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Biomethane Production and Methanogenic Microbiota Restoration After a pH Failure in an Anaerobic Sequencing Batch Reactor (A-SBR) Treating Tequila Vinasse

Adriana Serrano-Meza [1], Iván Moreno-Andrade [2], Edson B. Estrada-Arriaga [3], Sergio A. Díaz-Barajas [1], Liliana García-Sánchez [3] and Marco A. Garzón-Zúñiga [1,*]

[1] Laboratorio de Evaluación, Desarrollo e Innovación de Tecnología del Agua, CIIDIR-Durango, Instituto Politécnico Nacional, Sigma 119, 20 de Noviembre II, Durango 34220, Mexico; adriana.serrano.meza@gmail.com (A.S.-M.); chechoblaze@gmail.com (S.A.D.-B.)

[2] Laboratory of Research on Advanced Processes for Water Treatment, Juriquilla Academic Unit, Institute of Engineering, National Autonomous University of Mexico, Blvd. Juriquilla 3001, Querétaro 76230, Mexico; imorenoa@ii.unam.mx

[3] Subcoordinación de Sistemas de Saneamiento y Reutilización de Aguas Residuales, Instituto Mexicano de Tecnología del Agua, Paseo Cuauhnáhuac 8532, Jiutepec 62550, Mexico; edson_estrada@tlaloc.imta.mx (E.B.E.-A.); liliana_garcia@tlaloc.imta.mx (L.G.-S.)

* Correspondence: mgarzon@ipn.mx; Tel.: +52-618-814-2091 (ext. 82604)

Abstract: Precise control of operational parameters in anaerobic digestion reactors is crucial to avoid imbalances that could affect biomethane production and alterations in the microbiota. Restoring the methanogenic microbiota after a failure is essential for recovering methane production, yet no published strategies exist for this recovery. In this study, we restored the methanogenic microbiota in an anaerobic SBR reactor that operates with both biofilm and suspended biomass simultaneously, aiming to treat tequila vinasses. Four strategies were evaluated for restoring the methanogenic microbiota: reducing the initial vinasse concentration, increasing the reaction time (RT), adjusting the carbon/nitrogen (C/N) ratio, and progressively increasing the initial vinasse concentration. Among these, adjusting the C/N ratio emerged as a critical parameter for restoring organic matter removal efficiency and reestablishing methanogenic microbiota. The operational conditions under which the methanogenic activity and microbiota were restored were as follows: Operating the A-SBR with an initial vinasse concentration of 60%, an RT of 168 h, a pH of 6.9 ± 0.2, a temperature of 35 ± 2 °C, and a C/N ratio adjusted to 100/1.9 resulted in stable COD removal efficiency of 93 ± 3% over a year and a high percentage of methanogenic microorganisms in both the suspended microbiota (69%) and biofilm (52%). The normalized methane production (0.332 NL CH_4/g CODr) approached the theoretical maximum value (0.35 L CH_4/g CODr) after restoring the population and methanogenic activity within the reactor.

Keywords: bioenergy; biogas; biomethane; methanogenesis; anaerobic digestion; suspended microbiota; biofilm; tequila vinasses

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1. Introduction

Currently, the use of fossil fuels poses a problem due to the pollution caused by their extraction and use. The utilization of fuels such as hydrogen and methane derived from organic waste represents a sustainable energy alternative. These biogases can be obtained separately in anaerobic systems with different microbial communities, each requiring specific operating parameters for optimal production. For methane production, complete anaerobic digestion is necessary, with high hydraulic retention times (HRTs) of 4 days or more [1], a pH of 7–7.5 [2], and a carbon/nitrogen (C/N) ratio of 100/2.5 [3]. Among these parameters, pH is particularly important, as it can influence the microbiota generated in anaerobic digestion (AD). The highest methane production has been observed at a neutral

pH (7–7.5) [2]. A decrease in *Archaea* populations has also been observed when pH has risen above 8.5, along with resistance in the *Clostridium* genus at these pH values [4].

According to Bi et al. [1], operating with an HRT of 5–7 days favors CH_4 production, as it allows the microbiota more time to degrade a higher concentration of volatile fatty acids (VFAs) produced during acidogenesis before their complete transformation into methane. This increase in HRT can also enhance the degradation of organic matter (OM) within the system. As HRT increases, the efficiency of organic matter removal increases as well [5–8].

Microbial composition is also affected by HRT variations. For instance, Jiang et al. [9] observed a decrease in methanogenic species when HRT was reduced from 5 to 1 day. In contrast, authors such as Chen et al. [10] found that fermentative species like *Clostridium* predominated when operating an up-flow anaerobic sludge blanket (UASB) reactor with an HRT of less than one day.

The C/N ratio is another important parameter in anaerobic digestion. Carbon and nitrogen serve as essential organic substrates for this process: carbon influences the type of fermentation that occurs, while nitrogen is vital for microbial growth [3]. A high C/N ratio leads to rapid nitrogen consumption by acidogenic bacteria, leaving insufficient nitrogen available for methanogenic *Archaea* [11]. Conversely, a low C/N ratio results in a carbon shortage, leading to minimal acid formation, accumulation of NH_4^+, and an increase in pH [12]. Therefore, balancing the C/N ratio is important in anaerobic digestion, as it affects both organic matter degradation and biogas composition.

Tequila vinasses, a byproduct of tequila production, are liquid waste characterized by high organic content, including COD (25–100 g/L), total solids (20–50 g/L), low pH (3.4–4.5), and low concentrations of phenols (0.04–0.08 g/L) and salts (0.45–2.05 g/L). Additionally, they have a high C/N ratio (100/0.64) [13–15]. The high organic content and the low concentration of inhibitory compounds make tequila vinasses a potential substrate for methane production, even if an addition of nitrogen could be necessary to balance the C/N ratio.

In addition to presenting an environmental challenge due to its high organic load, tequila vinasse has considerable potential as a substrate for methane production via anaerobic digestion. As a byproduct of the tequila industry, it is produced abundantly; according to the Tequila Regulatory Council, 598 million liters of tequila were produced in 2023. For every liter of tequila, 10 to 15 L of vinasse are generated, equating to 5,980,000 to 8,970,000 m^3 annually [16].

However, toxic compounds present in tequila vinasse, particularly at elevated concentrations, have been shown to have adverse effects on methanogenic microorganisms in anaerobic digestion systems [17]. These compounds include, among others, high levels of organic compounds, volatile fatty acids, and ammonium, which can inhibit the growth and activity of the methanogenic *Archaea* responsible for methane production [18]. Over the long term, continuous exposure of methanogenic microorganisms to these compounds can cause permanent alterations in the microbial community of the reactor, complicating the recovery of the methanogenesis process. In this context, recent research suggests that manipulating operational parameters, such as adjusting the C/N ratio and hydraulic retention time (HRT), can stabilize the anaerobic digestion system [19,20]. However, when vinasse concentrations exceed 60%, severe inhibition of methanogenic activity has been observed due to the accumulation of toxic compounds such as volatile fatty acids and ammonium.

Sequential Biological Reactors (SBRs) have shown promise in generating biomethane from organic residues like tequila vinasses. Their construction requires a lower investment compared to continuous flow reactors, and they offer operational flexibility for varying influent flows [21]. Given these advantages, SBRs demand meticulous control over operating parameters such as organic load, temperature, pH, RT, alkalinity, and C/N ratio. Automated control is crucial in maintaining balance within SBRs; without it, the process can suffer from imbalances, such as VFA accumulation, pH fluctuations, and operation at suboptimal temperatures.

Imbalances in anaerobic digestion systems can lead to failures at various process stages, ultimately affecting methane production, as observed by Jeganathan et al. [22] and Hegde and Trabold [23]. They noted a 57% and 50% decrease in methane production, respectively, when operating a reactor treating food waste under high organic loads. This led to the accumulation of VFAs and a decrease in pH, resulting in overall failure characterized by severe sludge flotation and microbiota washout. Similarly, Sun et al. [24] found that in a reactor treating pig manure, an increase in VFA concentration caused methane content to decrease from 70% to 50%. Gupta et al. [25] also observed a decrease in methane proportion from 46% to 17% with an increase in the concentration of certain compounds, such as chlorophenol, in a glucose-fed reactor. Shi et al. [26] reported that increasing ammonium nitrogen concentration led to a reduction in methane content from 68% to 54% when working with food waste.

When it comes to the treatment of tequila vinasses, there are limited reports on failures in the anaerobic digestion process and their impact on biogas production. Arreola-Vargas et al. [27] documented a decrease in methane proportion in biogas from 90% to 75% when treating tequila vinasses due to pH and temperature variations (pH values from 7 to 8 and temperature from 32 °C to 38 °C). Jáuregui-Jáuregui et al. [28] reported a decrease in methane production from 0.319 to 0.268 NL CH_4/g CODr (a 16% reduction) after stopping feeding the reactor treating tequila vinasses for 6 months and then restarting it.

However, none of the studies reporting a decrease in biomethane production due to uncontrolled operational parameters provide information on specific operational actions to reverse the failure or demonstrate system recovery and biomethane production. There is a notable absence of published strategies for recovering methanogenesis after such failures. It is important to highlight that these imbalances can promote the growth of certain microbiota types over others, leading to a shift in microbial composition that significantly reduces biomethane production. Consequently, this study suggests that corrective actions must be implemented to restore the methanogenic microbiota and mitigate the effects of such failures. Despite this need, there is limited published evidence demonstrating the efficacy of various operational strategies in recovering the population of methanogenic microorganisms and biomethane production following a failure caused by pH fluctuations and the predominance of acidogenic microorganisms.

Thus, the aim of this study was to assess which operational parameters require adjustment and how to facilitate the transition from a predominantly acidogenic microbiota (H_2-producing) to a balanced microbiota inclusive of methane-producing *Archaea* in an A-SBR system following acidification-induced failure when treating tequila vinasses.

2. Materials and Methods

2.1. Transitioning a Methanogenic Microbiota to an Acidogenic One Within an A-SBR

To evaluate the recovery of methanogenic microbiota in a reactor containing both suspended microbiota and biofilm, microorganisms were subjected to an alkaline pH shock exceeding 11 pH units. This condition inhibits methanogenic microorganisms while favoring the presence of hydrogen producers from the *Clostridium* genus [29]. This experiment was conducted in a 4.17 L working volume anaerobic SBR with a 3 L exchange volume. The reactor was inoculated with sludge containing both acidogenic and methanogenic microorganisms and operated under stable conditions. These conditions included a volumetric organic loading rate (OLR) of 12.6 $\pm$ 1.3 kg COD/m^3 d, a temperature (T) of 35 $\pm$ 2 °C, a carbon/nitrogen ratio (C/N) of 100/0.5 (corresponding the C/N ratio of vinasse with no additional compounds added), and a pH of 6.9 $\pm$ 0.2. Each treatment cycle lasted 2 days, including a filling phase (0.025 d), a reaction time phase (1.9 d), a sedimentation of suspended microbiota phase (0.025 d), and an empty or draining effluent phase (0.05 d), favoring the suspended microbiota and biofilm to remain inside the reactor for the following treatment cycle. An automatic adjustment system with Master Flex 751800 peristaltic pumps (Cole-Parmer, Vernon Hills, IL, USA), was used to maintain pH, with a 20% NaOH

(weight-based) solution and H_2SO_4 acid solution. The reactor was fed with tequila vinasse, achieving a COD removal efficiency of 76 ± 5% under these conditions.

On the 59th day of operation, an alkaline pH shock was administered to the reactor by adding a 20% NaOH (weight-based) solution until reaching a pH of 12 for 3 h. Afterward, the basic pH vinasse was drained, and a new operational cycle commenced with vinasses at a pH of 6.9 ± 0.2, operating under regular conditions. The effect of the alkaline pH shock on COD removal efficiency was monitored, and microbial identification was conducted to assess the impact.

Microbiota Characterization Before and After the Basic pH Shock

At the start-up of the anaerobic SBR, a sample of sludge previously acclimated to tequila vinasse was collected. The basic pH shock took place on day 59, followed by the collection of a microbiota sample twelve days later, after six operation cycles, to monitor changes in microbial composition. Microbiota samples underwent analysis using an Illumina MiSeq system (Illumina, San Diego, CA, USA), following the protocol outlined by Serrano-Meza et al. [20].

2.2. Strategies for Restoring the Methanogenic Microbiota in the Reactor

2.2.1. Decreasing the Initial Concentration of Vinasse

After confirming the impact of the pH shock on both the microbiota composition and COD removal efficiency, the first strategy was implemented. This involved reducing the initial concentration (IC) of vinasses from 55 to 40% using tap water. This reduction resulted in a decrease of 6881 mg COD/L d and 3 kg COD/m^3 d of OLR (Table 1). Operating conditions, including reaction time (RT), temperature, (C/N) ratio, and pH remained consistent with those of the methanogenic reactor. The reactor was then monitored for 26 operation cycles (52 days) to assess COD removal efficiency under these conditions.

Table 1. Operation stages with their corresponding microorganism identification, volumetric organic loading rate (VOLR), influent and effluent COD, and COD removal efficiency.

Stage	Operating Conditions	Sampling Operation Day	Microorganism Communities	OLR (kg COD/m^3 d)	COD Influent (mg/L)	COD Effluent (mg/L)	COD Removal Efficiency (%)
Start-up	T = 35 ± 2 °C pH = 6.9–7.9 RT = 48 h C/N = 100/0.5 IC = 55%	Inoculum	Suspended microbiota *Clostridium* (20%) *Actinomyces* (6%) *Bacteroides* (7%) *Methanobacterium* (6%) *Desulfovibrio* (2%)	13 ± 1.4	25,096 ± 2844	5145 ± 1066	79 ± 4
Stable operation of A-SBR before pH shock	T = 35 ± 2 °C pH = 6.9–7.9 RT = 48 h C/N = 100/0.5 IC = 55%			12 ± 2.7	23,033 ± 2124	6616 ± 140	71 ± 2
A-SBR after pH shock > 11	T = 35 ± 2 °C pH = 6.9–7.9 RT = 48 h C/N = 100/0.5 IC = 55%	71	Suspended microbiota *Clostridium* (17%) *Bacteroides* (19%) *Desulfovibrio* (1.88%)	11 ± 1.3	21,902 ± 2772	7238 ± 1413	65 ± 6
A-SBR after decrease in IC of vinasse	T = 35 ± 2 °C pH = 6.9–7.9 RT = 168 h C/N = 100/0.5 IC = 40%			8 ± 0.5	15,021 ± 1028	6519 ± 344	56 ± 3
A-SBR after extension of RT		158	Suspended microbiota *Clostridium* 43% *Bacteroides* 21% Biofilm *Clostridium* 43% *Bacteroides* 4% *Methanosarcina* 9% *Desulfovibrio* 7%	2.3 ± 0.5	16,503 ± 3574	9196 ± 445	41 ± 11

Note: IC: initial concentration of vinasses.

2.2.2. Extension of Reaction Time (RT)

After decreasing the initial concentration of the vinasses and operating the anaerobic sequencing batch reactor (A-SBR) with a volumetric organic loading rate (OLR) of 8 ± 0.5 kg COD/m^3 d, the next step was to assess the impact of extending the reaction time (RT), which was prolonged from 48 to 168 h on day 100 of operation. The operating conditions were as follows: RT = 168 h, T = 35 ± 2 °C, IC = 40%. This adjustment in the RT resulted in a decrease in OLR to 2.3 ± 0.5 kg COD/m^3 d. Under these conditions, the reactor was monitored for 11 operation cycles (equivalent to 77 days) based on its COD removal efficiency. On cycle 9 (day 158) of operation, sludge samples were extracted to determine the microbial composition. Kinetic analyses of volatile fatty acid (VFA) degradation and COD were also performed to assess the reactor's performance.

2.2.3. Carbon/Nitrogen Ratio Adjustment

Before the pH shock failure, the reactor operated efficiently with a carbon/nitrogen (C/N) ratio of 100/0.5. However, this ratio fell below the reported optimal value (100/3.8). Therefore, the strategy at this stage was to increase the nitrogen concentration in the system. Four stages were tested for this purpose:

I. Addition of 2.4 g/L of NH_4Cl at the beginning of each operation cycle for 2 cycles.
II. Addition of 2.4 g/L of NH_4Cl at the beginning, plus an additional 2.4 g/L shot at 72 h into the operation cycle for 4 cycles.
III. Addition of 1.3 g/L of $CO(NH_2)_2$ for 2 operation cycles.
IV. Addition of 1.1 g/L of NH_4Cl for 11 operation cycles.

The removal of chemical oxygen demand (COD) was monitored to evaluate these stages.

2.2.4. Increase in the Initial Concentration of Vinasses

During this phase, the initial vinasse concentration increased from 50% to 65% across three stages, with incremental rises of 5% at each step. The efficiency of chemical oxygen demand (COD) removal was closely monitored throughout these stages. The increments in vinasse concentrations were continued until they began to adversely affect COD removal efficiency. Consequently, reactor operation was reverted to the previous concentration level, which had demonstrated the highest removal efficiency.

2.3. Statistical Analysis

The COD removal at various stages was subjected to statistical analysis using Analysis of Variance (ANOVA), Tukey's test, and a *t*-test, conducted with GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA) at a confidence level of 95%.

2.4. Physicochemical and Metagenomic Analysis

The evaluation of reactor performance included the measurement of physicochemical parameters such as chemical oxygen demand (COD) and alkalinity, conducted according to standard methods [30]. Additionally, volatile fatty acids (VFAs) were analyzed using the HACH™ protocol [31], and redox potential was measured using an ISE Orion (Thermo Scientific, Waltham, MA, USA).

2.5. Biogas Production and Composition

Biogas volume was measured using the volumetric method as described by Serrano-Meza et al. [20]. Biogas samples were collected individually in tightly sealed 20 mL glass bottles. The gas content and percentage were analyzed using an SRI 8610 gas chromatograph (Torrance, CA, USA), equipped with a thermal conductivity detector and a molecular sieve 13X column (Oude-Tonge, The Netherlands) with an internal diameter of 2.1 mm, a length of 6 ft, and a mesh size of 80/100. Chromatographic-grade helium served as the carrier gas, with 5 mL of the sample injected using a microsyringe. The measurement was conducted under optimized conditions reported by García-Sánchez [32]: run time: 5.5 min,

carrier gas flow rate: 20 mL/min, methane peak: 3.8 min, and temperature ramp initiated at 50 °C for 2 min, followed by a 20 °C per minute increment until reaching 120 °C.

3. Results and Discussion

3.1. Impact of Alkaline Shock on COD Removal and Methane Production

During the start-up period of A-SBR operation, spanning day 1 to day 29, the COD removal efficiency reached 79%. The biogas generated during this phase consisted of 56% CH_4, 0% H_2, and 43% CO_2, with a biomethane production rate of 0.07 NL CH_4/h and a yield of 0.31 NL CH_4/g CODr. The microbiota used for system inoculation included both acidogenic and methanogenic microorganisms (Table 1).

Throughout the stabilization stage, before the alkaline shock, from day 30 to day 54, the reactor maintained an average COD removal efficiency of 71 ± 2%, with the effluent showing minimal variation (6616 ± 140 mg/L) (Figure 1, Table 1). However, on day 59 of operation, following the administration of an alkaline pH shock, the COD removal efficiency decreased to 57% in subsequent cycles (Figure 1, Table 1). This decline marked a significant difference from the stable operational stage, as confirmed by the *t*-test ($p = 0.147$).

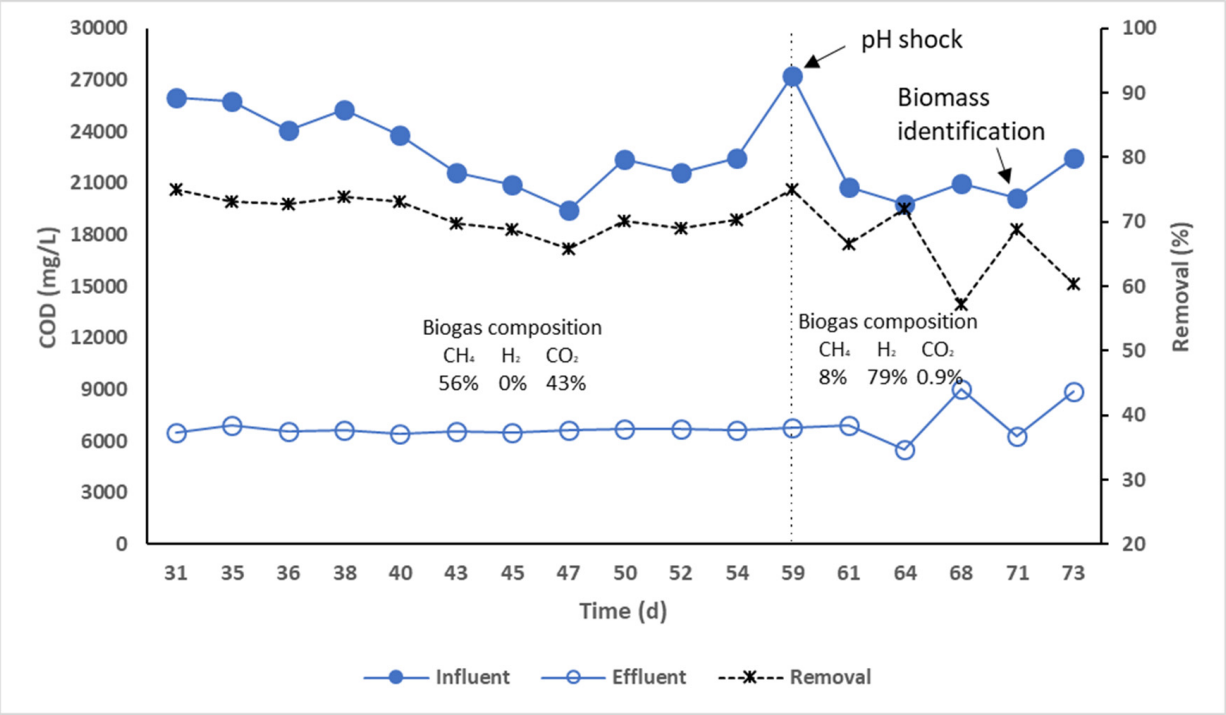

Figure 1. COD behavior during the transition from a methanogenic microbiota to an acidogenic one within an A-SBR.

In terms of biogas composition, it consisted of 8% CH_4, 0.9% CO_2, and 79% H_2, with a biomethane production rate of 0.00003 NL CH_4/h and a biomethane yield of 0.0107 NL CH_4/g CODr. Microorganism identification conducted on day 71 of operation, 12 days after the alkaline pH shock, revealed a notable impact on methanogens. Specifically, *Methanobacterium*, which comprised 6% of the population in the A-SBR before the pH shock, disappeared (Table 1).

While the percentage of acidogenic microorganisms remained stable, the proportions of certain populations varied (*Clostridium* 17%, *Bacteroides* 19%) (Table 1). These changes reflect the resilience of fermentative microbial communities to abrupt pH changes. The loss of methanogenic microorganisms likely contributed to the decrease in organic matter removal. The predominance of acidogenic microorganisms thereafter facilitated degrada-

tion of organic matter only to the production of volatile fatty acids, without proceeding to methanogenesis—a stage where greater degradation is achieved [33]. This shift aligns with the observed drastic decrease in biomethane production.

3.2. Evaluation of Strategies for Restoring the Methanogenic Microbiota

3.2.1. Decreasing the Initial Concentration of Vinasse

The initial vinasse concentration was reduced from 55% to 40% as a primary strategy to mitigate the presence of toxic compounds in the reactor and facilitate the recovery of the methanogenic microbiota. Additionally, the influent COD decreased from 21,902 ± 2772 to 15,021 ± 1028 mg/L. During this period, the reactor maintained conditions conducive to methanogenesis, with a pH of 6.9 ± 0.2 and a temperature of 35 ± 2 °C.

Despite these changes, the removal efficiency did not recover; instead, it declined compared to the previous stage. The average effluent concentration during this phase was 6519 ± 344 mg/L, resulting in a removal efficiency of 56 ± 3% (Table 1 and Figure 2). These results exhibited a statistically significant difference, as per the *t*-test, compared to the removal efficiency observed during the pH shock effect stage ($p = 0.0020$) and the initial stable methanogenesis stage ($p < 0.0001$). One potential factor contributing to the failure of recovering methanogenic activity and organic matter removal efficiency is the reaction time (RT). If the RT is shorter than the doubling time of acetoclastic methanogens, they cannot establish themselves in the reactor. Therefore, it was decided to evaluate the effect of increasing the RT.

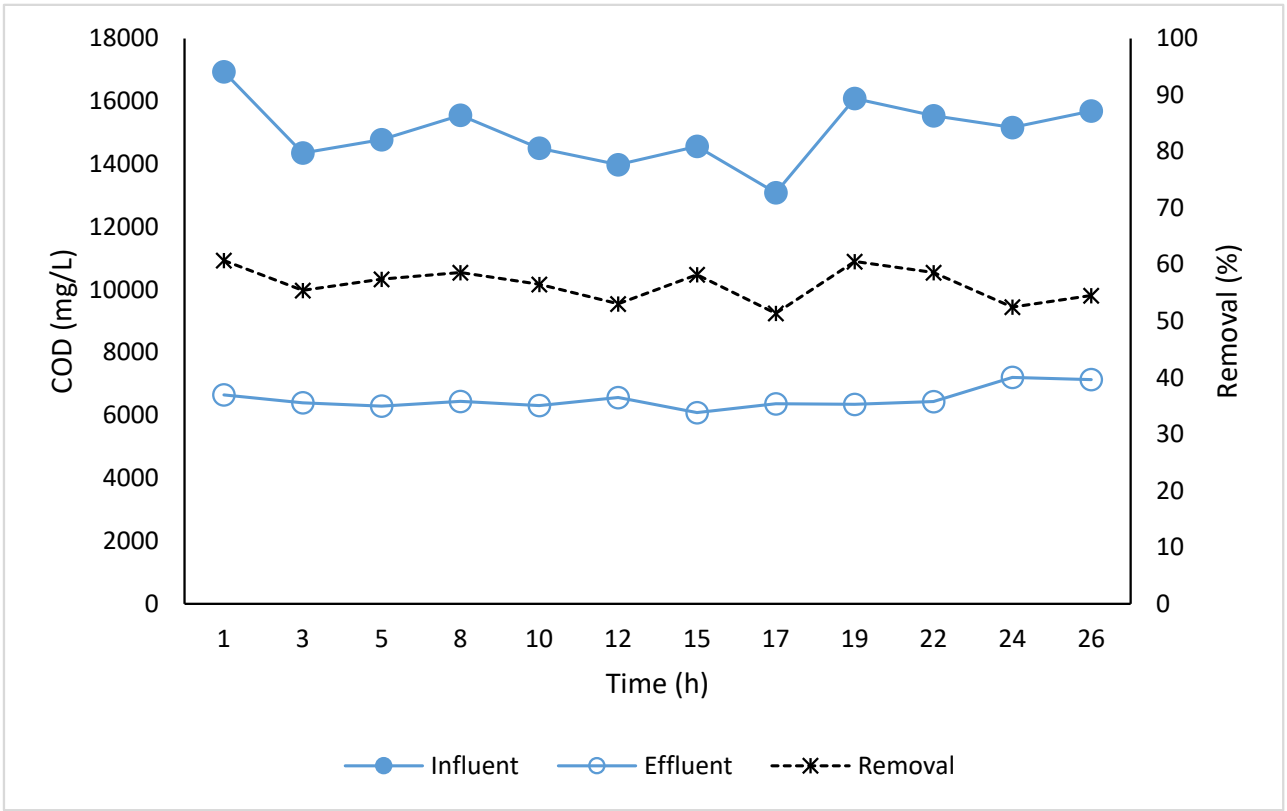

Figure 2. COD behavior when decreasing the initial concentration of vinasse from 55 to 40%.

3.2.2. Extension of Reaction Time (RT)

To allow sufficient time for the duplication of methanogenic microorganisms, which typically exceeds 2 days [34], and taking into account the findings by Alvillo-Rivera et al. [35], who achieved a 72% removal efficiency in treating tequila vinasses using an up-flow

anaerobic reactor with a 40% vinasse concentration and a reaction time (RT) of 7.16 days, the RT was extended to 7 days (168 h).

During this operational phase, the system's performance exhibited significant instability, with organic matter removal efficiencies fluctuating between 24% and 65% (Figure 3). This variability may be attributed to prolonged microorganism exposure to toxic compounds present in the vinasses, coupled with the microorganisms' incomplete recovery from the effects of the pH shock. Microorganism analysis conducted on day 36 of operation of this operational stage, revealed an increase in acidogenic microorganisms, particularly *Clostridium*, which accounted for 43% of the abundance in both the biofilm and suspended microbiota of the A-SBR. Additionally, *Methanosarcina* was observed in the biofilm, constituting 9% of the microbial community (Table 1). These findings suggest a partial recovery of methanogenic activity, despite COD removal rates remaining consistent at 40–45% (Table 1 and Figure 3).

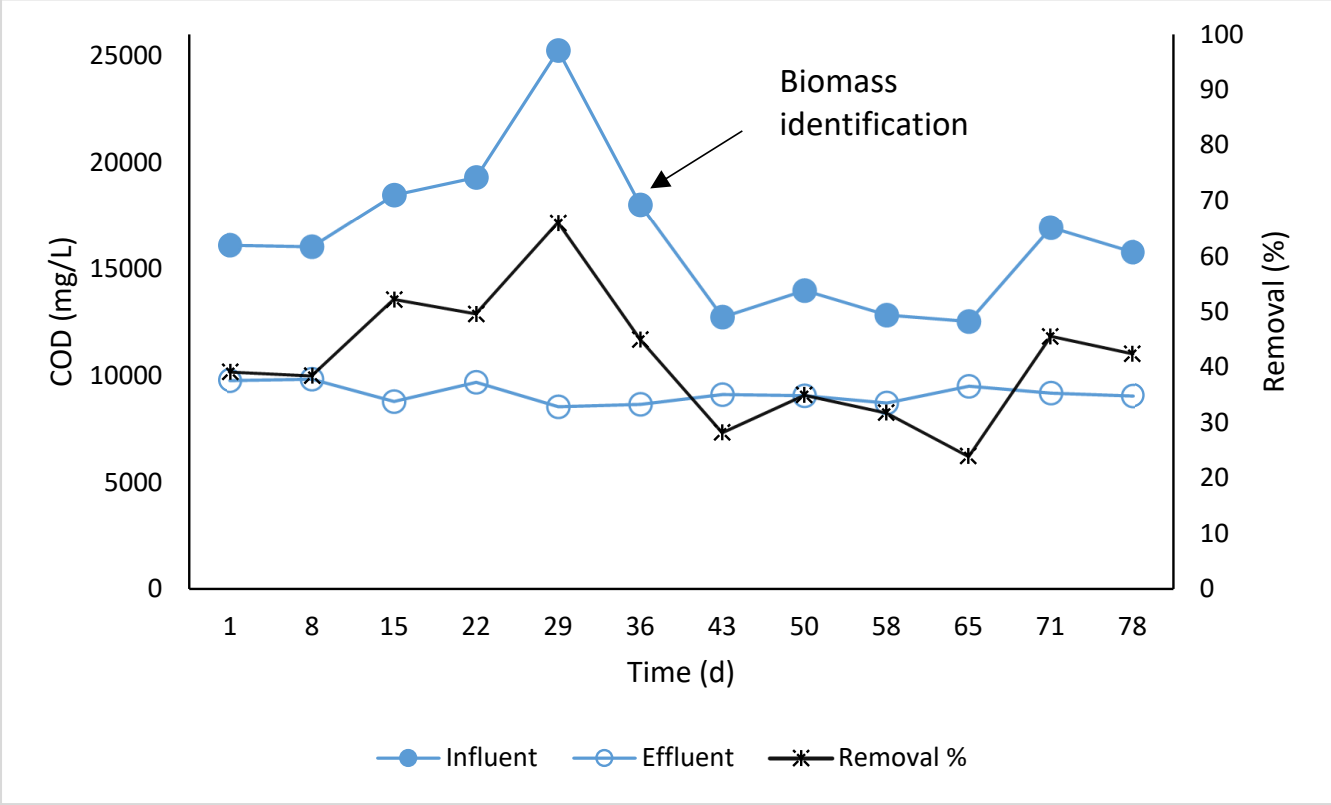

Figure 3. COD behavior with the increase of reaction time to 168 h.

When analyzing the kinetics of COD removal and volatile fatty acid (VFA) production (Figure 4) in the reactor operating under the specified conditions (IC = 40%, RT = 168 h, and OLR = 2.3 ± 0.5 kg COD/m^3 d), several observations were made. Initially, organic matter removal reached 5122 mg COD/L within the first 48 h, coinciding with an increase in VFA concentration from 2083 to 4756 mg/L (Figure 4). However, from 48 to 168 h, the rate of COD removal decelerated, resulting in an additional 2185 mg COD/L being removed (Figure 4). During this period, the VFA concentration decreased to a level almost equivalent to the initial concentration (2086 mg/L).

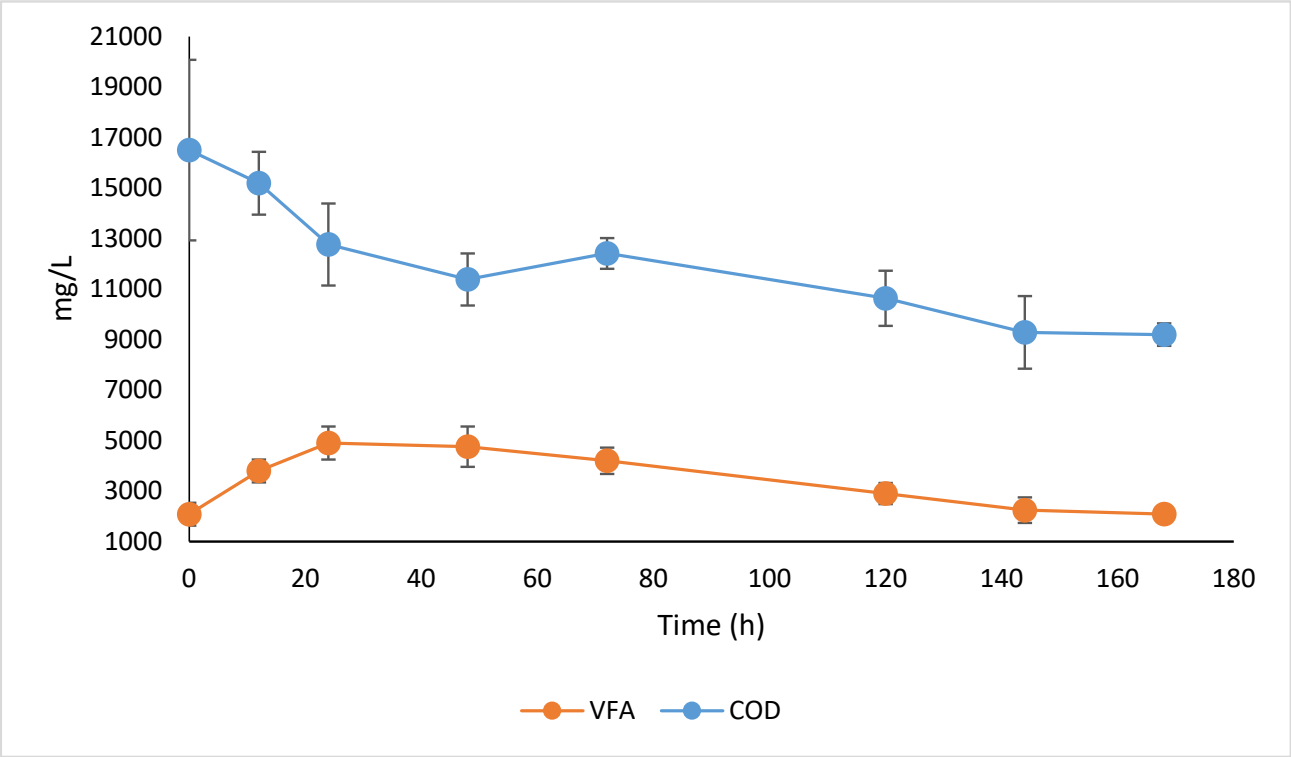

Figure 4. Degradation of VFA and COD in the operational cycles of increasing reaction time to 168 h.

After 48 h, VFA removal indicates limited methanogenic activity. This coincides with the emergence of a small population of methanogens in the biofilm, as previously mentioned. A possible explanation for this limited methanogenic activity, and consequently the modest removal of organic matter, with an average COD removal of 41 ± 1% (Table 2), could be a nutrient imbalance. Consequently, the carbon/nitrogen (C/N) ratio within the reactor was examined. The observed removal efficiency at this stage (41%) demonstrates a significant difference, as determined by the *t*-test (p = 0.0003), compared to the stage of initial vinasse concentration decrease (56%) and the stable methanogenic stage (71%) ($p < 0.0001$).

Table 2. Operational stages for C/N ratio adjustment in an anaerobic SBR treating tequila vinasses: compound addition, COD concentrations, and removal efficiency.

Stage	Added Compound	Concentration (g/L)	Influent COD (mg/L)	Effluent COD (mg/L)	Removal (%)
0	Without addition	0	16,503 ± 3574	9196 ± 445	41 ± 11
I	NH_4Cl	2.4	20,438 ± 811	7267 ± 1195	64 ± 4
IIa	NH_4Cl	4.6	19,133 ± 1288	9105 ± 2438	51 ± 16
IIb	NH_4Cl	0	19,686 ± 279	8840 ± 2661	55 ± 13
III	Urea	1.3	20,094 ± 576	6618 ± 2424	57 ± 13
IV	NH_4Cl	1.1	19,837 ± 931	4674 ± 481	76 ± 2

3.2.3. Adjustment of the Carbon/Nitrogen Ratio

Tequila vinasses had a C/N ratio of 100/0.5, indicating a low nitrogen concentration compared to the optimal ratio (100/3.8) reported by De-Lemos-Chernicharo [36]. Subsequently, an experimental period of C/N ratio adjustment was conducted over 134 days. Different nitrogen compounds, concentrations, and chemical forms were tested in four stages, based on the observed behavior of COD removal. The influent COD concentration remained consistent around 19,867 ± 968 mg/L across the four stages, with a correspond-

ing OLR of 2.84 ± 0.12 kg COD/m^3 d. Nonetheless, COD removal varied among stages (Table 2, Figure 5).

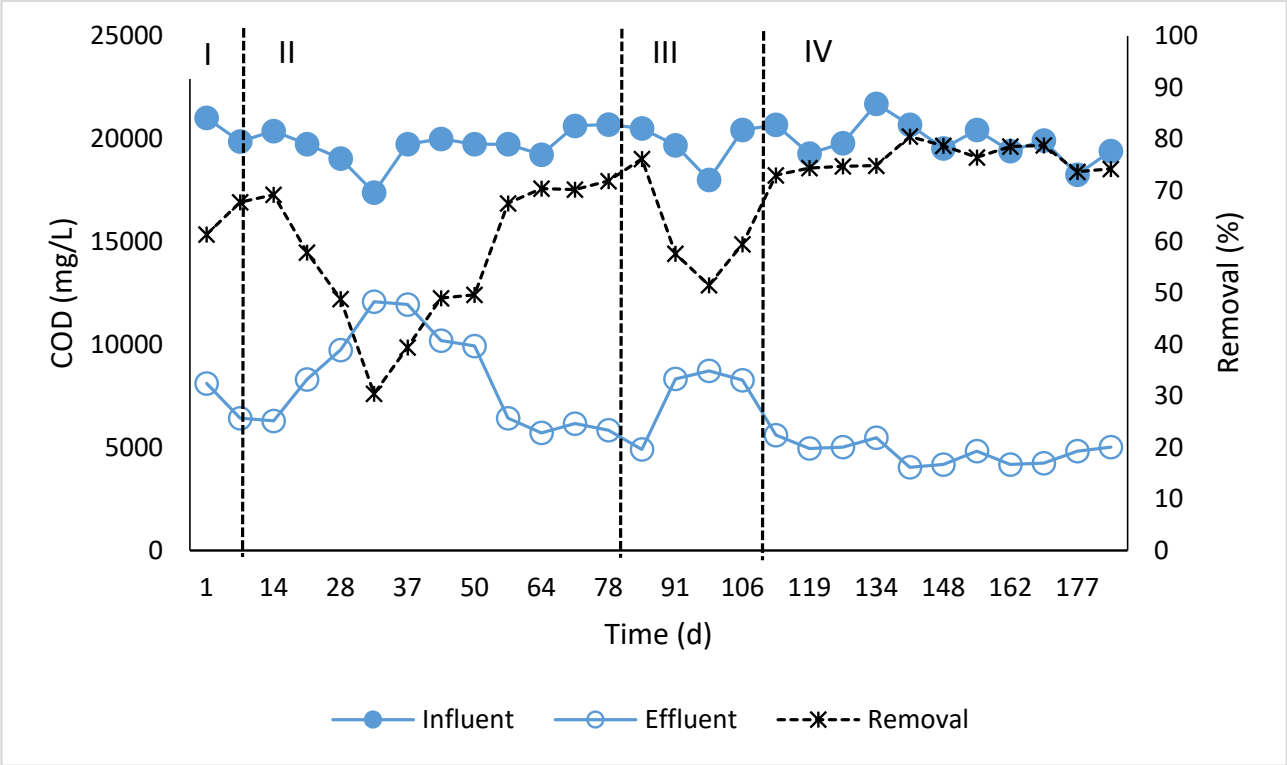

Figure 5. COD behavior during carbon/nitrogen (C/N) ratio adjustment: Stage I—Addition of 2.4 g/L NH_4Cl, Stage II—Two doses of 2.4 g/L NH_4Cl in the first 4 cycles, Stage III—1.3 g/L $CO(NH_2)_2$ in the first 3 cycles, Stage IV—Addition of 1.1 g/L NH_4Cl in all cycles.

Addition of Ammonium Chloride (NH_4Cl)

The addition of NH_4Cl was implemented in stages I, II, and IV, delineated by dashed lines in Figure 5.

In Stage I, 2.4 g/L of NH_4Cl was added to establish the C/N ratio of 100/3.8, considered optimal according to stoichiometric calculations and the literature [36]. Under these conditions, two cycles were conducted, resulting in an improvement in COD removal efficiency from 41 ± 11% to 64 ± 4%. This marks a 21% increase compared to the initial removal efficiency prior to nitrogen addition. Statistical analysis using a *t*-test between Stage I and the RT increment stage (Stage 0 in Table 2) revealed a significant difference (p = 0.0180). Examining the kinetics of average COD removal in these treatment cycles (Figure 6), it was observed that 53% of COD was removed within the first 72 h. However, subsequent removal over the remaining 96 h of the operation cycle was minimal (10%). This raised the question of whether nitrogen remained limiting after 72 h and necessitated a second nitrogen addition. Consequently, in Stage II, during the first 4 cycles, two doses of 2.4 g/L of ammonium chloride were introduced: the first at the beginning of each cycle and the second at 72 h.

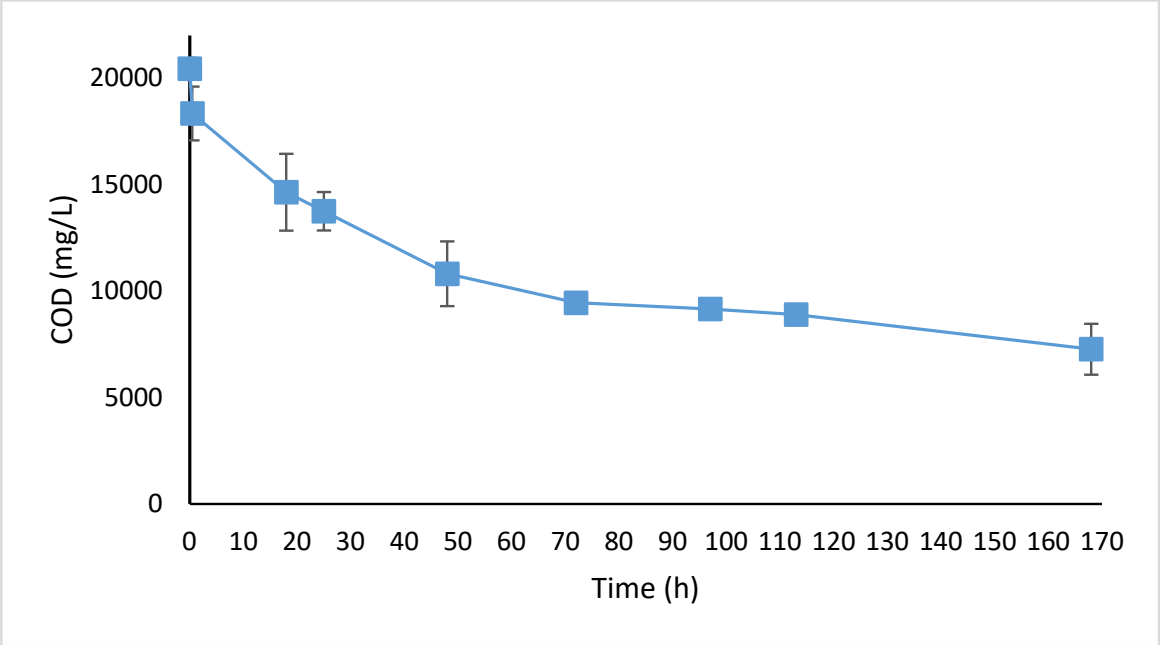

Figure 6. COD degradation in Stage I operational cycles with 2.4 g/L NH4Cl addition.

Under these operating conditions, COD removal efficiency consistently decreased over the subsequent four cycles, reaching 30% (Figure 5, Stage II), which showed a significant difference compared to Stage I (p = 0.0465), according to the Tukey test. Increasing the nitrogen concentration and adding a second dose of NH_4Cl led to an excess of nitrogen by 74% beyond what was needed to balance the C/N ratio. Although ammonium (NH_4) did not reach inhibitory levels, methane-producing microorganisms can tolerate up to 11 g/L of this compound according to Nakakubo et al. [37]. However, the addition of 4.8 g/L of NH_4Cl corresponded to the introduction of 3 g/L of Cl, a concentration well above the reported inhibitory concentration for methane-producing microorganisms of 0.06 g/L [38]. Consequently, the decline in organic matter removal could be attributed to chlorine inhibition. To restore the system, the reactor operated without NH_4Cl addition. After four cycles, the removal efficiency returned to 70% and remained stable for an additional three cycles without nitrogen addition. To reintroduce nitrogen and avoid chlorine inhibition, urea ($CO(NH_2)_2$) was tested to balance the C/N ratio in Stage III.

Urea Addition ($CO(NH_2)_2$)

Urea addition was performed only in Stage III for three operational cycles (Figure 5 and Table 2), where 1.3 g/L of $CO(NH_2)_2$ was added to establish the C/N ratio at 100:3.8 Initially, the removal efficiency improved from 71% to 76%, with a final COD concentration in the effluent of 4904 mg/L. However, in the subsequent two cycles, removal decreased to 58% and 51%, respectively (Figure 5). This reduction in removal efficiency might be attributed to urea, containing oxygen, which has been reported as an inhibitor of methane production at concentrations of 30 nM [39]. Adding 1.3 g/L of $CO(NH_2)_2$ corresponds to adding 3.1×10^7 nM of oxygen, potentially causing inhibition of methane-producing microorganisms and reducing organic matter degradation. Nonetheless, the average COD removal efficiency of Stage III did not significantly differ from Stage II (p = 0.4005), according to the Tukey test.

Observing that the addition of urea did not enhance removal efficiency but instead favored inhibition, the decision was made to reintroduce the missing nitrogen in the form of ammonium chloride in smaller quantities. However, the system was operated for 2 cycles without any nitrogen addition beforehand.

Addition of Ammonium Chloride (NH_4Cl) in Smaller Quantities

After observing a decrease in COD removal efficiency due to the excessive addition of ammonium chloride and urea, and observing good performance in the A-SBR with a lower dosage in Stage I, 1.1 g/L of NH_4Cl was added during eleven operation cycles in Stage IV. This resulted in an average efficiency of 76 ± 2%, which is very similar to the 75% COD removal observed by López-López et al. [17] in a UASB reactor with a methane production of 335 mL CH_4/g CODr from tequila vinasse. However, the A-SBR reactor in this study was operated with an OLR of 2.8 kg COD/m^3 d, slightly higher than the 2.5 kg COD/m^3 d used by López-López et al. [17], indicating that the A-SBR reactor can handle a higher organic load. According to the Tukey test, this stage showed a significant difference (p = 0.0355) compared to the 57% removal efficiency in Stage III.

The results indicate that with the recommended C/N ratio from the literature (100/3.8), a COD removal efficiency of 64 ± 4% was obtained in Stage I, while applying a C/N ratio of 100:1.9 in Stage IV achieved 76 ± 2%. Without balancing the C/N ratio (100/0.5), the maximum efficiency was 41 ± 1%, likely because, with limited nitrogen, acidogenic microorganisms are the first to utilize it [40], and methanogenic microorganisms have no access to nitrogen. Balancing the carbon/nitrogen ratio proved to be a crucial parameter in restoring the efficiency of organic matter removal and methanogenic microbiota. The added nitrogen likely facilitated the formation of new methanogenic microorganism cells, which have a slower growth rate than acidogenic microorganisms in a nitrogen-limited environment. From this point forward, the reactor was operated with a C/N ratio of 100/1.9. To further increase removal efficiency, the organic load applied to the system was increased.

3.2.4. Increase in OLR: Initial Concentration of Vinasse

To enhance the efficiency of organic matter removal, the effect of increasing the initial organic matter concentration, or the volumetric organic load (OLR) was studied. The initial concentration was first increased from 50 to 55%, raising the influent COD by 715 mg/L and decreasing the effluent COD from approximately 3000 to 2500 mg/L. This increased the removal efficiency from 75 ± 3% to 85 ± 2%, showing a significant difference ($p < 0.0001$), according to the Tukey test (Table 3). Subsequently, the initial vinasse concentration was increased to 60% (OLR = 3.3 ± 0.2%), achieving a removal efficiency of 90 ± 2%, significantly different (p = 0.00476) from the 55% initial vinasse concentration. This efficiency is higher than that obtained by López-Rivera et al. [41], who increased the OLR from 4 to 4.9 kg COD/m^3 d in a pilot-scale packed bed reactor treating tequila vinasse, achieving a removal efficiency increase from 87% to 89%. The increase in organic matter combined with the balanced C/N ratio favored the growth of methanogenic *Archaea* and the consumption of organic matter, as demonstrated by the results of this research. Serrano-Meza et al. [42] also reported that adjusting the C/N ratio is an effective strategy for facilitating the recovery and multiplication of microorganisms in anaerobic digestion. However, when the initial vinasse concentration was increased to 65%, the removal efficiency decreased to 82% over six cycles and then exhibited variable behavior (Figure 7), with no significant difference (p = 0.5326) compared to the 60% initial concentration, according to the Tukey test.

Table 3. COD concentration in influent and effluent, and organic matter removal efficiency when increasing initial vinasse concentration and volumetric organic load (OLR).

Initial Vinasse Concentration (%)	OLR (kg COD/m^3d)	Influent COD (mg/L)	Effluent COD (mg/L)	Removal (%)
50	2.8 ± 0.1	19,837 ± 931	4674 ± 481	75 ± 3
55	2.9 ± 0.1	20,552 ± 710	2930 ± 402	85 ± 2
60	3.3 ± 0.2	23,493 ± 1401	2240 ± 591	90 ± 2
65	3.5 ± 0.1	24,496 ± 945	2752 ± 740	88 ± 3
60	3.2 ± 0.2	22,758 ± 1234	1841 ± 411	92 ± 1

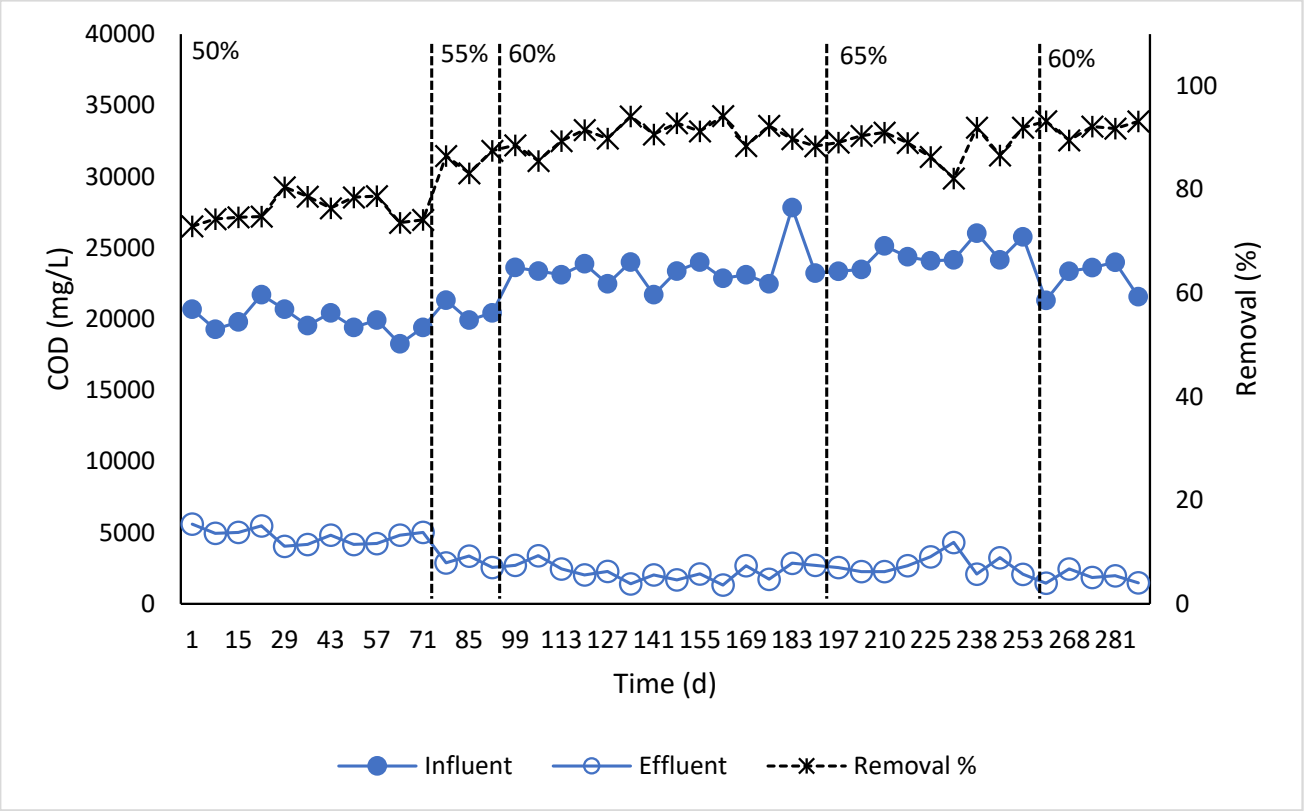

Figure 7. Behavior of COD with increasing initial vinasse concentration from 50% to 60%.

This was likely due to the increased concentration of toxic compounds, which affected the microorganisms and, consequently, their capacity for organic matter removal. As Rocha et al. [43] noted, anaerobic methane production systems tend to fail when the assimilable substrate concentration is exceeded. Therefore, the initial vinasse concentration was reduced from 65% to 60%, allowing the removal efficiency to increase to 92 ± 1%. This concentration represents the maximum vinasse concentration and OLR at which the highest organic matter removal efficiencies were achieved before the system was inhibited by toxic compounds (Table 3).

3.2.5. Operation and Stability of the System Under Optimal Conditions

Under these operating conditions—initial vinasse concentration of 60%, OLR of 3.29 ± 0.22 kg COD/m^3d, temperature of 35 ± 2 °C, pH of 6.9 ± 0.2, and a C/N ratio adjusted to 100:1.9—the reactor operated very stably, achieving an average COD removal efficiency of 93 ± 1.6% over nearly a year (353 days) (Figure 8). This demonstrated a significant difference (p = 0.0070) compared to the 88% COD removal efficiency obtained with an initial vinasse concentration of 65%. Throughout this period, the biogas composition was 60% CH_4, and 40% CO_2, with methane production at 0.075 NL CH_4/h and a yield of 0.33 NL CH_4/g CODr.

The long-term stability of an anaerobic reactor after recovering from a failure was maintained consistently for a year, except for one instance related to an external factor: a temporary failure in temperature control, during which the temperature dropped from 35 °C to 16 °C. This incident resulted in a temporary decrease in removal efficiency to 85% on day 78 (cycle 34) of operation.

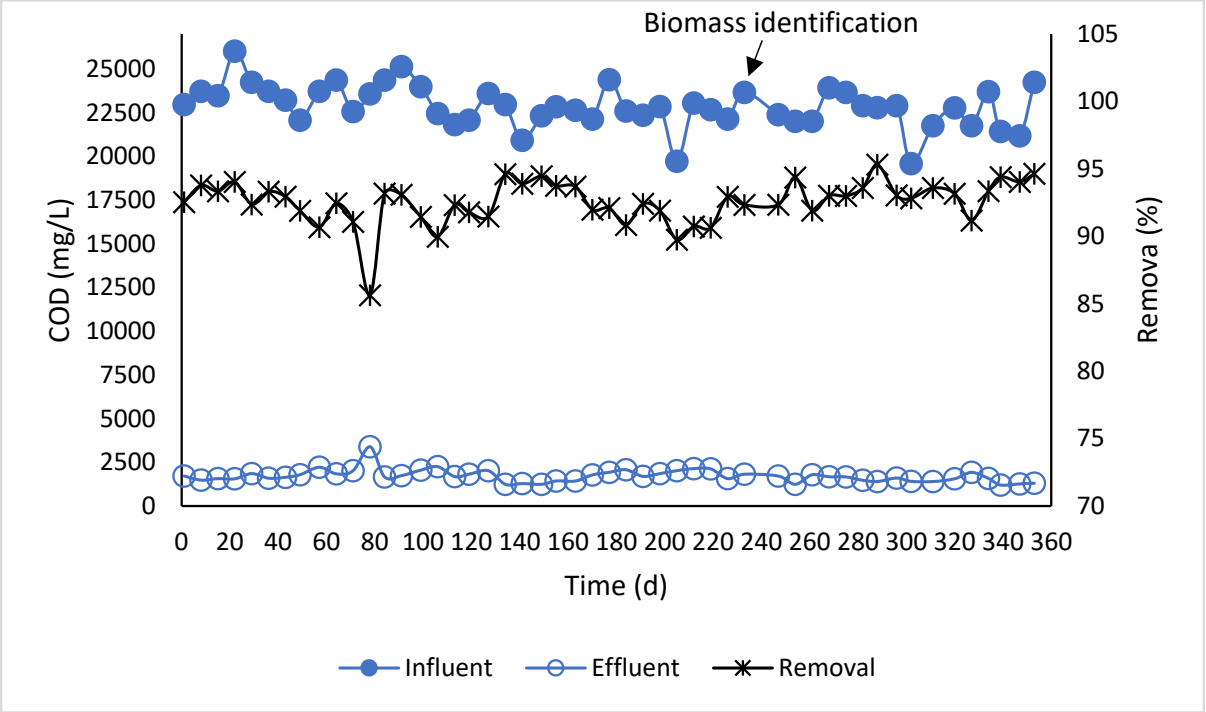

Figure 8. Behavior of COD with optimal conditions (RT = 168 h, initial concentration of vinasse = 60%, C/N ratio = 100/1.9).

On day 233 of operating the system under optimal conditions, a new identification of microorganisms was conducted. It was observed that 69% of the suspended microbiota were methanogenic microorganisms (Figure 9a) and 52% of the microorganisms in the biofilm were methanogenic microorganisms too (Figure 9b). The families observed were *Methanobacteriaceae* and *Methanosaetaceae*. *Methanobacteriaceae* had been previously observed in the A-SBR before the pH shock, with the genus *Methanobacterium* comprising 6%. *Methanosaetaceae* had not been previously observed in the A-SBR at a proportion greater than 1%. *Methanosaetaceae* was found at a proportion of 13% in the biofilm and 2% in the suspended microbiota, indicating that biofilm enhances *Methanosaetaceae* development. The small proportion observed in the suspended microbiota (2%) likely resulted from detachment from the biofilm, consistent with findings by Kim et al. [44], who observed *Methanosaetaceae* in the biofilm of an up-flow anaerobic sludge blanket reactor (UASB), and Morgan-Sugastume et al. [45], who noted this family in moving bed biofilm reactors (MBBR). The increase in the population of methanogenic *Archaea* in the reactor is noteworthy compared to the stage before the pH shock. A balanced population of acidogenic microorganisms (15% in suspended microbiota and 11% in the biofilm) was also observed, including *Carnobacteriaceae*, *Actinomycetaceae*, *Bifidobacteriaceae*, *Ruminococcaceae*, and *Acidaminococcaceae*, which are fatty acid producers [46], facilitating balanced acidogenesis in the anaerobic process. Additionally, microorganisms such as *Desulfovibrionaceae* and *Syntrophobacteraceae* were observed in the biofilm at proportions of 5% and 1%, respectively, corresponding to sulfate-reducing microorganisms [47,48].

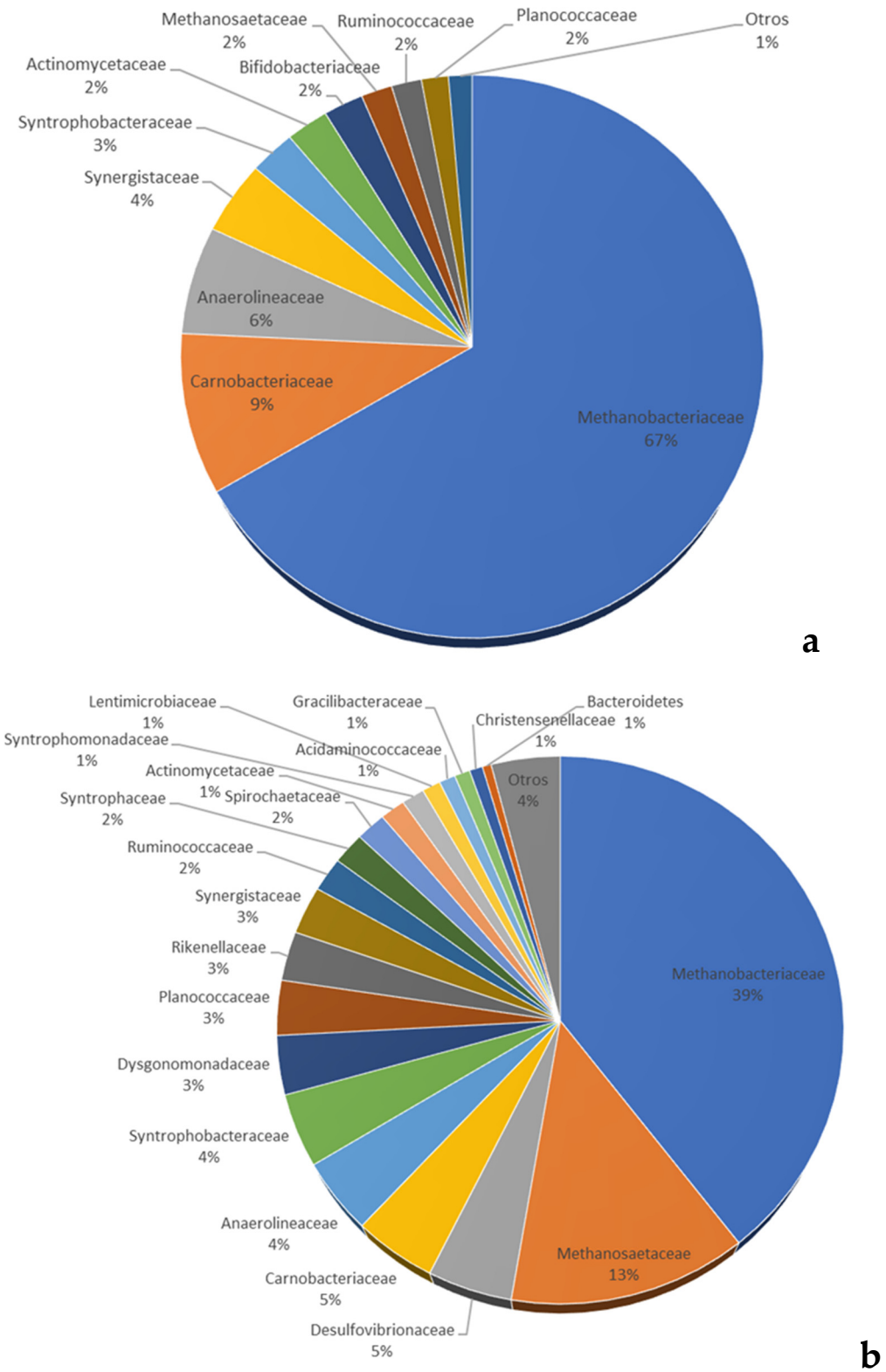

Figure 9. Families identified in the A-SBR operated under optimal conditions in (**a**) the suspended microbiota, and (**b**) the biofilm.

3.2.6. Biogas Production and Composition

As noted in Section 3.1, the pH shock induced a significant drop in the biomethane content of the biogas, dropping from 56% pre-shock to 8% post-shock. However, through the implementation of adjustments to the C/N ratio with NH_4Cl addition (Section Addition

of Ammonium Chloride (NH_4Cl) in Smaller Quantities) and adjustment of the initial vinasse concentration (Section 3.2.4), the biomethane proportion recovered to 60% (Table 4). During this phase of operation under optimal conditions, no hydrogen was detected, affirming the completion of anaerobic digestion up to methane production, aligning with the findings of Yin et al. [49] regarding methanogenesis pathways. Biogas production per unit COD degradation stood at 0.332 NL of CH_4/g COD degraded (CODr), close to the results of Jáuregui-Jáuregui et al. [28] (0.319 NL CH_4/g CODr, see Table 1), who processed tequila vinasse in a fixed bed anaerobic reactor.

Table 4. Composition, production, and yield of biogas generated.

Stage	Composition (%)			Production (NL CH_4/h)	Yield (NL CH_4/g COD_r)
	CH_4	CO_2	H_2		
Stable operation pre-pH shock	56	43	0	0.070	0.31
Post-pH shock > 11 (pre-recovery strategies)	8	0.9	79	0.00003	0.0107
Post-system recovery	60	40	0	0.075	0.33

The methane yield of the A-SBR (0.332 NL CH_4/g CODr) closely approximates the theoretical value of 0.35 L CH_4/g CODr reported by Torres-Lozada et al. [18] under standard temperature and pressure conditions. This alignment suggests that the methane output of the A-SBR nears the theoretical maximum, validating the efficacy of the employed recovery strategies.

3.2.7. Comparison of Anaerobic Activity Between Recovery Strategies

The removal efficiency of organic matter obtained after the application of each recovery strategy was compared with the organic matter removal efficiency obtained during the stable operation of the A-SBR before the pH shock using an ANOVA test. A *p*-value of less than 0.05 indicates a significant difference in this statistical analysis. The results showed significant differences after applying strategies 1 (decrease of initial concentration of vinasse) and 2 (extension of retention time), as the system's removal efficiency had not yet recovered. In strategy 3 (adjustment of C/N ratio), no significant difference was found, indicating that this strategy allowed the AD process to recover. The optimization of the operation parameters in strategies 4 and 5 showed a significant difference again in efficiency compared to the original stable stage, indicating optimal anaerobic digestion performance (Table 5).

Table 5. Statistical comparison between the organic matter removal from the stable phase and the effect of each recovery strategy.

Comparison Between the Stable Phase and the Effect of Each Recovery Strategy	Mean of Stable Period	Mean of Evaluated Strategy	Significant Difference	Adjusted *p*-Value
Stable period vs decrease of ICV	71.09	56.42	Yes	<0.0001
Stable period vs extension of RT	71.09	41.46	Yes	<0.0001
Stable period vs adjustment of C/N ratio	71.09	65.54	No	0.1386

Table 5. *Cont.*

Comparison Between the Stable Phase and the Effect of Each Recovery Strategy	Mean of Stable Period	Mean of Evaluated Strategy	Significant Difference	Adjusted *p*-Value
Stable period vs increase of OLR	71.09	86.16	Yes	<0.0001
Stable period vs optimal conditions	71.09	92.54	Yes	<0.0001

Note: ICV: initial concentration of vinasse, RT: reaction time, C/N: carbon/nitrogen, OLR: volumetric organic load.

3.2.8. Evolution of the Balance of Microbial Populations (Acidogenic–Methanogenic)

During the recovery of the A-SBR, a significant shift in acidogenic and methanogenic populations was observed with the application of each recovery strategy, as previously discussed (Table 1). However, it is important to summarize how these changes occurred. Initially, following an alkaline pH shock (of 11 units), the predominance of acidogenic bacteria was favored, with *Clostridium* at 17% and *Bacteroides* at 19%. This resulted in a substantial production of volatile fatty acids, increasing from 2083 to 4902 mg/L, and a decrease in the methanogenic population, with *Methanobacterium* dropping from 6% to 0%. When the initial concentration of vinasse was reduced and the retention time (RT) was increased, contrary to the desired effect, the proportion of acidogenic microorganisms in the suspended biomass increased, with *Clostridium* rising from 17% to 43% and *Bacteroides* from 19% to 21%. However, in the biofilm, a positive recovery effect was observed with the establishment of a small population of methanogenic microorganisms, as *Methanosarcina* reappeared at 9%. By balancing the C/N ratio, a total recovery of the balance between acidogenic and methanogenic populations was achieved, promoting a more favorable environment for the growth of methanogenic *Archaea*. This led to a significant abundance of 69% in the suspended microbiota (with *Methanobacteriaceae* at 67% and *Methanosaetaceae* at 2%) and an abundance of 52% in the biofilm (with *Methanobacteriaceae* at 39% and *Methanosaetaceae* at 13%) (Figure 9). This change was crucial for restoring methane production, which approached its theoretical maximum value, demonstrating the effectiveness of the applied strategies to restore equilibrium between acidogenic and methanogenic populations while enhancing long-term organic matter removal efficiency and biomethane production in the reactor.

3.2.9. Additional Considerations and Future Research

Extrapolation of Results

This work presents effective strategies for restoring the methanogenic microbiota in an A-SBR, along with the production of biomethane from tequila vinasse. However, the findings have broader implications that can be extrapolated to other types of waste and reactors. Some authors have demonstrated that manipulating operational parameters, such as hydraulic retention time (HRT) and carbon/nitrogen (C/N) ratio, is applicable not only in the anaerobic digestion of vinasse but also in the treatment of organic waste, such as sewage sludge and food waste. For example, Zhang et al. [50] observed that optimizing the HRT in a UASB reactor significantly improved methane production from sludge, highlighting that an extended HRT allows for greater degradation of volatile fatty acids (VFAs) and the stabilization of pH. Furthermore, the results of the recovery strategies evaluated for tequila vinasse can be applied to other waste streams, thereby promoting more efficient and sustainable management of biogas production across various industrial contexts.

Effect of Other Factors on the Recovery of Methanogenic Activity

The recovery of methanogenic activity in anaerobic digestion systems is also influenced by operational parameters such as alkalinity and external environmental factors. Alkalinity

plays a fundamental role in methanogenesis by acting as a buffer that stabilizes the pH of the medium. Bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions serve as buffering systems when acids are produced during the decomposition of organic matter; these compounds react with hydrogen ions (H^+) to form carbonic acid (H_2CO_3), which dissociates into water and carbon dioxide (CO_2), helping to maintain a stable pH [51]. The production of volatile fatty acids during anaerobic digestion can lead to acidification of the medium. If there is insufficient alkalinity to neutralize these acids, the pH may decrease, causing a reduction in the activity of methanogenic *Archaea*, resulting in lower methane production [52].

An external factor that could affect the recovery of methanogenic activity is the variation in influent composition during peaks of organic loading, toxic compounds, or temperature, as these can disrupt microbial balance. Peaks in organic loading can lead to excessive accumulation of volatile fatty acids (VFAs), resulting in a decrease in the medium's pH, which inhibits the activity of methanogenic *Archaea* [19]. Additionally, competition between methanogens and other microbial groups, such as sulfate-reducing bacteria (SRB), has been observed to intensify under conditions of high organic loading, potentially leading to increased production of hydrogen sulfide (H_2S) [53]. The presence of toxic compounds can also negatively impact methanogenic activity. These compounds may interfere with essential enzymes involved in the metabolic processes of methanogens. For example, cadmium and mercury have been documented to have significant inhibitory effects on methane production by altering enzymatic function and structure [54].

Future Studies

The literature mentions other strategies for recovering the activity of reactors that experience operational failure. Serrano-Meza et al. [42] present a discussion of some of these strategies: dilution with biomass (re-inoculation), addition of adsorbents and co-substrates (use of additives), and prolonged exposure to toxic compounds along with intermittent feeding of the system (modification in reactor operation). They conclude that the addition of adsorbents is the most reliable option, as this strategy results in greater biogas production; however, it also increases operational costs.

However, it would be important to study these strategies in future research, focusing on the recovery of biomethanogenic activity and considering, as in the present study, both their effects on organic matter removal efficiency and the reconfiguration of the microbiota (balance between acidogenic and methanogenic microorganisms), with the aim of continuing to reduce the existing lack of information regarding recovery actions.

4. Conclusions

The reduction in the initial vinasse concentration and the extension of reaction time (RT) were strategies that individually failed to fully restore a methanogenic microbiota in an anaerobic reactor treating tequila vinasse following a pH-related failure. However, achieving a balanced carbon/nitrogen (C/N) ratio emerged as a critical factor in re-establishing the efficiency of organic matter removal in the anaerobic digestion system post-pH failure, consequently leading to the recovery of methanogenic microbiota. Operating the A-SBR with an initial vinasse concentration of 60%, an RT of 168 h, a pH of 6.9 $\pm$ 0.2, a temperature of 35 $\pm$ 2 °C, and a C/N ratio adjusted to 100:1.9 resulted in a consistent COD removal efficiency of 93 $\pm$ 3% over a year. The A-SBR had a significant presence of methanogenic microorganisms in this period, constituting 69% of the suspended microbiota and 52% of the biofilm. In contrast, following the pH shock, methanogenic microorganisms were almost absent, with acidogenic microorganisms predominating. The normalized methane production (0.332 NL CH_4/g CODr) achieved under these conditions post-methanogenic population and activity restoration approached the theoretical maximum for normal temperature and pressure conditions (0.35 L CH_4/g CODr).

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