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Research paper

Anaerobic mono-digestion of lucerne, grass and forbs – Influence of species and cutting frequency

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ABSTRACT

In the present study, biogas potentials of multispecies swards including grass, lucerne, caraway, ribwort plantain and chicory from two- and four-cut regimes (Mix-2 and Mix-4) for mono-digestion applying batch and continuous modes under lab-scale conditions were investigated. The gas yields in terms of volatile solids (VS) loaded from Mix-2 and Mix-4 were compared with pure stand lucerne from the four cuts regime (Lu-4). The batch test results indicate that methane yield on a VS basis was highest from Mix-4 (295 $L\,kg^{-1}$), followed by Mix-2 (281 $L\,kg^{-1}$) and Lu-4 (255 $L\,kg^{-1}$). The results were confirmed with continuous experiments, during which the reactor digesting Mix-4 was stable throughout the experiment with low ammonia and volatile fatty acid (VFA) concentration. Meanwhile, mono-digestion of Lu-4 led to elevated VFA levels, even at a comparatively low organic loading rate of 1.76 $g\,L^{-1}\,d^{-1}$ but it was not possible to ascertain whether this was due to organic overload alone or if high ammonia levels during Lu-4 digestion were contributing to the reduced performance. It was found that four cuts per year was suitable for a lab-scale mono-digestion system as the substrate was less fibrous and has lower dry matter content, which minimize blockage during feeding and digestate unloading. Micronutrient concentrations, including cobalt, nickel and molybdenum decreased over time during the continuous experiments and were critically lower than the optimum concentration required by methanogens, particularly in Mix-4, but the gas yields of the reactor treating this substrate showed no decrease over time.

1. Introduction

In stockless organic plant production systems, it is a challenge to maintain adequate soil fertility, with stagnating or declining crop yields and weed problems as the consequences. In order to counteract this, it is necessary to incorporate perennial forage legumes, such as lucerne or clover, with the ability to fix nitrogen from the atmosphere into the crop rotation [1,2]. Inclusion of perennial legumes may improves the soil fertility of arable cropping systems that rely on internal nutrient cycling [3]. Nevertheless, grass-legume swards are characterized with a limited number of plant species. Increasing plant diversity is often beneficial to increase productivity and stability of the plant production systems [4]. Thus, there is a growing interest to explore the potential of multispecies swards to promote biomass yield and increase nutritive value by inclusion of forage forbs [4,5].

Recent studies have identified the positive influence of including forage forbs such as chicory, *Cichorium intybus* L.; caraway, *Carum carvi* L. and ribwort plantain, *Plantago lanceolata* L in the grass-legumes swards [3,4]. These forbs are characterized by deep-roots and have the ability to utilize the nutrient in deep soil layers when grown with grass-legume mixtures, causing improvement of the mineral nutrition [4]. Including forbs in grass-legume swards also enhances floristic diversity, which is beneficial for bees and pollinators [6]. Moreover, it was proved that incorporation of forbs such as plantain in grass-clover mixtures increases biomass yield [3,5], thus holding large potential for bioenergy production.

The use of different types of grasses as substrates for biogas production has been widely investigated [7–9], yet, the influence of integrating new and uncommon forage forbs in grass-legume swards on digestibility and methane production characteristics within mono-anaerobic digestion is still scarce. Anaerobic digestion (AD) may in this case serve a double purpose, namely production of energy in the form of methane and the possibility of using the digestate as a fertilizer or for producing biochar to be applied to annual crops in the crop rotation

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Abbreviations

AD	Anaerobic digestion
TS	Total solid
VS	Volatile solid
TAN	Total ammonium nitrogen
TN	Total nitrogen
VFA	Volatile fatty acids
FM	Fresh matter
DM	Dry matter
OLR	Organic loading rate
HRT	Hydraulic retention time
Lu-4	Lucerne silage from four cuts
Mix-2	Lucerne-forbs-grass silage from two cuts
Mix-4	Lucerne-forbs-grass silage from four cuts
D_{Lu-4}	Digester fed with Lu-4
D_{Mix-2}	Digester fed with Mix-2
D_{Mix-4}	Digester fed with Mix-4
NDF	Neutral detergent fiber
ADF	Acid detergent fiber
ADL	Acid detergent lignin

[10–12].

Methane yield from grassland biomass may vary depending on plant species, cutting period and management intensity [13]. Plant species may differ in terms of chemical compositions and hence could possibly influence methane yield [13]. Yet, the results of batch tests performed by Wahid et al. [9] indicated a contrasting observation, as specific methane yield of different pure stand forbs were comparable. Prochnow et al. [13], mentioned that results of test series with pure stand grass species demonstrated that grass species are mainly influenced on area-specific methane yield rather than grass-specific methane yield. However, grassland consists of a mixture of plant species, not only pure stands of single grass species. The question arises if multispecies plants may affects specific methane yields and this is indeed conceivable [13].

Management intensity of grassland such as cutting frequency may affect methane yields [13]. Traditionally, grass-legume mixtures are harvested four times during the year under temperate conditions in order to optimize feed quality. However, for methane production, previous studies with forbs have indicated that two harvests per year significantly decrease harvest costs without compromising methane yields [9] although grass-lucerne-forbs mixtures cut twice per year may have higher dry matter and fiber fraction compared to the mixture cuts four times yearly, since the time for plant growth is prolonged.

Grass-lucerne-forbs mixture is a seasonal biomass and must be preserved as biogas plants need to be fed continuously. Ensiling is a preferred preservation method that can preserve the biomass energy with minimal loss if good ensiling management is applied. The ensiling process has been reported to have positive effects on methane yields [14]. However, it has been suggested by Kreuger et al. [15] that perceived yield increases can often be explained by measurement errors due to volatilization of compounds during VS determination, and the authors found no increase in the methane yields of a variety of biomasses following correction for loss of volatiles.

This study is a continuation from previous work [9], following our preliminary investigation on the biogas potential of pure stand forbs and grass-clover mixture at different cutting regimes. In the present study, in addition to examining pure stand lucerne, a multispecies mixture consisting of lucerne, caraway, chicory, ribwort plantain and perennial ryegrass at different cutting regimes were investigated for mono-digestion in batch and continuous modes at mesophilic (35 °C) and thermophilic temperatures (52 °C) respectively. The AD of the silages were performed without addition of manure, tailored to the characteristics of stockless organic production systems. Without adding manure, the diminution of nutrients required by anaerobic microbes over time might be a concern, thus, macro- and micronutrients concentration of raw and digested materials were also analyzed.

2. Material and methods

2.1. Substrates

The plant material came from an experiment established in spring 2014 at the Foulumgaard Experimental Station, Aarhus University, Denmark (56°29′44 N, 9°34′3 E, elevation *ca.* 50 m) with different grassland mixtures and two cutting regimes (two and four cuts per year) in a split-plot design with four replicates. Only the last annual cut was used for this experiment. No fertilizer was applied in this experiment. To address the objectives of this study, the mixture consisting of perennial ryegrass, *Lolium perenne* L.; lucerne, *Medicargo sativa* L.; chicory, *Cichorium intybus* L.; caraway, *Carum carvi* L. and ribwort plantain, *Plantago lanceolata* L. was selected under two- and four-cut regimes and monoculture lucerne under four-cut regime. Plant material for this experiment was harvested on the 6th of October to a stubble height of 7 cm following a regrowth period since last harvest of 92 and 49 days in the two- and four-cut regimes, respectively. The foliar material from a four field replicates were mixed, chopped to approximately 5 cm and 5-kg portions were vacuum packed in polyamide/polyethylene bags immediately. The bags were stored at room temperature for three months for the material to ferment to a silage. In lucerne pure stand, the dry matter harvest yield was 1.1 Mg ha^{-1} (SE = 0.09), in the two- and four-cut regimes of the mixture, the yields were 4.0 (SE = 0.22) and 1.1 (SE = 0.06) Mg ha^{-1}, respectively. The botanical composition of the mixture under the two-cut regime was 49% lucerne, 33% chicory, 9% plantain, 3% grass, 1% caraway and 5% unsown species on a dry matter basis. Under four-cut regime, the dry matter mass fractions were 34% lucerne, 23% chicory, 19% plantain, 12% grass, 1% caraway and 11% unsown species.

2.2. Gas production

2.2.1. Biochemical methane potential

The batch test was conducted at mesophilic temperature (35 °C) to determine the ultimate methane yield. Inoculum used for the batch test was collected from a mesophilic post digester at the full-scale biogas plant in Research Center Foulum, Aarhus University, Denmark. The digester was mainly treating animal manure and agricultural residues such as cattle and pig manure, wheat straw and grass. The inoculum was filtered using a manual sieve with 2 mm mesh size and pre-incubated at 35 °C for 14 days to deplete the residual biodegradable organic material (degasification) [16]. The average total solids (TS) and VS of the inoculum were 3.0% and 2.1% mass fractions respectively.

The batch test was prepared according to the procedure described by Møller et al. [17]. Approximately 200 g of inoculum was added to each 500 mL infusion bottle, followed by the addition of substrate with a mass ratio of 1:1 ($VS_{inoculum}$: $VS_{substrate}$) in three replicates. A control with only inoculum was included. The bottles were incubated at 35 °C for 90 days. The measurement of biogas volume was achieved by inserting a needle connected to a tube with inlet to a column filled with acidified water (pH < 2) through the butyl rubber bottle cap. The biogas produced was calculated by the water displaced until the two pressures (column and headspace in bottles) were equal. Biogas compositions were analyzed using gas chromatography (7890 A, Agilent Technologies, USA). Methane produced from each sample was corrected by subtracting the volume of methane produced from the inoculum control. The resulting specific methane yields were normalized to standard conditions (gas volume adjusted to 273.15 K and 101.325 kPa). The decision to conduct the batch tests at the mesophilic temperature (whereas the continuous experiment was thermophilic) was because this was standard procedure within the laboratory. The batch tests were primarily used to determine ultimate methane yields and to compare these with continuous stirred tank reactor (CSTR) yields and these values should not be affected by reaction temperature after the long (90 day) digestion period.

2.2.2. Continuous stirred tank reactor (CSTR)

Mono-digestion of lucerne with or without forbs-grass mixture was conducted in three 20 L CSTRs with 15 L working volume for 70 days duration. Digester 1 was loaded with Lu-4 ($D_{Lu\text{-}4}$) while digester 2 and 3 were fed with Mix- 4 ($D_{Mix\text{-}4}$) and Mix-2 ($D_{Mix\text{-}2}$). Initially the reactors were filled with thermophilic inoculum sourced from pilot-scale digesters, which were mainly treating animal manure and agricultural residues. In Denmark, the majority of biogas plants are running under thermophilic condition, thus the reactors were running at 52 °C to simulate the common practice.

After several days, the reactors were fed with cattle manure and the reactors were monitored for 15 days to ensure process stability and no gas leaks (data not shown). When the process stabilized, the reactors were fed with a silage-water mixture with a mass ratio of 40% silage and 60% water. During the initial phase, the reactors were only fed during weekdays; by feeding 600 g of silage + water and unloading a corresponding amount of digestate manually prior to feeding. Starting from day 57 until the end of the experiment, feeding was done seven days per week. Thus, although the daily feed remained the same, the weekly mean organic loading rate (OLR) increased and the hydraulic retention time (HRT) decreased in the latter part of the experiment. The biogas production was measured continuously using an automatic biogas potential system (AMPTS II, Bioprocess Control, Sweden). The effluent from the reactors was sampled weekly and stored at -18 °C for further analysis.

2.3. Analytical methods

The raw silages and digestate samples were characterized for total ammonium nitrogen (TAN), VFA, pH, TS and VS following APHA [18]. The loss of VFAs prior to drying during VS determination was taking into account in this study. It is important since underestimated of VS of substrates may lead to overestimate of methane yield per unit VS [15]. The total nitrogen (TN) was analyzed following Dumas method [19] while TAN was determined using photometric kits (Spectroquant kit, Merck, USA). For macro- and micro-nutrient analysis, raw substrates and effluents from the middle and at the end of the experiment were analyzed at bonalytic GmbH Laboratory, Germany. The macro- and micronutrient concentration were determined using inductively coupled plasma atomic emission spectrometry (ICP-OES) following EN ISO 11885: 1997 method. The fiber content of raw silages and digestate at 70 days AD were examined following Van Soest et al. [20]. For this purpose, the samples were dried at 60 °C for 48 h and ground to < 0.8 mm particle size using a Foss mill (Cyclotec™ 1093, Foss, MN, USA).

2.4. Statistical data analysis

Biogas and methane yield data was analyzed statistically using Student's t-test and the analysis was performed using JMP Pro software version 13.

Table 1
Initial characteristics of the substrates and comparison of micronutrients values with raw grass silage.

Parameters	Units	Samples ID			
		Lu-4	Mix-4	Mix-2	Grass silage[◊]
TS*	%	17.20 ± 0.64	15.05 ± 0.55	21.81 ± 0.46	–
VS*	%	15.38 ± 0.61	13.08 ± 0.48	19.71 ± 0.45	–
TN+	%	3.50 ± 0.01	3.00 ± 0.02	2.25 ± 0.00	–
NDF+	%	33.32 ± 1.04	41.98 ± 0.00	51.94 ± 1.47	–
ADF+	%	30.11 ± 1.34	32.39 ± 0.07	42.72 ± 2.31	–
ADL+	%	4.84 ± 0.44	5.71 ± 0.11	8.91 ± 0.15	–
Phosphorus+	$g\ kg^{-1}$	3.09	3.81	2.50	–
Potassium+	$g\ kg^{-1}$	38.30	36.40	21.80	–
Magnesium+	$g\ kg^{-1}$	1.67	2.06	1.42	–
Calcium+	$g\ kg^{-1}$	17.00	18.90	15.50	–
Sulfur+	$g\ kg^{-1}$	3.33	4.72	2.31	–
Sodium+	$g\ kg^{-1}$	0.44	0.90	0.50	–
Aluminium+	$mg\ kg^{-1}$	37.30	274.00	66.40	–
Boron+	$mg\ kg^{-1}$	29.10	44.50	24.40	–
Barium+	$mg\ kg^{-1}$	15.30	21.80	21.30	–
Cadmium+	$mg\ kg^{-1}$	< 0.2	0.29	0.25	< 0.25
Cobalt+	$mg\ kg^{-1}$	< 0.3	< 0.3	< 0.3	–
Chrome+	$mg\ kg^{-1}$	0.47	0.98	1.82	–
Copper+	$mg\ kg^{-1}$	13.60	11.10	8.35	5.70
Iron+	$mg\ kg^{-1}$	113.00	444.00	121.00	277.00
Manganese+	$mg\ kg^{-1}$	59.80	112.00	53.8	38.40
Molybdenum+	$mg\ kg^{-1}$	< 1.0	< 1.0	< 1.0	13.60
Nickel+	$mg\ kg^{-1}$	2.60	2.74	2.65	3.60
Selenium+	$mg\ kg^{-1}$	< 15	< 15	< 15	–
Silicium+	$mg\ kg^{-1}$	124.00	506.00	206.00	–
Strontium+	$mg\ kg^{-1}$	51.30	55.20	55.80	–
Vanadium+	$mg\ kg^{-1}$	< 0.1	0.72	0.16	–
Zinc+	$mg\ kg^{-1}$	25.20	32.00	20.40	23.50

'<' Represents below the detection limits of particular element.
'±' Represents standard deviation of duplicates measurements.
[*] FM basis.
[+] DM basis.
[◊] Data from Wall et al. [7].

3. Results and discussion

3.1. Substrates characteristics

The initial characteristics of the three substrates used in the study are summarized in Table 1. The highest TS and VS content was observed from Mix-2, followed by Lu-4 and Mix-4. The TS values varied between 15 and 22% while between 13 and 20% for VS with regard to the substrates. The trend was also similar in term of fiber content; NDF concentration was higher in Mix-2 than Lu-4 and Mix-4. The observation was corroborated with the harvesting scheme of Mix-2, as the two-cut system prolonged the time for plant growth, and hence increased the dry matter and fiber fraction of Mix-2 [9]. The grass-lucerne-forbs mixtures have higher NDF concentration than lucerne. This is because the mixtures partly consisted of perennial ryegrass, a mono-cotyledonous plant, which normally has higher NDF content than di-cotyledonous plants [4,9]. Compared to Wahid et al. [9], NDF concentration of Mix-2 and Mix-4 were comparable with NDF content of grass-clover mixtures from two- and four-cut, while the NDF content of lucerne was within a similar range to red clover (legume) from four-cut. The crude protein content (TN x 6.25) of lucerne was around 22% (in term of DM), approximately 3 and 8% higher than Mix-4 and Mix-2, respectively. Protein concentration of lucerne examined in the present study was comparable with protein content of red-clover from four-cut measured by Wahid et al. [9].

Potassium and calcium were the two major macronutrients measured in the dry substrates with concentration ranging between 22 and 38 g kg^{-1} (potassium) and between 16 and 19 g kg^{-1} for calcium. Micronutrients or trace elements content were varied with respect to the substrates and generally, the concentration was higher in Mix-4 than Mix-2 and Lu-4. Aluminium, iron and silicon were the three dominant micronutrients. Compared to grass-silage reported by Wall et al. [7] in Table 1, the substrates used in present study, specifically Mix-4, have higher copper and manganese concentration. Meanwhile the grass silage contained more molybdenum and nickel than in the present study. The concentration of iron in Mix-4 was almost four times higher than Mix-2 and Lu-4, while nearly double the concentration of iron in grass silage.

3.2. Biomethane potential test

Cumulative methane yield of Lu-4, Mix-4 and Mix-2 at 30 and 90 days of AD are presented in Fig. 1a. At 90 days, methane yield in L per unit kg VS was maximum from Mix-4 (295), followed by Mix-2 (281)

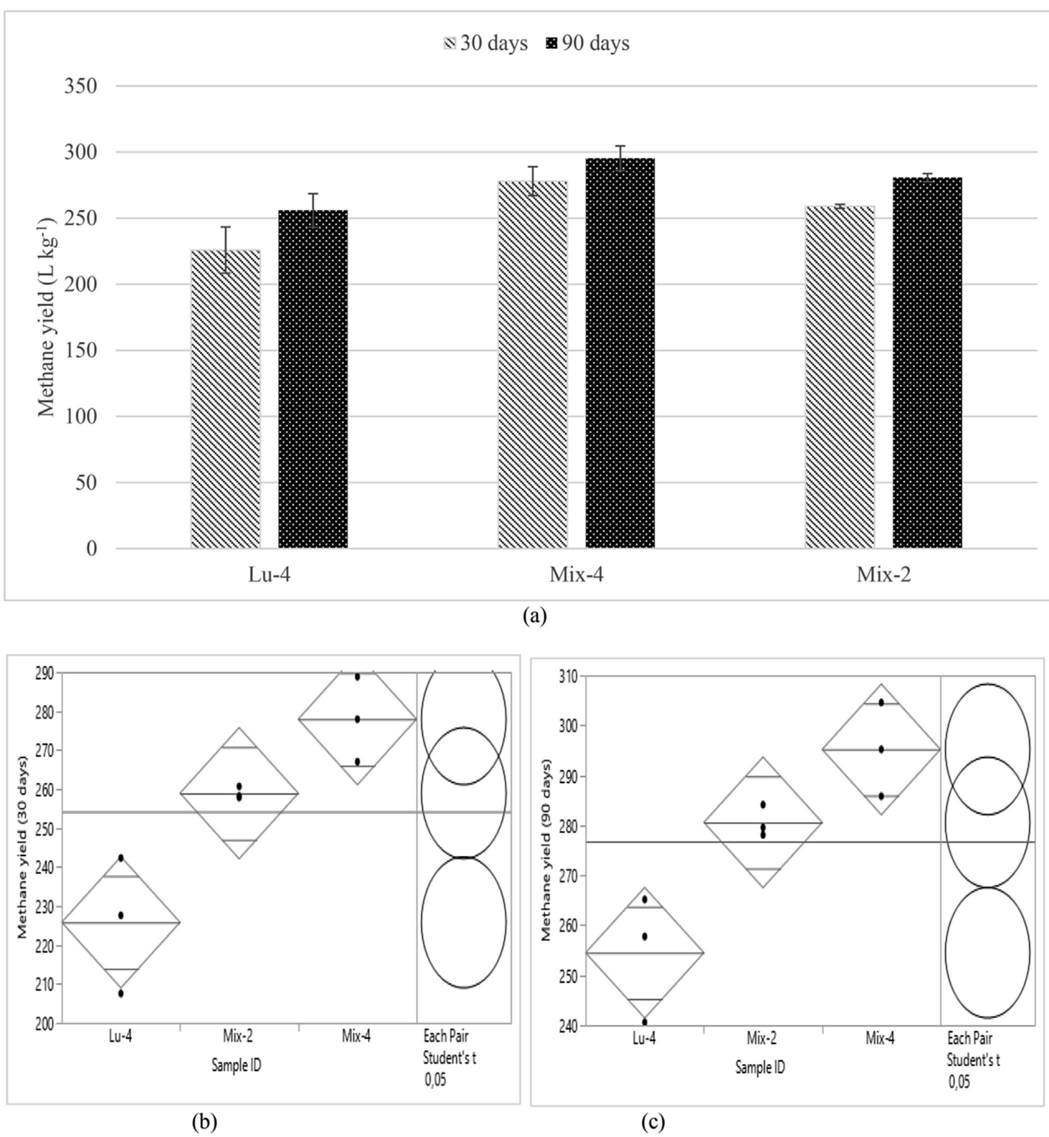

Fig. 1. (a) Average cumulative methane yield in L per kg VS and statistical analysis at (b) 30 days and (c) 90 days of D_{Lu-4}, D_{Mix-4} and D_{Mix-2}.

and Lu-4 (255). A similar trend was observed for methane yield at 30 days. According to Fig. 1b and c, AD of grass-lucerne-forbs mixture significantly increased methane yield, both at 30 and 90 days. Cutting strategy, (two vs. four cuts) did not influence the digestion of the mixture in a batch system. The observation was comparable with Wahid et al. [9]. However, the lower rate of methane production of Lu-4 could be attributed to a degree of inhibition within the batch test, which was shown by a more pronounced lag phase at the start (data not shown). The increased lag phase could suggest that Lu-4 is more rapidly degradable, with temporary accumulation of VFA to inhibitory levels although VFA was not measured in the batch test bottles. Some evidence to support this was found in the CSTR data and is discussed in section 3.3.

3.3. Continuous stirred tank reactors

3.3.1. Methane yield: $D_{Lu\text{-}4}$ vs. $D_{Mix\text{-}4}$

Biogas and methane production from $D_{Lu\text{-}4}$ and $D_{Mix\text{-}4}$ are shown in Fig. 2a and c. The data was further analyzed statistically and presented in Fig. 2b and d. The gas profile from $D_{Mix\text{-}2}$ was excluded because of technical problems occurring during the experiment. Some data were lost due to gas leaks (at the beginning of experiment) and blockages during sample feeding and unloading of the digestate. The Mix-2 tends to accumulate at the bottom of the reactor, causing blockage several times and the reactor was emptied a couple of times to fix the problem.

As observed in the figures, biogas and methane yields from $D_{Mix\text{-}4}$ were significantly higher than $D_{Lu\text{-}4}$. The average biogas and methane yield (from day 1–56) were 307 and 178 L kg^{-1} ($D_{Mix\text{-}4}$), and 291 and 163 L kg^{-1} ($D_{Lu\text{-}4}$), respectively. At the higher OLRs (based on continuous feeding, day 57–70), the average gas yield from $D_{Mix\text{-}4}$ and $D_{Lu\text{-}4}$ were 405 and 179 L kg^{-1} (biogas), and 236 and 95 L kg^{-1} (methane). Inhibition was observed in $D_{Lu\text{-}4}$ as the average methane yield was decreased during continuous feeding. The observation was also supported by declining methane volume fraction as later discussed in section 3.3.2., which also corresponds to the increased mean OLR during this period.

3.3.2. Reactor performance

Anaerobic digestion reduced VS in $D_{Lu\text{-}4}$, $D_{Mix\text{-}4}$ and $D_{Mix\text{-}2}$ as illustrated in Fig. 3. The percentage of VS removal for $D_{Lu\text{-}4}$, $D_{Mix\text{-}4}$ and $D_{Mix\text{-}2}$ at the end of the experiment were 45%, 66% and 37%. At the initial state of the experiment, VS removal of the reactors were quite similar, ranging from 58% to 66%. However starting day 18, the difference in VS reduction in $D_{Lu\text{-}4}$ and $D_{Mix\text{-}4}$ was more pronounced. The increased in OLR due to continuous feeding from day 57–70 disturb the AD process in $D_{Lu\text{-}4}$, led to lower VS reduction. As for $D_{Mix\text{-}2}$, no data was shown (from day 25–43) due to problem occurred mentioned in previous section. Dropped in VS removal was observed in $D_{Mix\text{-}2}$ towards the end of the experiment.

Methane content in biogas produced from each reactors are shown in Fig. 4a. It was observed that methane content in $D_{Lu\text{-}4}$ and $D_{Mix\text{-}4}$ were comparable from day 0–57, ranging between 50 and 62%. However, methane content in $D_{Lu\text{-}4}$ was reduced from 56 to 49% during continuous feeding, probably due to high substrate load as the OLR was increased. Methane content of Lu-4 and Mix-4 from batch test were higher than the values observed during continuous experiment. The average methane content of Lu-4 and Mix-4 (data not shown) from batch test were 68% and 66%, while 56% and 58% were observed from continuous experiment. However, it is not really possible to make a direct comparison of batch and continuous process gas compositions as the former process is very dynamic temporally with a high initial load, whereas the continuous process should maintain a steady state if the HRT and OLR are suitable.

pH is one of the important parameter to monitor process stability of

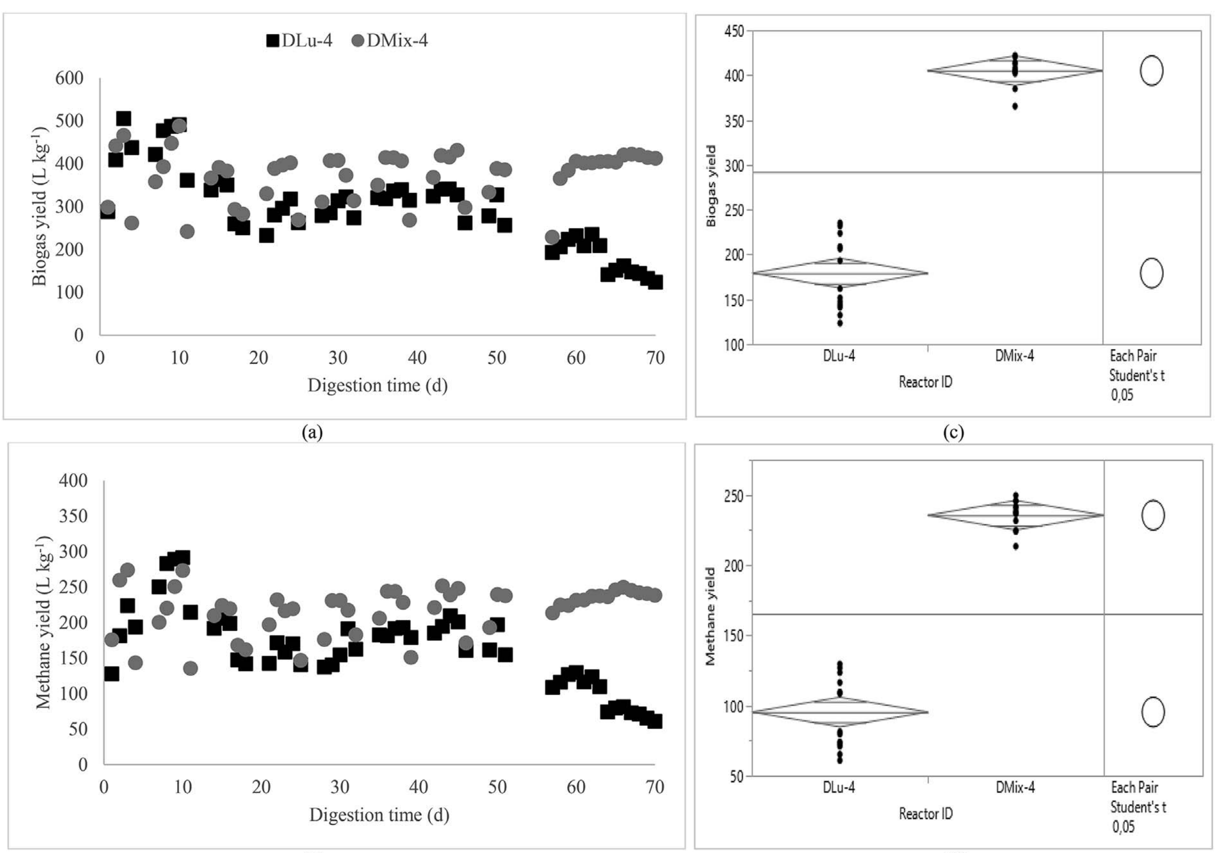

Fig. 2. Gas profiles for $D_{Lu\text{-}4}$ and $D_{Mix\text{-}4}$ in L per unit kg VS (a) Biogas yield (b) methane yield and (c, d) statistical data during continuous feeding.

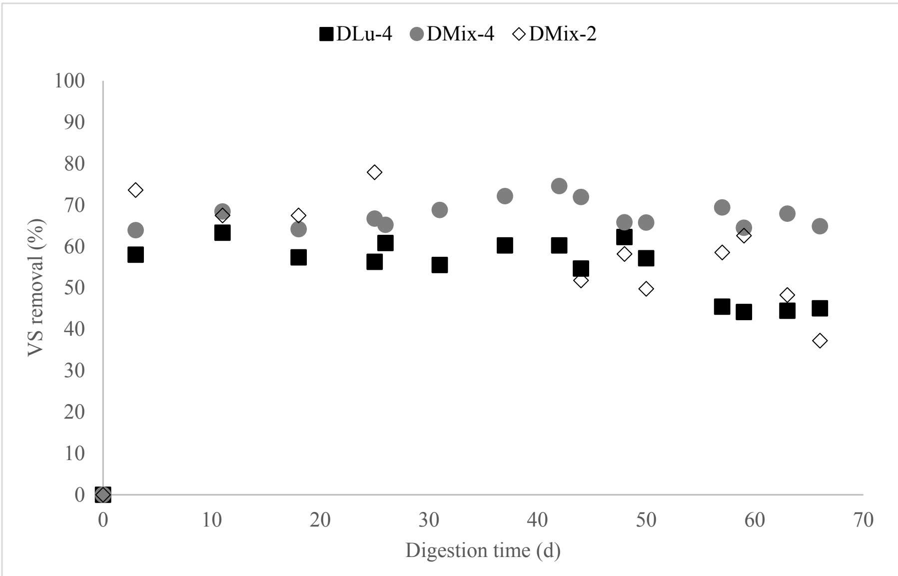

Fig. 3. The percentage of VS removal in D_{Lu-4}, D_{Mix-4} and D_{Mix-2}.

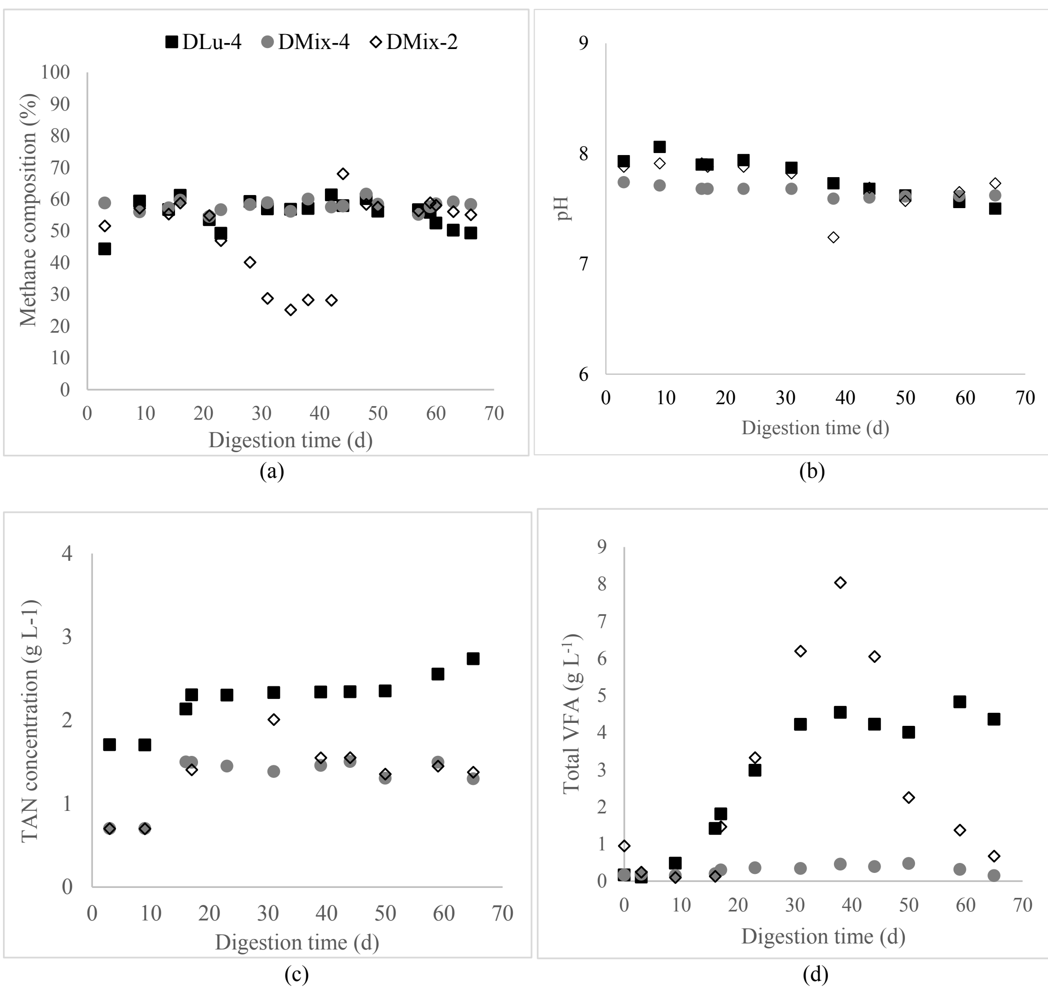

Fig. 4. Process parameters, (a) methane content, (b) pH, (c) TAN concentration, and (d) total VFA of D_{Lu-4}, D_{Mix-4} and D_{Mix-2}.

AD. The optimum pH value reported in the literature is ranging from 7.0 to 8.0 and the process is severely inhibited if the pH reduces below 6.0 or increases above 8.5 [21]. The pH value is strongly affected by VFA and buffering capacity, and the value decreased with the increase in VFA concentration [22]. Fig. 4b presents the pH value for each digester throughout the digestion period. The values lay between the optimum levels; varied from 7.2 to 8.0 with respect to the substrates. However, accumulation of VFA in D_{Lu-4} caused continuous pH reduction from 7.88 to 7.50. The drop in pH can be at least partly attributed to the reduced buffer capacity (alkalinity) during the mono-digestion of silages without a highly buffered co-substrate such as animal manure [23], although alkalinity was not measured in this study.

TAN is another essential parameter for assessing the reactor performance during AD [24]. A previous study reported that inhibitory TAN concentration ranged between 1.70 and 14.00 g L^{-1} [25]. The significant differences in inhibiting ammonia concentration depend on the substrates and inoculum used, process conditions (pH, temperature), and acclimation periods [25]. From Fig. 4c, the TAN level in D_{Lu-4} started to rise above 2.00 g L^{-1} after 15 days digestion and was stable from day 17–50, though slightly increased towards the end of AD (2.70 g L^{-1}) which corresponds with the increased OLR. Anaerobic digestion results in the mineralization of nitrogen, which consequently may reduce the C/N ratio in the reactor [26]. High amount of TN content may contribute to an increase in TAN concentration especially when mono-digestion was employed at high OLR. It is therefore possible that the high TAN value of Lu-4 induced ammonia inhibition in D_{Lu-4}, particularly when combined with a reduced alkalinity induced by a higher OLR. Inhibition by ammonia may result in increased VFA and a consequent reduction in methane yield [26]. Regarding D_{Mix-4}, the level of TAN was less than 1.50 g L^{-1}, indicating a reduced tendency for ammonia inhibition when a multi-species substrate was employed due to dilution of the TN value.

Volatile fatty acids including acetic acid, propionic acid and butyric acid that are produced during acidogenesis and/or acetogenesis steps are crucial for AD. Propionate and butyrate are degraded by syntrophic acetogenic bacteria to produce more acetate that is later degraded into methane and carbon dioxide by acetoclastic methanogens. The VFA plays an important role in maintaining the process stability as it strongly influences pH, alkalinity and methanogenic activity [22]. Fig. 4d illustrates total VFA of the respective substrates during the 70 days digestion period. The AD process in D_{Mix-4} was more stable than D_{Lu-4} and D_{Mix-2}, as the total VFA concentration was less than 1.00 g L^{-1}. The level of VFA in D_{Lu-4} elevated with digestion time and reached 5.29 g L^{-1} at 70 days. Regarding D_{Mix-2}, VFA concentration increased to 8.04 g L^{-1} due to technical problems (sudden increase in temperature).

The elevated VFA levels in D_{Lu-4} can also be attributed to a higher OLR when compared to D_{Mix-4}; the weekly mean OLR (as VS) between days 0–57 was 1.76 g L^{-1} d^{-1} for D_{Lu-4} and 1.49 g L^{-1} d^{-1} for D_{Mix-4}, after day 57 this rose to 2.46 and 2.09 g L^{-1} d^{-1} in D_{Lu-4} and D_{Mix-4} respectively. Fig. 5a shows that after an initial increase during the first ca. 20 days, the concentration of propionic acid in D_{Mix-4} remained stable below 0.13 g L^{-1} throughout the digestion period. Meanwhile as observed in Fig. 5b, propionic acid in D_{Lu-4} increased steadily to ca.

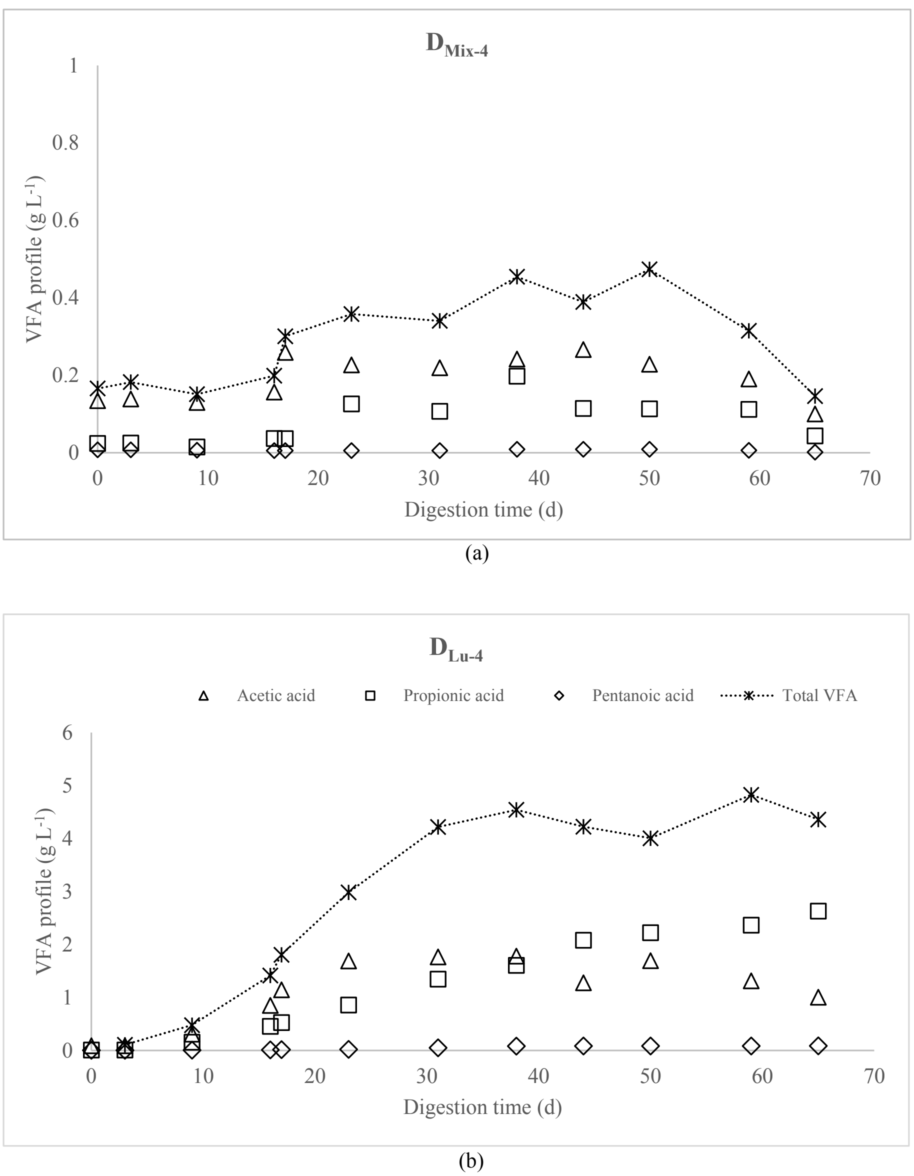

Fig. 5. VFA profile (total and individual VFA) of effluent from (a) D_{Mix-4} and (b) D_{Lu-4}.

$2.20\ g\ L^{-1}$. The increased OLR after day 57 further increased propionic acid concentration in D_{Lu-4} whereas in D_{Mix-4} the concentration dropped. Accumulation of propionic acid is indicative of problems within AD [27], as the process of oxidation of propionate to acetate is sensitive to inhibition. Comparing the OLRs of the two reactors, D_{Lu-4} suffered some propionic acid accumulation at an OLR of $1.76\ g\ L^{-1}\ d^{-1}$ whereas D_{Mix-4} did not, even at a higher OLR of $2.09\ g\ L^{-1}\ d^{-1}$ after day 57. It is apparent that Mix-4 could be digested at a higher OLR than Lu-4, which could be explained by Lu-4 being more rapidly degradable then Mix-4, as was evident in the longer lag phase in the former during batch tests. Faster degradability means the hydrolytic and acidogenic phases of AD are quickly accomplished, which can lead to production of VFA at a rate faster than it can be metabolized to acetate and ultimately to methane and therefore an accumulation of VFA. However, we cannot assume organic overload due to rapid degradability is the only reason for VFA accumulation as Lu-4 contained approximately 55% more organic nitrogen than Mix-2 and Mix-4. No nitrogen losses were observed due to AD, as the values were comparable with initial concentration.

Concerning D_{Mix-2}, 30% of new inoculum was added at day 37 to recover the process after an unintended temperature increase (65 °C) due to a technical problem in the heating system. Generally, methanogens are sensitive to temperature changes and it was expected that this conditions would inhibit them and disrupt methane formation. This was supported by low concentration of methane from D_{Mix-2} from day 31–42 as shown in Fig. 4a. The digester recovered after inoculum addition as the methane fraction in the gas increased to 58%. In addition, the VFA level was reduced to less than $1.00\ g\ L^{-1}$ and pH was elevated to 7.7.

3.3.3. Macro- and micronutrients

One of the concerns when running a digester without addition of animal excreta is paucity of nutrients over digestion time. As has been highlighted previously, optimal macro- and micronutrients supply in the digester is crucial for biogas production [28,29]. During AD, macronutrients play an important role as buffering agents while micronutrients are important co-factors in enzymatic reactions involved during methane production [28]. Insufficient nutrient availability during AD may inhibit microbial activity and jeopardize the process stability [29]. Carbon is an important element for anaerobic microbes and it is use for building up the cell structure. Apart from carbon, the other essentials macronutrients for AD are nitrogen, phosphorus and sulfur [29,30]. Nitrogen is important for protein synthesis while phosphorus provides energy carriers in the metabolism. Sulfur is an essential constituent of amino acids and nutrient for the growth of methanogens [29]. The requirements of macronutrients by the microbes however are generally low and based on bacterial composition and bacterial growth and biomass composition [29]. This fact tallied with the results (Table 2) as the concentration of macronutrients available in the feed and digestate at 70 days AD were comparable.

Anaerobic digestion did not influence phosphorus concentration as no phosphorus losses were determined. Generally only small amount of phosphorus is lost due to AD, approximately less than 10% [31]. Sulfur is mainly a required nutrient for methanogens as the relative level of sulfur was found to be higher in methanogens than other anaerobic microbes. The optimal level of sulfur reported in literature was given between 1 and $25\ mg\ L^{-1}$ [25]. The concentration of sulfur in this study lies within the optimum values, with variation of 8–$11\ mg\ L^{-1}$ in raw substrates and 2 to $4\ mg\ L^{-1}$ in the digestate. The sulfur losses due to AD have been reported and some studies observed less than 50% of sulfur was retained in the digestate after AD [31].

The incorporation of micronutrients especially cobalt, nickel and iron in enzyme systems enhance substrate degradation and maintain the efficient operation of the digester [30]. Wall et al. [7] observed a methane yield increment when cobalt, nickel and iron were added during mono-digestion of grass silage. Besides cobalt, nickel and iron, the importance of copper and zinc for methanogens in AD has also been highlighted [32,33]. Fig. 6 presents the micronutrients concentration in D_{Lu-4}, D_{Mix-4} and D_{Mix-2} at 30 and 65 days. The dotted line represents the minimum stimulatory concentration of the nutrients on the anaerobic biomass reported in Romero-Güiza et al. [28]. In general, the concentrations of micronutrients reduced over the course of the experiment. The micronutrients levels of nickel and cobalt are below the minimum and cobalt seems to be the most critical. Cobalt is important for acetogens and methanogens, and mainly used as a central atom in corrinoids and vitamin B12 enzyme [29]. Meanwhile, nickel plays a role to enhance the growth of methanogens and is involved in the formation of several enzymes which are essential for methanogenesis [29].

Pobeheim et al. [34] reported that deficiency in cobalt and nickel levels jeopardize the process stability of mono-digestion of maize silage. Addition of 50 and $600\ \mu g\ kg^{-1}$ of cobalt and nickel (respectively) improved the digester performance. Jarvis et al. [35] reported the stimulatory effects of cobalt addition during grass-clover digestion as a significant methane increase was observed. Weiland [21] highlights the necessity of micronutrients addition for mono-fermentation of energy crops for achieving stable process conditions and high loadings. However, nutrient concentrations in D_{Lu-4} were generally higher than in D_{Mix-4}, despite the latter performing at lower VFA levels, higher gas yields and a higher VS removal rate. Therefore, we cannot conclude that suboptimal nutrient concentrations were responsible for the inability of D_{Lu-4} to operate at the OLRs tested in this study.

4. Conclusions

Mono-digestion of a grass-lucerne-forbs silage at four-cut was stable at an OLR of $2.09\ g\ L^{-1}\ d^{-1}$, whereas lucerne silage showed signs of inhibition at an OLR of $1.76\ g\ L^{-1}\ d^{-1}$ due to either organic overload, ammonia inhibition or a combination of the two. Therefore, lower OLR is recommended if this substrate is to be mono-digested, or co-digestion with a substrate of lower nitrogen composition. Overall, grass-lucerne-

Table 2
Comparison of chemical characteristics of feeds (fresh silage + water) and digestate (at day 70).

Parameters	Lu-4		Mix-4		Mix-2	
	Feed	Digestate	Feed	Digestate	Feed	Digestate
TN (%)*	0.34 ± 0.01	0.33 ± 0.00	0.22 ± 0.02	0.22 ± 0.01	0.22 ± 0.00	0.24 ± 0.01
TAN (%)*	0.07 ± 0.00	0.24 ± 0.00	0.02 ± 0.00	0.13 ± 0.00	0.03 ± 0.01	0.13 ± 0.00
Phosphorus ($g\ kg^{-1}$)*	0.21	0.26	0.23	0.33	0.22	0.25
Potassium ($g\ kg^{-1}$)*	2.63	2.60	2.19	2.37	1.90	2.18
Magnesium ($g\ kg^{-1}$)*	0.12	0.12	0.12	0.15	0.12	0.16
Calcium ($g\ kg^{-1}$)*	1.17	0.99	1.14	1.16	1.35	1.34
Sulfur ($g\ kg^{-1}$)*	0.23	0.15	0.28	0.17	0.20	0.25
Sodium ($g\ kg^{-1}$)*	0.03	0.09	0.06	0.10	0.04	0.10

' ± ' Represents standard deviation of duplicates measurements.
* FM basis.

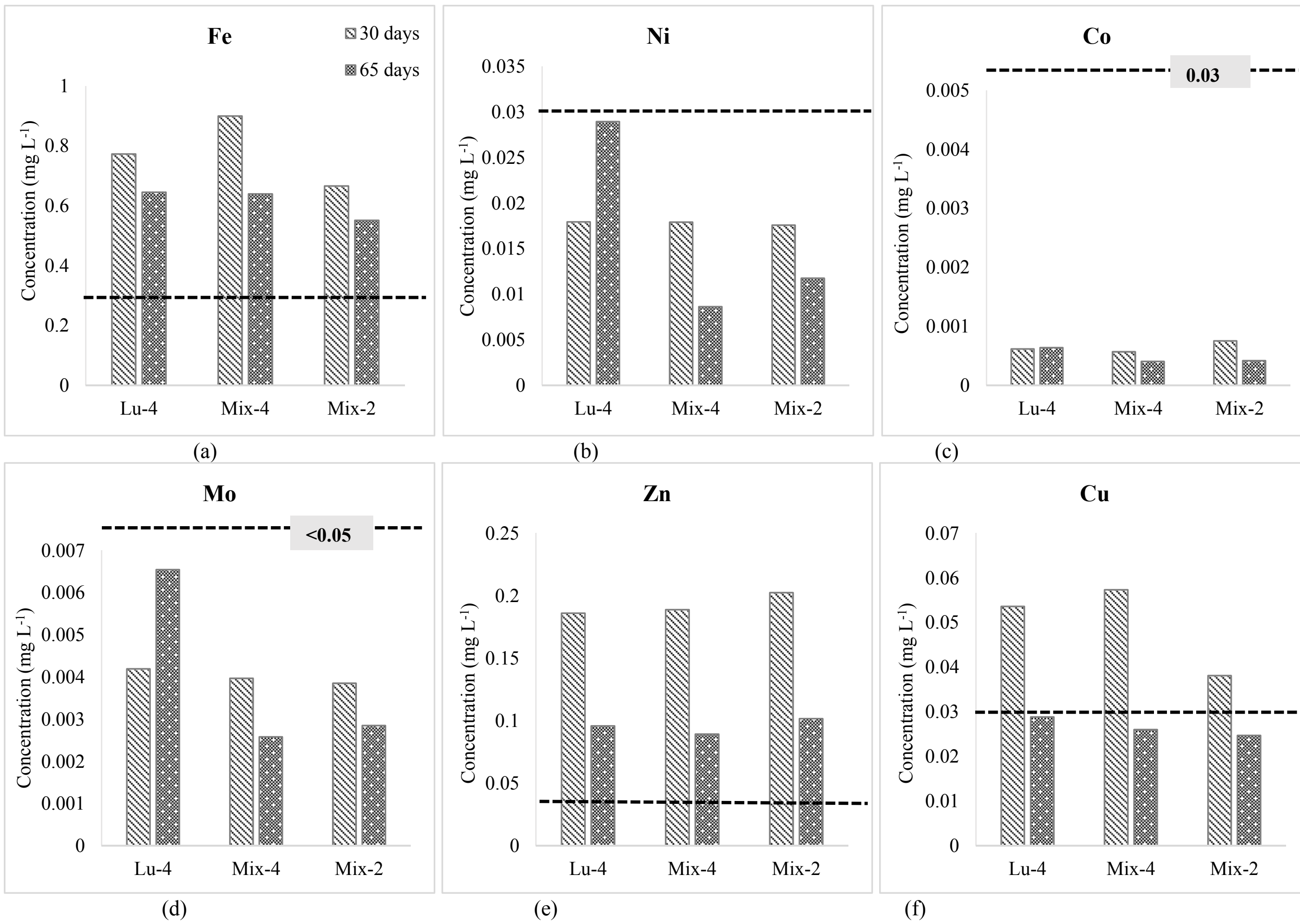

Fig. 6. Micronutrients content, (a) Fe, (b) Ni, (c) Co, (d) Mo, (e) Zn and (f) Cu in effluent samples of Lu-4, Mix-4 and Mix-2 at 30 and 65 days. represents minimum stimulatory concentration of nutrients on anaerobic biomass [26].

forbs at four cuts was found to be the best substrate for biogas production in this study, with good process stability and higher methane yield. Despite the levels of Co and Ni falling below recommended minimums during continuous digestion of all biomasses, no loss of process performance could be attributed to this in the current study, suggesting further work is required in this area.

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