

Article

Monensin Degradation and Methane Production from Sugarcane Vinassee in Two-Phase Thermophilic Anaerobic Fixed-Bed and Sludge Blanket Bioreactors

Sami Chatila  and Marcelo Zaiat 

Laboratory of Biological Processes (LPB), São Carlos School of Engineering, University of São Paulo (USP), 1100, João Dagnone Ave., Santa Angelina, São Carlos 13563-120, SP, Brazil; samichatila6@gmail.com

* Correspondence: zaiat@sc.usp.br

Abstract

Sugarcane vinassee, a byproduct of ethanol production, presents environmental challenges due to its high organic content and occasional contamination with antibiotics, such as monensin. This study successfully evaluated thermophilic two-phase anaerobic digestion for simultaneous monensin degradation and biogas production. The system, consisting of an acidogenic anaerobic structured-bed bioreactor (ASTBR) operating at with a hydraulic retention time (HRT) of 7.5 h followed by a methanogenic reactor at HRT = 24 h, with two options of the methanogenic phase, an upflow anaerobic sludge blanket (UASB), and an ASTBR, operated continuously for 254 days with incremental monensin concentrations (0–2000 ng·mL⁻¹). The acidogenic reactor consistently removed over 70% of monensin across all phases, demonstrating its effectiveness as a pretreatment step. At realistic residual concentrations (20–100 ng·mL⁻¹), monensin not only failed to inhibit biogas production but enhanced methane yield by up to 100% through selective pressure on the microbial community. This study demonstrated that anaerobic digestion can effectively degrade monensin while increasing the value of vinassee, providing a scalable solution for mitigating antibiotic contamination and enhancing bioenergy recovery in the sugarcane–ethanol industry.



Academic Editor: Diomi Mamma

Received: 11 August 2025

Revised: 25 August 2025

Accepted: 30 August 2025

Published: 3 September 2025

Citation: Chatila, S.; Zaiat, M. Monensin Degradation and Methane Production from Sugarcane Vinassee in Two-Phase Thermophilic Anaerobic Fixed-Bed and Sludge Blanket Bioreactors. *Fermentation* **2025**, *11*, 518. <https://doi.org/10.3390/fermentation11090518>

Copyright: © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Vinassee, as a liquid residue-turned-byproduct of the sugarcane–ethanol economy, represents both a challenge and an opportunity, as it is nutrient-rich, carbon-dense, and plentiful. Accordingly, it has been historically seen as a waste product destined for use as a fertilizer in a tight radius around ethanol distilleries, where its nutrients are a source of modest economy, and much of its content is leached into the soil and groundwater or left to decompose back into the atmosphere [1–4]. Considering that Brazil is expected to produce about 29 billion liters of ethanol in the 2024–2025 season [5], and considering that every liter of ethanol results in about 15 L of vinassee [6], better solutions than disposal on cropland must be established. As such, biogas production is the main candidate for extracting useful energy from sugarcane vinassee, while other methods for upgrading vinassee are under development [7–9].

Biogas production from vinassee has been extensively studied, and there have been some attempts to implement it on a large scale with varying results. One of the main issues

is the methanogenic process's sensitivity to high organic loading rates and toxic compounds, which is the reason the trend in vinasse biodigestion has been isolating methanogenesis from the rest of the metabolic process, specifically between acid production and conversion to biogas [1,10], but also sulfate removal [11–13]. In addition to the natural fermentation products and thermal degradation products resulting from the distillation process and inorganic additives, vinasse occasionally contains antibiotics dosed during fermentation to control bacterial populations, which compete with the yeast for substrate and produce organic acids, which inhibit yeast and diminish the yield of batch fermentation. The most common antibiotics used in sugarcane–ethanol plants are various penicillins and tetracycline [14,15], whose properties are extensively documented and studied, and monensin, which is commercialized as effective fermentation contamination control, based on a patent [16], and sold to ethanol plants that are concerned about environmental impacts. Monensin is known to be a very wide-spectrum antibiotic, to the extent that it presents toxicity to all living cells, to varying degrees, including vertebrates and invertebrates [17–21]. Monensin is also known to persist in the environment, resisting degradation in environmental water and soil [20]. Accordingly, its presence in vinasse presents a current environmental concern and could have implications for vinasse biodigestion, as producers have concerns about the viability of a biorefinery when a known toxin is present. Silva [22,23] has recently developed an analytical method to quantify antibiotics in vinasse, and established that monensin was found at $14 \text{ ng}\cdot\text{mL}^{-1}$ in sugarcane vinasse, while carrying out batch biodigestion experiments with $500 \text{ ng}\cdot\text{mL}^{-1}$ monensin added to conclude that monensin does, in fact, have an inhibitory effect on vinasse biodigestion. Chapter 2 of the present study establishes that real vinasse shows concentrations of $2\text{--}250 \text{ ng}\cdot\text{mL}^{-1}$ of vinasse. Accordingly, vinasse biodigestion under real concentrations of monensin in vinasse in a continuous reactor must be studied, and this study accomplishes this objective. This study aims to evaluate the effect of monensin on vinasse anaerobic digestion and evaluate the degradation of monensin in a continuous anaerobic reactor, which has not yet been reported in the literature.

2. Materials and Methods

2.1. Vinasse

Sugarcane–ethanol vinasse was collected from a sugarcane–ethanol plant located in Pradópolis, São Paulo, Brazil. This crude vinasse in three utilized collection lots was determined to have the following characteristics presented in Table 1. The vinasse was stored at -18°C and thawed for feeding the reactors. The vinasse was filtered with a viscose cloth filter before feeding the reactor to prevent clogging the pumps, tubes, and valves within the bench-scale reactors.

Table 1. Vinasse characteristics.

Collection Lot	21 July 2021	7 October 2021	24 June 2022	Method
COD Total ($\text{mg}\cdot\text{L}^{-1}$)	35,367	36,380	33,700	APHA [24]
COD Soluble ($\text{mg}\cdot\text{L}^{-1}$)	28,467	31,650	20,680	APHA [24]
Lactic Acid ($\text{mg}\cdot\text{L}^{-1}$)	2100	2120	720	Taylor [25]
Glycerol ($\text{mg}\cdot\text{L}^{-1}$)	3973	3956	2214	Valdez [26]
Carbohydrates ($\text{mg}\cdot\text{L}^{-1}$)	7760	7850	3750	Dubois [27]
pH	4.56	4.1	4.5	APHA [24]
Sulfate ($\text{mg}\cdot\text{L}^{-1}$)	2860	2000	1950	APHA [24]
NTK ($\text{mg}\cdot\text{L}^{-1}$)	1051.27	1205	1030	APHA [24]
NH_4^+ ($\text{mg}\cdot\text{L}^{-1}$)	154.09	147	92	APHA [24]
Phenol ($\text{mg}\cdot\text{L}^{-1}$)	1.29	3.49	2	APHA [24]
Phosphorous ($\text{mg}\cdot\text{L}^{-1}$)	47	53	60	APHA [24]

2.2. Thermophilic Bioreactors

While this study takes a two-phase bioreactor approach that involves separating the anaerobic digestion process into an acidogenic system and a methanogenic system, this study also evaluates two different configurations for the methanogenic bioreactor, specifically an upflow anaerobic sludge blanket (UASB) and an anaerobic structured-bed bioreactor (ASTBR). Accordingly, three reactors were operated, where one ASTBR acidogenic reactor fermented vinasse, converting organic material into organic acids, and the effluent of this reactor was used to feed the two following methanogenic reactors. The configuration of the reactors was based on the reactor development performed by Fuess 1 and is shown in Table 2 and Figures 1 and 2.

Table 2. Reactor configuration for all 3 reactors in this study.

Phase	Reactor	Configuration	Support Material	Total Height (mm)	Internal Diameter (mm)	Total Liquid Volume (L)
1— Acidogenic	1	ASTBR	Structured Hollow Polyethylene Cylinders	745	60	2.1
2— Methanogenic	2	UASB	None	400	50 (main body) 80 (3-phase separator and headspace)	1.6
	3	ASTBR	Vertically Arranged Polyurethane Foam Strips	745	60	2.1

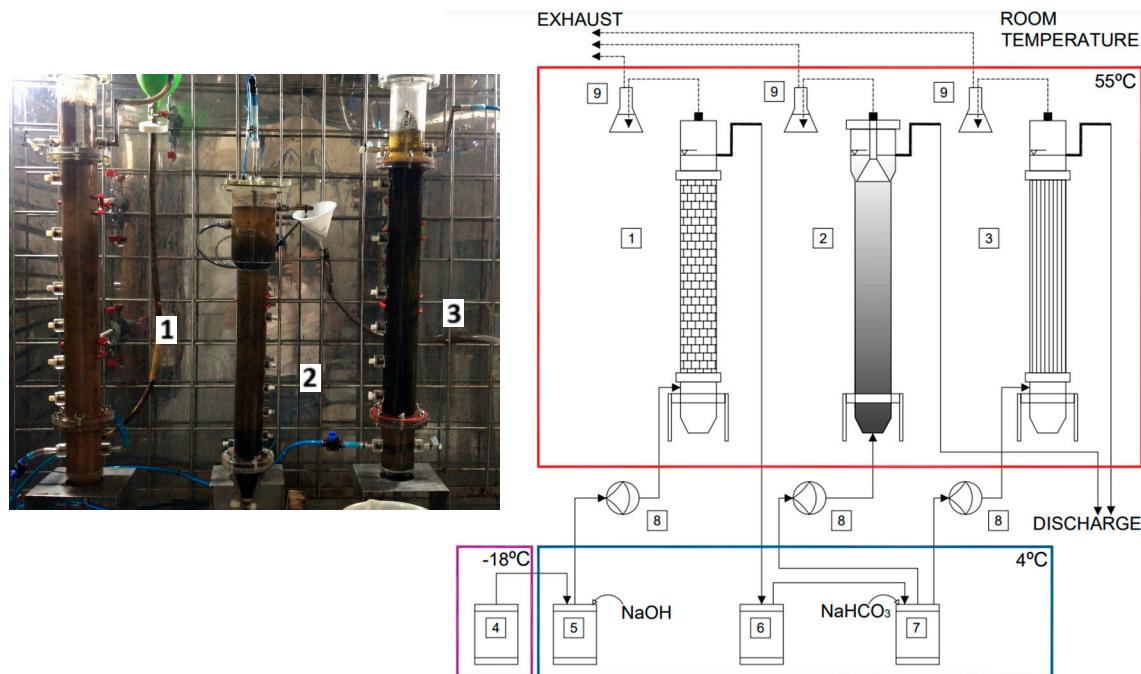


Figure 1. Experimental bench-scale thermophilic reactor setup in a 55 °C temperature-controlled chamber showing (1) the acidogenic ASTBR reactor, (2) the methanogenic UASB reactor, (3) the methanogenic ASTBR reactor, (4) stored crude vinasse, (5) vinasse alkalized with NaOH and acidogenic feedstock at 4 °C, (6) acidogenic reactor effluent at 4 °C, (7) acidogenic effluent alkalized with NaHCO₃ and methanogenic feedstock at 4 °C, (8) Grundfos diaphragm dosing pumps for

reactor feeding, and (9) 3M HCl bottle traps for atmospheric isolation. Solid lines are liquid flow. Dashed lines are gas flow.

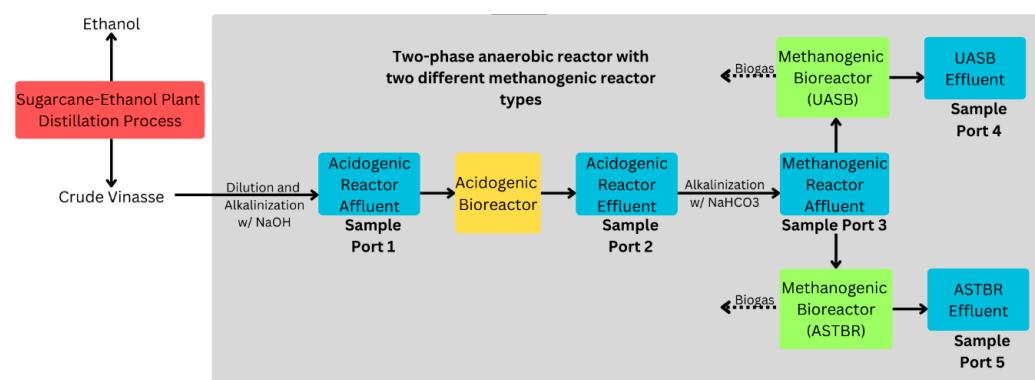


Figure 2. Experimental setup flowchart showing the flow of liquids (solid arrows) and gases (dashed arrows) and sampling ports used for regular monitoring.

2.3. Two-Phase Reactor Operating Conditions

The two-phase reactor system was operated for 254 days in a temperature-controlled chamber set at 55 °C. Vinassee was kept at 4 °C while being pumped into the reactors using one Grundfos DDA dosing pump for each reactor. The reactor headspace was sealed from the atmosphere using a bottle trap containing a 3 M HCl solution, preventing CO₂ dissolution from the produced biogas and ensuring reliable volumetric flow measurements. The reactor operation was divided into 5 phases with constant vinassee feeding characteristics with the incremental addition of monensin, as per Table 3. Phase 0 represented the establishment of a baseline of two-phase reactor performance without the presence of monensin. Each phase ended when a steady state of COD removal and biogas production was achieved, and the monensin concentration was increased, marking the beginning of the next phase. This was defined as when variation of COD removal and biogas volumetric production was observed to have stabilized following sharp drops at each monensin concentration range. Accordingly, reactor operation lasted for a total of 254 days.

Table 3. Phases of operation, their duration, and their respective continuously added monensin concentrations.

Phase	Duration (Days)	Nominal Monensin Concentration (ng·mL ⁻¹)
0	88	0
1	42	20
2	52	50
3	47	100
4	25	2000

2.3.1. Acidogenic Phase Reactor

The acidogenic ASTBR reactor (2.1 L liquid volume) was inoculated by auto-fermentation of vinassee [28] and posteriorly operated at a continuous hydraulic retention time (HRT) of 7.5 h (organic loading rate (OLR) of 70.0 COD/m³·d) with vinassee pH-adjusted to pH = 5.5 using 50% *m/v* NaOH [10]. This HRT was chosen based on the work performed by Fuess (2016) [29], albeit with a slightly lower OLR than optimal for hydrogen production, to ensure the stability of the acidogenic process, and since hydrogen production was not a priority and would not be optimized. Acid production and simplicity of operation as a pretreatment reactor were the priority, so an OLR of 70.0 kg COD·m⁻³·d⁻¹ would ensure the requirements of this study were met while remaining within the scope

of evaluating the performance of two-phase anaerobic digestion reactors for sugarcane vinasse. All effluents of this reactor were collected and stored for up to 3 days at 4 °C to be used to feed the methanogenic phase.

2.3.2. Methanogenic Phase Reactors

The methanogenic UASB (1.6 L liquid volume) and the ASTBR (2.1 L liquid volume) were inoculated with thermophilic sludge acquired from a full-scale methanogenic UASB reactor digesting vinasse from the same plant that supplied the vinasse used in this study. The UASB was filled to 40% of its volume with sludge. The ASTBR polyurethane support material was soaked in diluted and lightly ground sludge for 2 h prior to reactor final assembly [10]. Both reactors were then fed with fermented vinasse produced by the acidogenic phase alkalinized with 0.28 g NaHCO₃/g COD at a continuous OLR of 25 kg COD·m⁻³·d⁻¹ (HRT = 24 h). As the operation of the methanogenic reactors was aimed at the production of biomethane, the optimal OLR of 25 kg COD·m⁻³·d⁻¹ reported by Fuess [10] was maintained, and the HRT of 24 h was in line with previous research and fixed at an upper limit of the optimistic 20–24 h range for methanogenic metabolism to ensure that both reactors would enjoy stable long-term methanogenesis, even as the bench-scale reactors that accumulated biomass and HRT would effectively and gradually drop.

2.4. Experimental Monitoring

Throughout the operation, the two-phase reactor was monitored at five points shown in Figure 2: acidogenic affluent, acidogenic effluent, methanogenic affluent, methanogenic UASB effluent (including biogas output), and methanogenic ASTBR effluent (including biogas output).

All analyses were performed immediately, except for VOC and MON analysis, which were stored at –18 °C and analyzed in lots. Sampling of all affluents and effluents was performed by shaking the feedstock or collected effluent to homogeneity, and a primary sample of 50 mL was collected, of which 2 mL was used for immediate analysis, and 2 samples of 2 mL were stored at –18 °C for batch analysis of VOC and MON, respectively. Headspace sampling for biogas composition in the methanogenic reactors was taken through a septum-sealed sampler installed in-line on the gas outlet, while ensuring stable positive pressure in the headspace prior to sampling.

The parameters monitored for all reactors and their frequency are shown in Table 4.

Table 4. Reactor performance monitoring protocols.

Monitored Parameter	Frequency	Method
pH	Daily	Electrometry [24]
Total Chemical Oxygen Demand (TCOD) (mg·L ⁻¹)	3×/week	Closed Reflux Colorimetry [24]
Lactic Acid (HLA) (mg·L ⁻¹)	3×/week	Calorimetry [25]
Glycerol (mg·L ⁻¹)	3×/week	Enzymatic–Colorimetric [26]
Carbohydrates (mg·L ⁻¹)	3×/week	Colorimetry [27]
Total Suspended Solids (TSSs) (mg/mL)	1×/week	Gravimetry [24]
Volatile Suspended Solids (VSSs) (mg/mL)	1×/week	Gravimetry [24]
Biogas Volumetric Production (L/d)	5×/week	Mariotte's Bottle
Biogas Composition	5×/week	Gas Chromatography [30]
Volatile Organic Compounds (VOCs) (mg·L ⁻¹)	3×/week	Gas Chromatography [31]
Monensin (MON) (ng·mL ⁻¹)	3×/week	HPLC-MS/MS

Methane production in each phase of operation was evaluated by combining volumetric and composition data and was converted to volumetric methane production (VMP) (L CH₄·L⁻¹·d⁻¹) by dividing the average volume of methane produced daily in each phase

of operation by the liquid volume of each reactor. Methane yield (MY) ($\text{L CH}_4 \cdot \text{g}^{-1}$ COD removed) was calculated by dividing VMP by the average COD removal for each phase of operation.

3. Results and Discussion

In 254 days of operation and monitoring, the bench-scale two-phase anaerobic bioreactor had gone through several landmark events, starting with Phase 0, which can be further divided into a start-up phase and a steady-state phase, and Phases 1, 2, and 3, which represented the addition of incremental realistic concentrations of monensin to the vinasse feedstock, and ending with Phase 4, which represented an unrealistic or accidental concentration of monensin in the vinasse and quickly led to the collapse of the system and which demonstrated that despite its resilience, the bioreactor's capacity to absorb and degrade toxins has its limits.

Monensin removal was observed in both the acidogenic and methanogenic phases of the system, and it is notable that at a very low affluent concentration of monensin in Phase 1 of this study ($\text{MON} = 20 \text{ ng} \cdot \text{mL}^{-1}$), the acidogenic phase was able to eliminate monensin, and its effluent contained no detectable amounts. Accordingly, as far as the methanogenic reactors were concerned, no monensin arrived in the methanogenic affluent until Phases 2, 3, and 4 of this study, yet methanogenic performance in Phase 1 is still attributed to the presence of monensin in the two-phase reactor since the presence of monensin in the acidogenic reactor changes the character of the acidogenic effluent, and, consequently, the performance of the methanogenic reactors, as seen in Figure 3, and this will be discussed later.

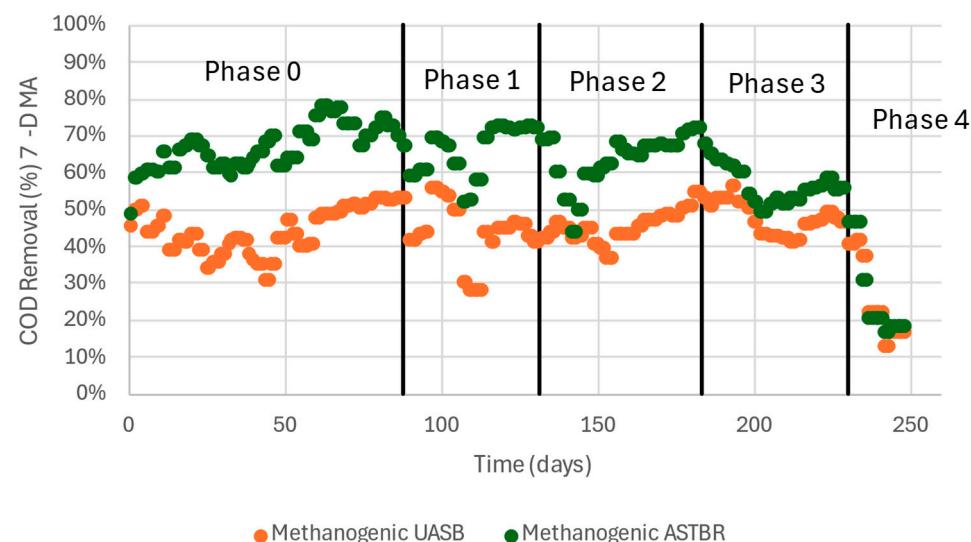


Figure 3. COD removal (%) 7-day moving average in all stages of this study across both methanogenic reactors divided into phases: Phase 0—feedstock not containing any monensin; Phase 1—addition of 20 ng monensin/ mL feedstock; Phase 2—addition of 50 ng monensin/ mL feedstock; Phase 3—addition of 100 ng monensin/ mL feedstock; Phase 4—addition of 2000 ng monensin/ mL feedstock.

3.1. Reactor Performance

The start-up phase is considered from the day the reactors were assembled and inoculated up to the day when day-to-day variations in performance became less significant; during operation, reactor performance was evaluated based on COD removal and biogas production in the methanogenic reactors since the target value-adding activity of the system, i.e., biogas production and organic material removal, is exclusive to the methanogenic

reactors in such a two-phase reactor. Accordingly, for the methanogenic UASB, steady-state performance was considered from day 51, when the 7-day moving average for COD removal permanently surpassed 40%, and for the methanogenic ASTBR, steady-state performance was taken from day 31, which is when the 7-day moving average for COD removal permanently surpassed 60%. This confirms previous results of superior performance of fixed-bed reactors over UASB reactors for methanogenesis, both in terms of efficiency and speed of start-up, especially in two-phase anaerobic digestion reactors [10].

3.2. Acidogenic ASTBR

Accordingly, the acidogenic reactor is initially viewed as a pretreatment component, which ferments vinasse and primes it for digestion in the methanogenic reactors by converting sugars and carbohydrates into organic acids and solvents (OASs), which include VOCs and lactic acid. Therefore, COD removal in the acidogenic reactor was not expected or observed, and no biohydrogen production was incentivized or observed, since this reactor was accumulating organic acids, both maintaining a high COD and a pH under 4.5 throughout operation. This inhibits biohydrogen production, as reported in other studies [29]. Additionally, while COD was measured in the affluent and effluent, values frequently implied the accumulation and release of organic material. Over the entire period of this study, the acidogenic reactor consumed just 3.5% of the COD fed into it due to cell maintenance and baseline metabolic activity. The success of acidogenesis is OAS production, shown in Figure 4 across each phase, and it was regularly monitored via glycerol removal from the vinasse. As a favored substrate in acidogenic systems [32–35], glycerol was completely consumed, without interruption, from the second week of operation until reactor collapse on the 254th day of operation. The acidogenic reactor was relatively consistent at OAS production across phases, albeit with less stability in the presence of monensin. At Phase 4, acid production seemingly increases, with much expanded instability, which implies widespread ecosystem collapse in the wake of monensin's bactericidal activity. Table 5 and Figure 5 demonstrate that the acidogenic ASTBR effectively removed MON across all phases, though removal efficiency declined as MON concentrations increased. A sharp drop was observed at the spike concentration in Phase 4; however, the system consistently achieved over 70% toxin removal. This performance highlights its ability to prime and pretreat vinasse for subsequent biodigestion in methanogenic reactors. Table 5 also shows that even though monensin addition to effluent was carefully micropipetted with the target concentrations shown in Table 3, real affluent concentrations of monensin are effectively lower, showing that even while at 4 °C, the feedstock itself was an environment that degraded or adsorbed some of the added monensin. This is unsurprising and expected since the vinasse itself contains an active microbiome, which was used for inoculating the acidogenic reactor; this is why affluent concentrations were monitored and not taken for granted.

Table 5. Acidogenic ASTBR average MON affluent concentration per phase of operation and COD removal (%) and MON removal (%).

	Phase 0	Phase 1	Phase 2	Phase 3	Phase 4
Average MON Affluent Concentration (ng·mL ⁻¹)	-	18.24 (5.79)	49.76 (16.25)	98.67 (29.68)	1763.04 (472.05)
MON Removal (%)	-	96% (9%)	88% (1%)	84% (19%)	71% (23%)
COD Removal (%)	2% (9%)	2% (8%)	3% (14%)	3% (7%)	7% (7%)

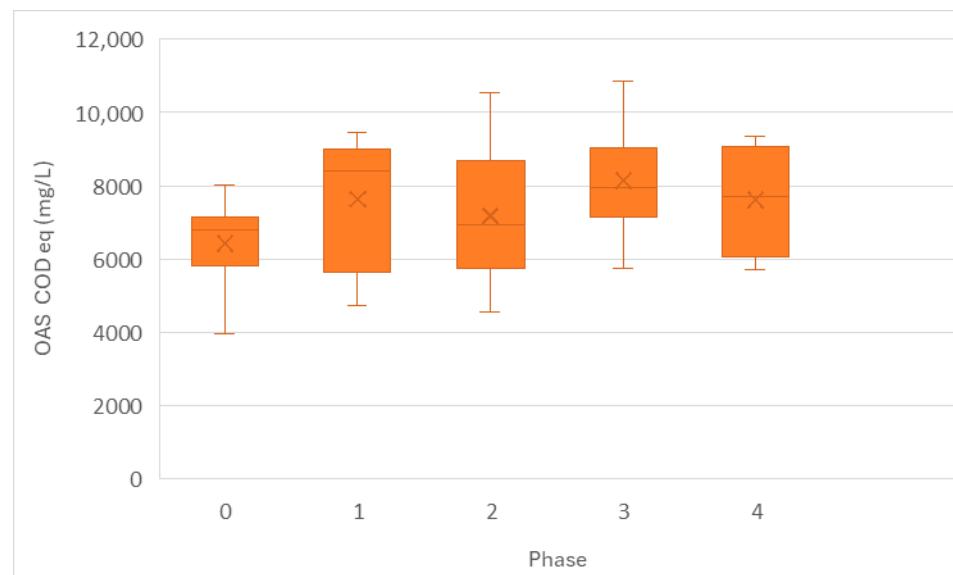


Figure 4. Average AOS released by the acidogenic ASTBR per phase of operation.

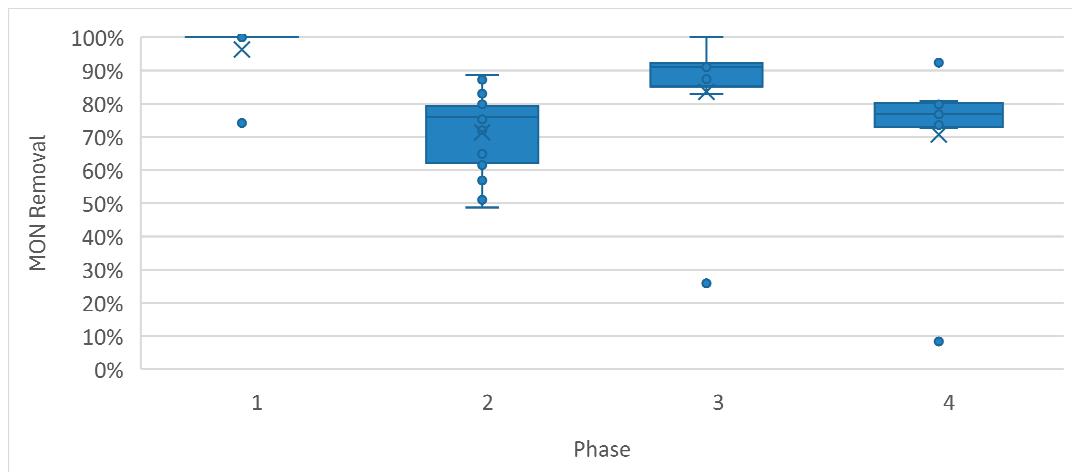


Figure 5. MON removal in the acidogenic phase showing a high capacity to reduce monensin presence in vinasse, whether in trace amounts, as in Phases 1–3, or in accidental high-dose discharge, as in Phase 4.

A breakdown of OAS production in COD equivalent per phase is shown in Figure 6. OAS production is affected by the presence of monensin, which can be inhibitory, as in the case of n-butanol, or potentiating, as in all other cases shown in this research project. Notably, n-butanol production increases in Phase 1, in comparison to Phase 0, and rapidly decreases in Phase 2. Similarly, propionic acid production peaks in Phase 3 and drops in Phase 4. This indicates that tolerance to monensin in the reactor ecosystem exists and that monensin imposes a selective pressure on bacteria in the reactor, even at residual concentrations. This has wide implications for monensin's (and possibly other bacterial control agents) use in the sugarcane–ethanol industry if residues are to be biologically upgraded into useful materials, such as biogas, VOCs, bioplastics, etc. Additionally, this result shows that monensin can be explored as a potential driver for biochemical pathways that produce specific compounds, in this case, VOCs, boosting the production of a solvent or acid and focusing the conversion of organic material into specific compounds, increasing yields by controlling pathways that compete for the substrate. Overall, VFA production in this system was much lower than in other studies, likely due to a pH of 4.5, while a pH above 5.5 is usually required for optimal VFA production [36].

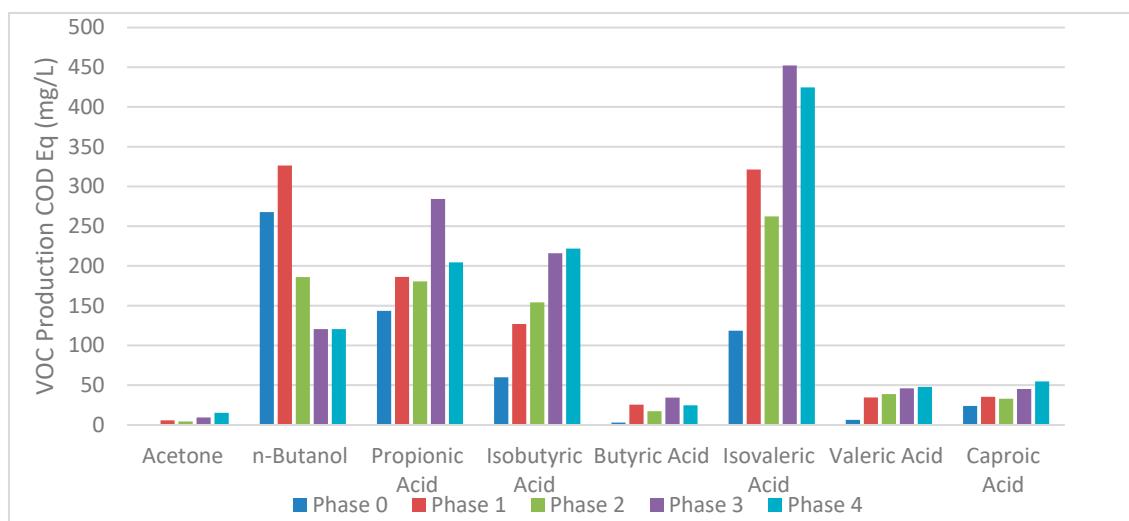


Figure 6. The average production of acetone, n-butanol, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and caproic acid in the acidogenic ASTBR reactor normalized as COD equivalent (mg/L).

3.3. Methanogenic UASB and ASTBR

As the nominally productive component of the two-phase anaerobic reactor, the methanogenic reactors were monitored for COD removal and biomethane production; a summary of reactor performance is presented in Table 6.

Table 6. Performance of the methanogenic UASB and ASTBR across all phases with average real monensin input ($\text{ng}\cdot\text{mL}^{-1}$) (as quantified), showing COD removal (%), monensin removal (%), volumetric methane production ($\text{L CH}_4\cdot\text{L}^{-1}\cdot\text{d}^{-1}$), and methane yield ($\text{L CH}_4/\text{g COD removed}$).

	Phase 0	Phase 1	Phase 2	Phase 3	Phase 4
Average MON Load ($\text{ng}\cdot\text{mL}^{-1}$)	-	7.25 (8.6)	43.83 (13.54)	56.14 (36.7)	839.83 (583.31)
COD Removal (%)	47 (7)	44 (15)	44 (7)	48 (6)	30 (13)
Average COD Removed ($\text{g}\cdot\text{L}^{-1}$)	13.11 (1.49)	10.88 (2.45)	9.93 (2)	10.37 (1.92)	7.05 (2.92)
Monensin Removal (%)	-	56 (38)	21 (10)	26 (13)	54 (23)
VMP ($\text{L CH}_4\cdot\text{L}^{-1}\cdot\text{d}^{-1}$)	1.94 (0.58)	2.54 (0.71)	2.17 (0.78)	2.6 (0.47)	1.51 (1.01)
MY ($\text{L CH}_4\cdot\text{g}^{-1}$ COD Removed)	0.148	0.233	0.219	0.25	0.214
UASB					
COD Removal (%)	68 (9)	65 (10)	63 (10)	58 (8)	33 (15)
Average COD Removed ($\text{g}\cdot\text{L}^{-1}$)	18.92 (2.65)	15.04 (2.85)	14.32 (2.79)	12.52 (2.23)	7.63 (3.17)
Monensin Removal (%)	-	59 (34)	30 (28)	20 (9)	37 (13)
VMP ($\text{L CH}_4\cdot\text{L}^{-1}\cdot\text{d}^{-1}$)	1.98 (0.63)	3.18 (1.1)	2.44 (0.73)	2.56 (0.56)	1.42 (0.83)
MY ($\text{L CH}_4\cdot\text{g}^{-1}$ COD Removed)	0.105	0.211	0.17	0.205	0.186
ASTBR					

Both reactors received the same affluent, and in Phases 1–4, containing the same concentration of monensin, throughout the operation at the same HRT of 24 h. Across all phases, and as predicted, the ASTBR significantly outperformed the UASB in terms of COD removal (41% higher removal from the same affluent), as shown in Figure 7, and in terms of volumetric methane production (6% higher production from the same affluent). This corroborates the results of previous studies on the performance of fixed-bed

versus suspended biomass reactors [10,32], albeit here considering the confirmed presence of the antibiotic monensin. As previous studies did not check for the presence of monensin in vinasse samples used as feedstock, its influence on previous reactor performance evaluation cannot be verified. For a nearly identical reactor setup, Fuess [10] reported that at $OLR = 25 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$, average VMP was $2.280 \pm 0.357 \text{ L CH}_4 \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ and $4.505 \pm 0.455 \text{ L CH}_4 \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ for the UASB and ASTBR methanogenic reactors, respectively, in a nearly identical configuration to the one presented in this study. MY was reported at $0.283 \pm 0.024 \text{ L CH}_4 \cdot \text{g}^{-1}$ COD removed and $0.301 \pm 0.012 \text{ L CH}_4 \cdot \text{g}^{-1}$ COD removed for the UASB and ASTBR reactors, respectively. These values show that the present study in all phases (Table 6) had very similar performance in the UASB to an optimized system, whereas the ASTBR had the potential for higher performance. This could be attributed to many factors, including fixed-bed support material, sludge inoculum quality, real OLR/HRT discrepancy, and solids accumulation resulting in useful volume loss, among others. Nevertheless, the present study still shows that the ASTBR, even when operationally less than optimal, still managed to remove COD at a much higher efficiency, a testament to the functional superiority of fixed-bed reactors in treating high-strength liquid residues.

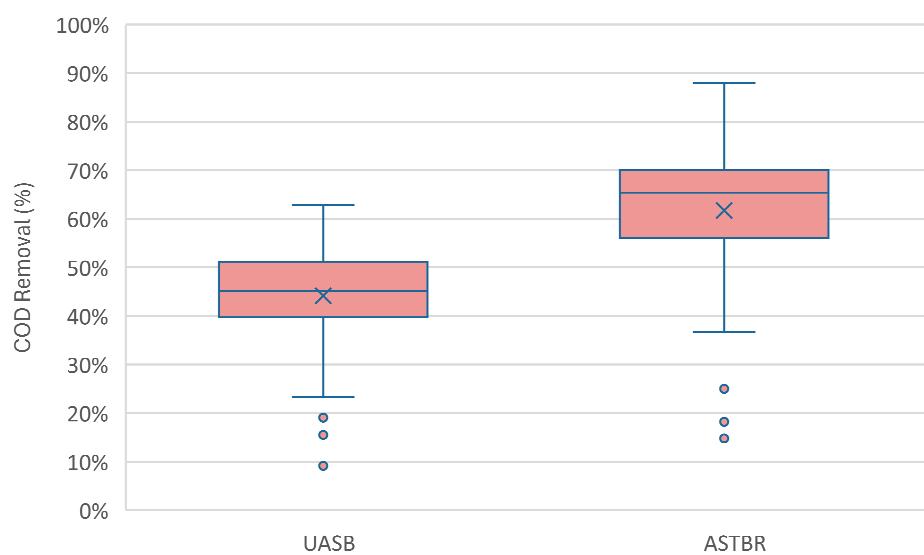


Figure 7. COD removal from fermented sugarcane vinasse in the methanogenic UASB (average = 44%) versus the methanogenic ASTBR (average = 62%) across 254 days of this study.

Figure 8 shows the removal of monensin in the methanogenic reactors. Removal efficiency is low, rarely surpassing 50%, whether at trace concentrations (Phases 1, 2, and 3) or at high concentrations (Phase 4), which implies that the methanogenic ecosystem is not well-suited for degrading this antibiotic, while being susceptible to its effects. This highlights the importance of the acidogenic reactor as a pretreatment reactor for the removal of toxins and the suitability of the two-phase anaerobic reactor concept for biodegradation of complex residues containing toxic organic compounds.

While across study phases the introduction of monensin at varying trace levels has a small effect on COD removal (Figure 9), VMP can be seen to increase when trace concentrations of monensin are introduced into the two-phase system since even in Phase 1, when monensin is undergoing degradation by the acidogenic reactor, VMP increases significantly, as shown in Figure 10. This implies that the gains in VMP are most likely due to increased OAS production, yielding a more accessible substrate to methanogenic species. This gain is lost at higher concentrations, as monensin arrives in high concentrations at the methanogenic reactors, directly affecting the stability of the reactor with a detrimental

outcome to COD consumption. This is in line with another study on batch reactors that demonstrates monensin's inhibitory effects at concentrations above $500 \text{ ng}\cdot\text{mL}^{-1}$ [22] and $1500 \text{ ng}\cdot\text{mL}^{-1}$ [37]. MY also increases at trace concentrations of monensin, doubling MY in the ASTBR at $20 \text{ ng}\cdot\text{mL}^{-1}$ of monensin (Table 3 and Figure 11). MY seems to remain high, even at an inhibitory concentration (Phase 4); however, this is misleading since COD removal drops, inflating apparent yield. This is highly reminiscent of monensin use for modifying ruminant livestock gut microbiomes to increase acid production and, consequently, improve the efficiency of conversion of animal feed into animal protein, just as monensin is justified to be used in cattle feed at low concentrations while exhibiting extreme toxicity at higher dosages [21,38].

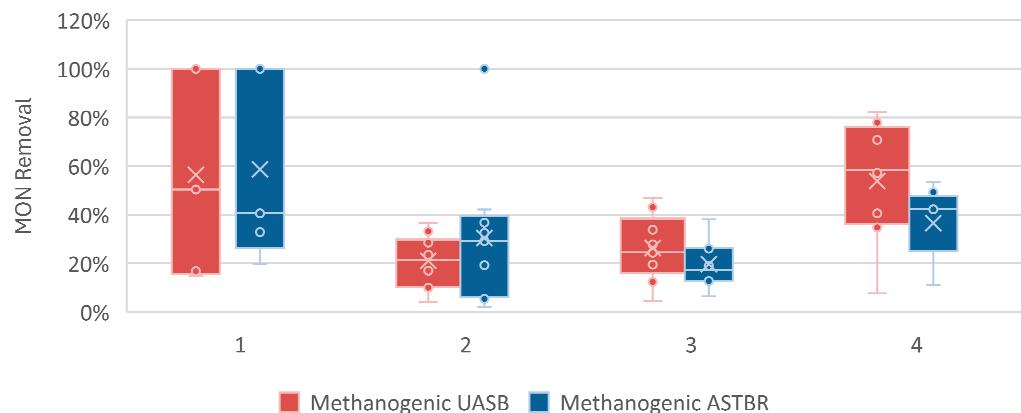


Figure 8. Monensin removal (%) in each phase of operation, per the methanogenic reactor.

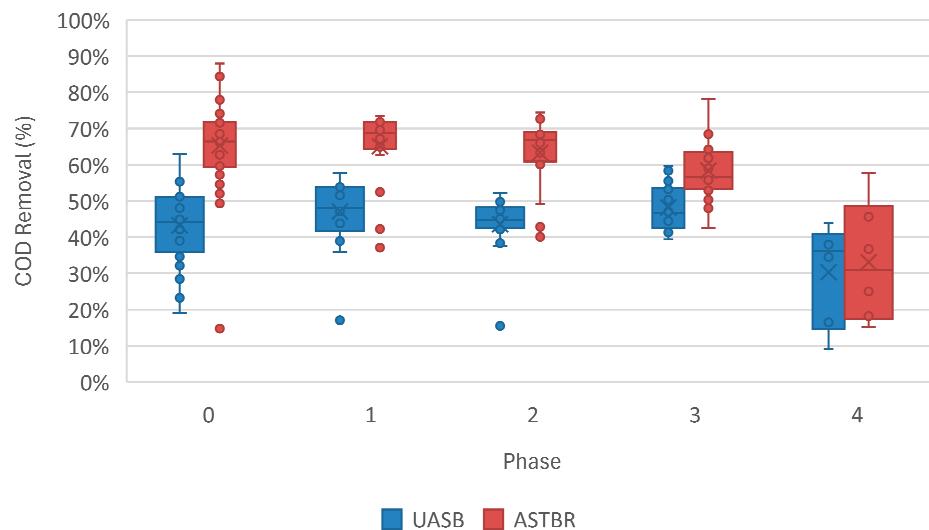


Figure 9. COD removal (%) in methanogenic reactors per phase showing consistent performance at varying trace concentrations of MON while suffering a significant drop at high concentrations.

These results demonstrate that trace levels of toxins in continuous anaerobic systems have extremely outsized effects, and the consequences of their residual presence should never be underestimated when considering their use in an industrial process. Amounts of monensin as low as $20 \text{ ng}\cdot\text{mL}^{-1}$ have the capacity to double MY in a fixed-bed thermophilic anaerobic reactor, for example, demonstrating the complex kinetics behind this interaction.

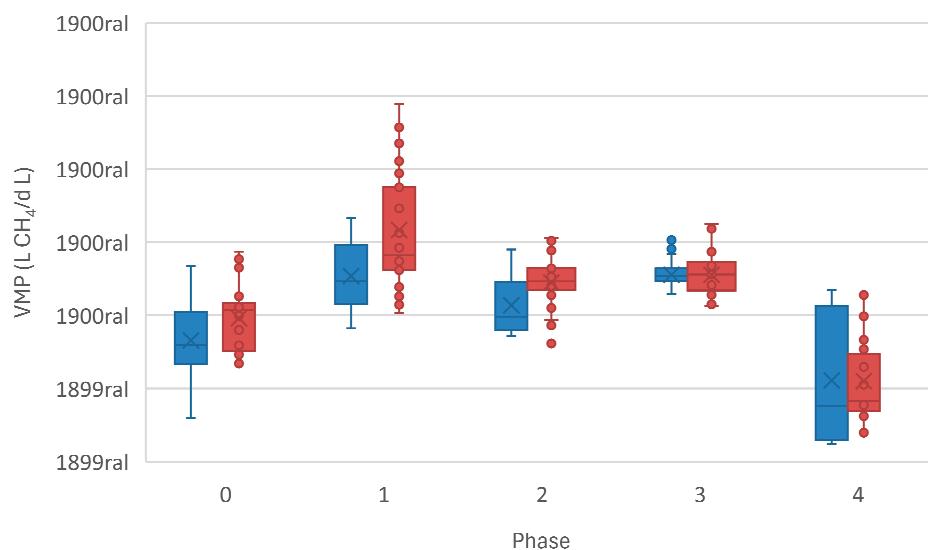


Figure 10. Showing increased VMP ($L\text{ CH}_4\cdot L^{-1}\cdot d^{-1}$) in the methanogenic reactors in the presence of trace concentrations of monensin in the two-phase system while dropping suddenly at higher concentrations.

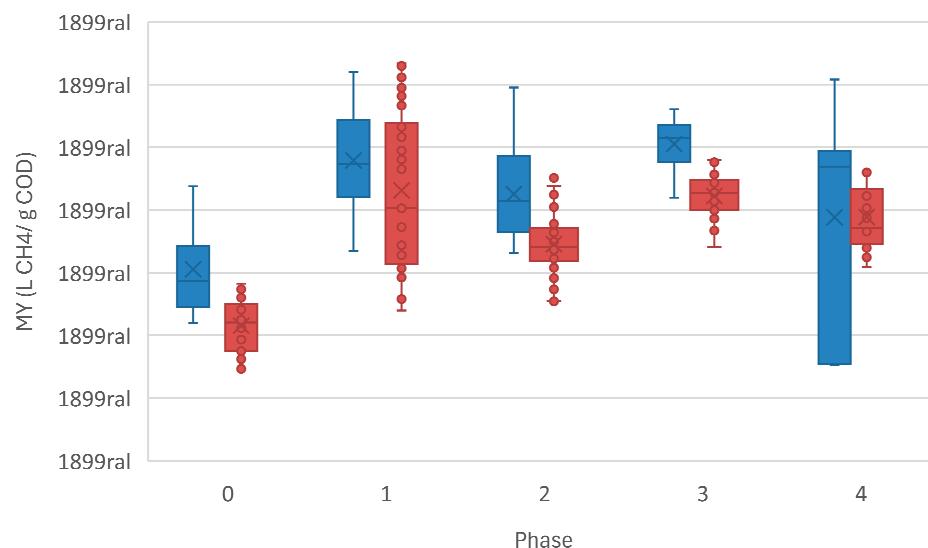


Figure 11. Methane yield (MY) ($L\text{ CH}_4/g\text{ COD}$) increase in the methanogenic reactors in the presence of monensin in the two-phase system.

3.4. Two-Phase Reactor Global Monensin Removal

The two-phase reactor as a unit, in both the ASTBR-UASB (A-U) and ASTBR-ASTBR (A-A) configurations, was shown to be capable of removing trace amounts of monensin to a similar degree (Figure 12). Overall, the A-U demonstrated less consistency than the A-A configuration, even if, when looking at averages, they might seem equally efficient. This highlights the stability of the fixed biomass system and its superiority in dealing with toxic substances. While the effluents would average out over a season to result in about the same amount of monensin being applied to soil in fertirrigation, the A-A system would result in lower variation and lower peak concentrations in the soil, and with optimization, perhaps higher removal rates of monensin and other toxic compounds.

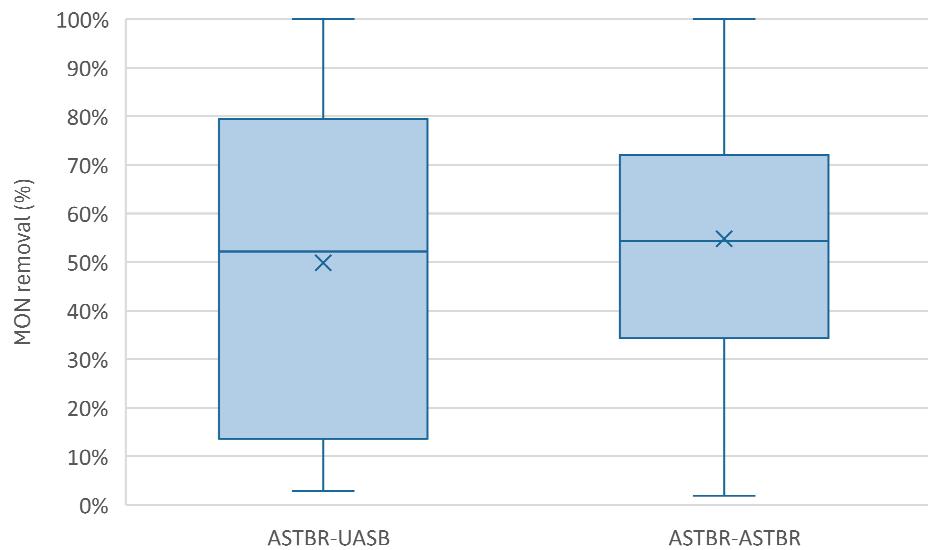


Figure 12. MON removal between A-U and A-A configurations overall.

While looking at the different phases of this study in Figure 13, the A-A configuration shines at the lowest concentrations, highlighting the importance of its role in removing the smallest trace amounts of micropollutants and highlighting the low reliability of the A-U configuration for this aim. At higher concentrations, the performance of both configurations tends to converge, showing that anaerobic digestion in general can improve the quality of vinasse heavily contaminated with monensin.

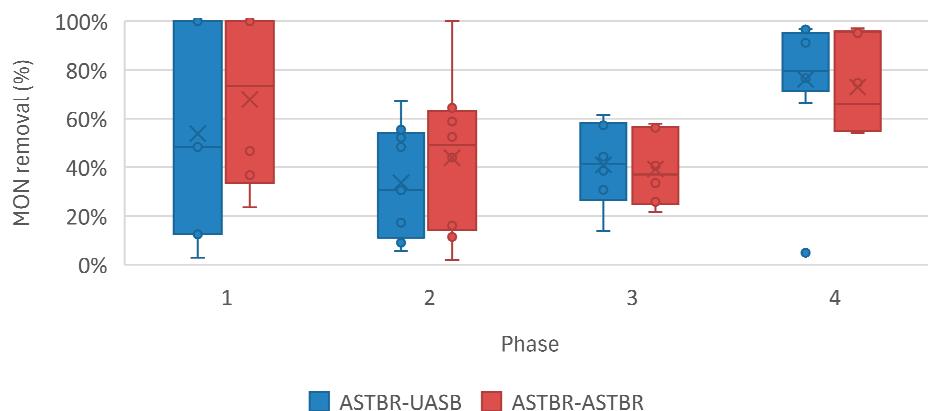


Figure 13. MON removal from sugarcane vinasse across different phases of this study in the two-phase anaerobic digestion reactor in both the A-U and A-A configurations.

4. Conclusions

Monensin use in sugarcane–ethanol production results in trace concentrations of the antibiotic in vinasse used for fertirrigation. This presents an environmental concern whose effects can range from the development of bacterial resistance in soil, returning to the sugarcane–ethanol production process and hindering the effectiveness of this antibiotic, to the persistence of toxicity in the environment and affecting wildlife, livestock, and public health. This creates a need for solutions to treat this issue, along with the general toxicity of vinasse fertirrigation. This study evaluated the effectiveness of simultaneous biogas production and removal of monensin using anaerobic digestion in a two-phase anaerobic reactor and showed that such a configuration provides a reliable way to both increase the value of vinasse as a byproduct while reducing its toxicity due to antibiotic persistence. This study also shows that the use of an antimicrobial agent does not necessarily imply the loss of viability of biodegradation as a form of upgrading vinasse. On the contrary, the pres-

ence of antimicrobial agents can potentially increase biogas production by disrupting the consumption of metabolites in the pretreatment phase and potentializing their conversion into biogas. As a novel study into a new way to view agro-industrial waste contaminants, this study has limitations, mainly pertaining to the uncertainties relating to the metabolic pathways and degradation mechanisms in mixed culture bioreactors. A lot remains to be understood in terms of which genes are present in the reactor ecosystem, when they are expressed, and how this can be leveraged to maximize biogas generation and toxicity resistance. Furthermore, the results obtained are on a bench scale and must be explored on a larger scale. Correlations of monensin with specific OAS products in the reactor should be explored, as they represent extensive economic value. Further studies can optimize this removal of monensin along with other toxins potentially present in vinasse and perhaps even proceed to use antimicrobial agents to increase the yield of anaerobic biodigestion systems. This can be performed by, for example, using monensin-contaminated vinasse as an additive in a two-phase reactor treating normally monensin-free vinasse, simultaneously degrading low-concentration monensin completely and increasing acid production in the acidogenic phase, for improving methane production in the methanogenic phase. A result would be both more value extraction from vinasse and a less harmful fertilizer byproduct discharged onto cropland. This study recommends modeling the kinetics of monensin intervention in continuous anaerobic reactors and an evaluation of the potential use of monensin or monensin-contaminated vinasse as an additive for enhanced methane production.

Author Contributions: S.C.: writing—original draft, writing—review and editing, methodology, investigation, formal analysis, and conceptualization. M.Z.: writing—review and editing, supervision, funding acquisition, and conceptualization. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financed, in part, by the São Paulo Research Foundation (FAPESP), Brasil, Process Numbers 2015/06246-7 and 17/02147-0. This study was financed, in part, by CNPq, Process 314731/2021-0. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES), Finance Code 001. The main author of this paper was a beneficiary of financial assistance from CAPES-Brasil.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

Acknowledgments: This study was made possible with the support and infrastructure of the Biological Processes Laboratory at the São Carlos School of Engineering of the University of São Paulo (LPB-EESC-USP) and the Chromatography Group at the São Carlos Institute of Chemistry of the University of São Paulo (CROMA-IQSC-USP).

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ASTBR	Anaerobic Structured-Bed Bioreactor
UASB	Upflow Anaerobic Sludge Blanket
COD	Chemical Oxygen Demand
OLR	Organic Loading Rate
HRT	Hydraulic Retention Time
VMP	Volumetric Methane Production
MY	Methane Yield
TSSs	Total Suspended Solids
VSSs	Volatile Suspended Solids
VOCs	Volatile Organic Compounds

MON	Monensin
VFAs	Volatile Fatty Acids
OASs	Organic Acids and Solvents
NTK	Total Kjeldahl Nitrogen (Nitrogênio Total Kjeldahl)
pH	Potential of Hydrogen (Acidity/Alkalinity Measure)

References

1. Fuess, L.T. Biodigestão Anaeróbia Termofílica de Vinhaça em Sistemas Combinados do Tipo Acidogênico-Metanogênico para Potencialização da Recuperação de Bioenergia em Biorrefinarias de Cana-de-Açúcar de Primeira Geração. Ph.D. Thesis, Universidade de São Paulo, São Paulo, Brazil, 2017. [\[CrossRef\]](#)
2. Poveda, M.M.R. Análise Econômica e Ambiental do Processamento da Vinhaça com Aproveitamento Energético. Master's Thesis, Universidade de São Paulo, São Paulo, Brazil, 2015. [\[CrossRef\]](#)
3. da Silva, G.A. Avaliação das Tecnologias de Disposição de Vinhaça de Cana de Açúcar Quanto ao Aspecto de Desenvolvimento Ambiental e Econômico. Ph.D. Thesis, Universidade de São Paulo, São Paulo, Brazil, 2011. [\[CrossRef\]](#)
4. Carpanez, T.G.; Moreira, V.R.; Assis, I.R.; Amaral, M.C.S. Sugarcane vinasse as organo-mineral fertilizers feedstock: Opportunities and environmental risks. *Sci. Total Environ.* **2022**, *832*, 154998. [\[CrossRef\]](#)
5. Conab-Produção de Cana-de-Açúcar na Safra 2024/25 Sofre Redução Devido a Condições Climáticas Adversas. Available online: <https://www.conab.gov.br/ultimas-noticias/5841-producao-de-cana-de-acucar-na-safra-2024-25-sofre-reducao-devido-a-condicoes-climaticas-adversas/> (accessed on 2 March 2025).
6. Silva, D.F.D.S.E.; Bomtempo, J.V.; Alves, F.C. Innovation opportunities in the Brazilian sugar-energy sector. *J. Clean. Prod.* **2019**, *218*, 871–879. [\[CrossRef\]](#)
7. Junior, A.D.N.F.; Etchebehere, C.; Perecin, D.; Teixeira, S.; Woods, J. Advancing anaerobic digestion of sugarcane vinasse: Current development, struggles and future trends on production and end-uses of biogas in Brazil. *Renew. Sustain. Energy Rev.* **2022**, *157*, 112045. [\[CrossRef\]](#)
8. Ramos-Reyes, M.F.; González-López, M.E.; Barajas-Álvarez, P.; Garcia-Garcia, C.E.; Tuesta-Popolizio, D.A.; Mussatto, S.I.; Gradilla-Hernández, M.S. Exploring the potential of distillery vinasses through upcycling: Pathways to a circular economy. *Environ. Technol. Innov.* **2025**, *38*, 104072. [\[CrossRef\]](#)
9. Sánchez, F.E.; Fuess, L.T.; Cavalcante, G.S.; Adorno, M.Á.T.; Zaiat, M. Value-added soluble metabolite production from sugarcane vinasse within the carboxylate platform: An application of the anaerobic biorefinery beyond biogas production. *Fuel* **2021**, *286*, 119378. [\[CrossRef\]](#)
10. Fuess, L.T.; Kiyuna, L.S.M.; Ferraz, A.D.N.; Persinoti, G.F.; Squina, F.M.; Garcia, M.L.; Zaiat, M. Thermophilic two-phase anaerobic digestion using an innovative fixed-bed reactor for enhanced organic matter removal and bioenergy recovery from sugarcane vinasse. *Appl. Energy* **2017**, *189*, 480–491. [\[CrossRef\]](#)
11. Borges, A.D.V.; Fuess, L.T.; Takeda, P.Y.; Rogeri, R.C.; Saia, F.T.; Gregoracci, G.B.; Damianovic, M.H.R.Z. Unleashing the full potential of vinasse fermentation in sugarcane biorefineries. *Renew. Sustain. Energy Rev.* **2025**, *208*, 115096. [\[CrossRef\]](#)
12. Fuess, L.T.; Rogeri, R.C.; Eng, F.; Borges, A.D.V.; Bovio-Winkler, P.; Etchebehere, C.; Zaiat, M. Thermophilic fermentation of sugarcane vinasse: Process flexibility explained through characterizing microbial community and predicting metabolic functions. *Int. J. Hydrogen Energy* **2024**, *77*, 1339–1351. [\[CrossRef\]](#)
13. Rogeri, R.C.; Fuess, L.T.; de Araujo, M.N.; Eng, F.; Borges, A.D.V.; Damianovic, M.H.R.Z.; da Silva, A.J. Methane production from sugarcane vinasse: The alkalinizing potential of fermentative-sulfidogenic processes in two-stage anaerobic digestion. *Energy Nexus* **2024**, *14*, 100303. [\[CrossRef\]](#)
14. de Oliva-Neto, P.; Yokoya, F. Susceptibility of *Saccharomyces cerevisiae* and lactic acid bacteria from the alcohol industry to several antimicrobial compounds. *Braz. J. Microbiol.* **2001**, *32*, 10–14. [\[CrossRef\]](#)
15. Hynes, S.H.; Kjarsgaard, D.M.; Thomas, K.C.; Ingledew, W.M. Use of virginiamycin to control the growth of lactic acid bacteria during alcohol fermentation. *J. Ind. Microbiol. Biotechnol.* **1997**, *18*, 284–291. [\[CrossRef\]](#)
16. De Miniac, M. US Patent for Use of Ionophoretic Polyether Antibiotics for Controlling Bacterial Growth in Alcoholic Fermentation Patent. U.S. Patent 5,888,788, 30 March 1999. Available online: <https://patents.justia.com/patent/5888788> (accessed on 18 August 2021).
17. Mollenhauer, H.H.; Morré, D.J.; Rowe, L.D. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim. Biophys. Acta (BBA)—Rev. Biomembr.* **1990**, *1031*, 225–246. [\[CrossRef\]](#)
18. Matsuoka, T.; Novilla, M.N.; Thomson, T.D.; Donoho, A.L. Review of monensin toxicosis in horses. *J. Equine Vet. Sci.* **1996**, *16*, 8–15. [\[CrossRef\]](#)
19. Zidar, P.; Kos, M.; Vogel-Mikuš, K.; van Elteren, J.T.; Debeljak, M.; Žižek, S. Impact of ionophore monensin on performance and Cu uptake in earthworm Eisenia andrei exposed to copper-contaminated soil. *Chemosphere* **2016**, *161*, 119–126. [\[CrossRef\]](#)

20. Watanabe, N.; Harter, T.H.; Bergamaschi, B.A. Environmental Occurrence and Shallow Ground Water Detection of the Antibiotic Monensin from Dairy Farms. *J. Environ. Qual.* **2008**, *37*, S-78–S-85. [\[CrossRef\]](#)

21. Melchior, E.; Hales, K.; Lindholm-Perry, A.; Freetly, H.; Wells, J.; Hemphill, C.; Wickersham, T.; Sawyer, J.; Myer, P. The effects of feeding monensin on rumen microbial communities and methanogenesis in bred heifers fed in a drylot. *Livest. Sci.* **2018**, *212*, 131–136. [\[CrossRef\]](#)

22. da Silva, J.J. Antibióticos na Produção de Etanol Combustível: Análise, Dispersão no Meio Ambiente e Efeitos na Biodigestão da Vinhaça. May 2021. Available online: <http://hdl.handle.net/11449/204933> (accessed on 1 March 2025).

23. da Silva, J.J.; da Silva, B.F.; Zanoni, M.V.B.; Stradiotto, N.R. Sample preparation and antibiotic quantification in vinasse generated from sugarcane ethanol fuel production. *J. Chromatogr. A* **2022**, *1666*, 462833. [\[CrossRef\]](#)

24. Baird, R.B.; Eaton, A.D.; Rice, E.W. *Standard Methods for the Examination of Water and Wastewater*, 23rd ed.; Water Works Association: Washington, DC, USA, 2017.

25. Taylor, K.A.C.C. A simple colorimetric assay for muramic acid and lactic acid. *Appl. Biochem. Biotechnol.* **1996**, *56*, 49–58. [\[CrossRef\]](#)

26. Valdez, H.d.C.; Amado, R.S.; de Souza, F.C.; D'ELia, E.; Vieira, E.d.C. Free and total glycerol determination in biodiesel samples by enzymatic method with colorimetric detection. *Química Nova* **2012**, *35*, 601–607. [\[CrossRef\]](#)

27. DuBois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28*, 350–356. [\[CrossRef\]](#)

28. Leite, J.A.C.; Fernandes, B.S.; Pozzi, E.; Barboza, M.; Zaiat, M. Application of an anaerobic packed-bed bioreactor for the production of hydrogen and organic acids. *Int. J. Hydrogen Energy* **2008**, *33*, 579–586. [\[CrossRef\]](#)

29. Fuess, L.T.; Kiyuna, L.S.M.; Garcia, M.L.; Zaiat, M. Operational strategies for long-term biohydrogen production from sugarcane stillage in a continuous acidogenic packed-bed reactor. *Int. J. Hydrogen Energy* **2016**, *41*, 8132–8145. [\[CrossRef\]](#)

30. Perna, V.; Castelló, E.; Wenzel, J.; Zampol, C.; Lima, D.F.; Borzacconi, L.; Varesche, M.; Zaiat, M.; Etchebehere, C. Hydrogen production in an upflow anaerobic packed bed reactor used to treat cheese whey. *Int. J. Hydrogen Energy* **2013**, *38*, 54–62. [\[CrossRef\]](#)

31. Adorno, M.A.T.; Hirasawa, J.S.; Varesche, M.B.A. Development and Validation of Two Methods to Quantify Volatile Acids (C2–C6) by GC/FID: Headspace (Automatic and Manual) and Liquid-Liquid Extraction (LLE). *AJAC* **2014**, *5*, 406–414. [\[CrossRef\]](#)

32. Carneiro, R.B.; Gomes, G.M.; Camargo, F.P.; Zaiat, M.; Santos-Neto, Á.J. Anaerobic co-metabolic biodegradation of pharmaceuticals and personal care products driven by glycerol fermentation. *Chemosphere* **2024**, *357*, 142006. [\[CrossRef\]](#)

33. Dias, M.E.S.; Lopes, J.C.; Carneiro, R.B.; Damianovic, M.H.R.Z.; Foresti, E. Using Compounds Derived from the Glycerol Fermentation as a Carbon Source for Denitrification and Biological Phosphorus Removal. *Water Air Soil Pollut.* **2021**, *232*, 339. [\[CrossRef\]](#)

34. Lopes, J.C.; Silva, B.G.; Dias, M.E.S.; Carneiro, R.B.; Damianovic, M.H.R.Z.; Foresti, E. Enhanced biological nitrogen and phosphorus removal from sewage driven by fermented glycerol: Comparative assessment between sequencing batch- and continuously fed-structured fixed bed reactor. *Environ. Sci. Pollut. Res.* **2023**, *30*, 11755–11768. [\[CrossRef\]](#)

35. Clomburg, J.M.; Gonzalez, R. Anaerobic fermentation of glycerol: A platform for renewable fuels and chemicals. *Trends Biotechnol.* **2013**, *31*, 20–28. [\[CrossRef\]](#)

36. Wang, K.; Yin, J.; Shen, D.; Li, N. Anaerobic digestion of food waste for volatile fatty acids (VFAs) production with different types of inoculum: Effect of pH. *Bioresour. Technol.* **2014**, *161*, 395–401. [\[CrossRef\]](#)

37. García, O.D.V. Toxicidade Metanogênica da Monensina Sódica e Beta-Ácidos de Lúpulo e Seus Efeitos na Biodigestão Anaeróbia da Vinhaça no Contexto da Indústria Sucroalcooleira Brasileira. Ph.D. Thesis, Universidade Federal do ABC, Santo André, Brazil, 2021.

38. Arikán, O.A.; Mulbry, W.; Rice, C.; Lansing, S. The fate and effect of monensin during anaerobic digestion of dairy manure under mesophilic conditions. *PLoS ONE* **2018**, *13*, e0192080. [\[CrossRef\]](#)

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.