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1 **Title:**

2 Integrating independent microbial studies to build predictive models of anaerobic digestion
3 inhibition by ammonia and phenol

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23 **Abstract**

24 Anaerobic digestion (AD) is a process that can efficiently degrade organic waste into
25 renewable energies. AD failure is however common as the underpinning microbial
26 mechanisms are highly vulnerable to a wide range of inhibitory compounds. Sequencing
27 technologies enable the identification of microbial indicators of digesters inhibition, but
28 existing studies are limited. They used different inocula, substrates, sites and types of reactors
29 and reported different or contradictory indicators. Our aim was to identify a robust signature
30 of microbial indicators of phenol and ammonia inhibitions across four independent AD
31 microbial studies. To identify such signature, we applied an original multivariate integrative
32 method on two in-house studies, then validated our approach by predicting the inhibitory
33 status of samples from two other studies with more than 90% accuracy. Our approach shows
34 how we can efficiently leverage on existing studies to extract reproducible microbial
35 community patterns and predict AD inhibition to improve AD microbial management.

36 **Key words**

37 Methane; Ammonia; Phenol; Microbial indicator; Inhibition prediction

38

39 1 Introduction

40 Anaerobic digestion (AD) is considered as the most efficient and sustainable technology
41 for organic waste treatment. It has the ability to enable simultaneously waste stabilization and
42 valorization through the production of methane rich biogas and of digestate used as an organic
43 amendment. Encouraged by the renewable energy policies, biogas production with AD has
44 increased in the European Union to reach 18 billion m³ methane (654 PJ) in 2015 (Scarlat et
45 al., 2018). However, The European Biomass Association (AEBIOM) estimates that AD still
46 has a considerable potential for expansion with a biogas potential at about 78 billion m³
47 methane. To reach this goal, the optimization of biogas production is essential to improve
48 high process stability and efficiency and lower susceptibility to disturbances. Indeed, process
49 failure reduces the economic and environmental performances of biogas technology as they
50 lead to decreased methane yields and thus reduce revenues. Therefore, it is important that
51 applied research on biogas technology improve robustness of these systems to stress factors,
52 such as altered operating conditions or inhibitory compounds.

53 Among the broad range of inhibitors from AD substrates, high concentrations of
54 ammonia and micro-pollutants such as phenol are considered as the primary cause of digester
55 failure (Gonzalez-Gil et al., 2018; Rajagopal et al., 2013). Commonly used feedstock such as
56 livestock manure, slaughterhouse byproducts and food industrial residues contain organic
57 nitrogen such as urea and proteins, which readily release ammonia during their anaerobic
58 degradation (Yenigün & Demirel, 2013). Previous studies addressing the topic of ammonia
59 inhibition in AD have reported a large disparity in the inhibitory limits, which range from 27
60 to 1450 mg of NH₃-N/L and from 1.1 to 11.8 g of NH₄-N/L (Capson-Tojo et al., 2020).

61 In addition, various natural or anthropogenic phenolic compounds are detected in
62 different types of effluents from coal gasification, coking, petroleum refining, petrochemical

63 manufacturing and paper (Rosenkranz et al., 2013). Phenols are also produced from
64 biodegradation of naturally occurring aromatic polymers such as humic acids and tannins or
65 from degradation of xenobiotic compounds such as pesticides (Veeresh et al., 2005). As a
66 result, contaminated sludge produced during the treatment of these various effluents can cause
67 digester imbalance. Phenol concentrations greater than 1000 mg/L were reported to severely
68 inhibit AD (Dong et al., 2019) and generally, half maximal inhibitory concentrations (IC₅₀)
69 were reported to be between 1.1 and 1.8 g/L (Chapleur et al., 2016). The stability and
70 efficiency of the overall AD process relies on tightly coupled synergistic activities between an
71 intricate community of microorganisms. But the understanding of biological mechanisms of
72 AD is still hampered by the extreme complexity of the microbial ecosystem involved in this
73 process (Li et al., 2019). New knowledge is needed to unravel bioindicators of digesters
74 inhibition which have the potential to guide and optimize operation management during
75 unexpected onset of inhibitors and prevent biogas production.

76 Different methods for characterizing AD microbiome have been proposed and reviewed
77 by Lim et al. (Lim et al., 2020). New molecular biology techniques have revealed the great
78 diversity of the biogas-producing microbiomes. Thus, these omics technologies provide
79 unprecedented opportunities to characterize microbiomes' diversity, composition, gene
80 expression or metabolism and can be used to evidence bioindicators. For example, Hao et al.
81 used quantitative PCR and 16S rDNA amplicon sequencing to evidence associations between
82 process parameters and the abundance of specific microbial phylotypes in full scale digesters
83 (Hao et al., 2016). De Vrieze et al. evaluated the microbial community in full-scale AD plants
84 through amplicon sequencing of both the 16S rRNA gene and the 16S rRNA transcripts to
85 compare the total and active microbial community directly (De Vrieze et al., 2018).

86 High-throughput sequencing technologies, including 16S rRNA amplicon sequencing
87 have enabled many studies to monitor microbial changes during steady state or the inhibition

88 of anaerobic digesters, for example with phenolic compounds (Chapleur et al., 2016; Madigou
89 et al., 2016; Poirier et al., 2016a) or ammonia (Lü et al., 2016; Poirier et al., 2016b). However,
90 given a same inhibitor, independent studies regularly identified inconsistent microbial
91 indicators, due to differences in inocula and substrates usage, geographical sites and/or at
92 different times, and types of digesters.

93 The lack of reproducibility is further accentuated when studies focus on the
94 identification of a single microbial bioindicator. Such univariate perspective is unlikely to
95 shed light into the global and complex ecosystem of AD.

96 Our study aims at identifying a robust microbial signature, consisting in multiple
97 microbial bioindicators, reflective of the interaction network within anaerobic digesters whilst
98 leveraging on in-house and other existing studies. The analytical challenge was to combine
99 such independent studies plagued by unwanted variation (e.g. different substrates and types of
100 digesters) that outweigh the interesting biological variation of ammonia and phenol
101 inhibitions. We applied a recently developed integration method MINT (Multivariate
102 INTegrative) (Rohart et al., 2017a) that provides an integrated view of anaerobic digester
103 microbiota subject to distinct types of inhibition. By integrating two independent in-house
104 experiments, we identified reproducible microbial bioindicators that characterize ammonia
105 inhibition, phenol inhibition and no inhibition. We evaluated this model by predicting AD
106 status on two external studies assessing the influence of ammonia on AD. By doing so, we
107 demonstrate the feasibility of detecting robust indicators evidencing the microbial symptoms
108 of AD process dysfunction in independent studies which suggests promising applications in
109 various biotechnologies thanks to the expansion of data deposited in public databases.

110 2 Materials and methods

111 2.1 Experimental data

112 Four studies assessing the influence of ammonia or phenol on AD were selected to build
113 the predictive models (Lü et al., 2016; Peng et al., 2018; Poirier & Chapleur, 2018a; Poirier &
114 Chapleur, 2018b). We selected these studies as they describe the effect of different levels of
115 the previously mentioned inhibitors on both the performances of the digesters and the
116 dynamics of the microbiome through 16S metabarcoding. Additionally, for each study, raw
117 sequencing data had been deposited in publicly available databases and could be linked to the
118 samples described in the original papers with no ambiguity.

119 In all studies, gas productions were measured to evaluate whether AD was inhibited. As
120 described in supplementary material, studies 1 and 2 were conducted in our laboratory with
121 the same type of substrate (biowaste) but with two different inocula collected one year apart
122 from an industrial mesophilic digester treating wastewater treatment sludge. Samples were
123 taken across time and under different inhibitory conditions. DNA was extracted and 16S
124 rRNA gene was sequenced providing datasets of raw sequences associated to different
125 inhibitory conditions. Study 1 aimed at assessing in parallel the effect of ten different levels of
126 total ammonia nitrogen (TAN) (from 0 to 50 g TAN/L) and phenol (from 0 to 5 g/L) on the
127 microbial community of batch anaerobic digester (Poirier & Chapleur, 2018b). Study 2
128 assessed the influence of support media addition to mitigate AD ecosystem inhibition in
129 presence of two inhibitory conditions (19 g/L of TAN and 1.5 g/L of phenol, respectively)
130 (Poirier & Chapleur, 2018a). Studies 3 and 4 were conducted in two distinct external
131 laboratories. Study 3 was conducted by Lü *et al.* using as inoculum an anaerobic granular
132 sludge collected from a plant-scale 35°C upflow anaerobic sludge bed reactor (Shanghai,
133 China) with liquid internal recirculation that was treating paper mill wastewater. They

134 evaluated the effectiveness of biochar of different particle sizes in alleviating different
135 ammonia inhibition levels (0, 3.5 and 7 g TAN/L) during AD of 6 g/L glucose (Lü et al.,
136 2016). Study 4 by Peng et al. used an inoculum collected from a lab-scale, high-solids
137 anaerobic digester and sought for microbial community changes during inhibition by
138 ammonia (from 2.7 to 3.6 g TAN/L) in high solid AD of food waste in a continuous stirred-
139 *tank* reactor (Peng et al., 2018).

140 In order to train an accurate and relevant MINT model, we removed some samples from
141 studies 1 and 2 that were deemed non-representative of our analytical objectives (listed in
142 supplementary material. In studies 1 and 2, only samples collected after at least 10 days of
143 incubation were retained to ensure that the microbial community was representative of the
144 inhibitory conditions. Samples taken after more than 60 days of incubation were removed as
145 biogas production was completed. Moreover, for studies 1 and 2, methane cumulated
146 production data were fitted to a modified Gompertz three-parameter model (Eq. (1)) where
147 $M(t)$ is the cumulative CH₄ production (mL) at time t (d); P is the ultimate CH₄ yield (mL);
148 R_{\max} is the maximum CH₄ production rate (mL/d); λ is the lag phase (d); e is the mathematical
149 constant (also known as Euler number):

$$150 \quad M(t) = P \times \exp \left\{ -\exp \left[\frac{R_{\max} \times e}{P} \times (\lambda - t) + 1 \right] \right\} \text{ (Eq.1)}$$

151 Reactors were deemed inhibited when R_{\max} was less than 80% of R_{\max} in the controls
152 without inhibitor, and not inhibited when R_{\max} was greater than 90% of R_{\max} in the controls
153 without inhibitor. In study 1, samples from reactors incubated with 250 or 500 mg/L of phenol
154 and 5.0 g/L of ammonium were discarded as the inhibition status (inhibited-non-inhibited)
155 was not well defined. Samples collected from batch digesters incubated with 50 g/L of
156 ammonium or 5 g/L of phenol were removed as these incubations were totally inhibited and
157 microbial community did not evolve. In total 81 samples remained in studies 1 and 2, taken in

158 35 different digesters. All samples from study 3 and bacterial samples from study 4 were
159 retained and their inhibition status predicted with the model (respectively 37 and 10, table
160 S2).

161 2.2 Data processing

162 In these four studies, sequencing of the V3-V4 or V4-V5 region of the 16S rRNA gene
163 was performed with three different approaches, as described in supplementary material . Data
164 from external studies were downloaded from NCBI with fastq-dump 2.8.1. Paired-end reads
165 from Lü *et al.*, and Peng *et al.*, studies were merged with pear v0.9.11 (Zhang et al., 2014).
166 Adapters from each study were specifically removed with cutadapt v1.12 (Martin, 2011). All
167 sequences were imported into FROGS pipeline (Find, Rapidly, Otus with Galaxy Solution)
168 (Escudié et al., 2018) . Samples from studies 1 and 2 were processed together while studies 3
169 and 4 were processed independently because of the differences in sequencing approaches.
170 Taxonomic assignment of Operational Taxonomic Units (OTUs) was performed using Silva
171 132 SSU as reference database. OTUs were trimmed by keeping only those present more than
172 10 times in the whole dataset (resp. 1133, 399, 158 OTUs for studies 1 and 2, 3, 4). For joint
173 analysis of data from studies 1 and 2, data was processed as obtained. For joint analysis of
174 studies 1, 2, 3 and 4, the three distinct biom files were concatenated and data were discussed
175 at the genus level. Sequences of interest were then assigned at the species level using the
176 Blastn+ algorithm (Camacho et al., 2009).

177 2.3 Statistical analyses and predictive model

178 OTUs abundances were scaled with total sum scaling to account for uneven sequencing
179 depth. OTUs that exceeded 3% in at least one sample were retained for the analysis. The total
180 relative abundance of these minor OTUs represented 17% of the total number of sequences.

181 Data were then transformed with centered log ratio (CLR) transformation to account for
182 compositional structure of the scaled data. All statistical analyses were implemented with
183 mixOmics R package, as described in (Rohart et al., 2017b).

184 In order to obtain a first understanding of the major sources of variation in the training
185 data (studies 1 and 2), and to obtain a first insight into the similarities between samples, we
186 conducted principal component analyses (PCA) on the 16S rRNA tags datasets (pca function).
187 A sparse Partial Least Squares Discriminant Analysis (Sparse PLS-DA) was then conducted
188 to assess the potential to discriminate the samples according to the type of inhibition (Lê Cao
189 et al., 2016) (sPLS-DA function) and identify microbial signatures characterizing inhibition
190 type. Classification accuracy was calculated based on the microbial signature identified by the
191 method, as described in (Rohart et al., 2017b). Finally, the MINT sPLS-DA method (referred
192 to as MINT in the following), that generalizes sPLS-DA while accounting for study-specific
193 effects was applied (Rohart et al., 2017a) (mint.splsda function). Parameters to tune in MINT
194 included the number of PLS-DA components, and the number of variables to select, which
195 was performed using 10-fold cross-validation. The final MINT model was then fitted on the
196 data, and the classification performance was estimated using the perf function and 10-fold
197 cross-validation repeated 10 times. Graphical display of the discriminative OTU signature
198 identified by MINT were output using clustered image maps (cim function). The multivariate
199 model not only identifies a microbial signature characterizing inhibition status, but it also
200 enables to predict the groups of samples from external data sets as described in detail in
201 (Rohart et al., 2017b). For prediction, we trained another MINT model on studies 1 and 2 for
202 conditions ammonia/no inhibition and predicted the inhibition status of the test samples
203 (studies 3 and 4) using the predict.mint.splsda function. The prediction area was visualized
204 with a colored background on the sample plot, as described in (Rohart et al., 2017b). Code
205 and functions used for data analysis are described in (Rohart et al., 2017a; Rohart et al.,

206 2017b) and available at <http://mixomics.org/> and <https://gitlab.irstea.fr/olivier.chapleur/mint->
207 [bioindicators/](#).

208 3 Results and discussion

209 3.1 Integration of independent studies to identify microbial bioindicators

210 3.1.1 Inhibition status classification of digesters according to methane production
211 performance

212 A total of 81 samples were selected from studies 1 and 2. These samples were collected
213 in 35 distinct digesters (Poirier & Chapleur, 2018a; Poirier & Chapleur, 2018b). Prior to
214 identifying potential bioindicators characteristic of both type of inhibition, inhibition status of
215 each digester (non-inhibited, inhibited by phenol or inhibited by ammonia) has been
216 characterized. For this purpose, maximum CH₄ production rate (mL CH₄/day) was chosen as
217 the most informative performance indicator. These values were calculated for each digester
218 using Grofit package of R software (version 3.1.2) in both previous studies(Poirier &
219 Chapleur, 2018a; Poirier & Chapleur, 2018b). To integrate both studies, we decided that
220 samples were inhibited as soon as maximum CH₄ production rate decreased by more than
221 20% compared to control and not inhibited if CH₄ production rate decreased by less than
222 10%. Figure 1 presents boxplots describing the distribution of the relative decrease of
223 maximum CH₄ production rate of each digester according to their inhibition status.

224 According to this threshold, we determined that 29 samples were non-inhibited whereas
225 24 samples were inhibited by phenol and 28 samples by ammonia. In study 1, samples were
226 non-inhibited as soon as initial inhibitor concentration remained lower than 0.1 g/L of phenol
227 or 2.5 g/L of TAN. In digesters inhibited by ammonia and phenol a decrease by respectively
228 20 to 60% and 20 to 80% of methanogenic activity was observed. In study 2, regardless
229 support addition, all digesters facing 19g/L of TAN were considered as inhibited (decrease of
230 methanogenic activity by 60 to 90%). In presence of 1.5g/L of phenol, only digesters
231 supplemented with activated carbons were considered as non-inhibited.

232 3.1.2 MINT modelling accounts for study effect

233 Considering, the inhibition status classification according to methane production
234 performance, PCA was performed on the data (Fig. 2A), for a first exploration of the major
235 sources of variation in the data. Sample distribution highlighted a strong study effect. Samples
236 on the left part of the individual plot were related to study 1 conducted with the inoculum A
237 while samples collected during study 2 conducted with inoculum B were on the right side of
238 the individual plot.

239 However, a clear influence of the type of inhibition on microbial community could still
240 be observed. For both studies, ecosystems facing ammonia inhibition were strongly
241 discriminated from samples that were non-inhibited or inhibited by phenol. Similarly, within
242 the study 1 conducted without support media, samples collected from batch digester inhibited
243 by phenol were separated from non-inhibited samples.

244 A supervised PLS-DA model was then fitted on the data. Sparse version of the method
245 was applied to select features and to identify discriminative OTUs that best described the
246 difference between groups of samples. In order to conduct sPLS-DA, parameters such as the
247 number of components, and the number of OTUs to select must be specified. We set these
248 parameters based on the classification performance of sPLS-DA using cross-validation.
249 Thirty-nine OTUs were thus selected by sPLS-DA and achieved a balanced error rate to 7.0%.
250 Samples distribution based on the first two components is presented on Fig. 2B. As expected,
251 sPLS-DA enabled to mitigate the study effect compared to the unsupervised PCA. However,
252 within each condition, the study effect was still present: each sample collected in Study 1 was
253 clearly separated from the ones collected in Study 2.

254 In order to counteract this bias, we applied MINT that combines independent studies
255 measured on the same OTU predictors and identifies reproducible bioindicator signatures

256 across heterogeneous studies. As described above for sPLS-DA, we chose the optimal number
257 of components and number of OTUs to select based on cross-validation, resulting in 45 OTUs
258 and achieved a balanced error rate to 9.2% (2 components) (supplementary material). Samples
259 representation from MINT is presented in Fig. 2C. It evidenced that the study effect was
260 accounted for, with the strongest separation observed according to inhibiting condition rather
261 than studies. The classification error rate of the final MINT model was 9.2% confirming the
262 good performance of MINT to classify our samples and identify a microbial signature.

263 3.1.3 Analysis of microbial community

264 Microbial signatures identified with MINT were output in a clustered image map (or
265 heatmap, 81 samples and 45 OTUs) in Fig. 3. This representation confirmed that, based on
266 their microbial community composition, samples could be grouped by inhibition type (non-
267 inhibited samples, samples inhibited by ammonia and samples inhibited by phenol).
268 Moreover, the 45 OTUs selected by MINT were clustered into five different groups with a
269 hierarchical clustering algorithm. The first group (Group A) was composed of 9 OTUs which
270 were specifically correlated to digesters inhibited by phenol. Similarly, a second group of 17
271 OTUs (Group E) was associated to samples inhibited by ammonia. In group D, 6 OTUs were
272 characteristic of both inhibitory conditions (phenol and ammonia). Group C included of 6
273 OTUs characterizing non-inhibited ecosystems while Group B was composed of 7 OTUs not
274 recovered under ammonia inhibition. Interestingly, 6 of the 7 OTUs recovered in Group B
275 were found in samples where phenol degradation was advanced. Consequently, the presence
276 of these OTUs in this group may be explained by the variability of the inhibitory pressure
277 throughout the incubation because of phenol degradation, and thus to their resilience capacity
278 after phenol inhibition. Our following results are reported at the genus level, which was the
279 most precise taxonomic level we could obtain with 16S rRNA sequencing.

280 3.1.3.1 Genera correlated to non-inhibitory conditions

281 The only discriminative archaeal OTU evidenced by the model, belonging to
282 *Methanosarcina* genus, was negatively correlated to ammonia inhibition. Its belonging to
283 Group B indicated that this genus was also inhibited by phenol but could still grow once
284 phenol was degraded, confirming that this genus is characteristic of non-inhibitory conditions.
285 *Methanosarcina* genus is known to play a key role in AD of many feedstocks (FitzGerald et
286 al., 2015). However, although its robustness has been evidenced during different disruption
287 situations (De Vrieze et al., 2012; Lins et al., 2014), it appeared that a drop of its relative
288 abundance can reveal the presence of inhibitors such as ammonia or phenol in digesters.
289 OTU_156 and OTU_6 assigned to *Clostridiaceae* 1 family and more precisely to
290 *Caloramator* and *Clostridium butyricum*, strictly anaerobic acetogenic species described as
291 butyrate producer (Cassir et al., 2016) were also correlated to non-inhibitory conditions. This
292 result confirmed the observation that was made for studies 1 and 2 during which butyrate
293 production was inhibited by phenol and ammonia (Poirier et al., 2016a; Poirier et al., 2016b).
294 While butyrate, acetate and propionate are regularly mentioned as indicators of process
295 imbalance (Ahring et al., 1995), our study tends to indicate that early variations in butyrate
296 producers abundances could also be used as a bioindicator of inhibition. Interestingly, a single
297 member of *Chloroflexi* phylum (OTU_26) kept for our model was also greatly associated to
298 digesters for which phenol degradation had occurred. It was only assigned to the family
299 *Anaerolinaceae*, which is widespread in full-scale digesters (Kirkegaard et al., 2017). Its
300 filamentous form was suggested to favor synergistic relationship with archaeal populations
301 such as *Methanosaeta* also known to be inhibited by phenol and ammonia (McIlroy et al.,
302 2017). *Cloacimonetes* phylum (formerly known as WWE1) was also specifically and strongly
303 correlated to digesters where phenol degradation was advanced. It was represented by three
304 distinct OTUs (OTU_11, OTU_35 and OTU_77). Only OTU_77 could be assigned at the

305 genus level as *Cloacomonas*. As *Anaerolinaceae*, this genus was recovered in various studies
306 analyzing methanogenic sludge which suggested it was a syntrophic bacterium capable of
307 degrading propionate, amino acids and cellulose (Regueiro et al., 2015). Another study
308 indicated that this genus was sensitive to different inhibitions and notably to the antibiotic
309 monensin which is released in cow manure and recovered in anaerobic digester (Spirito et al.,
310 2018). The last phylum that was specifically correlated to digesters where phenol was partly
311 degraded was *Thermotogaceae* with a single OTU (OTU_58) assigned to *Petrotogaceae*,
312 which is known to be involved in phenol degradation (Na et al., 2016).

313 3.1.3.2 Genera correlated to a single type of inhibition

314 The model permitted the identification of genera specifically related to each type of
315 inhibition. Interestingly, among the 17 OTUs that were correlated with ammonia inhibition, 9
316 of them belonged to 8 genera exclusively recovered in digesters inhibited by ammonia. They
317 were assigned *Caldicoprobacter*, *Clostridium sensu stricto* 15, *Defluviitalea*,
318 *Tepidimicrobium*, *Vagococcus*, *Tissierella*, *Lachnospiraceae* NK4136 group and to an
319 unknown genus of the *Family XI*. Among them, recent studies found that *Caldicoprobacter*
320 populations were dominant in reactors exposed to high levels of ammonia (Poirier et al.,
321 2016b). Similarly, Rui *et al.*, evidenced that *Tissierella* genus was positively correlated to
322 high concentration of TAN (Rui et al., 2015) while *Defluviitalea* were also dominant in
323 digesters treating animal manure (Ma et al., 2017).

324 For the phenol, three OTUs belonging to *Clostridiales* order and assigned to genus
325 *Sporoanaerobacter* (*Family XI*), to an unknown genus of the *vadinBB60* family and to an
326 unassigned genus of the *Ruminococcaceae* family were evidenced. However, these
327 cosmopolitan families are widely represented in anaerobic digesters.

328 On the other hand, we observed that some genera were similarly associated to digesters
329 inhibited by phenol and to non-inhibited digesters suggesting that they were specifically
330 sensitive to ammonia inhibition but not to phenol inhibition. It was notably the case for OTUs
331 belonging to *Syntrophomonadaceae*, one of the major families of AD ecosystems. It consisted
332 of five OTUs all assigned to *Syntrophomonas* genus but to unknown species. According to the
333 model, three of them were related to non-inhibited digesters while two others were linked to
334 phenol inhibition. *Syntrophomonas* are obligate anaerobic and syntrophic bacteria, which
335 have the ability to oxidize saturated fatty acids, which is expected to enhance VFAs
336 consumption. These functions are crucial in AD process. Thus, this result tended to confirm
337 that under inhibitory conditions, reorganizations within the *Syntrophomonas* populations
338 which carry these functions occur, thus confirming that the plasticity of the ecosystem is
339 directly responsible for its resistance and resilience capacities (Shade et al., 2012). Notably,
340 the high functional redundancy among *Syntrophomonas* species seemed to allow the
341 preservation of global metabolic chain and methane production.

342 3.1.3.3 Genera correlated to both inhibitions

343 A few bioindicators were also associated to both types of inhibition. It was notably the
344 case for the four OTUs belonging to genus *Bacteroidetes*, as well as for both couples of OTUs
345 belonging to family *Porphyromonadaceae* and assigned to *Petrimonas* and *Proteiniphilum*.
346 These three genera have been acknowledged to play an important role in degrading complex
347 carbohydrates and proteins and catalyzing the production of VFAs and CO₂. Furthermore, the
348 maintenance of important percentages of *Bacteroidetes* within a digester has already been
349 suggested to be responsible for the ability of the anaerobic microbiota to counteract
350 disturbances such as shock loadings (Regueiro et al., 2015). Similarly, within *Clostridiales*
351 order, genera *Anaerosalibacter*, *Mobilitalea*, *Peptostreptococcus* and two unknown genera of
352 *Lachnospiraceae* family were correlated to both inhibitions. *Lachnospiraceae* and particularly

353 to genus *Mobilitalea* as well as *Peptostreptococcus* and *Anaerosalibacter* were described as
354 resistant to phenol and ammonia inhibition and have been suggested to play important roles in
355 protein hydrolysis (Biddle et al., 2013). They were also reported to hydrolyze a variety of
356 polysaccharides by different mechanisms.

357 3.1.3.4 Clostridiales are key bioindicators of inhibition

358 Interestingly, 25 out of the 45 discriminant OTUs selected in our model belonged to
359 *Clostridiales* order. Among these 25 OTUs, 20 were related to inhibited ecosystems, which
360 tended to indicate that inhibitory pressure by phenol or by ammonia would preferentially
361 select more resistant bacteria affiliated to this order. A dominance of the *Clostridiales* order
362 has been reported by many studies at suboptimal conditions for methanogenesis (increased
363 ammonia or salt concentrations) (De Vrieze et al., 2015). Moreover, the importance of this
364 class is regularly mentioned as crucial in AD process (Joyce et al., 2018). However, the
365 majority of the OTUs belonging to this class could not be specifically correlated with only
366 one type of inhibition. It could be hypothesized that it may be due to the high functional
367 redundancy within these genera and diverse populations as mentioned previously. This
368 limitation was also observed for other phyla and notably for *Bacteroidetes* and *Spirochaetae*.
369 Furthermore, the lack of sequencing precision associated to the short length of 16S regions
370 amplified prevented the affiliation at taxonomic rank such as species or subspecies. Despite
371 the fact that we could not conclude about the correlation between these OTUs and the type of
372 inhibition, the model still highlighted that the emergence of these genera were associated to a
373 selection pressure caused by phenol or ammonia. Notably, it confirmed the negative
374 correlation observed by Heyer *et al.*, between *Methanosarcinales*, which is a marker of steady
375 state and bacterial orders such as *Clostridiales* and *Spirochaetales* (Heyer et al., 2016). It also
376 reinforced the link found by Lee *et al.* between *Spirochaetales* and inhibited ecosystems (Lee
377 et al., 2013).

378 3.2 A predictive model for ammonia inhibition validated in two external studies

379 Samples from external studies 3 and 4 were analyzed with distinct sequencing
380 techniques. Thus, different bioinformatic treatments were applied to raw sequences of each
381 dataset due to differences in sequencing primers and targeted regions. OTUs were
382 subsequently aggregated at the genus level (called ‘clusters’) in order to merge the three
383 datasets. Since both external studies were focused on inhibition by ammonia only, we trained
384 a new MINT model from our in-house samples where we removed the phenol condition. A
385 total of 55 samples collected during studies 1 and 2 were retained, including 29 samples
386 considered as non-inhibited and 26 as inhibited by ammonia.

387 3.2.1 Inhibition status prediction of external samples

388 In order to be consistent with the experimental strategy of the authors, samples from Lü
389 et al., study were categorized into four groups depending on the sampling time and on the
390 inhibitory pressure. Samples collected from digesters non-inhibited with ammonia were
391 gathered in the “No inhibition” group. Samples inhibited with 3g/L of TAN were clustered in
392 the “Ammonia moderate concentration” group. Samples inhibited with 7g/L of TAN were
393 divided into two groups: “Ammonia inhibition, early days” for samples collected at the end of
394 the lag phase and “Ammonia inhibition, final days” for samples collected near the end of the
395 methane-production phase. Similarly, samples from Peng et al., study were categorized into
396 four groups depending on the operational time when they were collected. Samples from day 0
397 to day 127, where methane yield remained stable circa 0.5 mL CH₄/g VS, were clustered in
398 the “No inhibition” group. Samples from day 139 to day 152 were clustered into the
399 “Ammonia inhibition start” group. During this phase, methane yield began to strongly
400 fluctuate and slightly decrease down to 0.4 mL CH₄/g VS. Samples from day 172 to day 212
401 during which methane yield dropped down to 0.25 mL CH₄/g VS, were clustered in the

402 “Ammonia inhibition” group. Both samples from day 223 to day 232 were clustered in the
403 “Ammonia inhibition decrease” group where biogas production restarted while methane yield
404 remained below 0.25 mL CH₄/g VS.

405 3.2.2 Ammonia inhibition model

406 As previously, PCA and sPLSDA were performed on the data (Fig. 4A and Fig. 4B). An
407 optimal number of 17 clusters were selected by the model, leading to a balanced error rate of
408 1.7% for sPLD-DA Sample distribution before applying MINT confirmed the strong study
409 effect. The efficiency of MINT to discriminate the in-house samples into inhibition / no-
410 inhibition conditions is depicted in Fig. 4C for the first two MINT components, with the first
411 (horizontal) component highly discriminative of the inhibitory status of the digester. Yet,
412 three of the inhibited samples were located on the left hand side of the plot. They
413 corresponded to the most inhibited samples of study 1 with the highest concentration of TAN
414 (25 g/L).

415 The OTUs selected in this second MINT model (supplementary material) were
416 consistent with the observation made with the first model in our previous section (Figure 5).
417 Among them, 10 clusters were positively correlated to non-inhibited digesters. Notably, it
418 confirmed that *Clostridium butyricum* (Cluster_6) could be considered as a robust
419 bioindicator of non-inhibitory conditions. Furthermore, clusters assigned to *Cloacimonetes*
420 phylum (Cluster_11, Cluster_43 and Cluster_49) and particularly to genus *Cloacomonas*
421 clearly related to non-inhibited digesters were re-evidenced. Nevertheless, due to the novelty
422 of this phylum, missing reference genomes most probably hampered a deeper taxonomic
423 classification of *Cloacimonetes* species. The ecological function of *Cloacimonetes* is thus not
424 established, but this group is suggested to be only present in mesophilic conditions, involved
425 in amino acid fermentation, syntrophic propionate oxidation and extracellular hydrolysis

426 (Muller et al., 2016). It was also evidenced that its abundance decreased with increasing
427 ammonia levels (Westerholm et al., 2018). We also confirmed that *Syntrophomonas*
428 (Cluster_17) was more represented in absence of ammonia. Moreover, the second model also
429 revealed three new genera considered as discriminant of non-inhibited digesters conditions.
430 These genera were assigned to two families belonging to *Clostridiales* order:
431 *Ruminococcaceae* (Cluster_34 and Cluster_69), and *Christensenellaceae* (Cluster_42).
432 Nevertheless, genera belonging to *Clostridiales* order are known to be involved in various
433 metabolic activities which prevent us from elucidating their specific role under non-inhibiting
434 conditions.

435 On the other hand, seven clusters were strongly linked to ammonia inhibition. Six of
436 them belonged to *Clostridiales* order and notably to genera that were previously identified in
437 the first model as specific markers of this type of inhibition (*Caldicoprobacter*, *Defluviitalea*,
438 *Anaerosalibacter*, *Tepidimicrobium* and *Tissierella*). Another unknown genus belonging to
439 *Family XI* was also discriminant confirming the great resistance of this family to ammonia
440 inhibitory pressure. The last OTU was assigned to *Sphaerochaeta*, which belongs to
441 *Spirochaetales* order. This order was associated to both types of inhibitions in the previous
442 model. Interestingly, the heatmap presented in Fig. 5 emphasized that six of these seven
443 clusters were significantly more abundant in samples collected in digesters inhibited with the
444 highest ammonia concentration, thus reinforcing the robustness of these bioindicators.

445 The bioindicators highlighted by the second model were consistent with those
446 evidenced by the model integrating phenol inhibition, thus confirming the robustness of this
447 statistical analysis.

448 3.2.3 Prediction of the inhibitory status of samples analyzed by external studies

449 This second model was built to predict the inhibitory status of samples collected in two
450 external studies 3 and 4. The prediction results are indicated in supplementary material . In
451 order to visualize external samples distribution in the model, Fig. 6 presents the test samples
452 from studies 3 and 4 projected on the two first components of the trained model, as well as
453 prediction areas (Rohart et al., 2017b).

454 As expected, inhibited samples were separated against the non-inhibited samples on the
455 first component. Two samples (11 and 39) were wrongly predicted as ‘inhibited’. Samples of
456 reactors that just started inhibition (“early days” in Lü et al. and ‘start of the inhibition’ in
457 Peng et al.) were mostly predicted at an intermediary position and predicted as either inhibited
458 or non-inhibited. We hypothesized that microbial community had started to change but was
459 not yet totally characteristic of inhibited reactors. Interestingly, sample 45 (Peng et al., day
460 223, just after inhibitory pressure was lowered) was predicted as inhibited while sample 46
461 (Peng et al. day 232, several days after inhibitory pressure was lowered) was predicted as non-
462 inhibited. This result may illustrate the progressive resilience of the microbial community
463 after the inhibition. Sample 14 (moderate ammonia, final days, no addition of activated
464 charcoal) was predicted as inhibited while samples 13, 15, 16 (moderate ammonia, but early
465 days or addition of activated charcoal) were predicted as non-inhibited, in agreement with the
466 conclusions of the authors. Finally, taking into account all the samples from digesters clearly
467 non-inhibited (15) or clearly inhibited by ammonia (13) we estimated that the model predicted
468 the inhibitory status of external samples with an accuracy of 93% as only two samples were
469 incorrectly predicted.

470 3.3 Further perspectives for anaerobic digesters management

471 16S rRNA gene sequencing have revolutionized environmental biotechnology research
472 and helped to progressively unravel the complexity of AD inhibition, but we still need to
473 improve the resilience of AD systems and promote its implementation at a larger scale to
474 avoid system failure. Our study focused on identifying robust microbial indicators from 16S
475 sequencing data by integrating two in-house studies. We built a multivariate model that was
476 highly predictive of inhibition status, as demonstrated in two external studies.

477 Despite the complexity and the functional redundancy of the microbial community
478 within digesters, our model revealed the feasibility of detecting key indicators evidencing the
479 state of the AD process whilst addressing the challenge of study-specific effects. The
480 microbial indicators we identified were separate from cosmopolitan OTUs that tended to co-
481 occur in all conditions. Thus, they can be considered as bioindicators to announce early signs
482 of process dysfunction in anaerobic digesters, or, on the contrary, indicate steady functioning.
483 Additionally these bioindicators are specific of one type of inhibitor, phenol or ammonia. Our
484 study also emphasized on the benefit of using multivariate methods to identify a microbial
485 signature from a training dataset (here our two in-house studies) then predict the inhibitory
486 status of external studies despite differences in sequencing primers and targeted regions.

487 Future investigation will include a finer definition of the role of the identified
488 microorganisms in AD process. As 16S reference databases are currently still incomplete,
489 taxonomic assignment at the genus level results in a substantial lack of data interpretation.
490 Consequently, the use of multiple marker genes DNA gyrase subunit B (*gyrB*) (Poirier et al.,
491 2018), RNA polymerase subunit B (*rpoB*) (Case et al., 2007), DNA recombinase protein
492 (*recA*), protein synthesis elongation factor-G (*fusA*), and dinitrogenase protein subunit D
493 (*nifD*) (Holmes et al., 2004) could improve the assignation and abundance estimation for
494 crucial taxonomic groups. Moreover, the use of shotgun metagenomic and metatranscriptomic

495 tools will also be useful to obtain different type of information such as functional
496 bioindicators.

497 When successful, integrative multi-studies analysis not only enables to increase sample
498 size and statistical power, but also to share and re-use data deposited in public databases. Our
499 proposed approach will benefit the research community interested in identifying reproducible
500 microbial signatures. Given the uncertainty related to inhibition thresholds in individual
501 reactors, identifying robust microbial biomarkers can improve digester management. An
502 increasing number of 16S sequences databases is becoming available to build predictive
503 models of different types of inhibition, irrespective of the digesters operating conditions.
504 Leveraging on these data will allow to optimize the prediction quality by recursively updating
505 monitoring model. We anticipate that a single metagenetic sequencing will reduce the number
506 and the complexity of analyses targeting different inhibitors, as bioindicators identified could
507 be correlated to different types of inhibitions. Moreover, miniaturization and portability of
508 sequencers will soon allow high frequency on-line measurements of microbial dynamics,
509 involving low workload, and limited sampling issues (Shaffer, 2019). Such tools could be
510 very useful in the daily management of AD systems and complement the existing
511 physicochemical-based management. They can provide support to detect early warning
512 indicators of process failure but also enhance the process performance by implementing the
513 most suitable operating conditions for microbial communities.

514 4 Conclusion

515 A robust microbial signature of AD inhibition by ammonia and phenol was identified by
516 integrating two independent 16S metabarcoding studies with the multivariate approach MINT.
517 The model based on the identified biomarkers was successful in predicting ammonia
518 inhibition in independent digesters. This outcome highlights the potential of our approach to
519 for other inhibitors and studies to discover new warning bioindicators. These data could
520 thereafter be aggregated and used to build robust statistical models of AD inhibition to detect
521 instabilities from various sources at early stages in industrial plants, and ultimately improve
522 management of AD.

523

524

525 **Data availability:** The sequencing data of study 1, 2, 3 and 4 are respectively available in the
526 bioprojects PRJNA450311, PRJNA450313, PRJNA253784 and PRJNA324313 in the NCBI
527 BioProject database.

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534 Link for this preprint is <https://www.biorxiv.org/content/10.1101/2020.03.16.993220v2.full>

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689 Figures legends:

690 Figure 1: Relative maximal specific methanogenic activity in the digesters of studies 1 and 2.
691 The different boxplots correspond to the different groups of digesters in each study. Relative
692 maximal specific methanogenic activity was calculated as the ratio of maximal specific
693 methanogenic activity in a digester divided by the maximal specific methanogenic activity in
694 controls without inhibitor.

695 Figure 2: (A) Principal Component Analyses (PCA), (B) Sparse Partial Least Squares
696 Discriminant Analysis, (C) Multivariate Integrative Sparse Partial Least Squares Discriminant
697 Analysis of OTUs distribution in samples from studies 1 and 2, inhibited by phenol, ammonia
698 or not inhibited. On the factorial maps each sample is represented with a coloured marker.
699 The colour scale represents the type of inhibitor. The type of marker represents the study.
700 OTU data was generated by 16S rRNA gene sequencing.

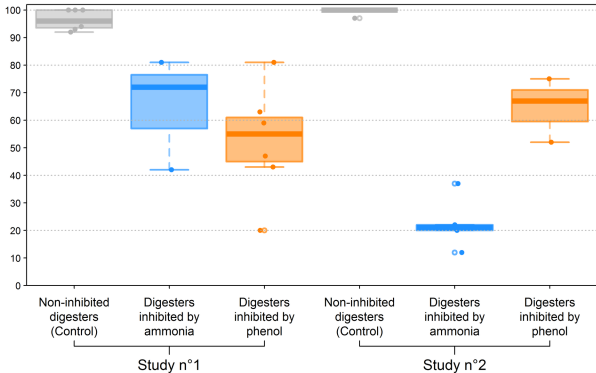
701 Figure 3: Heatmap of the most discriminant OTUs. Heatmap was built after selection of the
702 most discriminant OTUs with Multivariate Integrative Sparse Partial Least Squares
703 Discriminant Analysis of all OTUs generated by 16S rRNA gene sequencing for the different
704 samples of studies 1 and 2, inhibited by phenol, ammonia or not inhibited. Name of the OTUs
705 is indicated at the bottom. The color scale on the left represents the type of inhibitor. The
706 color key of the heatmap shows the abundance of the OTUs after CLR transformation (from
707 blue = low abundance to brown red = high abundance).

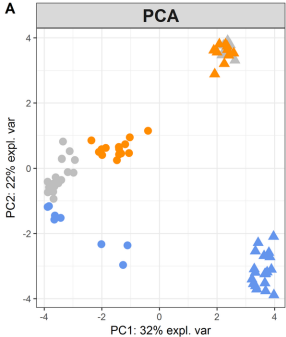
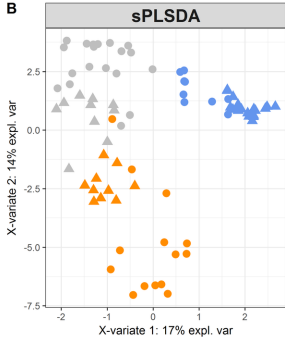
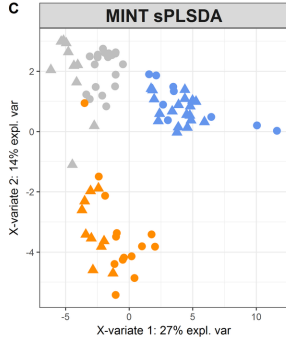
708 Figure 4: (A) Principal Component Analyses (PCA), (B) Sparse Partial Least Squares
709 Discriminant Analysis, (C) Multivariate Integrative Sparse Partial Least Squares Discriminant
710 Analysis of taxonomic distribution (genus level) in samples from studies 1 and 2, inhibited by
711 ammonia or not inhibited. On the factorial maps each sample is represented with a coloured
712 marker. The colour scale represents the type of inhibitors. The type of marker represents the
713 study. Taxonomic data was generated by 16S rRNA gene sequencing and aggregation of the
714 data at the genus level.

715 Figure 5: Heatmap of the most discriminant clusters of OTUs. Heatmap was built after
716 selection of the most discriminant clusters with Multivariate Integrative Sparse Partial Least
717 Squares Discriminant Analysis of all clusters generated after OTUs aggregation at the genus
718 level for the different samples of studies 1 and 2, inhibited ammonia or not inhibited. Name of
719 the clusters is indicated at the bottom. The color scale on the left represents the type of
720 inhibitor. The color key of the heatmap shows the abundance of the clusters after CLR
721 transformation (from blue = low abundance to orange = high abundance).

722 Figure 6: Projection of samples from studies 3 and 4 in the individual plot determined after
723 Multivariate Integrative Sparse Partial Least Squares Discriminant Analysis of samples from
724 studies 1 and 2. Each sample from studies 3 and 4 is represented by a marker. Type of marker
725 indicates the study. Color of the marker indicates the inhibition status in the reactor where the
726 sample was taken. A prediction area, based on studies 1 and 2 was calculated and is plotted on
727 the graph. The different figures indicate remarkable samples.

Relative maximal specific
methanogenic activity (expressed in %)



A**B****C**

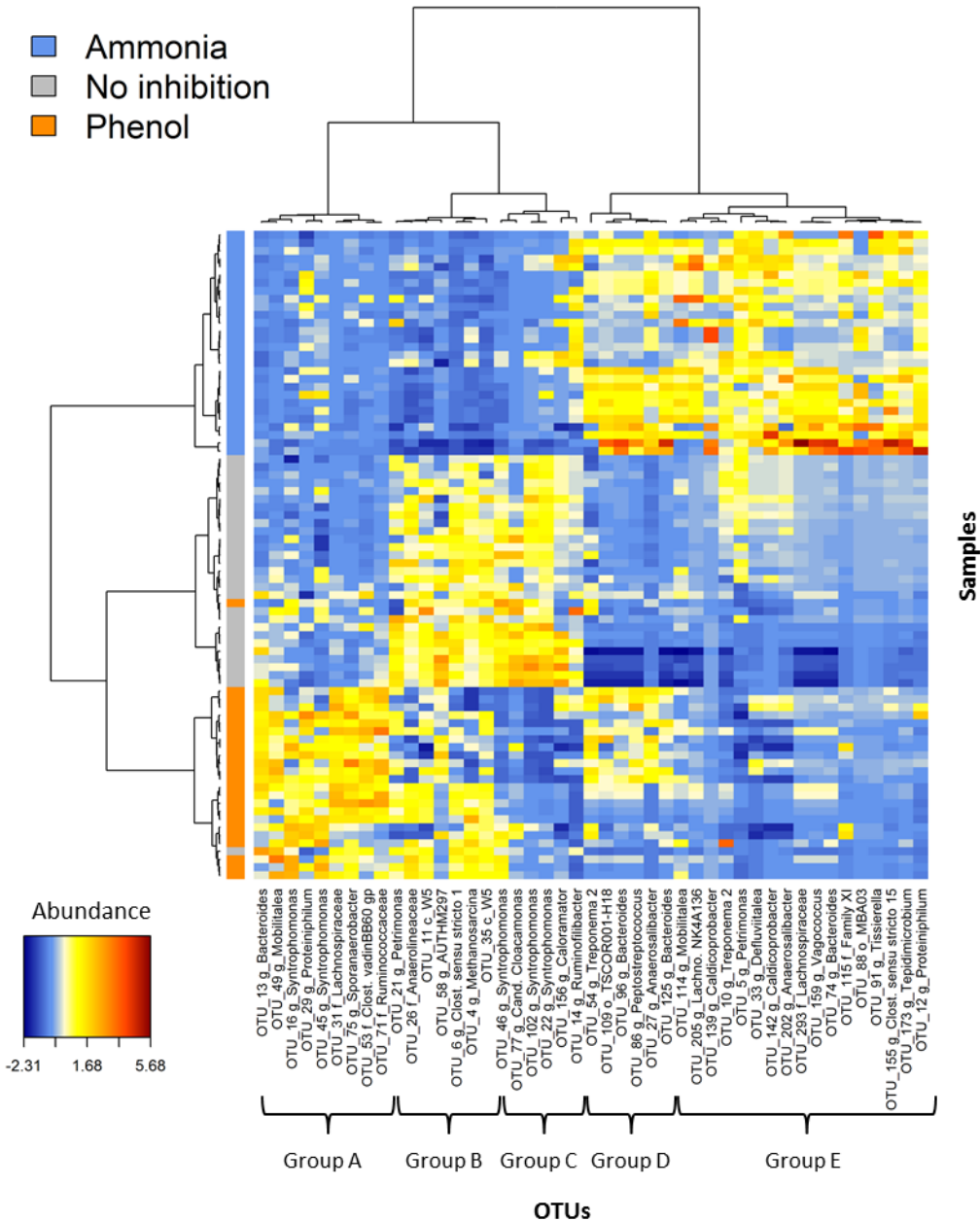
Inhibitor

- Ammonia
- No inhibition
- Phenol

Experiment

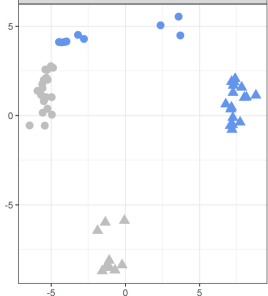
- Study 1
- ▲ Study 2

- Ammonia
- No inhibition
- Phenol



A

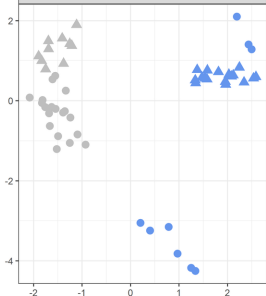
PC2: 24% expl. var

PCA

PC1: 46% expl. var

B

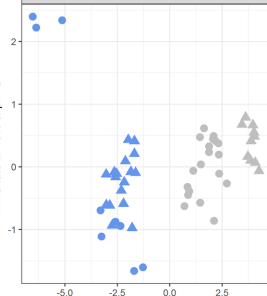
X-variate 2: 17% expl. var

sPLSDA

X-variate 1: 31% expl. var

C

X-variate 2: 10% expl. var

MINT sPLSDA

X-variate 1: 38% expl. var

Experiment

● Study 1

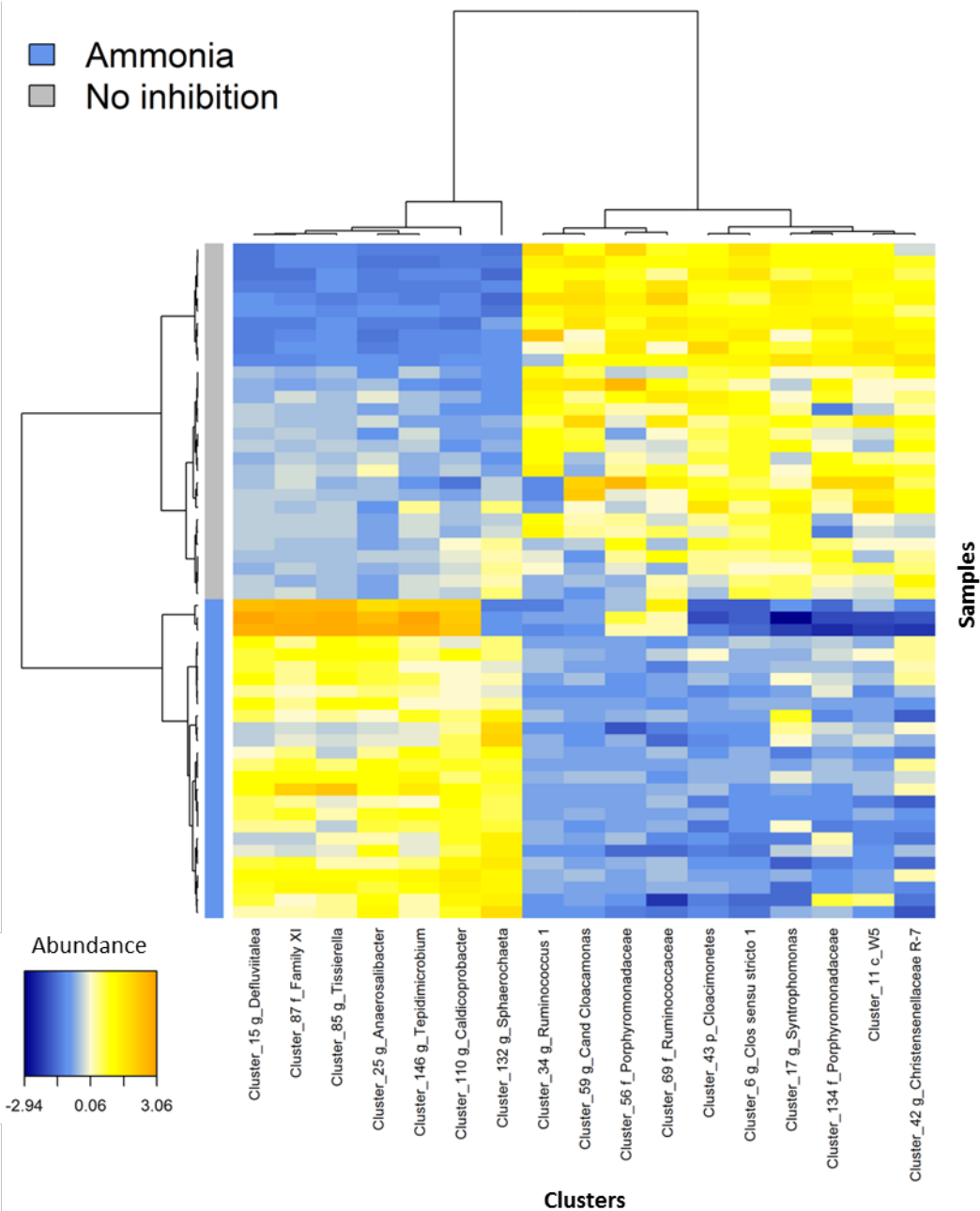
▲ Study 2

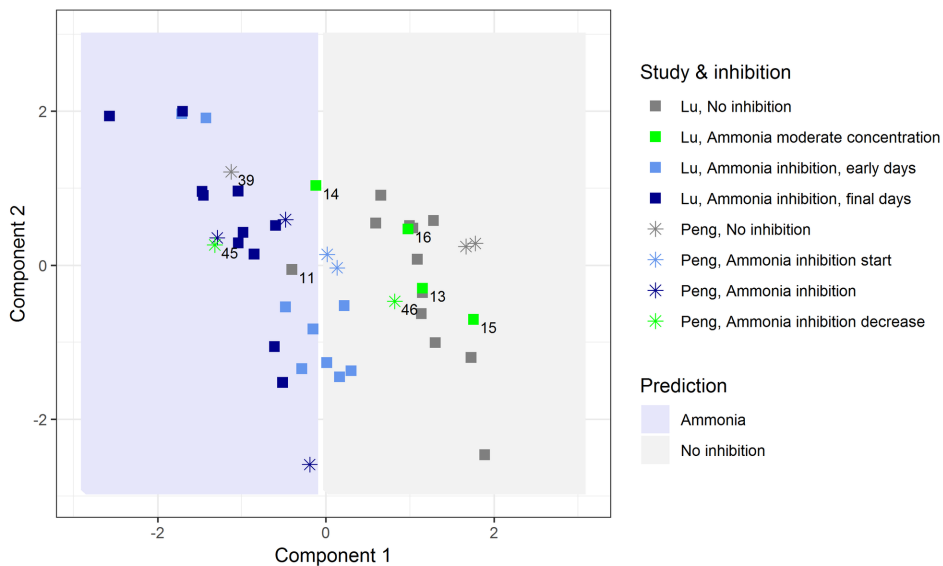
Inhibitor

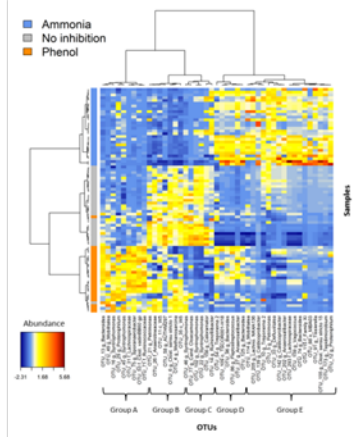
● Ammonia

● No inhibition

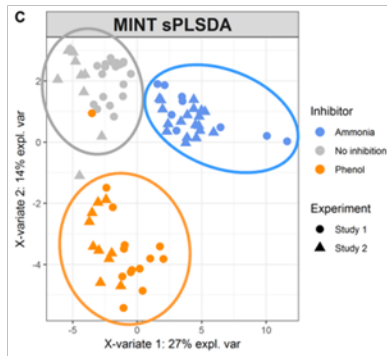
Ammonia
No inhibition



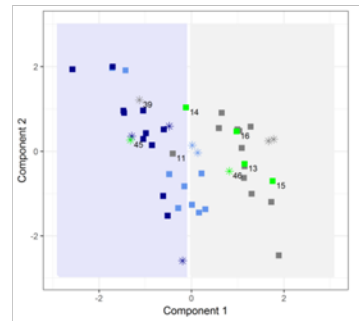
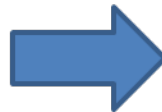




Identification of biomarkers of inhibition



Integrative analysis of data from independent studies



Prediction of the inhibition in external digesters