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1 **Methane yield optimization using mix response design and bootstrapping:**
2 **application to solid-state anaerobic co-digestion process of cattle manure**
3 **and damp grass**

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10 **Abstract**

11 Optimization of solid-state anaerobic digestion on cattle manure and damp grass were
12 performed simultaneously and combined to a bootstrapping tool to significantly decrease the
13 number of experiments for a methane yield optimization. 15 batch reactors at lab scale were
14 launched two times respecting a mix surface response methodology. A numerical method
15 called Bootstrapping was used to verify results significance. Results have shown a significant
16 influence with a p-value between 10^{-8} and 10^{-11} : the optimal parameter values depend on the
17 substrate composition with a maximal p-value of $5.60 \cdot 10^{-2}$. The methane yield reached 156.19
18 NL.kgVS-1 for a mixture of cattle manure and damp grass, and 142.92 NL.kgVS-1 for cattle
19 manure only. The bootstrapping were validated with a standard error lower than 3% in
20 comparison with ANOVA method, which confirms that the mix surface response methodology
21 combined to bootstrapping is an innovative and efficient way to optimize solid-state anaerobic
22 digestion process.

23 **Keywords**

24 ANOVA; Agricultural waste; Biogas; Experimental design

25 **1 Introduction**

26 The share of renewable energy in the European Union in gross final energy consumption
27 reached 18 % in 2018, twice the share of 2004, which was 8.5 %. The European Union's target
28 is to reach 20 % of its energy from renewable sources by 2020 and 32 % by 2030. 12 countries
29 have already reached a share equal or above their 2020 binding targets but many countries
30 have to increase their contribution, such as France's share from 16.6 % to 23 % ([Eurostat,](#)
31 [2020](#)). Anaerobic digestion (AD) of organic wastes is part of this handle. Two types of AD
32 process exist, depending on the total solid content (TS): the liquid AD and the solid-state
33 anaerobic digestion (SS-AD). The SS-AD is defined by a solid content higher than 15 % and is
34 more appropriate for degradation of substrates with varying composition and high solid
35 content ([Rocamora et al., 2020](#); [André et al., 2018](#); [Degueurce et al., 2016](#)). This process has
36 increased in recent years, but SSAD is less widespread compared to liquid AD due to the lack of
37 knowledge and the operational complexity to sustain a stable production ([Rocamora et al.,](#)
38 [2020](#)). In fact, the scientific and technological hurdles of the SS-AD are numerous as non-
39 representativeness of samples, biological process drifts, lack of monitoring indicators in solid
40 media, no hydrodynamic optimization, no comprehension of the process, nor inhibitions of the
41 process ([André et al., 2018](#); [André et al., 2019](#)).

42 Some of these obstacles as inhibition phenomena and hydrodynamic comprehension can be
43 studied using a type of design of experiments which is a Response Surface Methodology (RSM).
44 This statistical method allows to describe and explain the effect of several independent
45 variables on responses with multiple linear regression rules. The ultimate goal can be the
46 determination of variables impact on the response or the optimization of the response ([Goupy
47 and Creighton, 2007](#); [Goupy, 2012](#)). In literature, a couple of papers on the AD phenomenon
48 use this tool with surface response designs (SRD) e.g. for the impact of pretreatment ([Gunes et
49 al., 2021](#); [Jin et al., 2018](#); [Jackowiak et al., 2011](#)), for the recirculation parameters on the SS-AD
50 ([Degueurce et al., 2016](#)), for the average velocity optimization on account reactor
51 configuration parameters ([Leonzio et al., 2019](#)), for the methane yield optimization regarding
52 the Carbon to Nitrogen ratio (C/N), Food to Microbe ratio (F/M) and pH ([Kainthola et al., 2020](#);
53 [Kainthola et al., 2019](#); [Mortezaei et al., 2018](#)) on different reactors or other parameters
54 ([Jiménez et al., 2014](#); [Kumar et al., 2020](#); [Lee et al., 2020](#)). The mixture designs (MD) work in
55 the same way but consider compositions respecting the mixture properties. The main goal of
56 MD in the AD process is to study the substrate composition. Some papers use this last tool in
57 AD process ([Kashi et al., 2017](#); [Wang et al., 2013](#); [Bassard, 2015](#); [Rakotoniaina, 2012](#); [Rao et al.,
58 2011](#)). These RSM studies are very interesting and allow deepening AD knowledge. Although a
59 parameter optimization is only valuable for a specific substrate composition and a composition
60 optimization is only valuable for a specific set of parameters. Some adaptations of factorial
61 design are done in the literature ([Wang et al., 2013](#)) to consider mixture composition and
62 operational parameters at the same time in a SRD but there is no study about a mix design
63 between these two solutions. Moreover, these methods require a compromise between
64 minimizing the number of experiments and to obtain results significance. To determine
65 experimental repeatability, several reactors must be monitored under the same conditions and
66 the results must be processed by an analysis of variance (ANOVA). The number of runs
67 depends on the type of design used and the number of parameters studied. No information is
68 available in the literature concerning the number of experiments reduction by alternative
69 methods as numerical methods. The bootstrapping is a method which were invented by
70 Bradley Efron ([Efron, 1979](#); [Efron and Tibshirani, 1986](#)) and involves determining the
71 significance of the model coefficients and response by resampling residues and redefine the
72 model coefficients from a large number of iterations. A parametric bootstrapping method had

73 already been used to successfully estimate the distribution of the anaerobic digestion kinetic
74 parameter estimators (Ruiz et al., 2005) but there is still no RSM applications.

75 The novelty of this work is to combine a new surface response methodology for SS-AD process,
76 which is a mix design combining the response surface design and the mixture design with a
77 numerical tool to reduce the number of experiments by iterative resampling: the
78 bootstrapping. This study has been conducted on two substrates and two key parameters of
79 the SS-AD to maintain time and cost constraints on batch reactors. The substrates used were
80 cattle manure and damp grass. In fact, grass is a substrate with risk of inhibition by acidosis as
81 cattle manure may offset this effect. The chosen parameters have also been selected for their
82 impact on the acidosis and these parameters are immersion rate and recirculation frequency.
83 The chosen SRD is a full-factorial design and the chosen MD is a simplex-centroid design.

84

85 **2 Materials and methods**

86 *2.1 Characteristics of substrates and inoculum used*

87 Cattle manure (CM) was sampled from the farm of the UniLaSalle Polytechnic Institute (Beauvais,
88 France). CM has been sampled for each set of experiments to maintain its raw properties and avoid
89 any preliminary treatment. The liquid part of the manure or liquid bovin manure (LBM) was used as
90 inoculum to bring the microbial consortium. Damp grass (DG) was mowed from household gardens
91 in Les Clayes-sous-Bois (France) in September 2019 and has been aliquoted and frozen to be used
92 at each set of experiments to avoid any physicochemical differences with DG used. Two sets of
93 experiments were conducted in October 2019 and January 2020. The physicochemical analyses
94 were made on CM and DG for each data set.

95 For all experiments, all analyses were carried out in triplicate. The total solid content (TS) and the
96 volatile solid (VS) of the inoculum, CM and DG were determined by a drying at 105°C for 24h and a
97 combustion at 550°C for 2h (APHA, 1998). The pH of the inoculum has been determined using a pH
98 meter (Mettler Toledo, Switzerland). The total volatile fatty acid content (FOS) and the buffer
99 capacity (TAC) were determined by two titration using sulfuric acid. The first acidification at pH =
100 5.0 provides the TAC and the second one at pH = 4.4 provides the FOS. An automatic titrator
101 (Mettler Toledo, Switzerland) has been used to realize these analyses. These tests were done at the
102 beginning of each set of experiments and the results are presented in **Table 1**. The FOS/TAC value
103 is an indicator to recognize stable AD operation or acidosis risks, where an excessive accumulation
104 of acids leads to AD inhibition (Mcghee, 1968; Nordmann, 1977; Voß et al., 2009).

105 *2.2 Experimental set-up*

106 For this study, one of the fixed objectives was to denature substrates the less possible. This
107 means that no grinding is done on substrates and the CM is not frozen but sampled and

108 analyzed for each experiment. This choice is done to be as representative as possible of the
109 real experimental conditions of an industrial digester. Thus, used reactors must respect space
110 and cost constraints but it must be sufficiently large for samples studied. Moreover,
111 constraints must be respected: the experimental parameters tested must be adjustable.
112 Fifteen leach bed reactors (LBR) made of Plexiglas with a total volume of 2.65 L were used
113 enabling a compromise between lab scale and substrate representativeness. These reactors
114 were divided into two parts: the upper part containing the solid phase and the bottom part
115 holding the liquid phase. These parts were separated by a perforated grid with a pore size of 5
116 mm diameter. The grid height was adjustable along a central axis of 10 mm diameter by a
117 manual tightening ring screwed, allowing imposing the immersion rate. A manual peristaltic
118 pump was used on each LBR at constant flow speed range and allowed to adjust the
119 recirculation frequency depending on the experiment. Thus, the liquid phase was spread out
120 across the top of the solid phase in the LBR upper part and percolated to drop back into the
121 bottom part. According to the experiment, recirculation frequency was between once a day
122 and once a week. The top of the reactor was directly connected with manual biogas counters.
123 These bottles were graduated and operated according to the Mariotte's law through volume
124 displacement, allowing to measure the cumulative biogas production each day. A temperature
125 reading allowed to convert this volume into a normalized quantity. Biogas composition was
126 analyzed five times a week by a gas chromatography (MicroGC, SRA Agilent 3000A) to
127 determine the biogas composition on every reactor (weekends excepted) to measure
128 methane, carbon dioxide, nitrogen and hydrogen production rates. The system is represented
129 **Fig 1.**

130 At the beginning of the experiment, all the introduced inoculum was recirculated once. For
131 every other recirculation, the spread volume was 0.62 L, representing arbitrarily 2:3 of the
132 total liquid volume. The spreading period was 1 min, because $0.62 \text{ L}\cdot\text{min}^{-1}$ is the minimal flow
133 to insure a good liquid distribution over the whole surface of the solid matter. Every reactor
134 was filled with the same volatile solid content. The chosen quantity of substrates inserted in
135 each reactor contained the same volatile solid content than 250 g of cattle manure for the first
136 and second run and 300 g of cattle manure for the third run. This was done to have a sufficient
137 percolation. Previous investigations on the LBR permitted to determine this reference (data
138 not shown). DG was introduced on CM, imposing a two-stored solid matter. This successive
139 stratification was chosen to reduce the contact between grass and liquid phase and thus
140 acidosis risks (André et al., 2019). At the end of each run of experiments, mass balances were
141 calculated on each reactor.

142 The ideal gas law was used to obtain the biogas mass flow expression. Then biogas flow was
143 normalized and integrated throughout the experiment to obtain the total mass produced (g).
144 Equations (1) and (2) describe this method.

$$PQ(t)M(t) = m(t)RT(t) \quad (1)$$

$$\int_{m_0}^{m_f} dm = \frac{P}{R} \int_{t_0}^{t_f} \frac{M(t)Q(t)}{T(t)} dt \quad (2)$$

145 2.3 *Mix Response Surface Methodology*

146 A mix design was chosen to optimize the response variable. This RSM was composed by coupling a
147 response surface design and a mixture design. The first hypothesis made was the proportionality of
148 the responses on the mixture composition. Concerning MD, a simplex-centroid design was used to

149 determine the effect of mixture composition of the studied response. For this study, the response
 150 was the total methane yield Y_{CH_4} (NL.kg $_{VS}^{-1}$). The mixture factors γ_i were quoted as the ratio
 151 between the volatile solid content of the component i and the total volatile solid content (% VS $_i$.VS $^{-1}$). This unit ensures to obtain comparable results between experimentations and not to be
 152 dependent on experimental conditions as component density or moisture. The final equation (3) is
 153 the model used.

$$\begin{aligned}
 Y_{CH_4} = & (\alpha_{1,0} + \alpha_{1,1}\delta_1 + \alpha_{1,2}\delta_2 + \alpha_{1,12}\delta_1\delta_2)\gamma_1 & (3) \\
 & + (\alpha_{2,0} + \alpha_{2,1}\delta_1 + \alpha_{2,2}\delta_2 + \alpha_{2,12}\delta_1\delta_2)\gamma_2 \\
 & + (\alpha_{3,0} + \alpha_{3,1}\delta_1 + \alpha_{3,2}\delta_2 + \alpha_{3,12}\delta_1\delta_2)\gamma_1\gamma_2
 \end{aligned}$$

155 This design permits a better flexibility than classical designs and considers the interactions
 156 between SSAD process parameters variations and composition variations on the methane
 157 yield. Process factor values must be normalized between -1 and +1 to compare the effects of
 158 process factors with each other.

159 Each run is achieved with 15 LBR experiments. Five replications of the center point were done
 160 to estimate the experimental error and confirm the model significance. They are included into
 161 the first run to compare the bootstrapping method with the statistical method. For the second
 162 run, only the bootstrapping will be used to determine the confidence interval of methane
 163 yield. All the experiments are summarized in the **Table 2**.

164 The statistical analysis was established by a Fisher test for the model with a 95 % confidence
 165 level and a Student test for the coefficients value with a 90 % confidence level. The analysis
 166 was done with the data analysis tool from Excel, allowing to determine the coefficients value
 167 and confidence intervals associated. The adequacy of the model was checked by adjusted
 168 regression coefficients (R_{adj}^2). The regression models were determined by analysis of variance

169 (ANOVA) (Miller, 1974 ; O'Connor, 1991) and a regressor selection was done (Lind & Ljung,
170 2005). After this selection, only 4 to 5 parameters among 12 were maintained for each run.
171 This observation resulted from the high confidence level established. Only 14 experiments
172 were used to realize the analysis on each run because some LBR presented recirculation
173 failures and values were rejected. These LBR were the experiments R₁₅ and R₁₉ for the first run
174 and R₁₃ for the second one. Finally, the assumptions due to ANOVA method were proved by
175 plotting a residual plot and a residual normality plot.

176 2.4 Repeatability analysis

177 Residual error includes model adjustment error and experimental error. To identify each error
178 and verify the model significance, sum squares and degrees of freedom were calculated.
179 Dedicated experiments R₁₆ to R₂₀ were launched during the first run of experiments. These
180 reactors were loaded with the same substrate's mixture and in the same operating conditions
181 (Table 2). The experimental sum square was obtained by following the final methane yield
182 obtained y_{0k} for these k reactors and was compared to the mean \bar{y}_0 of these 5 experiments.
183 This is how experimental variability was determined. Once the model is fitted with
184 experimental points (R₁ to R₁₅, R₁₃ excluded), the difference between experimental methane
185 yield y_{ik} and predicted methane yield \hat{y}_i was done by the residual sum squares. The difference
186 between these two calculations allowed determining the adjustment error. The total number
187 of experiments N_{exp} was 18 because two experiments were deleted before (R₁₃ and R₁₉) and P
188 is the standardized regressor number, which value was 5 after regressor selection. An F-value
189 is determined for the model significance and allow creating the confidence interval for each
190 value included in the field of study. In numerical statistics, bootstrapping is a resampling
191 method that operates by random sampling with replacement. The main idea is to model the

192 inference of samples by resampling data and performing conclusions about a sample from
193 resampled results. In inferential statistical analysis, the real population is unknown but using
194 bootstrapping, the population is the sample and is known. The original data set is resampled
195 and create a new sample from the original. The process is repeated during a large number of
196 iterations N and each resampled result provides an estimate of the real population. This
197 method was published by [Efron \(1979\)](#) and was inspired by the jackknife method ([Miller,
198 1974](#)). It is a very practical tool concerning the determination of samples accuracy and allows
199 obtaining confidence intervals and estimating properties. The main advantage of this tool is
200 the implementation simplicity to determine complex parameters and also helps to check the
201 results stability. This method is more accurate than the standard confidence intervals obtained
202 using classic assumptions of inferential statistics and allows to reduce the number of
203 experiments. By contrast, the resampled results depend on the sample representativeness.
204 Concerning this work, bootstrapping is used for building new samples to offset the limited
205 number of experiments and to assess the significance of experimental results.

206 Bootstrapping was used on two major points of this study: the confidence intervals of
207 model coefficients and the confidence interval of predicted methane yield. The approach used
208 was the residual resampling. For the first point, the supposed model was fitted with
209 experimental data and fitted parameters and residues were retained. Then, randomly
210 resampled residues were added to the fitted response in order to create a new sample. The
211 model was fitted again with experimental data including the resampled results and all samples
212 and new samples were retained. These last two steps were repeated in a large number of
213 times to approach the empiric probability distribution. For the second point, once the model
214 was fitted with experimental data, the sample residues were resampled over the full field of

215 study. In addition to this attention point, the operating method was the same. The residues
216 were studentized to consider the standard deviation difference between residues. The
217 complete method is sketched **Fig 2**. Every calculation and plot were programmed with Scilab
218 6.0.0.

219 **3 Results and discussion**

220 *3.1 Batch reactor performance*

221 For the first run of experiments with the damp grass composition lower than 45%_{VS}, the
222 immersion rate and the recirculation frequency became higher as well as the methane yield.
223 The difference between immersion rates was clearly visible in the LBR when the immersion
224 rate was high, the methane yield was higher. However, the recirculation frequency induced
225 different compartments depending on the immersion rate and the mixture composition. For
226 the second run, when the damp grass composition was higher than 50%_{VS}, two main optimal
227 methane yield zones appeared: when the immersion rate was low and the recirculation
228 frequency was high and vice versa. Moreover, when the damp grass composition was higher
229 than 50%_{VS}, it was very difficult to construe the biogas flow and methane curve shapes. This is
230 a typical example justifying the use of response surface methodology. Three experiments were
231 removed from these two runs because there was some experimental bias like recirculation
232 blockages or sealing problems: R13 for each run of experiments and R19. All the
233 characteristics of the liquid phase and the solid phase are summarized in the Table given in
234 supplementary material.

235 For each run of experiments, mass balances were calculated on each reactor. Biogas flow has
236 been normalized and included in the mass balance to obtain the total mass produced (g). The
237 minimum and maximum differences are 0.20 % and 8.66 %. LBR are therefore representing

238 mass balance between 91.34 % and 99.80 %, attesting the absence of biogas leakage or reactor
239 failures. This difference may be explained by the mass losses during the reactors emptying and
240 mass weighing. Depending on experimental conditions, the VS removal ranged from 63% to
241 87% for the first run and from 62.9% to 83.5% for the second run, attesting a great
242 biodegradation of substrates by the microbial consortium. These results are illustrated in **Fig 3**.
243 This conclusion is echoed by the methane yield observed. These consumptions represent
244 between 51% and 95% of the BMP for the first run and between 20% and 57% for the second
245 run. These results show that the experimental conditions are not optimal, probably due to the
246 substrates accessibility. Generally, the experiments with a low recirculation frequency and with
247 more damp grass produce less biogas. Some experimental parameters could explain this
248 phenomenon as preferential path and dead zones during percolation of the liquid phase.
249 Moreover, the pH value is considerably higher than the beginning of experiments, between
250 7.79 and 8.05, indicating a good balance between produced and consumed volatile fatty acids
251 (VFA). For the first run, the FOS/TAC ratio is between 0.098 and 0.128 and between 0.112 and
252 0.296 for the second run. This result suggests an impact of damp grass on FOS concentration
253 and buffer capacity.

254 3.2 *Statistical analysis of the results*

255 **Table 3** gives the final model of methane yield coefficients after regressors selection (after
256 removing all nonsignificant regressors, with a p-value > 0.1). Two models were determined
257 according to the run of the experiment, therefore according to experimental conditions. This
258 model presents an adjusted R² of 88 ± 1 %, which attesting a great accuracy to describe the
259 reality. The residual variability could be explained by the uncertainty of measurement and by
260 the impact of external parameters variations which were not considered for this study. But

261 these results still remain good results in terms of accuracy. The model p-value is very low,
262 proving the model significance.

263

264 **Table 4** provides the regressors value. Each run of experiments presents different models and
265 thus different regressors according to the experimental conditions. In fact, the significant
266 coefficients are not the same depending on the inhibition phenomenon, the substrate mixture,
267 the *inoculum*/substrate ratio and the recirculation frequency. A positive sign of the coefficient
268 means that the coefficient has a favorable impact on the methane yield. Therefore, the cattle
269 manure γ_1 and damp grass γ_2 presence are beneficial to the methane production. The
270 immersion δ_1 and the recirculation frequency δ_2 are beneficial to the methane yield when
271 associated with the substrates mixture, according to the interaction results $\delta_1\gamma_1\gamma_2$ and $\delta_2\gamma_1\gamma_2$.
272 In contrast, when the damp grass composition is higher (during run 2), the interaction
273 coefficient between substrates $\gamma_1\gamma_2$ is negative. This means that a high composition rate of
274 damp grass induced a negative impact on the methane yield, probably due to inhibition
275 phenomenon. Finally, an interaction coefficient between process parameters and cattle
276 manure composition $\delta_1 \delta_2 \gamma_1$ is negative in the first run of experiments. This could be explained
277 by the substrate's stratification, inducting that cattle manure is easier to be submerged than
278 damp grass. As the damp grass has a higher methanogenic power, the process parameters may
279 not improve the methane yield of a mixture with a low damp grass composition. The residues
280 of each model were plotted and all the postulates may be graphically verified. There is still a
281 slight difference of residual variability between the experimental values and models and a few
282 points which present fractiles slightly apart the normality line. This is why all the residues were

283 normalized by dividing residues by the residual standard deviation. This point will also be
284 considered when the bootstrapping is used.

285 3.3 Optimization of the methane yield

286 The regression model obtained and the coefficients value are attained as illustrated in
287 equations (4) to (7).

$$Y_{CH4,Run1} = (\alpha_{1,0} + \alpha_{1,12}\delta_1\delta_2)\gamma_1 + \alpha_{2,0}\gamma_2 + (\alpha_{3,1}\delta_1 + \alpha_{3,2}\delta_2)\gamma_1\gamma_2 \quad (4)$$

$$Y_{CH4,Run2} = \alpha_{1,0}\gamma_1 + \alpha_{2,0}\gamma_2 + \alpha_{3,0}\gamma_1\gamma_2 + \alpha_{3,2}\delta_2\gamma_1\gamma_2 \quad (5)$$

$$Y_{CH4,Run1} = (147.95 - 8.31\delta_1\delta_2)\gamma_1 + 113.59\gamma_2 + (63.69\delta_1 + 27.48\delta_2)\gamma_1\gamma_2 \quad (6)$$

$$Y_{CH4,Run2} = 129.41\gamma_1 + 143.45\gamma_2 - 171.86\gamma_1\gamma_2 + 77.79\delta_2\gamma_1\gamma_2 \quad (7)$$

288 These equations were numerically solved in order to find the optimum coefficient values to
289 maximize the methane yield. The maximal production value obtained for each run is
290 respectively 156.19 NL.kg_{VS}⁻¹ and 143.45 NL.kg_{VS}⁻¹. This difference is due to the *inoculum*
291 properties and the differences between recirculation frequencies. Thus, this value could not be
292 compared. Therefore, the methane yield compartment can be analyzed and compared. These
293 conclusions are consistent with the result found in the literature (André et al., 2019; Feng et
294 al., 2017) considering the *inoculum*, cattle manure and damp grass TS, VS and composition
295 differences. The response surface obtained for the first run is illustrated in **Fig 4**. The predicted
296 methane yield depends on the process parameters and mixture parameters. In this way, when
297 the cattle manure composition increase (figures from the top to the bottom), the predicted
298 methane yield observed depending on the immersion rate and the recirculation frequency.
299 These observations indicate a rise of the methane yield when the damp grass and process
300 parameters rise until damp grass composition reaches 45 %_{VS}. If extrapolation is done, beyond

301 70 %_{VS} of damp grass, the predicted methane yield curve has a significantly different behavior.
302 This probably represents the impact of contact period between liquid phase and substrates.
303 Concerning the second run of experiments, the immersion rate had not been effectively
304 brought into focus. predicted methane yield is not depending on this parameter. In this way,
305 methane yield can be based only on damp grass composition and recirculation frequency as
306 illustrated in **Fig 5**. A red area means a higher methane yield and a blue area means a lower
307 methane yield. The methane yield surfaces are on the right and methane yield isovalues are
308 represented on the left. The predicted methane yield curve has a different behavior than the
309 first experiment. This is probably because the high damp grass percentage induced inhibitions,
310 potentially acidosis, that were not present during the first set of experiments and induce a
311 different behavior. This difference of behaviors highlights that extrapolation presents strong
312 uncertainties for RSM methods and should not be used for SS-AD with risks of inhibitions.

313 In these different experimental conditions, the same conclusion is done: in the mixture
314 considered, when the damp grass composition is upper than 70 %_{VS}, inhibitions by acidosis
315 could appear. In this case, immersion rate and recirculation frequency can limit this
316 phenomenon. This study highlighted that two process behaviors permit controlling acidosis
317 risks: high immersion rates and low recirculation frequency, or low immersion rates and high
318 recirculation frequency. This could be explained by an equilibrium between foster the contact
319 period between microbial consortium and the higher biodegradable DG substrate and limiting
320 the acidosis risks.

321 *3.4 Repeatability study*

322 Once the predicted methane yield was optimized and the response surface modeled, it is very
323 important to determine the prediction accuracy to know how these experiments are

324 repeatable. For this, two main tools were used: the classical analysis of variance and the
325 bootstrapping. ANOVA needs to respect some postulates done on the model residues: variance
326 is equal for all residues, each residue is independent, normally distributed, and the statistical
327 average of residues is zero. The postulate acceptance causes low bias and need a sufficient
328 number of experiments to be correctly verified. The confidence intervals were determined and
329 the bootstrapping tool were applied to the experimental results concerning two main points:
330 the confidence intervals on the model coefficients determination and the confidence intervals
331 on the predicted methane produced.

332 Concerning the model coefficients, the bootstrapping was used 200 times with 2000 iterations
333 to approach the real population. This represents 400,000 confidence interval simulations of
334 each coefficient or between 1,600,000 and 2,000,000 calculations. The results are shown **Table**
335 **5**. Beyond 100 uses of the bootstrapping tool, the simulations stay in the same domain. When
336 the simulations are over, the maximum upper confidence interval and the minimum lower
337 confidence interval are retained and compared with the ANOVA confidence intervals. The
338 results obtained provide the same confidence intervals with a maximal error of 2.9 %. This
339 error could be explained by the absence of postulates and the residual variability awareness by
340 the studentized residues. In this study, residue postulates were verified and enough
341 experimental data were used to obtain a robust analysis of variance. But for other design
342 experimentations where there is a lack of samples, bootstrapping could be a solution to
343 determine the repeatability of the model and may be useful to decrease the necessary number
344 of experiments without losing accuracy.

345 The same study has been done on the confidence intervals of predicted methane yield. The
346 confidence intervals on the first run have been determined for a damp grass composition

347 between 0.15 and 0.45. The extrapolation has been done for a damp grass composition
348 between 0.45 and 0.85. The same work has been done for the second run of experiments for a
349 damp grass composition between 0.15 and 0.85. The confidence intervals provided by the
350 ANOVA method and by the bootstrapping method are still comparable. Results are illustrated
351 in **Fig 6** for the second run. The green surface represents the predicted methane yield. The blue
352 surface represents the ANOVA confidence intervals and the red surfaces represent the
353 bootstrapping confidence intervals. It can be noticed that bootstrapping provides higher
354 confidence intervals than ANOVA. It could be explained by the consideration of residue
355 variability. Bootstrapping is a method that analyzes and reproduces a global error between
356 experimental points and predicted points, containing experimental errors and adjustment
357 errors. This is why experimental repetitions are not necessary to determine confidence
358 intervals. The predicted methane surface has a very simple shape, due to the predicted
359 methane yield non-dependency with the recirculation frequency. The ANOVA confidence
360 intervals are represented in blue and bootstrapping intervals are represented in red. This is
361 why the response surface had also been studied depending on the damp grass composition
362 and the recirculation frequency.

363 **Fig 5.** provides a response surface shape close to the studies already present in the literature
364 ([Degueurce et al., 2016](#)) and highlights the significance of recirculation frequency on the
365 methane yield. Moreover, for each experiment, bootstrapping provides larger confidence
366 intervals. This difference is probably due to the absence of study hypotheses, confirming its
367 use. The larger intervals allow a reduced risk of experimental errors and adjustment errors.

368 **4 Conclusions**

369 The results suggested that interactions between process parameters and mixture parameters
370 are significant and plays an important role in methane production, which validate the
371 usefulness of mix response designs. The repeatability could be studied with numerical tools as
372 bootstrapping for greater accuracy and minimizing the number of experiments. This work
373 proposes an optimal method in terms of time and accuracy to realize response surface studies.
374 However, it is only a first step and it is possible to go far beyond these results, notably using
375 more complex mixtures, more process parameters and different response designs.

376 **5 Acknowledgments**

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468 **Figure captions**

469 **Fig 1.** Schematic representation of the lab scale experimentation set up

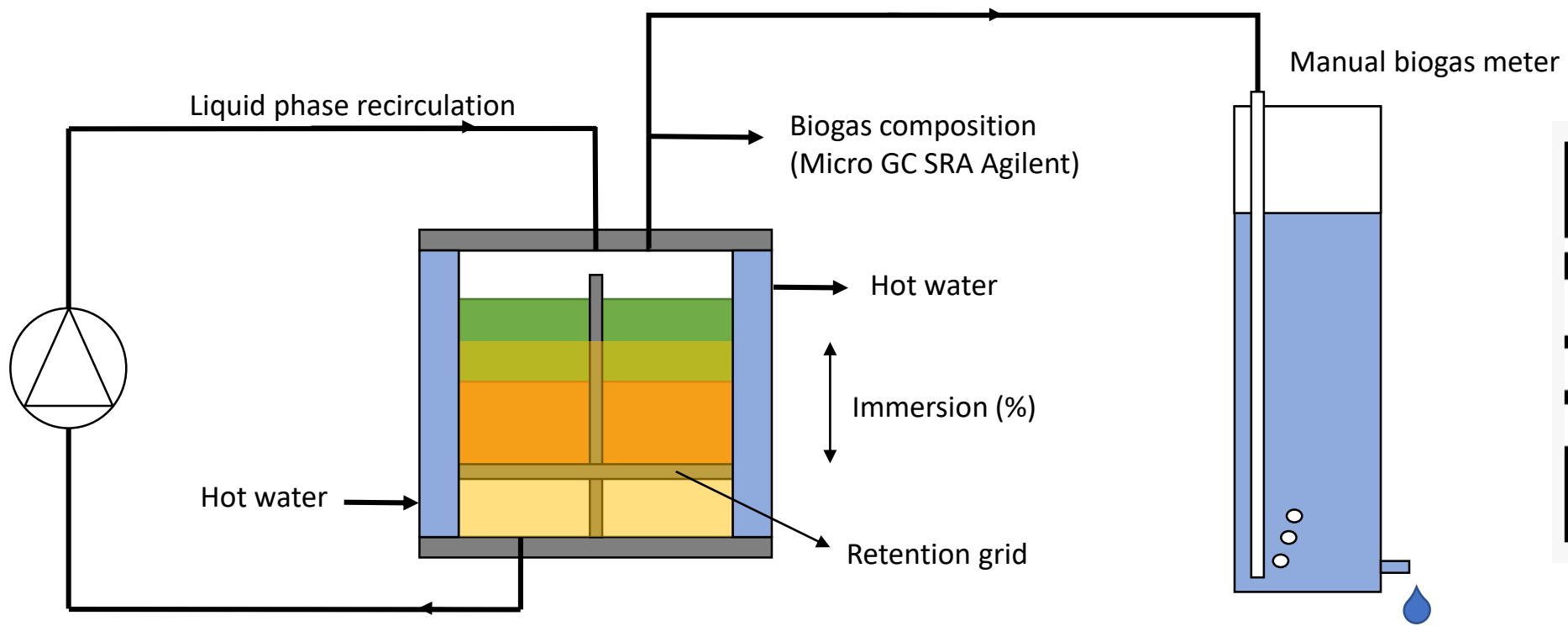
470 **Fig 2.** Global schema of bootstrapping method

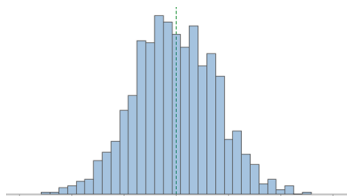
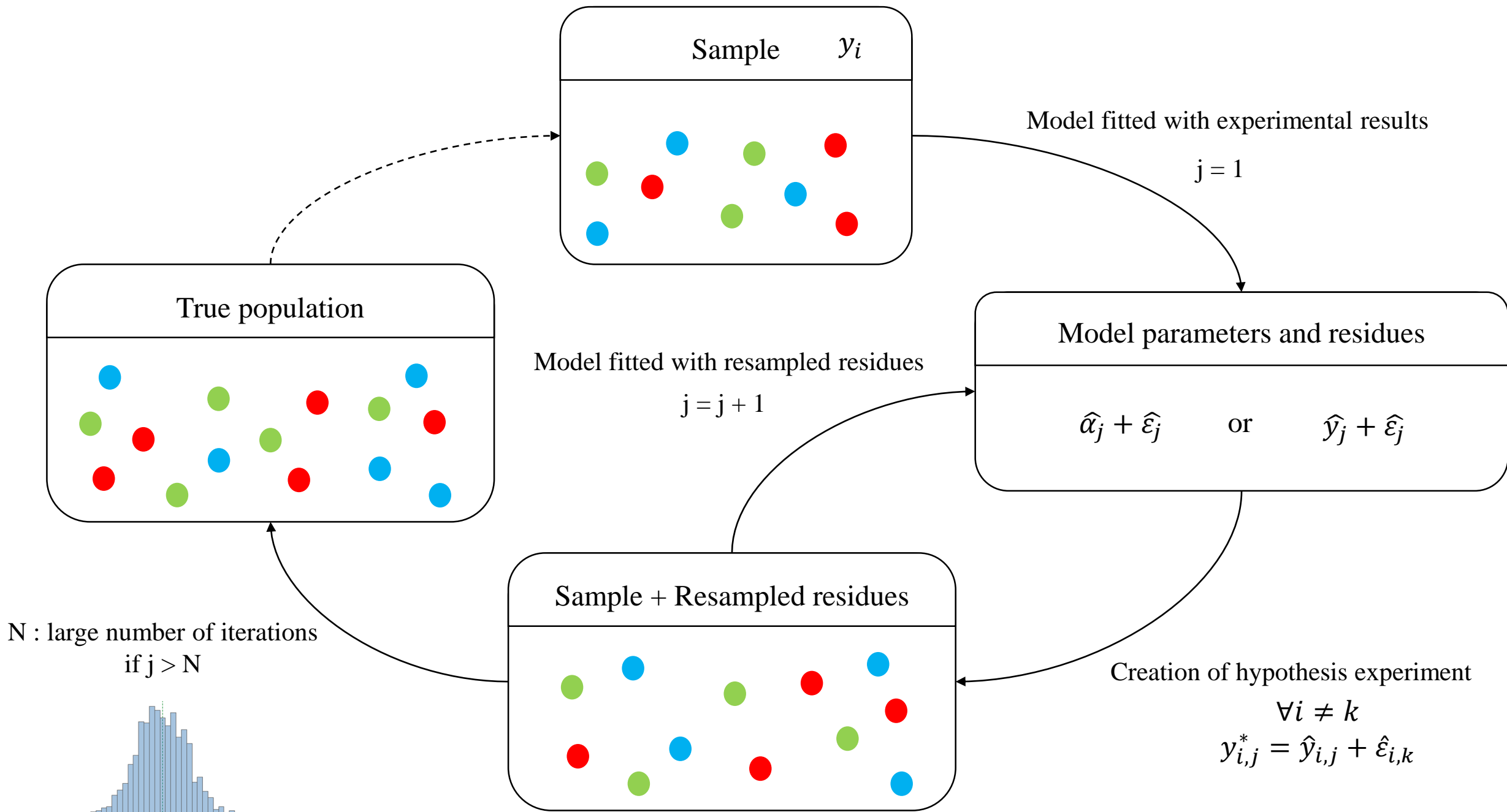
471 **Fig 3.** TS and VS content for solid and liquid phases for (a) : Run1 and (b) : Run 2

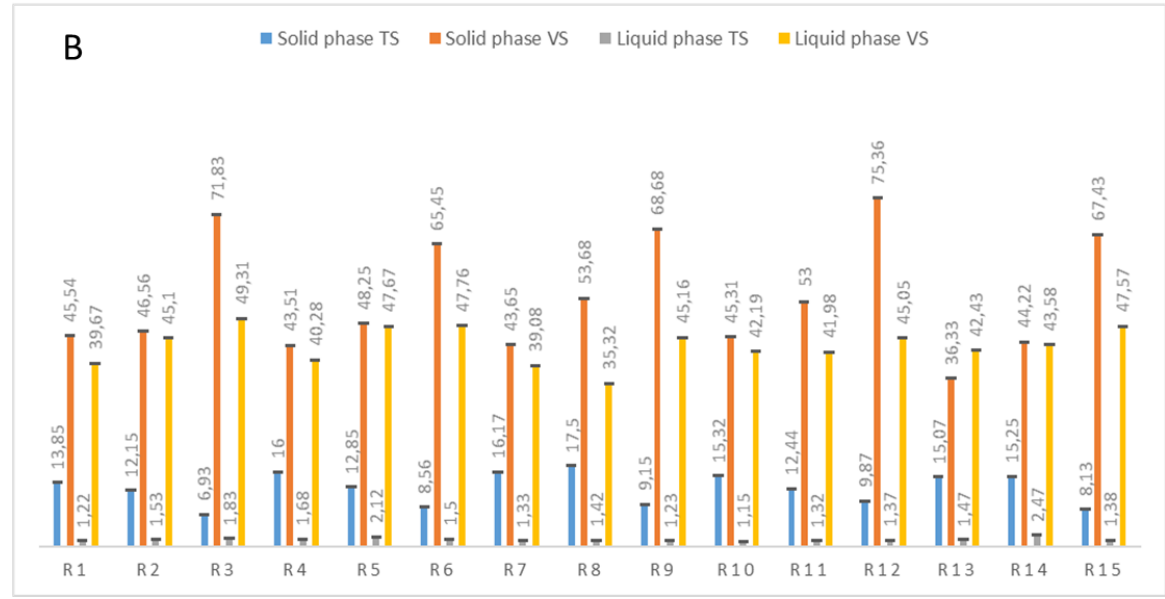
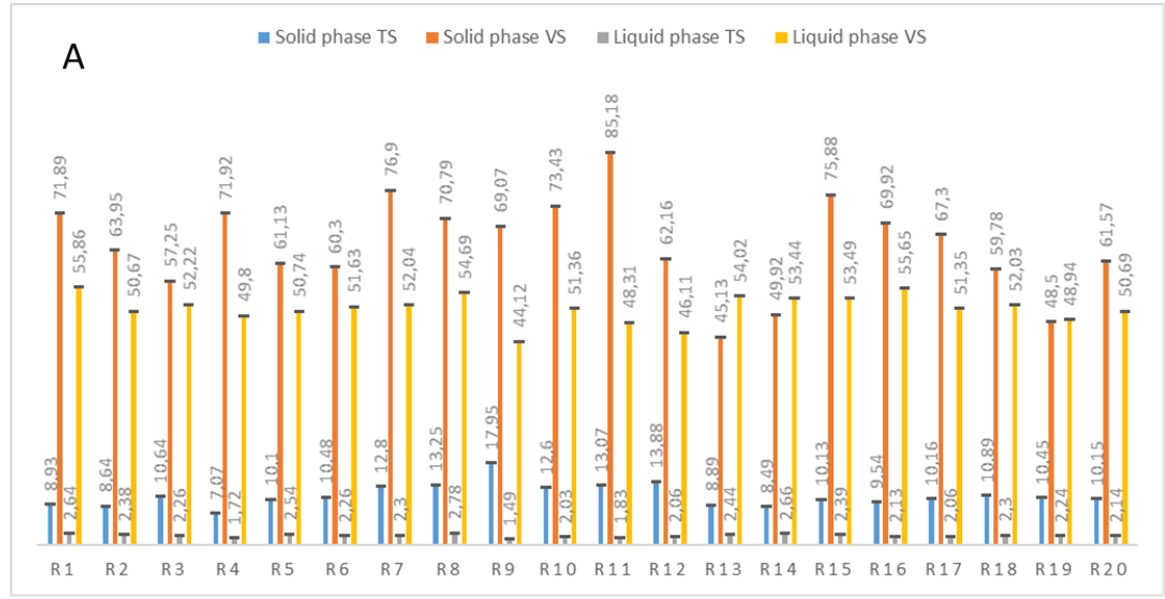
472 **Fig 4.** Predicted methane yield for Run 1, according to the recirculation frequency and the
473 immersion for a fixed composition

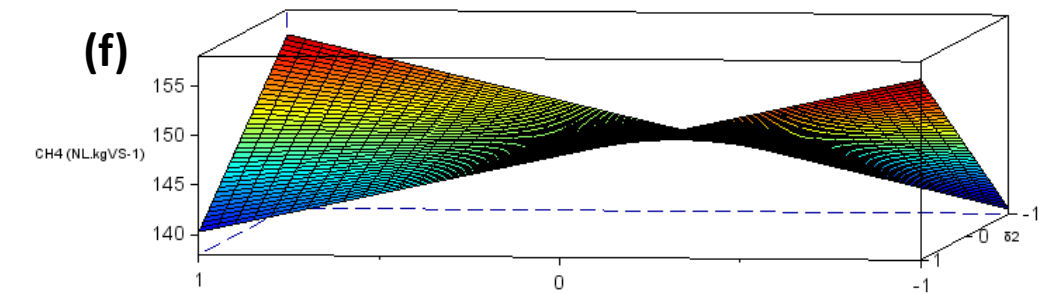
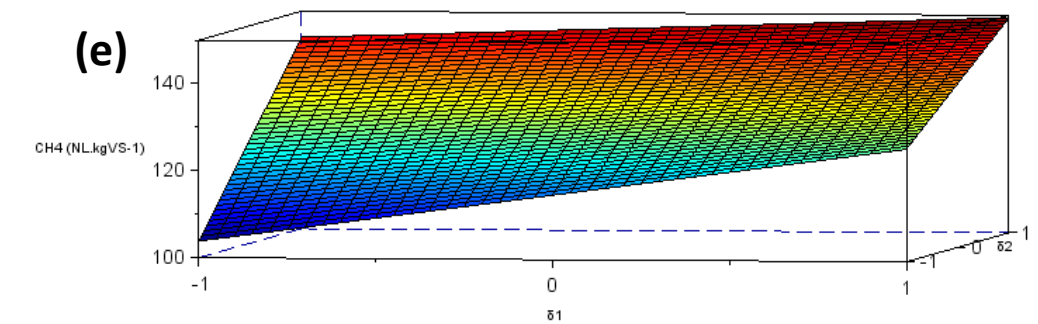
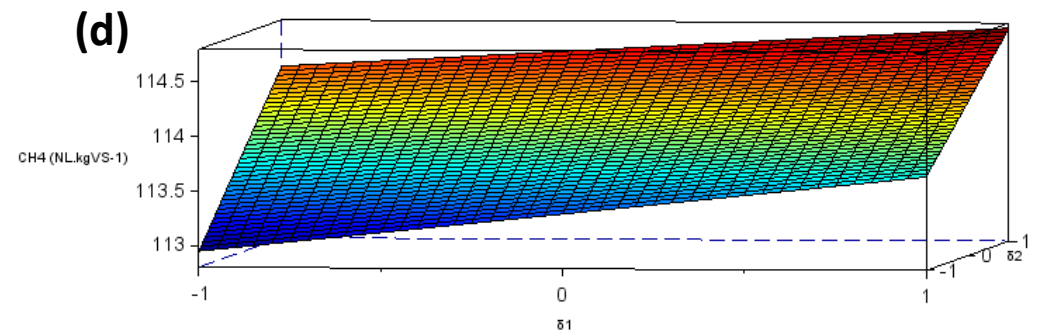
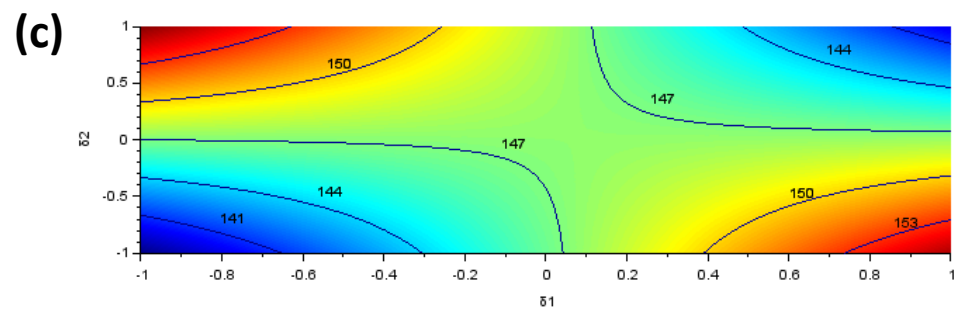
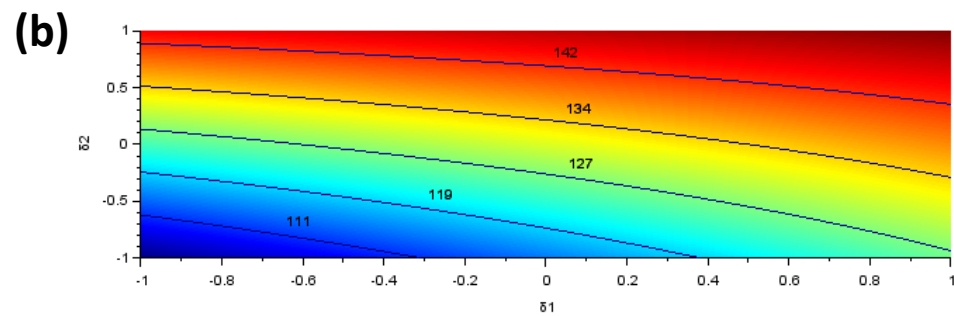
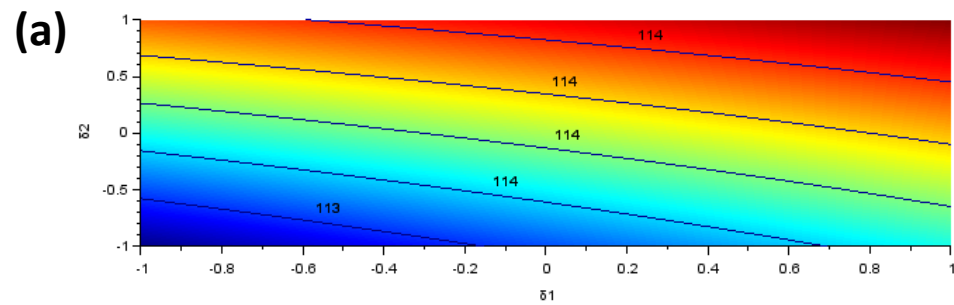
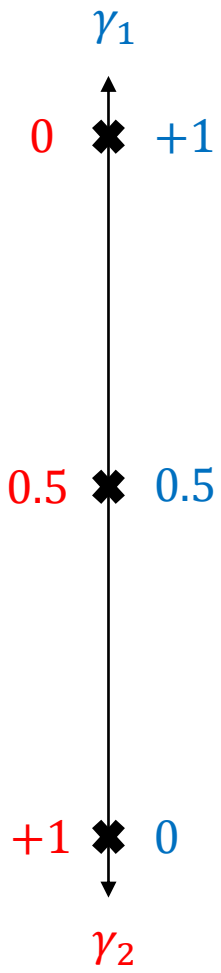
474 **Fig 5.** Predicted methane yield for Run 2, according to the recirculation frequency and the damp
475 grass composition

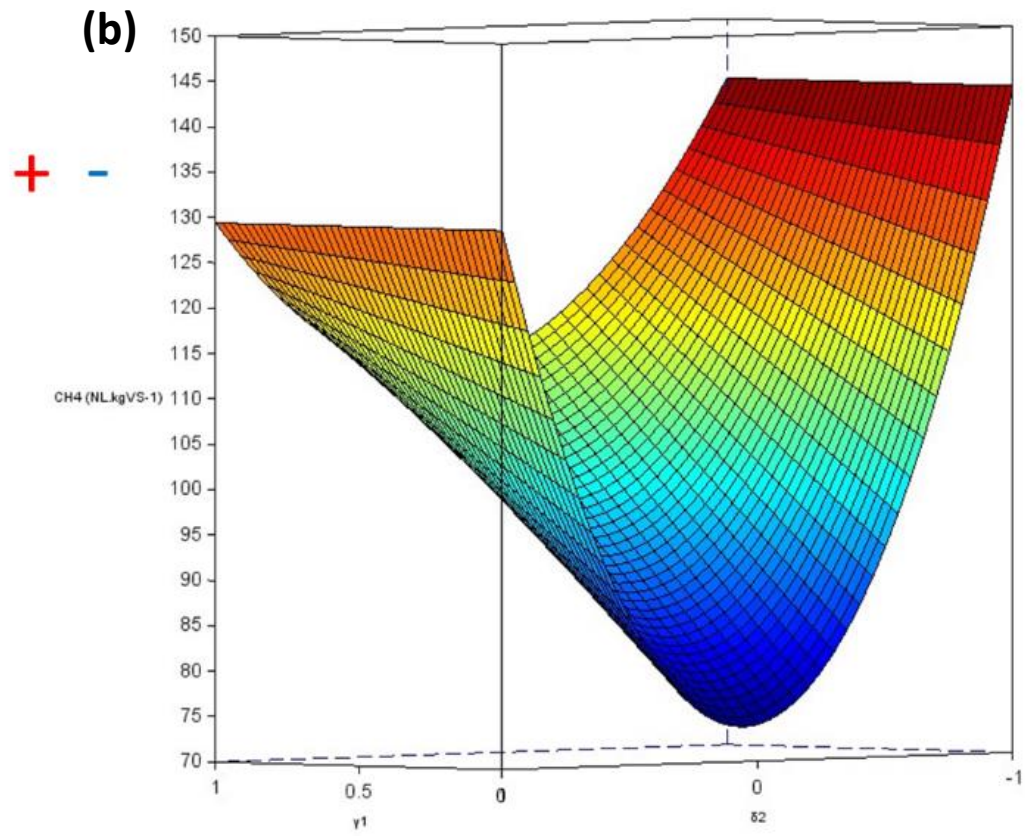
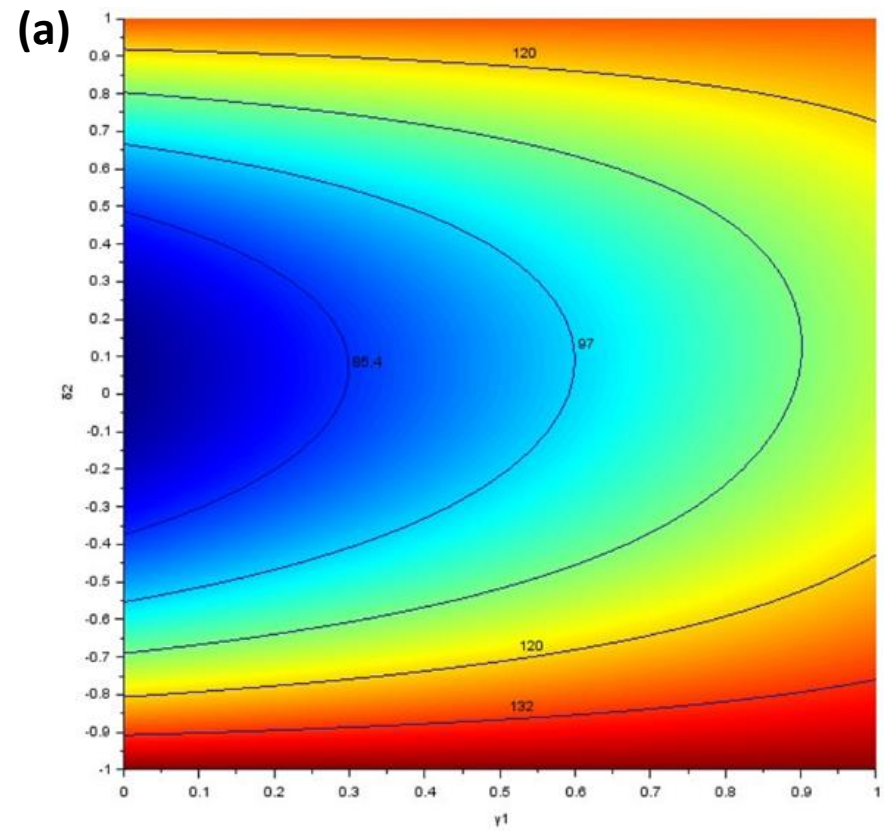
476 **Fig 6.** Predicted methane yield confidence intervals for the second run (Methane production:
477 green, ANOVA: blue, bootstrapping : yellow), depending on recirculation frequency and damp grass
478 composition











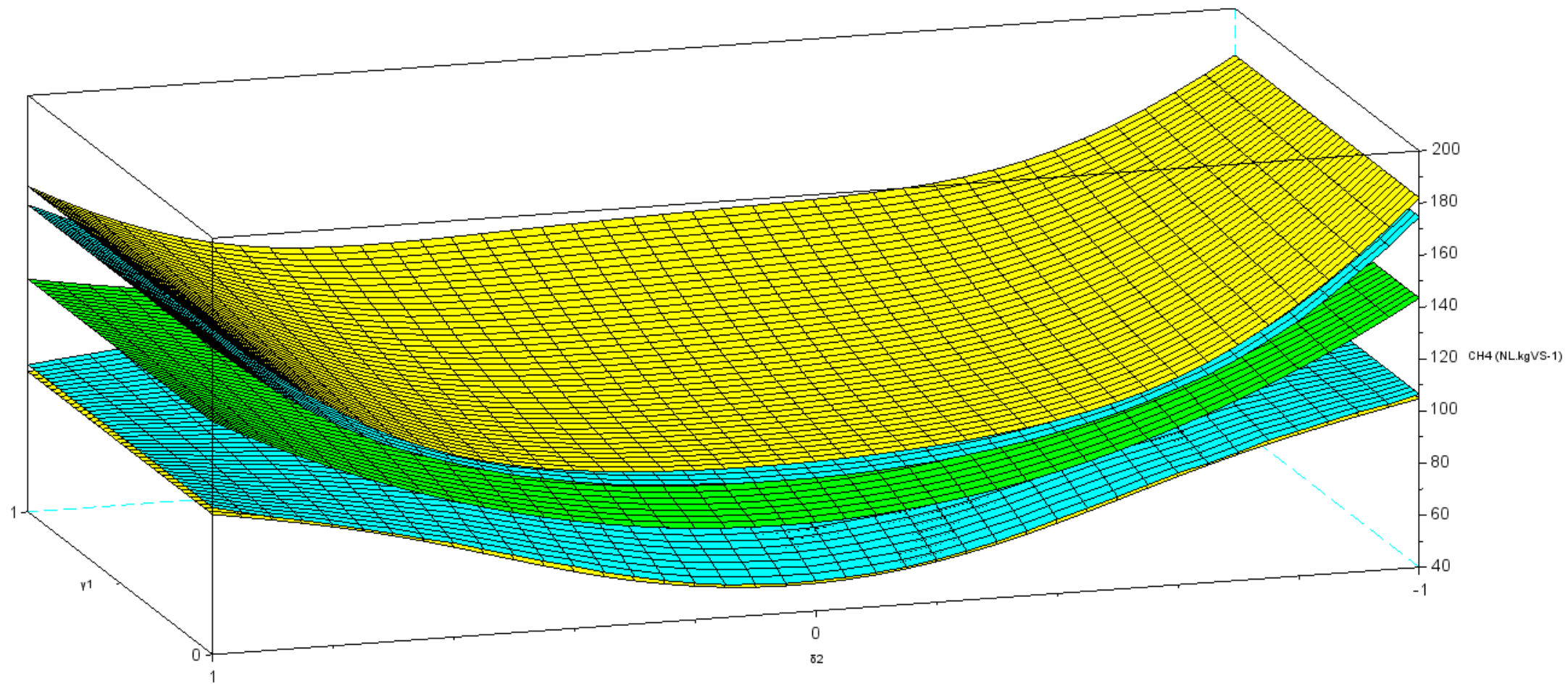


Table 1 Chemical characteristics of inoculum and initial substrates used

		TS	VS	pH	FOS	TAC	BMP
	Unit	%	% _{TS}	-	mg.L ⁻¹	mg.L ⁻¹	NL.kgvs ⁻¹
	Standard error	± 0.03	± 0.01	± 0.01	± 5.5	± 62	-
Run 1	Initial CM	22.0	87.7	7.51	-	-	142.92 ± 12.42
	Initial DG	42.2	71.7	5.81	-	-	240.39 ± 8.95
	Initial LM	1.61	62.7	7.92	96.81	3460	10.42 ± 0.01
Run 2	Initial CM	18.5	87.6	7.46	-	-	142.68 ± 16.93
	Initial DG	42.2	71.7	5.82	-	-	240.39 ± 8.95
	Initial LM	1.80	46.3	8.77	1021.21	7081	10.42 ± 0.01

Table 2 Mix design of experiment used for runs 1 & 2

Experiment	Standardized factor values		Compositions Run 1		Compositions Run 2		Methane yield Run 1	Methane yield Run 2
	δ_1	δ_2	γ_1	γ_2	γ_1	γ_2	(NL.kgvs ⁻¹)	(NL.kgvs ⁻¹)
R ₁	+1	-1	0.55	0.45	0.15	0.85	144.77	120.51
R ₂	+1	-1	0.70	0.30	0.5	0.5	154.86	85.41
R ₃	+1	-1	0.85	0.15	0.85	0.15	150.11	97.51
R ₄	+1	+1	0.55	0.45	0.15	0.85	148.28	126.01
R ₅	+1	+1	0.70	0.30	0.5	0.5	152.97	124.04
R ₆	+1	+1	0.85	0.15	0.85	0.15	143.42	130.04
R ₇	-1	-1	0.55	0.45	0.15	0.85	95.72	119.80
R ₈	-1	-1	0.70	0.30	0.5	0.5	121.27	47.63
R ₉	-1	-1	0.85	0.15	0.85	0.15	123.72	109.45
R ₁₀	-1	+1	0.55	0.45	0.15	0.85	129.99	111.42
R ₁₁	-1	+1	0.70	0.30	0.5	0.5	133.70	123.07
R ₁₂	-1	+1	0.85	0.15	0.85	0.15	144.56	88.00
R ₁₃	0	0	0.55	0.45	0.15	0.85	158.91	137.63
R ₁₄	0	0	0.70	0.30	0.5	0.5	147.8	87.19
R ₁₅	0	0	0.85	0.15	0.85	0.15	140.98	123.03
R ₁₆	0	0	0.70	0.30	-	-	151.93	-
R ₁₇	0	0	0.70	0.30	-	-	142.52	-
R ₁₈	0	0	0.70	0.30	-	-	136.70	-
R ₁₉	0	0	0.70	0.30	-	-	124.00	-
R ₂₀	0	0	0.70	0.30	-	-	138.66	-

Table 3 Analysis of variance on the final model (90 % confidence level)

Regression	DF	Sum of squares	Mean squares	F-value	p-value
Run 1					
Model	5	2.70.10 ⁵	5.39.10 ⁴	1421.22	1.44.10 ⁻¹¹
Residual	9	3.41.10 ²	3.79.10 ¹	-	-
Total	14	2.70.10 ⁵	-	-	-
R ²	-	0.999	-	-	-
R _{adj} ²	-	0.887	-	-	-
Standard error	-	6.16	-	-	-
Run 2					
Model	4	1.63.10 ⁵	4.08.10 ⁴	134.29	5.13.10 ⁻⁸
Residual	10	3.04.10 ³	3.04.10 ²	-	-
Total	14	1.66.10 ⁵	-	-	-
R ²	-	0.981	-	-	-
R _{adj} ²	-	0.876	-	-	-
Standard error	-	17.42	-	-	-

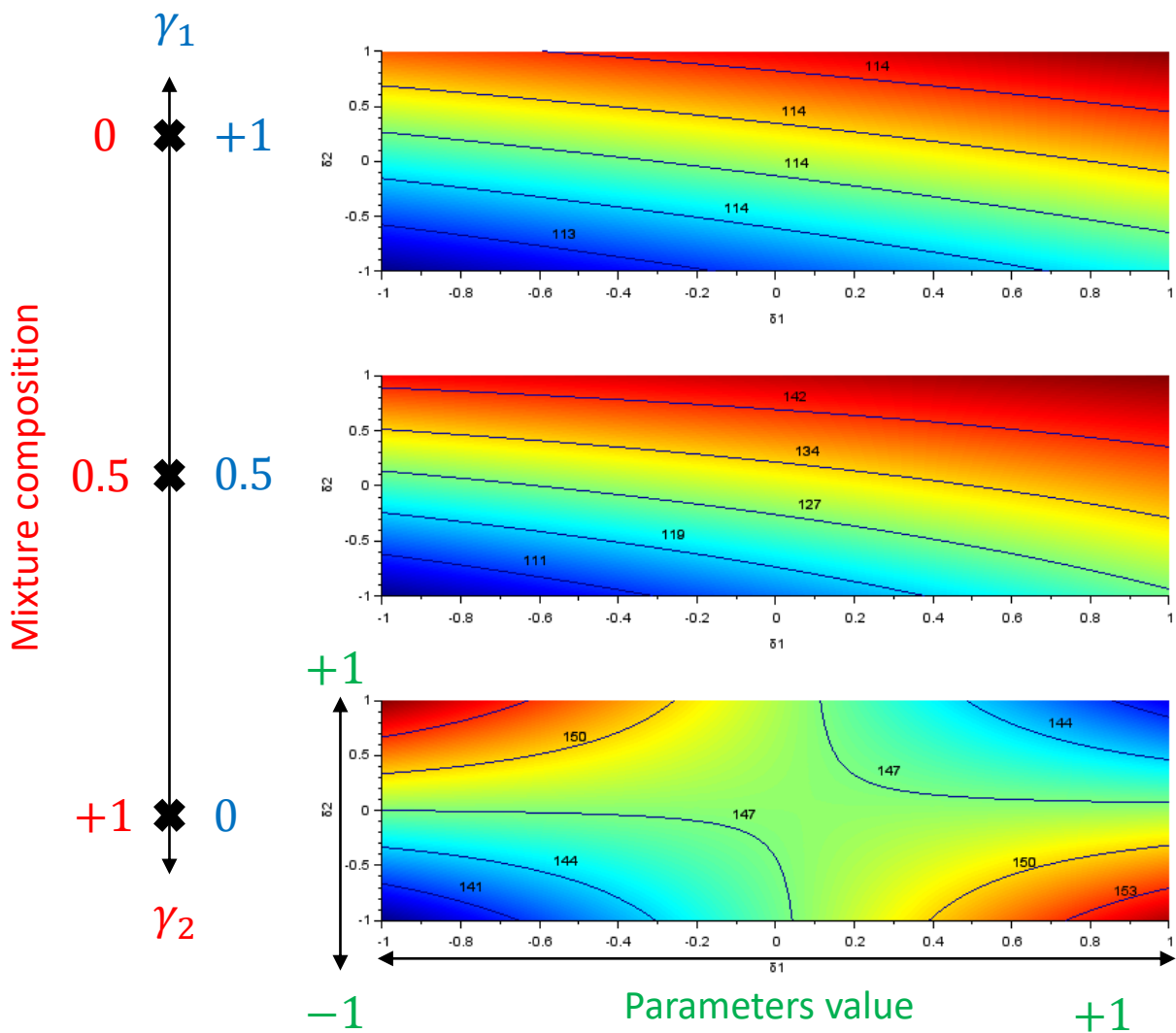
Table 4 Analysis of variance on the regressors (90 % confidence level)

Regressor	Coefficient value	Standard error	p-value	Lower boundary	Upper boundary
Run 1					
γ_1	147.95	4.90	$7.35 \cdot 10^{-11}$	140.06	155.84
γ_2	113.59	9.91	$1.13 \cdot 10^{-6}$	95.43	131.75
$\delta_1 \delta_2 \gamma_1$	-8.31	2.50	$8.95 \cdot 10^{-3}$	-12.89	-3.72
$\delta_1 \gamma_1 \gamma_2$	63.69	8.83	$5.02 \cdot 10^{-5}$	47.50	79.88
$\delta_2 \gamma_1 \gamma_2$	27.48	8.83	$1.25 \cdot 10^{-2}$	11.29	43.67
Run 2					
γ_1	129.41	15.99	$1.06 \cdot 10^{-5}$	100.43	158.39
γ_2	143.45	17.32	$8.67 \cdot 10^{-6}$	112.07	174.84
$\gamma_1 \gamma_2$	-171.86	79.52	$5.60 \cdot 10^{-2}$	-315.98	-27.73
$\delta_2 \gamma_1 \gamma_2$	77.79	28.27	$2.04 \cdot 10^{-2}$	26.56	129.02

Table 5 Analysis of variance and bootstrapping on the coefficients confidence intervals (90 % confidence level)

Regressor	Coefficient value	Lower ANOVA	Upper ANOVA	Lower Bootstrapping	Upper Bootstrapping	Relative error (%)
Run 1						
γ_1	147.95	140.06	155.84	140.03	155.51	1.91
γ_2	113.59	95.43	131.75	95.15	131.19	0.76
$\delta_1 \delta_2 \gamma_1$	-8.31	-12.89	-3.72	-12.76	-3.85	2.90
$\delta_1 \gamma_1 \gamma_2$	63.69	47.50	79.88	47.76	79.91	0.74
$\delta_2 \gamma_1 \gamma_2$	27.48	11.29	43.67	11.43	43.46	1.07
Run 2						
γ_1	129.41	100.43	158.39	98.13	157.26	2.01
γ_2	143.45	112.07	174.84	110.31	173.38	0.47
$\gamma_1 \gamma_2$	-171.86	-315.98	-27.73	-323.06	-30.90	1.36
$\delta_2 \gamma_1 \gamma_2$	77.79	26.56	129.02	25.24	130.54	2.77

Methane yield optimization with mix response Surface methodology



Repeatability study using Bootstrapping methods

