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Production of carboxylates from high rate activated sludge through fermentation

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Abstract

The aim of this work was to study the key parameters affecting fermentation of high rate activated A-sludge to carboxylates, including pH, 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. 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. Initial BOD of the sludge appeared to be key determining carboxylate yield from A-sludge. Whereas methanogenesis could be correlated linearly to the quantity of ferric used for coagulation, fermentation did not show a dependency on iron presence. This difference may enable a strategy whereby A-stage sludge is separated to achieve fermentation, and iron dosing for phosphate removal is only implemented at the B-stage.

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

1. Introduction

For domestic and industrial wastewater the focus for reuse has been on the water itself as well as the energy contained within the organics (Verstraete and Vlaeminck 2011). Domestic sewage could also be a source of products such as nutrients or volatile fatty acids (VFAs) that can be used as building blocks for the production of valuable products such as medium and long chain fatty acids, alcohols and *Polyhydroxyalkanoates (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 low organic concentration which impedes to obtain an economically feasible production process.

Several concentration technologies allow recovery of the clean water and increase the amount of organic matter for further processing (Meerburg et al. 2015, Verstraete et al. 2009). Best known is the “Adsorption-Belebungsverfahren” process (AB process, generally translated as Adsorption - Biodegradation or Adsorption - Bio-oxidation) (Boehnke et al. 1997), also termed “high-rate activated sludge”. The AB process is a two-stage treatment system where the first stage, the highly loaded biological adsorption stage or A-stage, is a modification of the conventional activated 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 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 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 (HRT 15 - 30 min) and solid retention time (SRT) between several hours and 1 day (Boehnke et al. 1998). These conditions determine bioflocculation with high biomass yields and minimal CO_2 formation, complemented with sorption and storage mechanisms that remove COD (Meerburg et al. 2015). The A-sludge is highly biodegradable and easily digested to biogas (Boehnke et al. 1998). The subsequent B-stage ensures polishing of the wastewater to meet discharge

standards. Typically, the A-stage has a COD removal efficiency of 26-52% (De Graaff and 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 A-sludge before digestion to achieve energy neutrality at the facility level. The process is applied at full scale in several municipal wastewater treatment plants (WWTPs) and industrial plants (Boehnke et al. 1997). The limited experience to date does lead to considerably different operational approaches and hence different sludge properties (De Graaff and Roest 2012).

Thus far the sludge valorization has been investigated through anaerobic digestion either as a single substrate (Huoqing et al. 2013, Meerburg et al. 2015) or in co-digestion with other organic waste streams (De Vrieze et al. 2013, Verstraete and Vlaeminck 2011). In recent years, another route has come to the fore, the carboxylate platform, whereby mixed populations are used to ferment organic matter carboxylates that are the end 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 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 suitable source. The main interest in A-sludge fermentation is that VFA can be used as perfect additional carbon source to enhance biological nitrogen and phosphorous removal in waste water treatment plants (Lee et al. 2014), with no requirement of previous extraction from the sludge, avoiding acetate or methanol expenses for wastewater's with low carbon content. The fermentation can then be followed by anaerobic digestion for removal of unfermented organics. The potential of the A-sludge for carboxylate production in terms of conversion efficiencies and product outcomes, and how this could relate to residual digestion has not been yet explored.

Therefore, the goal of this study was to identify key parameters affecting fermentation of an A-sludge (here temperature and pH, inoculum addition and retention time) and subsequently assess the fermentation potential of A-sludge originating from different sites and to correlate

this to different operational approaches. A prime example of a variable parameter is the iron content. Indeed considerable differences in iron concentration were found in the samples collected from the four WWTPs object of this study. Iron content was also different between the two time frames considered. In these sites salts of Fe (II) or (III) such as FeSO_4 and FeCl_3 are added in different concentrations to precipitate phosphates as $\text{Fe}_3(\text{PO}_4)_2(\text{s})$ or $\text{FePO}_4(\text{s})$ and to enhance the flocculation, coagulation and sedimentation of the A-sludge (Jiang and Graham 1998). Furthermore, iron is important as coenzyme or cofactor in several enzymes involved in the anaerobic digestion process (Zandvoort et al. 2006). Methanogens require iron for their metabolic activity, and their capability to continue producing methane is strictly dependent on the presence 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, 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 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 dilution and content of the domestic wastewater. Thus we measured key characteristics of different A-sludge and subjected them to fermentation and digestion.

2. Materials and methods

2.1. A-sludge collection

Auto-fermentation test without inoculum (see 2.2.1) were performed with A-sludge from Nieuwveer WWTP (Breda) in 11/2013 (COD $11.3 \pm 0.5 \text{ g L}^{-1}$, 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 $2.8 \pm 0.4 \text{ g L}^{-1}$) 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 during two different time frames (2/2014 and 7/2014) from four WWTPs in the Netherlands: Nieuwveer WWTP (Breda), Dokhaven WWTP (Rotterdam), Utrecht WWTP (Utrecht) and Garmerwolde WWTP (Groningen). The characteristics of the A-sludge on those two sampling points 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) 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 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 $45.1 \pm 5.9 \text{ g L}^{-1}$).

2.2. Fermentation batch tests

All tests were performed in triplicate in serum flasks (120 mL) sealed with a rubber stopper and aluminum sealer. Headspace was flushed with 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 samples were taken periodically decreasing the frequency over the experiment. Biogas production was monitored for every sampling point. Chemical analysis procedures are defined in supplementary information.

2.2.1. Determination of key parameters during auto-fermentation

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. Control tests without pH control were performed for each temperature tested for both A-sludge and WAS from Dendermonde WWTP. The pH was adjusted to the desired value by adding 1 M NaOH or 1 M HCl after each sampling time.

2.2.2. Determination of the inoculum impact

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 of 110 days) for VFA production was previously acclimated to A-sludge as substrate at either mesophilic or thermophilic conditions. The tests 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 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 (35 °C) or thermophilic (55 °C) temperatures. pH control was carried out by means of a pH buffer (strength 200 mM H⁺), prepared in tap water (pH = 6: 24.3 mg NaH₂PO₄ · H₂O L⁻¹ and 6.4 mg Na₂HPO₄ · 7 H₂O L⁻¹; pH = 7: 11.7 mg NaH₂PO₄ · H₂O L⁻¹ and 30.9 mg Na₂HPO₄ · 7 H₂O L⁻¹). *2.2.3. Comparison of different A-sludge fermentative capacity*

A-sludge from four different WWTP obtained either during the winter (February 2014) or the summer period (July 2014) were evaluated. The 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 were also included. The tests were run at 35 °C and pH 7.

2.2.4. Determination of iron impact on A-sludge fermentation

FeCl₃ was added to Breda A-sludge in order to obtain Fe concentrations of 2.5, 5, 10 and 20 mmol Fe L⁻¹ (as in Hoban and Van Den Berg (1979)), and evaluated to determine the effect of increasing iron concentration on VFA and CH₄ production during the fermentation. VFA

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-sludge where no iron was added were also carried out. The tests were run at 35 °C and pH 7. Methane production tests was evaluated by means of biochemical methane potential.

2.3. Biochemical methane potential (BMP) test

BMP tests were carried out to estimate the anaerobic biodegradability of the A-sludge from the four WWTPs (February and July 2014) and for 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. 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⁻¹ VS. The inoculum was obtained from a full scale anaerobic digester from Trevi (Belgium) and sieved at 2 mm to homogenize and remove solid materials. The inoculum biomass accounted for 5 g VS L⁻¹. Flasks were sealed with a rubber stopper and aluminum sealer and then connected to glass columns, in which biogas production was measured by means of water displacement. Biogas composition was evaluated at the end of the experiment, after 35 days. Methane yield was expressed as the volume of methane per gram of volatile solids of substrate. Values are reported at standard temperature and pressure (STP) conditions.

2.4. Analytical techniques

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

3. Results and discussion

3.1. Key parameters determining A-sludge auto-fermentation

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

3.1.1. Impact of retention time and temperature

Carboxylate production in all tests followed the same pattern, reaching a maximum VFA production at day 7. All fermentation conditions, except 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 ± 0 mg C VFA g⁻¹ VSS_{fed} at day 2 and 61 ± 1 mg C VFA g⁻¹ VSS_{fed} at day 4). The highest value obtained, up to 141 ± 15 mg C VFA g⁻¹ VSS_{fed}, equivalent to 420 mg COD g⁻¹ VSS_{fed}, (30% conversion of the COD supplied) was also higher than the greater values reported in literature for the fermentation of both primary and secondary WAS (298 and 368 mg COD g⁻¹ VSS_{fed} at mesophilic and thermophilic conditions, respectively) (Lee 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 and heat-alkaline pretreated sludge.

For the A-sludge, the highest VFA concentrations were observed on day 7 and 9 at all the pH values tested (Figure 1a). Carboxylate concentrations subsequently decreased due to the conversion of VFA to CH₄ (Figure 1b). CH₄ generation increased from day 7 and the highest 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 retention time for the A-sludge treated in batch mode was fixed at 7 days for all the following fermentation tests.

Temperature and pH range were chosen in this study based on applicability at WWTP level. In particular, thermophilic temperature can in some cases enhance the hydrolysis leading to higher biogas production rates at lower SRT (Levén et al. 2007, Veecken and Hamelers 1999). This fact

was also verified for the WAS fermentation test carried out at mesophilic and thermophilic conditions. Even though both temperatures lead to similar methane productions, thermophilic conditions enhanced hydrolysis of the secondary sludge and thus, increased the VFA concentration in 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 methane accumulation. In case of the A-sludge no significant differences in VFA production were observed between mesophilic and thermophilic conditions, confirming earlier findings in A-sludge digestion ((De Vrieze et al. 2013). In a continuous system, thermophilic fermentation would theoretically produce more methane after accumulation. Therefore, a low HRT (i.e. 2 days) is recommended to avoid methane generation.

3.1.1. Impact of pH

Although several studies in literature show an increase in VFA production when the fermentation is carried out at alkaline pH (8-10) (Cysneiros 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 A-sludge (pH 8, 9 and 10) yielded to lower VFA production than at neutral pH (Figure S1, Supplementary information). Therefore, the pH was varied between 4.5 and 7.

An increase in pH led to an increase in VFA production. However, at pH close to neutrality, methane production was enhanced. In several studies the inhibition of methanogens has been achieved controlling various parameters such as sludge retention time (SRT), pH and loading rate. pH is 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, Gujer and Zehnder 1983).

Fermentative bacteria are generally less sensitive and function in a pH range between 4 and 11 (Appels et al. 2008, Chen et al. 2007, Lee et al. 2014), depending on the carboxylate content. However, at pH values close to the equilibrium constant of the carboxylates (pKa 4.8 – 4.9)

also fermenters are impacted by product inhibition. We therefore assessed VFA production depending on pH and temperature only by values obtained after 7 days (Figure 2).

Cysneiros et al. (2012) demonstrated that increasing pH up to 6.5 improved hydrolysis and increased VFA production by 50% in leachate. Indeed, at 55 °C the VFA concentration almost doubled, when increasing from pH 5 ($77 \pm 5 \text{ mg C VFA g}^{-1} \text{ VSS}_{\text{fed}}$) to pH 7 ($141 \pm 15 \text{ mg C VFA g}^{-1} \text{ VSS}_{\text{fed}}$). The combination of thermophilic temperatures and pH 7 lead to an increased accumulation of VFA also due to less conversion to CH₄ under thermophilic conditions.

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 CH₄ was always higher at mesophilic conditions (Fig. 2b) due to optimal conditions for methanogenesis (35 °C and pH 6.8 - 7.5). Indeed, by increasing the pH from 5 to 6 and further to pH 7, the CH₄ production achieved values of $0.11 \pm 0.01 \text{ mg C VFA g}^{-1} \text{ VSS}_{\text{fed}}$, $0.44 \pm 0.00 \text{ mg C CH}_4 \text{ g}^{-1} \text{ VSS}_{\text{fed}}$ and $0.87 \pm 0.21 \text{ mg C CH}_4 \text{ g}^{-1} \text{ VSS}_{\text{fed}}$, respectively.

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, mesophilic tests yielded 128 and 110 mg C g⁻¹ VSS_{fed} and thermophilic tests yielded 114 and 155 mg C g⁻¹ VSS_{fed} at pH 6 and 7, respectively. Not much difference was presented within these conditions, thus they were chosen for further experiments in combination with a residence time of 7 days.

Table 2 summarizes the different carboxylates obtained for each condition. For all the A-sludge fermentation condition tested combining pH and temperature, acetate was the main product obtained ranging from 56 to 88% on the 7th day of operation when the maximum VFA concentration was detected. At 55 °C, other VFA from propionate to caproate were also detected at all the pH values tested. Propionate represented about 10% 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. 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 found and 87 - 89 % of the converted substrate ended up in acetate.

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

3.2. Fermentation in the presence of an adapted inoculum

The maximum carboxylate concentration detected for A-sludge auto-fermentation was 141 mg C VFA g⁻¹ VSS_{fed}, equivalent to 30 % conversion efficiency. According to previous work, the conversion efficiency during the digestion of A-sludge to CH₄ is around 50-70 % (De Graaff and Roest 2012, De Vrieze et al. 2013) which suggest that the potential for VFA production should be higher. In the second experiment we thus investigated the impact of an adapted fermentative inoculum acclimatized to A-sludge. Conditions with and without inoculum were tested both at 35 °C and 55 °C and at pH 6 and 7 (Figure S2).

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 agreement with the auto-fermentation tests.

The best VFA production was achieved with inoculum at 35 °C and pH 7, the production was significantly higher (P 0.046) than at pH 6.

The total VFA concentrations obtained in this second tests (Figure S2) were lower than the auto-fermentation tests (Figure 2). Batch tests in 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 production was found (data not shown). Therefore, the difference in total carboxylate production was attributed to differences in A-sludge composition due to day-by-day changes in operational parameters at WWTP level (see 3.3).

Although 35 °C and pH 7 appeared the most advantageous conditions for methanogenesis to occur, no CH₄ production was detected after 7 days of fermentation, thus confirming that a fermentative inoculum can direct the digestion specifically towards VFA production, when the residence time is kept low.

3.3. Influence of A-sludge composition

A-sludge samples from four different WWTPs in The Netherlands (Breda, Groningen, Rotterdam and Utrecht) were collected during winter and 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 A-sludge composition varied from one substrate to the other within a range of 3 – 15 g COD L⁻¹, 2 – 11 g TS L⁻¹ and 95 – 440 mg N L⁻¹, with winter values being higher for all with the exception of the organic content in Rotterdam (Table 1). Fermentation results are depicted in Figure 3.

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 92 ± 5 and 91 ± 3 mg C VFA g⁻¹ VSS_{fed}, respectively), although summer organics content was lower than winter (Table 1). The VFA production from Rotterdam and Groningen substrates was lower for the samples collected in summer. In particular, the VFA production of the Rotterdam sample was 143 ± 5 mg C VFA g⁻¹ VSS_{fed} in February 2014 and 88 ± 3 mg C VFA g⁻¹ VSS_{fed} in July 2014. Groningen VFA production values were 44 ± 1 mg C VFA g⁻¹ VSS_{fed} and 8 ± 5 mg C VFA g⁻¹ VSS_{fed} for the samples collected in February and July 2014, respectively. The 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⁻¹ that led to a decrease in VFA production from 9 % to 2 % g COD g⁻¹ COD_{fed}. However, this was not the case for the Rotterdam samples, as they showed a similar COD content in February and July of 5.1 ± 0.1 g L⁻¹ and 7.0 ± 0.3 g L⁻¹, respectively. The efficiency in VFA conversion for the A-sludge from Rotterdam was 18 % and 11 % for the samples collected in February 2014 and July 2014, respectively.

No CH₄ production occurred during the fermentation of A-sludge from Utrecht, Rotterdam or Breda WWTPs in February 2014 while only 1.00 ± 1.00 mg C CH₄ g⁻¹ VSS_{fed} was produced from the Groningen sample. Concerning the A-sludge collected in July 2014, 7.32 ± 0.47 mg C CH₄ g⁻¹ VSS_{fed} and 4.17 ± 0.19 mg C CH₄ g⁻¹ VSS_{fed} were produced respectively with A-sludge from Breda and Rotterdam while no CH₄ production occurred for Utrecht and Groningen substrates. CH₄ produced for A-sludge collected from Rotterdam WWTP in the summer period, only corresponds to 3 % of initial COD. Thus, methane production does not explain the lower VFA production.

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

production and organics/nutrient ratios were found within the analyzed sites and seasonal points (Table S2).

Rather than nutrient content, initial BOD₅ of the A-sludge appeared to be the key discriminant for VFA production, being these two parameters the ones presenting a higher correlation ($R^2=0.55$, Table S2). Different BOD₅ and BOD₅/COD ratio were found for A-sludge collected from 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 domestic wastewater (in terms of dilution and organics and nutrients content) determining presence of more/less biodegradable COD/BOD that can be converted into carboxylates. The higher BOD₅ the higher the VFA production (Table 3). Similar BOD₅ concentrations between Rotterdam and Breda led to similar amounts of carboxylates. Finally, the ratio between VFA produced and BOD₅ measured was low for all the A-sludge samples tested, i.e.: 2 % for Groningen, 7 % for Utrecht, 25 % for Rotterdam and 28 % for Breda. Therefore, in order to increase the production of carboxylates, one should increase the biodegradable organic matter content. A-stage sludge will likely need a combination of sludge pretreatment (Chen et al. 2007) that can enhance the BOD₅ by means of sludge hydrolysis and an enriched inoculum.

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 (in the case of Primary sludge (PS), co-fermentation with WAS increased the VFA production by 40 %), starch rich industrial wastewater, 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 inhibitors in the sludge by using a co-substrate might increase the hydrolytic capacity of the system helping to release biodegradable organic matter from the sludge itself.

3.4. Influence of iron concentration on carboxylate and methane production

Considerable differences in iron concentration were found in the samples collected from the four WWTPs and within the same WWTP at different time points (Table 1). Methanogens require iron for their metabolic activity, and increasing the iron content in the wastewater has been shown to boost CH₄ production (Hoban and Van Den Berg 1979, Ivanov et al. 2002, Lee and Shoda 2008). However, it was not clear if iron content affected only the last step of methane production or the entire anaerobic treatment. Thus, the digestibility and fermentability (VFA production) of all A-sludge samples in relation to the Fe content was explored, and results are depicted in Figure 4.

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 described in literature (Hoban and Van Den Berg 1979, Lee and Shoda 2008). Fermentation on the contrary did not show any dependency on the iron in the A-sludge samples (Figure 4b). Utrecht and Breda samples were not affected by the composition variability, in this case iron content, as they led to the same amount of VFA for winter and summer periods. Similar iron concentrations of around 300 mg Fe L⁻¹ gave three different carboxylate productions for Utrecht, Breda and Groningen A-sludge samples. Finally, similar iron concentrations (Table 1) within the same installation (Rotterdam), presented different VFA production values. Therefore, other variations in sludge composition different than iron were hypothesized as responsible for VFA production differences.

3.5. Iron concentration influence on A-sludge fermentation

In order to demonstrate the relationship of methanogenesis to the concentration of iron, 14-days batch tests with the same A-sludge were executed with addition of iron from 2.5 to 20 mmol L⁻¹. Results are depicted in Figure 5. VFA ranging between 151 ± 8 and 171 ± 3 mg C VFA g⁻¹ VSS_{fed} were produced in all the conditions tested with no significant difference in all sampling points analyzed along the days. An increase in the iron concentration increased the conversion

of VFA into CH_4 . This could be already observed after 4 days of fermentation, becoming more noticeable after 7 days. The highest methane production was obtained with 20 mmol Fe L^{-1} (AS_20), significantly higher than the control with no extra addition of iron (AS) (Figure 5b). Methane production after 14 days was $46 \pm 9 \text{ mg C CH}_4 \text{ g}^{-1} \text{ VSS}_{\text{fed}}$ for the A-sludge where no extra iron was added (AS) and 51 ± 3 , 67 ± 4 , 74 ± 10 and $114 \pm 12 \text{ mg C CH}_4 \text{ g}^{-1} \text{ VSS}_{\text{fed}}$ for the A-sludge with addition of 2.5, 5, 10 and 20 mmol Fe L^{-1} , respectively, showing a linear correlation ($R^2 = 0.98$) (Figure 5d).

Hoban and Van Den Berg (1979) demonstrated that addition of acetate and CO_2 to sewage enhance the conversion of acetate into methane increasing the iron concentration. Similarly, in our case, accumulation of VFA (acetate) produced from the A-sludge resulted in higher conversion of VFA into methane with higher concentration of iron. After 14 days the VFA concentration linearly decreased with higher concentration of iron (Figure 5a and 5b,) due to improved conversion of VFA into methane (Figure 5c).

In all the condition tested, similar VFA composition was obtained for the propionic, isobutyric and butyric and isovaleric and valeric acids (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 tested, and then converted into methane. In case of AS and AS_2.5, conversion of VFA of 22 % and 24 % to CH_4 , respectively, was obtained.

Increasing the iron content increased the conversion of VFA into CH_4 , resulting in a production up to 46 % ($118 \pm 12 \text{ mgC gVSS}_{\text{fed}}$) of CH_4 after 14 days of fermentation when 20 mmol Fe L^{-1} were added to the A-sludge. Acetate was the main VFA converted to methane while increasing the iron concentration (Figure S3), as indicated by Hoban and Van Den Berg (1979). Thus, iron is required for methanogenesis but again it does not seem to have any influence in the intermediate carboxylate production. This may relate to the low content of iron containing enzymes of many 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 sludge will be less likely to suffer from parasitic methanogenesis. If phosphorus is removed replacing the iron with polyelectrolytes or via the B-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 both fermentation and methanogenesis.

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4. Conclusions

Optimal pH, temperature and retention time for A-sludge fermentation to maximize VFA production and minimize methane generation was pH 7 and 35°C for 7 days. Production enhanced in the presence of an inoculum. Fermentation of A-sludge from four different WWTPs was executed to study the effect of different organics, nutrients and iron content on the fermentation. Initial BOD of the sludge appeared to be key determining VFA yield from A-sludge. Iron content could be correlated with CH₄ production but not with VFA production. This feature of iron limitation might be applied for further studies producing carboxylates to limit methane production.

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Figure captions

Figure 1. Specific VFA (a) and CH₄ (b) production at 55 °C and 35 °C and pH 4.5, 5, 6 and 7. C represents the controls where no pH adjustment was done. WAS represents the control where waste activated sludge was used.

Figure 2. Specific VFA (a) and CH₄ (b) production at 55 °C and 35 °C and pH 4.5, 5, 6 and 7 on day 7.

Figure 3. VFA (a) and CH₄ production (b) at day 7 for the A-sludge collected in February 2014 (winter) and in July 2014 (summer) from four WWTPs.

Figure 4. Dependency of iron content with CH₄ production (a) and VFA production (b) for Rotterdam (□), Utrecht (Δ), Breda (○) and Groningen (◇). Filled symbols originate from the winter period (February 2014) and empty symbols represent the summer period (July 2014).

Figure 5. Specific VFA (a) and CH₄ (c) production for A-sludge from Breda WWTP at different sampling days. Each bar represents iron concentrations of 2.5, 5, 10 and 20 mmol Fe L⁻¹ were added. AS represents the control where no iron was added. (b) and (c) represent respectively the dependency of the iron content with VFA and CH₄ produced after 14 days of fermentation.

Table captions

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

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

Table 3. BOD₅, BOD₅/COD ratio, VFA, and VFA/BOD_{fed} ratio for the A-sludge collected in summer (July 2014) from 4 WWTPs.

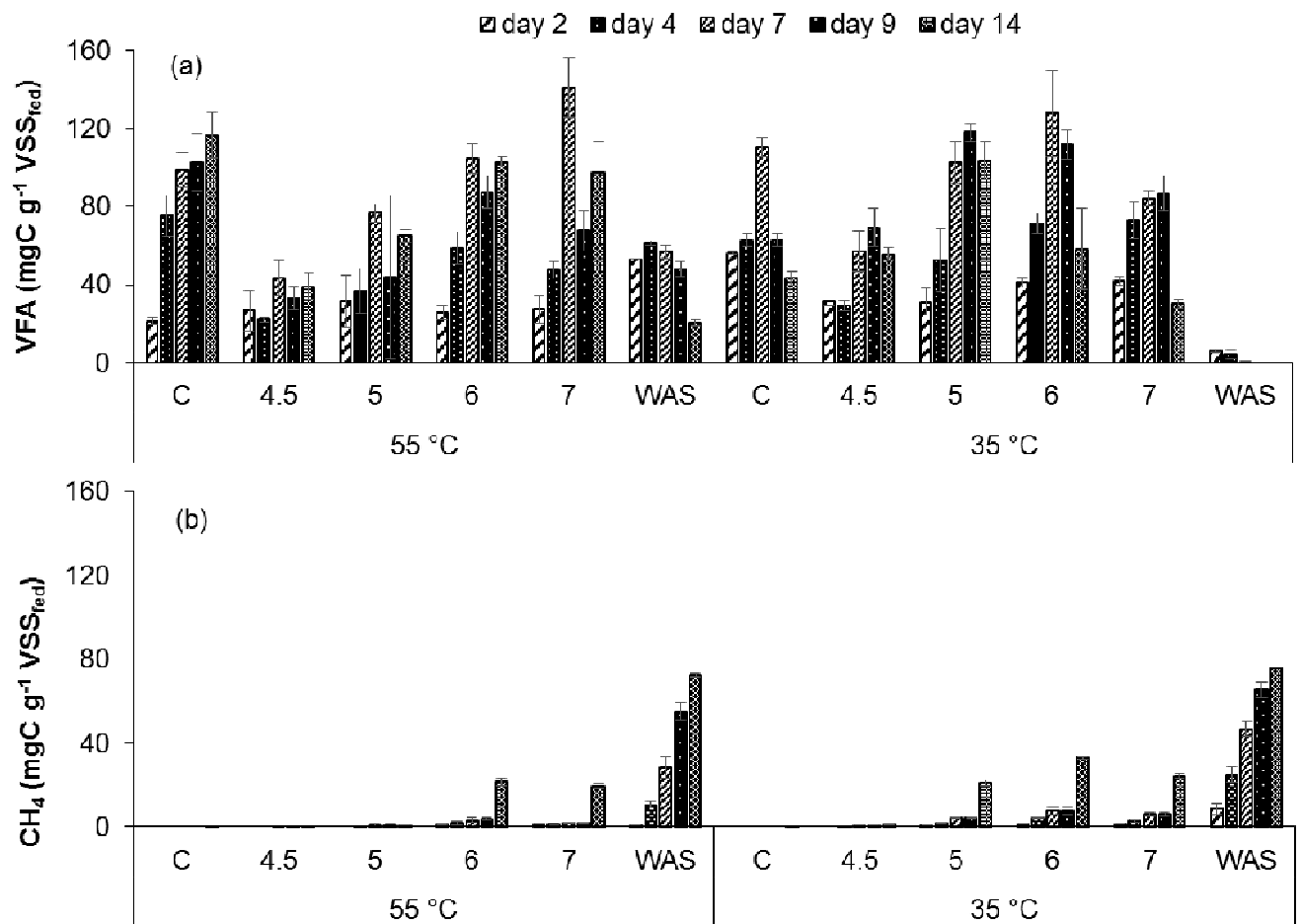


Figure 2. Specific VFA (a) and CH₄ (b) production at 55 °C and 35 °C and pH 4.5, 5, 6 and 7. C represents the controls where no pH adjustment was done. WAS represents the control where waste activated sludge was used.

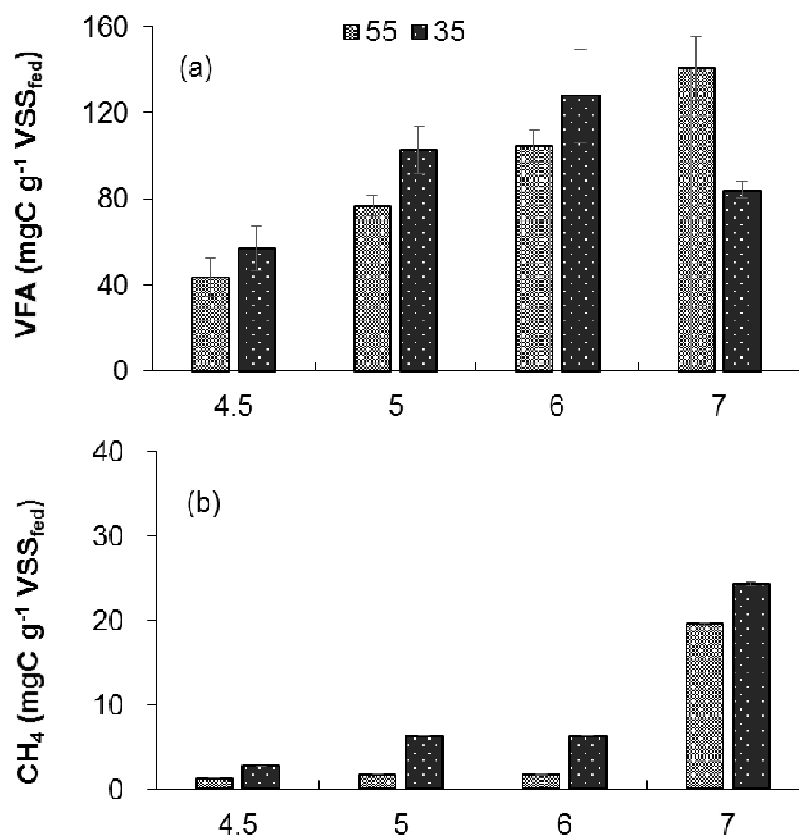


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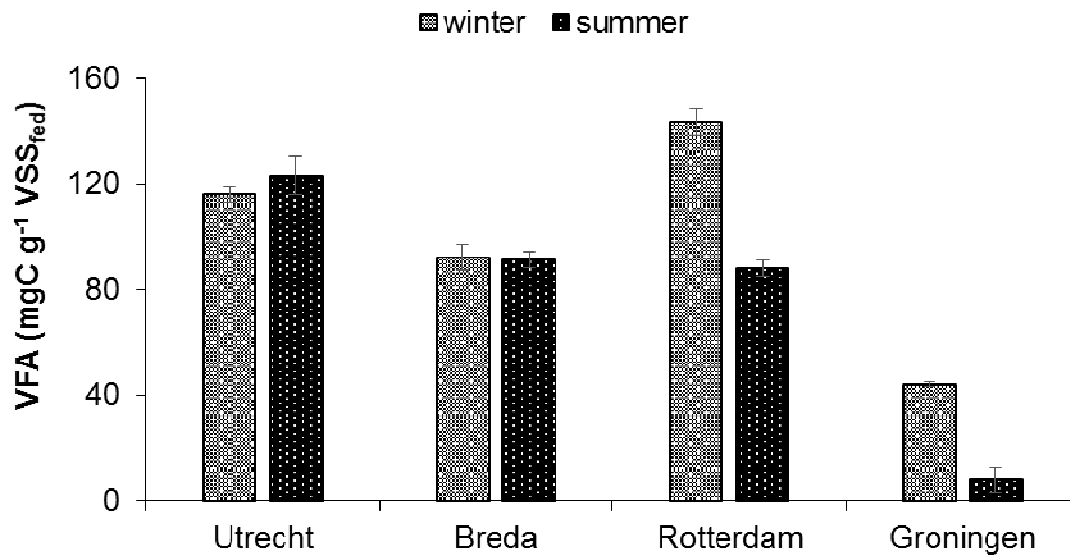


Figure 3. VFA production at day 7 for the A-sludge collected in February 2014 (winter) and in July 2014 (summer) from four WWTPs.

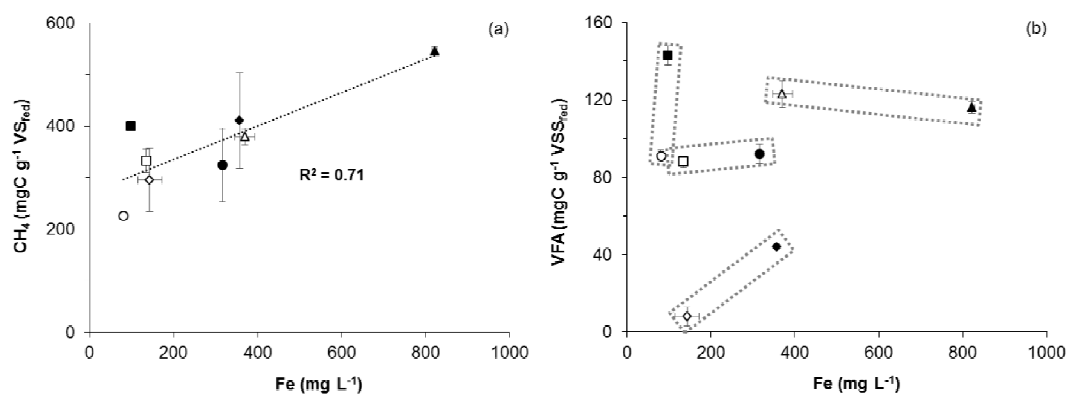


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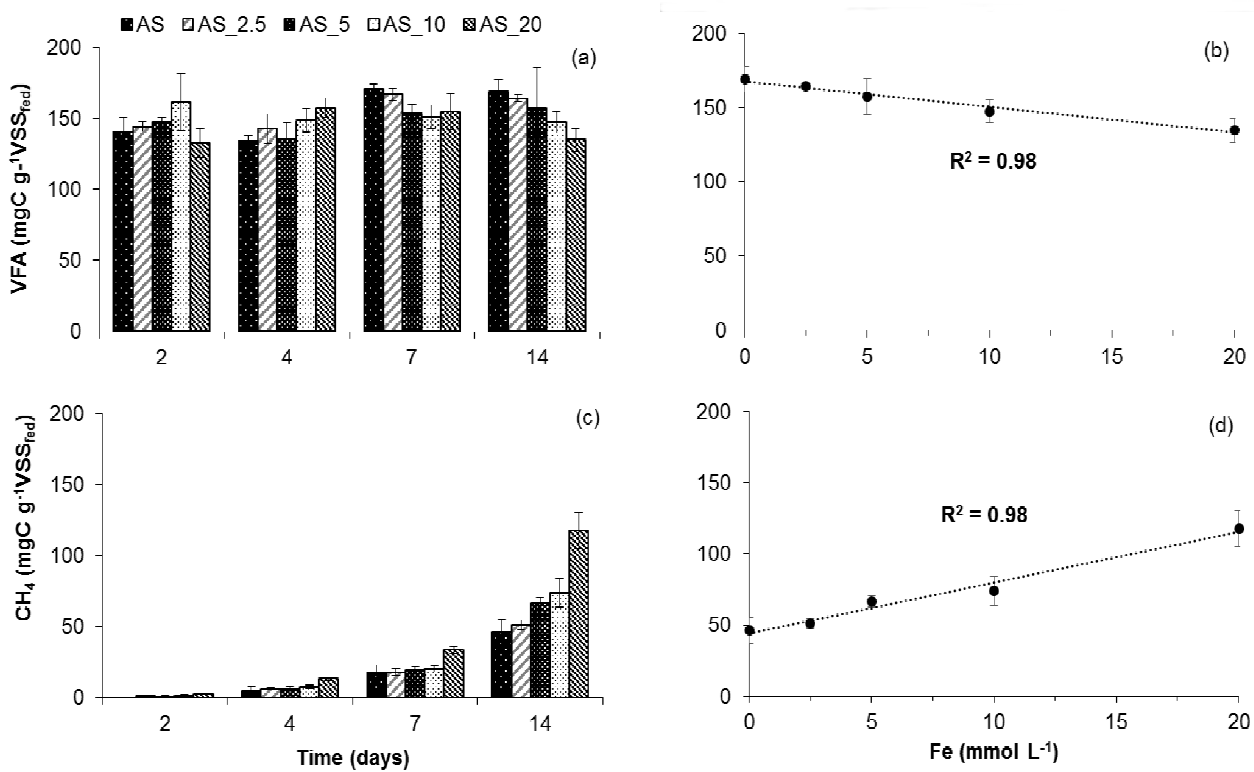


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Table 1. Characteristics of the A-sludge collected from the 4 WWTPs. All the analysis were carried out in triplicate

<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⁻¹)</i>	1.39	1.02	2.02	0.84	1.65	0.72	1.11	0.79
<i>Total COD (g L⁻¹)</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⁻¹)</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⁻¹)</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⁻¹)</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⁻¹)</i>	98 ± 2	135 ± 3	822 ± 6	370 ± 234	316 ± 2	80 ± 4	357 ± 66	143 ± 29
<i>P (mg L⁻¹)</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 4. BOD₅, BOD₅/COD ratio, VFA yield on day 7, and VFA/BOD_{fed} ratio (g C VFA g⁻¹ BOD_{fed}) for the A-sludge collected in summer (July 2014) from 4 WWTPs.

A-sludge	BOD₅ g L⁻¹	BOD₅/COD %	VFA mgC g⁻¹ VSSfed	VFA/BOD_{fed} %
Utrecht	3.78 ± 0.1	68	103 ± 5	7
Rotterdam	1.2 ± 0.0	17	78 ± 8	25
Breda	1.2 ± 0.1	17	72 ± 2	28
Groningen	0.7 ± 0.1	43	6 ± 1	2

Highlights

- pH of 7 and 35°C were found as optimal parameters for A-sludge fermentation.
- Fermentative inoculum presence enhances carboxylate production from A-sludge.
- A-stage sludge produces more VFA compared to waste activated sludge values.
- Iron content is directly proportional to CH₄ but not to carboxylate production.

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