed a value of 2,903.15 NMP/100 ml. These results are more favorable because only a 75% of

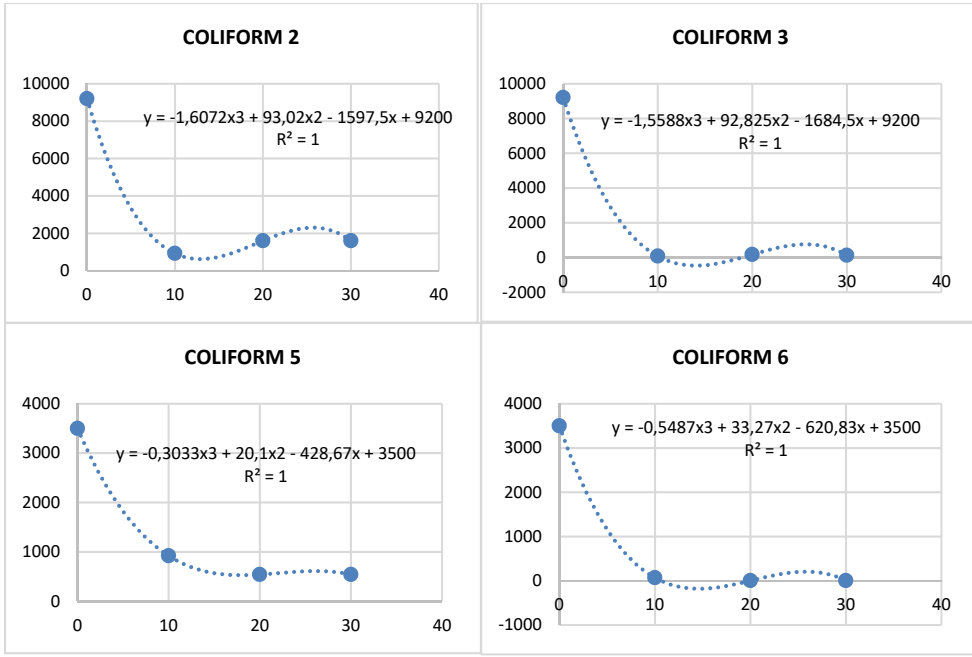

Figure 6: Polynomial degradation curve in the Coliform2 to Coliform6 reactors.

Table 11: Equation 1 cleared in the 4 reactors.

Coliform2	y= -1.6072x^3+93.02x^2-1597.5x+9200
Coliform3	y= -1.5588x^3+92.825x^2-1684.5x+9200
Coliform5	y= -0.3033x^3 20.1x^2-428.67x+3500
Coliform6	y= -0.5487x^3+33.27x^2-620.83x+3500

Table 12: Equation 2 of mathematical derivation in the 4 reactors.

Coliform2	-4.82x^2+186.04x-1597.5=0
Coliform3	-0.78x^2+31.18x-286.4=0
Coliform5	-0.909x^2+40.2x-428.67=0
Coliform6	-0.6015x^2+24.3x-232.9=0

Table 13: Day with the highest degradation for Coliforms in the 4 reactors.

Coliform2	12.89
Coliform3	10
Coliform5	17.94
Coliform6	10

Table 14: Equation 1 with values of Table 11 in the 4 reactors.

Coliform2	y= -1.6072(12.89)^3+93.02(12.89)^2-1597.5(12.89)+9200
Coliform3	y= -1.5588(5)^3+92.825(5)^2-1684.5(5)+9200
Coliform5	y= -1.6072(12.89)^3+93.02(12.89)^2-1597.5(12.89)+9200
Coliform6	y= -0.5487(5)^3+33.27(5)^2-620.83(5)+3500

Table 15: Coliform degradation of the four reactors in NMP/100 ml.

Coliform2	621.55 NMP/100 ml
Coliform3	2903.15 NMP/100 ml
Coliform5	613.9 NMP/100 ml
Coliform6	1159.02 NMP/100 ml

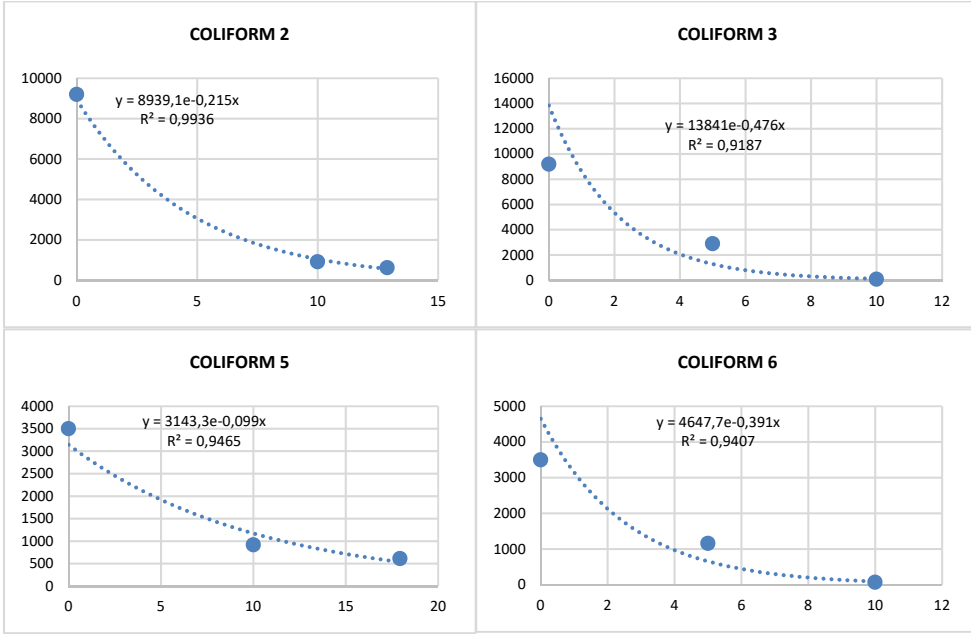

Figure 7: Exponential degradation curve in the Coliform2, Coliforms3, Coliforms5 and Coliforms6 reactors.

the bacteria dosage was used. Reactor Coliform5 with a dilution of 25% in the concentration of wastewater, 100% of bacteria dosage was used with an initial concentration of 3,500 NMP/100 ml. At day 10, there was a reduction in the coliforms with a value of 920 NMP/100 ml. At day 17 reached a final degradation of 613.9 NMP/100 ml. These results were favorable because a reduction of the coliforms in the dilution in the pollutant concentration were possible, however the time to reach this result is too long. Reactor Coliform6, concentration in the wastewater had a dilution of 25%, degradation at day 10 had a value of 70 NMP/100 ml. After this period a value of zero was recorded. The adjusted

exponential model only considered until day 5 with a value of 1,159.02 NMP/100 ml. The results from this reactor are very favorable, only a 75% of the bacteria dosage was used getting a higher level of degradation than reactor Coliform5 therefore, lower amount of wastewater concentration, faster degradation by the action of anaerobic bacteria.

3.3 Discussion

In the samples of wastewater that is used as a means for the identification of anaerobic bacteria, the necessary conditions for the growth of these bacteria are observed, this condition is evaluated by means of the laboratory techniques used. In similar studies such as Cyprowski et al. [24], it is analyzed that in the processes of wastewater treatment that have the anaerobic component, the presence of these bacteria will exist, for this research the objective is to validate those studies in wastewater present in Andean areas and use that type of bacteria to improve the degradation process of organic matter. The method of degradation of organic matter in anaerobic environments has certain limitations due to the performance of micro-organisms to degrade organic matter under these conditions, but the advantage is that it has the potential to obtain methane [25], with this premise when validating the presence of methane-producing bacteria the process is viable with possible subsequent uses.

In the treatment applied in the waters, in the organic matter, this is measured as COD and BOD, and that the anaerobic conditions are favored in the experiments, the bacteria characterized and inoculated, their metabolism [16], [19], in the analyzed data observe the degradation of organic matter in the set of parameters analyzed with cell retention times (CRT) of 15 days until its endogenous consumption begins.

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