This is a self-archived version of an original article. This version may differ from the original in pagination and typographic details.

Author(s): Jagadabhi, Padma Shanthi; Kaparaju, Prasad; Väisänen, Ari; Rintala, Jukka

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)

Copyright:

Rights: In Copyright

Rights url: http://rightsstatements.org/page/InC/1.0/?language=en

Please cite the original version:

4

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

7

8   **Padma Shanthi Jagadabhi [a*1], Prasad Kaparaju[b*], Ari Väisänen[c], Jukka Rintala[d*]**

9

10   *Previous address: Department of Biological and Environmental Science, PO Box 35,*

11   *University of Jyväskylä, FI 40014, Finland*

12   *[a] Current address: International Crops Research Institute for Semi-Arid Tropics*

13   *(ICRISAT), Hyderabad, Telangana, India*

14   *[b] Current address: Griffith School of Engineering, Environment 1 Building, Griffith*
15   *University, Brisbane 4111, Australia*
16   *[c] Department of Chemistry, PO Box 35, University of Jyväskylä, FI 40014, Finland*
17   *[d] Current address: Department of Chemistry and Bioengineering, Tampere University of*
18   *Technology, PO Box 527, FI 33101, Tampere, Finland*
19

20   *[1]Corresponding Author: J.Shanthi@cgiar.org*

21

22   **Abstract**

23

24   The effect of macro- (NH_4Cl) (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_4Cl and micro-nutrients improved COD solubilization by 18% (0.56 g SCOD g^{-1}

28   volatile solids, VS) and 7% (0.45 gSCOD g^{-1}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±0.02 m^3 CH$_4$ kg^{-1}VS$_{added}$) when

32   NH$_4$Cl was supplemented while Fe, Ni, Co and Mo addition improved methane yields by 15%

33   (0.33±0.005 m^3 CH$_4$ kg^{-1}VS$_{added}$) when compared to control.

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

35

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

2

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

3

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 NH_4Cl) 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

4

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$_{substrate}$ to VS$_{inoculum}$

113   ratio of 1 were added (Table 1). Grass silage with and without addition of NH$_4$Cl (set I) and micro-

114   nutrients (Fe, Ni, Co and Mo) (set II) was studied. Three different dosages of micro nutrients were

5

115 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})

116 and high (Fe-375, Ni-9, Co-3.75, Mo-0.75 mgL^{-1}) were tested and the concentrations of each of

117 the four micro-nutrients were based on the literature studies with substrates such as grass and

118 maize silage [18, 22]. Assays with inoculum alone, and no micro or macro nutrients addition were

119 used as controls. Distilled water was added to reach the liquid volume of 750 mL. NaHCO$_3$ (3 gL^{-1})

120 was used as buffer. The contents of the bottles were then flushed with nitrogen gas (98.8%) for

121 about 3 minutes before sealing with silicon stoppers. Prepared assays were statically incubated at

122 35°C. The produced biogas was collected into aluminium gas bags. The methane produced from

123 the control assays was subtracted from the sample (substrate) assays. The assays were manually

124 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$_4$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

6

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^{-1} of NH_4Cl 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^{-1}) 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^{-1}. The corresponding leachate

156   recirculation rate in set II run was 470 mL d^{-1} 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 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

8

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 NH_4-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 & Schhuell). NH_4-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_3 (pH < 2) and stored at $-20^{o}C$ until further analyses. Whilst, grass silage samples

205   (solids) were oven dried at $105^{o}C$ for 24 hours and then combusted in a muffle furnace (for 2 h) at

206      $550^{o}C$ 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^{-1}, plasma power of 1400W and

210   auxiliary gas flow of 15l m^{-1}).

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™ 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^3 CH_4 kg^{-1} VS_{added}. The methane production of the

222   control assays was subtracted from the sample assays. Specific SCOD production (g SCOD g^{-1} VS)

223   and specific NH_4-N (mg NH_4-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***

11

243   The effects of micro-nutrient (Fe, Ni, Co and Mo) and macro-nutrient (NH$_4$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±0.004 m^3 CH$_4$ kg^{-1} VS$_{added}$. Addition of NH$_4$Cl

246 improved the methane yield to 0.36±0.02 m^3 CH$_4$ kg^{-1} VS$_{added}$ 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$_4$Cl as a nitrogen source complementing high-carbon composition

250 (grass silage). Also, additional N from NH$_4$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±0.004 to

259 0.33±0.02 m^3 CH$_4$ kg^{-1} 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

12

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

13

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 NH_4Cl 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 NH_4Cl 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 (pH ≤ 4.5) compared to the LBR with NH_4Cl (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 NH_4Cl 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 ≤ 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 NH_4Cl 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 NH_4-N production*

322   Specific SCOD production in the LBR with NH_4Cl supplementation was about 18% higher

323   (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

324   specific SCOD production was attributed to the increased microbial growth and activity due to the

325   presence of additional nitrogen source, i.e., NH_4Cl [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

15

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 NH_4HCO_3 or NH_4Cl 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 g SCOD g^{-1} VS) than control LBR (0.42 g SCOD g^{-1} 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 g SCOD g^{-1} VS) but increased to 0.42 g SCOD g^{-1} VS in

341 control LBR and to 0.46 g SCOD g^{-1} 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 (NH_4Cl 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%,

16

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

17

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 NH_4Cl 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

18

396   *3.3.5. VFA and biogas production*

397   VFA production profiles are shown in Fig. 3 and 4. VFA production in LBR with NH_4Cl

398   addition started immediately from day 1 and peaked to a maximum value of 8 gL^{-1} by day 23. The

399   corresponding maximum value in control LBR was 4.8 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 CO_2, ≤ 15% of H_2 and ≤ 15% 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 gL^{-1} (day 13). The corresponding maximum value in control LBR was 7 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 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 CO_2 and up to 45% of 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 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.

19

418

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 ($0.33\pm$

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

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

4 Conclusions

448   The present study showed that addition of NH_4Cl 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

21

463   **References**

464   1.   EC 2009. Directive 2009/28/EC of The European Parliament and of The Council of 23 April 2009

465   on the promotion of the use of energy from renewable sources and amending and

466   subsequently repealing Directives 2001/77/EC and 2003/30/EC. Official Journal of the

467   European Union.

468   2.   Weiland P. Biogas production: current state and perspectives. Appl Microbiol Biotechnol 2010;

469   85: 849-860.

470   3.   Saady NM, Massè DI. Impact of organic loading rate on the performance of psychrophilic dry

471   anaerobic digestion of dairy manure and wheat straw: Long term operation. Bioresour Technol

472   2015; 182: 50-57.

473   4.   Thamisiriroj T, Nizami AS, Murphy JD. Why does mono-digestion of grass silage fail in long-

474   term operation? Appl Energ 2012; 95: 64-76.

475   5.   Elsamadony M, Tawfik A, Suzuki M. Surfactant - enhanced biohydrogen production from

476   organic fraction of municipal solid waste (OFMSW) via dry anaerobic digestion. Appl Energ

477   2015; 149: 272-282.

478   6.   Cysneiros D, Banks CJ, Heaven S, Kimon-Andreas GK. The role of phase separation and feed

479   cycle length in leach beds couple to methanogenic reactors for digestion of a solid substrate

480   (Part I): Optimisation of reactors performance. Bioresour Technol. 2012; 103: 56-63

481 7. Nizami, AS, Murphy, JD. What type of digester configurations should be employed to produce

482   biomethane from grass silage? Renew Sust Energ Rev 2010. 1558-1568.

483 8. Massè I D, Saady NMC, Gilbert Y. Psychrophilic dry anaerobic digestion of cow feces and wheat

484   straw: Feasibility studies. Biomass and Bioenerg 2015; 77: 1-8.

485 9. Wall DM, O'Kiely P, Murphy JD. The potential for biomethane from grass and slurry to satisfy

486   renewable energy targets. Bioresour Technol 2013; 149: 425-431.

487 10. Schroeder V, Schalau B, Molnarne M. Explosion protection in biogas and hybrid power plants.

488   Procedia Eng. 2014; 84: 259-272.

489 11. Braun R, Weiland P, Wellinger, A. Biogas from Energy crop digestion. IEA Bioenergy, 2010. Task

490   37.   Web   pages

491   http://www.en.esbjerg.aau.dk/digitalAssets/80/80448_energycrop_def_low_res.pdf

492   (accessed on 24 July 2014).

493 12. Nges IA, Björn A, Björnsson L. Stable operation during pilot scale anaerobic digestion of

494   nutrient-supplemented maize/sugar beet silage. Bioresour Technol 2012; 118:445-454

495 13. Demirel B, Neumann L, Scherer P. Microbial community dynamics of a continuous mesophilic

496   anaerobic biogas digester fed with sugar beet silage. Eng Life Sci 2008a; 8: 390-398.

497 14. Schattauer A, Abdoun E, Weiland P, Plöchl M, Heiermann M. Abundance of micro elements in

498   demonstration biogas plants. Biosyst Eng 2011; 108: 57-65.

499 15. Ortner M, Rachbauer L, Somitsch W, Fuch W. Can bioavailability of trace nutrients be

500   measured in anaerobic digestion. Appl Energ 2015 ; 126:190-198.

23

501   16. Wall DM, Allen E, Straccialini B, O'Kiely P, Murphy JD. The effect of trace element addition to

502   mono-digestion of grass silage at high organic loading rates. Bioresour Technol 2014a; 172:

503   349–355.

504   17. Zandavoort MH, Van Hullebusch ED, Fernando G Fermoso, Lens PNL. Micro metals in

505   anaerobic granular sludge reactors: Bioavailability and dosing strategies. Eng Life Sci 2006; 6:

506   293-301.

507   18. Pobeheim H, Munk B, Johansson J, Guebitz GM. Influence of micro elements on methane

508   formation from a synthetic model substrate for maize silage. Bioresour Technol 2010; 101:

509   836-839.

510   19. Pobeheim H, Munk B, Lindorfer H, Guebitz GM. Impact of nickel and cobalt on biogas

511   production and process stability during semi-continuous anaerobic fermentation of model

512   substrate for maize silage. Wat Res 2011; 45: 781-787.

513   20. Zhang Y, Banks CJ, Heaven S. Co-digestion of source segregated domestic food waste to

514   improve process stability. Bioresour Technol 2012; 114: 168-178.

515   21. Lehtomäki A, Huttunen S, Lehtinen TM, Rintala JA. Anaerobic digestion of grass silage in batch

516   leach bed processes for methane production. Bioresour Technol 2008; 99: 3267-3278.

517   22. Jarvis A, Nordberg A, Jarlsvik T. Mathisen B. Svensson BH. Improvement of a grass-clover

518   silage-fed biogas process by the addition of cobalt. Biomass Bioenerg 1997; 12: 453-460.

519   23. APHA. Standard Methods for the Examination of Water and Wastewater, 20th ed. 1998,

520   American Public Health Association, Washington, DC.

521   24. SFS Standards. 1988. Determination of chemical oxygen demand (CODCr) in water with the

522   closed tube method, oxidation with dichromate. SFS 5504, Finnish Standards Association,

523   Helsinki, Finland.

524   25. Väisänen A, Laatikainen P, Ilander A, Renvall S. Determination of mineral and micro element

525   concentrations in pine needles by ICP-OES: evaluation of different sample pretreatment

526   methods. Intern J Environ Anal Chem 2008; 88: 1005-1016.

527   26. SPSS Inc., 1999. *SPSS version 7.0 for Windows ®,* 233 S. Wacker Drive, 11th floor, Chicago, IL

528   60606-6307, USA.

529   27. Nizami A-S, Korres NE, Murphy JD. Review of the integrated process for the production of

530   grass biomethane. Environ Sci Technol 2009; 43:8496-8508.

531   28. Nges IA, Wang B, Cui Z, Liu J. Digestate liquor recycle in minimal nutrients-supplemented

532   anaerobic digestion of wheat straw. Biochem. Engg 2015; 94:106-114.

533   29. Gustavsson J, Yekta SS, Sundberg C, Karlsson A, Ejlertsson J, Skyllberg U. Bioavailability of

534   cobalt and nickel during anaerobic digestion of sulfur rich stillage for biogas formation. Appl

535   Energ 2013; 112: 473-477.

536   30. Isa HM, Anderson GK. Molybdate inhibition of sulphate reduction in two-phase anaerobic

537   digestion. Process Biochem. 2005; 40: 2079-2089.

538   31. M.S. Romero-Güiza , J. Vila, J. Mata-Alvarez, J.M. Chimenos, S. Astals. The role of additives on

539   anaerobic digestion: A review. Renew. Sustainable Energy Rev. 2016: 58 1486-1499.

540   32. Chen Y, Cheng JJ, Creamer KS. Inhibition of anaerobic digestion process: A review. Bioresour.

541   Technol. 2008; 99: 4044-4064.

542   33. Xu SY, Lam HP, Parthiba Karthikeyan O, Wong JWC. Optimization of food waste hydrolysis in

543   leach bed coupled with methanogenic reactor: Effect of pH and bulking agent. Bioresour

544   Technol 2011; 102: 3702-3708.

545   34. Wagner AO, Hohlbrugger P, Linns P, Illmer P. Effects of different nitrogen sources on biogas

546   production. A lab-scale investigation. Microbiol Res 2012;167: 630-636.

547   35. Demirel B, Scherer P. Production of methane from sugar beet silage without manure addition

548   by a single stage anaerobic digestion process. Biomass Bioenerg 2008b; 32: 203 209.

549   36. Demirel B. Scherer P. Micro element requirements of agricultural biogas digesters during

550   biological conversion of renewable biomass to methane. Biomass Bioenerg 2011; 35: 992-998

551   37. Kuo WC, Parking GF. Characterization of soluble microbial products from anaerobic treatment

552   by molecular weight distribution and nickel chelating properties. Wat Res 1996; 30: 915–922.

553   38. Jagadabhi PS, Kaparaju P, Rintala J. Effect of micro-aeration and leachate replacement on COD

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

555   bed reactors. Bioresour. Technol. 2010; 101, 2818–2824. .

556   39. Selling R, Håkansson T, Björnsson L. Two stage anaerobic digestion enables heavy metal

557   removal. Wat Sci Technol 2008; 57: 553-558.

558   40. Gustavsson J. Cobalt and Nickel Bioavailability for Biogas Formation. Linköping Studies in Arts

559   and Science, Linköping University, 2012. Linköping, Sweden.

26

560   41. Flemming H-C. Sorption sites in biofilms. Wat Sci Technol 1995; 32: 27-33.

561   42. Osuna MB, Zandavoort MH, Iza JM, Lettinga G, Lens PNL. Effects of micro element addition on

562   volatile fatty acid conversions in anaerobic granular sludge reactors. Environ Technol 2003;

563   24:573-587.

564   43. Jagadabhi PS, Kaparaju P, Rintala J. Effect of micro-aeration and leachate replacement on COD

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

566   bed reactors. Bioresour Technol 2010; 101: 2818-2824.

567

568

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

571

572

573

574   Set I LBRs   Set II LBRs

575

576

577   Grass silage

578   Glass beads

579   Foam layer

580   Steel mesh

581   Acrylic column

582   Nylon mesh

583

584   **Note:**

585   **Set I LBR experiments**
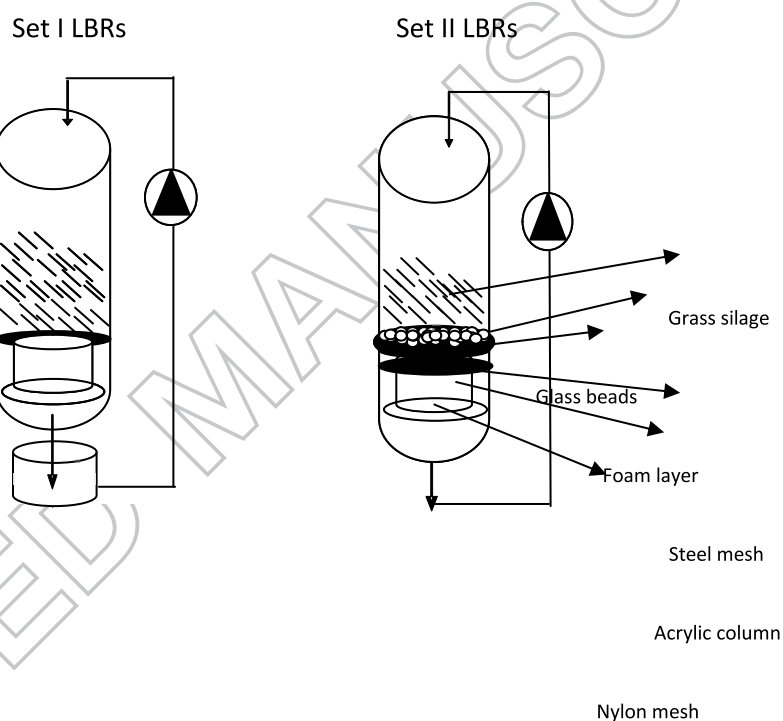
586   • On day 0, LBRs (L0 and L1) were filled with 50g VS of grass silage + 3g VS of
587   inoculum
588   • Reservoirs R0 and R1 filled with -750 mL water
589   • R1 was added with 2.1gL^{-1} of macro-nutrient NH$_4$Cl

28

590   •   Water was re-circulated at 750 mLd^{-1}

591   •   Leachate re-circulation started immediately

592

593   **Set II LBR experiments**

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

595   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

598   micro nutrients as determined in BMP assays)

599   ▪   No reservoirs for leachate collection and leachate collection system was

600   modified with additional foam layer and glass for retaining microbial

601   biomass

602   ▪   Leachate was allowed to be in contact with biomass

603   ▪   Leachate was re-circulated every 15 minutes (@470 mLd^{-1})
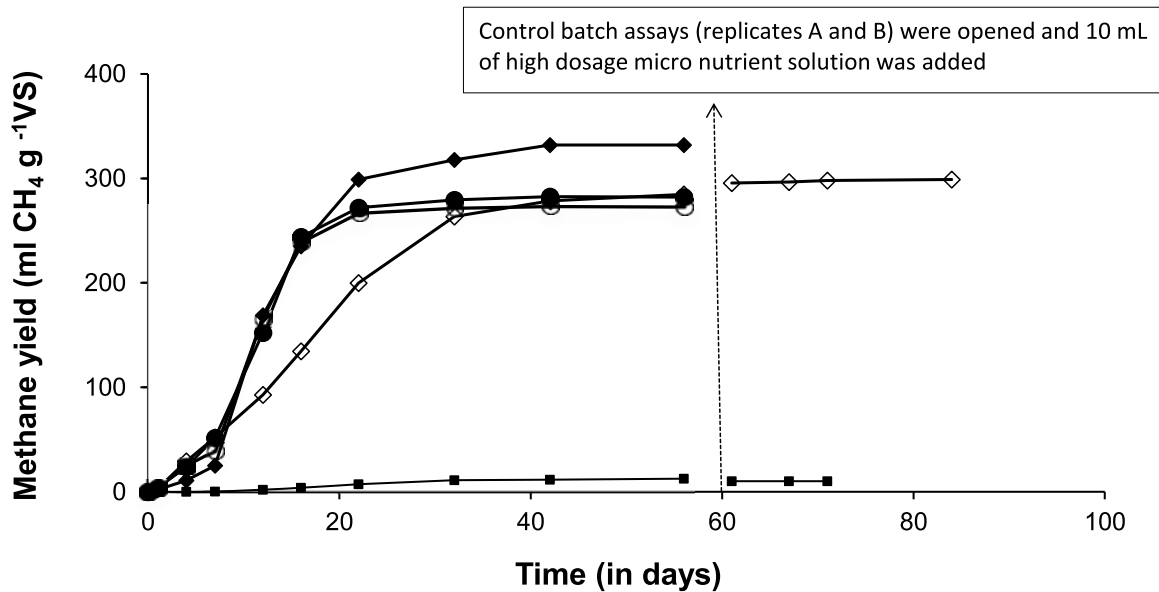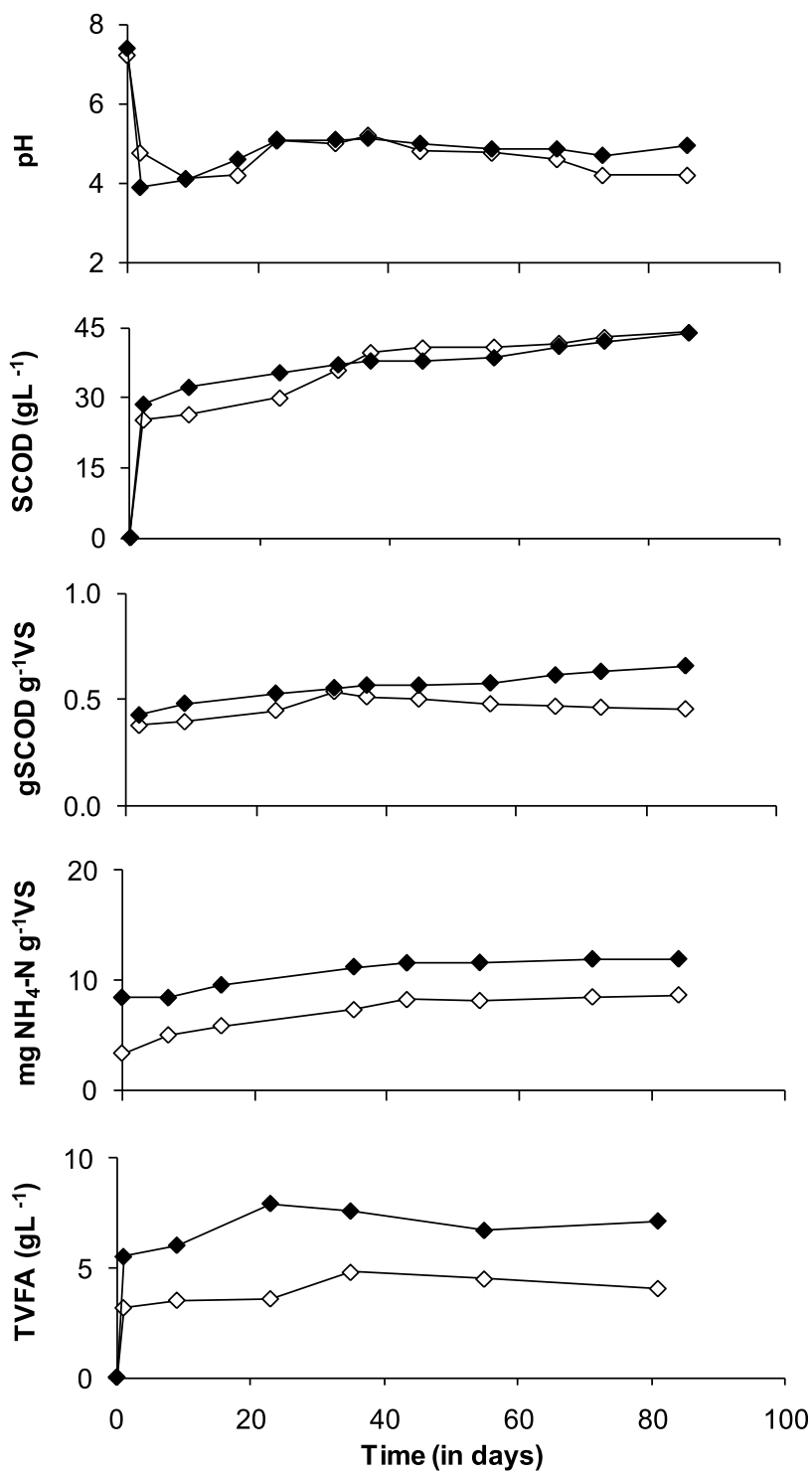
604

605

606

607

608

609

610   **Figure 2.**   Methane yields from grass silage in BMP assays supplemented with micro
611   nutrients and incubated at ±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.
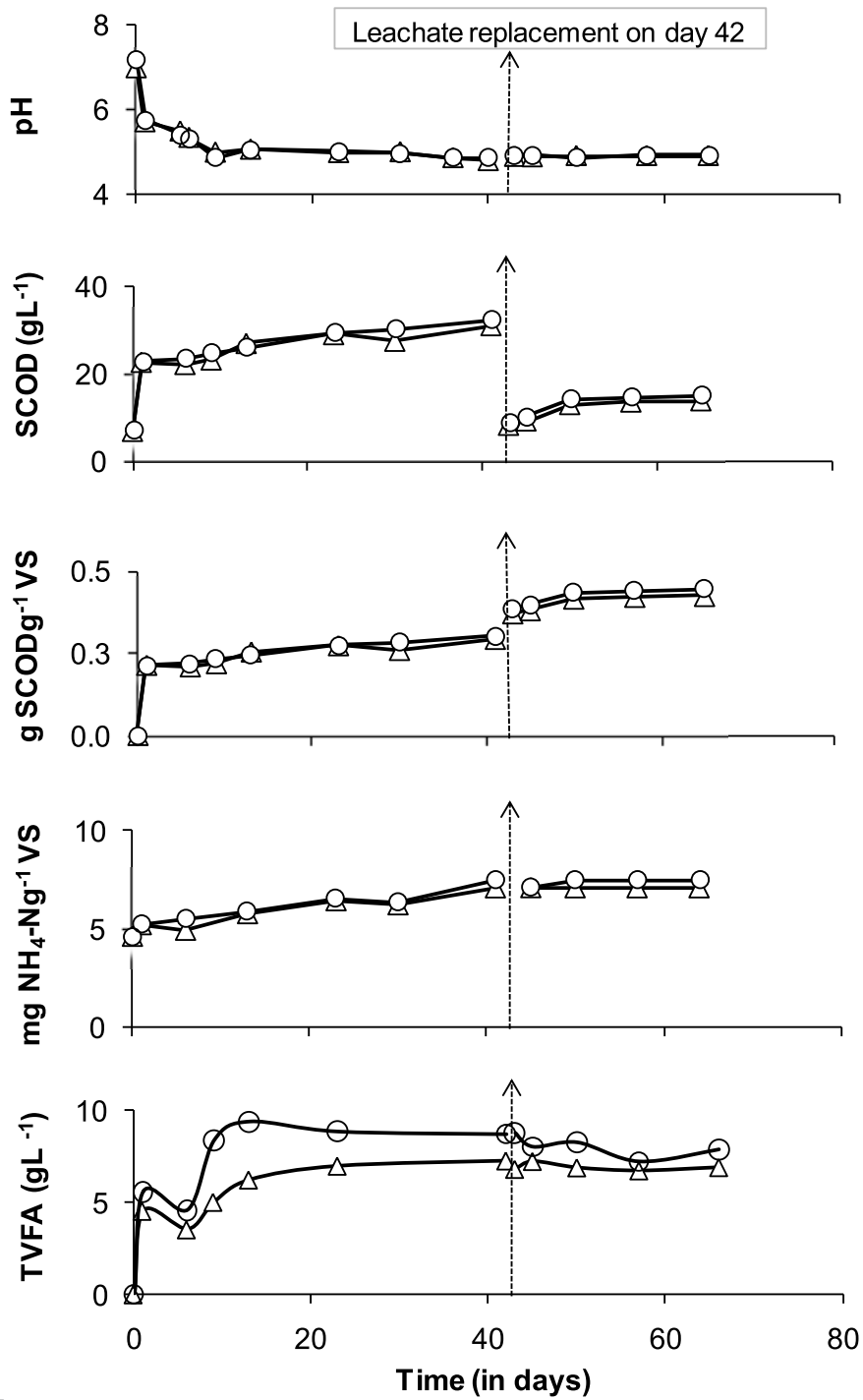
615

616

617

31

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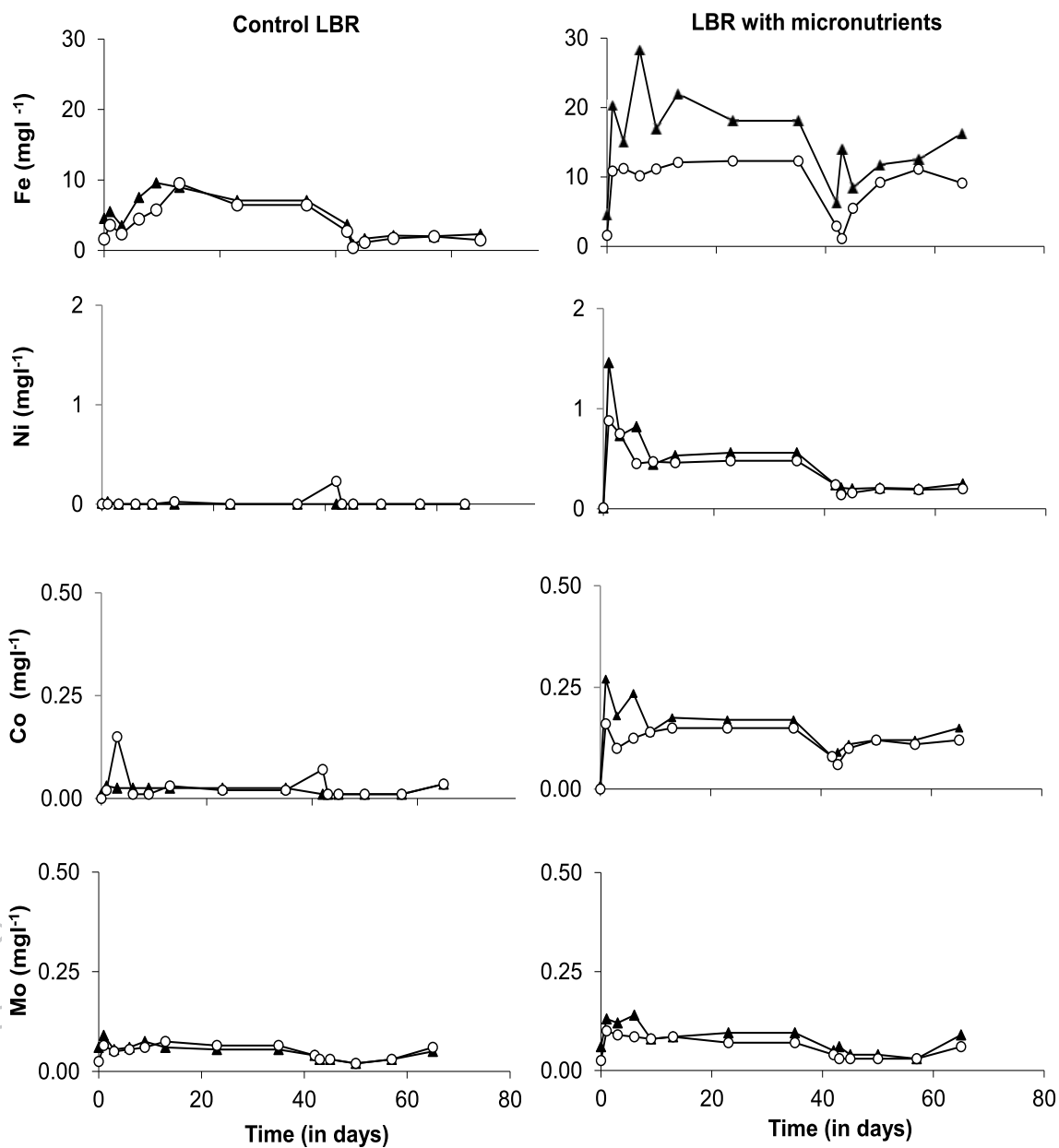620 NH_4Cl. Data shown here are "mean" values of replicate measurements.

621

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

627

628

629

630

34

631

632

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.

639

640

641 **Table 1**. Characteristics of the grass silage and inocula used in LBR studies for testing the
642 effect of NH_4Cl (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
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

647 *NA- Not analyzed

648

649

36

650

651

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

654

655

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

37

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.

662

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

38

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

664

665

666

667

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$_4$Cl 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

40

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

676

677   45.