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

**Year:** 2019

**Version:** Accepted version (Final draft)

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**Please cite the original version:**

Jagadabhi, P. S., Kaparaju, P., Väisänen, A., & Rintala, J. (2019). Effect of macro- and micro-nutrients addition during anaerobic mono-digestion of grass silage in leach-bed reactors. *Environmental Technology*, 40(4), 418-429. <https://doi.org/10.1080/09593330.2017.1393462>

1 **Publisher:** Taylor & Francis & Informa UK Limited, trading as Taylor & Francis Group

2 **Journal:** *Environmental Technology*

3 **DOI:** 10.1080/09593330.2017.1393462



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

7  
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21  
22 **Abstract**

23  
24 The effect of macro- (NH<sub>4</sub>Cl) (set I) and micro-nutrients (Fe, Ni, Co and Mo) (set II) addition on  
25 chemical oxygen demand (COD) solubilization during anaerobic mono-digestion of grass silage was  
26 investigated in two sets of leach bed reactor (LBR) experiments at 35°C. Results showed that  
27 addition of NH<sub>4</sub>Cl and micro-nutrients improved COD solubilization by 18% (0.56 g SCOD g<sup>-1</sup>

28 volatile solids, VS) and 7% ( $0.45 \text{ gSCOD g}^{-1}\text{VS}$ ) respectively than control. About 20-50% of the  
29 added micro-nutrients were bioavailable in the produced leachates, while the rest (50-80%) were  
30 adsorbed on to the grass silage. Results of biological methane potential (BMP) assays showed that,  
31 specific methane yields of grass silage were improved by 17% ( $0.36 \pm 0.02 \text{ m}^3 \text{ CH}_4 \text{ kg}^{-1} \text{VS}_{\text{added}}$ ) when  
32  $\text{NH}_4\text{Cl}$  was supplemented while Fe, Ni, Co and Mo addition improved methane yields by 15%  
33 ( $0.33 \pm 0.005 \text{ m}^3 \text{ CH}_4 \text{ kg}^{-1} \text{VS}_{\text{added}}$ ) when compared to control.

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

## 36 **1 Introduction**

37  
38 Agricultural biogas production has been progressively gaining attention for meeting EU's  
39 renewable energy targets set under Renewable Energy Directive [1]. The produced biogas can be  
40 used for heat and/or electricity production, and thereby replace the depleting fossil fuels and the  
41 associated greenhouse gas emissions. Depending upon the feedstock characteristics, biogas  
42 process can be classified as wet or dry anaerobic digestion systems [2]. Dry anaerobic digestion  
43 (AD) technology is most suitable for drier ligno-cellulosic substrates such as grass silage, hay, reed  
44 canary grass, common reed because of their high solids contents (30-50% of total solids [TS]).  
45 When compared to wet digestion technology, dry AD technology is simple to operate and can  
46 tolerate high solids content (as much as 50%) while overcoming the problems of scum formation,  
47 choking the gas lines, energy demand for mixing equipment etc. [4]. Further, dry AD technology  
48 requires less energy inputs and water and/or pretreatment [5]. Particularly, dry batch reactors  
49 such as leach bed reactors (LBRs) when operated in combination with high rate reactors such as

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

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

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

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

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

90 Therefore, the present study was conducted to investigate the effects of supplementation  
91 of macro- (N in the form of  $\text{NH}_4\text{Cl}$ ) and micro-nutrients (Fe, Ni, Co and Mo) on microbial hydrolysis  
92 indicated by COD solubilisation and subsequent production of volatile fatty acids (VFAs) and their  
93 conversion to biogas. This was particularly studied under low inoculum conditions (only 6% of the

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

98

## 99 **2 Materials and methods**

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

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

109

### 110 **2.2. Biological methane potential (BMP) assays**

111 Methane potentials were determined in triplicate 1L glass bottles with a liquid volume of  
112 750 mL (Table 2). To each assay, 250 mL of inoculum and grass silage at a  $VS_{\text{substrate}}$  to  $VS_{\text{inoculum}}$   
113 ratio of 1 were added (Table 1). Grass silage with and without addition of  $NH_4Cl$  (set I) and micro-  
114 nutrients (Fe, Ni, Co and Mo) (set II) was studied. Three different dosages of micro nutrients were

115 tested viz., low (Fe-50, Ni-0.1, Co-0.2, Mo-0.15 mgL<sup>-1</sup>) medium (Fe-75, Ni-1.7, Co-0.75, Mo-0.5 mgL<sup>-1</sup>) and high (Fe-375, Ni-9, Co-3.75, Mo-0.75 mgL<sup>-1</sup>) were tested and the concentrations of each of  
116 the four micro-nutrients were based on the literature studies with substrates such as grass and  
117 maize silage [18, 22]. Assays with inoculum alone, and no micro or macro nutrients addition were  
118 used as controls. Distilled water was added to reach the liquid volume of 750 mL. NaHCO<sub>3</sub> (3 gL<sup>-1</sup>)  
119 was used as buffer. The contents of the bottles were then flushed with nitrogen gas (98.8%) for  
120 about 3 minutes before sealing with silicon stoppers. Prepared assays were statically incubated at  
121 35°C. The produced biogas was collected into aluminium gas bags. The methane produced from  
122 the control assays was subtracted from the sample (substrate) assays. The assays were manually  
123 shaken twice a day and before each gas composition analyses.

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

129

### 130 **2.3. LBR experiments**

131 Two sets of LBR experiments were carried out and referred to as set I and set II (Fig. 1). In  
132 set I (control L0 and L1), the effect of macro-nutrient (NH<sub>4</sub>Cl) supplementation as a nitrogen  
133 source was tested. On the other hand, the effect of micro-nutrients (Fe, Ni, Co and Mo)  
134 supplementation was tested in set II (control L2 and L3). LBR experiments were carried out in two,  
135 1 L acrylic plastic column reactors with a working volume of 750 mL. No replicates for reactors  
136 were used. In set I experiments, leachate was collected for recirculation in two separate 1 L glass

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

147 On day 0, all LBRs (both set I and set II) were loaded with 50g VS of grass silage and 3g VS  
148 of inoculum. Thereafter, reservoirs R0 and R1 in set I experiment were filled with 750 mL of  
149 distilled water and 2.1 gL<sup>-1</sup> of NH<sub>4</sub>Cl was added as a nitrogen source in R1. For set II experiments,  
150 control LBR L2 was filled with 470 mL of distilled water, while LBR L3 was loaded with 470 mL of  
151 the medium dosage level micro-nutrients solution (Fe, Ni, Co and Mo). The concentration of the  
152 micro-nutrient solution is given in Table 2. The medium dosage level (Fe-75, Ni-1.7, Co-0.75, Mo-  
153 0.5 mgL<sup>-1</sup>) was chosen based on the best results obtained in the batch experiment, which was  
154 started 20 days prior to LBR experiment. Leachate re-circulation was started immediately in set I  
155 run by using a peristaltic pump at a flow rate of 750 mLd<sup>-1</sup>. The corresponding leachate  
156 recirculation rate in set II run was 470 mL d<sup>-1</sup> and was operated at an interval of 15 minutes (Fig.  
157 1). Sampling was performed 3 times in the first week and twice a week in the following  
158 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  $\text{NH}_4\text{OH}$ . 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 solubilisation 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,  $\text{NH}_4\text{OH}$  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  $\text{N}_2/\text{CO}_2$  mixture ( $\text{N}_2$  -98.8%,  $\text{CO}_2$  - 1.2%) for 3 minutes before sealing with butyl

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

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

191

## 192 **2.5. Analyses and calculations**

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

196       pH was measured with a Mettler Toledo S20 Seveneasy pH meter. Total solids (TS) and VS  
197 were determined according to the standard methods [23]. COD was measured according to  
198 Finnish standards [24]. Soluble COD (SCOD) and  $\text{NH}_4\text{-N}$  from crop samples were analysed after  
199 extraction according to Finnish standards (SFS-EN 12457-4) and after filtration through GF 50 glass  
200 fibre filter papers (Schleicher & Schuell).  $\text{NH}_4\text{-N}$  and Total Kjeldahl Nitrogen (TKN) were analysed  
201 according to Tecator application note as described elsewhere [21].

202 Total and soluble metal concentrations of Fe, Ni, Co and Mo in leachates were analysed  
203 using inductively coupled plasma optical emission spectrometry (ICP-OES). Leachate samples were  
204 acidified with HNO<sub>3</sub> (pH < 2) and stored at -20°C until further analyses. Whilst, grass silage samples  
205 (solids) were oven dried at 105°C for 24 hours and then combusted in a muffle furnace (for 2 h) at  
206 550°C to obtain dry ash. The dry ash samples were then digested using the ultrasound-assisted  
207 digestion method. The digested sample solutions were analyzed for the metal concentrations  
208 according to [25] using Perkin-Elmer (Norwalk, CT, USA), model Optima 4300 DV ICP-OES using the  
209 default parameters of the instrument (nebuliser flow 0.6l m<sup>-1</sup>, plasma power of 1400W and  
210 auxiliary gas flow of 15l m<sup>-1</sup>).

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

220 Specific methane yields of BMP assays were calculated as cumulative methane (mL) per g  
221 substrate VS added and were expressed in m<sup>3</sup> CH<sub>4</sub> kg<sup>-1</sup> VS<sub>added</sub>. The methane production of the  
222 control assays was subtracted from the sample assays. Specific SCOD production (g SCOD g<sup>-1</sup> VS)

223 and specific  $\text{NH}_4\text{-N}$  ( $\text{mg NH}_4\text{-N g}^{-1}$  VS) in LBR experiments were calculated by considering the total  
224 leachate volume and the sample volume removed during the operation of LBRs.

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

229

### 230 **3 Results and Discussion**

#### 231 **3.1. Substrate characteristics**

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

241

#### 242 **3.2. BMP assays**

243 The effects of micro-nutrient (Fe, Ni, Co and Mo) and macro-nutrient (NH<sub>4</sub>Cl) addition on  
244 cumulative methane production and methane yield of grass silage are presented in Fig. 2 and  
245 Table 3. Methane yield from control assays was  $0.30 \pm 0.004 \text{ m}^3 \text{ CH}_4 \text{ kg}^{-1} \text{ VS}_{\text{added}}$ . Addition of NH<sub>4</sub>Cl  
246 improved the methane yield to  $0.36 \pm 0.02 \text{ m}^3 \text{ CH}_4 \text{ kg}^{-1} \text{ VS}_{\text{added}}$  and was also found to be statistically  
247 significant ( $P < 0.05$ ). Increase in methane yield could be mainly due to (i) an increased pH in the  
248 assays when compared to control and (ii) a better state of C/N balance achieved in the assays due  
249 to the supplementation of NH<sub>4</sub>Cl as a nitrogen source complementing high-carbon composition  
250 (grass silage). Also, additional N from NH<sub>4</sub>Cl, could have enhanced the growth of anaerobic  
251 microbial consortia, particularly, methanogens which could have contributed to improved  
252 methanogenesis [12]. The presence of sufficient methanogenic populations within the assays  
253 would ensure sufficient buffering capacity in the assays as the produced VFAs were quickly  
254 converted to methane and thereby enabled a stable AD process with improved methane yields  
255 [12].

256

257 Among the three tested micro-nutrient dosages, assays supplemented with the highest  
258 dosage resulted in a significant ( $P < 0.05$ ) increase in methane yields (from  $0.28 \pm 0.004$  to  
259  $0.33 \pm 0.02 \text{ m}^3 \text{ CH}_4 \text{ kg}^{-1} \text{ VS}$ ). Previous research efforts [12, 28] suggest that micro-nutrients function  
260 as cofactors of enzymes or coenzymes that are responsible for the growth of anaerobic bacteria  
261 and this could be the reason for enhanced biosynthesis of methane observed in the current study.  
262 Particularly, it was reported that Fe acts as cofactors of enzymes, cytochrome and ferredoxin in  
263 methylotrophic methanogens [28] in an electron transport chain of metabolism and thus enhances  
264 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

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

299

### 300 **3.3. LBR experiments**

301 The effect of  $\text{NH}_4\text{Cl}$  and micro-nutrients addition on hydrolysis of grass silage was studied  
302 in LBRs for a period of 57 and 86 days, respectively. Process performance of LBRs is shown in Fig.  
303 3-5.

304

#### 305 **3.3.1. pH and SCOD production**

306 The pH and SCOD production trends of LBRs with  $\text{NH}_4\text{Cl}$  and micro-nutrients are presented  
307 in Fig. 3 and 4 respectively. In all the LBRs, pH dropped on day 2 (3.9-4.9) and remained in the  
308 range of 4-5 till the end of the experiments. Overall, an optimal pH of 4-5 for the

309 hydrolysis/acidogenesis was prevailing in the LBRs [32]. After day 45, pH in control LBR (L0) started  
310 to drop ( $\text{pH} \leq 4.5$ ) compared to the LBR with  $\text{NH}_4\text{Cl}$  (L1), which remained at 4.7-5.0. This small  
311 difference in pH between the two LBRs could be due to the ammonium buffering in the latter LBR.  
312 However, ammonium buffering in the LBR supplied with  $\text{NH}_4\text{Cl}$  was not enough to raise the pH to >  
313 5 even after 45 days. Hydrolysis of food waste was studied in LBRs and was reported to show a  
314 similar pH drop to  $\leq 5$  despite buffering the leachate before subjecting to recirculation in the LBR  
315 [33]. SCOD production was rapid during the first three days in all four LBRs and then stabilized  
316 thereafter. SCOD production in the LBR with  $\text{NH}_4\text{Cl}$  supplementation (L1) was consistently higher  
317 than its control LBR (L0) till day 45 but remained more or less the same thereafter. On the other  
318 hand, SCOD production in LBRs supplemented with micro nutrients was similar to control either  
319 before or after leachate replacement on day 42.

320

### 321 *3.3.2. Specific SCOD and $\text{NH}_4\text{-N}$ production*

322 Specific SCOD production in the LBR with  $\text{NH}_4\text{Cl}$  supplementation was about 18% higher  
323 ( $0.56 \text{ g SCOD g}^{-1} \text{ VS}$ , Fig. 3) than that obtained in control LBR ( $0.46 \text{ g SCOD g}^{-1} \text{ VS}$ ). This increase in  
324 specific SCOD production was attributed to the increased microbial growth and activity due to the  
325 presence of additional nitrogen source, i.e.,  $\text{NH}_4\text{Cl}$  [34]. Such enhanced microbial activity in the  
326 LBR resulted in an increased enzyme production (hydrolases) thus promoting higher polymer  
327 hydrolysis. Nitrogen is one of the macro-nutrients required for the anaerobic bacterial cell growth  
328 and specifically, when degrading ligno-cellulosic substrates such as grass silage. The demand for  
329 nitrogen grows higher than usual since these substrates have lower nitrogen contents. Nitrogen  
330 supplementation during the AD process is generally practiced when the crops digested are either



331 deficient in nitrogen or if the crops are highly acidic mainly to buffer the process and thus enhance  
332 the biogas production rates. For example, the effects of external addition of nitrogen in the form  
333 of  $\text{NH}_4\text{HCO}_3$  or  $\text{NH}_4\text{Cl}$  during the AD of sugar beet silage and fodder beet silage in different types of  
334 reactor systems were studied and were reported to improve VS degradation rates and up to 30 %  
335 increase in biogas production rates [ 35, 36].

336 Specific SCOD production obtained in LBR supplemented with micro-nutrients (L4) was 7%  
337 higher ( $0.46 \text{ g SCOD g}^{-1} \text{ VS}$ ) than control LBR ( $0.42 \text{ g SCOD g}^{-1} \text{ VS}$ ) (Fig. 4). However, it is difficult to  
338 attribute this small increase in specific SCOD production to either micro-nutrients addition or  
339 leachate replacement (day 42). Because, the specific SCOD production before leachate  
340 replacement was similar in both LBRs ( $0.3 \text{ g SCOD g}^{-1} \text{ VS}$ ) but increased to  $0.42 \text{ g SCOD g}^{-1} \text{ VS}$  in  
341 control LBR and to  $0.46 \text{ g SCOD g}^{-1} \text{ VS}$  in LBR with micro-nutrients after leachate replacement.  
342 Similar improvement in specific SCOD production after leachate replacement was also reported  
343 earlier [32]. Higher specific SCOD production obtained from grass silage in set I LBR experiments  
344 ( $\text{NH}_4\text{Cl}$  addition) than in set II LBR experiments (tested for micronutrients addition) could be  
345 attributed to the difference in the chemical nature of grass silage used and also due to the solid-  
346 liquid ratio applied in both sets of experiments.

347

### 348 *3.3.3. Micronutrients dynamics in the LBR experiments*

349 The results of micro-nutrient dynamics in the leachates (liquid fraction) with an initial and  
350 final concentrations in the grass silage (solid fraction) are given in Fig. 5 and Table 4, respectively.  
351 Eighty percent of the externally added Fe and Co concentration were found to be bioaccumulated  
352 (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

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

384

#### 385 *3.3.4. Solids destruction*

386 The results of solids destruction as TS and VS (%) are given in Table 3. Results showed that  
387 LBR with  $\text{NH}_4\text{Cl}$  supplementation had about 7% higher solids degradation than in control LBR. On  
388 the other hand, LBR with micro-nutrients mixture showed almost the same degradation efficiency  
389 as its control. However, the higher TS and VS (60%) degradation obtained in the LBR supplemented  
390 with micro-nutrients was attributed mainly to the leachate replacement (day 42). A similar  
391 observation of improved VS removal after leachate replacement (on day 11) was reported  
392 previously [43]. The author in the above study explained that the leachate replacement had  
393 diluted the inhibitory products of hydrolysis and acidogenesis thus enabling further hydrolysis and  
394 further VS reduction.

395

### 396 3.3.5. VFA and biogas production

397 VFA production profiles are shown in Fig. 3 and 4. VFA production in LBR with  $\text{NH}_4\text{Cl}$   
398 addition started immediately from day 1 and peaked to a maximum value of  $8 \text{ gL}^{-1}$  by day 23. The  
399 corresponding maximum value in control LBR was  $4.8 \text{ gL}^{-1}$ . Acetic and butyric acids accounted for  
400 80% of the total volatile fatty acid (TVFA) concentration in both LBRs. Biogas production in these  
401 two LBRs was noticed mainly during the first 20 days and biogas composition mainly contained 10-  
402 47% of  $\text{CO}_2$ ,  $\leq 15\%$  of  $\text{H}_2$  and  $\leq 15\%$   $\text{CH}_4$ . However, biogas production decreased after day 20. On  
403 the other hand, the maximum VFA concentration in the LBR with micro-nutrients supplementation  
404 was  $9.4 \text{ gL}^{-1}$  (day 13). The corresponding maximum value in control LBR was  $7 \text{ gL}^{-1}$  (day 23).  
405 However, leachate replacement on day 42 resulted in an increase in VFA production to reach a  
406 maximum concentration of  $8.8 \text{ gL}^{-1}$  by day 43. Thereafter, VFA concentration remained unchanged.  
407 The decrease in VFA concentration between day 1 and 6 was attributed to the conversion of VFA  
408 to methane. This was evident by the sharp increase in biogas production during these days with  
409 concentration of 45-76% of  $\text{CO}_2$  and up to 45% of  $\text{H}_2$  in both LBRs. The high VFA production  
410 pattern clearly confirms the absence of methanogenic activity in the process and the imbalance in  
411 the production and consumption of the VFAs in the LBRs. Similar observation was also reported by  
412 Jagadabhi et al., 2010 [38]. It was earlier reported that VFA production as high as  $9 \text{ gL}^{-1}$  with pH of  
413 4-5 in the LBRs could result in the reduction and inhibition of hydrolysis [43]. Accumulation of  
414 inhibitory products from polymer hydrolysis in the leachate and micro-nutrients in the solid  
415 fraction (grass silage) was considered as the reason for further reduction/inhibition in hydrolysis.  
416 Furthermore, low pH inhibited methanogenic activity causing VFA accumulation and thus resulted  
417 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 ( $0.31 \pm 0.04$   $0.05 \text{ m}^3 \text{ CH}_4 \text{ kg}^{-1} \text{ VS}$ ) than LBR  
423 supplemented with  $\text{NH}_4\text{Cl}$  ( $0.25 \pm 0.16 \text{ m}^3 \text{ CH}_4 \text{ kg}^{-1} \text{ VS}$ ) (Table 5). On the contrary, higher residual  
424 methane yields were obtained from the pH adjusted leachate collected from the LBR  
425 supplemented with  $\text{NH}_4\text{Cl}$  ( $0.36 \pm 0.14 \text{ m}^3 \text{ CH}_4 \text{ kg}^{-1} \text{ VS}$ ) compared to its control LBR material ( $0.33 \pm$   
426  $0.1 \text{ m}^3 \text{ CH}_4 \text{ kg}^{-1} \text{ VS}$ ). 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  $\text{NH}_4\text{Cl}$   
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

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

446

#### 447 **4 Conclusions**

448 The present study showed that addition of  $\text{NH}_4\text{Cl}$  and micro-nutrients during anaerobic  
449 digestion grass silage in the LBRs (Fe, Ni, Co and Mo) had improved specific soluble COD (18% and  
450 7% respectively) and VFA production (40% and 22% respectively). About 50-80% of the externally  
451 added micro-nutrients showed accumulation in the grass silage and about 20-50% were  
452 bioavailable in the leachates (in terms of soluble metal concentration analyzed). External addition  
453 of macro- and micro-nutrients under conditions of low inoculum supply could clearly aid in  
454 additional COD solubilization and VFA production rates from the LBRs. If LBRs could be connected  
455 to high-rate reactors such as UASBs higher methane yields could be obtained, although cost-  
456 benefit analysis for obtaining inoculum versus the chemicals and the corresponding methane  
457 benefits need to be further evaluated.

458

#### 459 **Acknowledgements**

460 Finnish Graduate School in Environmental Science and Technology (EnSte) is gratefully  
461 acknowledged for funding Mrs. Padma Shanthi Jagadabhi Ph.D. studies. We gratefully  
462 acknowledge Mrs. Mervi Koistinen for technical assistance in laboratory analyses.

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565 solubilization and VFA production on during mono-digestion of grass-silage in one-stage leach  
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568

569 **Figure 1:** LBR set up (Set I and II experiments) for macro- and micro-nutrient  
570 supplementation ( $\text{NH}_4\text{Cl}$ ).

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**Note:**

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**Set I LBR experiments**

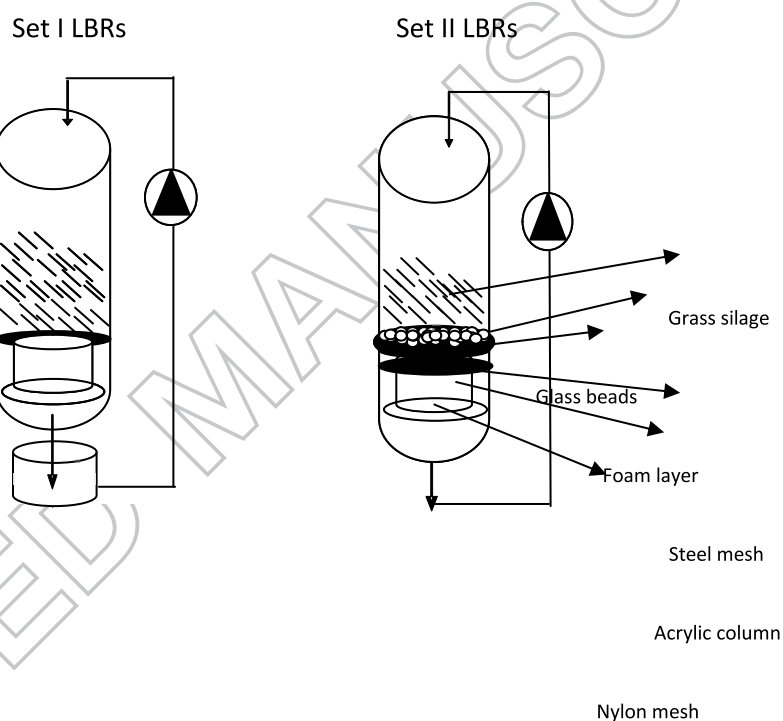
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- On day 0, LBRs (L0 and L1) were filled with 50g VS of grass silage + 3g VS of inoculum
- Reservoirs R0 and R1 filled with -750 mL water
- R1 was added with  $2.1\text{gL}^{-1}$  of macro-nutrient  $\text{NH}_4\text{Cl}$



590  
591  
592

- Water was re-circulated at 750 mLd<sup>-1</sup>
- Leachate re-circulation started immediately

593

### Set II LBR experiments

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595

- On day 0, LBRs (L2 and L3) were filled with 50gVS of grass silage + 3g VS of inoculum

596

- 470 mL of water was added into L2

597

- L3 was filled with 470 mL of micro-nutrient solution (with high dosage of micro nutrients as determined in BMP assays)

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- No reservoirs for leachate collection and leachate collection system was modified with additional foam layer and glass for retaining microbial biomass

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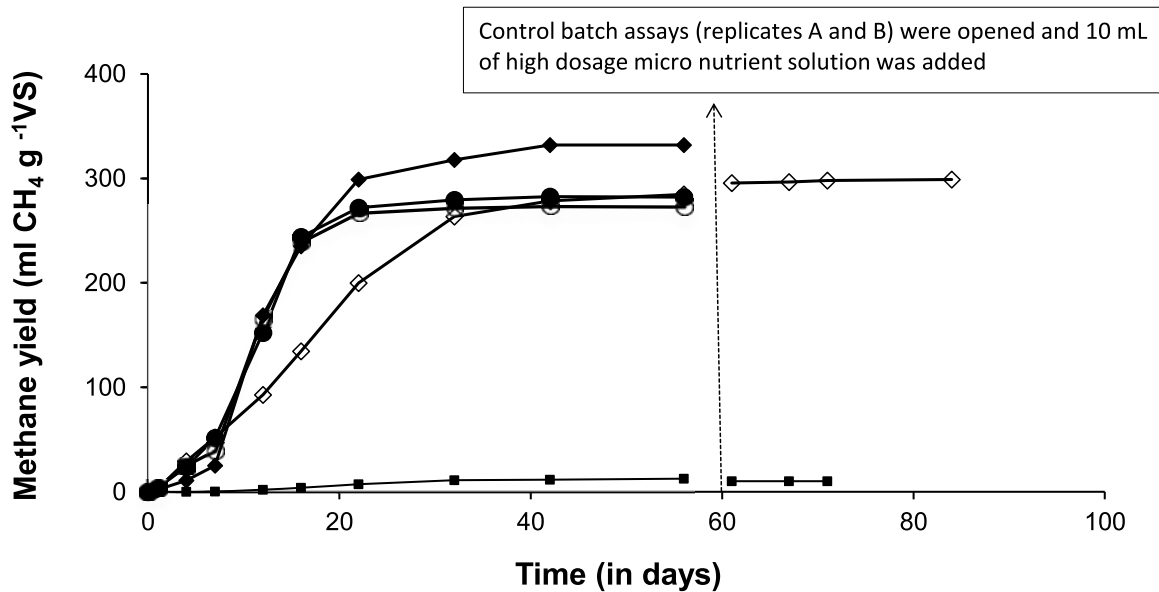
- Leachate was allowed to be in contact with biomass

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- Leachate was re-circulated every 15 minutes (@470 mLd<sup>-1</sup>)

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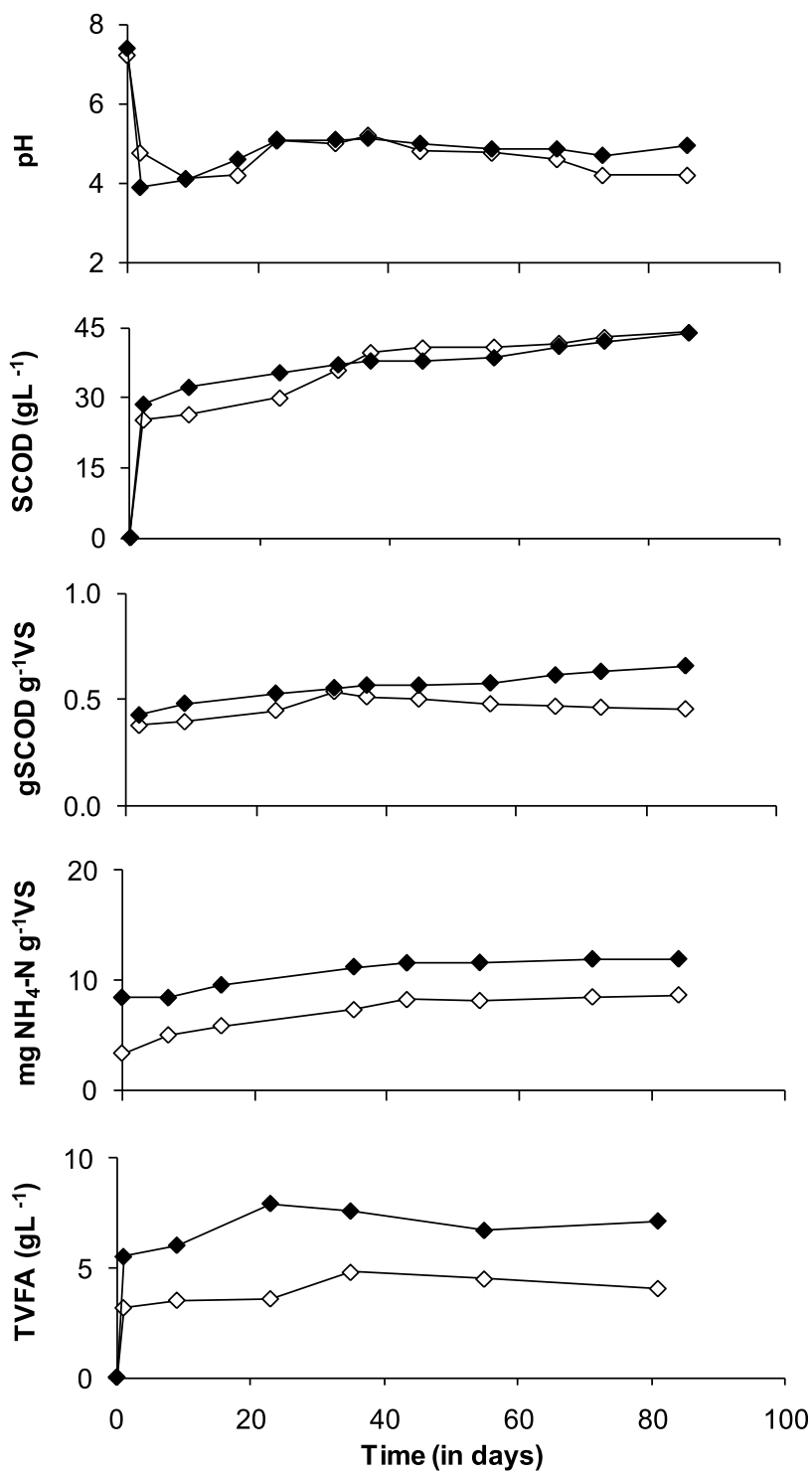
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610 **Figure 2.** Methane yields from grass silage in BMP assays supplemented with micro  
611 nutrients and incubated at  $\pm 35$  °C. ■ - Inoculum, ◇ - Control, ● - Low dosage (in  
612 mg/L, Fe - 50, Ni - 0.1, Co - 0.2 and Mo - 0.15), ○ - Medium dosage (Fe - 75, Ni -  
613 1.7, Co - 0.75, Mo - 0.5) and ◆ - High dosage (Fe - 375, Ni - 9, Co - 3.75, Mo -  
614 0.75). Standard Deviation (SD) data is shown in Table 3.

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SCRIPT

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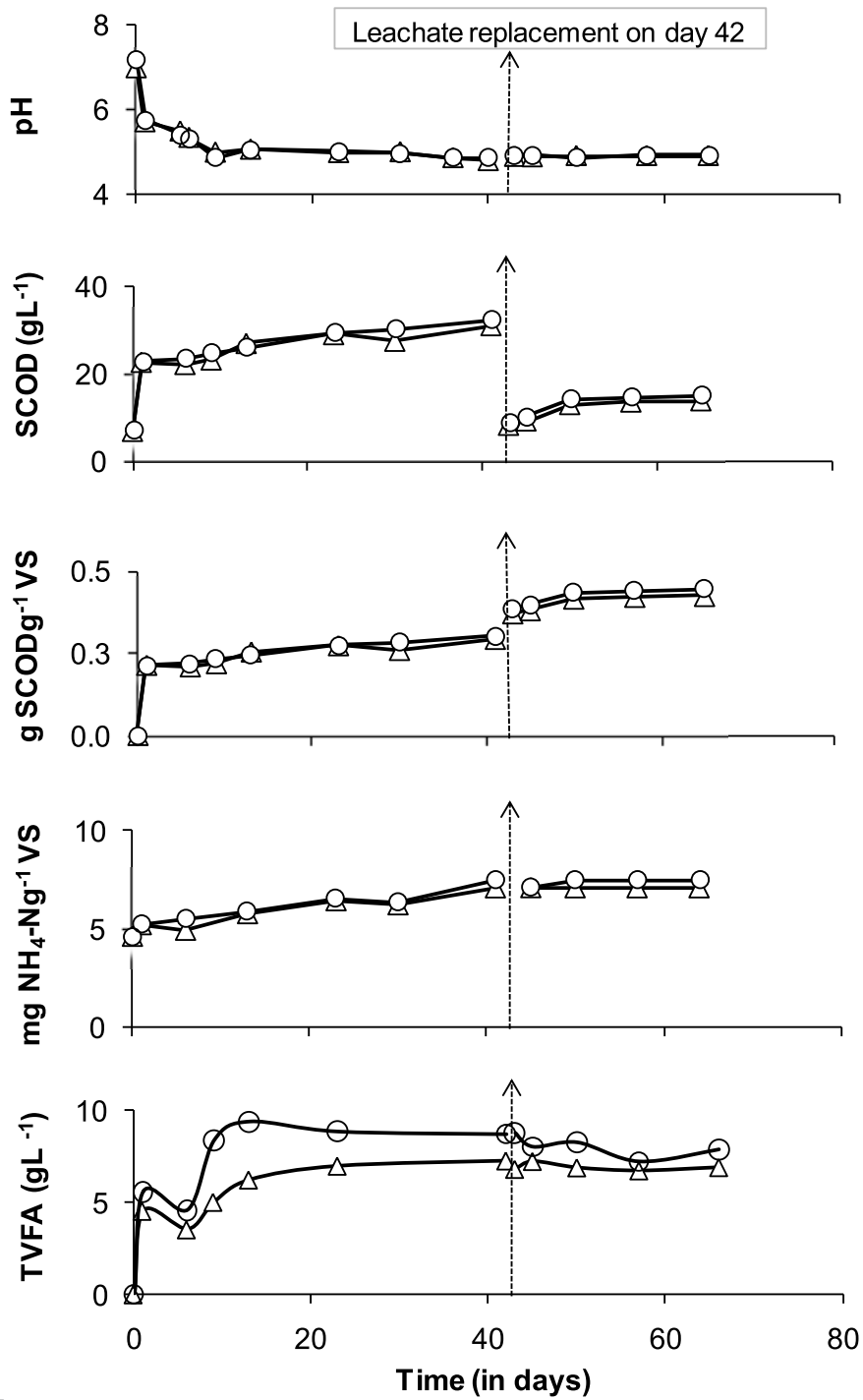
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618 **Figure 3.** Process performance during the mono-digestion of grass silage in LBR  
619 supplemented with  $\text{NH}_4\text{Cl}$  (set I experiments).  $\diamond$  - Control LBR,  $\blacklozenge$  - LBR with  
620  $\text{NH}_4\text{Cl}$ . Data shown here are “mean” values of replicate measurements.

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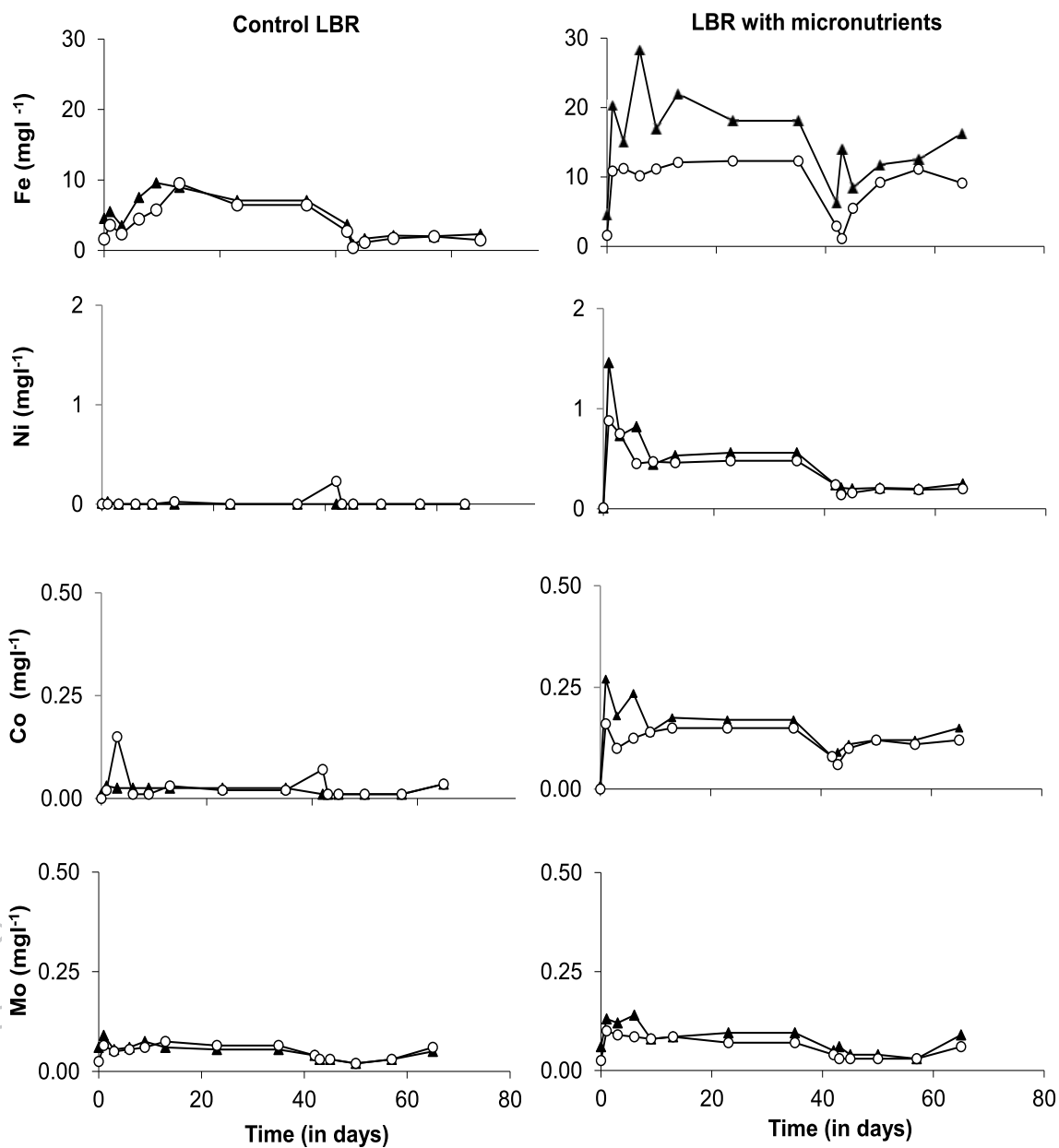
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624 **Figure 4.** Process performance of LBRs supplemented with micro-nutrients (set II).  $\Delta$ -  
 625 Control LBR,  $\circ$ - LBR with micro-nutrients. Data shown here are “mean” values of replicate  
 626 measurements.

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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.

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638 44.

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640

641 **Table 1.** Characteristics of the grass silage and inocula used in LBR studies for testing the  
 642 effect of  $\text{NH}_4\text{Cl}$  (set 1) and micro-nutrients (Fe, Ni, Co and Mo; set II)  
 643 supplementation during AD of grass silage. For set I and set II experiments, micro-  
 644 nutrient analyses of grass silage and inoculum were performed 1 month after  
 645 collection from the farm.

646

	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
$\text{NH}_4\text{-N}$ ( $\text{mg g}^{-1}\text{TS}$ )	0.3	0.2	7.3	5.0
Fe ( $\text{mg kg}^{-1}\text{TS}$ )	60 ± 4	1160 ± 280	NA	2300 ± 105
Ni ( $\text{mg kg}^{-1}\text{TS}$ )	1.1 ± 0.1	6.7 ± 1.7	NA	11.3 ± 0.1
Co ( $\text{mg kg}^{-1}\text{TS}$ )	<1	2.2 ± 1.4	NA	3.8 ± 0.3
Mo ( $\text{mg kg}^{-1}\text{TS}$ )	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).

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Micro-nutrient	Dosage level (mg L <sup>-1</sup> )*		
	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

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\*Concentrations in the medium

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**Table 3.** Specific methane yields obtained from grass silage supplemented with NH<sub>4</sub>Cl (set I) and micro-nutrients (Fe, Ni, Co and Mo) (set II) against respective controls (average  $\pm$ SD, n = 3) during BMP assays.

<b>Substrate</b>	<b>Methane yield (m<sup>3</sup> CH<sub>4</sub> kg<sup>-1</sup> VS)</b>
<b>Set I</b>	
Grass silage (Control)	0.30 $\pm$ 0.04
Grass silage + NH <sub>4</sub> Cl	0.36 $\pm$ 0.02
<b>Set II</b>	
Grass silage (Control)	0.28 $\pm$ 0.01
Grass silage + low dosage of micro-nutrients	0.29 $\pm$ 0.07
Grass silage + medium dosage of micro-nutrients	0.30 $\pm$ 0.05
Grass silage + high dosage of Micro-nutrients	0.33 $\pm$ 0.02
<b>Single micro nutrient addition (from control BMP assays)</b>	
Control	0.18 $\pm$ 0.00
Cobalt (Co)	0.21 $\pm$ 0.01

Iron (Fe)	0.21±0.01
Nickel (Ni)	0.20±0.00
Molybdenum (Mo)	0.20±0.01
<hr/>	
<b>Multiple micro-nutrient addition</b>	
<hr/>	
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).

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	Set I		Set II	
	L0	L1	L2	L3 (micro nutrients addition)
	(Control)	(NH <sub>4</sub> Cl addition)	(Control)	
Specific SCOD production (g SCOD g <sup>-1</sup> 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 <sub>4</sub> -N (mg g <sup>-1</sup> VS)	8.6	11.8	7.1	7.5
Hydrogen (mL H <sub>2</sub> g <sup>-1</sup> VS)	NA	NA--	26	26
CO <sub>2</sub> (mL CO <sub>2</sub> g <sup>-1</sup> VS)	-NA-	NA--	40	31
<b>Nutrient composition of LBRs</b>			L2	L2
			Start	End
				L3
				End
Fe (mg kg <sup>-1</sup> TS)			1500±120	2340±110
				7300±300

Ni		5.1±1	9.1±1	207±6
(mg kg <sup>-1</sup> TS)	NA			
Co		1.8±0.2	6.7±0.7	94±3
(mg kg <sup>-1</sup> TS)				
Mo		18.8±2.4	46±3	212±6
(mg kg <sup>-1</sup> TS)				

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672 NA- Not analyzed

673 **Table 5.** Specific methane yields of residual materials from control LBR and LBR  
 674 with supplemented  $\text{NH}_4\text{Cl}$  upon termination of set I experiments.

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<b>Set I</b>	<b><math>\text{m}^3 \text{CH}_4 \text{kg}^{-1}\text{VS}</math></b>	
<b>Assays with inoculum addition</b>	<b>Control LBR</b>	<b>LBR with <math>\text{NH}_4\text{Cl}</math></b>
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
<b>Assays without inoculum addition</b>		
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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