Exploring the potential of co-fermenting sewage sludge and lipids in a resource recovery scenario

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ABSTRACT

In this study, co-fermentation of primary sludge (PS) or waste activated sludge (WAS) with lipids was explored to improve volatile fatty acid production. PS and WAS were used as base substrate to facilitate lipid fermentation at 20 ºC under semi-aerobic conditions. Mono-fermentation tests showed higher VFA yields for PS (32-89 mgCOD gVS^{-1}) than for WAS (20-41 mgCOD gVS^{-1}) where propionate production was favoured. The principal component analysis showed that the base substrate had a notable influence on co-fermentation yields and profile. Co-fermentation with WAS resulted in a greater extent of oleic acid degradation (up to 4.7%) and evidence of chain elongation producing valerate. The occurrence of chain elongation suggests that co-fermentation can be engineered to favour medium-chain fatty acids without the addition of external commodity chemicals. BMP tests showed that neither mono-fermentation nor co-fermentation had an impact on downstream anaerobic digestion.

Keywords
Fermentation; Co-fermentation; Anaerobic digestion; Chain elongation; Fats, oil and grease

1. INTRODUCTION

Wastewater treatment plants (WWTP) are being transformed into resource recovery facilities (RRF). In RRF, clean water and nutrients are recovered from wastewater, and the production of value-added chemicals and biogas is maximised (Puyol et al., 2017). Fermentation is a crucial process of most RRF schemes due to its capacity to produce readily biodegradable organic matter to support other biotechnologies such as biological nutrient recovery, purple phototrophic bacteria, bioplastics production and chain elongation (Batstone and Virdis, 2014; Duber et al., 2018; Verstraete et al., 2016)

In traditional WWTP, primary sludge (PS) and waste activated sludge (WAS) are thickened and sent to anaerobic digestion (AD) for biogas production. However, both streams have the potential to be fermented before AD to produce volatile fatty acids (VFA), lactic acid and alcohols. PS has a higher potential for VFA production than WAS, with yields ranging from 30 to 400 mgCOD gVS^{-1} depending on the fermentation conditions (e.g., temperature, retention time, pH) (Ahn and Speece, 2006; Cokgor et al., 2009; Peces et al., 2016; Ucisik and Henze, 2008). However, the volume and composition of PS is somewhat variable since it is linked to the incoming raw wastewater and retention time in the primary settler. Consequently, VFA yields and VFA profile from PS fermentation are variable (Peces et al., 2016; Ucisik and Henze, 2008, Yasui et al., 2008). On the other hand, within the same WWTP, the production and composition of WAS are more constant, making it a more reliable substrate for VFA production despite the lower VFA yields. Without pre-treatment, WAS fermentation yields range from 18 to 240 mgCOD gVS^{-1} depending on the fermentation conditions (Chen et al., 2007; Ucisik and Henze, 2008; Xiong et al., 2012; Yuan et al., 2009). Considering the reported yields, PS and WAS fermentation efficiency is low since no more than 20% of the chemical oxygen demand (COD) contained in the sludge is converted to VFA.

VFA yields can be improved through intensification of fermentation conditions at the expense of energy, chemical use or both. Alternatively, other wastes can be fermented together with sludge (co-fermentation) to boost VFA production. The concept of co-fermentation is similar to anaerobic co-digestion, the simultaneous anaerobic digestion of various substrates to increase the methane production of anaerobic digesters (Macintosh et al., 2019; Mata-Alvarez et al., 2014).

Co-fermentation has the potential to improve fermentation efficiency by: (i) increasing the organic load, (ii) balancing macronutrients (e.g., C/N ratio), micronutrients and moisture, (iii) buffering the pH medium without chemicals addition, (iv) diluting inhibitory compounds, (v) providing an active fermentative microbial community, (vi) steering the fermentation towards a VFA profile, and (vii) making a better use of the current infrastructure by fermenting various wastes in a single reactor (Elefsiniotis et al., 2005; Fang et al., 2020; Feng et al., 2011; Garcia-Aguirre et al., 2019). Reported co-fermentation experiences for VFA production is limited to few publications. Among them, sewage sludge has been only co-fermented with food waste (Feng et al., 2011; Garcia-Aguirre et al., 2019) and starch-rich industrial wastewater (Elefsiniotis et al., 2005). Therefore, the potentials, limitations and impacts on downstream processes of co-fermentation remain mostly unexplored.

Fats, oils and grease (FOG) from the WWTP aerated grit chamber, from restaurants grease traps and from dissolved air flotation units in food processing industry is a potential substrate for fermentation due to their high COD density (around 3 gCOD gVS^{-1}) and high biodegradability (almost 100%). However, FOG lack of enough moisture, essential nutrients (e.g., nitrogen and phosphorus), trace elements (e.g., iron, cobalt, nickel) and microbes to be fermented by themselves. Nonetheless, FOG from the WWTP grease trap could be co-fermented with sludge to improve sludge fermentation yields and valorise this waste onsite. Despite the great potential to co-ferment FOG with other waste to produce VFA, this approach has received minimal attention. In a pilot-scale study operated at ambient temperature, Nicholson et al. (2013) and Long et al. (2014) showed that increasing a 20% the organic load of a PS fermenter with FOG from a food service grease trap increased the VFA concentration from 0.54 gCOD L^{-1} to 0.65 gCOD L^{-1}. Despite the modest improvement, the authors estimated that the extra VFA production would save up to 400,000 USD per year due to lower methanol purchasing costs for denitrification. Higher VFA production was reported by Varin et al. (2016) when FOG was co-fermented with mixed sewage sludge at mesophilic conditions. Specifically, the VFA concentration increased linearly from 2.4 gCOD L^{-1} to 4.2 gCOD L^{-1} as the FOG organic loading rate increased from 0 to 80%. Varin et al. (2016) identified that FOG fermentation was limited by the accumulation of saturated fatty acids which were not further degraded. Additionally, it should be taken into account that the anaerobic degradation of long chain fatty acids (LCFA) through β-oxidation is only thermodynamically possible at low H_2 concentration. Consequently, it is hypothesised that low H_2 partial pressures will trigger FOG fermentation into VFA. This experimental condition could be achieved by performing fermentation in an open vessel fermenter.

Open vessel fermentation is a relatively common practice for brewing beer in some countries (Briggs et al., 2004), but it is an unexplored practice for waste fermentation. However, Peces et al. (2016) reported that PS fermentation at 20 ºC and semi-aerobic conditions (i.e., open vessel) allowed both VFA recovery and improved methane yield. Peces et al. (2016) stated that fermenting under semi-aerobic conditions procured a slightly positive COD balance (4% improvement) when the fermented PS was anaerobically digested. Hence, it is hypothesised that semi-aerobic conditions could be a low-cost platform to co-ferment sludge and FOG to maximise VFA production in a RRF scenario.

This study aims to evaluate the potential and limitations of LCFA co-fermentation with PS or WAS under semi-anaerobic conditions. To this purpose, several mixtures between oleic acid (as a typical representative of lipids) and PS or WAS were co-fermented to evaluate the impact of co-fermentation on VFA yields and VFA profile. Finally, the effects of co-fermentation on downstream anaerobic digestion was assessed through biochemical methane potential (BMP) tests.

2. MATERIALS AND METHODS

2.1 Substrates and inoculum origin

Three batches of primary sludge (PS) and two batches of waste activated sludge (WAS) were obtained from a municipal WWTP in South East Queensland (Australia). PS and WAS were collected after being thickened by centrifugation and before being mixed and fed into the WWTP mesophilic anaerobic digesters. PS and WAS were stored at 4 ºC prior fermentation experiments (max. 1 day) and BMP testing (max. 5 days). Table 1 summarises the main characteristics of the different batches of PS and WAS. Technical grade oleic acid (Sigma-Aldrich, USA) was used as a model LCFA. Inoculum for the biochemical methane potential (BMP) tests was collected from the same WWTP. The inoculum was taken from a 5,500 m^3 digester that treats mixed sewage sludge (50% PS and 50% WAS in VS-basis) at a hydraulic retention time of 24 ± 1 days and a temperature of 37 ± 2 ºC. The inoculum was degassed at 37 ºC for 5 days prior its utilisation.

2.2 Experimental set-up

2.2.1 Mono-fermentation and co-fermentation experiments

All mono-fermentation (i.e. only PS or WAS) and co-fermentation (i.e. PS or WAS mixed with oleic acid) experiments were carried out under semi-aerobic conditions at 20 °C as described in Peces et al. (2016). Briefly, semi-aerobic conditions were attained by leaving the fermentation vessels open to the environment without agitation and without any forced aeration. Three sets of experiments were carried out in this study as detailed in Table 2.

The first set of experiments was conducted in 250 mL glass beakers. Mono-fermentation experiments contained 125 g of fresh PS or WAS. The co-fermentation experiments consisted of three mixtures between PS or WAS and oleic acid. Mixtures contained 125 g of PS or WAS, and approximately 3, 6 or 12 g of oleic acid. The tested amounts of oleic acid correspond to those that increased the VS concentration by 50%, 100% and 200%. No inoculum was added to the fermentation and co-fermentation tests. All tests were carried out in duplicate.

The second set of experiments was performed using two reactor types (i.e., 250 mL glass beakers vs. 300 mL glass bottles (Wheaton SKU-219437)) under the same experimental conditions to evaluate the effect of moisture loss on the VFA yields during the fermentation step. Mono-fermentation (125 g of PS or 125g of WAS) and co-fermentation mixture (PS-100% oleic or WAS-100% oleic) were carried out in duplicate without inoculum addition.

The third set of experiments was performed to study the reproducibility of PS mono-fermentation and co-fermentation (PS-100% oleic) results since previous PS fermentation results were variable. All the tests were carried out in duplicate.

In all test, 2 mL of fermented sludge liquor were withdrawn from the bottom of the reactor using a plastic dropper at 24, 48, 72 and 96 h. After withdrawal, the sample was immediately centrifuged at 10000 x *g* for 5 min. The liquid fraction was filtered through a 0.45 μm PES Millipore® filter prior chemical analyses. VFA and alcohols concentrations have been converted to COD equivalents using the theoretical oxygen demand equation (Koch et al., 2010). Additionally, all the reported VFA concentrations were normalised to account for moisture losses during fermentation using equation 1 (Eq. 1). Moisture losses were determined by weighting the bottle before and after each sampling event.

$$\mathrm{C_{VFA,n}} = \mathrm{C_{VFA,m}} \cdot \frac{\mathrm{m_t}}{\mathrm{m_0}} \qquad \text{Eq.1}$$

where $C_{VFA,n}$ is the normalised VFA concentration (mg COD L^{-1}), $C_{VFA,m}$ is the measured VFA concentration (mg COD L^{-1}), m_0 is the initial mass of the sample (g), and m_t is the mass of the sample at the measuring time (g).

The conversion of oleic acid to VFA (% conversion) was estimated by subtracting the final VFA production in the mono-fermentation tests ($VFA_{f,mF}$) to the final VFA production in the co-fermentation tests ($VFA_{f,CoF}$), normalised by the initial oleic acid concentration ($COD_{oleic\ acid}$) as per equation 2 (Eq. 2):

$$\%\ \mathrm{conversion} = \frac{\mathrm{VFA_{f,CoF}} - \mathrm{VFA_{f,mF}}}{\mathrm{COD_{oleic\ acid}}} \cdot 100 \qquad \text{Eq. 2}$$

2.2.2 Biochemical methane potential (BMP) test

The effect of mono-fermentation and co-fermentation on subsequent AD was evaluated using BMP tests. Mono-fermentation and co-fermentation samples were compared to freshly prepared mixtures (un-fermented) with PS and WAS belonging to the same batch (batch 1). Un-fermented PS and WAS were stored at 4 ºC until BMP preparation (max 5 days). The un-fermented mixtures had the same proportion of PS, WAS and oleic acid than the fermented ones. BMP tests were carried out following the procedure defined by Holliger et al. (2016). Specifically, BMP tests were performed in triplicate in 160 mL serum bottles sealed with rubber septa and aluminium caps. All tests contained 70 g of inoculum and different quantities of substrates required to achieve an initial inoculum to substrate ratio of 2 (VS-basis). Blank assays containing inoculum only were used to correct for the inoculum background methane potential. The headspace of each bottle was flushed with 99.9% N_2 for one minute (4 L min^{-1}). Finally, the bottles were placed in an incubator set at 37 ± 1 ºC. Serum bottles were manually mixed by swirling before each sampling event. At each sampling event, headspace pressure was measured using a manometer (Sper Scientific 840064) and composition (i.e., H_2, N_2, CH_4 and CO_2) with a gas chromatograph (Shimadzu GC-2014). Methane yields (mLCH_4 gVS^{-1}) are calculated using the VS of the substrate measured prior BMP testing (i.e. VS from un-fermented mixtures or VS after fermentation tests). Methane yields are reported at normal conditions (i.e., 0 ºC, 1 bar and dry). Error bars indicate 95% confidence in the average of the triplicate. BMP calculations were cross-validated with the OBA web application (Hafner et al., 2018).

BMP tests were carried out for all the mono-fermentation and co-fermentation conditions of the first experimental set after 96 hours of fermentation. Besides, un-fermented PS, WAS, or freshly prepared sludge - oleic mixtures (VS increase of 50, 100 and 200%) were tested for comparison purposes. A BMP test with oleic acid was used as a positive control. BMP tests were run until the methane production during three consecutive days was less than 1% of the accumulated methane produced (Holliger et al., 2016).

2.3 Analytical methods

Total solids (TS) and volatile solids (VS) were measured according to Standard Method 2540G (Baird et al., 2017), volatile fatty acids (VFA) and alcohols losses were taken into account to correct the final TS and VS value (Peces et al., 2014). Total chemical oxygen demand (tCOD) and soluble chemical oxygen demand (sCOD) were measured using a Merck COD Spectroquant® test kit (range 0.5 - 10 g L^{-1}) and a Move 100 colorimeter (Merck, Germany). Individual VFA (acetate, propionate, butyrate, valerate and caproate) and alcohols (methanol, ethanol, butanol and hexanol) were analysed with an Agilent 7890A gas chromatograph equipped with an Agilent DB-FFAP column. Total Kjeldahl nitrogen (TKN) and total phosphorus (TP) were determined using sulfuric acid, potassium sulfate and copper sulfate catalyst in a block digester (Lachat BD-46, Hach, USA) followed by flow injection analysis (FIA). Ammonia and soluble phosphorus were determined by FIA using a Lachat QuickChem800 flow injection analyser. Biogas composition (i.e., H_2, N_2, CH_4 and CO_2) was determined using a Shimadzu GC-2014 gas chromatograph equipped with a HayeSep Q column and argon as a carrier gas.

2.4 BMP modelling and data analysis

BMPs tests were modelled to assess the impact of fermentation on the subsequent anaerobic digestion step. As hydrolysis is considered the rate-limiting step of PS and WAS degradation, BMPs were modelled using first-order kinetics as for equation 3 (Eq. 3) (Batstone et al., 2002). For tests containing oleic acid, an inhibition function was included to model LCFA inhibition on the anaerobic digestion process, Eq. 4 (Arnell et al., 2016).

$$\mathrm{r = 350 \cdot f_i \cdot k_{hyd,i} \cdot X_i \cdot C_i \cdot V \cdot I_{li}} \qquad \text{Eq. 3}$$

$$\left.\begin{array}{r} I_{li} = \exp\left(-2.77259 \cdot \left(\frac{S_{li} \cdot K_{I,min}}{K_{I,max} - K_{I,min}}\right)^2\right)_{S_{li}>K_{I,min}} \\ I_{li} = 1_{S_{li}<K_{I,min}} \end{array}\right\} \qquad \text{Eq. 4}$$

In Eq 3, r is the methane production rate (mL CH_4 d^{-1}), 350 is the conversion ratio based on the maximum theoretical methane yield at standard conditions (mLCH_4 $gCOD^{-1}$), f_i is the substrate biodegradability (-), $k_{hyd,i}$ is the first order hydrolysis rate constant of the substrate (d^{-1}), X_i is the sludge concentration (g VS L^{-1}), C_i is the measured COD-to-VS ratio of the sludge, V is the liquid volume (L), and I_{li} is the LCFA inhibition factor, which varies from 0 (total inhibition) to 1 (no inhibition). In Eq. 4, S_{li} is the lipid concentration (g VS L^{-1}), $K_{I,min}$ is the lower level inhibition constant (g VS L^{-1}) and $K_{I,max}$ is the upper-level inhibition constant (g VS L^{-1}).

The model was implemented in Aquasim 2.1d. Parameter estimation and uncertainty analysis were simultaneously estimated, with a 95% confidence limit, as described in Batstone et al. (2009). Parameter uncertainty was estimated based on a two-tailed t-test on parameter standard error around the optimum, and non-linear confidence regions were tested to confirm the linear estimate was representative of true confidence. The objective function used was the sum of squared errors ($\chi2$), where average data from triplicate experiments were used. Principal component analysis (PCA) was used to explore associations between all the fermentation results and experimental conditions in reduced ordination space. The variables (individual VFA concentration and yields) were z-score standardised to compare variables with different magnitudes. Permutational multivariate analysis of variance (PERMANOVA) was applied to determine the significance of the fermentation conditions on either the overall fermentation response or the individual VFA responses. The null hypothesis corresponds to equivalent dispersion and centroid between groups. Similarity was based on Euclidean distance. Degrees of freedom (df) depended on the groupings and are reported with the PERMANOVA results. Confidence level was set at 95%, and 999 permutations. Linear regression was used to determine the relationship between the individual VFA profile and the addition of oleic acid (n = 4). All statistical analyses were carried out in the software R, version 3.6.1.

3. RESULTS AND DISCUSSION

3.1 Mono-fermentation and co-fermentation experiments

3.1.1 VFA production

The mono-fermentation at 20 ºC under semi-aerobic conditions reached higher total VFA yields for PS (32 – 89 mgCOD gVS^{-1}) than for WAS (20 – 41 mgCOD gVS^{-1}). pH changes during the fermentation tests were minimal drifting from 5.2 ± 0.2 to 4.9 ± 0.2 for PS and from 6.7 ± 0.1 to 6.4 ± 0.1 for WAS. The obtained PS and WAS yields fall within mid-low range of the reported values for sludge fermentations carried out at 20 °C under anaerobic conditions. Reported PS yields range between 32 – 390 mgCOD gVS^{-1} (Ahn and Speece, 2006; Bouzas et al., 2002; Cokgor et al., 2009; Ferreiro and Soto, 2003; Peces et al., 2016; Ucisik and Henze, 2008) while WAS yields range between 11 – 104 mgCOD gVS^{-1} (Ucisik and Henze, 2008; Yuan et al., 2011).

The co-fermentation of oleic acid with PS or WAS at 20 ºC under semi-aerobic conditions showed different product yield and trends depending on the sludge type (Figure 1, Figure 2). The influence of oleic acid in the co-fermentation can be easily visualised in the PCA plot (Figure 3) and further confirmed by PERMANOVA analyses with a significant contribution for PS (R^2 =0.4432, P = 0.001, df = 5) and WAS (R^2 = 0.1070, P = 0.044, df = 5). Figure 3 shows that each base substrate had its own separate cluster, indicating that the properties of the base substrate have a clear impact on fermentation performance. PS fermentation tests reached higher VFA concentration due to its inherent higher biodegradability, while WAS showed a higher capability to ferment oleic acid. The latter is shown by the growing distance between the initial sample and subsequent time points along the first principal component (PC1 in Figure 3).

For PS, the VFA concentration after 96 h did not follow a trend with the addition of oleic acid (Figure 1). Specifically, PS without oleic acid addition reached a total VFA concentration of 3,044 ± 72 mgCOD L^{-1}, while the PS-50%, PS-100% and PS-200% mixtures reached a VFA concentration of 2,680 ± 98 mgCOD L^{-1} (-12%), 4,516 ± 90 mgCOD L^{-1} (+48%) and 4,155 ± 92 mgCOD L^{-1} (+36%), respectively. On the one hand, the lower VFA production of the PS-50% test compared to the mono-fermentation can be explained by the consumption of acetate (see Section 3.1.3 for discussion). On the other hand, the lower VFA production of the PS-200% test, compared to the PS-100%, could be related to inhibition phenomena as a result of the higher concentration of oleic acid. After 96 hours of co-fermentation it was estimated that 3.0% and 1.2% of the initial oleic acid COD was fermented to VFA at PS-100% and PS-200%, respectively. The oleic acid conversion in the PS-50% could not be estimated due to the lower VFA production compared to the PS mono-fermentation. Regardless of the oleic acid concentration in the mixture, PS co-fermentation tests showed a 48-hour lag phase, i.e., the period when the VFA concentration and distribution in the co-fermentation tests did not significantly differ from the PS mono-fermentation test (R^2 =0.2219, P = 0.7210, df = 3). After a 48 h lag-phase, the concentration of acetate, propionate and butyrate was higher in the co-fermentation tests (PS-100% and PS-200%) than in the mono-fermentation test (Figure 1A). A lag phase was also observed in the replication experiments (batch 2 and 3), where the difference in VFA concentration in the co-fermentation tests only increased after 48 hours (Figure 1A, Figure 2A, Figure 2B). These consistent results suggest that the biomass present in the PS needed an adaptation time to grow or activate the enzymes and metabolic pathways required for oleic acid β-oxidation.

For WAS, the total VFA concentration after 96 hours of fermentation increased from 740 ± 113 mgCOD L^{-1} (WAS-0%) to 1,600 ± 133 mgCOD L^{-1} (WAS-50%), 1,937 ± 47 mgCOD L^{-1} (WAS-100%) and 2,871 ± 133 mgCOD L^{-1} (WAS-200%) (Figure 1B). The estimated conversion of oleic acid into VFA was 4.7%, 3.3% and 3.1% for the WAS-50%, WAS-100% and WAS-200% co-fermentation tests, respectively. These results indicate the absence of noticeable inhibition effects over the WAS biomass under the tested oleic acid concentrations. In contrast to PS co-fermentation tests, no lag phase was observed in the WAS co-fermentation test regardless of the oleic acid concentration. Indeed, the total VFA concentration after 24 hours fermentation was already higher in the co-fermentation tests than in the WAS mono-fermentation test, indicating a high affinity of WAS biomass towards oleic acid (Figure 1B).

Overall, these experimental results show that WAS is a preferable base substrate to produce VFA from oleic acid. This improvement can be related to several factors, such as sludge composition, biomass concentration, microbial sludge composition, and improved nutrient balance (e.g., C/N ratio or micronutrients). It should be noted that these experiments relied on the activity of non-acclimatised biomass, and therefore, semi-continuous or continuous experiments may reach higher VFA production due to biomass acclimation. However, the present results indicate that the inherent characteristics of the base substrate play a role in co-fermentation performance.

3.1.2 VFA profile

The VFA profile was affected by both the base substrate and the amount of oleic acid while alcohols were not detected during the mono- or co-fermentation tests (Figure 1, Figure 2). A comprehensive table with all the experimental VFA concentrations for each fermentation test can be found in the electronic supplementary material. For the three PS mono-fermentation batches, propionate was the main VFA after 96 hours of fermentation contributing between 37% (first batch) and 68% (second batch) to the total VFA concentration (COD-basis). The enrichment of propionate in PS mono-fermentation was also reported in Peces et al. (2016) using the same fermentation conditions although the enrichment was lower (27% fresh PS vs. 32% fermented PS after 72 hours of fermentation). Propionate was also enriched in the two WAS mono-fermentation batches increasing its contribution from 11% to 30% in the first batch and from 0% to 34% in the second batch. The contribution of acetate to the VFA distribution decreased in both PS and WAS mono-fermentation tests. Butyrate and valerate contribution were variable depending on the sludge type and the collected batch. Butyrate and valerate were enriched in the first and second PS batch, increasing by an average of 7% and 4% after 96 h fermentation, respectively, but no increment or decrease was observed for the third PS batch. Butyrate contribution consistently decreased in WAS mono-fermentation assays from 22% to 15% at the end of the test. Valerate contribution was variable in WAS mono-fermentation, increasing in the first WAS batch (from 9% to 21%), but remaining constant in the second WAS batch. The absence of clear trends for butyrate and valerate indicates that the applied fermentation conditions are not stringent to provide a reproducible VFA profile. However, the consistent enrichment of propionate in all tests suggests that these fermentation conditions (i.e., 20 ºC and semi-aerobic) favour propionate accumulation. The proportion of propionate in the fermentation liquor depends on the sludge type and sludge composition where the proportion of propionate in PS was more variable than in WAS mono-fermentation batches despite the minor differences in pH between sludge batches. This high variability was related to the PS inherent compositional heterogeneity.

The addition of oleic acid as co-substrate led to a different response on the VFA profile depending on the base substrate. In the PS co-fermentation tests there was not a clear shift in the VFA profile when compared to PS mono-fermentation results (Figure 1, Figure 2). The only noticeable phenomenon was a slightly higher proportion of acetate in the PS-100% and PS-200% tests once the lag phase was over (38% and 33%, respectively versus 31% in PS mono-fermentation). Other intermediates from the β-oxidation pathway such as butyrate were not further accumulated, suggesting that they were rapidly converted to acetate. Contrariwise, WAS co-fermentation tests showed a distinct profile compared to the mono-fermentation tests reaching a higher concentration of butyrate, and to a lesser extent caproate, in the fermentation liquor (Figure 1B and Figure 2B). The final concentration of butyrate ($R^2 = 0.9662$, $P = 0.017$) and caproate ($R^2 = 0.9552$, $P = 0.022$) was correlated with the concentration of oleic acid added to the co-fermentation test indicating an expressed β-oxidation pathway with a lower conversion rate to acetate. Interestingly, valerate was also enriched during WAS co-fermentation compared to WAS mono-fermentation (Figure 1B, Figure 2B). Odd-number VFA is predominantly produced from protein degradation (Ramsay and Pullammanappallil, 2001), however, in the co-fermentation tests, the WAS amount (the proteinaceous substrate) was constant while the valerate concentration produced positively correlated with the amount of oleic acid in the test ($R^2 = 0.9474$, $P = 0.026$). These results were attributed to the occurrence of chain elongation during WAS and oleic co-fermentation, as discussed in the following paragraph.

Oleic acid is an even-numbered LCFA that produces even number VFA through the β-oxidation pathway (i.e., loss of 2 carbon) (Kim et al., 2004). Moreover, unsaturated even-number LCFAs such as oleic acid, is reported to be unable to undergo α-oxidation (i.e., loss of 1 carbon) (Su et al., 2004) and, to date, there is no evidence of bacteria or archaea capable of expressing an α-oxidation pathway (Jimenez-Diaz et al., 2017). Odd-number LCFA is also fermented through the β-oxidation pathway, with the distinction that the terminal VFA are acetate and propionate. The addition of oleic acid could have stimulated the β-oxidation metabolism non-selectively degrading any LCFA present. However, odd-number LCFAs are rarely occurring in nature, and if found in WAS, they are a structural component in some of the bacterial cell membranes (O'Leary, 1962). Therefore, it is hypothesised that valerate being produced from odd-number LCFA is very unlikely. The most plausible explanation is that valerate was produced from acetate and propionate through a reverse β-oxidation pathway, also known as chain-elongation. In chain elongation studies, ethanol or lactate are typically supplemented to the fermentation broth to favour the reverse β-oxidation pathway (Chwialkowska et al., 2019), although other alcohols, sugars, and amino acids have also been successfully used (Angenent et al., 2016). Additionally, Carvajal-Arroyo et al. (2019) have recently demonstrated the production of caproate by chain elongation without the addition of an external electron donor, solely relying on the fermentation of solid-free stillage. Importantly, the present results show that co-fermentation can be engineered to produce medium-chain fatty acids through chain elongation.

3.1.3. Acetate consumption during PS fermentation

In some PS mono-fermentation and co-fermentation tests, the net acetate concentration decreased during fermentation tests (Figure 1A and 2A). These results show that the rate of acetate consumption was higher than the rate of acetate production. In WAS mono-fermentation and co-fermentation tests the net acetate concentration was always positive, except for the slight decrease in acetate (from 289 ± 6 mgCOD L^{-1} to 235 ± 28 mgCOD L^{-1}) observed in the first WAS mono-fermentation test (Figure 1B, Figure 2B).

A decrease in acetate concentration has been typically reported in fermentation experiments carried out under anaerobic conditions, neutral pH and long fermentation time (i.e., higher than 5 days). This trend has been associated with the growth of methanogenic archaea (Miron et al., 2000; Xiong et al., 2012). However, a priori, 20 °C, pH around 5 and semi-aerobic fermentation conditions are not favourable for methanogenic archaea. Consequently, the consumption of acetate is related to the presence of other microorganisms that use acetate for their growth and metabolism such as denitrifying bacteria, polyphosphate accumulating organisms, sulphate-reducing bacteria, or ordinary heterotrophs. However, since the decrease in acetate concentration occurred randomly in some of the tests, the present results do not allow to elucidate if the consumption of acetate relates to the chemical sludge composition or the presence of acetate consuming microorganisms inherent in the sludge microbial community. Due to the negative impact of this phenomena on fermentation performance, there is a strong need to conduct in-depth research targeting this topic.

3.1.4 Effect of moisture loss due to water diffusion

Mono-fermentation and co-fermentation tests progressively lost weight during the experiment (data for all fermentation tests shown in the electronic supplementary material). The weight loss during the fermentation test was solely attributed to water diffusion since no TS or VS losses were detected. However, minor TS or VS losses due to sludge and oleic acid mineralisation or VFA volatilisation cannot be discarded. The amount of water diffused was sensitive to the reactor type with beakers losing more moisture than serum bottles due to the higher contact area. The water loss in experiments carried out in beakers averaged 35% (average 45 g of weight loss during fermentation), while the water loss in the serum bottles tests was around 8% (average 6 g of weight loss during fermentation).

The mono-fermentation and co-fermentation experiments carried out in beakers (with higher moisture loss) recurrently showed a lower VFA yield, and total VFA concentration, than the ones carried out in serum bottles (Figure 2). The progressive moisture loss during the fermentation tests implied a higher TS concentration which could had influenced the VFA yields. Studies testing the influence of TS on fermentation performance have shown that the VFA yield linearly decreased with the increase in the initial sludge TS concentration (Banister and Pretorius, 1998; Ferreiro and Soto, 2003; Skalsky and Daigger, 1995; Yuan et al., 2009). These authors attributed the higher VFA yields at low TS concentration (i.e., higher water content) to the dilution of inhibitory compounds or the improved biomass-substrate contact due to the better mixing. Interestingly, Ferreiro and Soto (2003) also reported that the TS concentration affected the VFA profile when diluting PS from 10 to 1 gTS L^{-1}. However, in this study, a change in the VFA profile was not observed with changes on TS concentration for both mono-fermentation and co-fermentation tests.

3.2 Anaerobic digestion

BMP tests were used to determine the impact of oleic acid co-fermentation on the downstream anaerobic digestion process. It is well known that lipids are both beneficial and problematic since they have a high methanogenic potential (~1000 mLCH4 gVS^{-1}), but they can also lead to process inhibition due to LCFA accumulation (Alves et al., 2009; Long, et al., 2012). Importantly, the impact of co-fermentation performance over the methanogenic potential and inhibition is still not well studied. The two main LCFA inhibition mechanisms have been proposed for a long time as reviewed in O’Leary (1962), yet it remains unclear the precise nature of the inhibition due to LCFA (Churchward et al., 2018). One mechanism is associated with a mass transfer limitation, i.e., LCFA adsorbs onto the microbial cells membranes, changing the cell membrane permeability that prevents the uptake of essential nutrients. The second mechanism is related to the biochemical properties of the LCFA that inhibit internal microbial metabolic reactions. Therefore, two hypotheses were considered prior experimental runs: (1) fermenting the LCFA prior AD will breakdown the LCFA chains to VFA, hence reducing the inhibitory potential, (2) during co-fermentation, LCFA could adsorb in the sludge particles reducing the further attachment onto the active AD biomass.

Experimental BMP results showed, as expected, a methane yield increase under co-digestion conditions proportional to the amount of lipids in the mixture. The methane yields and curve profile obtained from the co-fermentation effluents were very similar to the ones obtained from the freshly prepared mixtures (Figure 4, Table 3). These results indicate that there were no COD losses during the co-fermentation. These observations are further supported by the modelling outputs since the co-fermentation effluents and the freshly prepared mixtures showed invariant parameter values, i.e., degradation extent (fi), kinetic values ($k_{h,i}$) and inhibition constants ($K_{i,min}$ and $K_{i,max}$). Similar $K_{i,min}$ and $K_{i,max}$ values across all experimental conditions indicated that the co-fermentation of sludge (PS and WAS) with oleic acid did not decrease the inhibitory effects of LCFA in AD (Figure 4, Table 3). From the low extent of fermentation (max. 4.7% from the initial COD concentration) and the invariant inhibition constants derived from the BMP tests, it is deduced that most of the oleic acid might have been still in its current form or other LCFA. Therefore, the hypothesis that the inhibitory potential of lipids could be improved as fermentation yields increase cannot be discarded. Finally, it is worth to state that no synergistic or antagonistic interactions have been observed between the different substrates since all the BMP curves could be reliably modelled using a single set of parameters for each compound (PS, WAS and oleic acid).

CONCLUSIONS

Mono-fermentation batch tests at 20 ºC under semi-aerobic conditions reached higher VFA yields for PS (32-89 gCOD gVS^{-1}) than for WAS (20-41 gCOD gVS^{-1}) and favoured propionate production. Co-fermentation of oleic acid with PS or WAS showed that the base substrate has a notable influence on the co-fermentation performance, i.e., VFA production and profile. Co-fermentation with WAS resulted in a greater extent of oleic acid degradation (up to 4.7%) and evidence of chain elongation. Finally, BMP tests showed that neither mono-fermentation nor co-fermentation had an impact on downstream anaerobic digestion.

E-SUPPLEMENTARY MATERIAL

E-supplementary data of this work can be found in the e-version of this paper online

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Highlights

- 20°C semi-aerobic fermentation conditions promoted propionate production for sludge
- Co-fermentation of sewage sludge and lipids improved fermentation performance
- Waste activated sludge was a preferable base substrate for lipid co-fermentation
- Chain elongation occurred in waste activated sludge and lipid co-fermentation tests
- Mono- and co-fermentation had no impact on downstream anaerobic digestion

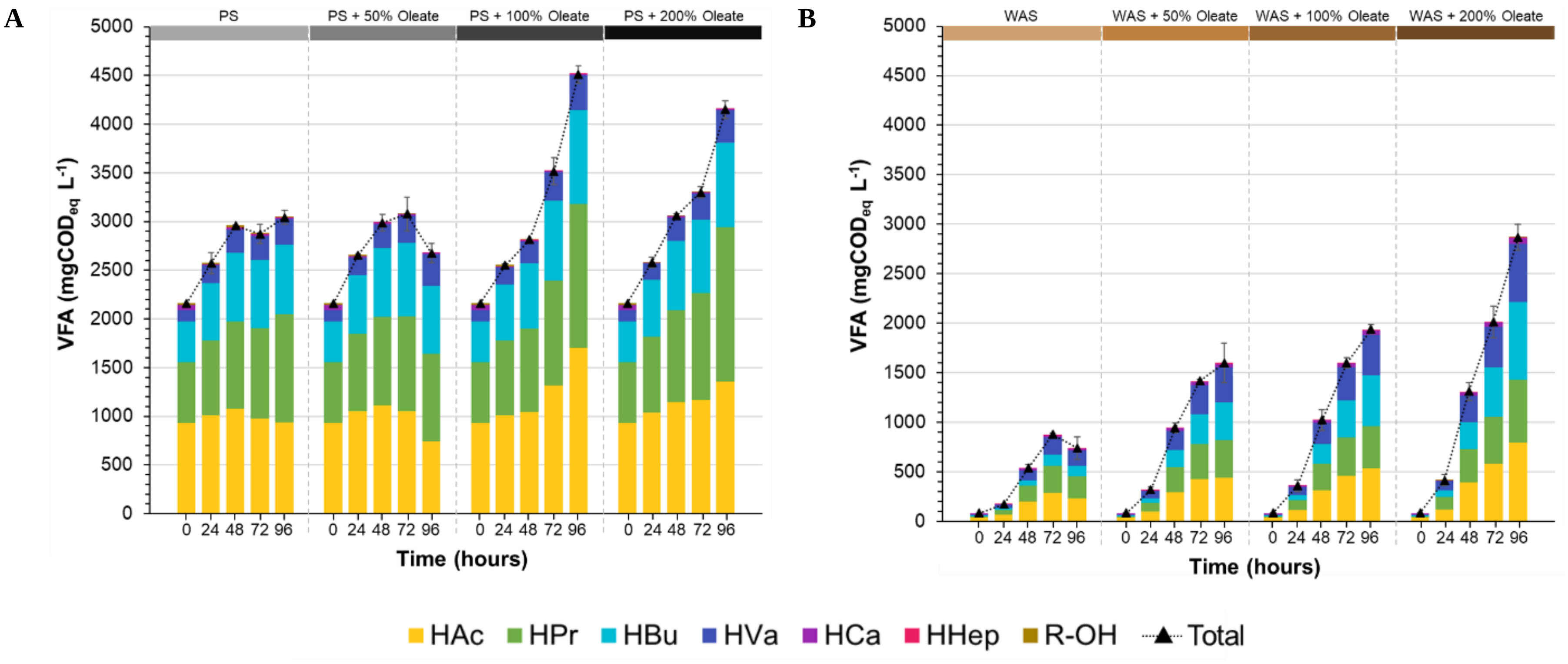

Figure 1. VFA distribution during 20 °C semi-aerobic fermentation experimental time and amount of oleate in the mixture for batch 1 in beakers. (A) Primary sludge. (B) Waste activated sludge. Each colour represents one VFA. Black markers represent the sum of total VFA. All VFA concentrations have been corrected to account for moisture losses during the fermentation tests.

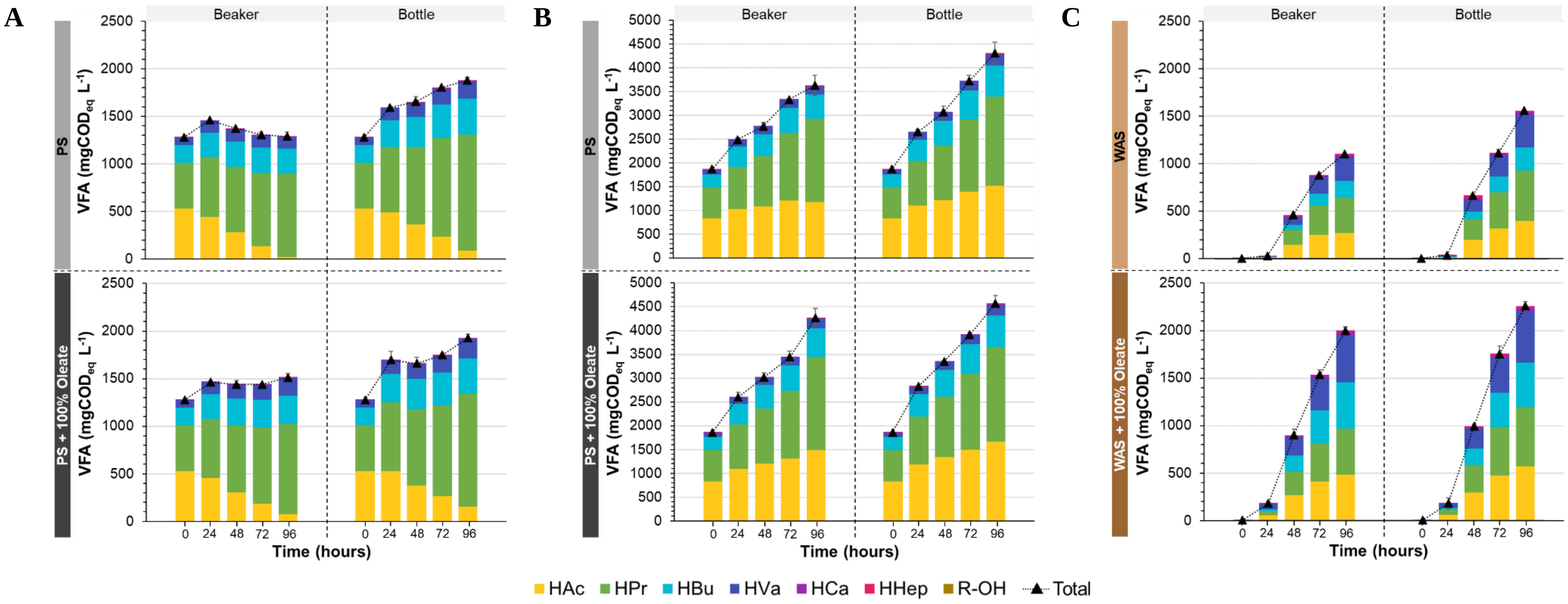

Figure 2. VFA distribution during 20°C semi-aerobic fermentation experimental time and amount of oleate in the mixture and fermentation vessel. (A) Primary sludge batch 2. (B) Primary sludge batch 3 (B) Waste activated sludge batch 2. Each colour represents one VFA. Black markers represent the sum of total VFA. All VFA concentrations have been corrected to account for moisture losses during the fermentation tests.

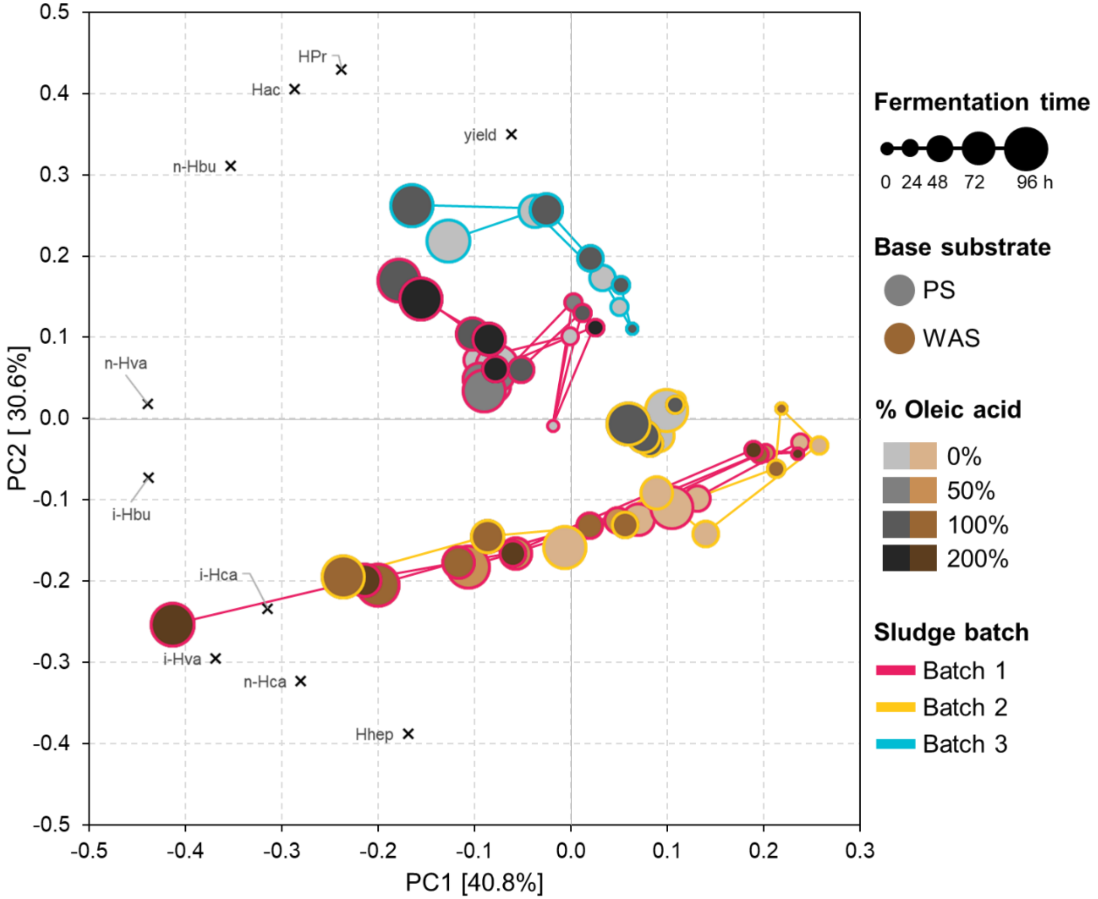

Figure 3. PCA plot summarises the results for all the fermentation conditions tested (in beakers). Each sample is represented by a single circle coloured according to the sludge type. Colour intensity reflects the amount of oleic acid in the test and circle size the fermentation time. Circle contour colour represents each sludge batch tested. Variables (VFA and yields) are represented by black crosses.

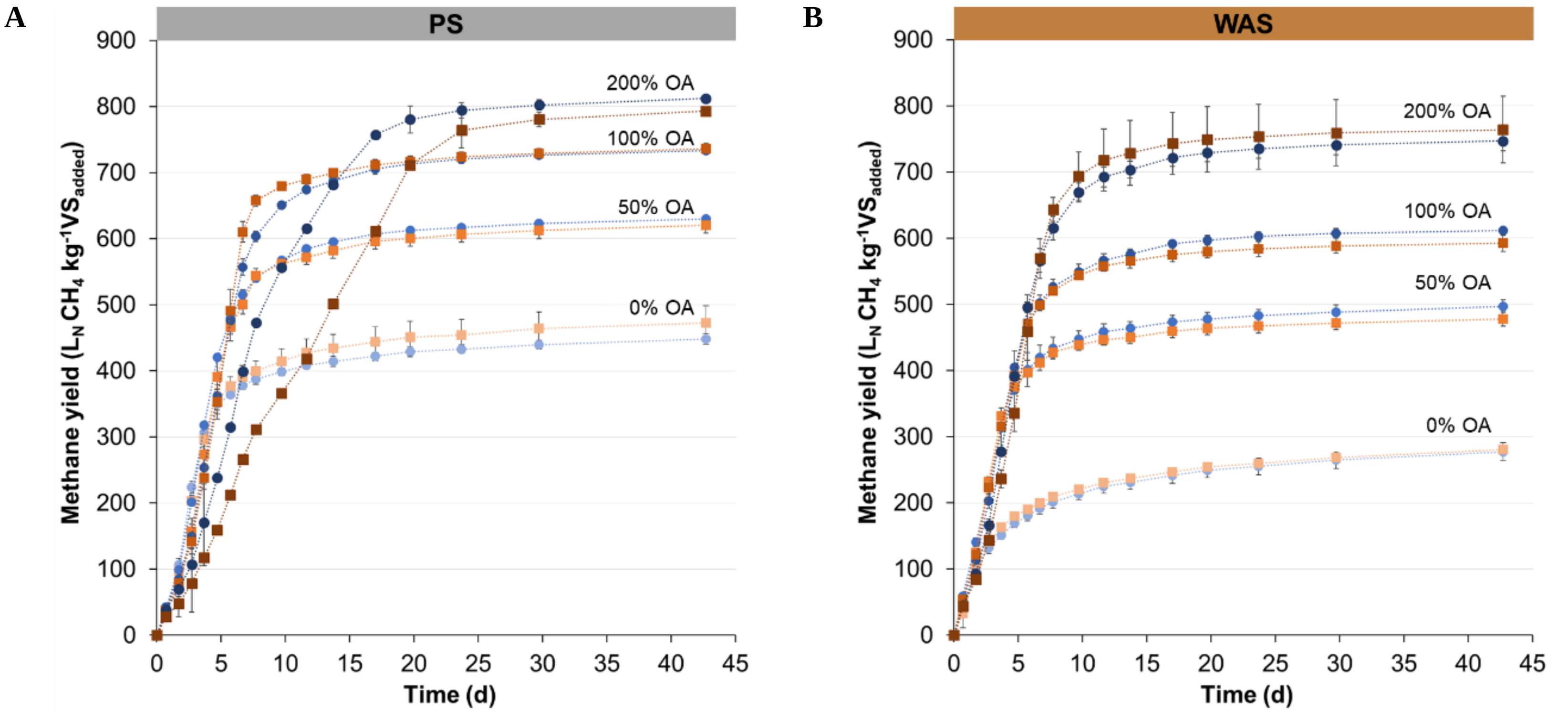

Figure 4. Experimental BMP curves for the mono-fermented, co-fermented and fresh mixtures. (A) Primary sludge. (B) Waste activated sludge. Blue circles represent freshly prepared mixtures, and orange squares represent fermented mixtures. Colour intensity increases with the addition of oleic acid in the mixture. Error bars for PS + 200% Oleic acid (highly inhibited tests) have been removed to facilitate visualisation from day 4 to day 17, the complete figure can be found in the online material.

Table 1. Characterisation of the different PS and WAS batches (average ± SD)

	PS (batch 1)	PS (batch 2)	PS (batch 3)	WAS (batch 1)	WAS (batch 2)
TS (g L^{-1})	59.1 ± 0.7	49.1 ± 0.4	55.2 ± 0.1	44.4 ± 1.0	46.0 ± 1.1
VS (g L^{-1})	51.0 ± 0.7	40.9 ± 0.4	48.4 ± 0.2	36.3 ± 1.0	38.4 ± 1.1
tCOD (g L^{-1})	81.1 ± 2.2	68.3 ± 4.8	80.2 ± 2.3	52.0 ± 2.3	58.2 ± 1.9
sCOD (mg L^{-1})	2,580 ± 17	1,720 ± 23	2,270 ± 13	270 ± 28	280 ± 12
tVFA (mgCOD L^{-1})	2,145 ± 78	1,282 ± 16	1,869 ± 41	82 ± 4	4.1 ± 0.1
HAc (mgCOD L^{-1})	926 ± 48	530 ± 8	833 ± 19	38 ± 8	2.2 ± 0.1
HPr (mgCOD L^{-1})	629 ± 22	483 ± 4	655 ± 22	9.1 ± 3.2	n.d
HBu (mgCOD L^{-1})	418 ± 15	184 ± 7	276 ± 12	16 ± 4	1.0 ± 0.1
HVa (mgCOD L^{-1})	118 ± 11	79 ± 3	88 ± 4	7.1 ± 1.4	0.9 ± 0.1
HCa (mgCOD L^{-1})	50 ± 4	4.9 ± 0.8	11 ± 1	12 ± 2	n.d
HHep (mgCOD L^{-1})	3.6 ± 0.2	1.4 ± 0.3	6.0 ± 0.7	n.d	n.d
Ethanol (mgCOD L^{-1})	14 ± 2	n.d	n.d	n.d	n.d
TKN (mgN L^{-1})	1,568 ± 66	-	-	3,101 ± 79	-
TP (mgP L^{-1})	317 ± 20	-	-	973 ± 42	-

HAc : acetate, HPr: propionate, HBu: butyrate, HVa: valerate, HCa: caproate, HHep: heptanoate, n.d: below detection limit (<0.5 mgCOD L^{-1})

Table 2. Experimental set-up of the co-fermentation assays

	Sample code	**Sludge (g)**	**Oleic acid (g)**	**VS sludge (g)**	**VS oleic acid (g)**
Batch 1	PS[a]-0%	125	0	6.4	0
	PS[a]-50%	125	3.2	6.4	3.2
	PS[a]-100%	125	6.4	6.4	6.4
	PS[a]-200%	125	12.8	6.4	12.8
	WAS[a]-0%	125	0	4.5	0
	WAS[a]-50%	125	2.3	4.5	2.3
	WAS[a]-100%	125	4.7	4.5	4.7
	WAS[a]-200%	125	9.3	4.5	9.3
Batch 2	PS[a]-0%	125	0	5.1	0
	PS[b]-0%	125	0	5.1	0
	PS[a]-100%	125	5.1	5.1	5.1
	PS[b]-100%	125	5.1	5.1	5.1
	WAS[a]-0%	125	0	4.8	0
	WAS[b]-0%	125	0	4.8	0
	WAS[a]-100%	125	5.2	4.8	5.2
	WAS[b]-100%	125	5.2	4.8	5.2
Batch 3	PS[a]-0%	125	0	6.0	0
	PS[b]-0%	125	0	6.0	0
	PS[a]-100%	125	6.0	6.0	6.1
	PS[b]-100%	125	6.0	6.0	6.1

a; tests carried out in 250 mL glass beaker

b: tests carried out in 300 mL glass serum bottles

Table 3. Model parameter outputs and 95% confidence interval (in brackets) of all experiments carried out in this study.

	PS Fresh	PS Fermented	WAS Fresh	WAS Fermented
$K_{I,max}$	20.78 (18.96 - 23.00)	16.45 (13.83 - 19.75)	22.25 (19.59 - 25.28)	20.63 (17.38 - 26.19)
$K_{I,min}$	1.09 (0.00 - 3.99)	1.87 (0.09 - 4.95)	1.41 (0.00 - 6.29)	1.83 (0.02 - 8.95)
$K_{I,50}$	10.93	9.16	11.83	11.23
$k_{hyd,li}$	0.25 (0.18 – 0.32)	0.29 (0.15 – 0.44)	0.32 (0.23 – 0.39)	0.35 (0.21 – 0.49)
$k_{hyd,s}$	0.23 (0.17 – 0.28)	0.21 (0.16 – 0.28)	0.17 (0.11 – 0.23)	0.18 (0.16 – 0.21)
f_{li}	0.94 (0.99 – 0.91)	0.84 (0.91 – 0.76)	0.92 (0.95 – 0.88)	0.88 (0.93 – 0.85)
f_s	0.74 (0.78 – 0.69)	0.79 (0.88 – 0.70)	0.45 (0.49 – 0.39)	0.43 (0.44 – 0.41)