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**Research Article**

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**Posted Date:** October 30th, 2026

**DOI:** https://doi.org/10.21203/rs.3.rs-11239550/v1

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**Additional Declarations:** No competing interests reported.

# Fe(II) and Cu(II)-Mediated Enhancement of Biogas Yield and BioMethane Content in Cow Dung - Chicken Manure Co-Digestion: A Kinetic Study

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**Abstract.**
Trace metal supplementation is an exciting, low-cost approach to enhancing anaerobic digestion (AD) performance, yet the concentration-dependent effects of individual metals on co-digestion kinetics and biogas quality has remained incompletely resolved for heterogeneous livestock-waste mixtures. This study investigated the impacts of Fe(II) and Cu(II) supplementation at concentration of 0.02, 0.05, and 0.10 mM as $FeSO_4 \cdot 7H_2O$ and $CuCl_2 \cdot 2H_2O$) on the anaerobic co-digestion of cow dung (CD) and chicken manure (CM) at a 50:50 ratio, in terms of both digestion kinetics and biogas compositional quality over a 50-day hydraulic retention time. Physicochemical characterization of the CD and CM was conducted by standard protocols. The result proved that compositionally, CM was richer than CD in nitrogen, protein, and macro-minerals, whereas CD contributed majorly a carbon-dominant, fibrous fraction. Across all bioreactors, cumulative biogas yield followed a clear biphasic (diauxic) trajectory, which was accurately described by a two-phase logistic model ($R^2$ = 0.992 - 0.999).This pattern reveals sequential use of readily degradable soluble organics, followed by the refractory lignocellulosic materials. Fe(II) displayed a typical hormetic dose - response, and 0.05 mM demonstrated the best performance (29.90 $dm^3$ cumulative biogas, which 21.16% above control; 75.34% $CH_4$) while 0.10 mM turned out to be inhibitory, with only 23.51 $dm^3$ (with 63.27% $CH_4$, lower than control). In contrast, Cu(II) produced a monotonically positive response in all the tested range, with 0.10 mM producing the overall highest cumulative yield (30.25 $dm^3$, 22.56% above control) and biomethane content (76.79%), alongside near-complete suppression of sulfurous off-gas, consistent with copper-mediated sulfide precipitation. Furthermore, gas chromatographic analysis revealed a concurrent increase in residual hydrogen under high-dose Cu(II), suggesting possible a partial uncoupling of hydrogenotrophic and acetoclastic methanogenic pathways. These findings demonstrate that micro-dosing of Fe(II) (0.05 mM) or Cu(II) (0.10 mM) can enhance both the yield and quality of biogas substantially from CD/CM co-digestion, while highlighting that effective trace-metal

concentrations are strongly substrate- and matrix-dependent, underscoring the value of two-phase kinetic modelling for optimizing metal-amended anaerobic digestion systems.

**Keywords:** Anaerobic co-digestion; Trace metal supplementation; Diauxic biogas production; Diphasic logistic model; Biogas composition; Circular bioeconomy

## 1. Introduction.

Driven by the unsustainable nature of petroleum-based fuels, the need for energy security, and environmental concerns, especially mitigation of greenhouse gas (GHG) emissions, researchers are now focusing more attention on carbon neutral and sustainable bioenergy resources to replace fossil fuels (Lora *et al.,* 2026; Aromolaran *et al.,* 2023). Biogas is an excellent renewable and sustainable bioenergy resource that can effectively serve as a substitute for non-renewable and unsustainable fossil fuels. Anaerobic digestion (AD) has been viewed as an efficient and technological approach to sustainable conversion of organic wastes such as animal manure, municipal solid waste (MSW), agricultural crop residues and industrial waste into biogas, a bioenergy source (Darmey *et al.,* 2025; Ignatowicz *et al.,* 2023). In addition to optimization of sustainable energy consumption of communities, this technology offers other exciting benefits, including an ecofriendly waste management technique, improvement in the ecology of rural areas, expanding food supplies and improvement in health conditions via elimination/decrease of pathogenic microorganisms (Nwokolo *et al.,* 2020). Anaerobic digestion is a controlled biodegradation process that allows cost-effective and efficient capturing and utilization of biogas (approximately 40 - 75% methane and 30 - 40% carbon dioxide) for energy generation (Heikala *et al.,* 2022). More so, AD technology mitigates pollution from organic wastes generated via agricultural practices, while alleviating energy crisis (Wang, *et al.,* 2022).

Meat from poultry is globally the second most consumed, and in order to meet the continuously growing demand, global production of poultry meat and eggs have skyrocketed. The increased production has, over the past decades, consequently led to the generation of large quantities of poultry waste. One of the major challenges confronting animal husbandry, including piggery, poultry and fish farming is effective management of the waste generated (Yuana et al., 2025; Ankathi et al., 2024), and anaerobic digestion (AD) is the panacea to this problem, restoring clean and green environment.

However, to ensure the process performance of AD, several parameters such as the nature of the substrates: carbon-to- nitrogen (C: N) ratio, total volatile solids (VS), total solids (TS), as well as

the macro- and micro- nutrient concentration must be considered as fundamental. Animal manures such as poultry dropping, cow and pig dung are considered very attractive for renewable and sustainable energy production because they are natural resources. Poultry droppings are very rich in important macro- and micro-elements, particularly large amounts of nitrogen, phosphorus, magnesium and trace elements such as copper and zinc. However, mono-digestion of poultry manure can particularly encounter some inhibition problems as a result of their high nitrogen content. When a certain threshold is exceeded (1500 and 7000 $mg.L^{-1}$), the AD process would be inhibited (Mansour *et al.,* 2023). Co-digestion, therefore, is recommended to forestall the inhibitory effect; however, the co-substrate should be chosen carefully while taking into consideration its composition and influence on the major feedstock, and the mixture ratio should also be optimized

Cow dung has some limitations in AD, including low degradability due to the presence of lignocellulosic biomass, low volatile solids (VS) and C/N ratio (Orlando and Borja 2020). Recently, research interests have been directed towards improving anaerobic digestion of animal manure. Anaerobic co-digestion of two or more digester feeds is the alternative to resolve the problems of mono-substrate digestion such as unfavorable nutrient imbalance, recalcitrant compounds in the substrate, and highly inhibitory or toxic substances (Wongarmat *et al.,* 2020).

Anaerobic co-digestion (AcoD) of cow dung mixed with other carbon and nitrogen-rich organic substrates such as poultry manure has been proven to reduce the possibilities of ammonia inhibition and volatile fatty solids (VFA) accumulation, while improving the process (Hammad *et al.,* 2018).

Co-digestion of organic wastes with other feed stocks and supplementation with trace elements have been carried out to overcome bioreactor failures as well as to enhance the process performance to maximize the bioconversion of organics to biomethane (Liang et al., 2026). Some of the times the observed instability in the AD process for biomethane production may be due to the total lack of, or very low level of trace elements. One of the methods of improving biogas yield is by supplementation of bioreactor feeds with small amounts of trace elements. Trace elements including Iron, Nickel and Cobalt etc. have been proven to stimulate bacterial activities in AD (Abdel-Shafy and Mansour, 2014).

Iron plays several essential roles in AD process, basically due to its large reduction capacity. The importance of Fe and its involvement in energy metabolism is dependent on its redox properties.

Fe is part of the transport system of the methanogenic bacteria for the conversion of $CO_2$ to $CH_4$, and functions both as an electron acceptor and donor. Its role is vital in the composition of enzymes such as hydrogenases, CO-dehydrogenase, methane monooxygenase, NO-reductase, superoxide dismutase, nitrite and nitrate reductase, etc. (Golub *et al.,* 2022). Nickel and Iron are present in the form of Ni-Fe-S and Fe-S clusters that are the primary subunits of the enzymes, hydrogenase and acetyl-CoA synthase (Myszograj *et al*., 2018).

Cu, Zn, and Ni constitute part of hydrogenase enzymes and have been reported to speed up metabolism of biogas formation at concentrations not exceeding the threshold standards (Matheri *et al.,* 2016). Although many trace elements/heavy metals example; Fe, Ni, Co, Cu, Zn, Se etc. are required for the activation or normal functioning of many enzymes and coenzymes in anaerobic digestion (Tian *et al.,* 2019), high amounts of heavy metals have been reported to exert inhibitory effects on anaerobic microorganisms (Alrawashdeh *et al.,* 2020; Bui *et al.,* 2020; Dokulilová *et al.,* 2018). As the desired threshold where copper stimulates or inhibits AD process has not been clearly defined, experimental determination of the required concentration or threshold to achieve enhanced yield in biogas is very imperative. This study therefore aimed to: (i) evaluate the impact of supplementation of animal manure with different concentrations (0.02, 0.5 and 0.1mM) of $FeSO4.7H_2O$ and $CuCl_2.2H_2O$ on the anaerobic co-digestion for biogas production. (ii) Provide insight on the process kinetics of diphasic anaerobic digestion pattern and assess the fitting of two-phase logistic function model to the experimental data.

## 2.0. Materials and Methods.

### 2.1. Feedstock Collection, Preparation and Characteristics.

The cow dung (CD) and chicken (CM) were collected from a local poultry farm and an abattoir, respectively, both in Owerri municipal, Imo State Nigeria. The samples were sorted to remove objectionable and non-biodegradable materials and sun-dried afterwards to reduce the moisture content. The dried samples were milled with automated milling machine, sieved to a specific range of particle size (1- 2mm).

### 2.2 Reagents.

Analytical grade iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) and Copper (II) chloride dihydrate ($CuCl_2.2H_2O$) used in this study were sourced from Sigma-Aldric (Germany). As described by

Opurum *et al.,* (2023), stock solution (10mM concentration) of each of the salts was prepared with distilled from which the varying concentrations required for the experiment was obtained.

**2.3. Experimental Procedure.**

The experiment was conducted in closed system custom-built bioreactors of ten liter capacity made of high-density polyethylene (HDPE) material. Slurry of 50:50 ratio of cow dung (CD) and chicken manure (CM) were prepared in replicates with distilled water. Distinct volumes (16, 40 and 80 ml) of each of the 10 mM stock solution of the metal salts (Fe and Cu) were added separately to achieve specific concentrations of 0.02, 0.05, and 0.1 mM, while the control bioreactor contained CD/CM alone. The preparations were thoroughly mixed and loaded into bioreactors and properly labeled. Subsequently, 1.6 L of active inoculum (aliquot of freshly strained cow rumen waste) was introduced into each bioreactor. The initial pH was adjusted to 7.5 ± 0.2 using appropriate buffering agents. The working volume of each bioreactor was adjusted to 8.0 L with distilled water, and the systems were tightly sealed to ensure anaerobic conditions. Each bioreactor was fitted to a 12 liter biogas collection system via outlet hose, and biogas production was measured by downward water displacement method. The bioreactors were operated under mesophilic conditions (25 - 35 °C ± 0.2) for a hydraulic retention time of 50 days.

**2.4 Biogas Composition Analysis.**

The composition of the produced biogas including the major components: methane ($CH_4$), carbon dioxide ($CO_2$), carbon monoxide (CO), and hydrogen sulfide ($H_2S$), was analyzed using gas chromatography equipped with a thermal conductivity detector (GC–TCD). Helium was used as the carrier gas at a pressure of 5 psi. Biogas samples collected in airtight tubes were injected into the GC system at a flow rate of 20 ml $min^{-1}$ for compositional analysis.

**2.5 Two - Phase Logistic Model Kinetic Studies.**

Cumulative biogas production data were modeled using a two-phase logistic function to account for the diauxic anaerobic digestion pattern observed during the process. The model was fitted to the experimental data using SigmaPlot software (version 10.0).

$$y = \frac{P_{b1}}{1+\exp\left[\frac{4R_{m1}(\lambda_1 - t)}{P_{b1}} + 2\right]} + \frac{P_{b2} - P_{b1}}{1+\exp\left[\frac{4R_{m2}(\lambda_2 - t)}{P_{b2} - P_{b1}} + 2\right]} \qquad (2)$$

### 2.6 Statistical Analysis.

Statistical analysis was performed to evaluate the effects of Fe(II) and Cu(II) supplementation on biogas yield. Differences in mean cumulative biogas production were assessed using one-way analysis of variance (ANOVA) implemented in IBM SPSS Statistics version 27.0. Statistical significance was determined at $P \leq 0.05$.

## 3.0. Results.

### 3.1 Physicochemical Composition of the Bioreactor Feedstocks.

Chicken manure (CM) had much higher moisture content of 19.80% compared to cow dung (CD) (12.38%), implying a greater and effective water-holding capacity. The ash contents of the two feed-stocks are quite similar, with values of 39.46 and 38.74% for CD and CM, respectively. However, CM had reasonably higher fiber content (10.77%) relative to CD (4.10%). In general, CM showed a stronger profile in terms of protein-related parameters. It had a protein content of 9.94%, compared to CD that only had 8.49%. This difference is also evident in the crude protein contents, with CM at 14.89% and CD at 8.67%. The CM also demonstrated a higher percentage of total organic carbon (TOC) of 16.32% relative to CD (14.96%). The carbon-to- nitrogen ratio of CM was 10.30%, comparatively close to the CD (11.0%). Considering the total solids (TS), CM displayed a notable advantage with TS of 80.19 and volatile solids (VS) of 41.48%. CD in contrast, had lower percentages 47.80 and 28.15, respectively. There was slightly higher nitrogen in CM (1.59%) compared to CD (1.36%).

Mineral composition evaluation clearly showed that CM was much richer in essential nutrients relative to CD. For instance, the potassium concentration in CM was impressively 1882.53 mg/kg, approximately twice that of CD, which measured 931.79 mg/kg. Sodium levels were also higher in CM at 2133.53 mg/kg, while CD had only 746.07 mg/kg. Phosphorus contents were 17.07 mg/kg in CM compared to 11.37 mg/kg in CD. More so, CM had greater amounts of calcium and magnesium, with values of 1.817 and 3.47%, respectively, while CD had 0.977 and 0.149%.

Potassium concentration in CM was found to be 1882.53 mg/kg, approximately twice that of CD (931.79 mg/kg). Similarly, sodium content of CM was considerably higher (2133.53 mg/kg) compared to 746.07 mg/kg found in CD. Phosphorus levels measured at 17.07 mg/kg CM and 11.37 mg/kg in CD. Additionally, CM boasted higher calcium and magnesium contents, with

values of 1.82 and 3.47 %, respectively, compared to 0.977% and 0.149% in CD. Generally, CM clearly demonstrated richer nutrient profile, more organic matter, and higher mineral concentrations relative to CD, highlighting its greater potential as a valuable bioresource for possible application in composting, biomethane and biofertilizer production.

**Table 1. Proximate Composition of the Bioreactor Feedstocks.**

| Parameters % | CD | CM |
|---|---|---|
| Moisture Content | 12.38 | 19.80 |
| Ash Content | 39.46 | 38.74 |
| Fibre | 4.10 | 10.77 |
| Protein | 8.49 | 9.94 |
| Fat | 6.90 | 5.90 |
| Crude Protein | 8.67 | 14.89 |
| Total Organic Carbon | 14.96 | 16.32 |
| C/N | 11.00 | 10.30 |
| Total Solid | 47.80 | 80.19 |
| Volatile Solid | 28.15 | 41.48 |
| Nitrogen | 1.36 | 1.59 |
| Potassium | 931.79 | 1882.52 |
| Phosphorus | 11.37 | 17.07 |
| Sodium | 746.08 | 2133.54 |
| Calcium | 0.98 | 1.82 |
| Magnesium | 0.15 | 3.48 |

### 3.2 Anaerobic Digestion and Biogas Production Profile.

#### 3.2.1 Daily Biogas Production under the various Fe (II) Concentrations.

Figure 1 below shows the daily biogas production profiles of the bioreactors supplemented with Fe(II) at the tested concentrations (0.02, 0.05, and 0.10 mM). Biogas production pattern typically followed the well-known stages of anaerobic digestion process (lag phase, active biogas production phase, and decline phase). In the bioreactors amended with 0.02 mM Fe (II), there was

a short lag period that lasted between 1-6days, with biogas production that fluctuated between 7 to 37cm³.

However, from Day 7, a notable spike in biogas production was observed, signaling the beginning of the active production phase. It can be seen that the active phase lasted until Day 24, with an impressive peak in daily yield reaching 2205 cm³ on Day 18. Biogas production afterwards, gradually began to reduce. Though there was a secondary increase in gas production was observed between Days 33 and 42, the overall trend suggested substrate depletion. The decline phase which was evident by well decreased gas yield from Day 25 onward, eventually dropped as low as 175 cm³ by Day 50.

In bioreactor with supplemented with Fe (II) 0.05 mM, a lag phase that lasted for the first 6 days also observed, during which biomethane production varied between 24 and 89 cm³. Active biomethane production commenced on Day 7, and strongly continued until approximately Day 24. The peak daily production which hit an impressive 2232.5 cm³ was recorded on Day 17, slightly earlier than that recorded in the 0.02 mM treatment. Following the peak, biomethane production gradually started to taper off, although some moderate production of gas persisted until about Day 43. The decline phase began post- Day 24, with production reducing substantially to 54 cm³ by Day 50.

Similar to the trend noted in 0.02 mM Fe (II) concentration, approximately 6 days of lag phase was also observed in bioreactors containing 0.10 mM Fe (II), during this period, daily biogas yield were below 20 cm³. Active production phase kicked off on Day 7, and quickly ramped up to a peak on Day 16 with 1480 cm³ daily yield, followed by another impressive peak of 1390 cm³ on Day 20. The active period lasted from Day 7 to Day 23, and subsequently a gradual decline in gas generation was observed. The decrease became more pronounced after Day 35, with output steadily dropping to 95 cm³ by Day 50.

In terms of overall performance, the 0.05 mM Fe(II) addition achieved the highest daily biogas yield of 2232.5 cm³, which trailed closely by the 0.02 mM Fe treatment (2205 cm³), whereas the 0.10 mM Fe treatment produced the lowest yield of 1480 cm³. All the treatments had similar lag duration of about six days, implying that the various concentrations Fe (II) tested did not remarkably impact the adaptation time but affected the level and duration of active biogas production.

The control bioreactor (CD/CM co-digestion alone) demonstrated a relatively long lag duration which lasted between Days 1 - 18. During this period, there was unpredictable and generally low biogas yield that fluctuated between 0 - 319 $cm^3$. There was very minimal gas production for several days, particularly Days 5, 6, and 17. This is an indication that the microbial populations were still acclimatizing with the prevailing bioreactor environment and establishing their metabolic activity. The active biogas production phase was recorded on Day 19, with a marked increase in daily yield of 583 $cm^3$. Subsequent to this this surge, gas production soared to 1669 $cm^3$ on Day 20, then reached 2025 $cm^3$ on Day 21, and finally attained an impressive peak on Day 22 at 2035 $cm^3$. This peak gas yield of 2035 $cm^3$ on Day 22 clearly highlighted the efficiency of co-fermentation of CD and CM in improving utilization of available substrates, while boosting biogas production.

Biogas production remained robust for about 25 days, with biogas yield exceeding 400 $cm^3$ daily. Interestingly, another spike in gas yield was notated between Days 38 - 43, with biogas yields that fluctuated between 770 and 905 $cm^3$, indicating continued biodegradation of residual organic matter. However, after Day 45, a reduction in yield became apparent, with biogas output decreasing quickly from 675 $cm^3$ on Day 45 to just 240 $cm^3$ by Day 46, and steadily continued to decline to 35 $cm^3$ by Day 50. This decline highlights depletion of biodegradable substrates and reduced activity of the microbes toward the end of the digestion process.

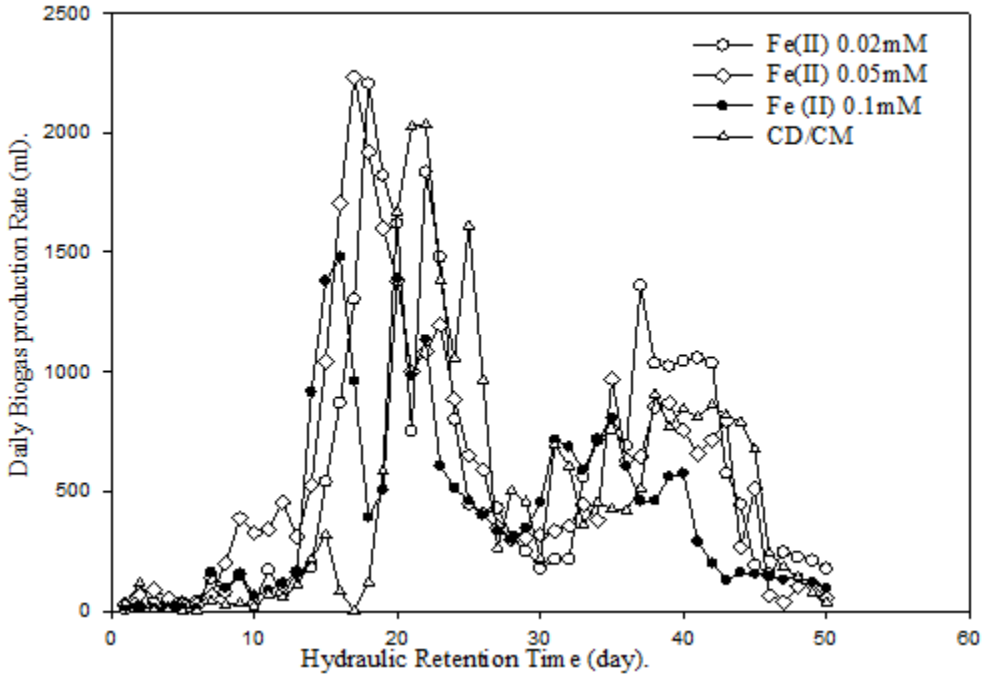


**Figure 1. Daily Biogas Production Pattern of 50:50 CD/CM with various Concentrations of Fe.**

### 3.2.2 Daily Biogas Production with the various Cu (II) Concentrations.

Similarly, the daily biogas production profiles of bioreactors treated with Cu (II) at the experimented (Figure 2) concentrations (0.02, 0.05, and 0.10 mM) followed the typical phases of anaerobic digestion discussed earlier. However, there was variations in the intensity and duration of these phases with the various Cu (II) concentrations. For instance, the reactor treated with 0.02 mM Cu experienced a notably extended lag phase from Days 1 - 12, during which daily biogas production was quite low, fluctuating between 14 to 54 $cm^3$ $day^{-1}$. On Day 13, a significant spike in gas production was observed, reaching 359 $cm^3$, which marked the start of the active methanogenic phase.

Biogas production continued to rise, peaking at an impressive 1212.5 $cm^3$ on Day 18. Afterwards, a relatively high production levels were maintained until around Day 45. However, there were some noticeable fluctuations all through the period of digestion. After Day 45, biogas yield declined sharply, and plummeted from 250 $cm^3$ on Day 45 to 60 $cm^3$ on Day 46, and finally

stabilized at 100 $cm^3$ by Day 50. Thus, the active phase of production stretched from Days 13 and 45, whereas the decline phase was evident from Days 46 - 50.

In the bioreactors supplemented with 0.05 mM concentration of Cu(II), the lag period also spanned from Days 1 – 12, during which the daily gas production was well below 355 $cm^3\ day^{-1}$. The active stage of biogas production started on Day 13, and increased steadily from 455 $cm^3$ to an optimal value of 2230 $cm^3$ on Day 18. This treatment impressively sustained biogas yield over a long period, as daily yields generally remained above 400 $cm^3$ until Day 45. This period was followed by a sharp downturn in production, declining from 360 $cm^3$ on Day 45 to 170 $cm^3$ on Day 46 and finally 140 $cm^3$ by Day 50. Thus, the active phase lasted from Days 13 to 45, while the decline phase spanned from Days 46 to 50.

A lag phase similar to other treatments was also observed for the bioreactor with 0.10 mM Cu, lasting between Days 1 - 12. During this time, the biomethane production was relatively low, fluctuating between 0 to 223 $cm^3\ day^{-1}$. A rapid surge in biogas yield started from Day 13, peaking at an impressive 2327.5 $cm^3$ on Day 18, which was recorded as the highest yield across all the Cu treatments. Increased biogas production was maintained until Day 45, with several secondary peaks recorded between Days 21 - 24, and again between Days 34 - 44. Thereafter, biogas yield substantially reduced, dropping from 551.5 $cm^3$ on Day 45 to 350 $cm^3$ on Day 46 and finally to 145 $cm^3$ on Day 50. Therefore, the active production stage for this treatment was between Days 13 - 45, and the decline stage occurred from Days 46 - 50.

The treatment with Cu (II) made a remarkable impact on daily biogas production, but a slight effect on the duration of lag phase. All the Cu-amended bioreactors displayed similar acclimatization period of approximately 12 days before they kicked into the exponential methanogenic phase. When the Cu (II) concentration was increased from 0.02 - 0.10 mM, the maximum daily biogas yield was improved, with the 0.10 mM treatment reaching the highest peak at 2327.5 $cm^3\ day^{-1}$. This was followed closely by 0.05 mM concentration of at 2230 $cm^3\ day^{-1}$ and 0.02 mM at 1212.5 $cm^3\ day^{-1}$. The observed prolonged active phase between Days 13 and 45 suggests sustained microbial activity and efficient substrate breakdown. These findings in all, indicate that supplementation with Cu (II), especially at 0.10 mM, improved anaerobic digestion performance and stimulated biogas production without substantially prolonging the acclimatization period of the microbial community.

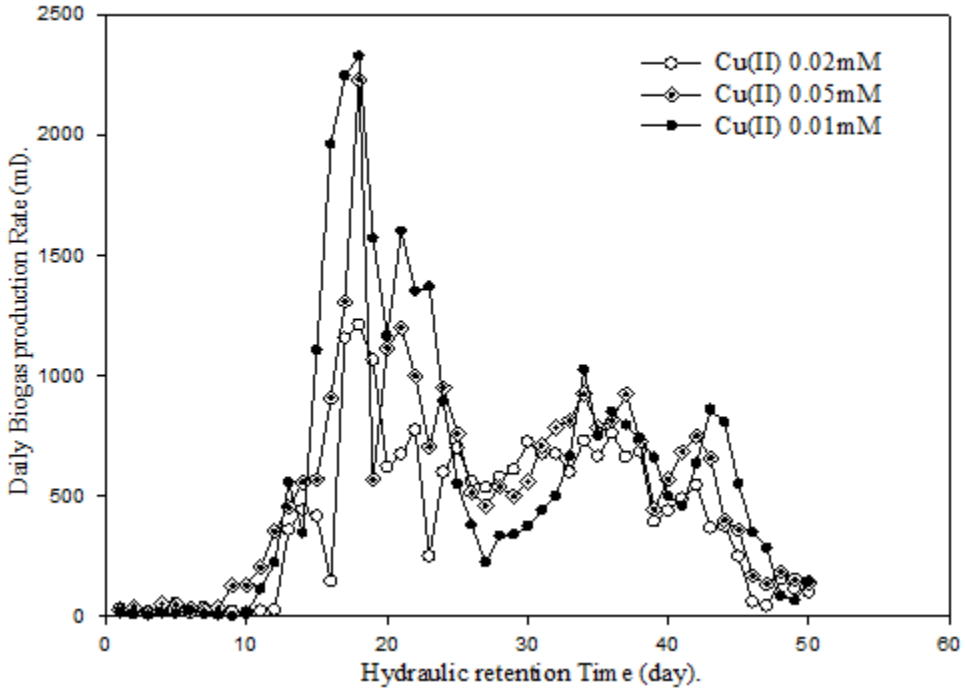


**Figure 2. Daily Biogas Production Pattern of 50:50 CD/CM with various Cu(II) Concentrations.**

### 3.2.3 Cumulative Biogas Yield from Cu(II) and Fe(II) -Supplemented Co-digestion with CD/CM Alone: A comparative Insight.

The untreated CD/CM system achieved a cumulative biogas yield of 24.68 ± 2.04 $dm^3$, a specific biogas yield of 0.092 $dm^3\ g^{-1}$ VS (Table 2). The kinetic parameters were $Pb_1$ = 13.752 $dm^3$, $Rm_1$ = 1.751 $dm^3\ d^{-1}$, $\lambda_1$ = 18.028 days, $Pb_2$ = 25.552 $dm^3$, $Rm_2$ = 0.835 $dm^3\ d^{-1}$, and $\lambda_2$ = 32.240 days (Table 4). The amendment with Cu(II) and Fe(II) notably impacted both the biomethane production performance and kinetic behavior of the anaerobic digestion process. Specifically, adding 0.05 mM Cu(II) boosted cumulative biomethane yield to 26.99 ± 1.77$dm^3$, marking an 8.35% increase, while 0.10 mM Cu(II) further enhanced it to 30.25 ± 1.83 $dm^3$, a 22.56% increase. Notably, the highest of Cu(II) concentration also improved the specific biogas yield from 0.092 to 0.113 $dm^3\ g^{-1}$ VS. From the kinetic perspective, 0.10 mM Cu(II) improved both the first-phase and second-phase biogas potentials ($Pb_1$ = 16.549 $dm^3$ and $Pb_2$ = 31.312 $dm^3$) compared to the control (13.752 and 25.552 $dm^3$, respectively).

Additionally, the first-phase production rate ($Rm_1$ = 2.116 $dm^3$ $d^{-1}$) surpassed that of the control (1.751 $dm^3$ $d^{-1}$), implying accelerated conversion of substrate. Cu(II) supplementation, however, led to in a longer second-phase lag period ($\lambda_2$ = 27.71 days) compared with lower of Cu(II), although it was still shorter than what was observed in the control group. Treatment with 0.02 mM Cu(II) notably produced less biogas (20.91 ± 11.99 $dm^3$) than the control (24.68 ± 2.04 $dm^3$), indicating that microbial activity effectively was not stimulated this concentration. In contrast, Fe(II) treatment generally demonstrated greater improvements than Cu(II) at lower concentrations. At 0.02 mM Fe(II) and 0.05 mM Fe(II), cumulative biomethane yields increased to 26.99 ± 4.97 $dm^3$ and 29.90 ± 2.55 $dm^3$, representing improvements of 9.36% and 21.16%, respectively, over the control.

Treatment with 0.05 mM concentration of Fe(II) showed the best first-phase biogas potential ($Pb_1$ = 18.943 $dm^3$), exceeding the untreated control by almost 38%. It also lowered the first-phase lag duration from 18.03 days to 12.63 days, which indicates faster microbial adaptation and earlier onset of active phase of production biogas.

The second-phase biogas potential ($Pb_2$ = 30.47 $dm^3$) was also substantially higher than that of the control. On the flip side, increase in the concentration of Fe(II) to 0.10 mM reduced the cumulative biogas yield to 23.51 ± 9.44 $dm^3$, falling short of the control. This decrease in the values of $Pb_1$ and $Pb_2$ relative to control suggests that excessive dose of Fe(II) negatively affected microbial metabolism and methanogenic activity. This comparison clearly illustrates that introducing trace metals at certain concentrations can substantially influence anaerobic digestion performance compared to untreated CD/CM. The control set up showed moderate biogas yield and relatively long lag periods, but when appropriate concentrations of Cu(II) and Fe(II) were added, it really improved substrate utilization, increased biogas production potential, and enhanced production rates.

Among all the treatments, 0.10 mM Cu(II) achieved the highest cumulative biogas yield of 30.25 $dm^3$ and specific biogas yield of 0.113 $dm^3$ $g^{-1}$ VS. Meanwhile, 0.05 mM Fe(II) displayed the best kinetic characteristics, including the highest first-phase biomethane potential of 18.943 $dm^3$ and the shortest lag phase of 12.632 days. In contrast, 0.10 mM Fe(II) proved to be inhibitory, leading to lower biogas yield than the control, which suggests that higher concentration of trace metals can be toxic to the system. These results indicate that while adding trace metals can substantially enhance the anaerobic co-digestion of CD and CM, the positive effects are concentration-

dependent. Optimal levels of Cu(II) and Fe(II) enhance microbial and enzymatic activity, while excessively high concentrations may negatively affect microbial communities responsible for biogas production. At final ingestate stabilization on Day 50, the cumulative biomethane yield ($dm^3$) established the following performance hierarchy: Cu(II) 0.10 mM (30.25) > Fe(II) 0.05 mM (29.90) > Fe(II) 0.02 mM (26.99) > Cu(II) 0.05 mM (26.74) > Control (24.68) > Fe(II) 0.01 mM (23.51) > Cu(II) 0.02 mM (20.91).

**Table 2. Effect of Fe(II) and Cu (II) Treatments on the Final Cumulative Response.**

| Treatments | Metal Conc. (mM) | Mean ± SD Cumulative Biogas Yield ($dm^3$) | Yield/gVS | % Increase. |
|---|---|---|---|---|
| CD/CM | Cu(II) 0.02 | 20.91 ± 11.99a | 0.078 | -15.29 inhibition |
| | Cu(II) 0.05 | 26.74 ± 1.77a | 0.100 | 8.35 |
| | Cu(II) 0.1 | 30.25 ± 1.83a | 0.113 | 22.56 |
| CD/CM | Fe(II) 0.02 | 26.99 ± 4.97a | 0.100 | 9.36 |
| | Fe(II) 0.05 | 29.90 ± 2.55a | 0.111 | 21.16 |
| | Fe(II) 0.1 | 23.51 ± 9.44a | 0.064 | - 4.75 (inhibition) |
| | CD/CM alone | 24.68 ± 2.04a | 0.092 | - |

### 3.2.4 Compositional Analysis of the Biogas by Gas Chromatography (GC)

In all the experimental setups, the profile of gaseous output all clearly confirmed that supplementation with trace metals like $Cu^{2+}$ and $Fe^{2+}$ remarkably affected both the elemental distribution of the biogas produced and purity (Table 3). In the CD/CM alone (control set-up), we observed a baseline biomethane ($CH_4$) purity of 67.20% with 29.76% $CO_2$, which is very typical for mixtures of lignocellulosic - proteinaceous waste.

**Dose-dependent enrichment with Copper ($Cu2^+$):** The addition of Cu(II) apparently displayed a positive linear dose-response relationship with biomethane concentration. Notably, increasing the concentration of Cu(II) from 0.02 - 0.10 mM progressively enhanced biomethane content from 71.59% to a peak of 76.79%, representing a 14.27% increase compared to the non-supplemented

control. $CO_2$ levels concurrently plummeted from 29.76% to just 6.03% at the 0.10 mM Cu(II) concentration.

Particularly fascinating is that the reduction in $CO_2$ coincided with a substantial increase in residual molecular hydrogen ($H_2$), which reached 16.19% in the 0.10 mM Cu(II) treatment. This metabolic shift suggests that $Cu^{2+}$ ions acted as important enzymatic cofactors, stimulating hydrogenotrophic pathways and accelerating carbon dioxide conversion into biomethane without causing any issues in the system.

**Biphasic Threshold of Iron ($Fe^{2+}$):** In contrast to copper, treatment with (Fe(II) displayed a classic biphasic or hormetic metabolic response.

**Optimal Dosing (0.05 mM):** The optimal dose was recorded at 0.05 mM Fe(II), where we achieved peak performance with 75.34% CH4 and $CO_2$ levels dropping to 10.96%. $Fe^{2+}$ plays a vital role as a structural component of cytochromes and ferredoxins, which are essential for both acetoclastic and hydrogenotrophic methanogenesis.

**Inhibitory Dosing (0.10 mM):** However, increasing the Fe(II) concentration to 0.10 mM led to a marked decline in performance with CH4 content dropping to 63.27%, even lower than the control, while $CO_2$ level rose to 30.64%. This decline shows that higher iron concentrations may have induced enzymatic toxicity or precipitated important phosphate nutrients, thereby disrupting methanogenic cell membranes.

**Trace Volatile Organic Compounds (VOCs) and Off-Gases.**

In most bioreactors, the carryover of volatile fatty acids, measured specifically as the gas phase equivalent of acetic acid, remained, clocking in at less than 0.8%. The only exception was the bioreactor with 0.05mM Fe(II), which showed a higher carryover of 2.99%. This indicates that, overall, there's a strong buffering capacity at play. Moreover, trace solvents such as methanol, acetone, and acetonitrile accounted for less than 1.5% of the total volume across all treatments. Importantly, the level hydrogen sulfide ($H_2S$) reduced to non-detectable limits under moderate-to-high Cu(II) supplementation, highlighting that copper addition provides an added benefit of suppressed corrosive off-gas generation.

**Table 3. Compositional Analysis of the Biogas using GC.**

| Treatments Metal Conc. (mM) | | Biogas Components (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **CO** | **$H_2$** | **$CH_4$** | **Acetic Acid** | **Methanol** | **$CO_2$** | **Acetone** | **Acetonitrile** | **$H_2S$** |
| CD/CM | Cu(II) 0.02 | 1.29 | 5.04 | 71.59 | 0.78 | 0.07 | 19.98 | 0.50 | 0.09 | 0.02 |
| | Cu(II) 0.05 | - | 5.89 | 73.15 | 0.11 | 0.56 | 20.25 | 0.03 | 0.05 | - |
| | Cu(II) 0.1 | 0.90 | 16.19 | 76.79 | 0.10 | 0.06 | 6.03 | 0.02 | 0.03 | - |
| CD/CM | Fe(II) 0.02 | 0.17 | 6.45 | 71.57 | 0.04 | 0.45 | 21.22 | 0.03 | 0.08 | 0.02 |
| | Fe(II) 0.05 | 0.98 | 1.85 | 75.34 | 2.99 | - | 10.96 | 1.40 | 1.50 | 3.48 |
| | Fe(II) 0.1 | 0.46 | 3.30 | 63.27 | 0.04 | 0.25 | 30.64 | 0.32 | 0.27 | 0.34 |
| | CD/CM Only | 0.08 | 0.50 | 67.20 | 0.08 | - | 29.76 | 1.06 | 1.09 | 1.18 |

**3.2.5 Data Analysis.**

A one-way analysis of variance (ANOVA) was conducted to determine whether the final cumulative response differed significantly among the control, iron-treated, and copper-treated groups. Before performing ANOVA, the normality assumption was assessed using the Shapiro–Wilk test on the ANOVA residuals. The residuals showed no significant deviation from normal distribution (Shapiro–Wilk W = 0.977, P = 0.969 > 0.05), indicating that the normality assumption required for ANOVA was satisfied. The Normal Q–Q plot also showed that the residual points followed the expected diagonal trend, further supporting the assumption of normality (Figure 5).

The one-way ANOVA showed that there was no significant difference in the final cumulative response among the seven treatments ($F(6,7) = 0.608$, $P = 0.720 > 0.05$). Therefore, the null hypothesis was not rejected, indicating that the application of copper and iron at different concentrations did not significantly affect the final cumulative response compared with the control.

Duncan's multiple range test at $P = 0.05$ further confirmed this result by placing all treatments into a single homogeneous subset (Group a). The treatments (Cu 0.02, Fe 0.10, Control, Cu 0.05, Fe 0.02, Fe 0.05, and Cu 0.10) were statistically similar, with no significant differences observed among their final cumulative means (Table3). Although Cu 0.10 mM produced the numerically highest cumulative mean (30.25 ± 1.83a cm3) and Cu 0.02 mM recorded the numerically lowest

(20.91 ± 11.99a cm3), a spread of roughly 32% between the extremes. Overall, the data met the normality requirement for parametric analysis, and the ANOVA results indicate that neither copper nor iron treatment produced a statistically significant change in final cumulative response under the conditions tested.

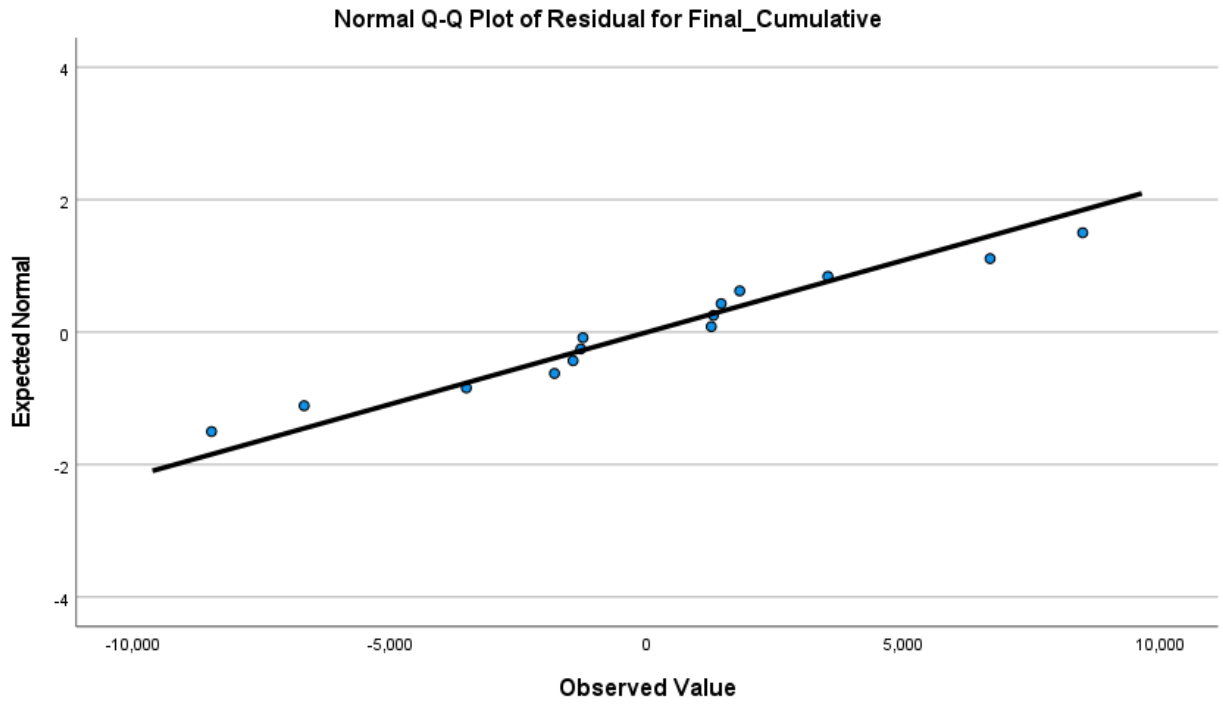


**Figure 5 Normal Q-Q plot of Residual for Cumulative Response**

### 3.2.6 Kinetic Dynamics and Diphasic Modeling of Fe(II) - and Cu(II) - Mediated Co-Digestion.

To explain how trace metals influence multi-phasic substrate utilization during anaerobic co-digestion of CD and CM, generated daily cumulative biogas data were fitted to a diphasic logistic simulation model over a 50-day hydraulic retention time (HRT) as shown in Figures 3 and 4. Across the all bioreactor setups, daily cumulative biogas yield demonstrated very distinct biphasic (diauxic) trajectory, indicating a multi-phasic metabolic breakdown:

**Phase 1 (P1: Soluble Degradation, Days 1 - 15):** The surge in initial biogas production was fueled by the digestion of bioavailable, soluble organics and proteins from CM. Interestingly, Fe(II) at a concentration of 0.05 mM triggered the most rapid initial reaction, generating 1.266 $dm^3$ by Day 10, about six-fold higher than the control which was only 0.215.00 $dm^3$.

**Diauxic Transition Phase (Days 15 - 25):** In this phase, a significant change in kinetics was observed as the primary soluble substrate pool started to deplete. During this transition, the population of microbes underwent metabolic re-adaptation to produce extracellular enzymes like lignases and cellulases that could break the recalcitrant lignocellulosic fibers present in cow dung.

**Phase 2 (P2: Lignocellulosic Degradation, Days 25 - 50):** The second phase of digestion showed very striking divergence, largely influenced by the presence of trace metal. A high concentration of copper Cu(II), at 0.10 mM enhanced Phase 2 performance, reaching to 17.429 $dm^3$ at Day-25 and ultimately achieving a peak yield of 30.25 $dm^3$.

**Comparative Trace Metal Performance.**

**The Copper (Cu2+) Response.** The response to Copper (Cu2+) was particularly impressive, displaying a clear positive dose-dependent trajectory. A lower copper dose of 0.02 mM caused some inhibition, resulting in 20.91 $dm^3$, which declined by 15.29 % relative to control. However, Increase in the dose to 0.10 mM unlocked maximum methanogenesis, leading to a yield of 30.25 $dm^3$, which was a 22.56% increase over the control.

**The Iron Response (Fe 2+).** Treatment with Fe(II) revealed an optimal dosage around 0.05 mM, producing 29.90 $dm^3$, which represented a +21.16% increase over the control. However, further increasing the iron concentration to 0.10 mM resulted to severe inhibition of microbial kinetics, causing a decline in the final cumulative yield to 23.51 dm3 (a 14.43% decrease below the control. The diphasic logistic model captured accurately the multi-phasic substrate utilization trends, demonstrating a very strong correlation coefficients ($R^2$ = 0.992–0.999). The model also confirmed that targeted micro-dosing of Fe(II) (0.05 mM) or Cu(II) (0.10 mM) mitigates diauxic lag delays during substrate shifts, ensuring uninterrupted carbon-nitrogen metathesis and maximizing biomethane potential in decentralized bioreactors.

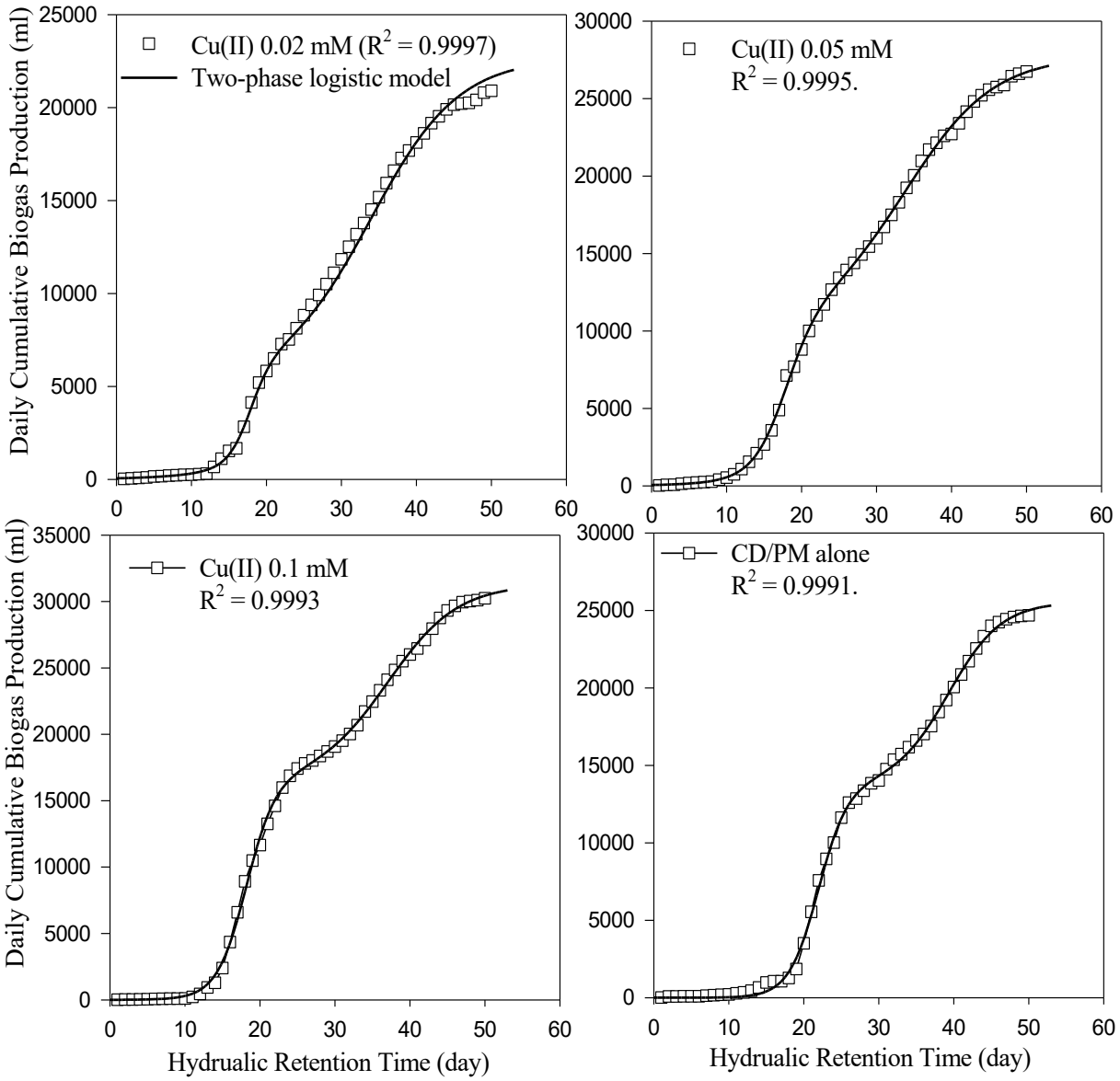


Figure 3. Plots of Experimental Biogas production data from Cu(II) Treatment and Control fitted with the two-phase logistic model.

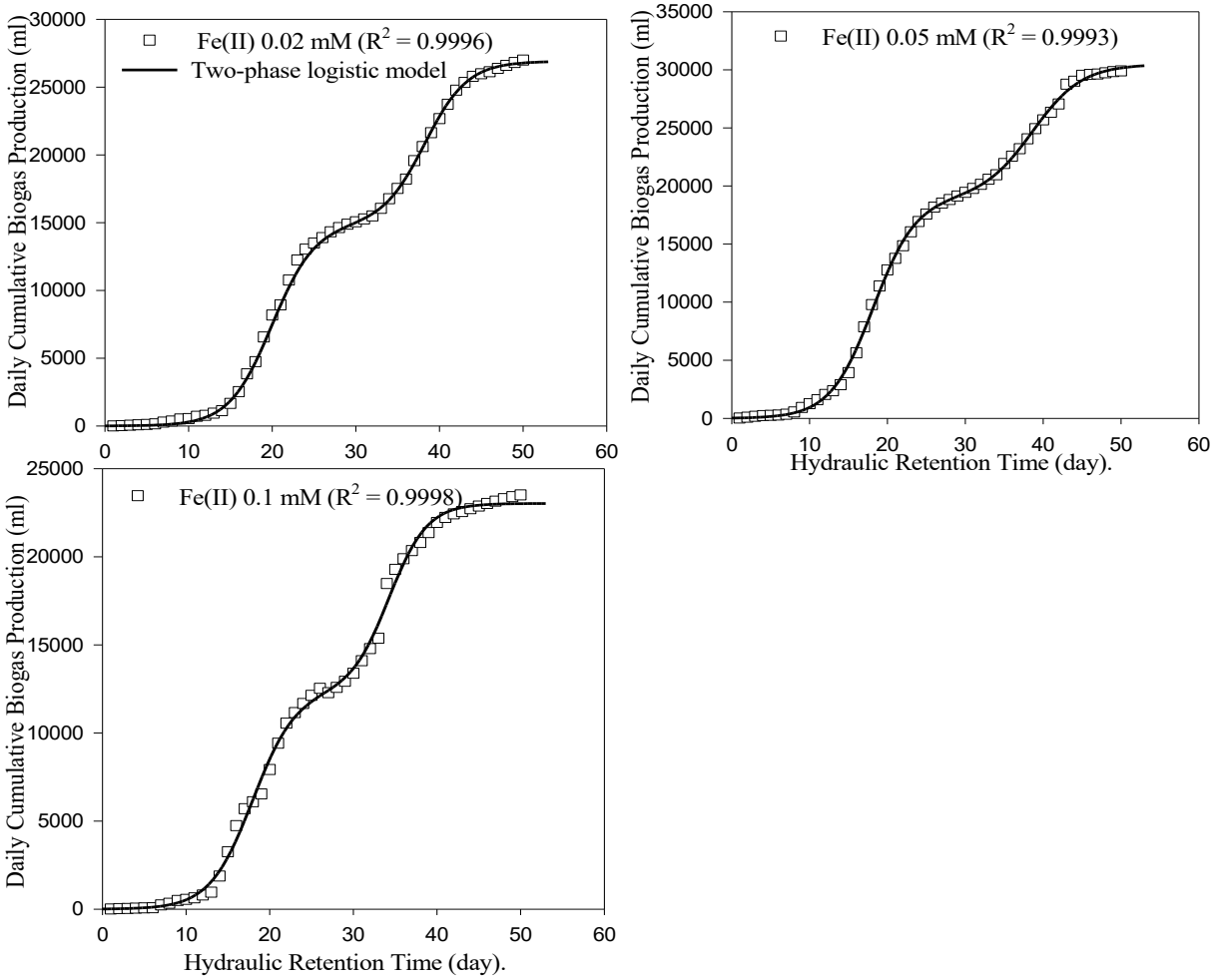


Figure 4 Plots of Experimental Biogas production data from Fe(II) Treatment fitted with the two-phase logistic model.

### 3.2.7 The Two-Phase Logistic Model Parameters for Biogas Production

The kinetic parameters derived from the two-phase logistic model for the anaerobic co-digestion of CD/CM supplemented with varying concentrations of Cu(II) and Fe(II) are detailed in Table 4. This model offers a concise and effective description of biogas production in two distinct and sequential phases. Each phase is characterized by unique metrics: the biogas production potentials ($Pb_1$ and $Pb_2$), maximum production rates ($Rm_1$ and $Rm_2$), and lag phases ($\lambda_1$ and $\lambda_2$).

In the treatments where Cu(II) was added, the first-phase biogas production potential ($Pb_1$) showed a marked increase from 5.308 $dm^3$ at 0.02 mM Cu(II) to an impressive 16.549 $dm^3$ at 0.10 mM Cu(II). This highlights an enhanced microbial activity with increasing Cu(II) concentration. Similarly, the maximum biogas production rate during the first phase ($Rm_1$) rose from 0.915 $dm^3$

$d^{-1}$ to 2.116 $dm^3$ $d^{-1}$. The lag period in the first phase ($\lambda_1$) ranged between 13.583 and 14.949 days, indicating a relatively consistent time for microbial adaptation. In the second phase, $Pb_2$ increased from 21.538 $dm^3$ at 0.02 mM Cu(II) to 31.312 $dm^3$ at 0.10 mM Cu(II), while $Rm_2$ slightly increased from 0.723 $dm^3$ $d^{-1}$ to 0.790 $dm^3$ $d^{-1}$. However, lag period ($\lambda_2$) in the second-phase increased from 21.122 days to 27.710 days as concentration of Cu(II) increased, implying a delay in the onset of the second biogas production phase.

In Fe(II)-amended treatments, $Pb_1$ accelerated from 14.838 $dm^3$ at 0.02 mM to 18.943 $dm^3$ at 0.05 mM before dropping to 12.336 $dm^3$ at 0.10 mM Fe(II). A similar trend was recorded for $Rm_1$, with a peak of 1.719 $dm^3$ $d^{-1}$ at 0.05 mM Fe(II). Interestingly, the shortest first-phase lag period (12.632 days) was also noted at 0.05 mM Fe(II), indicating enhanced microbial acclimatization and activity. In the second phase, $Pb_2$ increased from 26.899 $dm^3$ at 0.02 mM Fe(II) to 30.473 $dm^3$ at 0.05 mM Fe(II), and eventually decreased to 23.025 $dm^3$ at 0.10 mM Fe(II). The second-phase production rate ($Rm_2$) hit its peak at 1.180 $dm^3$ $d^{-1}$ for 0.02 mM Fe(II), while $\lambda_2$ period varied between 29.696 and 33.169 days.

On the other hand, the control treatment (CD/CM without metal addition) showed a first-phase biogas potential ($Pb_1$) of 13.752 $dm^3$, a production rate ($Rm_1$) of 1.751 $dm^3$ $d^{-1}$, and a lag phase ($\lambda_1$) of 18.028 days. Correspondingly, the corresponding second-phase parameters were 25.552 $dm^3$, 0.835 $dm^3$ $d^{-1}$, and 32.240 days for $Pb_2$, $Rm_2$, and $\lambda_2$, respectively.

Generally, the kinetic analysis highlighted that trace metals supplementation positively influenced the biogas production process. The 0.10 mM Cu(II) treatment produced the highest biogas potentials in both phases ($Pb_1$ = 16.549 $dm^3$ and $Pb_2$ = 31.312 $dm^3$) and the highest first-phase production rate ($Rm_1$ = 2.116 $dm^3$ $d^{-1}$), suggesting strong stimulation of anaerobic microbial activity. In Fe(II), the optimum concentration was 0.05 mM, which produced the highest first-phase biogas potential of 18.943 $dm^3$ and improved production rates, whereas increasing Fe(II) to 0.10 mM resulted in decreased biogas potential and production performance, indicating inhibitory effects at elevated concentrations. These findings clearly demonstrate that appropriate concentrations of Cu (II) and Fe(II) can enhance both the kinetics and overall efficiency of anaerobic digestion.

**Table 4. Two-phase Logistic Model Parameters for Biogas Production.**

| Treatment | Two-phase logistic model parameters |
|---|---|

| | Metal ion Concentration (mM) | $Pb_1$ (dm$^3$) | $R_{m1}$(dm$^3$.d) | $\lambda$ (days) | $P_{b2}$ (dm$^3$) | $R_{m2}$ (dm$^3$.d) | $\lambda_2$(days) |
|---|---|---|---|---|---|---|---|
| CD/CM | Cu(II) 0.02 | 5.308 | 0.915 | 14.949 | 21.538 | 0.723 | 21.122 |
| | Cu(II) 0.05 | 10.385 | 1.205 | 13.583 | 27.760 | 0.739 | 22.147 |
| | Cu(II) 0.1 | 16.549 | 2.116 | 14.263 | 31.312 | 0.790 | 27.710 |
| CD/ CM | Fe(II) 0.02 | 14.838 | 1.551 | 14.869 | 26.899 | 1.180 | 33.169 |
| | Fe(II) 0.05 | 18.943 | 1.719 | 12.632 | 30.473 | 0.906 | 32.274 |
| | Fe(II) 0.1 | 12.336 | 1.204 | 12.839 | 23.025 | 1.170 | 29.696 |
| CD/CM | None | 13.752 | 1.751 | 18.028 | 25.552 | 0.835 | 32.240 |

**4.0. Discussion.**

The bioconversion efficiency and biomethane yield in anaerobic digestion is strongly influenced by feedstock composition (Table 1). For instance, CM exhibited remarkably higher total solids (TS = 80.19%) and volatile solids (VS = 41.48%) compared to CD, which has a TS of 47.80% and VS of 28.15%. The elevated percentage VS suggests a greater concentration of biodegradable organics, which means a potentially richer pool of biomethane-forming substrate in chicken manure (CM). This finding is in line with recent studies that highlights the high-solids and organic-rich characteristics of chicken manure and its promising potential for biomethane production (Xiao et al., 2025).

Similarly, CM showed a higher total organic carbon content of 16.32%, surpassing that of cow dung (CD) at 14.96%, indicating a greater abundance of carbon-rich organic matter. Such variation may likely stem from the unique digestive physiology and feed utilization pattern of monogastric poultry compared to ruminants, as well as the distinct disparity in manure composition and organic-matter transformation. Recent studies have highlighted notable differences in the composition, biodegradability, and transformation of organic matter between chicken and cattle manures (Wang et al., 2014).

Both substrates exhibited very narrow C/N ratios, with CD at 11.00 and CM at 10.30, both of which are below the optimal range of 20:1 - 30:1. The elevated total nitrogen of 1.59% and crude protein of 14.89% in CM driven by uric acid excretion in poultry pose a high risk of Free Ammonia Nitrogen (FAN) inhibition during mono-digestion (Wei et al., 2026). Conversely, CD contains

4.10% of recalcitrant lignocellulosic fibers that may slow down hydrolysis but provide structural stability. Due to low C/N stoichiometry, mono-digestion of CM risks ammonia-induced failure. However, co-digesting CM with CD can lead to improved substrate complementarity by moderation of organic loading, enhancing nutrient balance, and reducing the risk of ammonia-related inhibition. This creates a more favorable environment for microbial activity and biomethanation. This interpretation is alignment with recent studies showing that co-digestion can help reduce inhibitory compounds and boost methane production from high-solids chicken manure, while balancing the C/N ratio contributes to process stability and production of methane-rich biogas (Xiao et al., 2026; Karumanchi et al., 2026).

Regarding the macro-minerals, CM stand out with its impressively higher concentrations of potassium (1882.52 mg/kg), sodium (2133.54 mg/kg), calcium (1.82%), and magnesium (3.48%). These light metal cations play a crucial role as enzyme co-factors, while divalent ions ($Ca^{2+}$, $Mg^{2+}$) improve system buffering capacity by forming carbonate precipitates that mitigate ammonia toxicity.

The daily cumulative biogas production pattern observed in all CD/CM bioreactor configurations demonstrated a clear biphasic (diauxic) trajectory, with a relatively rapid first phase that could be attributed to the breakdown of soluble, bioavailable organics contributed majorly by CM. This was followed by a kinetic transition and a slower second phase which was driven by microbial degradation of the very recalcitrant lignocellulosic fraction from CD.

This pattern is consistent with earlier findings of diauxic-like, multi-peak biogas kinetics in co-digested livestock manures of heterogeneous composition. Opurum et al. (2021) studied binary mixtures of poultry manure, pig dung, and cow dung, and similarly observed that cumulative biogas yield followed a diauxic-like, biphasic trajectory with multiple peaks. They attributed this to the multi-component nature of the mixed substrates, and successfully captured this behaviour by using a bi-logistic function along with the modified Gompertz equation. The strong fit achieved in this study ($R^2$ = 0.992 - 0.999) for the diphasic logistic model supports the applicability of two-phase kinetic formulations in substrate systems that combine both labile and fibrous organic fractions, and extends this framework by explicitly linking the inter-phase lag to cellulase-mediated metabolic re-adaptation rather than treating the transition purely as a curve-fitting artefact.

In this study, Fe(II) supplementation produced a narrow optimum at 0.05 mM (about 2.8 mg $L^{-1}$), beyond which increasing the dose to 0.10 mM (approx. 5.6 mg $L^{-1}$) sharply declined cumulative production. This biphasic (stimulation - inhibition) dose - response is in consistence with the broader trace-metal literature, which recognizes iron as an important cofactor for hydrogenases and ferredoxin-mediated electron transfer at low concentrations but it can become inhibitory once a system-specific threshold is exceeded (Salazar-Batres & Moreno-Andrade, 2025). Interestingly, the effective concentration range identified here is much lower than optima reported in several recent studies.

A micronutrient mix that featuring 1 mg $L^{-1}$ Fe (II) in addition to Cu, Zn, Mn, Mg, and Ni, was the ideal combination for maximizing biomethane yield from food waste and cow dung (Mary et al.,2024). Tukanghan et al. (2025) on the other hand, reported that during a two-stage anaerobic digestion of palm oil mill effluent, 10 mg $L^{-1}$ $Fe^{2+}$ was the optimum concentration. These findings were by Salazar-Batres et al. (2023), highlighting that higher concentrations of $Fe^{2+}$ also positively impacted the two-stage digestion of organic solid waste.

Interestingly, the lower effective concentration of Fe(II) observed in the CD/CM system might stem from variations in trace metal availability, sulfide-buffering capacity, or even the specific characteristics of the substrate itself. This divergence from the wider literature deserves careful consideration rather than being dismissed as a mere discrepancy. The response to Cu(II) treatment was substantially different from what is typically reported. Copper displayed a positive, dose-dependent trajectory across the range experimented, with the lowest dose (0.02 mM) being inhibitory, and resulting in a 15.29% decline compared to the control. In contrast, the highest dose (0.10 mM) led to the greatest overall enhancement, showing a 22.56% increase over the control, with no evidence of inhibition at the upper end of the range tested.

This finding stands out because it challenges the consensus view that copper is one of the more narrowly tolerated trace metals in anaerobic digestion. Typically, $Cu^{2+}$ is thought to exert stimulatory effects only within a relatively limited low-concentration window before inhibitory effects associated with enzyme and cell-membrane binding begin to dominate (Salazar-Batres & Moreno-Andrade, 2025). The absence of inhibitory effect, even at the highest Cu(II) dose tested in this study suggests either that the actual inhibitory threshold for this substrate mix lies beyond 0.10 mM. Alternatively, it could be that the organic-rich CD/CM matrix provided enough chelating

capacity to buffer copper bioavailability compared to the simpler or higher-loading systems often used in previous dose - response studies.

This result represents one of the more literature-discordant findings of the present study and, definitely, calls for further investigation at higher Cu(II) concentrations to establish the upper inhibitory boundary. Interestingly, the stimulatory effect of both metals at their respective optimal concentrations aligns with their well-established roles as cofactors for metalloenzymes governing hydrolysis, acidogenesis, and methanogenesis (DelaVega-Quintero et al., 2025). Looking beyond the overall cumulative biogas yield, the parameters of the two-phase logistic model ($Pb_1$, $Pb_2$, $Rm_1$, $Rm_2$, $\lambda_1$, $\lambda_2$) offers underlying mechanistic understanding of how Cu(II) and Fe(II) play a role digestion process.

For Cu(II), it was observed that both phase-specific biogas potentials increased monotonically with dose ($Pb_1$: 5.308 →16.549 $dm^3$; $Pb_2$: 21.538 →31.312 $dm^3$ across 0.02–0.10 mM), and the first-phase rate constant ($Rm_1$) more than doubled in that same range (0.915 →2.116 $dm^3\ d^{-1}$). This indicated that higher Cu(II) concentrations accelerated substrate conversion during the labile-organics phase without triggering the metabolic slowdown usually associated with copper toxicity. However, it is worth noting that the second-phase lag ($\lambda_2$) extended from 21.122 to 27.710 days as Cu(II) levels increased, even as $Pb_2$ and $Rm_2$ continued to increase, a decoupling in which the lignocellulosic-phase potential improved while its onset was delayed. This pattern where an increased production ceiling is accompanied by a longer lag before the second phase kicks in, is not commonly observed in single-metal dose - response studies, suggesting Cu(II) may be acting differently on the microbial communities responsible for phase-1 (hydrolytic/acidogenic) versus phase-2 (cellulolytic/methanogenic) activity, rather than uniformly accelerating the whole community.

Fe(II) displayed a classic non-monotonic (optimum-then-inhibition) pattern in both phases: $Pb_1$ increased from 14.838 $dm^3$ in 0.02 mM to a peak of 18.943 $dm^3$ at 0.05 mM and eventually dropped to 12.336 $dm^3$ at 0.10 mM. $Rm_1$ and $\lambda_1$ mirrored this same trend, both hitting their optimum at 0.05 mM (with $Rm_1$ peaking at 1.719 $dm^3\ d^{-1}$ and $\lambda_1$ reaching its shortest duration of 12.632 days, compared to 18.028 days for the control). The reduction in the lag-phase at a moderate, non-toxic metal dose is in consistence with the findings of Tian et al. (2020).They found that appropriate concentrations of heavy metals actually expedited the biogas peak and reduced the lag phase ($\lambda$) during co-fermentation of cow dung and Phragmites straw, while this positive

effect was reversed by excessive concentrations. The results observed with Fe(II) The results we observed with Fe(II): at 0.05 mM, Fe(II) most plausibly boosted the pool of reduced-ferredoxin and hydrogenase available to acidogens and hydrolytic bacteria, accelerating the onset of active biogas production. However, at 0.10 mM the metal probably exceeded the system's chelation/buffering capacity, and began to interfere with the same enzymatic machinery it had previously supported.

The overall quality of fit ($R^2$ = 0.992 - 0.999 for the two-phase logistic model) is in concordance with recent kinetic-modelling studies on trace-metal-supplemented digestion. Wang et al. (2021), investigated binary and ternary combinations of Fe/Co/Ni in cattle manure digestion and found that both the logistic model and modified Gompertz model more closely fit cumulative biogas data than a first-order kinetic model. The prediction errors for the sigmoidal models were only 0.32–5.3%, compared to the larger deviations for the first-order model. Thisupports the preference for a logistic-family model over first-order kinetics for metal-amended substrates in general, and by extension supporting the two-phase logistic approach adopted in the present study for a substrate combination (CD/CM) with an even more heterogeneous composition than the single-manure systems typically modelled.

Reconciling the Cu(II) and Fe(II) responses with known metal-toxicity rankings**,** the biogas cumulative-yield comparison revealed 0.10 mM Cu(II) to be the single best-performing treatment overall, achieving a cumulative yield of 30.25 $dm^3$ (with a specific yield of 0.113 $dm^3\ g^{-1}$ VS). This performance even surpasses the best results achieved by the Fe(II) optimum, which hit 29.90 $dm^3$ at a concentration of 0.05 mM. It's interesting to note this finding in light of the broader literature on heavy-metal toxicity, where copper is often cited as one of the more toxic trace metals to anaerobic microbial communities. For instance, Alrawashdeh et al. (2020), examined heavy-metal inhibition during anaerobic digestion of olive mill waste, ranked toxicity in increasing order as Cu > Ni > Pb > Cr > Zn > Fe, very explicitly pointing out that iron, by contrast, actually tends to exhibit stimulatory effects at concentration, but only up to a certain level. If this ranking is taken at face value, we would predict that Cu(II) should become inhibitory at a lower concentration than Fe(II), the opposite of the result obtained here: Fe(II) showed inhibitory effects at 0.10 mM, while Cu(II) continued to promote growth at that same concentration.

This apparent contradiction can be better explained by examining the substrate-matrix effects rather than assuming a genuine reversal of intrinsic metal toxicity. The CD/CM matrix in this study

is rich in organic ligands, sulfide-generating capacity, and particulate organic matter. All of these factors can reduce the bioavailable (free ionic) fraction of $Cu^{2+}$ when compared to the simpler or more mineral-dominated systems, like olive mill wastewater, in which the classical toxicity rankings were established. If a substantial portion of the Cu(II) forms complexes or precipitates (like CuS) instead of remaining bioavailable, then the effective exposure at 0.10 mM might actually be lower than the concentration needed to trigger the inhibition reported in other studies. This suggests that the present data simply may not have attained the true inhibitory threshold for copper in this matrix. This interpretation is consistent with the broader principle noted by Salazar-Batres and Moreno-Andrade (2025), which emphasizes that the optimal and inhibitory concentrations of trace metals are highly matrix- and system-dependent rather than fixed values.

To accurately assess whether the differences in cumulative biogas yield across various treatments genuinely reflect treatment effects rather than just random variation, a one-way ANOVA was conducted on the final cumulative response from the six metal-supplemented treatments and the control. Residual diagnostics confirmed the assumptions underlying parametric analysis: the Shapiro–Wilk test showed no significant deviation from normality ($W = 0.977$, $p = 0.969$), and this was further validated by a Normal Q–Q plot where the residuals closely followed the expected diagonal.

The ANOVA results however, revealed no statistically significant difference in final cumulative biogas response among the seven treatments ($F(6,7) = 0.608$, $p = 0.720$). Duncan's multiple range test grouped all seven treatments: Cu 0.02, Fe 0.10, Control, Cu 0.05, Fe 0.02, Fe 0.05, and Cu 0.10 into a single homogeneous subset at $P = 0.05$. Although the yield from Cu (II) at 0.10 mM had the numerically highest cumulative mean ($30.25 \pm 1.83$ $dm^3$) and Cu(II) at 0.02 mM the lowest ($20.91 \pm 11.99$ $dm^3$), showing a spread of about 32% between the extremes, this difference was statistical significance.

The data from GC compositional analysis further support the kinetic findings, showing that trace-metal supplementation not only influenced the quantity of biogas produced but also its elemental quality. The control $CH_4$:$CO_2$ ratio (67.20%:29.76%) falls within the range typically reported for co-digested lignocellulosic - proteinaceous manure mixtures. Notably, the progressive $CH_4$ enrichment recorded with increasing Cu(II) (71.59% → 76.79% across 0.02–0.10 mM) is in consistence with Hao et al. (2017), who observed that $Cu^{2+}$ supplementation (as $CuCl_2$, at 30 and 100 mg $L^{-1}$) increased both cumulative biogas yield and $CH_4$ content relative to control during co-

fermentation of cow dung and Phragmites straw, while only the much higher 500 mg $L^{-1}$ dose proved inhibitory. Converting the Cu(II) doses of the present study to a mass basis yields values of 0.02 - 0.10 mM ≈ 1.3 - 6.4 mg $L^{-1}$, which is an order of magnitude lower than the stimulatory concentrations reported by Hao et al., yet yields a comparable level of $CH_4$ enrichment, this again points to a substrate - and inoculum-specific sensitivity to copper that is worth discussing rather than being dismissed as a minor numerical detail.

The simultaneous increase in residual $H_2$ (up to 16.19% at 0.10 mM Cu(II)) along with the significant drop in $CO_2$ really calls for a deeper look into the mechanisms at play, rather than just a straightforward interpretation. Hydrogenotrophic methanogenesis consumes both $CO_2$ and $H_2$ in a fixed 4:1 stoichiometry ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$); if this pathway were solely responsible for the drawdown $CO_2$ observed, $H_2$ would be expected to decrease in parallel, not accumulate.

The observed trend here, in which falling $CO_2$ levels alongside rising, not falling, $H_2$ is more consistent with Cu(II) selectively favouring acetoclastic methanogenesis and/or fermentative $H_2$-producing acidogens over hydrogenotrophic methanogens. This means $CO_2$ is likely being reduced primarily through the acetate pathway, while $H_2$ produced during acidogenesis is not being as consumed rapidly. Essentially, this indicates a partial uncoupling of the hydrogenotrophic pathway instead of its acceleration. This kind of Cu-associated shift in the relative balance of methanogenic and acidogenic/homoacetogenic pathways has some precedent: Wang et al. (2020), investigating inhibitory conditions on acetate-degrading methanogenic communities, observed that copper stress can impact coexisting microbial groups in varying ways, rather than uniformly stimulating or suppressing the entire consortium as reported by Gadow et al., 2026, which aligns with the divergent $CO_2/H_2$ trajectories reported here.

The response of Fe(II) composition shows that $CH_4$ levels peak at 75.34% at an optimum concentration of 0.05 mM, but then drop to 63.27% (which is below the control) at 0.10 mM.

Meanwhile, $CO_2$ levels show an inverse relationship, reflecting the biphasic pattern already established for cumulative yield and kinetic parameters. This aligns with findings from Zhang et al. (2016), who noted that adding ferrous chloride during anaerobic digestion of cow dung and Phragmites straw enhanced biogas properties and enzymatic activity at moderate levels, but led to adverse effects once an optimum level was exceeded. The explanation provided here suggests that $Fe^{2+}$ serves as a structural cofactor for cytochromes and ferredoxins at 0.05 mM, but at 0.10 mM, it may induce enzymatic toxicity or lead to phosphate co-precipitation. This interpretation fits the

established dose-dependency, but it would be even more convincing with direct evidence of enzyme activity or microbial community responses, like coenzyme $F_{420}$ or hydrogenase assays, similar to those conducted by Hao et al. (2017) and Zhang et al. (2016), to move from a plausible inference to a confirmed mechanism.

The observed decrease in sulfide off-gas with moderate-to-high Cu(II) addition aligns perfectly with a well-established mechanism in the anaerobic digestion literature. Essentially, free $Cu^{2+}$ ions can readily react with dissolved sulfide to form copper sulfide (CuS), which is highly insoluble. This reaction effectively removes sulfide from solution before it can escape into the gas phase as hydrogen sulfide ($H_2S$). This mechanism was illustrated clearly in a recent study by Gadow et al. (2026), which showed that employing $Ni^{2+}$/$Cu^{2+}$ metal precipitation strategies effectively controlled in-situ sulfide levels in a UASB reactor treating sulfate-rich wastewater. Likewise, Pornmai et al. (2023) found that Fe supplementation, whether alone or in combination with Co, Cu, and Ni led to reduction in both dissolved sulfide and gas-phase $H_2S$, while also slightly boosting $H_2$ and $CH_4$ production.

The near-total elimination of $H_2S$ to undetectable levels under moderate-to-high Cu(II) dosing in this study strongly supports the CuS-precipitation mechanism. This finding is particularly valuable, as it highlights an additional benefit of copper supplementation beyond just enhancing methane yield. $H_2S$ is a significant factor in biogas corrosivity and can cause fouling in downstream equipment in large-scale digesters. The increased acetate signal at the Fe(II) optimum is plausibly linked to the accelerated first-phase substrate conversion documented earlier in this discussion (with the highest $Rm_1$ and shortest $\lambda_1$), where faster acidogenesis may transiently outpace acetoclastic consumption before the system re-stabilises again.

Generally, the diphasic kinetic behaviour reported this study reinforces the increasing recognition that co-digestion of compositionally heterogeneous substrates such as CD and CM is inherently multi-phasic, and that two-phase logistic modelling captures this response more accurately compared to single-phase formulations. The Fe(II) response aligns qualitatively, though not quantitatively, with optimal ranges recently documented (Mary et al., 2024; Tukanghan et al., 2025). On the other hand, the Cu(II) response diverges from the commonly documented low-threshold inhibition pattern, highlighting a substrate- and matrix-dependent component to trace-metal bioavailability that is not yet fully resolved in the anaerobic digestion literature (Salazar-Batres & Moreno-Andrade, 2025).

**Conclusion**

This study clearly demonstrates that CD and CM form a compositionally complementary co-digestion feedstock, with CM contributing a nitrogen, minerals, and easily digestible organic-rich fraction while CD contributes a carbon-dominant, fibrous fcomponent, and that this heterogeneity manifests kinetically as a robust, well-resolved diphasic (diauxic) biogas production profile ($R^2$ = 0.992 - 0.999) rather than the single-phase behaviour typically assumed in conventional kinetic modelling of manure-based substrates. Fe(II) and Cu(II) supplementation markedly reshaped both the rate and quality of this diphasic process. Fe(II) demonstrated a narrow, biphasic (hormetic) response, with 0.05 mM producing the overall best kinetic performance: the shortest lag phase, the highest first-phase production rate, and the most robust methane enrichment (75.34% $CH_4$), whereas 0.10 mM was clearly inhibitory across all the kinetic and compositional metric measured, including a cumulative yield that fell below the control.

By contrast, Cu(II) consistently showed a monotonically positive dose–response throughout the tested range, with 0.10 mM Cu(II) achieving the highest cumulative biogas yield of 30.25 ± 1.83$dm^3$, the bests pecific biogas yield (0.113 $dm^3\ g^{-1}$ VS), and the greatest methane enrichment (76.79% $CH_4$) compared to any treatment. It also nearly completely suppressed hydrogen sulfide ($H_2S$) off-gas, which is consistent with copper-mediated sulfide precipitation as CuS**.** Since the Cu(II) dose - response had not yet turned inhibitory at the highest concentration evaluated, the genuine upper tolerance threshold for copper in this substrate system remains to be established. Additionally, the observed concurrent increase in residual hydrogen alongside decreasing $CO_2$ under Cu(II) supplementation suggests to a partial uncoupling of the hydrogenotrophic and acetoclastic methanogenic pathways. This warrants further investigation into microbial and enzymatic processes rather than being treated as fully resolved by the current dataset.

Overall, these findings suggest that micro-dosing of Fe(II) at 0.05 mM or Cu(II) at 0.10 mM offers a practical, low-cost approach to boost both the yield and the quality of biogas from CD/CM co-digestion. It also underscores that the optimal concentration for any given trace metal is substrate- and matrix-specific rather than being universally applicable across different feedstock systems. Future research should extend the range of Cu(II) dose to identify its inhibitory threshold in this substrate, explore the impact of combined Fe(II) - Cu(II) co-supplementation across the two kinetic phases identified, and integrate gas composition analysis with microbial community and enzyme-

activity assessments to mechanistically resolve the metal-specific shifts in methanogenic pathway balance observed in this study.

**Authors' contributions:**

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**Acknowledgements** The authors do hereby declare that there was no form of external funding or financial support received for the research or publication of this article. This study was self-funded entirely by the authors. However, the authors acknowledge with gratitude, New Concept Analytical Laboratory and Environmental Services Limited, Owerri, Imo State, Nigeria and the Department Microbiology, School of Biological Sciences, Federal University of Technology, Owerri, Nigeria for providing the necessary reagents, access to laboratory space, equipment, and analytical software required to conduct this research.

**Funding: Not applicable.**

**Data availability:** The raw datasets used in this study are presented as additional supporting file.

**Declaration:**

**Competing interests:** The authors declare that there are no form of competing interests.

**Consent for Publication**: Not applicable.

**Ethics and Consent to Participate declarations:** Not applicable

**Clinical trial number:** Not applicable

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