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Effect of macro and micro nutrients addition during anaerobic mono-digestion of grass silage in leach-bed reactors

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Abstract

The effect of macro- (NH_4Cl) (set I) and micro-nutrients (Fe, Ni, Co and Mo) (set II) addition on chemical oxygen demand (COD) solubilization during anaerobic mono-digestion of grass silage was investigated in two sets of leach bed reactor (LBR) experiments at 35°C. Results showed that addition of NH_4Cl and micro-nutrients improved COD solubilization by 18% (0.56 g SCOD g^{-1}

volatile solids, VS) and 7% (0.45 gSCOD g^{-1}VS) respectively than control. About 20-50% of the added micro-nutrients were bioavailable in the produced leachates, while the rest (50-80%) were adsorbed on to the grass silage. Results of biological methane potential (BMP) assays showed that, specific methane yields of grass silage were improved by 17% (0.36 ± 0.02 m^3 CH_4 $kg^{-1}VS_{added}$) when NH_4Cl was supplemented while Fe, Ni, Co and Mo addition improved methane yields by 15% (0.33 ± 0.005 m^3 CH_4 $kg^{-1}VS_{added}$) when compared to control.

Key words: leach bed reactor; micro nutrients; Anaerobic digestion, grass silage, methane

1 Introduction

Agricultural biogas production has been progressively gaining attention for meeting EU's renewable energy targets set under Renewable Energy Directive [1]. The produced biogas can be used for heat and/or electricity production, and thereby replace the depleting fossil fuels and the associated greenhouse gas emissions. Depending upon the feedstock characteristics, biogas process can be classified as wet or dry anaerobic digestion systems [2]. Dry anaerobic digestion (AD) technology is most suitable for drier ligno-cellulosic substrates such as grass silage, hay, reed canary grass, common reed because of their high solids contents (30-50% of total solids [TS]). When compared to wet digestion technology, dry AD technology is simple to operate and can tolerate high solids content (as much as 50%) while overcoming the problems of scum formation, choking the gas lines, energy demand for mixing equipment etc. [4]. Further, dry AD technology requires less energy inputs and water and/or pretreatment [5]. Particularly, dry batch reactors such as leach bed reactors (LBRs) when operated in combination with high rate reactors such as

Upflow Anaerobic Sludge Blanket (UASB) reactors or Upflow Anaerobic Filters (AF) offer the benefits of handling high loading rates as well as producing high volumetric methane yields [6,7]. Finally, the higher nutrient content in high-solid substrates enhances the prospects of agronomic valorization of the digested residues (effluent) of biogas plants. Digested residues/effluents with high nutrient content can thus be easily transported for agricultural and economic benefits [8].

In Europe, grass-silage produced in excess at the farm is diverted to biogas production for meeting the on-farm energy needs in terms of heat and electricity. For instance, co-digestion of grass silage, harvested from 1.1% of grassland, and dairy manure at 1:1 volatile solids (VS) ratio could contribute to over 10% of renewable energy supply in transport in Ireland [9]. In wet AD systems, grass silage is commonly co-digested with animal manures using continuously mixed reactor systems or digested alone (mono-digestion) in percolating leach bed reactor systems (LBRs). Currently, there are more than 9000 biogas plants in Germany [10] and most of these plants are co-digesting livestock manures with a mixture of energy crops (83% of biogas plants), energy crops alone (15%) or manure alone (2%) [11]. In recent times, mono-digestion of energy crops has been gaining attention mainly due to high volumetric yields of biogas [6] and regional inaccessibility of livestock manures [12]. Thus, availability and accessibility (in terms of distance) of manure is a limitation of these farms to practice co-digestion.

Anaerobic mono-digestion of energy crops or co-digestion of low nutrient organic wastes with high proportion of energy crops as feedstock over longer periods of time may lead to insufficient availability of macro- (Nitrogen - N, Sulphur - S, Phosphorus - P) and micro-nutrients (Iron-Fe, Nickel-Ni, Cobalt-Co, Molybdenum-Mo and Tungsten-W) resulting in process imbalance and low methane yields [12,13,14,15]. During AD, micro- and macro-nutrients play a key role in

digester performance and process stability. Macronutrients are mainly associated with the digested residues because they substitute as a liquid/solid fertiliser in crop production while, micronutrients are essential for the operational performance of the anaerobic digester and their deficiency will negatively affect the methane yields [16].

Macro-nutrients are essential for the growth and metabolism of all anaerobic microbial consortia including, methanogens (*Methanosarcina barkeri, Methanospirillum hungatii, Methanocorpusculum parvum, Methanobacterium Thermoautotrophicum* etc.) [12]. On the other hand, micro-nutrients are required in very small amounts as they form the essential components of enzymes and cofactors that are involved in the biochemistry of methane formation [17]. Deficiency of micro-nutrients may impede cell function and thus the microbial degradation process during AD [18]. For instance, a 10% and 25% decrease in biogas production in response to Co and Ni deficiency respectively was reported during semi-continuous operation of 5 anaerobic reactors using a model substrate for maize silage [19]. Several studies reported improvement of methane yields from energy crops when macro (N, P and S) and micro-nutrients (Fe, Ni, Co, Mo, Se and W) were supplemented during the one-stage or two-stage process, particularly during the co-digestion [13, 14, 20]. However, studies dedicated to mono-digestion of energy crops and the effects of nutrients addition on process performance (particularly, microbial hydrolysis) and methane yield when operated in dry-anaerobic digesters are very limited.

Therefore, the present study was conducted to investigate the effects of supplementation of macro- (N in the form of NH_4Cl) and micro-nutrients (Fe, Ni, Co and Mo) on microbial hydrolysis indicated by COD solubilisation and subsequent production of volatile fatty acids (VFAs) and their conversion to biogas. This was particularly studied under low inoculum conditions (only 6% of the

substrate VS) mimicking unavailability of inocula/seed materials/cow manure and thus lower supply of nutrients during the AD process. The current study also aimed to understand and evaluate the nutrient requirements as well as nutrient dynamics during the AD of grass silage specifically, in dry AD systems such as, batch LBRs.

2 Materials and methods

Grass silage was obtained as grab samples from a dairy farm (Kalmari Farm, Laukaa Central Finland). The feedstock, a mixture of 75% timothy grass (*Phleum pratense*) and 25% of meadow fescue (*Festuca pratensis*) was prepared at the farm as described in [21]. The grass silage was stored at the farm for 4-5 months in an open bunker silo under ambient conditions before collection for set I experiments and for >12 months before collection for set II experiments. In the laboratory, grass silage was cut to 2-3 cm length by using a scissors and stored at -20^{o}C until further use.

Digestate from the above dairy farm's mesophilic digester co-digesting cow manure, energy crops and by-products from confectionery industry was used as inoculum.

2.2. Biological methane potential (BMP) assays

Methane potentials were determined in triplicate 1L glass bottles with a liquid volume of 750 mL (Table 2). To each assay, 250 mL of inoculum and grass silage at a $VS_{substrate}$ to $VS_{inoculum}$ ratio of 1 were added (Table 1). Grass silage with and without addition of NH_4Cl (set I) and micro-nutrients (Fe, Ni, Co and Mo) (set II) was studied. Three different dosages of micro nutrients were

tested viz., low (Fe-50, Ni-0.1, Co-0.2, Mo-0.15 mgL^{-1}) medium (Fe-75, Ni-1.7, Co-0.75, Mo-0.5 mgL^{-1}) and high (Fe-375, Ni-9, Co-3.75, Mo-0.75 mgL^{-1}) were tested and the concentrations of each of the four micro-nutrients were based on the literature studies with substrates such as grass and maize silage [18, 22]. Assays with inoculum alone, and no micro or macro nutrients addition were used as controls. Distilled water was added to reach the liquid volume of 750 mL. $NaHCO_3$ (3 gL^{-1}) was used as buffer. The contents of the bottles were then flushed with nitrogen gas (98.8%) for about 3 minutes before sealing with silicon stoppers. Prepared assays were statically incubated at 35°C. The produced biogas was collected into aluminium gas bags. The methane produced from the control assays was subtracted from the sample (substrate) assays. The assays were manually shaken twice a day and before each gas composition analyses.

In the control assays, two of the three replicate bottles were opened (day 60) and supplemented with 10 ml of high micro-nutrient dosage solution to verify whether the high micro-nutrient dosage resulted in the increased methane yields. Thereafter, the incubation of the prepared assays was continued.

2.3. LBR experiments

Two sets of LBR experiments were carried out and referred to as set I and set II (Fig. 1). In set I (control L0 and L1), the effect of macro-nutrient (NH_4Cl) supplementation as a nitrogen source was tested. On the other hand, the effect of micro-nutrients (Fe, Ni, Co and Mo) supplementation was tested in set II (control L2 and L3). LBR experiments were carried out in two, 1 L acrylic plastic column reactors with a working volume of 750 mL. No replicates for reactors were used. In set I experiments, leachate was collected for recirculation in two separate 1 L glass

reservoirs and referred to as R0 and R1. The leachate collection system in set I experiment consisted of 3 cm cylindrical acrylic column and steel mesh (pore size about 2 mm) to support the biomass weight. Several layers of nylon mesh (pore size <1 mm) were placed underneath the acrylic column to prevent clogging and thereby prevent solids entering into the reservoir (Fig. 1). On the other hand, leachate in set II experiment was not collected but was allowed to remain in the reactor and thereby increase the contact between grass silage and microbes. Further, leachate collection system in set II was modified by including a layer of foam (~1cm thickness) and glass beads. This was done to provide additional surface area for microbial adherence, to prevent microbial washout (that occurred during re-circulation in Set I experiments) and thus retain the microbes within the reactor to facilitate improved hydrolysis (Fig. 1).

On day 0, all LBRs (both set I and set II) were loaded with 50g VS of grass silage and 3g VS of inoculum. Thereafter, reservoirs R0 and R1 in set I experiment were filled with 750 mL of distilled water and 2.1 gL^{-1} of NH_4Cl was added as a nitrogen source in R1. For set II experiments, control LBR L2 was filled with 470 mL of distilled water, while LBR L3 was loaded with 470 mL of the medium dosage level micro-nutrients solution (Fe, Ni, Co and Mo). The concentration of the micro-nutrient solution is given in Table 2. The medium dosage level (Fe-75, Ni-1.7, Co-0.75, Mo-0.5 mgL^{-1}) was chosen based on the best results obtained in the batch experiment, which was started 20 days prior to LBR experiment. Leachate re-circulation was started immediately in set I run by using a peristaltic pump at a flow rate of 750 mLd^{-1}. The corresponding leachate recirculation rate in set II run was 470 mL d^{-1} and was operated at an interval of 15 minutes (Fig. 1). Sampling was performed 3 times in the first week and twice a week in the following experimental period. About 25 mL of the leachate sample was collected and to keep the liquid

159 volume in the reactor/reservoir constant, the same volume of distilled water (25 mL) was replaced
160 back into the reactors.

161

162 ***2.4. Residual methane potential assays***

163 Upon termination of set I LBR experiments, the digested material was collected and
164 separated into solid and liquid fractions (leachate). Thereafter, residual methane potential of the
165 solid and liquid fractions was compared with the mixed fraction (solid + liquid fraction) in batch
166 experiments. Batch experiment was carried out in triplicate serum bottles (120 mL) with 40 mL
167 working volume. At the start of the experiment, assays with solid and liquid fractions were
168 inoculated and pH was adjusted with NH_4OH. On the contrary, assays with mixed fraction were
169 incubated as such with and without pH adjustment. These residual methane potential experiments
170 were performed to simulate LBR experiment and understand the effect of hydrolysis in LBR when
171 grass silage is completely submerged in leachate compared to wetting of grass silage during
172 leachate recirculation. Further, the influence of pH adjustment and inoculation on COD
173 solubilsation and VFA conversion to methane was also studied.

174 For the solid fraction assays, 25 mL of inoculum, 10 mL of distilled water (based on
175 substrate dry matter) and 5 g of solid fraction (w/w) were added whereas for the liquid fraction
176 assays, 25 mL of inoculum and 15 mL of liquid fraction were added and incubated with and
177 without pH adjustment. In the assays with pH adjustment, NH_4OH was used to adjust the pH to
178 7.5-7.6. For the assays with mixed fraction, 7 g of solid fraction and 33 mL of liquid fraction were
179 added. Assays with inoculum alone were used as controls. Finally, the prepared assays were
180 flushed with N_2/CO_2 mixture (N_2 -98.8%, CO_2 – 1.2%) for 3 minutes before sealing with butyl

rubber stoppers and aluminium crimps. Methane produced from control assays was subtracted from the sample assays.

Upon termination of BMP experiments (conducted after set II LBR experiments), the residual materials of control BMP assays were analysed to determine the micro-nutrient responsible for the increased methane yield. Further, control BMP assays were also analysed to verify the results obtained from low, high and medium dosage micro-nutrients supplementation. The experiment was conducted in 60 mL serum bottles in triplicates, with 25 mL working volume. Assays with multiple micro-nutrient addition were supplemented with three micro-nutrients in four different combinations. On the other hand, assays with single micro-nutrient addition were analysed separately. Assays without micro-nutrients addition was used as control.

2.5. Analyses and calculations

In LBR experiments (set I and set II), 25-50 ml of leachate was sampled periodically for chemical analyses. This removed sample volume was replaced by distilled water and considered in further leachate dilution.

pH was measured with a Mettler Toledo S20 Seveneasy pH meter. Total solids (TS) and VS were determined according to the standard methods [23]. COD was measured according to Finnish standards [24]. Soluble COD (SCOD) and NH_4-N from crop samples were analysed after extraction according to Finnish standards (SFS-EN 12457-4) and after filtration through GF 50 glass fibre filter papers (Schleicher & Schhuell). NH_4-N and Total Kjeldahl Nitrogen (TKN) were analysed according to Tecator application note as described elsewhere [21].

Total and soluble metal concentrations of Fe, Ni, Co and Mo in leachates were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES). Leachate samples were acidified with HNO_3 (pH < 2) and stored at -20^{o}C until further analyses. Whilst, grass silage samples (solids) were oven dried at 105^{o}C for 24 hours and then combusted in a muffle furnace (for 2 h) at 550^{o}C to obtain dry ash. The dry ash samples were then digested using the ultrasound-assisted digestion method. The digested sample solutions were analyzed for the metal concentrations according to [25] using Perkin-Elmer (Norwalk, CT, USA), model Optima 4300 DV ICP-OES using the default parameters of the instrument (nebuliser flow 0.6l m^{-1}, plasma power of 1400W and auxiliary gas flow of 15l m^{-1}).

Methane and hydrogen contents in the biogas were analyzed using a gas chromatograph (Perkin Elmer Clarus 500 GC with thermal conductivity detector and Supelco Carboxen™ 1010 PLOT fused silica capillary column 30m×0.53mm, carrier gas argon, oven temperature 100°C, injection port 250°C, detector 225°C). A pressure lock syringe was used for sampling gas. VFAs were analyzed with gas chromatograph (PE Autosystem XL GC equipped with flame-ionization detector and PE FFAP column 30 m x 0.32 mm x 25 µm, carrier gas helium, injection port 225°C, oven temperature 100-160 °C). Biogas was collected in aluminium gas bags (Teseraux, TECOBAG, PETP/AL/PE 12/12/75) (5L capacity). The volume of collected biogas was measured by downward displacement of water using a volumetric gas meter (100L acrylic column).

Specific methane yields of BMP assays were calculated as cumulative methane (mL) per g substrate VS added and were expressed in m^3 CH_4 kg^{-1} VS_{added}. The methane production of the control assays was subtracted from the sample assays. Specific SCOD production (g SCOD g^{-1} VS)

and specific NH_4-N (mg NH_4-N g^{-1} VS) in LBR experiments were calculated by considering the total leachate volume and the sample volume removed during the operation of LBRs.

The data obtained in the BMP assays was subjected to analysis of variance (ANOVA) using the SPSS program [26]. The treatment means were separated by Tukey test if the *F*-test was significant at $P \leq 0.05$. Before performing ANOVA, data was subjected to Welch's test to evaluate the homogeneity of variance.

3 Results and Discussion

3.1. Substrate characteristics

The chemical characteristics of grass silage are shown in Table 1. Grass silage used for the set I LBR experiment had higher pH and lower solids content (TS, VS) than the grass silage used in set II LBR experiment. On the other hand, micro-nutrients concentration of grass silage in set II LBR experiment was much higher than in set I. The differences in the physico-chemical characteristics between the two grass silage samples could be due to differences in agronomic factors such as physiological maturity and harvest time of grass as well as due to difference in ensilage process, storage time, nutrient losses, maturation etc. [27]. The above differences in chemical composition may have also affected the rates of hydrolysis and the subsequent chemical parameters (pH, SCOD and VFA production) during the digestion in the LBRs.

3.2. BMP assays

The effects of micro-nutrient (Fe, Ni, Co and Mo) and macro-nutrient (NH_4Cl) addition on cumulative methane production and methane yield of grass silage are presented in Fig. 2 and Table 3. Methane yield from control assays was 0.30±0.004 m^3 CH_4 kg^{-1} VS_{added}. Addition of NH_4Cl improved the methane yield to 0.36±0.02 m^3 CH_4 kg^{-1} VS_{added} and was also found to be statistically significant ($P < 0.05$). Increase in methane yield could be mainly due to (i) an increased pH in the assays when compared to control and (ii) a better state of C/N balance achieved in the assays due to the supplementation of NH_4Cl as a nitrogen source complementing high-carbon composition (grass silage). Also, additional N from NH_4Cl, could have enhanced the growth of anaerobic microbial consortia, particularly, methanogens which could have contributed to improved methanogenesis [12]. The presence of sufficient methanogenic populations within the assays would ensure sufficient buffering capacity in the assays as the produced VFAs were quickly converted to methane and thereby enabled a stable AD process with improved methane yields [12].

Among the three tested micro-nutrient dosages, assays supplemented with the highest dosage resulted in a significant ($P < 0.05$) increase in methane yields (from 0.28±0.004 to 0.33±0.02 m^3 CH_4 kg^{-1} VS). Previous research efforts [12, 28] suggest that micro-nutrients function as cofactors of enzymes or coenzymes that are responsible for the growth of anaerobic bacteria and this could be the reason for enhanced biosynthesis of methane observed in the current study. Particularly, it was reported that Fe acts as cofactors of enzymes, cytochrome and ferredoxin in methylotrophic methanogens [28] in an electron transport chain of metabolism and thus enhances methane productivity. Previous studies also reported that when sufficient quantities of Ni and Co

265 were present in the medium the microbial community was dominated by acetogenic methanogens
266 resulting in a stable process conversion of VFAs to methane. On the other hand, hydrogenotrophic
267 methanogens increased together with VFA accumulation under nutrient deficient conditions of Ni
268 and Co [29]. Similarly, Mo is a component of the enzyme formate dehydrogenase and plays a
269 crucial role in the biochemical process of AD as it inhibits sulphate reducing bacteria and reduces
270 the competition for methanogenic bacteria [30].

271 The increase in methane yield in the present study was however lower than those
272 reported in the literature. Several researchers have previously reported 35-60% increase in the
273 biogas/methane yields when anaerobic digesters operating with crops and crop residues were
274 supplemented with micro nutrients such as Fe, Ni, Co, Mo, Se and W [19, 12]. In the above studies,
275 the difference in response to micro-nutrients was attributed to the differences in the chemical
276 nature of the substrates (grass silage), micro-nutrient requirements and uptake, differences in the
277 process and digestates quality etc. [27]. Thus, further research is necessary to determine the
278 optimum concentration of these micro-nutrient combinations to obtain higher methane yields
279 from mono-digestion of grass silage in LBRs. Moreover, the concentration of single or multiple
280 micro-nutrients used in the above studies were process-specific and also substrate-specific.
281 Nevertheless, results from the present study suggest that anaerobic microbial consortia on grass
282 silage were able to tolerate/withstand the supplemented high concentrations of the micro
283 nutrients (Table. 2). On the other hand, two replicates of control assays that were supplemented
284 with high dosage of micro nutrients (to verify the high methane yield obtained) showed only a
285 small (5%) increase in methane yield over a period of 24 days. This small increase in methane yield
286 was attributed to the fact that, by day 60, easily degradable fraction of the substrate in these
287 assays was already converted to methane and the hydrolysis of less degradable and/or recalcitrant

fraction would have been limited. BMP assays tested with single micro-nutrient additions of Co and Fe showed about 15% increase in methane yield when compared to control while addition of Ni and Mo resulted in a 10% increase in methane yield (Table 3). On the other hand, when combinations of micro-nutrients were tested (Table 3) to know which missing nutrient significantly affects methane yields, it was observed that, when Fe and Mo were missing there was a 10% increase in methane yield whereas when Co and Ni were missing, there was only a 6% increase in methane yield compared to control. Overall, in the current study, the addition of Fe was found to be promising in enhancing methane production in the assays than Co and Ni during the methanogenic process (31). Furthermore, these assays showed that there is a positive, synergistic effect when all the micro-nutrients (Fe, Ni, Co and Mo) are added together and that they contribute to an enhanced methanogenic activity (Table 3).

3.3. LBR experiments

The effect of NH_4Cl and micro-nutrients addition on hydrolysis of grass silage was studied in LBRs for a period of 57 and 86 days, respectively. Process performance of LBRs is shown in Fig. 3-5.

3.3.1. pH and SCOD production

The pH and SCOD production trends of LBRs with NH_4Cl and micro-nutrients are presented in Fig. 3 and 4 respectively. In all the LBRs, pH dropped on day 2 (3.9-4.9) and remained in the range of 4-5 till the end of the experiments. Overall, an optimal pH of 4-5 for the

hydrolysis/acidogenesis was prevailing in the LBRs [32]. After day 45, pH in control LBR (L0) started to drop (pH ≤ 4.5) compared to the LBR with NH_4Cl (L1), which remained at 4.7-5.0. This small difference in pH between the two LBRs could be due to the ammonium buffering in the latter LBR. However, ammonium buffering in the LBR supplied with NH_4Cl was not enough to raise the pH to > 5 even after 45 days. Hydrolysis of food waste was studied in LBRs and was reported to show a similar pH drop to ≤ 5 despite buffering the leachate before subjecting to recirculation in the LBR [33]. SCOD production was rapid during the first three days in all four LBRs and then stabilized thereafter. SCOD production in the LBR with NH_4Cl supplementation (L1) was consistently higher than its control LBR (L0) till day 45 but remained more or less the same thereafter. On the other hand, SCOD production in LBRs supplemented with micro nutrients was similar to control either before or after leachate replacement on day 42.

3.3.2. Specific SCOD and NH_4-N production

Specific SCOD production in the LBR with NH_4Cl supplementation was about 18% higher (0.56 g SCOD g^{-1} VS, Fig. 3) than that obtained in control LBR (0.46 g SCOD g^{-1} VS). This increase in specific SCOD production was attributed to the increased microbial growth and activity due to the presence of additional nitrogen source, i.e., NH_4Cl [34]. Such enhanced microbial activity in the LBR resulted in an increased enzyme production (hydrolases) thus promoting higher polymer hydrolysis. Nitrogen is one of the macro-nutrients required for the anaerobic bacterial cell growth and specifically, when degrading ligno-cellulosic substrates such as grass silage. The demand for nitrogen grows higher than usual since these substrates have lower nitrogen contents. Nitrogen supplementation during the AD process is generally practiced when the crops digested are either

deficient in nitrogen or if the crops are highly acidic mainly to buffer the process and thus enhance the biogas production rates. For example, the effects of external addition of nitrogen in the form of NH_4HCO_3 or NH_4Cl during the AD of sugar beet silage and fodder beet silage in different types of reactor systems were studied and were reported to improve VS degradation rates and up to 30 % increase in biogas production rates [35, 36].

Specific SCOD production obtained in LBR supplemented with micro-nutrients (L4) was 7% higher (0.46 g SCOD g^{-1} VS) than control LBR (0.42 g SCOD g^{-1} VS) (Fig. 4). However, it is difficult to attribute this small increase in specific SCOD production to either micro-nutrients addition or leachate replacement (day 42). Because, the specific SCOD production before leachate replacement was similar in both LBRs (0.3 g SCOD g^{-1} VS) but increased to 0.42 g SCOD g^{-1} VS in control LBR and to 0.46 g SCOD g^{-1} VS in LBR with micro-nutrients after leachate replacement. Similar improvement in specific SCOD production after leachate replacement was also reported earlier [32]. Higher specific SCOD production obtained from grass silage in set I LBR experiments (NH_4Cl addition) than in set II LBR experiments (tested for micronutrients addition) could be attributed to the difference in the chemical nature of grass silage used and also due to the solid-liquid ratio applied in both sets of experiments.

3.3.3. Micronutrients dynamics in the LBR experiments

The results of micro-nutrient dynamics in the leachates (liquid fraction) with an initial and final concentrations in the grass silage (solid fraction) are given in Fig. 5 and Table 4, respectively. Eighty percent of the externally added Fe and Co concentration were found to be bioaccumulated (immobilised) in the solid fraction (grass silage) compared to Ni and Mo (72 and 53%,

353 respectively). These results are in accord with the previous results in literature confirming the
354 importance of Fe and Co during the AD process [22, 36]. The remaining micro-nutrients
355 concentration (20-46%) was bioavailable and found in the liquid fraction (leachates). The micro-
356 nutrients accumulation in the solid fraction was further evident by the high concentrations found
357 in the grass silage at the end of the LBR experiments (Table. 4). This could be attributed to multiple
358 reasons such as metals binding to the accumulated soluble microbial products due to the absence
359 of methanogenic activity [37, 38]. The absence of methanogenesis contributed to an imbalance in
360 the process further causing low pH, accumulation of solubilized or intermediary products (SCOD)
361 and accumulation of VFAs as found in previous LBR studies [21].

362 Accumulation of intermediary products caused saturation of leachate thus inhibiting
363 further hydrolysis and metal (micro-nutrients) mobilization into leachate despite low pH
364 conditions prevailing in the LBRs [39]. At low pH conditions, bioavailability of metals depends on
365 complex interactions between solid fraction and liquid fraction in anaerobic reactors and the
366 uptake of metals by microbes proceeds mainly by the transport of free metal ions across the cell
367 membranes [40]. These metals have to encounter complex biochemical processes in the leachate
368 such as precipitation and the formation of organic and inorganic complexes before actually
369 reaching the biomass. Therefore, accumulation of soluble intermediary products in the leachates
370 might have reduced the free metal ion concentrations available to the microbes affecting the
371 metal uptake in the LBRs.

372 Furthermore, carbohydrate and protein components of extracellular polymeric substances
373 (EPS) were also reported to sorb dissolved micro metals from aqueous media in biotechnological
374 applications suggesting greater ability of these groups to metal binding [41]. Such metal

accumulation in metal supplemented reactors and metal leaching in metal depleted reactors (Table. 4) was previously reported while studying the effect of absence of micro metals during conversion of a mixture of VFAs by distillery granular sludge in up flow anaerobic sludge blanket (UASB) reactors [42]. In the present study, total metal concentrations in grass silage and total and soluble metal concentrations in leachates were analyzed. This data is still insufficient to determine how and what percentage of the analyzed metal concentrations were actually bioavailable to the microbes since bioavailability of the metals is more accurately understood by carrying out more complex "metal speciation" studies such as organic or inorganic, free ion or chelated forms analyses [43].

3.3.4. Solids destruction

The results of solids destruction as TS and VS (%) are given in Table 3. Results showed that LBR with NH_4Cl supplementation had about 7% higher solids degradation than in control LBR. On the other hand, LBR with micro-nutrients mixture showed almost the same degradation efficiency as its control. However, the higher TS and VS (60%) degradation obtained in the LBR supplemented with micro-nutrients was attributed mainly to the leachate replacement (day 42). A similar observation of improved VS removal after leachate replacement (on day 11) was reported previously [43]. The author in the above study explained that the leachate replacement had diluted the inhibitory products of hydrolysis and acidogenesis thus enabling further hydrolysis and further VS reduction.

3.3.5. VFA and biogas production

VFA production profiles are shown in Fig. 3 and 4. VFA production in LBR with NH_4Cl addition started immediately from day 1 and peaked to a maximum value of 8 gL^{-1} by day 23. The corresponding maximum value in control LBR was 4.8 gL^{-1}. Acetic and butyric acids accounted for 80% of the total volatile fatty acid (TVFA) concentration in both LBRs. Biogas production in these two LBRs was noticed mainly during the first 20 days and biogas composition mainly contained 10-47% of CO_2, ≤ 15% of H_2 and ≤ 15% CH_4. However, biogas production decreased after day 20. On the other hand, the maximum VFA concentration in the LBR with micro-nutrients supplementation was 9.4 gL^{-1} (day 13). The corresponding maximum value in control LBR was 7 gL^{-1} (day 23). However, leachate replacement on day 42 resulted in an increase in VFA production to reach a maximum concentration of 8.8 gL^{-1} by day 43. Thereafter, VFA concentration remained unchanged. The decrease in VFA concentration between day 1 and 6 was attributed to the conversion of VFA to methane. This was evident by the sharp increase in biogas production during these days with concentration of 45-76% of CO_2 and up to 45% of H_2 in both LBRs. The high VFA production pattern clearly confirms the absence of methanogenic activity in the process and the imbalance in the production and consumption of the VFAs in the LBRs. Similar observation was also reported by Jagadabhi et al., 2010 [38]. It was earlier reported that VFA production as high as 9 gL^{-1} with pH of 4-5 in the LBRs could result in the reduction and inhibition of hydrolysis [43]. Accumulation of inhibitory products from polymer hydrolysis in the leachate and micro-nutrients in the solid fraction (grass silage) was considered as the reason for further reduction/inhibition in hydrolysis. Furthermore, low pH inhibited methanogenic activity causing VFA accumulation and thus resulted in extremely low biogas production from the LBRs.

418

419 *3.3.6. Residual methane potential assays*

420 The results of residual methane potential assays carried out at the end of LBR experiments
421 are shown in Tables 3 and 5. For set I experiments, adjusting the pH of the reactor materials
422 resulted in higher methane yields from control LBR (0.31±0.04 0.05 m^3 CH_4 kg^{-1}VS) than LBR
423 supplemented with NH_4Cl (0.25±0.16 m^3 CH_4 kg^{-1}VS) (Table 5). On the contrary, higher residual
424 methane yields were obtained from the pH adjusted leachate collected from the LBR
425 supplemented with NH_4Cl (0.36±0.14 m^3 CH_4 kg^{-1}VS) compared to its control LBR material (0.33±
426 0.1 m^3 CH_4 kg^{-1}VS). However, no significant difference (*P > 0.05*) was noticed between these two
427 results indicating discrepancies among the assays. Both these results reflect the hydrolysis and
428 specific SCOD production in these LBRs. For instance, control LBR with lower SCOD solubilization
429 rate resulted in higher residual methane potential from the solids fraction and lower residual
430 methane potential from the liquid fraction (leachate). The opposite was true for LBR with NH_4Cl
431 addition. On the other hand, pH adjustment did not contribute to any increase in methane yield
432 from mixed material and leachate indicating that inoculum plays a crucial role as a nutrient source
433 as well as buffering agent for the process. Previous studies also reported that pH adjustment
434 resulted in inhibition of hydrolysis/acidification and methanogenesis while operating grass silage
435 in LBRs [21].

436 The results of residual methane potential of reactor material (in set II) with single and
437 multiple micro-nutrient additions are presented in Table 3. Results showed that there was no
438 significant difference (*P > 0.05*) in residual methane potentials among the tested single micro-
439 nutrient additions or their combinations. The probable reason for this discrepancy could be due to

the fact that the substrate used in these assays was taken from the control BMP assay on day 60 when the assays were about to be terminated. Thus, by day 60, most of the easily degradable fraction of the substrate in these assays was already converted to methane and the hydrolysis of less degradable and/or recalcitrant fraction would have been limited despite addition of external micro nutrients. These assays would have probably yielded meaningful results if fresh grass silage was used.

4 Conclusions

The present study showed that addition of NH_4Cl and micro-nutrients during anaerobic digestion grass silage in the LBRs (Fe, Ni, Co and Mo) had improved specific soluble COD (18% and 7% respectively) and VFA production (40% and 22% respectively). About 50-80% of the externally added micro-nutrients showed accumulation in the grass silage and about 20-50% were bioavailable in the leachates (in terms of soluble metal concentration analyzed). External addition of macro- and micro-nutrients under conditions of low inoculum supply could clearly aid in additional COD solubilization and VFA production rates from the LBRs. If LBRs could be connected to high-rate reactors such as UASBs higher methane yields could be obtained, although cost-benefit analysis for obtaining inoculum versus the chemicals and the corresponding methane benefits need to be further evaluated.

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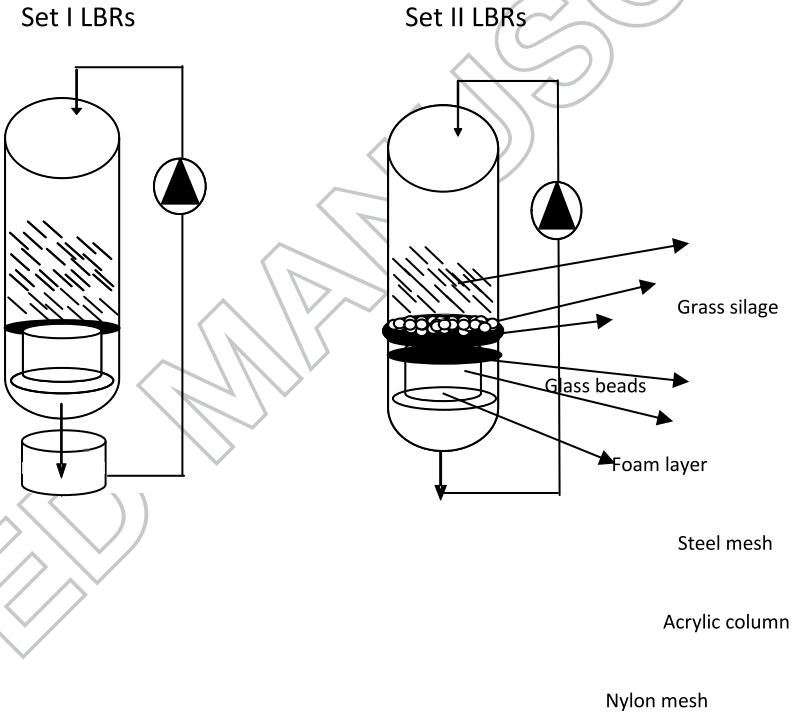
Figure 1: LBR set up (Set I and II experiments) for macro- and micro-nutrient supplementation (NH_4Cl).



Note:

Set I LBR experiments

- On day 0, LBRs (L0 and L1) were filled with 50g VS of grass silage + 3g VS of inoculum
- Reservoirs R0 and R1 filled with ~750 mL water
- R1 was added with 2.1gL^{-1} of macro-nutrient NH_4Cl

- Water was re-circulated at 750 mLd^{-1}
- Leachate re-circulation started immediately

Set II LBR experiments

- On day 0, LBRs (L2 and L3) were filled with 50gVS of grass silage + 3g VS of inoculum
- 470 mL of water was added into L2
- L3 was filled with 470 mL of micro-nutrient solution (with high dosage of micro nutrients as determined in BMP assays)
- No reservoirs for leachate collection and leachate collection system was modified with additional foam layer and glass for retaining microbial biomass
- Leachate was allowed to be in contact with biomass
- Leachate was re-circulated every 15 minutes (@470 mLd^{-1})

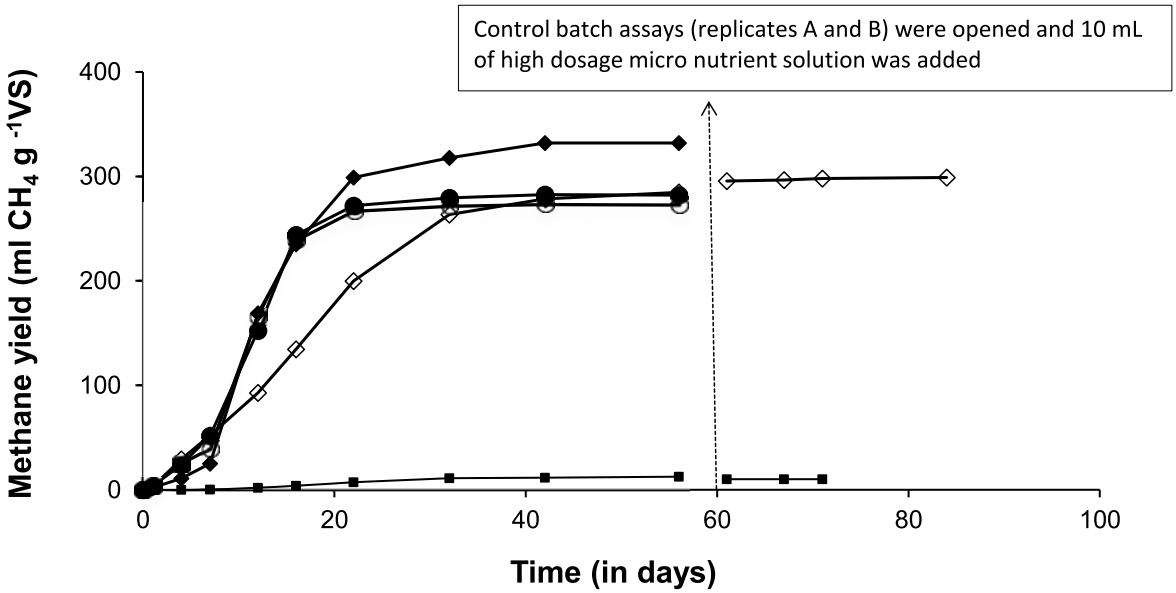


Figure 2. Methane yields from grass silage in BMP assays supplemented with micro nutrients and incubated at ±35 °C. ■ - Inoculum, ◇- Control, ●- Low dosage (in mg/L, Fe - 50, Ni - 0.1, Co - 0.2 and Mo - 0.15), ○ - Medium dosage (Fe - 75, Ni - 1.7, Co - 0.75, Mo - 0.5) and ◆ - High dosage (Fe - 375, Ni - 9, Co - 3.75, Mo – 0.75). Standard Deviation (SD) data is shown in Table 3.

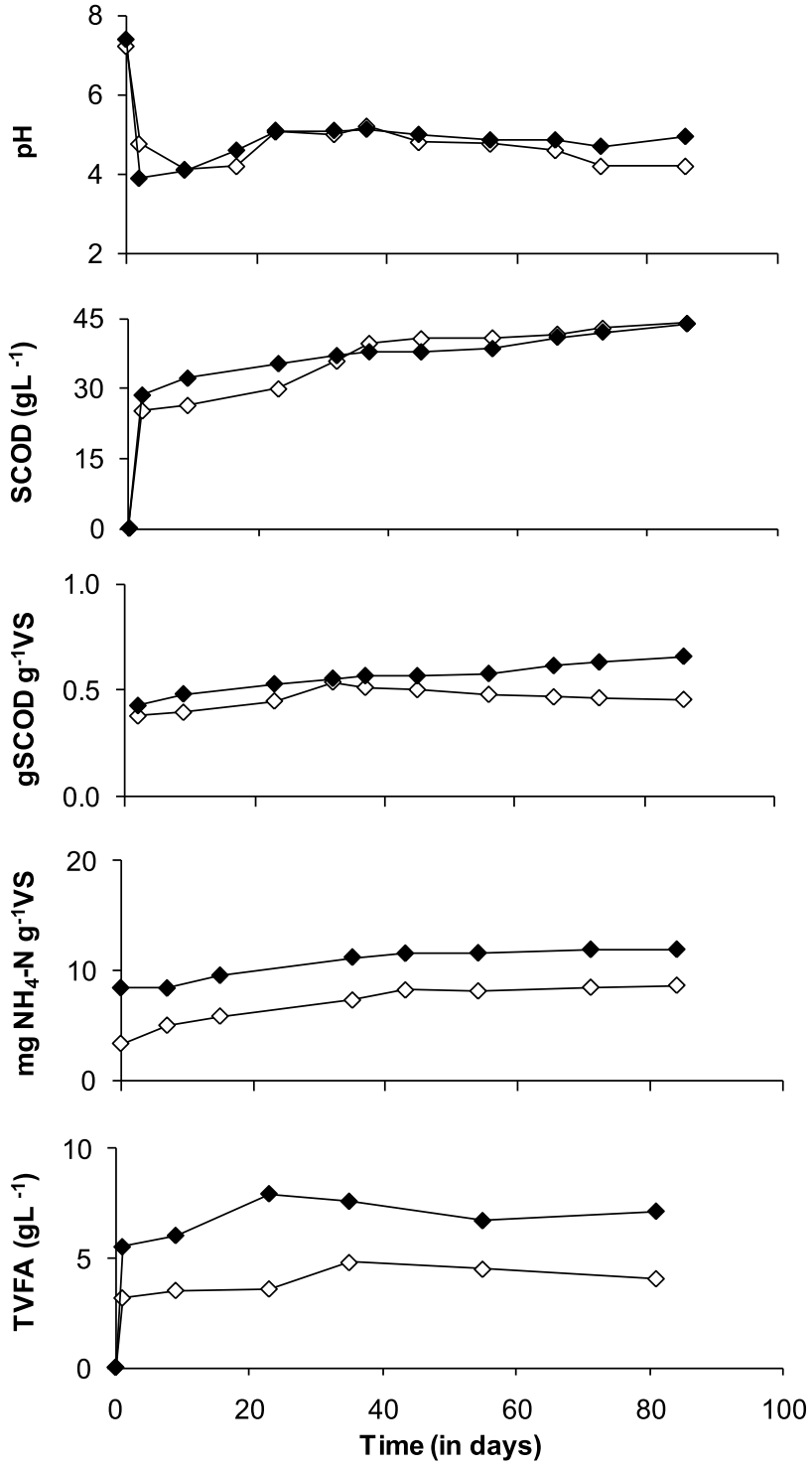


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Figure 3. Process performance during the mono-digestion of grass silage in LBR supplemented with NH_4Cl (set I experiments). ◇ - Control LBR, ◆ - LBR with NH_4Cl. Data shown here are "mean" values of replicate measurements.

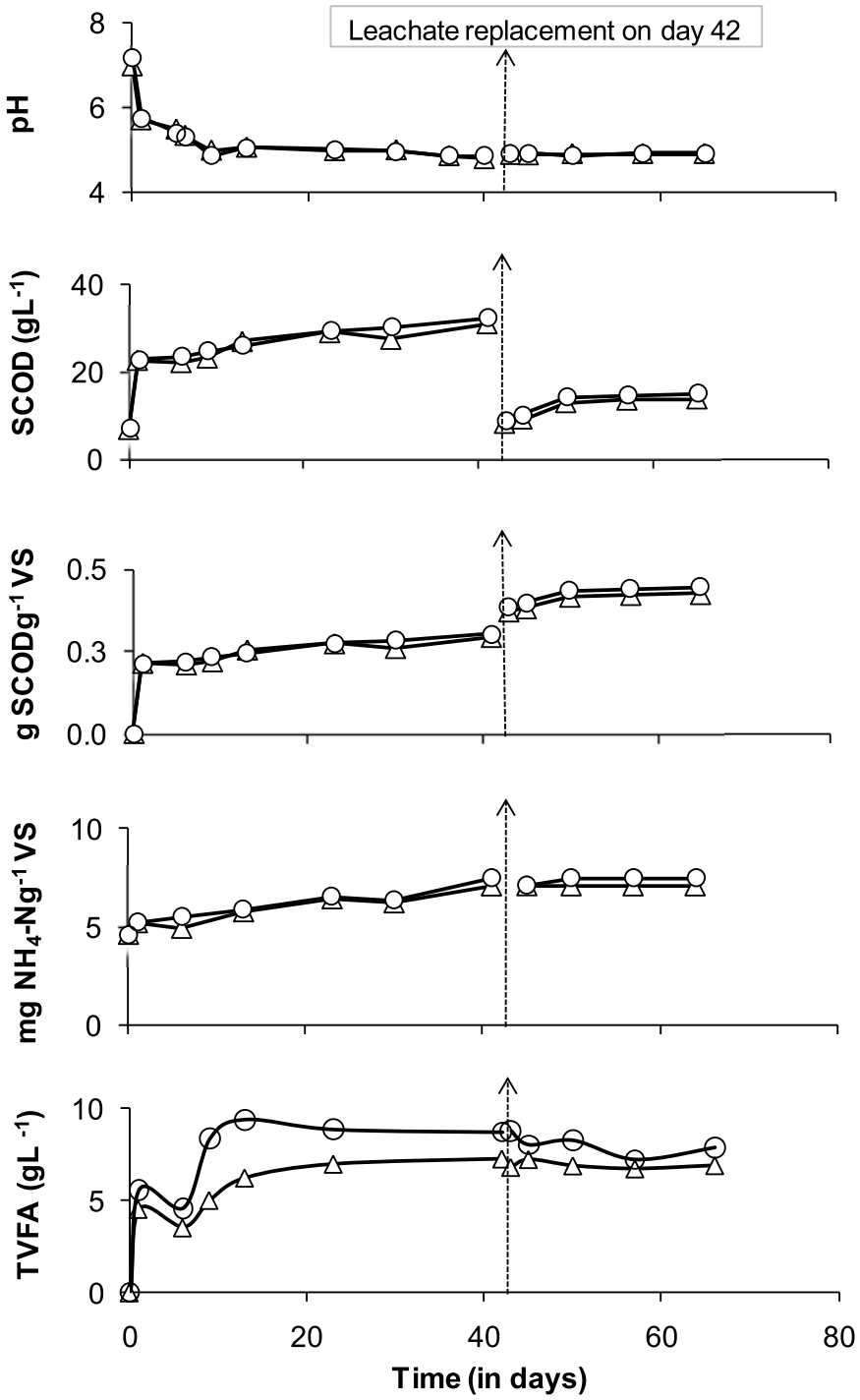

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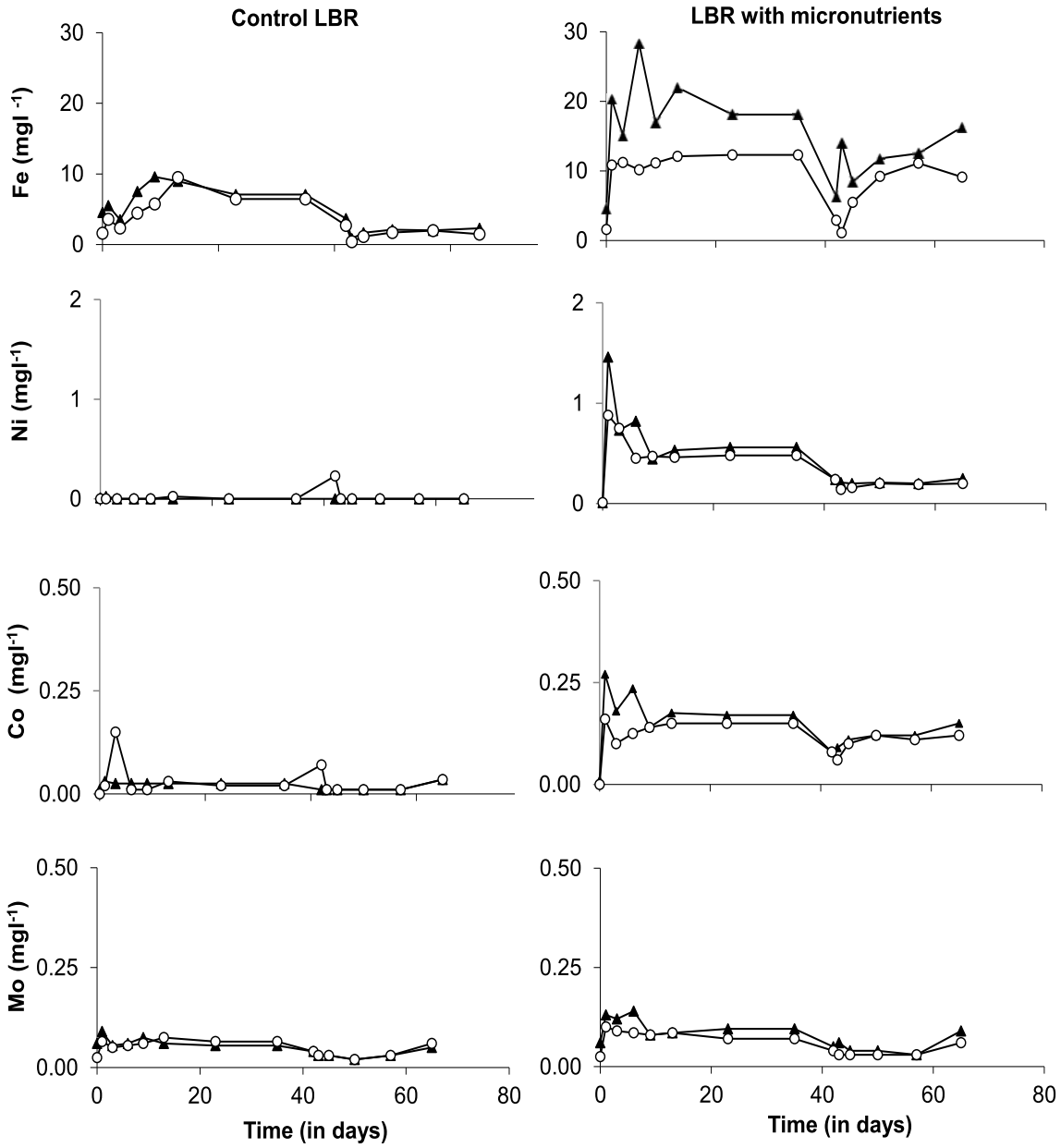
Figure 4. Process performance of LBRs supplemented with micro-nutrients (set II). Δ- Control LBR, ○- LBR with micro-nutrients. Data shown here are "mean" values of replicate measurements.



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633

634 **Figure 5.** Soluble (○) and total (▲) micro-nutrient concentrations in leachates of control
635 LBR and in LBR with micro-nutrients addition (set II). Data shown here are "mean"
636 values of replicate measurements.

637

638 44.

Table 1. Characteristics of the grass silage and inocula used in LBR studies for testing the effect of NH_4Cl (set 1) and micro-nutrients (Fe, Ni, Co and Mo; set II) supplementation during AD of grass silage. For set I and set II experiments, micro-nutrient analyses of grass silage and inoculum were performed 1 month after collection from the farm.

	Grass silage		Inoculum	
	Set I	Set II	Set I	Set II
pH	4.6	3.9	8.6	8.2
TS (%)	27	39	6.8	3.2
VS (%)	26	36	5.6	2.1
NH_4-N (mg g^{-1} TS)	0.3	0.2	7.3	5.0
Fe (mg kg^{-1}TS)	60 ±4	1160±280	NA	2300±105
Ni (mg kg^{-1}TS)	1.1± 0.1	6.7±1.7	NA	11.3±0.1
Co (mg kg^{-1}TS)	<1	2.2±1.4	NA	3.8±0.3
Mo (mg kg^{-1}TS)	5.5 ± 0.4	26.7±9.7	NA	32.1±1.1

*NA- Not analyzed

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652 **Table 2.** Micro-nutrient dosages supplemented in the BMP assays and LBR experiments
653 (set II).

654

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Micro-nutrient	Dosage level (mg L^{-1})*		
	Low	**Medium**	**High**
Fe	50	75	375
Ni	0.1	1.7	9
Co	0.2	0.75	3.75
Mo	0.15	0.5	0.75

656 *Concentrations in the medium

657

658

659 **Table 3.** Specific methane yields obtained from grass silage supplemented with
660 NH_4Cl (set I) and micro-nutrients (Fe, Ni, Co and Mo) (set II) against
661 respective controls (average ±SD, n = 3) during BMP assays.

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663

Substrate	**Methane yield (m^3 CH_4 kg^{-1} VS)**
Set I	
Grass silage (Control)	0.30±0.04
Grass silage + NH_4Cl	0.36±0.02
Set II	
Grass silage (Control)	0.28±0.01
Grass silage + low dosage of micro-nutrients	0.29±0.07
Grass silage + medium dosage of micro-nutrients	0.30±0.05
Grass silage + high dosage of Micro-nutrients	0.33 ±0.02
Single micro nutrient addition (from control BMP assays)	
Control	0.18±0.00
Cobalt (Co)	0.21±0.01

Iron (Fe)	0.21±0.01
Nickel (Ni)	0.20±0.00
Molybdenum (Mo)	0.20±0.01
Multiple micro-nutrient addition	
Fe + Ni + Co	0.20±0.01
Ni + Co + Mo	0.20±0.00
Fe + Ni + Mo	0.19±0.02
Fe + Co + Mo	0.19±0.02

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668 **Table 4.** Specific SCOD production, solids destruction, gas production and micro-nutrient
669 composition in control LBR and in the LBR with micro-nutrients (Fe, Ni, Co and Mo)
670 supplementation (set II).

671

	Set I		Set II	
	L0 (Control)	L1 (NH_4Cl addition)	L2 (Control)	L3 (micro nutrients addition)
Specific SCOD production (g SCOD g^{-1} VS)	0.46	0.56	0.42	0.45
TS removal (%)	38.4	41.5	60.8	60.8
VS removal (%)	38.3	41.2	59.8	60.1
NH_4-N (mg g^{-1} VS)	8.6	11.8	7.1	7.5
Hydrogen (mL H_2 g^{-1} VS)	NA	NA--	26	26
CO_2 (mL CO_2 g^{-1} VS)	-NA-	NA--	40	31

Nutrient composition of LBRs	L2 Start	L2 End	L3 End
Fe (mg kg^{-1} TS)	1500±120	2340±110	7300±300

Ni		5.1±1	9.1±1	207±6
(mg kg^{-1} TS)	NA			
Co		1.8±0.2	6.7±0.7	94±3
(mg kg^{-1} TS)				
Mo		18.8±2.4	46±3	212±6
(mg kg^{-1} TS)				

672 NA- Not analyzed

673 **Table 5.** Specific methane yields of residual materials from control LBR and LBR

674 with supplemented NH_4Cl upon termination of set I experiments.

675

Set I	**m^3 CH_4 $kg^{-1}VS$**	
Assays with inoculum addition	**Control LBR**	**LBR with NH_4Cl**
pH adjusted reactor material (solids -grass silage)	0.31±0.05	0.25±0.16
pH adjusted leachate	0.33± 0.10	0.36±0.14
Assays without inoculum addition		
Mixture of reactor material (grass silage) and leachate with pH adjustment	0.03±0.03	0.03±0.01
Mixture of reactor material and leachate without pH adjustment	0.03± 0	0.02±0.01

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677 45.