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1 **Unraveling the literature chaos around free ammonia inhibition in anaerobic digestion**

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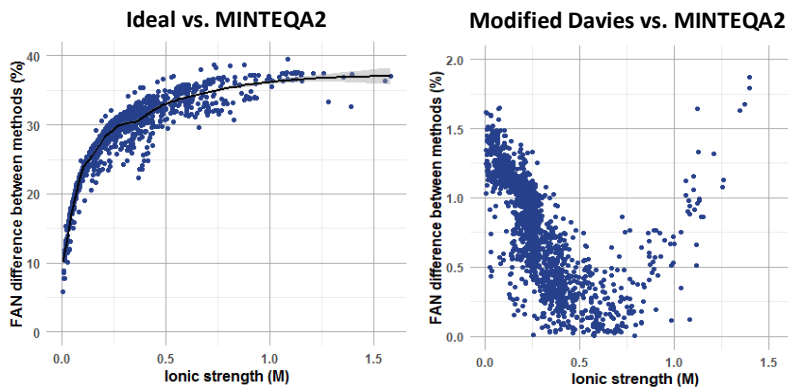
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16 **Abstract**

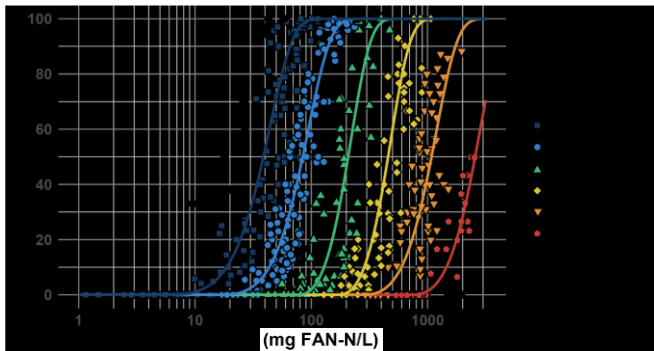
17 This review aims at providing a unified methodology for free ammonia nitrogen (FAN)
18 calculation in anaerobic digesters, also identifying the factors causing the huge disparity in
19 FAN inhibitory limits. Results show that assuming ideal equilibria overestimates the FAN
20 concentrations up to 37 % when compared to MINTEQA2 Equilibrium Speciation Model,
21 used as reference. The Davies equation led to major improvements. Measuring the
22 concentrations of NH_4^+ , Na^+ and K^+ was enough to achieve major corrections. The best

23 compromise between complexity and accuracy was achieved with a novel modified Davies
24 equation, with systematic differences in FAN concentrations of 2 % when compared to
25 MINTEQA2. Applying this modified Davies equation, data from the literature (1,590 data
26 points from over 50 scientific studies) were used to recalculate FAN inhibitory limits using a
27 clustering approach. This procedure allowed to link inhibition resilience with operational
28 conditions and microbial communities, providing also generalized values of inhibitory
29 constants. The results showed that pH and temperature are the main factors affecting FAN
30 inhibition. Consequently, thermophilic systems have a higher resilience towards FAN
31 inhibition. The clustering results showed that *Methanosaeta*-dominated reactors have the
32 lowest resilience towards FAN, verifying the relatively low inhibition limits for acetoclastic
33 archaea. Mixotrophic *Methanosarcina* dominated at intermediate FAN concentrations, being
34 more resistant than *Methanosaeta* but more vulnerable than hydrogenotrophic archaea.
35 *Methanoculleus* appeared as the most resilient methanogen. This article provides general
36 guidelines for accurate FAN calculation, explaining also how FAN resilience relates to the
37 operational conditions and the microbial communities, underlying the importance of microbial
38 adaptation.

39 **Graphical abstract**



- Assuming ideal equilibrium overestimates FAN concentrations up to 37 %
- A proposed modified Davies equation reduced this error to below 2 %



- FAN concentrations from literature were recalculated using the modified Davies equation
- Clusters identified according to FAN resilience
- Temperature and pH (rather than TAN contents) as main factors affecting FAN resilience
- Acetoclastic methanogens as least resilient archaea. Mixotrophs dominated at intermediate levels. Hydrogenotrophs as most resilient

40

41

42 **Highlights**

- 43 • Ideal equation overestimates the FAN concentration up to 37 %
- 44 • Davies equation considering few ions leads to a mayor improvement in most cases
- 45 • A more accurate modified Davies equation is proposed
- 46 • pH and temperature as main factors determining the resilience to FAN inhibition
- 47 • *Methanosaeta* replaced by hydrogenotrophic archaea at high FAN concentrations

48

49 **Keywords**

50 Biogas; Davies equation; toxicity; ionic strength; modelling; methanogenic archaea

51

52 **Abbreviations and symbols**

53 Ac^- , acetate; AD, anaerobic digestion; ADM1, anaerobic digestion model no. 1; Bu^- , butyrate;
54 C_i , concentration of species i ; COD, chemical oxygen demand; CSTR, continuous stirred tank
55 reactor; f , activity coefficient; FAN, free ammonia nitrogen; GSA, global sensitivity analysis;
56 HRT, hydraulic retention time; I , ionic strength; IC_{50} , 50 % inhibitory concentration; K_a , acid
57 dissociation constant; $K_{a_{25}}$, acid dissociation constant at 25 °C; KI_{max} , concentration where
58 inhibition is almost complete; KI_{min} , concentration where inhibition starts; LHS, Latin
59 Hypercube Sampling; MSW, municipal solid waste; N , number of Monte Carlo runs;
60 OFMSW, organic fraction of municipal solid waste; OLR, organic loading rate; Pr^- ,
61 propionate; R^2 , coefficient of determination; RMSE, root-mean-square error; SAO, syntrophic
62 acetate oxidation; SAOB, syntrophic acetate oxidizing bacteria; SMA, specific methanogenic
63 activity; SRC, standardised regression coefficient; T , temperature; TAN, total ammoniacal
64 nitrogen; TS, total solids; UASB, upflow anaerobic sludge blanket reactor; Val^- , valerate;
65 VFA, volatile fatty acids; z_i , charge of ion i ; β_i , standardised regression slope; ϵ , dielectric
66 constant of water; ϵ_{FAN} , accepted error on X-axis; ϵ_{SMA} , accepted error on Y-axis; ν , reactant or
67 product stoichiometric coefficient.

68

69 **1. Introduction**

70 Anaerobic digestion (AD) is a key technology in the field of environmental engineering. It is
71 widely applied for the treatment of several organic wastes, such as sewage sludge, municipal
72 solid waste, green waste, food waste and animal manure [1, 2,3]. AD is a multistage
73 biological process that besides a proper waste treatment and stabilization, also allows to
74 produce renewable energy in the form of biogas and to recover nutrients (*e.g.* N, P and K) via

75 digestate application. These advantages, together with lower costs and environmental impacts
76 when compared to other treatment options, make AD a major technology for wastewater and
77 waste treatment [4].

78 Nevertheless, AD is a complex process and several factors can affect its performance and
79 stability, such as temperature (T), organic loading rate (OLR), hydraulic retention time
80 (HRT), structure of microbial communities and presence of inhibitors. Ammoniacal nitrogen,
81 including free ammonia nitrogen (FAN, NH_3) and ammonium ion (NH_4^+), is an inhibitor often
82 encountered in AD. Ammoniacal nitrogen is produced during AD due to the reduction of the
83 organic N present in the substrate in the form of proteins, amino acids, urea or nucleic acids
84 [5,6,7]. Thus, high concentrations of total ammoniacal nitrogen (TAN) in AD appear when
85 treating N-rich substrates (*e.g.* manure, slaughterhouse waste or food waste). Besides,
86 modifications in the operational conditions, waste pre-treatment, digestate recirculation and/or
87 co-digestion can also lead to high TAN concentrations [8]. On one hand, TAN is a key
88 macronutrient and therefore a certain concentration is required for a proper microbial growth.
89 TAN also contributes to increase the media buffer capacity and keep the pH stable [1,9,10].
90 On the other hand, TAN concentrations above 1,000-1,500 mg TAN-N·L⁻¹ have been often
91 reported as the primary cause of AD failure due to FAN inhibition [5,11]. However, as
92 addressed in this review, there are no universally applicable inhibition threshold
93 concentrations for FAN inhibition (neither for any inhibitory compound), since the inhibition
94 resilience of a microbial community is influenced by several factors.

95 Although there has been a debate about which microorganisms are more affected by
96 TAN/FAN inhibition in AD, nowadays, it is widely accepted that methanogenic archaea are
97 more vulnerable than most of bacteria, with the exception of some syntrophic acetate

98 oxidizing bacteria (SAOB) [12,13]. Therefore, a decline in the methanogenic activity and AD
99 performance is often observed at high FAN/TAN concentrations [5]. This reduced
100 methanogenic activity typically results in the accumulation of intermediate compounds, such
101 as volatile fatty acids (VFAs), since the decline in activity of fermentative bacteria does not
102 occur to the same extent. This will further inhibit the methanogenic archaea and reduce the
103 methane production rates, causing a feedback inhibition loop that might lead to AD failure
104 [14,15,16]. It has been observed that high levels of both FAN and/or NH_4^+ can inhibit the
105 methanogenic communities [8,17,18]. However, it is generally recognized that FAN is the
106 most inhibitory species. For this reason, the inhibition of AD processes due to the presence of
107 FAN has been widely researched in the last decades, aiming at understanding the toxicity
108 mechanisms involved and developing solutions to this issue [19,20,21].

109 Many studies have researched the influence of both TAN and FAN concentrations on the AD
110 performance. Generally, the change in the specific methanogenic activity (SMA) is measured
111 at different inhibitor concentrations, resulting in an inhibition coefficient representing the
112 concentration at which the SMA is half the maximum (IC_{50}). Previous reviews addressing the
113 topic of FAN/TAN inhibition in AD have reported a huge disparity in the inhibitory limits,
114 which range from 27 to 1,450 mg FAN-N·L⁻¹ and from 1.1 to 11.8 g TAN-N·L⁻¹
115 [5,6,7,8,11,13,22,23] (Table 1). These wide ranges of inhibitory concentrations hinder the
116 extrapolation of the obtained results and restrain the applicability of the given limits to almost
117 each case study. Thus, it is important to include batch inhibition tests as part of AD studies,
118 which provide specific inhibition data for the microbial communities under study [8,23]. It
119 has been suggested that this large variability on FAN inhibition is caused by: (i) the different
120 microbial communities used in the studies (some archaea and/or bacteria are more vulnerable

121 to FAN/TAN than others), (ii) particular operational factors such as temperature, pH and ionic
122 strength (I), (iii) microbial acclimation strategies, and (iv) the different mathematical methods
123 used for FAN calculation [5,8,13,24,25,26].

124 **Table 1.** IC_{50} values for both FAN and TAN reported in the literature (adapted and extended from Chen *et al.* [6] and Yenigün and Demirel [7])

Substrate	Reactor operation	Temperature (°C)	pH	IC_{50} TAN (mg TAN-N·L ⁻¹)	IC_{50} FAN (mg FAN-N·L ⁻¹)	Main archaea after AD	Reference
Acetate + Ethanol	Semi-continuous	35	6.6-7.2	11,780	nr	<i>Methanosarcina</i>	[16]
CO ₂ + H ₂	Batch	nr	6.5	4,200	nr	<i>Methanospirillum</i>	[27]
Acid mixture	Batch	35	nr	nr	106-183	nr	[28]
Sodium butyrate	Batch	nr	6.9-7.0	6,000	nr	<i>Methanobacterium</i>	[29]
OFMSW	CSTR	55	6.5-7.6	1,500	nr	nr	[30]
Cattle manure	UASB	55	7.2	4,000	280	Acetoclastic	[31]
Cattle manure	UASB	55	7.2	7,500	520	Hydrogenotrophic	[31]
OFMSW	CSTR	37	6.7-7.5	3,000	220	nr	[32]
OFMSW	CSTR	55	6.7-7.5	3,000	690	nr	[32]
MSW and sludge	Semi-continuous	39	5.7-8.7	2,300-2,400	nr	nr	[33]
Sewage sludge	Batch	37	6.5-9.0	4,090-5,500	nr	nr	[34]
Glucose	Batch	35	6.8-8.0	2,350-3,650	nr	nr	[35]
Pig manure	CSTR	51	8.0	11,000	1450	nr	[22]
Meat industry wastes	Batch	35	7.6-7.8	1,130	70	nr	[36]
OFMSW	Batch	Mesophilic; thermophilic	7.0-8.5	3,860; 5,600 ¹	215; 468	nr	[37]
Yeast extract	Batch	35	7.7	1,445	27	nr	[38]
Acid mixture	Batch	37	7.0	nr	40	nr	[39]
Slaughterhouse waste	CSTR	55	7.5	5600	635	nr	[40]
Glucose	Batch	nr	7.5-7.7	4000	275	nr	[41]
Food waste	Batch	32	7.2	3800	146	nr	[42]
Acetate	Batch	37	7.0-7.9	nr	86	<i>Methanosaeta</i>	[23]
Acetate	Batch	37	6.8-7.7	2,642-4,385 ¹	53-78	<i>Methanosaeta</i>	[8]
Acetate	Batch	37; 55	6.7-8.4	nr	32-175	<i>Methanosaeta</i>	[43]

125 FAN stands for free ammonia nitrogen, TAN for total ammoniacal nitrogen, nr for “non-reported”, OFMSW for organic fraction of municipal solid waste, CSTR for continuous
 126 stirred-tank reactor, UASB for up-flow anaerobic sludge blanket reactor and OFMSW for municipal solid waste
 127 1. mg NH₄⁺-N·L⁻¹

128 The goal of this review is to analyze and understand the impacts of the environmental and
129 microbial factors causing the disparity in FAN inhibition of AD systems. For this purpose,
130 different algorithms for FAN calculation have been compared, aiming at finding the best
131 compromise between simplicity and accuracy. Recommendations for the application of each
132 method have also been discussed. Applying the FAN calculation method considered to be the
133 most convenient, the FAN inhibitory limits have been recalculated via a clustering approach
134 using data from the literature (1,590 data points). The resulting inhibitory limits were
135 compared in terms of substrate fed in the experiments, operational conditions (*i.e.* T and pH)
136 and predominant archaeal communities. This study gives general indications to achieve an
137 accurate FAN quantification in N-rich AD systems. In addition, the results presented in the
138 literature have been unified, giving inhibitory limits that can be extrapolated to general AD
139 conditions and linking the observed values with the operational conditions and the microbial
140 communities. Although different reviews have already addressed ammonia inhibition in AD
141 [5,44], this is the first study using large amounts of experimental data to produce general,
142 non-specific, results. A unified methodology for FAN calculation and general FAN inhibitory
143 limits can have significant implications in both laboratory and industrial applications,
144 reducing the errors in FAN estimation and helping to explain the behaviors of AD systems
145 working at high FAN concentrations.

146

147 **2. Data collection and treatment**

148 *2.1. Data collection*

149 Both quantitative and qualitative data were retrieved from 50 scientific studies carried out at
150 different operational conditions (*i.e.* T , pH, reactor operation, etc.) and using different

151 microbial inocula. The data retrieved combined with 339 values from an internal industrial
152 project with Suez (Suez S.A., France) as partner (already used for research purposes, see Hao
153 *et al.* [45]), generated a database containing 1,590 data points.

154 To produce a homogeneous database that would allow the analysis of the data retrieved,
155 different categories were defined, *i.e.* inocula type, substrate digested, reactor operation (*e.g.*
156 batch, semi-continuous, CSTR, etc.) and dominant archaeal genus. In addition, different
157 hypotheses were made:

158 - No nitrates were present in the reactors or the substrates (Total Kjeldahl Nitrogen
159 equals total nitrogen).

160 - If not reported otherwise, the oxidation states of the ions were assumed to be the
161 predominant species at anaerobic conditions (Na as Na^+ , Ca as Ca^{2+} , K as K^+ , P as
162 PO_4^{2-} , Mg as Mg^{2+} , Fe as Fe^{2+} , Mn as Mn^{2+} , Mo as Mo^{2+} , Cu as Cu^{2+} , Zn as Zn^{2+} , Co
163 as Co^{2+} , Ni as Ni^{2+} , Se as Se^{2+} and Cl as Cl^-).

164 - If the concentrations of soluble species in continuous reactors were not reported, the
165 values were calculated according to the initial amounts in the reactor and the substrate,
166 the substrate loads, and the retention times given.

167 - If not reported otherwise, the total solids (TS) contents in the reactors were assumed to
168 be constant.

169 - Concentrations below detection limits were considered to be zero.

170 - The reductions in the SMAs were calculated, when possible, as a percentage of
171 decrease in the specific methane production rates when compared to a control reactor
172 or a previous steady state where no (or negligible amounts of) FAN was present.

173 A table comprising all the bibliographic data used in this study and the categories used is

174 given in the supplementary material (Table S1).

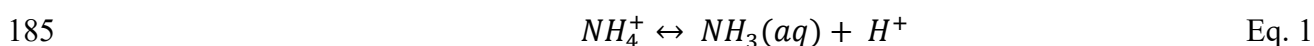
175 2.2. Methods for free ammonia calculation

176 Three different approaches were compared: (i) assuming an ideal solution (ideal equilibrium),
177 (ii) considering a non-ideal solution behavior by taking into account the ionic strength (I) of
178 the media (Davies equation) and (iii) using MINTEQA2 Equilibrium Speciation Model (see
179 Section 2.2.3).

180 2.2.1. Ideal equilibrium

181 The FAN concentrations were calculated combining equations 2 and 3 [46]. This approach
182 assumes that the solute behavior is ideal and that the only species in equilibrium with FAN is
183 NH_4^+ .

184



$$186 \quad \text{FAN} = \frac{K_a \cdot \text{TAN}}{K_a + 10^{-\text{pH}}} \quad \text{Eq. 2}$$

$$187 \quad K_a = K_{a_{25}} \cdot e^{\frac{51965}{R} \left(\frac{1}{298.15} - \frac{1}{T} \right)} \quad \text{Eq. 3}$$

188

189 Where FAN is the FAN concentration in $\text{mg FAN-N} \cdot \text{L}^{-1}$, R is the ideal gas constant (8.314
190 $\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$), TAN is the TAN concentration in $\text{mg TAN-N} \cdot \text{L}^{-1}$, K_a is the acid dissociation
191 constant, $K_{a_{25}}$ is the acid dissociation constant at 25°C ($10^{-9.25}$), and T is the temperature in
192 K.

193 2.2.2. Davies equation

194 The FAN concentrations were calculated using the Davies equation. This approach considers
195 the pH, the T and the I of the media [5]. The Davies equation is based upon the introduction of

196 an activity coefficient (f) as correction factor into the ideal equilibrium equation (Eq. 2),
197 resulting in Equation 4 [47]. The set of expressions used is as follows:

198

$$199 \quad FAN = \frac{K_a \cdot f \cdot TAN}{K_a \cdot f + 10^{-pH}} \quad \text{Eq. 4}$$

$$200 \quad f = 10^{\left(-A \cdot z_i^2 \cdot \left(\frac{\sqrt{I}}{1 + \sqrt{I}}\right) - \lambda \cdot I\right)} \quad \text{Eq. 5}$$

$$201 \quad A = 1.82 \cdot 10^6 \cdot (\varepsilon \cdot T)^{-\frac{3}{2}} \quad \text{Eq. 6}$$

$$202 \quad I = \frac{1}{2} \sum_{i=1}^n C_i \cdot z_i^2 \quad \text{Eq. 7}$$

203

204 Where I is the ionic strength (M), T is the temperature in K, λ is an empirically determined
205 constant (0.2), ε is the dielectric constant of water at the working temperature (74.828 and
206 68.345 at 35 and 55 °C, respectively), C_i is the concentration of the species i (M), and z_i is the
207 corresponding charge. Among the different possibilities for calculating f (see for instance
208 Stumm and Morgan [47]), Equation 5 with λ equal to 0.2 was selected because it is the value
209 valid at the widest range of I values (up to 0.5 M) [47].

210 Four different approaches using the Davies equation were evaluated, comparing the FAN
211 concentrations given by each option.

212 1. *Comprehensive approach*: this method considered the contribution of the main
213 chemical species to I (*i.e.* Na^+ , Ca^{2+} , K^+ , Mg^{2+} , Fe^{2+} , Mn^{2+} , Mo^{2+} , Cu^{2+} , Zn^{2+} , Co^{2+} , Ni^{2+} , Se^{2+} ,
214 PO_4^{2-} , Cl^- , $\text{C}_2\text{H}_3\text{O}_2^-$, $\text{C}_3\text{H}_5\text{O}_2^-$, $\text{C}_4\text{H}_7\text{O}_2^-$ and $\text{C}_5\text{H}_9\text{O}_2^-$). These species were selected because
215 they are the most common ions present in anaerobic media [5,48]. The VFAs were also
216 considered. To account for the counterions that are generally not measured (*i.e.* HCO_3^- or

217 CO_3^{2-}), a monovalent ion was added into the calculation of I . The concentration of this
218 counterion was calculated as the missing amount required to close the charge balance. In
219 addition, since the concentration of NH_4^+ affects the value of I , an iterative process was
220 implemented by a loop recalculating the NH_4^+ concentration according to the previous FAN
221 content.

222 2. *Simplified I calculation with iterative NH_4^+ correction:* this approach is similar to the
223 previous one but considers that the single ionic species contributing to I were NH_4^+ and its
224 counterion (a monovalent ion used to close the charge balance). The NH_4^+ concentration was
225 calculated using the aforementioned iterative process. This simplification was tested to assess
226 the error caused by the lack of input data if only few ions are considered. In addition, this
227 approach also allowed assessing which ions are to be measured to correct most of the error in
228 FAN concentration calculations.

229 3. *Simplified I calculation without iterative NH_4^+ correction:* previous simplified
230 approach but avoiding the iterative processes to determine I by calculating the contribution of
231 NH_4^+ using the ideal equilibrium equation (Eq. 2) and assuming it to be constant. This
232 simplification was tested to elucidate if iterative processes could be avoided without causing a
233 significant error. If the iterative process is not needed, the computational requirements of the
234 FAN calculation method would be much lower, facilitating its integration in complex AD
235 models.

236 4. *Modified Davies equation:* the original Davies equation for f calculation (Eq. 5) was
237 recalibrated to fit the results given by MINTEQA2 using a non-linear least square analysis
238 (considering the value of I as constant and as the one used in MINTEQA2). This approach
239 simply modifies the empirical parameter of the original equation (λ) to obtain the best

240 possible fit of the experimental data [49]. Calibrating λ using the MINTEQA2 FAN data
241 resulted in a value of 0.1276, instead of 0.2. The corresponding modified Davies equation is:

242

$$243 \quad f = 10^{\left(-A \cdot z_i^2 \cdot \left(\frac{\sqrt{I}}{1+\sqrt{I}}\right)^{-0.1276 \cdot I}\right)} \quad \text{Eq. 8}$$

244

245 2.2.3. MINTEQA2 Equilibrium Speciation Model

246 Among the different geochemical models available (see Di Bonito *et al.* [50]), MINTEQA2
247 has been used in this study as reference scenario for equilibrium speciation calculations.

248 MINTEQA2 is a geochemical equilibrium speciation model revised in 2006 that was
249 developed by the United States Environmental Protection Agency (EPA) to calculate the

250 equilibrium composition of aqueous systems [51,52]. MINTEQA2 can be easily accessed
251 using the Visual MINTEQ package, which is one of the chemical equilibrium software

252 applications most used among researchers publishing in Elsevier journals [53]. This
253 geochemical equilibrium speciation model allows computing mass distribution equilibria

254 among dissolved species, adsorbed species, and multiple solid phases under a variety of
255 conditions including a gas phase with constant partial pressures. MINTEQA2 includes a

256 comprehensive database of reliable thermodynamic data that is adequate for solving a broad
257 range of problems without the need of additional user-supplied equilibrium constants. The

258 model employs a predefined set of components that includes free ions such as Cl^- or NH_4^+ and
259 neutral and charged complexes (*e.g.*, $\text{C}_2\text{H}_3\text{O}_2^-$, $\text{C}_3\text{H}_5\text{O}_2^-$, $\text{C}_4\text{H}_7\text{O}_2^-$). The database of reactions

260 is written in terms of these components as reactants.

261 Chemically, the system is modelled from a set of components and a set of species, as

262 described by Allison *et al.* [51]. MINTEQA2 allows calculating the equilibrium composition
263 with precision since the model takes into account different factors such as T and activity
264 coefficient corrections of equilibrium constants (through the van't Hoff and Davies/modified
265 Debye-Hückel equations, respectively), charge and activity coefficients for organic species,
266 complexation of metals by dissolved organic matter, activity of water, total alkalinity, pH and
267 I of the media, and saturation index and formation/dissolution of chemical precipitates. A
268 precise description of the set of equations defining MINTEQA2 can be found in Appendix B.
269 The chemical components considered in MINTEQA2 were the same as those used in the
270 *comprehensive approach* applied with the Davies equation. Also as previously, to account for
271 the counterions generally not measured (*i.e.* HCO_3^- or CO_3^{2-}), a monovalent ion was
272 considered (its concentration was calculated as the missing amount required to close the
273 charge balance).

274 The described MINTEQA2 can be used for different purposes, such as (i) calculating the
275 speciation of inorganic ions and complexes in water, (ii) evaluating the effect of dissolving or
276 precipitating solids on water chemistry, (iii) investigating the equilibrium speciation of
277 common redox couples, (iv) simulating the change in chemical composition of a water sample
278 during a titration, or (v) estimating the binding of ions to (hydr)oxide surfaces and organic
279 matter using state-of-the-art complexation models (SHM or NICA-Donnan for organic
280 complexation, CD-MUSIC, TLM, CCM or DLM for surface complexation). Indeed,
281 MINTEQA2 has been widely applied for different purposes within the waste treatment field
282 (*e.g.*, [54,55,56,57,58,59]).

283 It must be mentioned that, as MINTEQA2 applies the Davies equation using a λ of 0.3, the
284 manual warns the user that the activity correction model is generally not intended to be used

285 at I values greater than 0.7-1.0 M (cut-off for effective use treated as 1.0 M in the last
286 manual). However, it also mentions that these correction equations may still provide usable
287 results that should be verified for the specific modelled system, even I values higher than 1.0
288 M. As an alternative to the Davies equation, MINTEQA2 offers the possibility of applying the
289 modified Debye-Hückel equation for activity corrections [60]. Nevertheless, the basic Debye-
290 Hückel correction has been reported to lead to substantial errors at I above 0.1 M, even for
291 simple solutions [61]. In addition, the Debye-Hückel method has been found to be particularly
292 inaccurate for correcting the activities of monovalent ions (such as NH_4^+) [62]. To ensure that
293 the best method for activity corrections was used, both the Davies and the modified Debye-
294 Hückel equations were used in MINTEQA2 (data not shown), concluding that the Davies
295 equation was the most convenient approach (more coherent results in agreement with the
296 literature were obtained).

297 Considering the aforementioned statements, the FAN results given by MINTEQA2 were
298 considered as the reference scenario for comparison and validation purposes. The wide
299 application of this model, its correct predictions with I values up to 1.0 M and the fact that it
300 considers the interactions between all the components considered in the media (including
301 dissolved species, adsorbed species, multiple solid phases and gas phases), as well as the
302 complexation of metals by dissolved organic matter (ion-binding), are main supporters of this
303 choice.

304 *2.3. Validation of the proposed modified Davies equation*

305 Using MINTEQA2 as reference scenario, an uncertainty framing was defined for validation of
306 the proposed modified Davies equation (Eq. 8) as a method for FAN calculation in a wide
307 range of I values. In addition, a global sensitivity analysis (GSA) based on Monte Carlo

308 simulations was conducted for identifying the influential factors affecting MINTEQA2
309 results. Monte Carlo results were used to assess uncertainties between the results from the
310 modified Davies equation and MINTEQA2.

311 For this purpose, the concentrations of the different ionic species considered were varied
312 according to the average experimental concentrations found in the database. Average
313 experimental concentrations and uncertainty factors are both shown in supplementary material
314 (Table S2). The FAN concentration given by MINTEQA2 was used as output variable.

315 The GSA was conducted using the standardised regression coefficient (SRC) method and
316 Monte Carlo was conducted using the semi-random Latin Hypercube Sampling (LHS) method
317 [63]. A coefficient of determination (R^2) above 0.7 was obtained, validating the standardised
318 regression slope (β_i) as sensitivity measure [64]. Input factors resulting in β_i higher than 0.1
319 were selected as influential factors. To reduce stratification of sampling, the number of Monte
320 Carlo runs (N) was set to 10,000 by performing batches of 100 runs 100 times.

321 *2.4. Calculation of inhibitory constants and data clustering*

322 This section aimed at obtaining representative/generalized values of the inhibitory constants
323 given in the literature. This was done by identifying clusters of the data retrieved from the
324 literature according to their FAN resilience.

325 The values corresponding to the TAN concentrations, temperatures, pH and SMAs, together
326 with the FAN concentrations calculated using the modified Davies equation were used to
327 determine generalized values of the inhibitory constants (IC_{50}). The modified Davies equation
328 was used for FAN calculation because it was the approach showing the best compromise
329 between accuracy and complexity (see Section 3.2). The inhibition function considered was
330 the threshold inhibition function proposed by Astals *et al.* [8]:

331

$$332 \quad SMA \text{ reduction } (\%) = \begin{cases} 0 & ; \text{ if } FAN \leq KI_{min} \\ 100 \cdot \left(1 - e^{-2.77259 \left(\frac{FAN - KI_{min}}{KI_{max} - KI_{min}} \right)^2} \right) & ; \text{ if } FAN > KI_{min} \end{cases} \quad \text{Eq. 9}$$

333

334 Where KI_{min} and KI_{max} representing the FAN concentrations where inhibition starts (onset
335 concentration) and when it is almost complete ($SMA = 0.06 \cdot SMA_{max}$), respectively [8]. The
336 arithmetic average of KI_{min} and KI_{max} equals IC_{50} (*i.e.* $IC_{50} = (KI_{min} + KI_{max})/2$). Thus, results
337 from this approach can be extrapolated and compared to the IC_{50} obtained when using a non-
338 competitive inhibition function.

339 As the microbial resilience can vary greatly among different studies, different IC_{50} can be
340 extrapolated from the collected data. Therefore, when plotting the reduction of the SMA (Y-
341 axis) against the FAN concentrations (X-axis), it can be expected that more than one
342 inhibition curve (given by Eq. 9) could fit the collected data. Therefore, a procedure was
343 tailored to cluster the data according to a set of inhibition curves. This clustering approach
344 depended on two parameters: ε_{FAN} and ε_{SMA} , which correspond to the accepted error around
345 inhibition curves on the X-axis (*i.e.* FAN concentrations) and on the Y-axis (*i.e.* SMA
346 reduction), respectively. Hence, it was possible to define an area around each inhibition curve
347 inside which all the points could be considered to belong to the respective inhibition curve.
348 This approach was implemented to consider and mitigate FAN and SMA measurement errors.
349 A precise description of the iterative approach followed to define each cluster can be found in
350 Appendix C.

351 Once the optimal clusters were defined, the corresponding quantitative and qualitative data

352 (e.g. temperature, AD substrate, pH or main archaeal genus) were compared. Prior analysis,
353 data points with 0 and 100 % of SMA reduction were removed from the intermediate and last
354 clusters and from the first and intermediate clusters, respectively, as they could not be
355 attributed to any cluster with certitude (e.g. 100 % inhibition for the second cluster could also
356 belong to the first one). Statistical differences between clusters for quantitative data was
357 assessed on R 3.5.0 (2018) using the non-parametric Kruskal-Wallis test with the *kruskal.test*
358 function. Then, multiple pairwise comparisons were carried out using the Dunn's test [65]
359 applying the *dunnTest* function of the package "FSA". All the p-values were adjusted
360 according to the Holm's method [66] and statistical significance was assumed for p-values <
361 0.01.

362 This approach allowed (i) identifying clusters of data that represented the values belonging to
363 each curve and (ii) obtaining the values of the IC_{50} corresponding to each cluster.

364 2.5. Sensitivity analysis of the clustering procedure

365 The number of clusters and the overall quality of the approach strongly depend on the values
366 of the parameters ε_{FAN} and ε_{SMA} . Thus, a sensitivity analysis was carried out to assess their
367 optimal values according to the minimization criterion presented in Equation 10. The
368 complete clustering procedure was carried out for ε_{FAN} and ε_{SMA} ranging from 0.05 to 0.25
369 $\log_{10}(\text{FAN})$ and 5 to 25 % SMA reduction, respectively. Smaller values of ε_{FAN} and ε_{SMA} were
370 not considered to ensure the stability of the clustering process. The quality of the clustering
371 result was quantitatively assessed considering three parameters to be minimized: (i) the
372 number of clusters (to avoid overfitting of the data); (ii) curve overlapping, *i.e.* the number of
373 points that could be attributed to two different clusters according to ε_{FAN} and ε_{SMA} (expressed
374 as % of the total points); and (iii) the total number of non-attributed points, not belonging to

375 any cluster (expressed as % of the total points). For instance, overlapping could occur when a
376 new cluster was defined with an IC_{50} lower than the previous $IC_{50} + 2 \varepsilon_{SMA}$, while non-
377 attributed points were typically points above the final inhibition curve during the clustering
378 loop.

379 The optimal ε_{FAN} and ε_{SMA} couple was determined by minimizing the following function:

380

$$381 \quad \text{Clustering score} = \frac{\text{Number of clusters}}{\max(\text{Number of clusters})} + \frac{\text{Overlapping}}{\max(\text{Overlapping})} + \frac{\text{Non-attributed points}}{\max(\text{Non-attributed points})} \quad \text{Eq. 10}$$

382

383 **3. Evaluation of methods for FAN calculation**

384 *3.1. Importance of considering the ionic strength for FAN calculation*

385 Amongst the different mathematical methods used to calculate FAN concentration, the ideal
386 equilibrium equation and the Anthonisen equation are the most used formulas in the literature
387 [5,67]. In an ideal solution, the FAN concentration will depend on the TAN concentration, the
388 T and the pH (equations 1 to 3) [46]. Nevertheless, these methods do not take into account
389 that the AD media is a non-ideal solution. Therefore, equations 2 and 3 should be applied only
390 in diluted systems, where the chemical behavior can be simplified to that of an ideal pure
391 water-ammonia solution (*e.g.* wastewater) [25,68]. During the treatment of more concentrated
392 substrates, this approach is not appropriate as it is critical to account for the effect of I on the
393 chemical activity of the ions (*e.g.* animal manure and food waste) [5,25,58,69]. This implies
394 that in most cases the ideal equilibrium equation is not applicable and other approaches for the
395 calculation of the FAN concentration must be used.

396 Therefore, activity corrections must be considered and, if required, chemical species other
397 than NH_4^+ that might be in equilibrium with FAN must be accounted for. Hafner and Bisogni

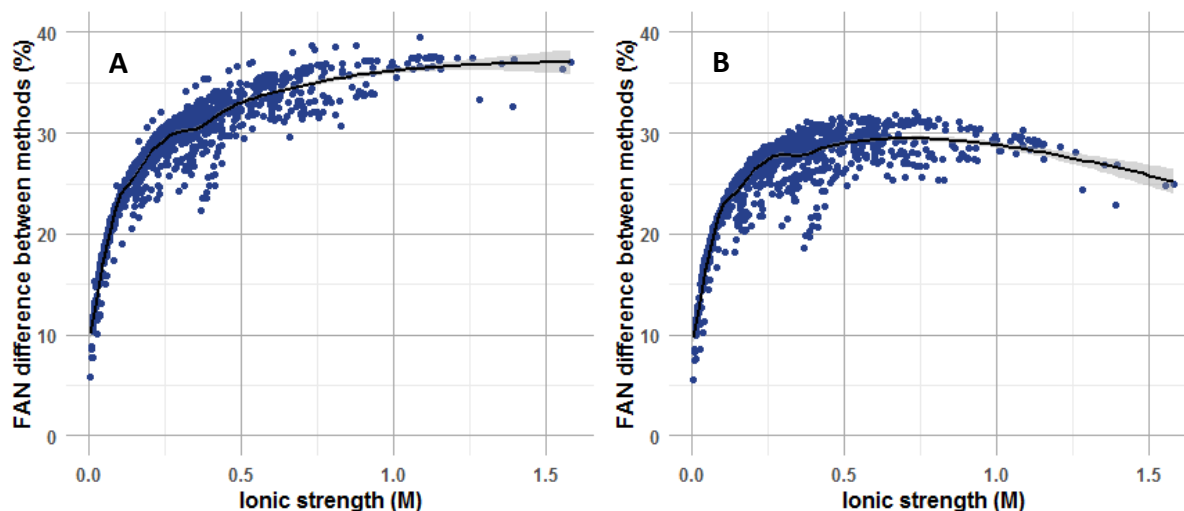
398 [25] showed that if activity corrections were not considered in reactors digesting animal
399 manure, significant overestimations of the FAN concentrations occurred, with errors up to 40
400 %. Hafner and Bisogni [25] proposed a comprehensive speciation model based on the Pitzer's
401 ion-interaction approach. The authors concluded that, although the complex model was more
402 precise, simply including an activity coefficient for NH_4^+ in the ideal equilibrium equation
403 already resulted in much more accurate estimates. The influence of including ion activity
404 corrections has also been studied for AD modelling. Solon *et al.* [58] included activity
405 coefficients into the anaerobic digestion model no. 1 (ADM1) using the Davies equation.
406 They observed that the salinity and the pH were significantly different when compared to the
407 default ADM1 and recommended the application of activity corrections at values of I higher
408 than 0.2 M. In a recent study, Patón *et al.* [70] evaluated the impact of neglecting activity
409 corrections on the prediction of key AD processes. They concluded that the systematic
410 incorporation of activity corrections in AD models was needed if errors in the alkalinity
411 ratios, the inhibition factors (*i.e.* for H_2S and FAN) and phosphorus speciation were to be
412 avoided. Patón *et al.* [70] also used the Davies equation to account for non-ideality due to its
413 fair compromise between simplicity and accuracy. Even though the Davies equation is only
414 valid up to a certain ionic strength, this approach has been the most widely applied method to
415 account for non-ideality. Other authors have used modelling software such as MINTEQA2
416 [54,55,56,57]. This latter method is the best option to use when a precise knowledge of the
417 physicochemical processes involved is required (*e.g.* for pH modelling, for modelling biogas
418 composition or gas stripping through liquid-gas equilibria, or for modelling chemical
419 precipitations, among others) [54,55,56]. Nevertheless, the effectiveness of this approach
420 increases with the level of characterization of the media, giving comparable results to other

421 simpler approaches when the number of ionic species measured is reduced (e.g. NH_4^+).
422 Regardless of the methodology used, to date, only few authors have considered non-ideality in
423 FAN calculations, which represents a source of data misinterpretation as discussed in the
424 following subsection.

425 3.2. Comparison of different methods for FAN calculation

426 In this section, the FAN concentrations given by the ideal equilibrium equation (no I
427 considered) are compared with those given by MINTEQA2 and by the Davies equation,
428 which consider the influence of I . Figure 1 shows the results corresponding to the application
429 of MINTEQA2 and the Davies equation considering the main chemical species in the media.
430 The differences between the FAN concentrations given by each of these methods and the
431 ideal equilibrium equation (Eq. 2) are presented.

432



433

434 **Figure 1.** Differences in the FAN concentrations at different ionic strengths: (A) the ideal
435 equilibrium equation vs MINTEQA2, (B) the ideal equilibrium equation vs the comprehensive
436 Davies equation. A Loess local regression curve is included (black curves; 1,590 data points

437 presented). FAN stands for free ammonia nitrogen

438

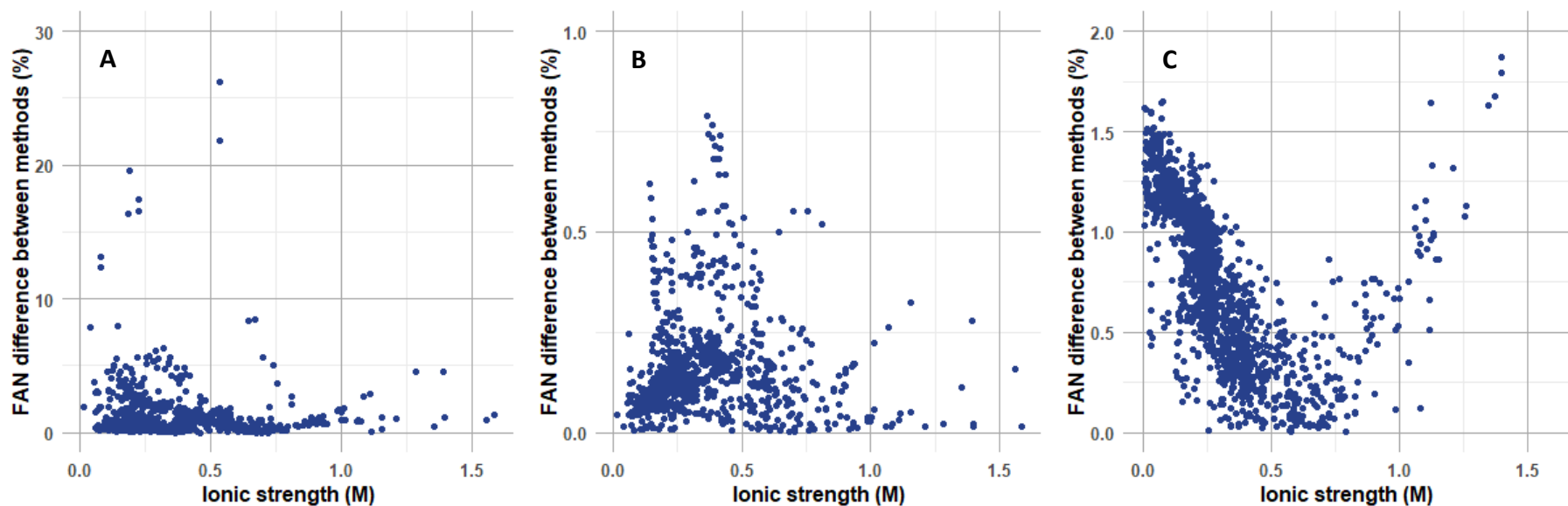
439 In both cases, the obtained curves followed a similar trend, with higher FAN differences at
440 increasing values of I . This was expected since higher I values lead to lower f values (Eq. 4 to
441 7) [25]. Starting with MINTEQA2 (Figure 1A), FAN differences higher than 25 % were
442 found for values of $I > 0.25$ M, which indicates the importance of considering this parameter
443 for FAN calculation even at low concentrations of ionic species. At higher values of I , the
444 FAN differences continued to increase, with differences up to 37 % at values of I over 1.00
445 M. This is in agreement with previous studies, where differences of 30-40 % were found
446 when accounting for the effect of I for FAN calculation [25,71]. Taking the results given by
447 MINTEQA2 as the most realistic approach (but also the most analytic-demanding to obtain
448 enhanced results), the large errors associated to the application of the ideal equilibrium
449 equation for FAN determination are evident. It can be concluded that the application of Eq. 2
450 for FAN calculation in AD systems is not recommended.

451 The main reasons leading to FAN overestimation using the ideal equilibrium equation are
452 related to the assumption of a pure solution with FAN and NH_4^+ as single species in
453 equilibrium. According to Equation 1, the presence of ionic species in the media (thus higher
454 values of I) shifts the equilibrium towards the formation of NH_4^+ , thus reducing the FAN
455 concentration. In addition, interactions with other ionic species reduce the NH_4^+
456 concentrations (*e.g.* via precipitation or complexation), further decreasing the FAN contents.

457 Regarding the comprehensive Davies equation (Figure 1B), the FAN differences were
458 always slightly lower when compared to MINTEQA2. The main reason for this is that the
459 Davies method considers that the only species in equilibrium with NH_4^+ is FAN, which leads

460 to a slight overestimation of the FAN concentrations. At low values of I (< 0.5 M), both
461 curves were almost identical with differences between the FAN values given by both methods
462 between 0 and 6 %. However, as it can be observed in Figure 1B, at higher values of I a
463 maximum appeared when applying the Davies equation. Differences in FAN around 30 %
464 were reached, remaining relatively constant until values of I of 0.75-0.80 M. At this point, the
465 FAN differences compared to the ideal equilibrium equation started to follow a slightly
466 decreasing trend. This caused an increase in the differences between the FAN values given by
467 the Davies equation and those calculated with MINTEQA2, with values up to 16 % (Davies
468 vs MINTEQA2). The decreasing trend in Figure 1B (thus the increase in the differences
469 between both methods) appears due to the limited application range of the equation for
470 calculating f when using the Davies method (Eq. 5). This expression is an empirical equation
471 that should not be applied at values of I over 0.5 M [47]. However, it must be mentioned that
472 Figure 1B shows that, despite of this limitation, the Davies equation gives in any case a better
473 estimation of the FAN concentrations than the ones obtained applying the ideal method,
474 which clearly overestimates much more significantly the FAN concentrations. The value of I
475 was lower than 0.7 M for 92 % of the data and lower than 0.5 M for the 84 %, implying that
476 the Davies equation could be applicable for most of the conditions found in AD systems, even
477 when treating concentrated substrates. The I in AD influents generally range from 0.1 to 1.0
478 M [72]. Diluted substrates such as diluted industrial wastewaters and domestic wastewaters
479 generally have an I below 0.1-0.2 M [72,73]. However, for high solids and manure digesters I
480 is generally above 0.2 M [58]. For instance, values of 0.5-0.6 M and 0.6-0.8 M have been
481 reported during food waste and poultry manure AD, respectively [21,74].
482 As previously mentioned, two simplifications using the Davies equation were compared with

483 the comprehensive approach: (i) a simplified I calculation considering that the only two
484 contributors to I were the NH_4^+ and its counterion, and (ii) this latter approach but avoiding
485 the iterative processes to determine the contribution of NH_4^+ to I . The differences between the
486 FAN concentrations due to each simplification are shown in Figure 2.



487

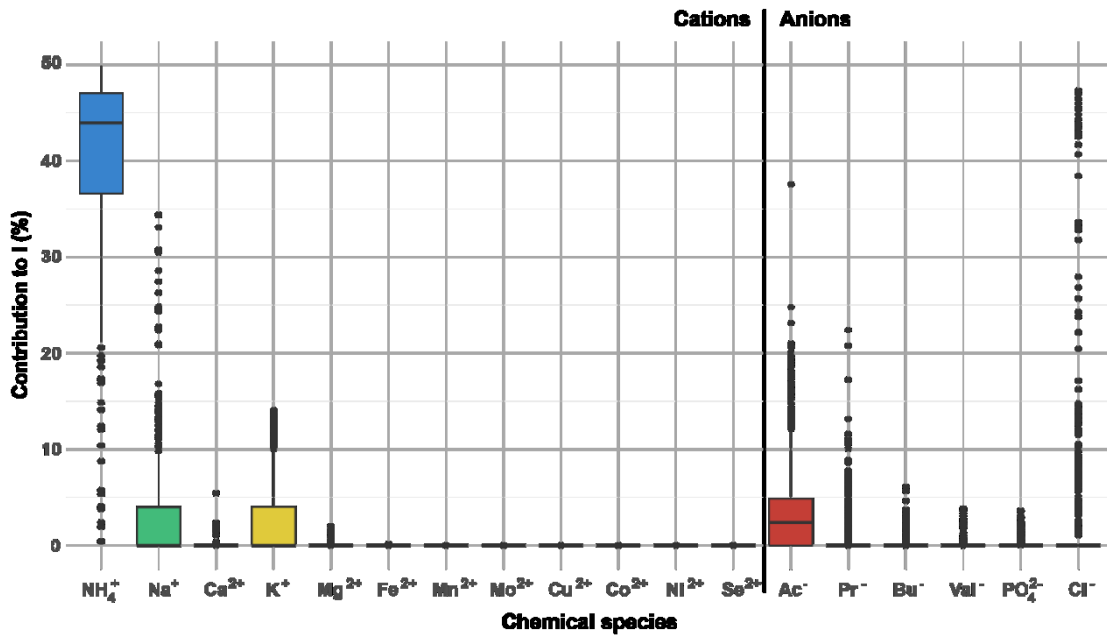
488 **Figure 2.** Differences in the FAN concentrations at different ionic strengths: (A) the comprehensive Davies method vs the simplified Davies
 489 method considering only the NH_4^+ and its counterion, (B) the simplified Davies method considering only the NH_4^+ and its counterion vs the same
 490 method without iterative NH_4^+ calculation, and (C) the modified Davies method (Eq. 8) vs MINTEQA2. Only data from experiments with
 491 significant concentrations of ions other than NH_4^+ have been considered in A and B (1,132 data points). 1,590 data points are presented in C.
 492 Different y-axis scales are presented to allow a precise data interpretation. FAN stands for free ammonia nitrogen

493 The first simplification led to significant differences in the FAN concentrations in some cases
494 (up to 26 % when compared to the comprehensive Davies method considering all the main
495 ions; Figure 2A). However, avoiding the iterative step did not further increase the error
496 significantly, with the differences between the application of the iterative process or not
497 (when considering only the NH_4^+) up to 0.76 % (Figure 2B). This simplification can be
498 particularly useful for integration with existing models that do not aim at predicting
499 extensively the physicochemical interactions in the media (*e.g.* for inclusion with the ADM1
500 or simpler kinetic models), as it would reduce the time required to run a simulation. It must be
501 mentioned that the error caused by removing the iterative process when applying the
502 comprehensive Davies method considering all the ions was also negligible, with differences
503 always below 1 %.

504 The FAN differences due to considering only the NH_4^+ and its counterion as single
505 contributors to I are presented in Figure 2A. Although relatively high errors were observed
506 due to this simplification, the differences were below 3 % for 93 % of the data and below 5 %
507 for 97 % of the data. These results indicate that, in most of the cases, it would not be
508 necessary to measure all the ionic species in the media to correct most of the error in FAN
509 concentration. To elucidate which were the ions responsible for the differences observed in
510 Figure 2A, the relative contribution to the total I of each ionic species were calculated (as %
511 of total I). The results, shown in Figure 3, further prove that the NH_4^+ is the main species
512 contributing to I in AD of N-rich substrates. Considering both NH_4^+ and its counterion, they
513 represent most of the I in most of the experiments. However, when looking at the
514 contributions of other ions, it is clear that other species also contributed to I . More precisely,
515 while the impact of the trace elements (*i.e.* Fe^{2+} , Mn^{2+} or Cu^{2+}) was clearly negligible, some

516 ions (*i.e.* Na⁺, K⁺ and Cl⁻) and some VFAs (*i.e.* acetate and propionate) had an impact on the
517 value of *I* due to their relatively high concentrations.

518



519

520 **Figure 3.** Contributions of each chemical species to the total ionic strength of the media. Only
521 data from experiments reporting significant concentrations of ions other than NH₄⁺ have been
522 considered (1,097 data points). *I* stands for ionic strength and Ac⁻, Pr⁻, Bu⁻ and Val⁻ stand for
523 acetate, propionate, butyrate and valerate, respectively

524

525 Considering the results shown in Figure 3, it can be assumed that in most cases simply
526 measuring the NH₄⁺ concentration to account for its contribution (and that of its counterion)
527 in the Davies equation will already account for most of the error in the FAN concentration
528 calculation. However, in experiments where the Na⁺/K⁺/Cl⁻ concentrations are above 1 g·L⁻¹
529 and/or VFA concentrations are above 1 g COD·L⁻¹, the contribution of these ionic species to *I*
530 must not be neglected. Nevertheless, it must be considered that as Cl⁻ and VFAs are anions,

531 their impact on I would not modify the FAN value when applying the Davies method used in
532 this study (its concentration would belong to the pool of monovalent counterions used to close
533 the charge balance). Thus, measuring the concentrations of NH_4^+ , Na^+ and K^+ should be
534 enough to account for most of I in most of the studies.

535 A modification of the Davies equation was carried out, calibrating the experimental parameter
536 λ in Equation 5 to fit the obtained FAN concentrations to the ones given by MINTEQA2. The
537 Davies equation is itself the result of an empirical modification of the Debye-Hückel theory,
538 by adding the linear term $-\lambda \cdot I$ to the formula. Davies estimated the parameter λ by optimizing
539 the measured activity coefficients at I below 0.1 M, obtaining values of 0.2-0.3. Although
540 these values provide a quasi-constant value of the activity coefficients at I ranging from 0.3 to
541 0.7 M, a I of 0.5 M is generally assumed as the application limit for this equation [47,61].

542 Over this limit, the linear term becomes predominant and the value of λ must be recalibrated.
543 The corresponding modified Davies equation corresponds to Equation 8, with a λ of 0.1276.
544 This value is lower than those used in the Davies equation (0.2) or in MINTEQA2 (0.3).

545 As it can be observed in Figure 2C, this modified Davies equation resulted in differences
546 between the FAN values below 2 % (below 1 % for 72 % of the data) when compared to those
547 given by MINTEQA2. It must be commented that, according to the results from the previous
548 simplifications, the value of I for the calibration was estimated considering all the main ions
549 in the media and avoiding the iterative approach for NH_4^+ calculation (using the I values
550 obtained from the Davies equation for the calibration). When considering the iterative
551 recalculation of I , the error was below 1 % for 70 % of the data (Figure S1).

552 To verify that the obtained equation could be applied at different operating conditions (pH, T
553 and concentrations of ionic species, thus I values), the modified Davies equation was

554 validated by comparing the resulting FAN concentrations with the ones given by MINTEQA2
555 at several conditions (see Section 2.3). GSA results revealed that pH, TAN and T are
556 influential factors affecting FAN calculation using MINTEQA2 (β_i higher than 0.1; see Figure
557 S2). This finding further justifies the possibility of applying a simplified equation considering
558 these parameters, such as the Davies equation. The Monte Carlo simulations validate the
559 proposed equation. A regression analysis of the FAN values given by MINTEQA2 and those
560 calculated using the modified Davies equation on the 10,000 conditions studied resulted in a
561 R^2 of 0.9999 and a systematic error of 2.1 % (slope of 0.978) (Figure S3). The differences
562 between the FAN concentrations obtained with the modified Davies equation and MINTEQA
563 were always below 5 % (below 3 % for 69.5 % of the conditions simulated), confirming the
564 applicability of the proposed equation (Figure S4). For an equal I , these errors were generally
565 higher in thermophilic conditions (53-57 °C; up to 4.9 %) when compared to mesophilic
566 temperatures (33-37 °C; up to 3.6 %). This might be related to the higher FAN concentrations
567 at increasing temperatures and due to the predominance of mesophilic conditions in the
568 database. Nevertheless, it must be considered that the Monte Carlo simulations based on
569 average conditions led to extreme concentrations of FAN and I values (particularly high in
570 thermophilic conditions), much higher than what can be usually found in AD systems. These
571 results show that the modified Davies equation is a step forward for increasing the accuracy of
572 the Debye-Hückel theory for FAN estimation in AD systems at high I values (up to around
573 1.0 M).

574 To simplify the presentation of the conclusions drawn, a comparison of the tested methods for
575 FAN determination is presented in Table 2. Recommendations for their application are also
576 given.

577 **Table 2.** Comparison of the tested methods for FAN calculation and recommendations

Calculation method	Advantages	Disadvantages	Recommendations
MINTEQA2	<ul style="list-style-type: none"> - Allows the most precise FAN calculation - Allows pH modelling through proton balance - Provides physico-chemical knowledge - Interactions between different components considered - Precipitations considered 	<ul style="list-style-type: none"> - Input-demanding for enhanced outputs - Interface required to integrate with existing models - Requires expertise and programming knowledge for automatic on-line calculations 	<ul style="list-style-type: none"> - To be used for comprehensive modelling purposes - Recommended for I values > 0.5 M - Useful when the formation of precipitates is relevant
Davies¹	<ul style="list-style-type: none"> - Simple to implement - Low computational requirements - Easy to integrate in existing models - I can be estimated from conductivity measures 	<ul style="list-style-type: none"> - I should be below 0.5 M for precise results - Extensive data input required for I calculation - Interactions between different components and precipitations not considered 	<ul style="list-style-type: none"> - To be used for generalized process engineering and modelling applications if interactions between NH_4^+ and other ionic species are not significant
Davies considering only NH_4^+ and counterion¹	<ul style="list-style-type: none"> - Simple to implement - Low computational requirements - Easy to integrate in existing models - Low data input needed: the single ionic species to be measured is NH_4^+ 	<ul style="list-style-type: none"> - I should be below 0.5 M for precise results - Significant errors at high Na^+/K^+ concentrations - Interactions between different components and precipitations not considered 	<ul style="list-style-type: none"> - To be used as substitute of the regular Davies application when the contributions of other ions to I are expected to be negligible
Modified Davies equation¹	<ul style="list-style-type: none"> - Simple to implement - Low computational requirements - Easy to integrate in existing models - Applicable at higher values of I (up to 1.0 M) - I can be estimated from conductivity measures 	<ul style="list-style-type: none"> - Extensive data input required for I calculation (reduced if only NH_4^+, Na^+ and K^+ are considered) - Interactions between different components and precipitations not considered 	<ul style="list-style-type: none"> - To be used as substitute of the Davies equation in AD of N-rich substrates for everyday process engineering and modelling applications

578 1. Without iterative calculation of the NH_4^+ concentration

579 Basically, if a precise determination of the FAN concentrations and/or if interactions between
580 other ionic species (including the formation of precipitates) are needed, the use of a
581 comprehensive geochemical model such MINTEQA2 is recommended. This approach is
582 clearly the most convenient option for comprehensive modelling of AD systems.
583 Nevertheless, to profit of its advantages, MINTEQA2 demands a broader analytical input, as
584 well as programming knowledge for automatic on-line calculations. For everyday FAN
585 calculation, the Davies equation appears as a much simpler and straight-forward approach
586 whose implementation can be generalized for almost any AD system, excluding only those
587 presenting an I above 0.5 M. In addition, its low computational requirements, together with its
588 simplicity if the iterative process is avoided, allow an easy integration with existing AD
589 models. The application of the Davies equation considering just the NH_4^+ and its counterion
590 appears as a simplification that can be applied without causing significant errors in systems
591 working at low concentrations of other ionic species. If the concentrations of other ions
592 cannot be neglected, the most relevant species to be considered are Na^+ and K^+ . Finally, the
593 modified Davies equation represents a much more precise calculation method for FAN
594 estimation in AD systems when compared to the classic algorithm. This latter option appears
595 as the method providing the best compromise between simplicity and accuracy (for I values
596 up to 1.0 M). The simplifications tested with the classic Davies equation (*e.g.* considering
597 only the NH_4^+ and its counterion) can also be applied with the proposed modified Davies
598 equation.

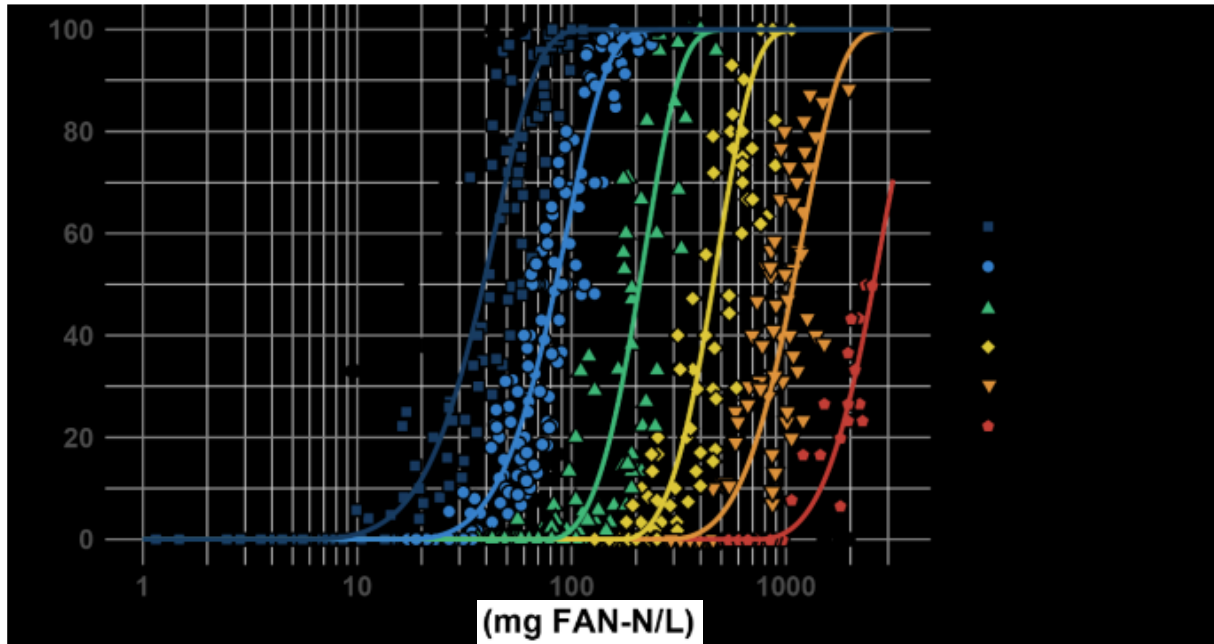
599

600 **4. Understanding the variability in FAN inhibitory limits**

601 *4.1. Cluster definition and IC_{50} estimation*

602 The gathered values of the SMAs at different FAN concentrations, together with the results of
603 the clustering process described in Section 2.4 are presented in Figure 4.

604



605

606 **Figure 4.** Reduction of the SMA at different FAN concentrations reported in the literature.
607 The set of inhibition curves and IC_{50} values resulting from the clustering approach are also
608 shown. SMA stands for specific methanogenic activity and FAN for free ammonia nitrogen

609

610 As it can be observed, the clustering process resulted in six different inhibition curves, with
611 IC_{50} values ranging from 39 to 2,565 mg FAN-N·L⁻¹. The corresponding values of K_{min} , K_{max} ,
612 RMSE and the number of points belonging to each cluster are presented in Table 3.

613

614 **Table 3.** Results of the clustering method: values of the IC_{50} , K_{min} , K_{max} , RMSE and number
 615 of points for each cluster

Cluster #	IC_{50} (mg FAN-N·L ⁻¹)	K_{min} (mg FAN-N·L ⁻¹)	K_{max} (mg FAN-N·L ⁻¹)	RMSE (% SMA reduction)	# of points
1	39	6	72	18.1	129
2	84	17	152	11.7	143
3	208	78	337	18.0	109
4	458	175	741	15.4	106
5	1,082	285	1,878	16.1	90
6	2,565	743	4,387	8.4	40

616 FAN stands for free ammonia nitrogen, RMSE for root-mean-square error and SMA for specific methanogenic
 617 activity
 618

619 First, it must be mentioned that the results presented in Figure 4 and Table 3 were obtained
 620 using the values of ε_{FAN} and ε_{SMA} that were found to minimize the clustering score from
 621 Equation 10 (see Section 2.5). They corresponded to values of 0.205 log₁₀(FAN) and 5 %
 622 SMA reduction, respectively. These were the values showing the lowest clustering score
 623 (0.32), with only 5.9 % of non-attributed points and an overlapping of 16.2 %. It is important
 624 to mention that the distribution of the values of the clustering score followed a normal trend,
 625 indicating that the optimum found did not correspond to a local minimum. This also
 626 reinforces the obtained results, as it proves that the collected data have been correctly
 627 gathered. The values of the clustering score resulting from the sensitivity analysis, the
 628 distribution of the clustering scores and the values of each parameter considered for its
 629 calculation (*i.e.* number of clusters, number of non-attributed points and overlapping) at the
 630 different values of ε_{FAN} and ε_{SMA} that were tested can be found in the supplementary material
 631 (Figures S5 to S9).

632 The obtained clustering results show the divergence existing in the literature regarding FAN

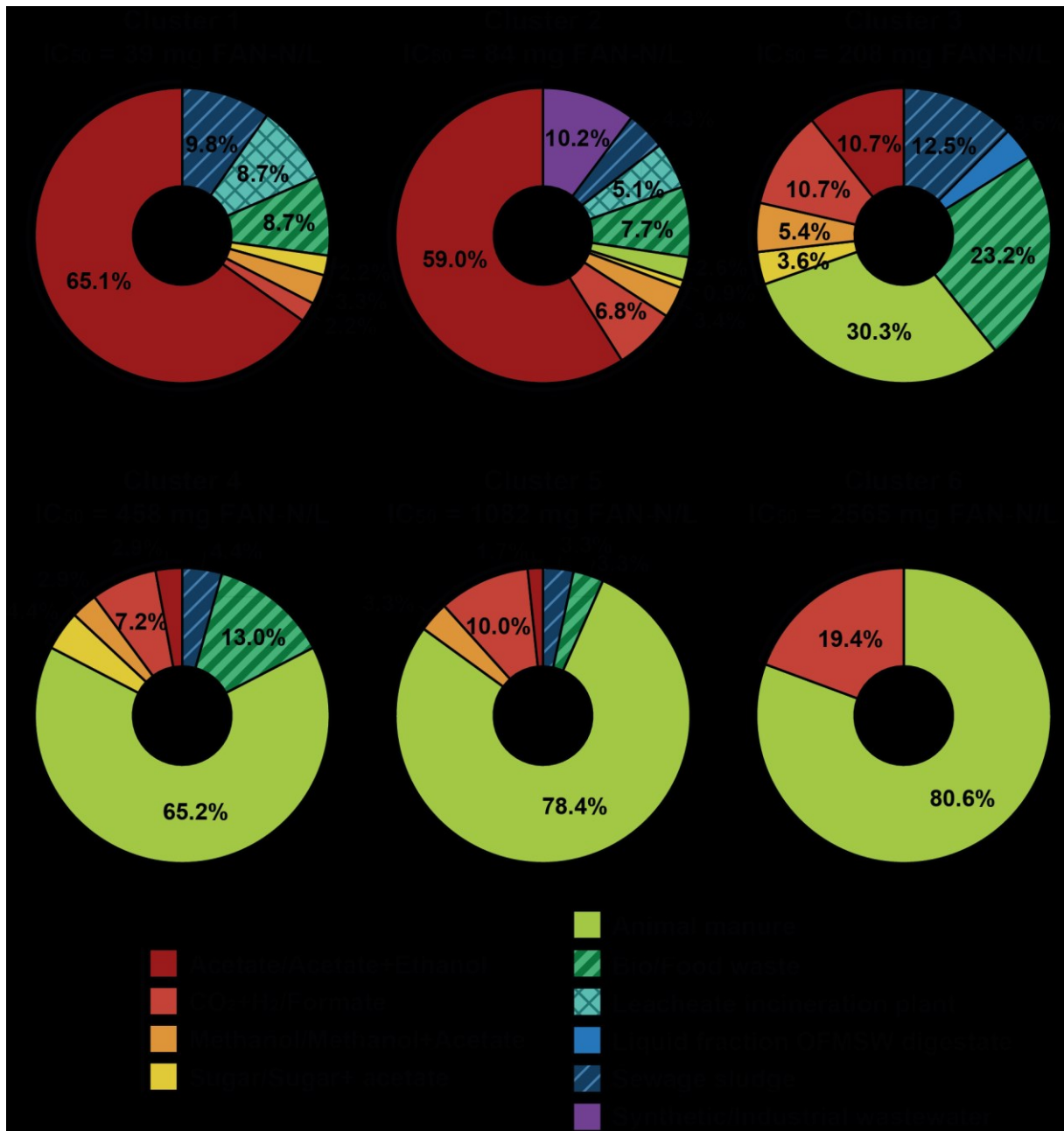
633 inhibition. This disparity in the resilience of a microbial community towards FAN inhibition
634 has been linked to several factors, such as background FAN concentration, acclimation
635 strategy, temperature, pH, and substrate-type [11,26,43,75,76,77]. The first cluster, with an
636 IC_{50} of 39 mg FAN-N·L⁻¹ could correspond to non-adapted microbial inocula with low FAN
637 background concentrations, thus vulnerable to inhibition at low FAN contents. As shown in
638 Table 1, similar values have been previously reported. IC_{50} values of 27 and 40 mg FAN-N·L⁻¹
639 ¹ have been previously reported using digested sewage sludge as inoculum [38,39,43]. Using
640 similar inocula, a maximum allowable FAN concentration of 55 mg FAN-N·L⁻¹ was found by
641 Bhattacharya and Parkin [78]. Regarding the second cluster (IC_{50} of 84 mg FAN-N·L⁻¹),
642 similar values have been reported using inocula acclimated to slightly higher FAN
643 concentrations. IC_{50} values of 70 and 86 mg FAN-N·L⁻¹ were found using digested sewage
644 sludge as inoculum but treating substrates with higher N contents [23,36]. The IC_{50} resulting
645 from the third cluster (208 mg FAN-N·L⁻¹) was close to values found when treating N-rich
646 biowastes and manure. Benabdallah El Hadj *et al.* and Borja *et al.* reported IC_{50} values of 215
647 and 280 mg FAN-N·L⁻¹ for digesters treating OFMSW and cattle manure, respectively
648 [31,37]. The fourth cluster (458 mg FAN-N·L⁻¹) mainly consists of digesters treating N-rich
649 wastes at thermophilic conditions. IC_{50} values of 468, 520, 635 and 690 mg FAN-N·L⁻¹ have
650 been reported in thermophilic AD when treating OFMSW, manure, slaughterhouse waste and
651 OFMSW, respectively [31,32,37,40]. Regarding the fifth cluster, with an IC_{50} of 1,082 mg
652 FAN-N·L⁻¹, similar results have only been achieved by Nakakubo *et al.* [22], who reported an
653 IC_{50} of 1,450 mg FAN-N·L⁻¹ when digesting pig manure at thermophilic conditions. Finally,
654 no study has so far given an IC_{50} as high as the one obtained for the last cluster, with an
655 estimated IC_{50} value of 2,565 mg FAN-N·L⁻¹. However, several studies have shown that

656 acclimated microbial communities can carry out efficient AD without signs of inhibition at
657 very high TAN (8.0-10 g TAN-N·L⁻¹) and/or FAN (1,000-1,500 mg FAN-N·L⁻¹)
658 concentrations [21,22,26,79,80]. Therefore, it can be expected that the IC₅₀ values
659 corresponding to these systems would be much higher than those previously reported.

660 *4.2. Influence of the operational conditions on the resilience to FAN inhibition*

661 To elucidate which were the main factors driving FAN resilience, the substrate type,
662 operational conditions, and microbial communities of each cluster were evaluated. These
663 variables/parameters have been previously identified as key factors influencing FAN
664 resilience. Figure 5 shows the repartition of AD substrates for each cluster.

665



666

667 **Figure 5.** Repartition of the substrates used as AD feed in each of the resulting clusters.

668 OFMSW stands of organic fraction of municipal solid waste

669

670 The first two clusters corresponded to experiments where the substrates were generally low in

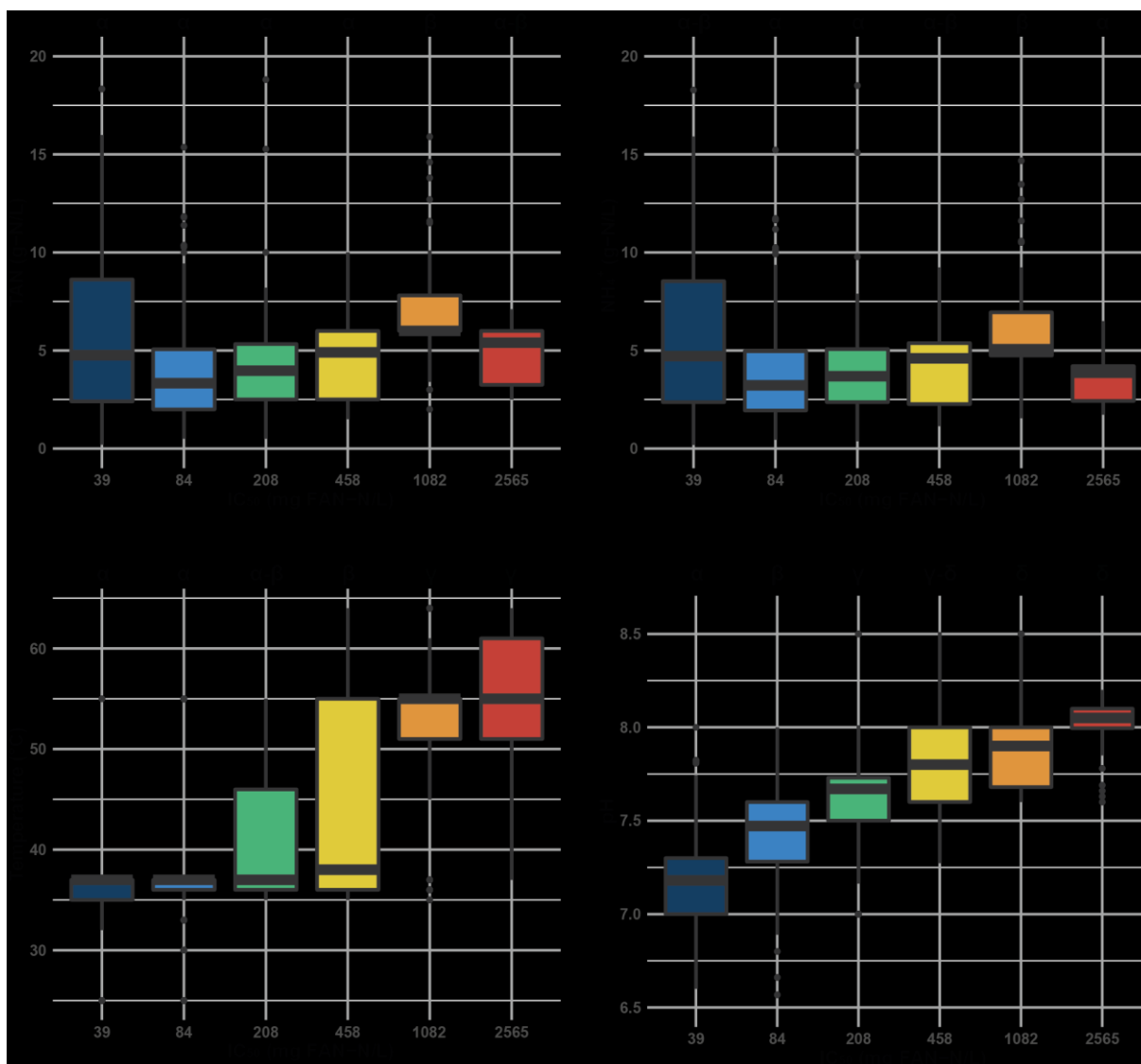
671 N, such as VFAs, sewage sludge, or wastewater. The third cluster corresponded to the AD of

672 substrates such as animal manure and biowaste/food waste, feedstocks that are richer in N
673 than the previous ones [2,81,82]. The last three clusters were fed with similar N-rich
674 substrates, mainly consisting of animal manure and biowaste/food waste. Nevertheless, it
675 must be mentioned that in many of the experiments used to determine IC_{50} values, N is
676 externally added to synthetic carbon and electron sources, such as acetate, CO_2 , hydrogen,
677 methanol or sugars [8,23,83]. This is the reason for the presence of experiments fed with
678 hydrogen and CO_2 and/or acetate in the last three clusters. In addition, many studies dealing
679 with nitrogen inhibition, particularly those operated at relatively high TAN concentrations (>
680 4-6 g TAN-N·L⁻¹), usually increase the nitrogen concentration in the reactors by adding an
681 external source of TAN, such as NH_4Cl or NH_4CO_3 . Substrate type has been reported as a
682 factor influencing FAN resilience (independent of the background FAN concentration) [40].
683 However, Figure 5 shows that microbial communities can be acclimated to high FAN
684 concentrations for a wide variety of substrates.

685 In fact, while a compositional pattern can be observed in Figure 5, the corresponding TAN
686 concentrations in each cluster were quite similar (Figure 6A). Although the TAN
687 concentration medians followed a moderate increase with higher IC_{50} values (Figure 6A and
688 Table 4), no significant differences were found except for a slightly higher value in Cluster 5.
689 The same occurred with the NH_4^+ concentrations, although they were increasingly lower than
690 the TAN contents at higher IC_{50} values due to the increasing proportions of FAN (Figure 6B).
691 On the other hand, the temperature and the pH medians for each cluster did increase
692 significantly ($p < 0.01$) with the obtained IC_{50} values (Figure 6C and Figure 6D). These
693 results suggest that the speciation of TAN and FAN (FAN proportion) is the main factor
694 determining inhibition of AD by FAN. As microorganisms able to grow at high pH and

695 temperatures are more likely to be exposed to high FAN concentrations, it is logical to expect
696 higher IC_{50} values under these conditions, *i.e.* a greater resistance to inhibition. These results
697 highlight that thermophilic microorganisms have higher FAN inhibitory limits than
698 mesophilic ones, which is agreement with previous results presented in the literature [32]. The
699 debate regarding the impact of temperature on FAN resilience has been ongoing for years,
700 with studies confirming that thermophiles are more resilient to FAN inhibition and others
701 showing that thermophilic AD reactors are more vulnerable to TAN/FAN increases than
702 mesophilic systems [5]. This latter observation is usually a consequence of the higher FAN
703 proportions at higher temperatures, which implies that smaller TAN increments are more
704 detrimental to thermophilic reactors non-adapted to high TAN/FAN contents because more
705 FAN is actually added. Nevertheless, this observation is not against the claim of adapted
706 thermophiles being more resilient to FAN than adapted mesophilic archaea, as this finding
707 simply confirms that microorganisms more likely to live at higher FAN levels are more
708 resilient to FAN inhibition. Because of this, adaptation of the archaeal community to high
709 FAN concentrations is even more relevant at high temperatures.

710



711
 712 **Figure 6.** Values of the (A) TAN concentrations, (B) NH₄⁺ concentrations, (C) temperatures
 713 and (D) pH for each cluster. For each parameter, conditions with different upper Greek letters
 714 are significantly different. The boxplots shown provide the values for the minimum, first
 715 quartile, median, third quartile and maximum. As the data were generally non-normally
 716 distributed, non-parametric tests were applied to assess significant differences (see Section
 717 2.4). TAN stands for total ammoniacal nitrogen

718

719 **Table 4.** Features of the clusters: main substrate fed, median TAN concentrations, median
 720 temperatures, median pH and predominant archaeal genus after AD

Cluster #	IC_{50} (mg FAN-N·L ⁻¹)	Main substrate	TAN (g TAN-N·L ⁻¹)	Temperature (°C)	pH	Predominant archaeal genus
1	39	Acetate	4.8	37	7.2	<i>Methanosaeta</i>
2	84	Acetate	3.3	37	7.5	<i>Methanosaeta</i>
3	208	Manure	4.0	37	7.7	<i>Methanosarcina</i>
4	458	Manure	4.9	38	7.8	<i>Methanoculleus</i> ¹
5	1,082	Manure	6.0	55	7.9	<i>Methanoculleus</i> ¹
6	2,565	Manure	5.4	55	8.1	<i>Methanoculleus</i> ¹

721 FAN stands for free ammonia nitrogen and TAN for total ammoniacal nitrogen

722 1. The microbial communities were characterized in less than 50 % of the datasets in the cluster

723

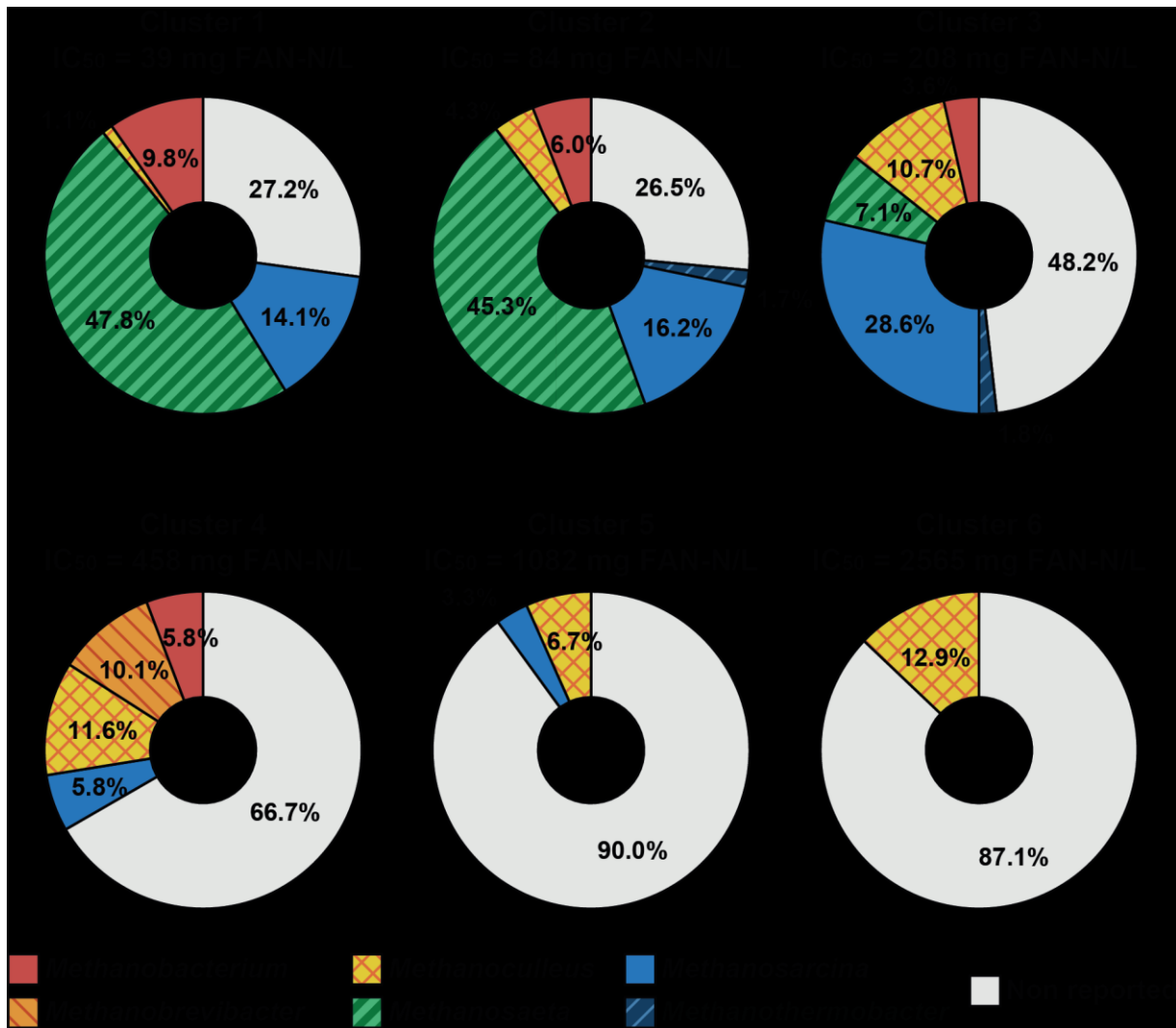
724 Although FAN is the main inhibitor of digesters operated at high TAN concentrations, several
 725 publications have shown that NH₄⁺ is also an inhibitor of the AD process [8,17,18,34,37].
 726 Indeed, the results presented in Figure 6B show that the resilience to FAN inhibition is
 727 independent of the NH₄⁺ concentrations, which is agreement with Benabdallah *et al.* [37] and
 728 Astals *et al.* [8], who were able to model the separate impact of both inhibitors. For digested
 729 sewage sludge (a non-adapted inocula belonging to clusters 1 and 2), Lay *et al.* [34] and
 730 Astals *et al.* [8] observed that FAN starts inhibiting as soon as its concentration was increased
 731 (1-10 mg FAN-N·L⁻¹), whereas a certain threshold concentration had to be reached prior
 732 observing NH₄⁺ inhibition (~1 g NH₄⁺-N·L⁻¹). The results from Lay *et al.* [34] and Astals *et*
 733 *al.* [8] also showed that the difference between the starting inhibition concentration and the
 734 concentration where inhibition is complete is an order of magnitude for FAN (from 10 to 150
 735 mg FAN-N·L⁻¹) and 2 to 5-folds for NH₄⁺ (from 1 to 5 g NH₄⁺-N·L⁻¹). FAN and NH₄⁺
 736 inhibition can only be distinguished by carrying out experiments at different TAN and pH
 737 values. In practice, this is only achievable in batch inhibition tests where a range of conditions

738 can be tested for a given inoculum [8,34,37]. Astals *et al.* [8] proposed a methodology to
739 differentiate the impact of both inhibitors. However, the long legacy of FAN as main AD
740 inhibitor and the fact that FAN is, in most cases, a stronger inhibitor than NH_4^+ , means that
741 NH_4^+ inhibition in AD systems remains overlooked. It must be commented that the
742 mechanisms behind NH_4^+ toxicity remain unknown. The reported decrease in methanogenic
743 activities could be related to higher osmotic pressures due to higher concentrations of ions in
744 the media. Experiments should be carried out to differentiate between a potential specific
745 NH_4^+ toxicity and the impact of high osmotic pressures.

746 *4.3. Influence of the microbial community on FAN resilience*

747 Regarding the structure of the microbial communities in each cluster, Figure 7 and Table 4
748 show the repartition of dominant archaeal genus (archaea are the most vulnerable
749 microorganisms to FAN inhibition) for each cluster.

750



751

752 **Figure 7.** Repartition of the main archaeal genus after AD for each of the resulting clusters.

753 Only the predominant genus (that with the highest percentage in relative abundance) was
 754 considered for the counting. “Non-reported” corresponds to studies in which the microbial
 755 communities were not analyzed

756

757 The first two clusters (IC_{50} of 39 and 84 mg FAN-N·L⁻¹), the most vulnerable to FAN
 758 inhibition, were mostly dominated by acetoclastic methanogens (mainly *Methanosaeta*).

759 Different studies have demonstrated that the inhibitory effect due to the presence of FAN is

760 generally stronger for acetoclastic methanogens when compared to hydrogenotrophic
761 methanogens [20,21,79,83,84,85,86,87,88]. *Methanosaeta* is a genus formed by acetoclastic
762 methanogens able to perform a more efficient acetate conversion to methane than
763 hydrogenotrophic archaea [13]. Therefore, they are usually the predominant archaea under
764 non-stressed AD conditions (*i.e.* low TAN/FAN and VFA concentrations). However,
765 *Methanosaeta* have been reported to be particularly sensitive to FAN inhibition, with IC_{50}
766 values up to 175 mg FAN-N·L⁻¹ [5,43,85]. Studies working at increasing FAN concentrations
767 have reported that acetoclastic archaea dominate at low FAN/TAN concentrations, while
768 mixotrophic and hydrogenotrophic archaea dominate at higher FAN concentrations. Thus,
769 high FAN concentrations cause a shift from acetoclastic to hydrogenotrophic methanogenesis.
770 Under these circumstances, syntrophic acetate oxidation (SAO) is the predominant acetate-
771 consuming pathway [43,76,79,89,90,91,92,93,94].

772 Consistently, at increasing values of the IC_{50} , the mixotrophic *Methanosarcina* and
773 hydrogenotrophic methanogens (*i.e.* *Methanoculleus*, *Methanobacterium* and
774 *Methanobrevibacter*) started to gain importance (Cluster 3, IC_{50} of 208 mg FAN-N·L⁻¹). The
775 role of the genus *Methanosarcina* in this transition from low to high FAN levels has been
776 reported to be particularly important, being more resistant to FAN than strict acetoclastic
777 methanogens (*e.g.* *Methanosaeta*). *Methanosarcina* are able to degrade acetate, which allows
778 this genus to thrive even if an effective SAO community is not present [13,85]. In agreement
779 with our results, a total failure of *Methanosaeta*-dominated reactors has been observed at
780 TAN concentrations over 4,100-4,200 mg TAN-N·L⁻¹, corresponding to FAN contents of
781 150-175 mg FAN-N·L⁻¹ at the corresponding working conditions [8,84,95,96]. The K_{max} for
782 the *Methanosaeta*-dominated clusters (1 and 2) further verify these inhibition limits for

783 acetoclastic archaea, with values of 72 and 152 mg FAN-N·L⁻¹, respectively. The K_{min}
784 corresponding to the third cluster (78 mg FAN-N·L⁻¹) further proves the greater resistance of
785 *Methanosarcina* to FAN inhibition, with a K_{max} of 337 mg FAN-N·L⁻¹. This is also supported
786 by the presence of this archaea in clusters 4 and 5, with K_{max} of 741 and 1,878 mg FAN-N·L⁻¹,
787 respectively. Consistently, efficient methane production has been achieved in
788 *Methanosarcina*-dominated systems working at TAN concentrations over 7,000 mg TAN-
789 N·L⁻¹ (FAN contents up to 600-700 mg FAN-N·L⁻¹) [26,71,97,98].

790 At increasing values of IC_{50} (*i.e.* last three clusters) a total disappearance of *Methanosaeta*
791 occurred, which can be explained by the higher K_{min} of these clusters (> 175 mg FAN-N·L⁻¹)
792 when compared to the K_{max} of the first two (< 152 mg FAN-N·L⁻¹), where *Methanosaeta* was
793 the dominant methanogen. In addition, *Methanosarcina* was no longer the dominant
794 methanogen, being replaced by hydrogenotrophic archaea. While *Methanosarcina* was no
795 longer dominant in any study after the fifth cluster (K_{max} of 1,878 mg FAN-N·L⁻¹),
796 *Methanoculleus* appeared as the most resistant methanogen, dominating the last cluster, with a
797 K_{min} of 743 mg FAN-N·L⁻¹ and a K_{max} of 4,387 mg FAN-N·L⁻¹. Recent studies indicate that
798 when working at these high FAN concentrations hydrogenotrophs become dominant due to
799 their higher tolerance to FAN, confirming the metabolic shift towards hydrogenotrophic
800 methanogenesis [21,79,83]. Amongst the most relevant genus of hydrogenotrophic archaea
801 reported in the literature are *Methanoculleus*, *Methanobacterium*, *Methanothermobacter* and
802 *Methanobrevibacter* [21,79,87,99]. In agreement with the results presented in this document,
803 *Methanosarcina* has been reported to be predominant at intermediate FAN concentrations (*i.e.*
804 FAN contents up to 600-700 mg FAN-N·L⁻¹), being more resistant to FAN than acetoclastic
805 methanogens but more vulnerable than hydrogenotrophs [13,71,85,96,97,98,100,101].

806 *Methanosarcina* have been found to be responsible for a partial resistance of acetoclastic
807 methanogenesis to sudden increases of the FAN levels and they have also been found to be
808 the archaea allowing the recovery of methane production after severe FAN inhibition and
809 VFA accumulation [85,99,102]. The ability of *Methanosarcina* to consume both acetate and
810 hydrogen allow this genus to thrive when an effective SAO community is not present.
811 Therefore, transitional periods might be a niche where *Methanosarcina* thrive, being
812 outcompeted afterwards when a functional SAO-hydrogenotrophic syntrophy is developed.

813 *Methanoculleus* has been reported as the predominant archaea at extreme FAN levels
814 [26,91,92]. *Methanoculleus* have been found to be more tolerant to FAN than
815 *Methanosarcina*, which is in agreement with the results presented in Figure 7 and Table 4.
816 Shifts from *Methanosarcina*-dominated reactors towards *Methanoculleus*-dominated systems
817 have been found at increasing high FAN concentrations [83,103]. In addition, experiments
818 applying bioaugmentations strategies using *Methanoculleus bourgensis* have demonstrated
819 that the addition of this archaea can alleviate the effect of FAN toxicity on the AD
820 performance [104,105].

821 It has also been found that mesophilic hydrogenotrophic methanogens are more sensitive to
822 FAN toxicity compared to thermophilic methanogens, being *Methanoculleus thermophilus*
823 more tolerant than *Methanoculleus bourgensis* [12]. *Methanothermobacter* is another
824 thermophilic archaeal genus that has been found to be dominant at high FAN levels [21,106].
825 This is in agreement with the results presented in Figure 6C, showing increasing IC_{50} values
826 at higher temperatures. Unfortunately, most of the studies that operated digesters at high FAN
827 concentrations did not report the microbial community (see “non-reported” proportions in
828 clusters 3 to 6 in Figure 7). More research should be carried out to elucidate which are the

829 archaea dominating at these conditions and to determine their FAN inhibition limits and their
830 practical growth rates.

831 It can be concluded from the data that acclimation of the microbial communities to high FAN
832 concentrations is a key factor affecting their resilience. Recently, Tian et al. [77], who
833 compared three different acclimation strategies (batch, fed-batch, continuous reactor),
834 reported that a fed-batch reactor was the most efficient method to enrich a FAN-resistant
835 microbial community since it allowed a stepwise increase of FAN concentration while
836 preventing biomass washout. This is in agreement with other studies showing the
837 effectiveness of consecutive batch operation for microbial acclimation [21,76,107,108,109].
838 The variation of the microbial distribution with time before, during and after inhibition (light
839 or severe) must be always considered if a resilient AD system is to be achieved.

840

841 **5. Practical implications and industrial relevance**

842 In the last decades, the industrial application of AD has been extended from the treatment of
843 waste activated sludge in wastewater treatment plants to the valorization and stabilization of
844 several waste streams, such as municipal solid waste, food waste and animal manure [1,2,3].
845 The introduction of a more diverse variety of influents has boosted tremendously the
846 development of AD as treatment technology but, at the same time, it has raised novel
847 challenges. In the case of concentrated, N-rich, substrates, the main challenge has been
848 process inhibition due to ammonia-N accumulation, which has frequently led to reactor
849 failure. The most common practical solution to maintain stable operation is substrate dilution,
850 which is associated to significant economic losses and different process complications (*e.g.*
851 larger equipment and digestate volumes). Because of this, several research and industrial

852 projects have been focused on the subject, aiming at understanding the issue and at finding a
853 solution.

854 This review aimed at unifying all the previous efforts to provide a methodology that can serve
855 to correctly calculate the FAN concentrations in digesters, producing comparable, accurate
856 data. The proposed modified Davies equation can serve as a straight-forward option to reduce
857 the errors in FAN calculations to negligible values in both industrial and research purposes. A
858 main limitation for its application is the necessity of determining the I of the media, which
859 requires the measurement of different chemical species. Nevertheless, the results presented in
860 Figure 3 confirm that few ions need be measured to account for most of the I (*e.g.* NH_4^+ , Na^+
861 and K^+). Therefore, a routine analytical set could easily serve for FAN monitoring for
862 industrial applications, simply measuring NH_4^+ and pH. If required, estimating the Na^+ and K^+
863 concentrations could be done via conductivity measurements (after previous calibration).
864 Another limitation of the modified Davies equation is the range of I values where it can be
865 applied (up to 1.0 M). However, these conditions are very rarely reached neither in industrial
866 nor in research applications, so the proposed equation is potentially applicable in most
867 working conditions.

868 Another outcome with both academic and industrial interest is the definition of general IC_{50}
869 values for different working conditions (*i.e.* TAN concentrations, temperature and pH).
870 Although the most precise approach is the experimental determination of the IC_{50} in each
871 particular set-up, the values given here can act as indicators of the FAN resilience for given
872 working conditions, even if the microbial communities are not known. The given values could
873 obviously be used for modelling purposes either as model parameters or as initial estimates
874 for calibration purposes. The given values can be directly applied in commonly used

875 mechanistic models, such as the ADM1.

876 Finally, the results presented in Section 4.3 confirm the importance of the adaptation of the
877 archaeal communities to high FAN concentrations, mainly related to a shift in the population
878 dynamics. Although the microbial communities are rarely monitored in industrial digesters,
879 the importance of adaptation must be considered in plants where the substrates are often
880 changed, such as territorial digesters than frequently modify co-digestion proportions or add
881 new influent streams. If feeding modifications lead to significant increases of the FAN
882 concentrations, transient VFA peaks are to be expected, which might lead to reactor
883 acidification if not properly managed. This is particularly relevant in thermophilic reactors, as
884 increases in the TAN input are translated into much higher FAN levels when compared to
885 mesophilic conditions. The utilization of additives, such as trace elements to favor the growth
886 and development of particular archaea might be also useful for process stabilization and to
887 favor microbial adaptation [21,88].

888 Although the amount of data available on both performance and microbial communities of
889 AD at high TAN/FAN contents has increased significantly in the last years, it is clear when
890 looking at the obtained results (*i.e.* “non-reported” fractions for clusters 4, 5 and 6 in Figure 7;
891 66.7-87.1 %) that more research must be carried out to study the microbial communities in N-
892 rich systems at high FAN levels.

893

894 **6. Conclusions**

895 The presented results show that the ideal equilibrium equation should not be applied for FAN
896 calculation in AD. This method overestimates significantly the FAN concentrations (up to 37
897 % when compared with MINTEQA2). The application of the Davies equation considering

898 only few ions (e.g. NH_4^+ , Na^+ and K^+) can lead to major improvements in most cases. The
899 proposed modified Davies equation appears as the method presenting the best compromise
900 between complexity and accuracy. The differences in the FAN concentrations obtained by this
901 approach and the ones given by MINTEQA2 were below 1 % for 70 % of the data (with a
902 systematic error of 2 %). Analysis of data from the literature dealing with FAN inhibition
903 resulted in six clusters. The results of the clustering procedure proved that pH and
904 temperature, rather than the TAN content itself, are the main factors affecting inhibition of
905 AD by FAN. *Methanosaeta*-dominated reactors presented lower inhibition limits when
906 compared to mixotrophic and hydrogenotrophic systems. The mixotroph *Methanosarcina* was
907 dominant at intermediate FAN concentrations, being more resistant than *Methanosaeta* but
908 more vulnerable than hydrogenotrophic methanogens. *Methanoculleus* appeared as the most
909 resistant archaea, being predominant in AD systems working with the highest FAN levels
910 (mainly fed with manure).

911

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917

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1236 **Figure and table captions**

1237 **Figure 1.** Differences in the FAN concentrations at different ionic strengths: (A) the ideal
1238 equilibrium equation vs MINTEQA2, (B) the ideal equilibrium equation vs the comprehensive
1239 Davies equation. A Loess local regression curve is included (black curves; 1,590 data points
1240 presented). FAN stands for free ammonia nitrogen

1241 **Figure 2.** Differences in the FAN concentrations at different ionic strengths: (A) the
1242 comprehensive Davies method vs the simplified Davies method considering only the NH_4^+
1243 and its counterion, (B) the simplified Davies method considering only the NH_4^+ and its
1244 counterion vs the same method without iterative NH_4^+ calculation, and (C) the modified
1245 Davies method (Eq. 8) vs MINTEQA2. Only data from experiments with significant
1246 concentrations of ions other than NH_4^+ have been considered in A and B (1,132 data points).
1247 1,590 data points are presented in C. Different y-axis scales are presented to allow a precise
1248 data interpretation. FAN stands for free ammonia nitrogen

1249 **Figure 3.** Contributions of each chemical species to the total ionic strength of the media. Only
1250 data from experiments reporting significant concentrations of ions other than NH_4^+ have been
1251 considered (1,097 data points). *I* stands for ionic strength and Ac, Pr, Bu and Val stand for
1252 acetate, propionate, butyrate and valerate, respectively

1253 **Figure 4.** Reduction of the SMA at different FAN concentrations reported in the literature.
1254 The set of inhibition curves and IC_{50} values resulting from the clustering approach are also
1255 shown. SMA stands for specific methanogenic activity and FAN for free ammonia nitrogen

1256 **Figure 5.** Repartition of the substrates used as AD feed in each of the resulting clusters.
1257 OFMSW stands of organic fraction of municipal solid waste

1258 **Figure 6.** Values of the (A) TAN concentrations, (B) NH_4^+ concentrations, (C) temperatures
1259 and (D) pH for each cluster. For each parameter, conditions with different upper Greek letters
1260 are significantly different. The boxplots shown provide the values for the minimum, first
1261 quartile, median, third quartile and maximum. As the data were generally non-normally
1262 distributed, non-parametric tests were applied to assess significant differences (see Section
1263 2.4). TAN stands for total ammoniacal nitrogen

1264 **Figure 7.** Repartition of the main archaeal genus after AD for each of the resulting clusters.
1265 Only the predominant genus (that with the highest percentage in relative abundance) was
1266 considered for the counting. “Non-reported” corresponds to studies in which the microbial
1267 communities were not analyzed

1268 **Table 1.** IC_{50} values for both FAN and TAN reported in the literature (adapted and extended
1269 from Chen *et al.* [6] and Yenigün and Demirel [7])

1270 **Table 2.** Comparison of the tested methods for FAN calculation and recommendations

1271 **Table 3.** Results of the clustering method: values of the IC_{50} , K_{min} , K_{max} , RMSE and number
1272 of points for each cluster

1273 **Table 4.** Features of the clusters: main substrate fed, median TAN concentrations, median
1274 temperatures, median pH and predominant archaeal genus after AD