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Effects of sludge age on anaerobic acidification of waste activated sludge: Volatile fatty acids production and phosphorus release

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ABSTRACT

Effects of sludge age on volatile fatty acids (VFAs) production and Phosphorus (P) release during anaerobic acidification of waste activated sludge (WAS) were investigated. Sequencing batch reactors (SBR) fed with simulating domestic sewage were applied to produce WAS of different sludge ages, and batch tests were used for anaerobic acidification. The maximum dissolved total organic carbon, release of $PO_4^{3+}-P$, and accumulation of acetate (C2), propionate (C3), butyrate (C4), and valerate (C5) decreased by 56.2%, 55.8%, 52.6%, 43.7%, 82.4% and 84.8%, respectively, as the sludge age of WAS increased from 5 to 40 days. Limited degradation of protein played a dominating role in decreasing DTOC and VFAs production. Moreover, the increase in molecular weight of organics and organic nitrogen content in the supernatant after acidification suggested that the refractory protein in WAS increased as sludge age extended. Although the production of C2, C3, C4, and C5 from WAS decreased as the sludge age increased, the proportions of C2 and C3 in VFAs increased, which might be due to the declined production of C5 from protein and the faded genus *Dechlorobacter*. Keeping sludge age of WAS at a relatively low level (<10 days) is more appropriate for anaerobic acidification of WAS as internal carbon sources and P resource.

Introduction

The amount of waste activated sludge (WAS) produced from wastewater treatment plants (WWTPs) in China has been growing rapidly in recent years (Chen et al., 2020a). Since lots of pollutions including perishable organic matters, heavy metal and pathogens were accumulating in WAS (Appels et al., 2008), how to treat and dispose it to avoid secondary pollution has been an essential topic in the literatures. Anaerobic digestion (AD) can reduce the volume of sludge and simultaneously produce bio-products, such as methane, volatile fatty acids (VFAs), and hydrogen, is a widely used method treating WAS (Appels et al., 2008; Gao et al., 2021). Among the bio-products, VFAs produced from WAS through anaerobic acidification has attracted lots of attention since they are not only efficient external carbon sources for removing denitrification and biological phosphorus (P) in WWTPs (Chen et al., 2004; Thomas et al., 2003), but also the key intermediates for methane production during AD (Schievano et al., 2012). In addition, the release and recovery of P from WAS during anaerobic acidification (accompanied by the destruction of organic matters) has also

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attracted lots of attention (Mehta et al., 2015) due to the increasing P-fertilizer prices, limited availability of natural P and negative environmental impacts of P on natural waterways (Mehta et al., 2015).

Moreover, due to the significant difference in chemical oxygen demand (COD) content in influents at different areas as a result of poor sewage collection system, differed sludge ages (5~30 days) operated in different WWTPs were found to be a widespread phenomenon (Chen et al., 2020b; Yang et al., 2015). When the sludge age changed, the characteristics of WAS shifted. For example, more regular morphology, increased hydrophobicity and flocculation ability were found for WAS of longer sludge age (Bolzonella et al., 2005; Liao et al., 2001). Since both substrates and microorganisms influence anaerobic acidification, it can be deduced that changes in characteristics of WAS caused by different sludge ages during wastewater treatment would make bio-production of VFAs and release of P full of uncertain during anaerobic acidification. Most of the previous studies about anaerobic acidification of WAS focused on the effects of the operating conditions (including temperature, pH, and reactor structure), pretreatment methods (including physical, chemical, biological treatment or their combinations), and the composition of organic matters (including protein, carbohydrate, and lipid) (Duan et al., 2016; Ma et al., 2019; Rajagopal and Béline, 2011). However, to the best of our knowledge, there has been rarely attention being paid on how sludge age of WAS affects the accumulation of bio-products, especially VFAs, and the release of P during anaerobic acidification.

The main objective of this study is to investigate the effect of sludge age on accumulation and composition of VFAs and release of P during anaerobic acidification of WAS. First, the different levels of VFAs and orthophosphate ($PO_4^{3+}-P$) produced during anaerobic acidification due to difference in sludge age (5 to 40 days) of WAS were investigated. Second, the diversity of anaerobic microorganisms responsible for VFAs production in WAS of different sludge ages during anaerobic acidification were examined. This work clarified the effects of sludge age on anaerobic acidification of WAS from macro and micro perspective, and would be helpful in applying bio-products from WAS of different sludge ages as internal carbon sources and also the nutrients recovery, especially P.

1. Materials and methods

1.1. *The production of activated sludge of different sludge ages*

WAS were obtained by anoxic/oxic (A/O) process using simulating domestic sewage as influent, to avoid the influence of other factors such as micron-sized silica particles, metal irons and so on (Chen et al., 2020a; Dai et al., 2017; Xu et al., 2017). Five SBR systems (effective volume:10 L) were used and the daily discharged sludge of R1, R2, R3, R4 and R5 was 2 L, 1 L, 0.5 L, 0.33 L and 0.25 L, respectively, so that the sludge ages of WAS were 5 days, 10 days, 20 days, 30 days and 40 days, respectively (**Table S2**). The SBR reactors (**Fig. S1**), formulation of simulated domestic sewage including COD, NH_4^+-N, and total phosphorus of the influent (**Table S1**), operating scheme of SBR systems (**Table S2**) and effluent properties (**Fig. S2**) are presented in **Appendix A. Supplementary data**.

1.2. *The design of acidification experiment*

Fifty identical reactors, with a working volume of 100 mL each, were kept at a temperature of 35 °C in an air bath shaker (130 r/min). WAS of different sludge ages obtained from the five reactors were divided into five groups for the anaerobic acidification test, with 6 hr static settlement to remove supernatant and increase the solid content. Before the experiment, deionized water was used to adjust the solid content of different sludge. The inoculated sludge with total solids (TS, w/w) of 0.90%±0.02% and volatile solids content (VS/TS, w/w) of 56.99%±0.21% was taken from an AD digester of sludge that does not produce methane and VFAs anymore. And it was heated under 102 °C for 30 min to inhibit the activity of methanogens before it was used (Feng et al., 2014). Each group was set up with 10 parallel samples for a total of 50 samples, and the total VS content of sludge initially added to each reactor was consistent. After adding sludge, the reactors were purged with nitrogen for 2 min and then sealed, with air pocket connected to check methane production. The test was carried out under conditions of inoculation ratio ($VS_{substrate}$: $VS_{inoculated\ sludge}$) of 4/1, and the added mass of the inoculated sludge in each bottle was 10 g (total weight). The total weight and characteristics of substrates that were added in flasks are shown in Table 1. The duration of the acidification experiment was 9 days, and at each sampling time (once a day) a flask from each group was analyzed (that is, 5 samples were analyzed on each day).

1.3. *Analytical methods*

The detection of TS, VS, total polysaccharides, protein and lipids in WAS were the same as those reported in Chen et al. (2018). Prior to analyzing dissolved total organic carbon (DTOC), NH_4^+-N, dissolved Kjeldahl nitrogen (DKN), VFAs and molecular weight (MW), sludge samples were centrifuged under 10,000 g for 15 min and then, filtered by microfiber filters (0.45 μm). DTOC was analyzed by a TOC analyzer (TOC-L, Shimadzu, Japan). DKN, NH_4^+-N, total P and dissolved $PO_4^{3+}-P$ were detected according to Standard Methods (APHA/AWWA/WEF, 2012). A high-performance size exclusion chromatography (Agilent 1100 series, USA) was used to analyze the molecular weight distribution of soluble organic matters in the supernatant, and a GC (2010 plus, Shimadzu, Japan) with flame ionization detector (FID) was used to determine VFAs. A pH meter (S210, METTLER, Switzerland) was used to determine the pH values of the sludge samples.

During anaerobic acidification, sludge of different sludge ages (5, 10, 20, 30, and 40 days) were sampled on days 0 and 4 to analyze the changes in taxonomic patterns of microbial communities. Moreover, day 4 was chosen since the MW of organic matters in supernatant began to reduce and VFAs production rate reached maximum on this day (**Fig. 3**). DNA extraction, barcode attachment and 16S rRNA sequencing analysis by Illumina MiSeq platform were conducted and the data analysis was consistent with that of Chen et al. (2018). The pyrosequencing data of the bacterial communities on days

Table 1 – The total weight and characteristics of substrates added in flasks.

Groups	1	2	3	4	5
Substrate	Sludge 1	Sludge 2	Sludge 3	Sludge 4	Sludge 5
Sludge age (days)	5	10	20	30	40
TS (%)	1.0 ± 0.02	1.0 ± 0.04	1.0 ± 0.02	1.0 ± 0.04	1.0 ± 0.03
VS/TS (%)	88.00 ± 0.04	88.25 ± 0.02	87.40 ± 0.01	85.36 ± 0.05	86.83 ± 0.03
Total Kjeldahl nitrogen (mg/g VS)	83.7 ± 0.1	90.2 ± 0.2	90.6 ± 0.1	88.2 ± 0.2	82.8 ± 0.1
Total organic carbon (mg/g VS)	470.6 ± 0.1	465.5 ± 0.2	466.2 ± 0.4	475.4 ± 0.1	455.3 ± 0.2
Total phosphorus (mg/g VS)	56.8 ± 0.5	50.6 ± 0.8	47.9 ± 0.9	44.6 ± 0.8	41.9 ± 0.7
Total weight added (g)	23	23	23	24	24

TS: total solids; VS/TS: volatile solids content.

0 and 4 were transformed to an individual quantitative matrix, and analyzed by non-metric multidimensional scaling (NMDS) based on the Sorensen (Bray-Curtis) distance in R with the Vegan package (Li et al., 2017). The NMDS analysis transforms and condenses a complex community data set to points in a low dimensional space in which similar data sets are closely plotted together, providing a clear view of the relationships between the tested profiles of the bacterial and archaeal communities (Li et al., 2017). The raw datasets have been deposited in the database of NCBI Short Read Archive (Accession numbers: SRR10338905, SRR10338904, SRR10338903, SRR10338902, SRR10338901).

2. Results and discussions

2.1. *Hydrolysis of organic matters*

Sludge hydrolysis can be expressed by the changes in DTOC concentrations (Abelleira et al., 2012; Imbierowicz and Chacuk, 2012). The effect of sludge age on DTOC at different fermentation time is shown in **Fig. 1a**. After two days of adaption, the DTOC concentration in each group gradually increased with time, which indicated that more and more organics in the particles transformed into soluble substrates. Then, the DTOC concentration reached a maximum value and the pH value continued to decrease (**Fig. 1b**), then both remained stable from day 8, with no methane detected in the gas collected in the air pocket, indicating the hydrolytic potential of sludge and un-conspicuous interruption of acidification caused by methane production. According to Fig. 1a, when sludge age increased from 5 to 40 days, the maximum dissolution concentration decreased from 124.64 ± 3.74 to 52.90 ± 1.05 mg/g VS_{added}. Moreover, the maximum TOC dissolution percentage (DTOC/TOC) was the highest (26.5 ± 0.7%) with a sludge age of 5 days and decreased with an increase in sludge age. When the sludge age increased to 10 days, the maximum DTOC/TOC significantly decreased to 19.0% ± 0.4% (−28.3%). Then, the change in maximum DTOC/TOC became unconspicuous (from 17.5% ± 0.4% to 16.7% ± 0.3%) as the sludge age increased from 10 to 30 days. The maximum DTOC/TOC decreased to 11.6% ± 0.2% as the sludge age was increased to 40 days (decreased by 56.2% compared to that of 5 days). It was apparent that the extension of sludge age limited the hydrolysis and dissolution of liquefaction of sludge, and the limiting degree was slightly differed when the sludge age changed from 10 to 30 days.

2.2. *Transformation of protein, carbohydrate and lipid, as well as P release*

Protein, carbohydrate, and lipid are the main components of sewage sludge (Chen et al., 2018; Liu et al., 2020; Luo et al., 2020). In this study, the organic matters in sludge consisted of 52%~56% protein, 16%~18% carbohydrate, 16%~18% lipid and about 10%~15% unknown components. It was found that sludge age had no significant effect on the organic components in WAS, and the different degradability of WAS of different sludge ages might be due to changed characteristics of those organic matters. The TOC dissolution and hydrolysis was therefore further analyzed by focusing on the transformation of protein, carbohydrate and lipid into VFAs during the anaerobic acidification. Generally, the organic matters are first transformed to simple soluble products, such as amino acids, sugars, and long-chain fatty acids; this is commonly known as hydrolysis step (Khanal, 2011). Then, the products from the first step are fermented to form a mixture of VFAs, carbon dioxide and hydrogen, including the processes of acidogenesis (production of VFAs, such as acetate (C2), propionate (C3), butyrate (C4) and valerate (C5)) and acetogenesis (production of C2 by VFAs with $C > 2$) (Khanal, 2011). Meanwhile, the release of P was widely reported with the degradation of organic matters during anaerobic fermentation (Latif et al., 2015). The transformation of protein, carbohydrate and lipid in the above two processes would determine the production of VFAs and the release of P.

As shown in **Table 2**, sludge age almost had the similar effect on the transformation percentage of protein, carbohydrate and lipid, that is, the transformation amount and percentage of these three substrates gradually decreased with the increase of sludge age, and with slightly change between sludge ages of 10 to 30 days. When the sludge age increased from 5 to 40 days, the transformation percentage of total protein, carbohydrate and lipid gradually decreased from 29.8% ± 0.3%, 33.0% ± 0.2%, and 21.6% ± 0.2% to 12.9% ± 0.2% (−56.7%), 12.1% ± 0.2% (−63.3%), and 12.2% ± 0.2% (−43.5%),

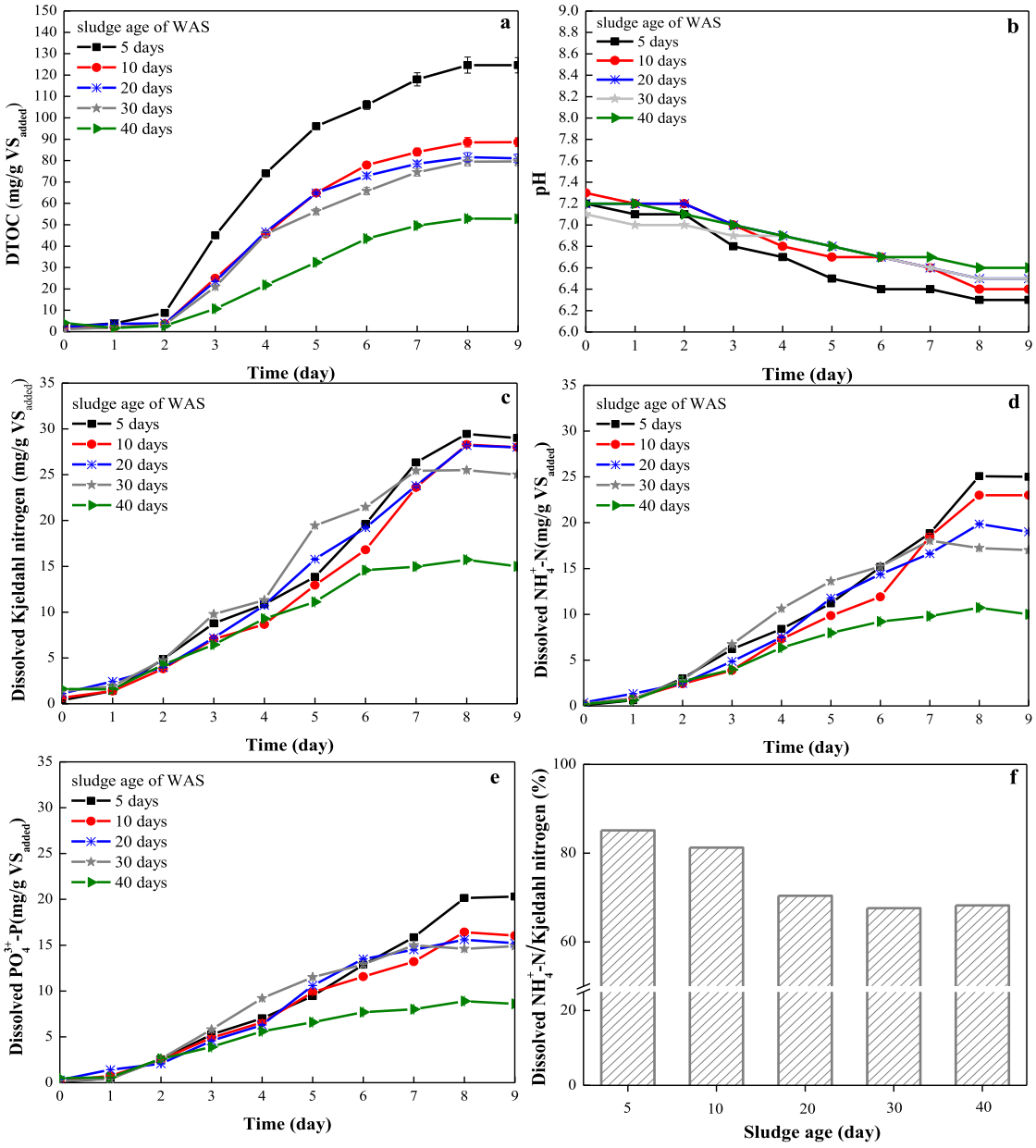

Fig. 1 – The variations concentration of dissolved total organic carbon (a), pH (b), dissolved Kjeldahl nitrogen (c), NH_4^+-N (d) and PO_4^{3+} – P (f) during anaerobic acidification, as well as the ratio of dissolved NH_4^+-N in Kjeldahl nitrogen on day 8 (e).

respectively, and their transformation amount decreased from 156.2 ± 1.2, 58.9 ± 0.4 and 38.8 ± 0.5 mg/g VS_{added} to 66.9 ± 2.4, 20.2 ± 0.8 and 21.1 ± 1.2 mg/g VS_{added}, respectively. It can be seen that the limited degradation of protein played dominating roles in limited anaerobic acidification of WAS of longer sludge age. The decline in the transformation percentage of protein and carbohydrates in WAS were in accord with the limited degradation of protein and carbohydrates in sludge of longer sludge age due to their less proportion distributed extracellularly (Bougrier et al., 2008; Liao et al., 2001; Matsuda et al., 1993). Lipid is the main component of cell membrane, and the limited degradation of extracellular organic matters (protein and carbohydrate) might resist the access of microbes and enzymes to lipids in the cell membrane (Coskun and Simons, 2011; Spector and Yorek, 1985). Thus, the hydrolysis of lipid is deduced to be limited under the indirect impact of sludge age, and the hypothesis was supported by the results of this study.

Hydrolysis, acidogenesis and acetogenesis of organic matters led to significant increase in DKN (Fig. 1b), NH_4^+–N (Fig. 1c) and PO_4^{3+} – P (Fig. 1d). Dissolved N and P in supernatants depended on the fermentation time and sludge age for WAS. As seen in Fig. 1c, Fig. 1d, and Fig. 1f, during anaerobic acidification, the concentration of dissolved N (Kjeldahl Nitrogen and NH_4^+–N) and PO_4^{3+} – P in five group all kept increasing. Nevertheless, the accumulation of Kjeldahl nitrogen and NH_4^+–N and PO_4^{3+} – P in the supernatant were the highest (29.46 ± 0.15, 25.08 ± 0.12 and 20.15 ± 0.10 mg/g VS_{added}) in the sludge age

Table 2 – The maximum transformation percentage of organic matters and P release in sludge 1~5 in anaerobic acidification test.

Substrate Sludge age (days)	Sludge 1 5	Sludge 2 10	Sludge 3 20	Sludge 4 30	Sludge 5 40
Total protein (day 0) (mg/g VS_{added})	522.5 ± 0.6	563.1 ± 1.2	565.6 ± 0.6	550.6 ± 1.2	516.9 ± 0.6
Total protein (day 8) (mg/g VS_{added})	366.3 ± 0.6	419.4 ± 0.6	429.4 ± 1.2	424.7 ± 1.2	450.0 ± 1.8
Transformation amount of protein (mg/g VS_{added})	156.2 ± 1.2	143.7 ± 1.8	136.2 ± 1.8	125.9 ± 2.4	66.9 ± 2.4
Transformation percentage of protein/organic nitrogen (%)	29.9 ± 0.3	25.5 ± 0.2	24.1 ± 0.1	22.9 ± 0.1	12.9 ± 0.2
Total carbohydrate (day 0) (mg/g VS_{added})	178.2 ± 0.3	173.5 ± 0.2	167.5 ± 0.1	166.3 ± 0.3	166.8 ± 0.4
Total carbohydrate (day 8) (mg/g VS_{added})	119.3 ± 0.1	130.2 ± 0.2	131.6 ± 0.2	130.9 ± 0.3	146.6 ± 0.4
Transformation amount of carbohydrate (mg/g VS_{added})	58.9 ± 0.4	43.3 ± 0.4	35.9 ± 0.3	35.4 ± 0.6	20.2 ± 0.8
Transformation percentage of carbohydrate (%)	33.0 ± 0.2	24.9 ± 0.3	21.4 ± 0.2	21.3 ± 0.5	12.1 ± 0.2
Total lipid (day 0) (mg/g VS_{added})	179.5 ± 0.4	167.3 ± 0.3	157.6 ± 0.1	160.6 ± 0.5	168.5 ± 0.8
Total lipid (day 8) (mg/g VS_{added})	140.7 ± 0.1	137.8 ± 0.2	132.8 ± 0.2	134.4 ± 0.3	147.6 ± 0.4
Transformation amount of lipid (mg/g VS_{added})	38.8 ± 0.5	29.5 ± 0.5	24.8 ± 0.3	26.2 ± 0.8	21.2 ± 1.2
Transformation percentage of lipid (%)	21.6 ± 0.2	17.6 ± 0.3	16.0 ± 0.2	16.1 ± 0.5	12.2 ± 0.2
P release percentage (%)	35.7 ± 0.2	31.6 ± 0.2	31.7 ± 0.1	33.4 ± 0.2	20.5 ± 0.1

of 5 days, which gradually decreased to 15.73 ± 0.15 (−46.6%), 10.73 ± 0.12 (−57.2%) and 8.90 ± 0.09 (−55.8%) mg/g VS_{added} as the sludge age increased to 40 days. Apparently, the release of N and P during anaerobic acidification would be significantly limited as the increase in sludge age for WAS, which is similar to that of DTOC, protein, carbohydrate and lipid.

Especially, at the end of acidification fermentation, there were still certain amount of organic nitrogen (Kjeldahl nitrogen minus NH_4^+–N) in the supernatant that had not been fermented into NH_4^+–N in all the five groups. As shown in **Fig.1e**, the ratio of dissolved NH_4^+-N in Kjeldahl nitrogen at day 8 declined from 84.9% to 68.2% as the sludge age prolonged from 5 to 40 days, that is, the ratio of organic nitrogen to Kjeldahl Nitrogen increased from 15.1% to 31.8%. It was suggested that there was some refractory protein in sludge; although they were transformed from solid into supernatant during acidification, some dissolved portion was not accessible for deamination and utilization by microbes (Appels et al., 2008). And with the extension of sludge age from 5 to 40 days, the content of refractory protein was found to increase, which would play a vital role in the downgraded fermentation of protein in WAS of longer sludge age.

2.3. *Molecular weight*

The distribution of the MW of organic matters in the supernatant in the five groups during anaerobic acidification is shown in Fig. 2. With little residual organic matters from effluent (with STOC low as 1~4 mg/L), the MW of supernatant among five sludge at day 0 showed difference but would not influence the results for the subsequent acidification process. Clearly, although organic matters presented no significant dissolution on the first two days of acidification process (Fig. 1), the MW of the dissolved organic matters reduced, namely, the dissolved organic matters began hydrolyzing at day 1. From day 2 to 8, the macromolecular organic matters were hydrolyzed into small molecules and the content of organic matters with MW below 1000 Da in the supernatant in all the five groups gradually increased and became the dominating component (58%~66%). Moreover, with the prolongation of sludge age from 5 to 40 days, the proportion of organic molecules with MW below 1000 Da in the supernatant on day 8 gradually decreased from 66.9% to 58.1%, and that between 1000 and 100,000 Da gradually increased from 14.8% to 23.0%. It was indicated that the longer the sludge age, the lower the proportion of small molecular organic matter in the supernatant after acidification. Organic molecules with MW below 1000 Da could be utilized directly by anaerobic microorganisms for deamination, acidification, hydrogen production and methanogenesis, thus the higher MW would result in a harder utilization by microorganisms (Morgenroth et al., 2002; Kafkewitz, 1996). Therefore, a higher proportion of large molecular organic matters in the supernatant at the end of fermentation could support the lower decomposition (deamination) of dissolved protein (lower ratio of NH_4^+ – N to Kjeldahl nitrogen) in sludge of longer sludge age.

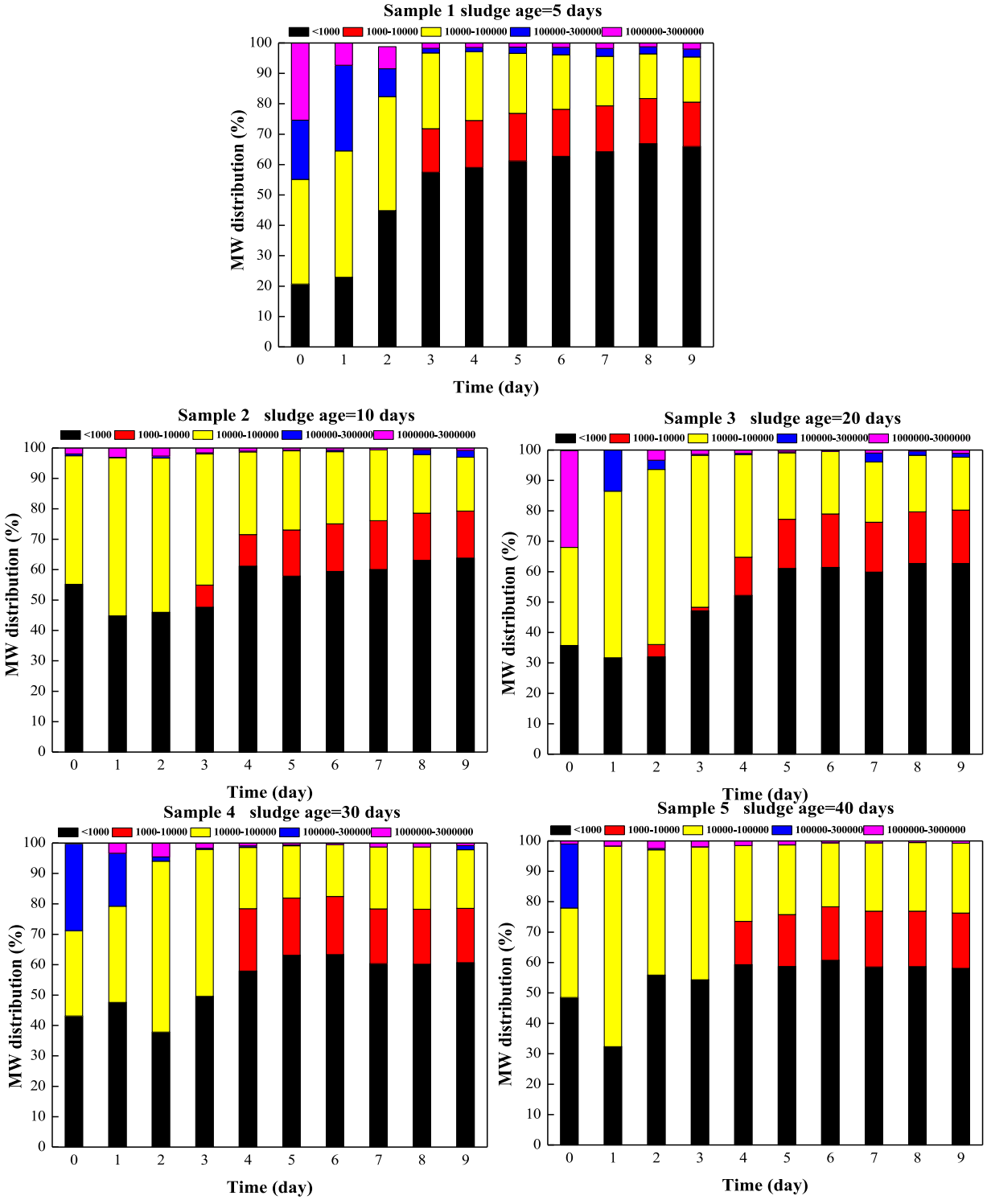

Fig. 2 – Organic molecular weight (MW) distribution of dissolved organic matters in the supernatants during anaerobic acidification test of sludge 1~5 of different sludge ages.

2.4. *VFAs production and composition*

Fig. 3 shows the effects of sludge age and fermentation time on VFAs production and VFAs production rate. It was observed that the concentration was the highest on day 8 to 9 and became stable in all the five groups as time increased (Fig. 3a~3e), and all the VFAs production rates reached the maximum at day 4. The accumulation (highest value on day 8) was 221.0 ± 6.6, 154.6 ± 4.6, 138.9 ± 4.2, 132.7 ± 4.0 and 78.3 ± 2.4 mg/g VS_{added} for sludge ages of 5, 10, 20, 30 and 40 days, respectively (Fig. 3f). Obviously, the extension of sludge from 5 to 10 days would sharply decline the production

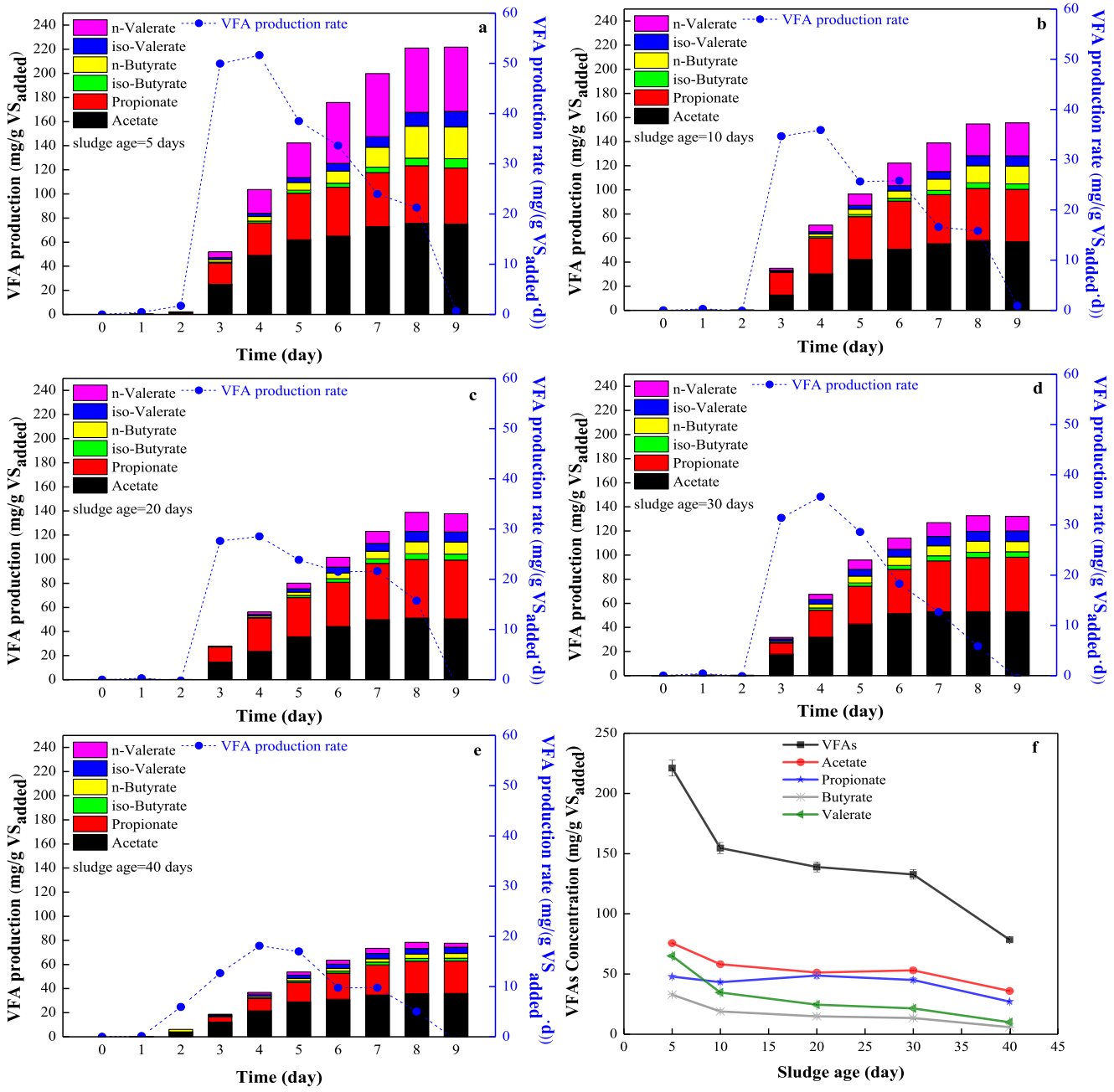

Fig. 3 – (a~e) The cumulative amount and production rate of volatile fatty acids (VFAs) during the anaerobic acidification test of sludge 1~5 in different sludge ages; (f) The maximum value of VFAs during the anaerobic acidification test of sludge 1~5 of different sludge ages.

performance of VFAs, and the decreasing trend was slight when the sludge age was among 10 to 30 days, with a sharp decrease from 30 to 40 days, which was similar to its influence on the dissolution of TOC. And the ratios of organic carbon in VFAs to dissolved TOC in the supernatant on day 8 for sludge 1 to 5 were 96.0%, 93.3%, 89.8%, 88.1% and 77.8%, respectively. It was further indicated that there was dissolved organic matters not transformed by microbes to VFAs as mentioned above. And with the extension of sludge age, the untransformed portion increased, which was in accord with the lower decomposition (deamination) of dissolved organic matters in sludge (especially protein).

According to Fig. 3f, when the sludge age increased from 5 to 40 days, the accumulation of C2, C3, C4, and C5 decreased by 52.6%, 43.7%, 82.4%, and 84.8%, respectively. And the decrease in their accumulation contributed 27.9%, 14.6%, 18.9% and 38.6%, respectively, to the decrease in the total accumulation of VFAs as the sludge age extended from 5 to 40 days. It was indicated that the main reason for declined VFAs accumulation as the sludge age increased was the decrease in the

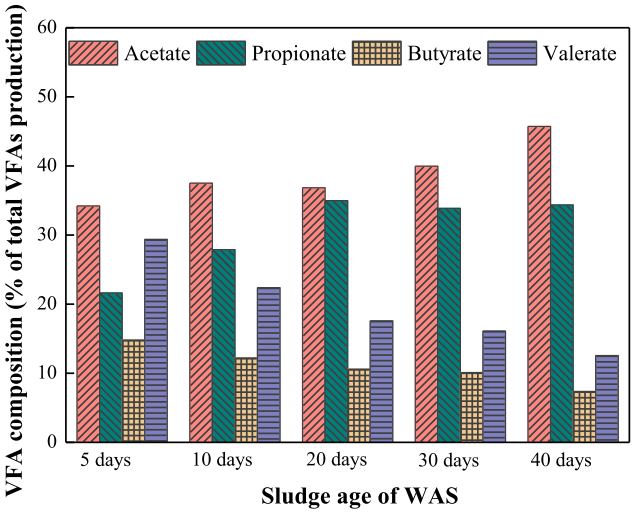

Fig. 4 – Percentage of individual VFA accounted for total VFAs at maximum values in the anaerobic acidification test.

production of C5 and C2, especially C5. C2 to C4 can be formed directly from the fermentation of protein, carbohydrate and lipid (McInerney, 1988). While C5 is mainly associated with the fermentation of protein because of their little production from acidogenesis of non-proteinaceous (Chen et al., 2007; Zoetemeyer et al., 1982). Therefore, the lower VFAs production driven by the extension of sludge age could be attributed to the reduction in transformation of protein, carbohydrate and lipid reported above, and mainly the declined degradability of protein. The results were consistent with the main contribution of limited protein hydrolysis to the decreased transformation of organic matters, and further verified the increased content of refractory protein in WAS of longer sludge age discussed above.

A significant difference was also detected in the distribution of VFAs components in the five groups of sludge on day 8 (Fig. 4). C2 and C3 were the two main products (accounted for 55.8~80.1% in total) despite how long the sludge age was; this was in accord with previous studies in which these two products were the two main components of VFAs (Wang et al., 1999). Fig. 4 shows that the percentage of C2 and C3 increased from 34.2% and 21.6% to 45.7% and 34.4%, respectively (with the greater increase in percentage of C3 than that of C2), and that of C4 and C5 which decreased from 14.8% and 29.3% to 7.4% and 12.56%, respectively, as the sludge age extended from 5 to 40 days. C3 and C4 were reported to be oxidized into C2 with by-products of H_2 and CO_2, by hydrogen-producing acetogenic bacteria (Khanal, 2011), and C5-degrading acetogenic step was followed by the β-oxidation mechanism, producing 1 mol of C2 and 1 mol of C3 from 1 mol of C5 (Flotats et al., 2003). The increased percentage of C2 and C3 (especially C3), and the declined percentage of C4 and C5 from WAS of longer sludge age might be attributed to the sharply declined production of C4 and C5 due to the limited protein hydrolysis (Fig. 3f), as well as the inhibited oxidation of C3 to C2. The changes in the production of VFAs were usually attributed to the different metabolic pathways conducted by bacteria (Rajagopal and Béline, 2011; Ucisik and Henze, 2008). Thus, the changes in bacterial communities were further analyzed.

2.5. *Key microbes induced by different sludge ages*

The microbial community on day 4 (when the MW of organic matters in the supernatant began to reduce and VFAs production rate reached their maximum values) during anaerobic acidification were analyzed. The reads and operational taxonomic unit (OTU) numbers of the test are shown in Appendix A **Table S3**. The microbial composition of sludge of different sludge ages differed on day 0 (Fig. 5a), and with the application of inoculum and operation of acidification process the microbial community shifted into a significantly different group for sludge samples on day 4 (indicated by NMDS map in Fig. 5a). Considering the difference in the microbial composition in the five sludge samples on day 0 was attributed to sludge age in SBR systems, and the same inoculum was added for quick start of anaerobic acidification, the change in microbial composition among the five sludge samples on day 4 was due to the comprehensive influence of sludge age, including microbial community and organic matters. It was found that the bacterial community of the five sludge samples on day 4 were dominantly composed of *Proteobacteria, Bacteroidetes* and *Actinobacteria* at phylum level (accounted for 88.9~93.6% in total) (Fig. 5a). All the three phyla are important microbes involved in hydrolysis and acidification of sludge and conversion of organic matters to VFAs (Chen et al., 2016; Kindaichi et al., 2004; Liu et al., 2016). The relative abundance of *Proteobacteria* significantly decreased from 57.0% to 40.6% as the sludge age increased from 5 to 40 days, and the total relative abundance of *Bacteroidetes* and *Actinobacteria* showed an opposite trend. It was indicated that the dominating bacteria related to acidification of organic matters in sludge gradually shifted from *Proteobacteria* to *Bacteroidetes/Actinobacteria*, especially *Bacteroidetes,* when the sludge age gradually increased from 5 to 40 days.

As to the detailed shift of bacterial composition, the main 23 bacterial genera in sludges on day 4 were ranked (Fig. 5b). The propagation and recession of each genus are presented by a heatmap. It can be seen that the general distributions of the five sludge samples were in significant differences, which could explain the differences in the VFAs distributions among the five sludge samples. Some genera, such as *Propioniciclava, Candidatus Competibacter, AAP99* and *Dechlorobacter* faded as the sludge age increased. Among them, *Candidatus Competibacter* belonging to phylum *Proteobacteria* was a typical glycogen-accumulating organism (GAO), which uses glycogen as energy source and converts VFA simultaneously to intracellular poly-β-hydroxyalkanoate under anaerobic condition (Saunders et al., 2003). Its fading was in accord with the declined converted carbohydrate for energy and the lower concentration of C2~C5 for uptake when the sludge age was prolonged. Especially, genera *Propioniciclava* and *Dechlorobacter* are known as typical anaerobes and correlated to the anaerobic acidification of organic matters (Fraj et al., 2013; Sugawara et al., 2011; Thrash et al., 2010). *Propioniciclava* belonging to phylum Actinobacteria has been reported to ferment various carbohydrates and produces C2 and C3 from glucose

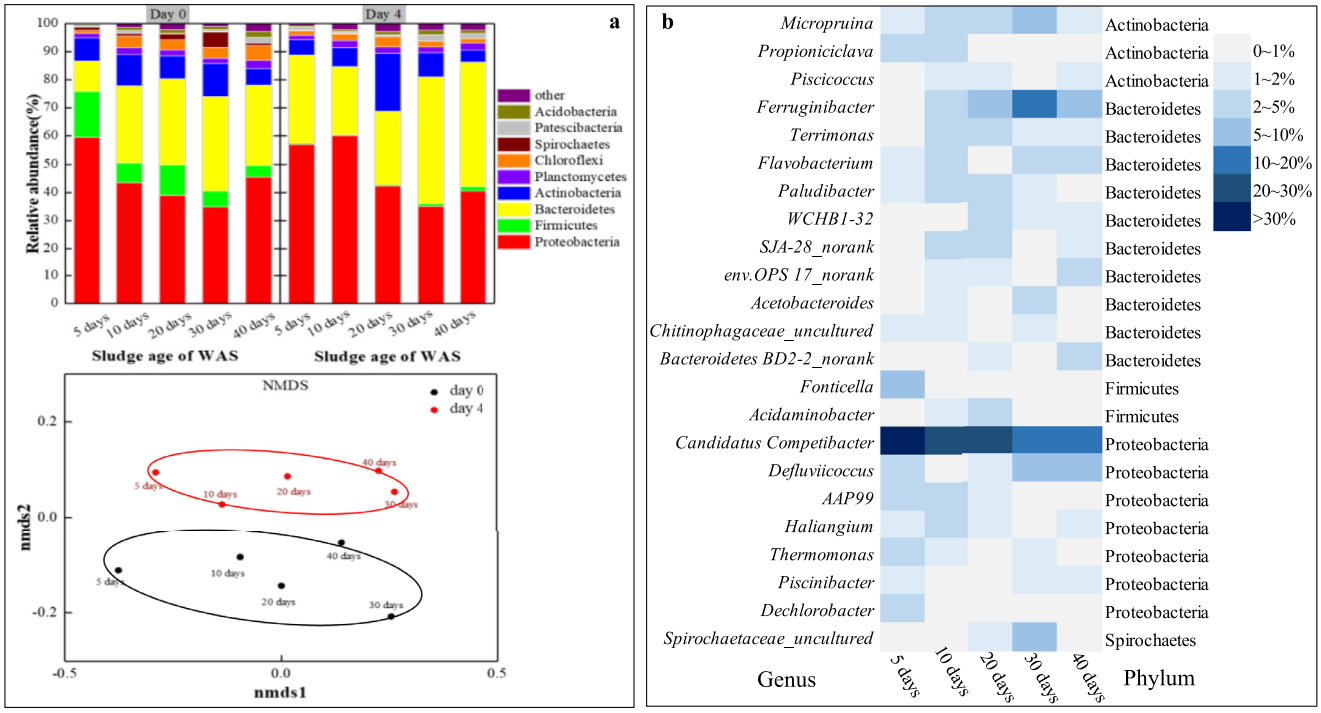

Fig. 5 – (a) Relative abundance of the main bacterial community of five sludge samples at phylum level on day 0 and day 4, and the non-metric multidimensional scaling (NMDS) map of bacterial community of five sludge of different sludge ages on day 0 and day 4;(b) relative abundance of the main 23 bacterial genera (accounted for 63.6% of all sequences) in five sludge of different sludge ages on day 4.

(Sugawara et al., 2011). *Dechlorobacter* belonging to phylum *Proteobacteria* can use organic substrates especially C2 and grow mixotrophically with hydrogen as the electron donor (Thrash et al., 2010). The significant decrease in abundance of *Proteobacteria* would cause the decrease in VFAs production. And the decrease in abundance of *Dechlorobacter* might cause the less utilization of C2 and then the high percentage of C2 from WAS of longer sludge age.

However, there were still anaerobic genera presenting obviously increasing trend as the sludge age increased, such as *WCHB1–32* belonging to phylum *Bacteroidetes*. This contributed to the more dominating role of *Bacteroidetes* in the acidification of sludge of longer sludge age reported above. *WCHB1–32* was found from a hydrocarbon-and chlorinated-contaminated aquifer (Dojka et al., 1998), suggesting that the bacterium might be related to the utilization of refractory organic matters. And its abundance was found to be strongly positively correlated to the percentage of C3 ($p<0.05$), suggesting its role during the acidogenesis.

3. Conclusion

The extension of sludge age during wastewater treatment would significantly limit VFAs accumulation and P release from WAS during the anaerobic acidification. When sludge age increased from 5 to 40 days, the maximum DTOC/TOC decreased by 56.2%, the transformation percentage of total protein, carbohydrate and lipid decreased by 56.7%, 63.3% and 43.5%, respectively, the release of $PO_4^{3+}-P$ decreased by 55.8%, and the accumulation of C2, C3, C4 and C5 decreased by 52.6%, 43.7%, 82.4% and 84.8%, respectively. The content of refractory protein was suggested to increase with the extension of sludge age, and the limited degradation of protein played dominating roles in declined DTOC and VFAs accumulation. Even though the production of C2~C5 from WAS were all limited as the sludge age increased, the C2 and C3 proportions in VFAs were found to increase, and this might be related to the significantly declined production of C5 from protein, as well as the faded *Dechlorobacter*. In summary, selecting or keeping sludge age of WAS at a relatively lower level (<10 days) is more appropriate for the application of WAS as internal carbon sources and P resource through anaerobic acidification.

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Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.12.030.

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