

**This item is the archived peer-reviewed author-version of:**

Production of carboxylates from high rate activated sludge through fermentation

**Reference:**

Cagnetta C., Coma M., Vlaeminck Siegfried, Rabaey K.- Production of carboxylates from high rate activated sludge through fermentation  
Bioresource technology - ISSN 0960-8524 - 217(2016), p. 165-172  
International Conference on Solid Waste Knowledge Transfer for Sustainable Resource Management (ICSWHK2015)  
Full text (Publisher's DOI): <https://doi.org/10.1016/J.BIORTECH.2016.03.053>  
To cite this reference: <https://hdl.handle.net/10067/1399120151162165141>

1 **Production of carboxylates from high rate activated sludge through fermentation**

2 Cagnetta, C. <sup>a</sup>, Coma, M. <sup>a□</sup>, Vlaeminck, S.E. <sup>ab</sup>, Rabaey, K. <sup>a\*</sup>

3

4 <sup>a</sup> *Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Coupure Links 653, B-9000 Gent, Belgium*

5 <sup>b</sup> *Research group of Sustainable Energy, Air and Water Technology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerpen, Belgium*

6 Authors: Cristina Cagnetta (cristina.cagnetta@ugent.be)

7 Marta Coma

8 Siegfried Vlaeminck

9 Korneel Rabaey (korneel.rabaey@ugent.be)

10

11

12

13 \*Corresponding author:

14 Prof. Dr. Ir. Korneel Rabaey

15 Laboratory of Microbial Ecology and Technology, Faculty of Bioscience Engineering; Coupure Links 653, 9000 Ghent, Belgium. Tel. +32 9 264

16 5976; Mob. +32 468 118476; Fax. +32 9 264 6248; Korneel.rabaey@ugent.be ; <http://labmet.ugent.be>

17

18     Present address of Marta Coma ([m.coma@bath.ac.uk](mailto:m.coma@bath.ac.uk)): Centre for Sustainable Chemical Technologies, University of Bath, Claverton Down,

19    Bath, BA2 7AY, United Kingdom

20

21

22 **Abstract**

23 The aim of this work was to study the key parameters affecting fermentation of high rate activated A-sludge to carboxylates, including pH,  
24 temperature, inoculum, sludge composition and iron content. The maximum volatile fatty acids production was  $141 \text{ mg C g}^{-1} \text{ VSS}_{\text{fed}}$ , at pH 7.  
25 Subsequently the potential for carboxylate and methane production for A-sludge from four different plants at pH 7 and  $35 \text{ }^{\circ}\text{C}$  were compared.  
26 Initial BOD of the sludge appeared to be key determining carboxylate yield from A-sludge. Whereas methanogenesis could be correlated linearly  
27 to the quantity of ferric used for coagulation, fermentation did not show a dependency on iron presence. This difference may enable a strategy  
28 whereby A-stage sludge is separated to achieve fermentation, and iron dosing for phosphate removal is only implemented at the B-stage.

29 **Keywords:** *AB process; Anaerobic digestion; A-sludge; iron; volatile fatty acids (VFA).*

## 30 **1. Introduction**

31 For domestic and industrial wastewater the focus for reuse has been on the water itself as well as the energy contained within the organics  
32 (Verstraete and Vlaeminck 2011). Domestic sewage could also be a source of products such as nutrients or volatile fatty acids (VFAs) that can be  
33 used as building blocks for the production of valuable products such as medium and long chain fatty acids, alcohols and *Polyhydroxyalkanoates*  
34 (*PHA*) (Agler et al. 2011, Kleerebezem et al. 2015, Lee et al. 2014) among the others. However, the typical issue with domestic wastewater is its  
35 low organic concentration which impedes to obtain an economically feasible production process.

36 Several concentration technologies allow recovery of the clean water and increase the amount of organic matter for further processing (Meerburg  
37 et al. 2015, Verstraete et al. 2009). Best known is the “Adsorption-Belebungsverfahren” process (AB process, generally translated as Adsorption  
38 - Biodegradation or Adsorption - Bio-oxidation) (Boehnke et al. 1997), also termed “high-rate activated sludge”. The AB process is a two-stage  
39 treatment system where the first stage, the highly loaded biological adsorption stage or A-stage, is a modification of the conventional activated  
40 sludge system (CAS) (Constantine et al. 2012). CAS is typically performed with, loadings of  $0.25 \text{ kg BOD kg}^{-1} \text{ VSS d}^{-1}$  (PaDEP 2014), high  
41 aeration and SRT of 8 - 20 days, with a low digestion efficiency of the sludge (waste activated sludge (WAS)) due to the high aeration and high  
42 sludge age. (Bolzonella et al. 2005). The A-stage is operated with high loadings ( $2 - 10 \text{ kg BOD kg}^{-1} \text{ VSS d}^{-1}$ ), low hydraulic retention time  
43 (HRT 15 - 30 min) and solid retention time (SRT) between several hours and 1 day (Boehnke et al. 1998). These conditions determine  
44 bioflocculation with high biomass yields and minimal  $\text{CO}_2$  formation, complemented with sorption and storage mechanisms that remove COD  
45 (Meerburg et al. 2015). The A-sludge is highly biodegradable and easily digested to biogas (Boehnke et al. 1998). The subsequent B-stage  
46 ensures polishing of the wastewater to meet discharge standards. Typically, the A-stage has a COD removal efficiency of 26-52% (De Graaff and

47 Roest 2012), and 80 % of the excess sludge of the overall plant is withdrawn from the A-stage. The excess B-sludge is combined with the excess  
48 A-sludge before digestion to achieve energy neutrality at the facility level. The process is applied at full scale in several municipal wastewater  
49 treatment plants (WWTPs) and industrial plants (Boehnke et al. 1997). The limited experience to date does lead to considerably different  
50 operational approaches and hence different sludge properties (De Graaff and Roest 2012).

51 Thus far the sludge valorization has been investigated through anaerobic digestion either as a single substrate (Huoqing et al. 2013, Meerburg et  
52 al. 2015) or in co-digestion with other organic waste streams (De Vrieze et al. 2013, Verstraete and Vlaeminck 2011). In recent years, another  
53 route has come to the fore, the carboxylate platform, whereby mixed populations are used to ferment organic matter carboxylates that are the end  
54 product instead of biogas (Agler et al. 2011, Angenent et al. 2004). As past studies on hydrolyzed secondary sludge have shown that it is possible  
55 to ferment it although to a limited extent (Morgan-Sagastume et al. 2011, Pratt et al. 2012), the highly degradable A-sludge could be a more  
56 suitable source. The main interest in A-sludge fermentation is that VFA can be used as perfect additional carbon source to enhance biological  
57 nitrogen and phosphorous removal in waste water treatment plants (Lee et al. 2014), with no requirement of previous extraction from the sludge,  
58 avoiding acetate or methanol expenses for wastewater's with low carbon content. The fermentation can then be followed by anaerobic digestion  
59 for removal of unfermented organics. The potential of the A-sludge for carboxylate production in terms of conversion efficiencies and product  
60 outcomes, and how this could relate to residual digestion has not been yet explored.

61 Therefore, the goal of this study was to identify key parameters affecting fermentation of an A-sludge (here temperature and pH, inoculum  
62 addition and retention time) and subsequently assess the fermentation potential of A-sludge originating from different sites and to correlate this to  
63 different operational approaches. A prime example of a variable parameter is the iron content. Indeed considerable differences in iron

64 concentration were found in the samples collected from the four WWTPs object of this study. Iron content was also different between the two  
65 time frames considered. In these sites salts of Fe (II) or (III) such as  $\text{FeSO}_4$  and  $\text{FeCl}_3$  are added in different concentrations to precipitate  
66 phosphates as  $\text{Fe}_3(\text{PO}_4)_2(s)$  or  $\text{FePO}_4(s)$  and to enhance the flocculation, coagulation and sedimentation of the A-sludge (Jiang and Graham 1998).  
67 Furthermore, iron is important as coenzyme or cofactor in several enzymes involved in the anaerobic digestion process (Zandvoort et al. 2006).  
68 Methanogens require iron for their metabolic activity, and their capability to continue producing methane is strictly dependent on the presence  
69 and availability of the metal (Demirel and Scherer 2011). The presence of iron in the sludge flocs can have a key impact for the methanogenesis,  
70 as the process is limited at low iron concentrations (Schattauer et al. 2011, Zandvoort et al. 2006). It is as yet unknown how this would impact  
71 fermentation. Similarly, A-sludge will vary in their BOD content and BOD/COD ratio, due to day-by-day variances at WWTPs level and in  
72 dilution and content of the domestic wastewater. Thus we measured key characteristics of different A-sludge and subjected them to fermentation  
73 and digestion.

74

75 **2. Materials and methods**

76 ***2.1. A-sludge collection***

77 Auto-fermentation test without inoculum (see 2.2.1) were performed with A-sludge from Nieuwveer WWTP (Breda) in 11/2013 (COD  $11.3 \pm$   
78  $0.5 \text{ g L}^{-1}$ , TSS  $9.1 \pm 0.4 \text{ g L}^{-1}$ , VSS  $6.7 \pm 0.3 \text{ g L}^{-1}$ ). In 1/2014 new sludge was collected (COD  $4.3 \pm 0.4 \text{ g L}^{-1}$ , TSS  $3.7 \pm 0.5 \text{ g L}^{-1}$ , VSS  $2.8 \pm 0.4$   
79  $\text{g L}^{-1}$ ) to ferment in the presence of an inoculum (2.2.2). To compare the fermentative capacity of different A-sludge (2.2.3), they were collected  
80 during two different time frames (2/2014 and 7/2014) from four WWTPs in the Netherlands: Nieuwveer WWTP (Breda), Dokhaven WWTP  
81 (Rotterdam), Utrecht WWTP (Utrecht) and Garmerwolde WWTP (Groningen). The characteristics of the A-sludge on those two sampling points  
82 are summarized in Table 1. To determine the effect of iron on the fermentation (2.2.4), A-sludge was collected from Nieuwveer WWTP (Breda)  
83 in 10/2015 (COD  $5.3 \pm 0.5 \text{ g L}^{-1}$ , TSS  $2.2 \pm 0.3 \text{ g L}^{-1}$ , VSS  $1.7 \pm 0.0 \text{ g L}^{-1}$ , Fe  $171 \pm 2 \text{ mg L}^{-1}$ ). Waste activated sludge (WAS) was collected from  
84 Dendermonde WWTP in 8/2015 (TS  $58.7 \pm 7.1 \text{ g kg}^{-1}$ , VS  $32.4 \pm 7.3 \text{ g kg}^{-1}$ , COD  $45.1 \pm 5.9 \text{ g L}^{-1}$ ).

85

86 ***2.2. Fermentation batch tests***

87 All tests were performed in triplicate in serum flasks (120 mL) sealed with a rubber stopper and aluminum sealer. Headspace was flushed with  
88  $\text{N}_2$  at day 0 of all experiments which were kept temperature controlled and shaking at 120 rpm for a period of 7 or 14 days. Liquid and gas  
89 samples were taken periodically decreasing the frequency over the experiment. Biogas production was monitored for every sampling point.  
90 Chemical analysis procedures are defined in supplementary information.

91



92 *2.2.1. Determination of key parameters during auto-fermentation*

93 Eight conditions with combination of different pH (4.5, 5, 6, 7) and temperatures (35 °C and 55 °C) were carried with only 80 mL A-sludge.  
94 Control tests without pH control were performed for each temperature tested for both A-sludge and WAS from Dendermonde WWTP. The pH  
95 was adjusted to the desired value by adding 1 M NaOH or 1 M HCl after each sampling time.

96

97 *2.2.2. Determination of the inoculum impact*

98 Mixed culture inoculum from a fermenter (CSTR) treating diluted molasses (HRT 5 days, pH 5.5, T 35 °C, obtained after a stable working period  
99 of 110 days) for VFA production was previously acclimated to A-sludge as substrate at either mesophilic or thermophilic conditions. The tests  
100 were prepared by mixing 10 mL of inoculum, 30 mL of substrate and 40 mL of a pH buffer. Controls with only inoculum or only substrate by  
101 replacing either one of them with water were performed to normalize the final results. Batch tests were run combining pH (6 or 7) and mesophilic  
102 (35 °C) or thermophilic (55 °C) temperatures. pH control was carried out by means of a pH buffer (strength 200 mM H<sup>+</sup>), prepared in tap water  
103 (pH = 6: 24.3 mg NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O L<sup>-1</sup> and 6.4 mg Na<sub>2</sub>HPO<sub>4</sub> · 7 H<sub>2</sub>O L<sup>-1</sup>; pH = 7: 11.7 mg NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O L<sup>-1</sup> and 30.9 mg Na<sub>2</sub>HPO<sub>4</sub> · 7 H<sub>2</sub>O L<sup>-1</sup>).

104 *2.2.3. Comparison of different A-sludge fermentative capacity*

105 A-sludge from four different WWTP obtained either during the winter (February 2014) or the summer period (July 2014) were evaluated. The  
106 tests consisted of a mixture of substrate, inoculum and pH buffer as defined in section 2.2.2. Controls with only inoculum and only substrate  
107 were also included. The tests were run at 35 °C and pH 7.

108

109 *2.2.4. Determination of iron impact on A-sludge fermentation*

110 FeCl<sub>3</sub> was added to Breda A-sludge in order to obtain Fe concentrations of 2.5, 5, 10 and 20 mmol Fe L<sup>-1</sup> (as in Hoban and Van Den Berg  
111 (1979)), and evaluated to determine the effect of increasing iron concentration on VFA and CH<sub>4</sub> production during the fermentation. VFA  
112 production tests consisted of a mixture of substrate, inoculum and pH buffer as defined in section 2.2.2. Controls with only inoculum and A-  
113 sludge where no iron was added were also carried out. The tests were run at 35 °C and pH 7. Methane production tests were evaluated by means of  
114 biochemical methane potential.

115

116 ***2.3. Biochemical methane potential (BMP) test***

117 BMP tests were carried out to estimate the anaerobic biodegradability of the A-sludge from the four WWTPs (February and July 2014) and for  
118 iron impact evaluation (see 2.2.4). The test was performed in triplicate under mesophilic conditions (35 °C) with a working volume of 80 mL.  
119 Inoculum or substrate were replaced by tap water in the control tests. The substrate to inoculum ratio was maintained at 0.3 g COD g<sup>-1</sup> VS. The  
120 inoculum was obtained from a full scale anaerobic digester from Trevi (Belgium) and sieved at 2 mm to homogenize and remove solid materials.  
121 The inoculum biomass accounted for 5 g VS L<sup>-1</sup>. Flasks were sealed with a rubber stopper and aluminum sealer and then connected to glass  
122 columns, in which biogas production was measured by means of water displacement. Biogas composition was evaluated at the end of the  
123 experiment, after 35 days. Methane yield was expressed as the volume of methane per gram of volatile solids of substrate. Values are reported at  
124 standard temperature and pressure (STP) conditions.

125

126 **2.4. Analytical techniques**

127 All the analysis were carried out as described in the Supplementary information.

## 128 **3. Results and discussion**

### 129 ***3.1. Key parameters determining A-sludge auto-fermentation***

130 In a first phase, we evaluated the impact of key parameters such as temperature, pH and retention time of the sludge towards auto-fermentation  
131 purpose (without inoculum) (Figure 1).

132

#### 133 ***3.1.1. Impact of retention time and temperature***

134 Carboxylate production in all tests followed the same pattern, reaching a maximum VFA production at day 7. All fermentation conditions, except  
135 pH 4.5 and 55 °C, presented maximum VFA productions higher than the obtained from the WAS at both 35 °C and 55 °C (respectively  $6 \pm 0$  mg  
136 C VFA g<sup>-1</sup> VSS<sub>fed</sub> at day 2 and  $61 \pm 1$  mg C VFA g<sup>-1</sup> VSS<sub>fed</sub> at day 4). The highest value obtained, up to  $141 \pm 15$  mg C VFA g<sup>-1</sup> VSS<sub>fed</sub>,  
137 equivalent to 420 mg COD g<sup>-1</sup>VSS<sub>fed</sub>, (30% conversion of the COD supplied) was also higher than the greater values reported in literature for the  
138 fermentation of both primary and secondary WAS (298 and 368 mg COD g<sup>-1</sup>VSS<sub>fed</sub> at mesophilic and thermophilic conditions, respectively) (Lee  
139 et al. 2014, Zhang et al. 2009). VFA production from A-sludge was also higher than what reported from Ma et al. (2016) for WAS both untreated  
140 and heat-alkaline pretreated sludge.

141 For the A-sludge, the highest VFA concentrations were observed on day 7 and 9 at all the pH values tested (Figure 1a). Carboxylate  
142 concentrations subsequently decreased due to the conversion of VFA to CH<sub>4</sub> (Figure 1b). CH<sub>4</sub> generation increased from day 7 and the highest  
143 concentrations were obtained at pH 6 and 7 (including the control test with a pH around 6.5). Therefore, to maximize the VFA production, the  
144 retention time for the A-sludge treated in batch mode was fixed at 7 days for all the following fermentation tests.

145 Temperature and pH range were chosen in this study based on applicability at WWTP level. In particular, thermophilic temperature can in some  
146 cases enhance the hydrolysis leading to higher biogas production rates at lower SRT (Levén et al. 2007, Veecken and Hamelers 1999). This fact  
147 was also verified for the WAS fermentation test carried out at mesophilic and thermophilic conditions. Even though both temperatures lead to  
148 similar methane productions, thermophilic conditions enhanced hydrolysis of the secondary sludge and thus, increased the VFA concentration in  
149 the fermentation broth. In a continuous system, if thermophilic fermentation is performed, HRT should be kept low (e.i. 2 days) in order to avoid  
150 methane accumulation. In case of the A-sludge no significant differences in VFA production were observed between mesophilic and  
151 thermophilic conditions, confirming earlier findings in A-sludge digestion ((De Vrieze et al. 2013). In a continuous system, thermophilic  
152 fermentation would theoretically produce more methane after accumulation. Therefore, a low HRT (i.e. 2 days) is recommended to avoid  
153 methane generation.

154

### 155 *3.1.1. Impact of pH*

156 Although several studies in literature show an increase in VFA production when the fermentation is carried out at alkaline pH (8-10) (Cysneiros  
157 et al. 2012), the use of such high pH involves high concentration of chemicals such as NaOH to raise the pH. Moreover, alkaline fermentation of  
158 A-sludge (pH 8, 9 and 10) yielded to lower VFA production than at neutral pH (Figure S1, Supplementary information). Therefore, the pH was  
159 varied between 4.5 and 7.

160 An increase in pH led to an increase in VFA production. However, at pH close to neutrality, methane production was enhanced. In several studies  
161 the inhibition of methanogens has been achieved controlling various parameters such as sludge retention time (SRT), pH and loading rate. pH is

162 considered to be the most useful parameter since most of the methanogens perform optimally in a narrow pH range of 6.8-7.5 (Appels et al. 2008,  
163 Gujer and Zehnder 1983). Fermentative bacteria are generally less sensitive and function in a pH range between 4 and 11 (Appels et al. 2008,  
164 Chen et al. 2007, Lee et al. 2014), depending on the carboxylate content. However, at pH values close to the equilibrium constant of the  
165 carboxylates (pKa 4.8 – 4.9) also fermenters are impacted by product inhibition. We therefore assessed VFA production depending on pH and  
166 temperature only by values obtained after 7 days (Figure 2).

167 Cysneiros et al. (2012) demonstrated that increasing pH up to 6.5 improved hydrolysis and increased VFA production by 50% in leachate.  
168 Indeed, at 55 °C the VFA concentration almost doubled, when increasing from pH 5 ( $77 \pm 5$  mg C VFA  $g^{-1}$  VSS<sub>fed</sub>) to pH 7 ( $141 \pm 15$  mg C VFA  
169  $g^{-1}$  VSS<sub>fed</sub>). The combination of thermophilic temperatures and pH 7 lead to an increased accumulation of VFA also due to less conversion to  
170 CH<sub>4</sub> under thermophilic conditions.

171 At 35 °C the carboxylate production was always higher than at 55 °C at all the pH values considered, except at pH 7. Conversion of VFA to CH<sub>4</sub>  
172 was always higher at mesophilic conditions (Fig. 2b) due to optimal conditions for methanogenesis (35 °C and pH 6.8 - 7.5). Indeed, by  
173 increasing the pH from 5 to 6 and further to pH 7, the CH<sub>4</sub> production achieved values of  $0.11 \pm 0.01$  mg C VFA  $g^{-1}$  VSS<sub>fed</sub>,  $0.44 \pm 0.00$  mg C  
174 CH<sub>4</sub>  $g^{-1}$  VSS<sub>fed</sub> and  $0.87 \pm 0.21$  mg C CH<sub>4</sub>  $g^{-1}$  VSS<sub>fed</sub>, respectively.

175 Considering this first set of experiments, a pH of 6 - 7 gave the highest productions. If production is considered as the sum of VFA and methane,  
176 mesophilic tests yielded 128 and 110 mg C  $g^{-1}$  VSS<sub>fed</sub> and thermophilic tests yielded 114 and 155 mg C  $g^{-1}$  VSS<sub>fed</sub> at pH 6 and 7, respectively.  
177 Not much difference was presented within these conditions, thus they were chosen for further experiments in combination with a residence time  
178 of 7 days.

179 Table 2 summarizes the different carboxylates obtained for each condition. For all the A-sludge fermentation condition tested combining pH and  
180 temperature, acetate was the main product obtained ranging from 56 to 88% on the 7<sup>th</sup> day of operation when the maximum VFA concentration  
181 was detected. At 55 °C, other VFA from propionate to caproate were also detected at all the pH values tested. Propionate represented about 10%  
182 at all pH values, butyrate decreased from 12 to 6% with increasing the pH. Traces of caproate were found above pH 6, increasing to 3 % at day 7.  
183 At 35 °C the specificity of acetate production improved and propionate was only detected at low pH. At pH values above 6 no propionate was  
184 found and 87 - 89 % of the converted substrate ended up in acetate.

185 Despite the fact that low pH is often used in fermentation processes to inhibit methanogenesis, auto-fermentation of A-sludge improved at pH  
186 values between 6 and 7. Ma et al. (2016) also demonstrated that neutral pH could be favor to the acidogenesis reaction improving VFA  
187 production. Mesophilic conditions (35 °C) increased the total product concentration and the product specificity, acetate, in the final mixture.  
188 Propionate was mainly detected in non-optimal conditions (thermophilic or lower pH). Methane production was still one of the limitations in  
189 sludge fermentation, although it might be reduced when fixing the retention time to 7 days.

190

### 191 ***3.2. Fermentation in the presence of an adapted inoculum***

192 The maximum carboxylate concentration detected for A-sludge auto-fermentation was 141 mg C VFA g<sup>-1</sup> VSS<sub>fed</sub>, equivalent to 30 % conversion  
193 efficiency. According to previous work, the conversion efficiency during the digestion of A-sludge to CH<sub>4</sub> is around 50-70 % (De Graaff and  
194 Roest 2012, De Vrieze et al. 2013) which suggest that the potential for VFA production should be higher. In the second experiment we thus

195 investigated the impact of an adapted fermentative inoculum acclimatized to A-sludge. Conditions with and without inoculum were tested both at  
196 35 °C and 55 °C and at pH 6 and 7 (Figure S2).

197 Inoculum presence enhanced VFA production at both temperatures. In all cases the production at 35 °C was higher than at 55 °C (Figure S2), in  
198 agreement with the auto-fermentation tests. The best VFA production was achieved with inoculum at 35 °C and pH 7, the production was  
199 significantly higher (P 0.046) than at pH 6.

200 The total VFA concentrations obtained in this second tests (Figure S2) were lower than the auto-fermentation tests (Figure 2). Batch tests in  
201 which the buffer was replaced by tap water were carried out to test if phosphate buffer could affect the fermentation, yet no difference in VFA  
202 production was found (data not shown). Therefore, the difference in total carboxylate production was attributed to differences in A-sludge  
203 composition due to day-by-day changes in operational parameters at WWTP level (see 3.3).

204 Although 35 °C and pH 7 appeared the most advantageous conditions for methanogenesis to occur, no CH<sub>4</sub> production was detected after 7 days  
205 of fermentation, thus confirming that a fermentative inoculum can direct the digestion specifically towards VFA production, when the residence  
206 time is kept low.

207

### 208 ***3.3. Influence of A-sludge composition***

209 A-sludge samples from four different WWTPs in The Netherlands (Breda, Groningen, Rotterdam and Utrecht) were collected during winter and  
210 summer periods to evaluate their potential for production of carboxylates through fermentation at pH 7 and 35 °C in 7-day batch tests. The main



211 A-sludge composition varied from one substrate to the other within a range of 3 – 15 g COD L<sup>-1</sup>, 2 – 11 g TS L<sup>-1</sup> and 95 – 440 mg N L<sup>-1</sup>, with  
212 winter values being higher for all with the exception of the organic content in Rotterdam (Table 1). Fermentation results are depicted in Figure 3.  
213 The A-sludge collected from Utrecht and Breda WWTPs had a similar production for both winter and summer periods (116 ± 3 and 123 ± 7, and  
214 92 ± 5 and 91 ± 3 mg C VFA g<sup>-1</sup> VSS<sub>fed</sub>, respectively), although summer organics content was lower than winter (Table 1). The VFA production  
215 from Rotterdam and Groningen substrates was lower for the samples collected in summer. In particular, the VFA production of the Rotterdam  
216 sample was 143 ± 5 mg C VFA g<sup>-1</sup> VSS<sub>fed</sub> in February 2014 and 88 ± 3 mg C VFA g<sup>-1</sup> VSS<sub>fed</sub> in July 2014. Groningen VFA production values  
217 were 44 ± 1 mg C VFA g<sup>-1</sup> VSS<sub>fed</sub> and 8 ± 5 mg C VFA g<sup>-1</sup> VSS<sub>fed</sub> for the samples collected in February and July 2014, respectively. The  
218 decrease in VFA production in the sample from Groningen might be explained by the reduction of organic matter from 7.2 to 3.1 g COD L<sup>-1</sup> that  
219 led to a decrease in VFA production from 9 % to 2 % g COD g<sup>-1</sup> COD<sub>fed</sub>. However, this was not the case for the Rotterdam samples, as they  
220 showed a similar COD content in February and July of 5.1 ± 0.1 g L<sup>-1</sup> and 7.0 ± 0.3 g L<sup>-1</sup>, respectively. The efficiency in VFA conversion for the  
221 A-sludge from Rotterdam was 18 % and 11 % for the samples collected in February 2014 and July 2014, respectively.  
222 No CH<sub>4</sub> production occurred during the fermentation of A-sludge from Utrecht, Rotterdam or Breda WWTPs in February 2014 while only 1.00 ±  
223 1.00 mg C CH<sub>4</sub> g<sup>-1</sup> VSS<sub>fed</sub> was produced from the Groningen sample. Concerning the A-sludge collected in July 2014, 7.32 ± 0.47 mg C CH<sub>4</sub> g<sup>-1</sup>  
224 VSS<sub>fed</sub> and 4.17 ± 0.19 mg C CH<sub>4</sub> g<sup>-1</sup> VSS<sub>fed</sub> were produced respectively with A-sludge from Breda and Rotterdam while no CH<sub>4</sub> production  
225 occurred for Utrecht and Groningen substrates. CH<sub>4</sub> produced for A-sludge collected from Rotterdam WWTP in the summer period, only  
226 corresponds to 3 % of initial COD. Thus, methane production does not explain the lower VFA production.

227 The specificity of VFA production was also evaluated for each substrate and period (Table S1). Utrecht and Breda samples, with similar VFA  
228 productions between seasons, also presented similar profiles for both winter and summer. In both cases the acetate proportion ranged between 43  
229 % and 59 % while propionate was detected in a range of 26 % and 33 %. This differed from the ones obtained from auto-fermentation with the  
230 same pH and temperature (Table 2), in which mainly acetate and residual other carboxylates were detected. No repeatability in the percentage of  
231 each VFA was found for samples collected during winter and summer from Rotterdam and Groningen, which also presented different VFA  
232 production values. Variations in sludge composition might affect the carboxylate production as well as composition. A large nutrient variation  
233 was detected between each plant and between both periods tested, representing the major variability within a plant, from the parameters analyzed  
234 for each substrate (Table 1). Further investigations are required as no correlation between VFA production and organics/nutrient ratios were  
235 found within the analyzed sites and seasonal points (Table S2).

236 Rather than nutrient content, initial BOD<sub>5</sub> of the A-sludge appeared to be the key discriminant for VFA production, being these two parameters  
237 the ones presenting a higher correlation ( $R^2=0.55$ , Table S2). Different BOD<sub>5</sub> and BOD<sub>5</sub>/COD ratio were found for A-sludge collected from  
238 different WWTPs and in different time points (Table 3). This high variability is consequent to day-by-day variances in the WWTPs and in the  
239 domestic wastewater (in terms of dilution and organics and nutrients content) determining presence of more/less biodegradable COD/BOD that  
240 can be converted into carboxylates. The higher BOD<sub>5</sub> the higher the VFA production (Table 3). Similar BOD<sub>5</sub> concentrations between Rotterdam  
241 and Breda led to similar amounts of carboxylates. Finally, the ratio between VFA produced and BOD<sub>5</sub> measured was low for all the A-sludge  
242 samples tested, i.e.: 2 % for Groningen, 7 % for Utrecht, 25 % for Rotterdam and 28 % for Breda. Therefore, in order to increase the production

243 of carboxylates, one should increase the biodegradable organic matter content. A-stage sludge will likely need a combination of sludge  
244 pretreatment (Chen et al. 2007) that can enhance the BOD<sub>5</sub> by means of sludge hydrolysis and an enriched inoculum.

245 Another approach to increase the VFA production could be the co-fermentation of the A-sludge with other streams, such as other kinds of sludge  
246 (in the case of Primary sludge (PS), co-fermentation with WAS increased the VFA production by 40 %), starch rich industrial wastewater,  
247 kitchen waste (Lee et al. 2014) or food waste (De Vrieze et al. 2013). Increasing the BOD and/or the micro-nutrients and diluting possible  
248 inhibitors in the sludge by using a co-substrate might increase the hydrolytic capacity of the system helping to release biodegradable organic  
249 matter from the sludge itself.

250

### 251 ***3.4. Influence of iron concentration on carboxylate and methane production***

252 Considerable differences in iron concentration were found in the samples collected from the four WWTPs and within the same WWTP at  
253 different time points (Table 1). Methanogens require iron for their metabolic activity, and increasing the iron content in the wastewater has been  
254 shown to boost CH<sub>4</sub> production (Hoban and Van Den Berg 1979, Ivanov et al. 2002, Lee and Shoda 2008). However, it was not clear if iron  
255 content affected only the last step of methane production or the entire anaerobic treatment. Thus, the digestibility and fermentability (VFA  
256 production) of all A-sludge samples in relation to the Fe content was explored, and results are depicted in Figure 4.

257 Digestibility obtained from the biomethane production tests correlated with the iron content of each substrate ( $R^2$  0.71, p 0.009) (Figure 4a) as  
258 described in literature (Hoban and Van Den Berg 1979, Lee and Shoda 2008). Fermentation on the contrary did not show any dependency on the  
259 iron in the A-sludge samples (Figure 4b). Utrecht and Breda samples were not affected by the composition variability, in this case iron content,

260 as they led to the same amount of VFA for winter and summer periods. Similar iron concentrations of around 300 mg Fe L<sup>-1</sup> gave three different  
261 carboxylate productions for Utrecht, Breda and Groningen A-sludge samples. Finally, similar iron concentrations (Table 1) within the same  
262 installation (Rotterdam), presented different VFA production values. Therefore, other variations in sludge composition different than iron were  
263 hypothesized as responsible for VFA production differences.

264

### 265 ***3.5. Iron concentration influence on A-sludge fermentation***

266 In order to demonstrate the relationship of methanogenesis to the concentration of iron, 14-days batch tests with the same A-sludge were  
267 executed with addition of iron from 2.5 to 20 mmol L<sup>-1</sup>. Results are depicted in Figure 5. VFA ranging between 151 ± 8 and 171 ± 3 mg C VFA  
268 g<sup>-1</sup> VSS<sub>fed</sub> were produced in all the conditions tested with no significant difference in all sampling points analyzed along the days. An increase in  
269 the iron concentration increased the conversion of VFA into CH<sub>4</sub>. This could be already observed after 4 days of fermentation, becoming more  
270 noticeable after 7 days. The highest methane production was obtained with 20 mmol Fe L<sup>-1</sup> (AS\_20), significantly higher than the control with no  
271 extra addition of iron (AS) (Figure 5b). Methane production after 14 days was 46 ± 9 mg C CH<sub>4</sub> g<sup>-1</sup> VSS<sub>fed</sub> for the A-sludge where no extra iron  
272 was added (AS) and 51 ± 3, 67 ± 4, 74 ± 10 and 114 ± 12 mg C CH<sub>4</sub> g<sup>-1</sup> VSS<sub>fed</sub> for the A-sludge with addition of 2.5, 5, 10 and 20 mmol Fe L<sup>-1</sup>,  
273 respectively, showing a linear correlation (R<sup>2</sup> = 0.98) (Figure 5d).

274 Hoban and Van Den Berg (1979) demonstrated that addition of acetate and CO<sub>2</sub> to sewage enhance the conversion of acetate into methane  
275 increasing the iron concentration. Similarly, in our case, accumulation of VFA (acetate) produced from the A-sludge resulted in higher

276 conversion of VFA into methane with higher concentration of iron. After 14 days the VFA concentration linearly decreased with higher  
277 concentration of iron (Figure 5a and 5b,) due to improved conversion of VFA into methane (Figure 5c).

278 In all the condition tested, similar VFA composition was obtained for the propionic, isobutyric and butyric and isovaleric and valeric acids  
279 (Figure S3). Acetic acid was produced and accumulated in the first 2 days of fermentation, and reached between 46 to 48 % in all the conditions  
280 tested, and then converted into methane. In case of AS and AS\_2.5, conversion of VFA of 22 % and 24 % to CH<sub>4</sub>, respectively, was obtained.

281 Increasing the iron content increased the conversion of VFA into CH<sub>4</sub>, resulting in a production up to 46 % (118 ± 12 mgC gVSS<sub>fed</sub>) of CH<sub>4</sub> after  
282 14 days of fermentation when 20 mmol Fe L<sup>-1</sup> were added to the A-sludge. Acetate was the main VFA converted to methane while increasing the  
283 iron concentration (Figure S3), as indicated by Hoban and Van Den Berg (1979). Thus, iron is required for methanogenesis but again it does not  
284 seem to have any influence in the intermediate carboxylate production. This may relate to the low content of iron containing enzymes of many  
285 fermenters, and may be exploited as a strategy. If the A-stage separation can be performed at minimal or no Fe addition, fermentation of the  
286 sludge will be less likely to suffer from parasitic methanogenesis. If phosphorus is removed replacing the iron with polyelectrolytes or via the B-  
287 stage sludge, and mixing of A- and B-sludge occurs post-fermentation, a full process removing C, N and P is still possible while maximizing  
288 both fermentation and methanogenesis.

289 **4. Conclusions**

290 Optimal pH, temperature and retention time for A-sludge fermentation to maximize VFA production and minimize methane generation was pH 7  
291 and 35°C for 7 days. Production enhanced in the presence of an inoculum. Fermentation of A-sludge from four different WWTPs was executed  
292 to study the effect of different organics, nutrients and iron content on the fermentation. Initial BOD of the sludge appeared to be key determining  
293 VFA yield from A-sludge. Iron content could be correlated with CH<sub>4</sub> production but not with VFA production. This feature of iron limitation  
294 might be applied for further studies producing carboxylates to limit methane production.

---

295

296 **Acknowledgements**

297 CC is supported by the FWO project (Research Foundation Flanders) “The Domestic Biorefinery”. MC and KR are supported by Ghent  
298 University Multidisciplinary Research Partnership (MRP) - Biotechnology for a sustainable economy (01 MRA 510W). SEV is supported as  
299 postdoctoral fellow by the Research Foundation Flanders (FWO-Vlaanderen). Authors acknowledge Nieuwveer WWTP (Breda), Dokhaven  
300 WWTP (Rotterdam), Utrecht WWTP (Utrecht) and Garmerwolde WWTP (Groningen) for the provision of the A-sludge; Francis A. Meerburg  
301 for the help in samples collection and first substrate characterization; Dries Seuntjens for provision of A-sludge from Breda, Jo De Vrieze, Ruben  
302 Props and Alberto Scoma for the inspiring scientific discussions. Stephen J. Andersen, Ramon Ganigué and Emilie Courtens for their feedback  
303 on the manuscript.

304

305 **References**

- 306  
307  
308  
309  
310  
311  
312  
313  
314  
315  
316  
317  
318  
319  
320  
321  
322  
323  
324  
325  
326  
327  
328  
329  
330  
331  
332  
333  
334  
335  
336  
337  
338  
339
1. Agler, M.T., Wrenn, B.A., Zinder, S.H. and Angenent, L.T. (2011) Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. *Trends in Biotechnology* 29(2), 70-78.
  2. Angenent, L.T., Karim, K., Al-Dahhan, M.H., Wrenn, B.A. and Domínguez-Espinosa, R. (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends in Biotechnology* 22(9), 477-485.
  3. Appels, L., Baeyens, J., Degrève, J. and Dewil, R. (2008) Principles and potential of the anaerobic digestion of waste-activated sludge. *Progress in Energy and Combustion Science* 34(6), 755-781.
  4. Boehnke, B., Diering, B. and Zuckut, S.W. (1997) Cost-effective wastewater treatment process for removal of organics and nutrients. *Water-Engineering & Management* 144(5), 30-&.
  5. Boehnke, B., Schulze-Rettmer, R. and Zuckut, S.W. (1998) Cost-effective reduction of high-strength wastewater by adsorption-based activated sludge technology. *Water-Engineering & Management* 145(12), 31-34.
  6. Bolzonella, D., Pavan, P., Battistoni, P. and Cecchi, F. (2005) Mesophilic anaerobic digestion of waste activated sludge: influence of the solid retention time in the wastewater treatment process. *Process Biochemistry* 40(3-4), 1453-1460.
  7. Chen, Y., Jiang, S., Yuan, H., Zhou, Q. and Gu, G. (2007) Hydrolysis and acidification of waste activated sludge at different pHs. *Water Research* 41(3), 683-689.
  8. Constantine, T., Houweling, D. and Kraemer, J. (2012) Doing the Two-Step - Reduced Energy Consumption Sparks Renewed Interest in Multistage Biological Treatment. *Proceedings of the Water Environment Federation* 2012(10), 5771-5783.
  9. Cysneiros, D., Banks, C.J., Heaven, S. and Karatzas, K.A.G. (2012) The effect of pH control and 'hydraulic flush' on hydrolysis and Volatile Fatty Acids (VFA) production and profile in anaerobic leach bed reactors digesting a high solids content substrate. *Bioresource Technology* 123, 263-271.
  10. De Graaff, M. and Roest, K. (2012) Inventarisatie van AB-systemen - optimale procescondities in de A-trap. *Nieuwegein, KWR*.
  11. De Vrieze, J., De Lathouwer, L., Verstraete, W. and Boon, N. (2013) High-rate iron-rich activated sludge as stabilizing agent for the anaerobic digestion of kitchen waste. *Water Research* 47(11), 3732-3741.
  12. Demirel, B. and Scherer, P. (2011) Trace element requirements of agricultural biogas digesters during biological conversion of renewable biomass to methane. *Biomass and Bioenergy* 35(3), 992-998.
  13. Gujer, W. and Zehnder, A.J.B. (1983) Conversion processes in anaerobic digestion. *Water Science and Technology* 15(8-9), 127-167.
  14. Hoban, D.J. and Van Den Berg, L. (1979) Effect of Iron on Conversion of Acetic Acid to Methane During Methanogenic Fermentations. *Journal of Applied Bacteriology* 47(1), 153-159.
  15. Huoqing, G., Batstone, D.J. and Keller, J. (2013) Operating aerobic wastewater treatment at very short sludge ages enables treatment and energy recovery through anaerobic sludge digestion. *Water Res* 47(17), 6546-6557.
  16. Ivanov, V.N., Stabnikova, E.V., Stabnikov, V.P., Kim, I.S. and Zubair, A. (2002) Effects of Iron Compounds on the Treatment of Fat-Containing Wastewaters. *Applied Biochemistry and Microbiology* 38(3), 255-258.
  17. Jiang, J.Q. and Graham, N.J.D. (1998) Pre-polymerised inorganic coagulants and phosphorus removal by coagulation - A review. *Water Sa* 24(3), 237-244.
  18. Kleerebezem, R., Joosse, B., Rozendal, R. and Van Loosdrecht, M.M. (2015) Anaerobic digestion without biogas? *Reviews in Environmental Science and Bio/Technology* 14(4), 787-801.

340 19. Lee, H. and Shoda, M. (2008) Stimulation of anaerobic digestion of thickened sewage sludge by iron-rich sludge produced by the fenton method.  
341 Journal of Bioscience and Bioengineering 106(1), 107-110.

342 20. Lee, W.S., Chua, A.S.M., Yeoh, H.K. and Ngoh, G.C. (2014) A review of the production and applications of waste-derived volatile fatty acids. Chemical  
343 Engineering Journal 235(0), 83-99.

344 21. Levén, L., Eriksson, A.R.B. and Schnürer, A. (2007) Effect of process temperature on bacterial and archaeal communities in two methanogenic  
345 bioreactors treating organic household waste. FEMS Microbiology Ecology 59(3), 683-693.

346 22. Ma, H., Chen, X., Liu, H., Liu, H. and Fu, B. (2016) Improved volatile fatty acids anaerobic production from waste activated sludge by pH regulation:  
347 Alkaline or neutral pH? Waste Management 48, 397-403.

348 23. Meerburg, F.A., Boon, N., Van Winckel, T., Vercamer, J.A.R., Nopens, I. and Vlaeminck, S.E. (2015) Toward energy-neutral wastewater treatment: A  
349 high-rate contact stabilization process to maximally recover sewage organics. Bioresource Technology 179(0), 373-381.

350 24. Morgan-Sagastume, F., Pratt, S., Karlsson, A., Cirne, D., Lant, P. and Werker, A. (2011) Production of volatile fatty acids by fermentation of waste  
351 activated sludge pre-treated in full-scale thermal hydrolysis plants. Bioresource Technology 102(3), 3089-3097.

352 25. PaDEP (2014) Module 17: The Activated Sludge Process Part III. Pennsylvania Department of Environmental Protection.

353 26. Pratt, S., Liew, D., Batstone, D.J., Werker, A.G., Morgan-Sagastume, F. and Lant, P.A. (2012) Inhibition by fatty acids during fermentation of pre-  
354 treated waste activated sludge. Journal of Biotechnology 159(1-2), 38-43.

355 27. Schattauer, A., Abdoun, E., Weiland, P., Plöchl, M. and Heiermann, M. (2011) Abundance of trace elements in demonstration biogas plants.  
356 Biosystems Engineering 108(1), 57-65.

357 28. Veeken, A. and Hamelers, B. (1999) Effect of temperature on hydrolysis rates of selected biowaste components. Bioresource Technology 69(3), 249-  
358 254.

359 29. Verstraete, W., Van de Caveye, P. and Diamantis, V. (2009) Maximum use of resources present in domestic "used water". Bioresource Technology  
360 100(23), 5537-5545.

361 30. Verstraete, W. and Vlaeminck, S.E. (2011) ZeroWasteWater: short-cycling of wastewater resources for sustainable cities of the future. International  
362 Journal of Sustainable Development & World Ecology 18(3), 253-264.

363 31. Zandvoort, M.H., van Hullebusch, E.D., Feroso, F.G. and Lens, P.N.L. (2006) Trace Metals in Anaerobic Granular Sludge Reactors: Bioavailability and  
364 Dosing Strategies. Engineering in Life Sciences 6(3), 293-301.

365 32. Zhang, P., Chen, Y. and Zhou, Q. (2009) Waste activated sludge hydrolysis and short-chain fatty acids accumulation under mesophilic and  
366 thermophilic conditions: Effect of pH. Water Research 43(15), 3735-3742.



368 **Figure captions**

369 **Figure 1.** Specific VFA (a) and CH<sub>4</sub> (b) production at 55 °C and 35 °C and pH 4.5, 5, 6 and 7.  
370 C represents the controls where no pH adjustment was done. WAS represents the control where waste activated sludge was used.

371 **Figure 2.** Specific VFA (a) and CH<sub>4</sub> (b) production at 55 °C and 35 °C and pH 4.5, 5, 6 and 7 on day 7.

372 **Figure 3.** VFA (a) and CH<sub>4</sub> production (b) at day 7 for the A-sludge collected in February 2014 (winter) and in July 2014 (summer) from four  
373 WWTPs.

374 **Figure 4.** Dependency of iron content with CH<sub>4</sub> production (a) and VFA production (b) for Rotterdam (□), Utrecht (Δ), Breda (○) and  
375 Groningen (◇). Filled symbols originate from the winter period (February 2014) and empty symbols represent the summer period (July 2014).

376 **Figure 5.** Specific VFA (a) and CH<sub>4</sub> (c) production for A-sludge from Breda WWTP at different sampling days. Each bar represents iron  
377 concentrations of 2.5, 5, 10 and 20 mmol Fe L<sup>-1</sup> were added. AS represents the control where no iron was added. (b) and (c) represent  
378 respectively the dependency of the iron content with VFA and CH<sub>4</sub> produced after 14 days of fermentation.

379

380

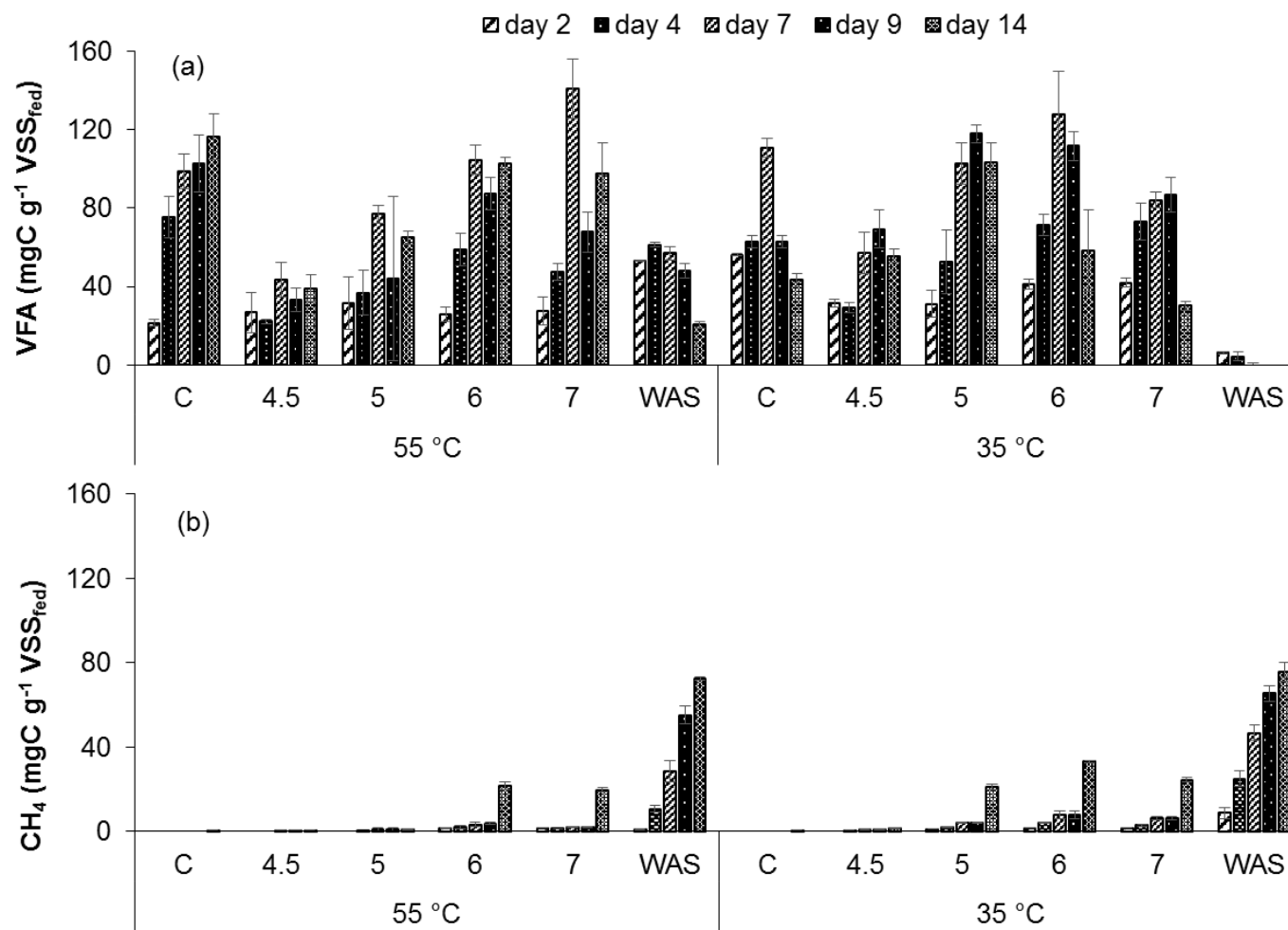
381 **Table captions**

382 **Table 1.** Characteristics of the A-sludge collected from the 4 WWTPs. All the analysis were carried out in triplicate

383 **Table 2.** VFA composition in the auto-fermentation tests at different pH and temperatures measured at day 7.

384 **Table 3.** BOD<sub>5</sub>, BOD<sub>5</sub>/COD ratio, VFA, and VFA/BOD<sub>fed</sub> ratio for the A-sludge collected in summer (July 2014) from 4 WWTPs.

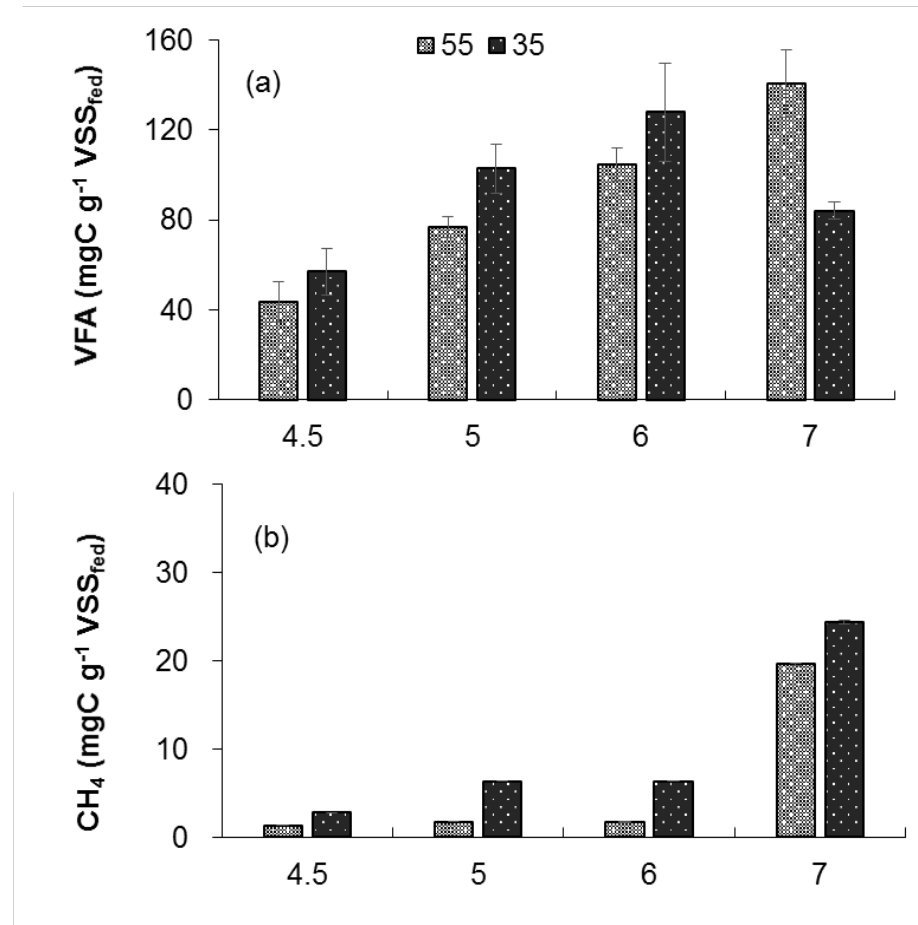
385



388 **Figure 2.** Specific VFA (a) and CH<sub>4</sub> (b) production at 55 °C and 35 °C and pH 4.5, 5, 6 and 7.

389 C represents the controls where no pH adjustment was done. WAS represents the control where waste activated sludge was used.



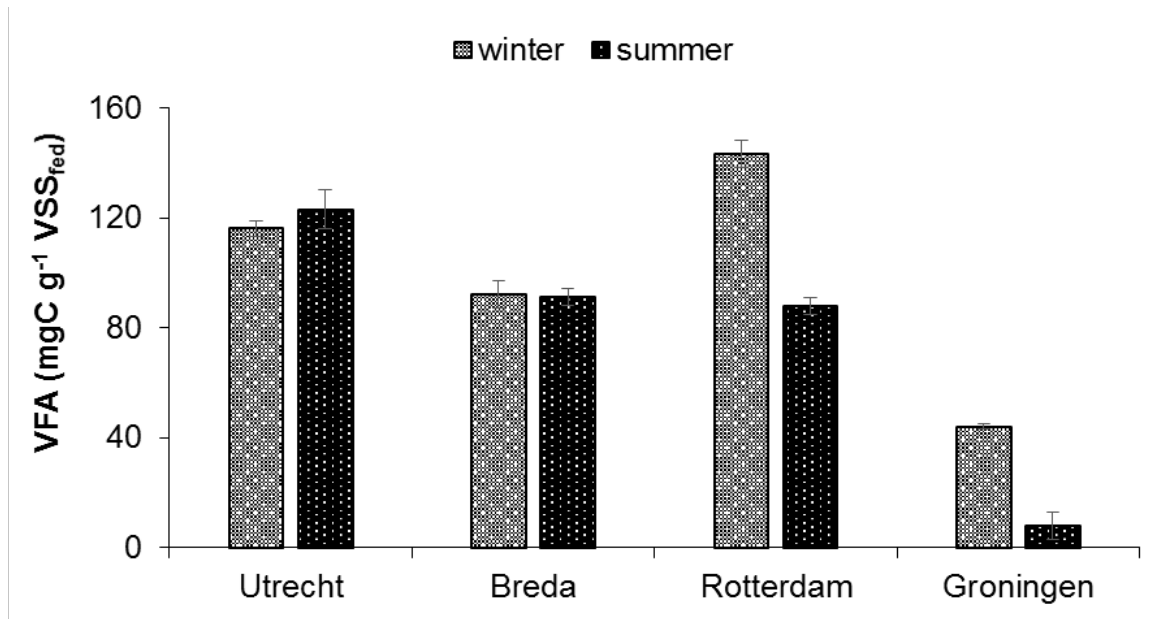


392

393 **Figure 2.** Specific VFA (a) and CH<sub>4</sub> (b) production at 55 °C and 35 °C and pH 4.5, 5, 6 and 7 on day 7.

394

395



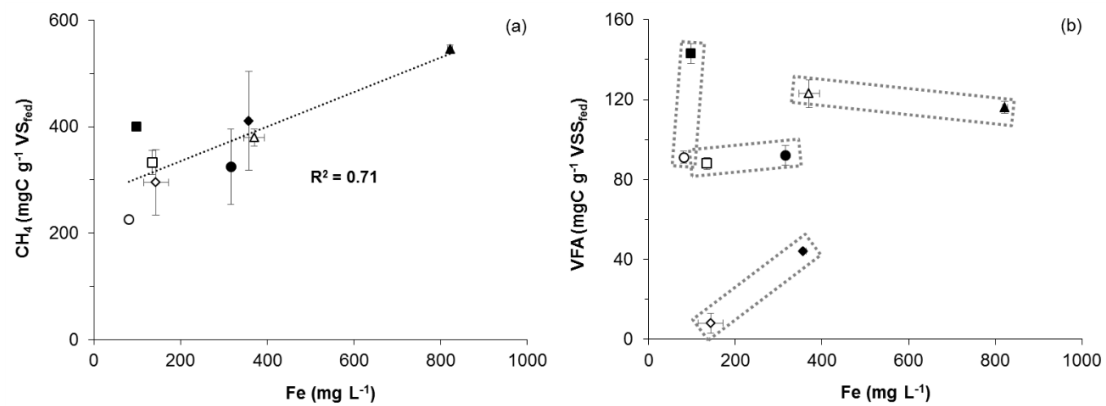
396

397 **Figure 3.** VFA production at day 7 for the A-sludge collected in February 2014 (winter) and in July 2014 (summer) from four WWTPs.

398

399

400

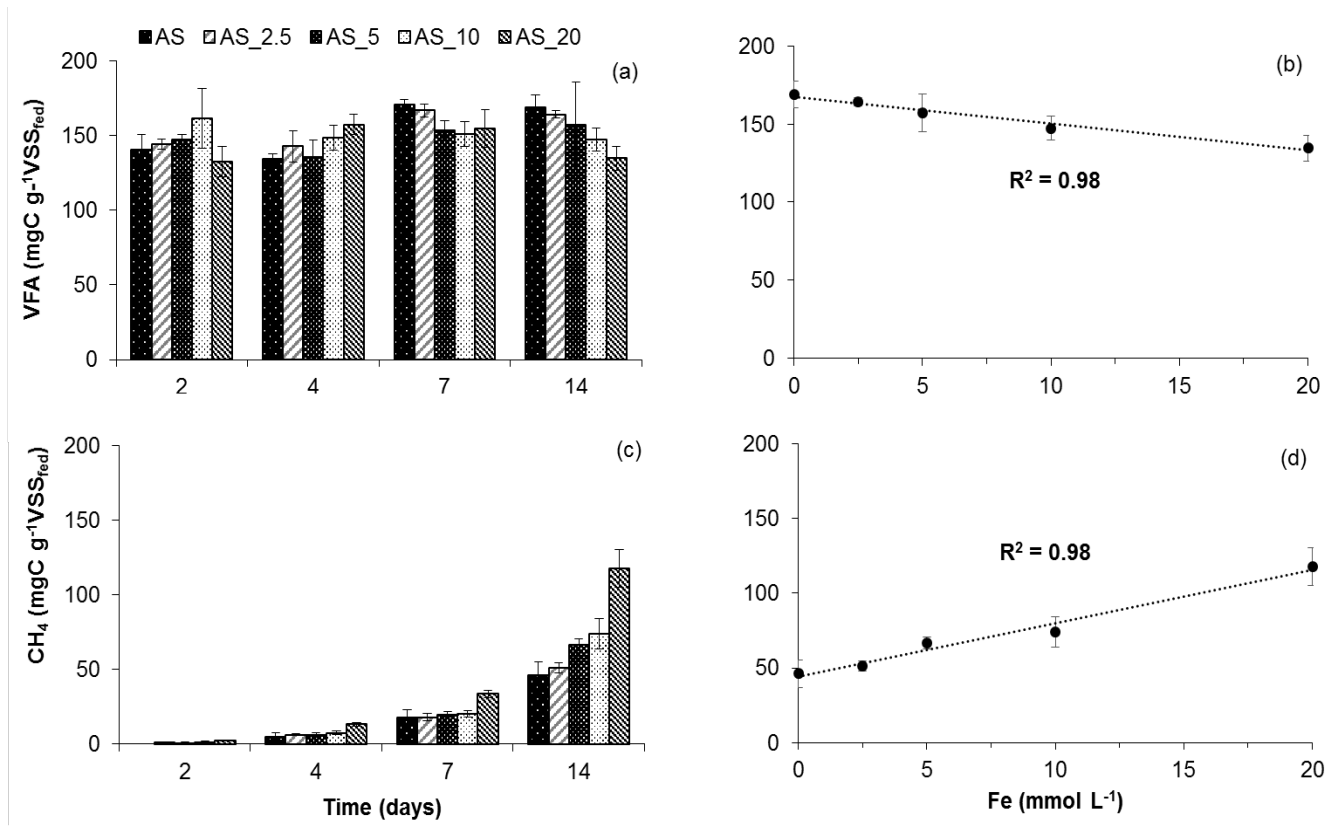


401

402 **Figure 4.** Dependency of iron content with CH<sub>4</sub> production (a) and VFA production (b) for Rotterdam (□), Utrecht (Δ), Breda (○) and  
403 Groningen (◇). Filled symbols originate from the winter period (February 2014) and empty symbols represent the summer period (July 2014).

404

405



406

407

408 **Figure 5.** Specific VFA (a) and CH<sub>4</sub> (c) production for A-sludge from Breda WWTP at different sampling days. Each bar represents iron  
 409 concentrations of 2.5, 5, 10 and 20 mmol Fe L<sup>-1</sup> were added. AS represents the control where no iron was added. (b) and (c) represent  
 410 respectively the dependency of the iron content with VFA and CH<sub>4</sub> produced after 14 days of fermentation.

411

412

**Table 2.** Characteristics of the A-sludge collected from the 4 WWTPs. All the analysis were carried out in triplicate

<i>Parameter</i>	<i>Rotterdam</i>		<i>Utrecht</i>		<i>Groningen</i>		<i>Breda</i>	
	<i>February 2014</i>	<i>July 2014</i>	<i>February 2014</i>	<i>July 2014</i>	<i>February 2014</i>	<i>July 2014</i>	<i>February 2014</i>	<i>July 2014</i>
<i>pH</i>	6.2	6.7	5.6	6.0	7.0	6.9	6.3	6.8
<i>Conductivity (mS cm<sup>-1</sup>)</i>	1.39	1.02	2.02	0.84	1.65	0.72	1.11	0.79
<i>Total COD (g L<sup>-1</sup>)</i>	5.1 ± 0.1	3.4 ± 0.1	15.1 ± 0.5	2.7 ± 0.1	7.0 ± 0.2	0.9 ± 0.0	11.7 ± 0.2	3.5 ± 0.2
<i>Total solids (g L<sup>-1</sup>)</i>	4.6 ± 0.2	3.1 ± 0.1	14.6 ± 0.2	1.8 ± 0.0	16.4 ± 0.7	2.7 ± 0.0	10.9 ± 0.2	3.8 ± 0.1
<i>Volatile solids (%)</i>	75	77	73	66	74	65	79	74
<i>Kjeldahl nitrogen, TKN (mg N L<sup>-1</sup>)</i>	440 ± 22	125 ± 21	204 ± 92	310 ± 16	321 ± 2	95 ± 40	120 ± 2	182 ± 7
<i>Total ammonia nitrogen, TAN (mg N L<sup>-1</sup>)</i>	66 ± 3	103 ± 1	100 ± 3	130 ± 29	99 ± 7	28 ± 1	50 ± 2	25 ± 7
<i>COD:VS ratio</i>	1.5 ± 0.1	1.4 ± 0.0	1.4 ± 0.0	2.3 ± 0.1	0.6 ± 0.0	0.49 ± 0.0	1.4 ± 0.0	1.25 ± 0.1
<i>COD:P ratio</i>	66.5 ± 1.9	79.3 ± 2.9	30.8 ± 1.7	32.2 ± 1.9	135.5 ± 6.5	40.0 ± 4.0	5.1 ± 0.1	59.7 ± 15
<i>COD:N ratio</i>	11.7 ± 0.7	55.9 ± 9.5	73.7 ± 33.2	17.7 ± 1.2	21.9 ± 0.6	18.5 ± 7.9	97.6 ± 2.4	38.2 ± 8.1
<i>Fe (mg L<sup>-1</sup>)</i>	98 ± 2	135 ± 3	822 ± 6	370 ± 234	316 ± 2	80 ± 4	357 ± 66	143 ± 29
<i>P (mg L<sup>-1</sup>)</i>	77 ± 0.2	88 ± 1	488 ± 25	170 ± 7	52 ± 2	44 ± 2	2293 ± 38	116 ± 16
<i>Fe:P ratio</i>	1.27 ± 0.02	1.52 ± 0.04	1.68 ± 0.09	2.18 ± 0.16	6.09 ± 0.25	1.84 ± 0.13	0.16 ± 0.00	1.23 ± 0.30
<i>Fe:S ratio</i>	1.95 ± 0.04	2.45 ± 0.07	4.29 ± 0.07	2.66 ± 0.23	3.18 ± 0.03	2.14 ± 0.13	5.72 ± 0.10	3.29 ± 0.87
<i>Fe:VS ratio</i>	0.03 ± 0.00	0.06 ± 0.00	0.08 ± 0.00	0.31 ± 0.02	0.03 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.01



---

<i>Mean particle size D [4,3] (μm)</i>	88.0	58.8	270.1	86.3	125.2	152.8	80.0	160.6
--	------	------	-------	------	-------	-------	------	-------

---

**Table 2.** VFA composition in the auto-fermentation tests at different pH and temperatures measured at day 7.

Temperature pH VFA	55 °C					35 °C				
	C (6.5)	4.5	5 %	6	7	C (6.5)	4.5	5 %	6	7
<b>Acetate</b>	64 ± 9	64 ± 12	66 ± 5	62 ± 3	72 ± 9	89 ± 7	56 ± 14	69 ± 10	87 ± 12	88 ± 5
<b>Propionate</b>	11 ± 1	12 ± 3	11 ± 1	13 ± 2	8 ± 1	0 ± 0	18 ± 4	14 ± 2	0 ± 0	0 ± 0
<b>Isobutyrate</b>	5 ± 1	3 ± 1	5 ± 1	5 ± 1	4 ± 0	3 ± 1	3 ± 1	2 ± 0	3 ± 1	4 ± 0
<b>Butyrate</b>	7 ± 1	12 ± 3	7 ± 1	8 ± 1	6 ± 1	2 ± 0	10 ± 2	9 ± 1	3 ± 1	1 ± 0
<b>Isovalerate</b>	10 ± 1	8 ± 2	10 ± 1	11 ± 1	6 ± 1	5 ± 1	6 ± 1	3 ± 0	4 ± 1	6 ± 0
<b>Valerate</b>	1 ± 1	2 ± 1	2 ± 1	2 ± 0	1 ± 0	2 ± 0	6 ± 1	3 ± 0	2 ± 1	1 ± 0
<b>Caproate</b>	2 ± 0	0 ± 0	0 ± 0	0 ± 0	3 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

**Table 3.** BOD<sub>5</sub>, BOD<sub>5</sub>/COD ratio, VFA yield on day 7, and VFA/BOD<sub>fed</sub> ratio (g C VFA g<sup>-1</sup> BOD<sub>fed</sub>) for the A-sludge collected in summer (July 2014) from 4 WWTPs.

<b>A-sludge</b>	<b>BOD<sub>5</sub></b> <b>g L<sup>-1</sup></b>	<b>BOD<sub>5</sub>/COD</b> <b>%</b>	<b>VFA</b> <b>mgC g<sup>-1</sup> VSSfed</b>	<b>VFA/BOD<sub>fed</sub></b> <b>%</b>
<b>Utrecht</b>	3.78 ± 0.1	68	103 ± 5	7
<b>Rotterdam</b>	1.2 ± 0.0	17	78 ± 8	25
<b>Breda</b>	1.2 ± 0.1	17	72 ± 2	28
<b>Groningen</b>	0.7 ± 0.1	43	6 ± 1	2