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**Interpreting the synergistic effect in combined ultrasonication-ozonation sewage
sludge pre-treatment**

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Abstract

The sequential combination of ultrasonication and ozonation as sewage sludge treatment prior to anaerobic digestion was investigated. Synergistic volatile suspended solids (VSS) solubilization was observed when low energy ultrasonication ($\leq 12 \text{ kJ g}^{-1} \text{ TS}$) was followed by ozonation. $0.048 \text{ g O}_3 \text{ g}^{-1} \text{ TS}$ ozonation induced the maximum VSS solubilization of 41.3% when the sludge was pre-ultrasonicated at $9 \text{ kJ g}^{-1} \text{ TS}$; while, the same ozone dosage applied without prior ultrasonication only induced 21.1% VSS solubilization. High molecular weight (MW) components ($\text{MW} > 500 \text{ kDa}$) were found to be the main solubilization products when sludge was only ozonated. However, solubilization products by ozone were mainly in the form of low MW components ($\text{MW} < 27 \text{ kDa}$) when sludge was pre-ultrasonicated. The high MW products generated by ultrasound were effectively degraded in the subsequent ozonation. Anaerobic biodegradability increased by 34.7% when ultrasonication ($9 \text{ kJ g}^{-1} \text{ TS}$) and ozonation ($0.036 \text{ g O}_3 \text{ g}^{-1} \text{ TS}$) were combined sequentially. The maximum methane production rate increased from 3.53 to 4.32, 4.21 and $4.54 \text{ mL CH}_4 \text{ d}^{-1}$ after ultrasonication, ozonation and ultrasonication-ozonation pre-treatments, respectively.

Keywords: Sewage sludge; ultrasound; ozone; pre-treatment; molecular weight; anaerobic digestion

1. Introduction

Sludge produced in wastewater treatment plants needs to be stabilized before it can be safely disposed of. This is because of its high organic as well as pathogen content (Bitton, 2005). Anaerobic digestion is generally accepted as an appropriate way to stabilize

41 sludge, reduce the final amount of solids requiring disposal as well as produce methane
42 for energy recovery (Zhang et al., 2007). However, anaerobic digestion is a slow process
43 and its performance is typically limited by hydrolysis of the particulate organic matters in
44 the sludge (Pavlostathis and Giraldo-Gomez, 1991). Therefore, sludge can be pre-treated
45 to accelerate the hydrolysis step and to enhance the overall anaerobic process before it is
46 fed into an anaerobic digester..

47 Sludge pre-treatment technologies can be categorized into mechanical, thermal and
48 chemical treatments. The aim of sludge pre-treatment is to solubilize the particulate
49 organics and make them more accessible for subsequent microbial action (Tiehm et al.,
50 1997). Conventional pre-treatments such as thermal and alkaline treatments have been
51 reported since late 1970s (Stuckey and McCarty, 1978; Stuckey and McCarty, 1984).
52 Compared to these conventional pre-treatments, ultrasound and ozone pre-treatments are
53 relatively newer technologies and have only been widely reported in the last decade.
54 Ultrasonic sludge disintegration had been preferred at lower ultrasonic frequency (Tiehm
55 et al., 2001). The predominant sludge disintegration mechanism in low frequency
56 ultrasonication, a mechanical method, is of the hydro-mechanical shear force caused by
57 collapse of cavitation bubbles (Wang et al., 2005). Ultrasound readily reduces biological
58 floc sizes, rupturing microorganism cells as well as significantly increasing sludge
59 biodegradability in the subsequent anaerobic digestion (Tiehm et al., 1997; Lehne et al.,
60 2001; Zhang et al., 2007). Ozonation has also been reported as an effective sludge pre-
61 treatment technology but with a different disintegration mechanism. Ozone chemically
62 reacts with sludge and destroys microorganism cell components (Chu et al., 2009; Yan et
63 al., 2009). Ozone also attacks the extracellular polymeric substances and breaks down the

complex macromolecules in soluble phase (Yan et al., 2009). In addition, ozone is able to convert refractory organic matters into biodegradable form (Volk et al., 1993; Nishijima et al., 2003). Most importantly sludge biodegradability is reported to be remarkably improved after ozonation (Weemaes et al., 2000; Goel et al., 2003).

Ultrasound and ozone act differently on sludge (Bougrier et al., 2006). Comparison between sludge ultrasonication and ozonation have been conducted by previous researchers (Bougrier et al., 2006; Braguglia et al., 2012). Bougrier et al. (2006) found that ultrasonication and ozonation resulted in different physical and biochemical characteristics in the same sludge. Due to the supplementary effects of these two pre-treatments, combination of ultrasonication and ozonation has been suggested for a larger impact (Xu et al., 2010; Yang et al., 2012; Yang et al., 2013). However, information in the published literatures on such combination is relatively scarce and the synergistic mechanisms between the two pre-treatment are still ambiguous. Eskicioglu et al. (2006) have indicated the molecular weight (MW) of the solubilized substances is an important indicator of the sludge pre-treatment performance. However, the size of the solubilized substances by ultrasound, ozone and their combination has not been reported previously. Given the information discussed above, this work aims to investigate the interaction between the two pre-treatment processes and seek explanations for the synergistic effects. The optimum combination sequence was determined and changes in the sludge characteristics after individual and combined pre-treatments were measured. In addition to the conventional parameters such as SCOD for measurement of sludge solubilization, size exclusion chromatography measurement was conducted to determine the change in MW distribution after each pre-treatment. Batch anaerobic digestion tests were then

conducted to evaluate the influence of the pre-treatment on sludge anaerobic biodegradability and methane production kinetics.

2. Materials and Methods

2.1. Sludge samples

Samples of a mixture of primary sludge (PS) and thickened waste activated sludge (WAS) (ratio around 1:1 based on dry solids) were collected from a local municipal wastewater reclamation plant. Properties of the raw sludge used in this study are listed in Table 1.

2.2. Analytical methods

The total solids (TS), volatile solids (VS), total suspended solids (TSS), volatile suspended solids (VSS), soluble COD (SCOD) and total COD (TCOD) were measured as described in Standard Methods (APHA, 1998). The soluble fraction was obtained by first centrifuging the sludge at 10,000 rpm for 10 min. The supernatant was then filtered through a 0.45 µm membrane filter for SCOD, proteins and carbohydrates analysis. COD solubilization was quantified as $SCOD_+$ which was the SCOD difference before and after pre-treatment as shown in Eq. 1:

$$SCOD_+ = SCOD_{after} - SCOD_{before} \quad (1)$$

Solids solubilization was calculated in Eq. 2:

$$VSS \text{ solubilization} = \frac{VSS_b - VSS_a}{VSS_b} \times 100\% \quad (2)$$

Where VSS_b stands for the VSS concentration before each pre-treatment; VSS_a stands for the VSS concentration after each pre-treatment.

Protein concentration was determined with the Lowry's method (1951) using bovine serum albumin as standard and a UV spectrophotometer (Shimadzu, UV-1800) against the blank at a wavelength of 750 nm. Carbohydrate concentration was determined with the sulfuric-phenol method using D-Glucose as standard (DuBois et al., 1956). Proteins and carbohydrates concentrations were converted to equivalent COD concentration with factors of 1.5 and 1.07, respectively as described by Trzcinski (2009). Sludge pH was measured with a pH meter (Agilent, model 3200P) with an accuracy of 0.001. Buffer solution of pH 4, pH 7 and pH 10 (Fisher, UK) were used to calibrate the pH meter every time before measurement.

2.3. Pre-treatment conditions

Ultrasonication was performed with an ultrasonicator (Misonix, Q700). The ultrasound frequency was 20 kHz and the maximum power input was 700 Watts. The power input was around 130 Watts and the treated sludge volume (TS: 16.2 – 17.2 g L⁻¹) was 200 mL. The ultrasonication process was performed in a 250 mL glass beaker containing the sludge. Ultrasonication energy was quantified in terms of ultrasonic energy received per gram solids and the calculation is as shown in Eq. 3 (Lehne et al., 2001):

$$\text{Specific energy input} = \frac{P \times t}{V_{\text{sludge}} \times TS} \quad (3)$$

Where P is power input of the ultrasonicator (W), t is the time of ultrasonication (s), V_{sludge} is volume of treated sludge (L) and TS is the total solids concentration of treated sludge (g L^{-1}). During ultrasonication the temperature was monitored and maintained at about 30 °C with an ice-water bath.

Ozonation was performed with an ozone generator (Wedeco, GSO 30). Pure oxygen was used as feed gas and converted to ozone with a high voltage converter. The power input of the ozone generator was 180 Watts. The applied ozone dosage was quantified according to the potassium iodide method (Konsowa, 2003). Two hundred mL of sludge sample (TS: 16.2 – 17.2 g L^{-1}) was placed in a 1 L glass bottle during the ozonation process. A stone diffuser was installed to produce fine ozone bubbles and to enhance ozone mass transfer. The applied ozone was quantified in terms of ozone dosage ($\text{g O}_3 \text{ g}^{-1}$ TS). The maximum applied ozone dosage was 0.12 $\text{g O}_3 \text{ g}^{-1}$ TS.

Optimum combination sequence of ultrasonication and ozonation was determined before commencing the main experiment. The Ultrasonication-Ozonation (ULS-Ozone) sequence was performed by applying ozonation after ultrasonication and the Ozonation-Ultrasonication (Ozone-ULS) sequence was conducted by dosing ozone prior to ultrasonication.

2.4. Molecular weight distribution

An HPLC (Agilent Technologies, 1260 LC system) was used for the MW distribution analysis using the PL aquagel-OH 8 μm MIXED-M column. Milli-Q water was used as mobile phase with a flow rate of 1 mL min^{-1} . Calibration was done using polyethylene glycol and polyethylene oxide standards with molecular weight of 500 kDa, 70 kDa, 4

kDa, 600 Da and 106 Da with the refractive index (RI) detector. A linear relationship was derived between the log value of MW (Da) and retention time (Rt: min) with a correlation coefficient of 99.2% as shown in Eq. 4:

$$\text{Log}(MW) = 9.8223 - 0.6748(Rt) \quad (4)$$

Both RI and UV (254 nm) detector were used for the detection of the eluted substances. The sample was first centrifuged at 10,000 rpm for 10 min. The supernatant was then filtered through a 0.2 µm membrane filter for injection.

2.5. Anaerobic digestion

Sludge anaerobic biodegradability was quantified with biochemical methane potential (BMP) assays according to a modified method of Owens et al. (1979). Ten mL of substrate sludge and 30 mL of degassed inoculum (VS: 9.9 g L⁻¹) were added to serum bottles. The food to inoculum ratios were around 0.43 g VS g⁻¹ VS in the serum bottles containing raw sludge and ultrasonicated sludge, while it was around 0.4 g VS g⁻¹ VS for the ozonated sludge and ultrasonicated-ozonated (ULS-Ozonated). In parallel, 30 mL of inoculum sludge were also anaerobically incubated following the same procedure to obtain background gas production due to the anaerobic inoculum. A mixture of 20% CO₂ and 80% N₂ was used to purge each bottle for 3 min to create an anaerobic environment. All bottles were incubated in an orbital shaker at 35 °C. The biogas volumes were regularly measured using a wetted glass syringe. The biogas composition was determined with gas chromatography (Agilent Technologies, 7890A GC system). The methane produced from the substrate was calculated by subtracting the background gas production

from the gas produced from the test bottles. The TCOD concentration of the substrate sludge varied due to the pre-treatment at around 16.8, 16.9, 15.3 and 14.6 g COD L⁻¹ for the raw, ultrasonicated, ozonated and ULS-Ozonated sludge, respectively. Therefore, the anaerobic biodegradability was compared by normalizing the methane production from each gram of COD added as introduced by Owens et al. (1979).

The energy balance was then analyzed by subtracting the energy input during the pre-treatment from the energy recovered from the combined heat and power (CHP) production of the produced methane. In order to keep the consistency, the calculation was based on one litre of sludge that was used. During the CHP production process, it was assumed that 30% of the methane calorific energy was converted to electricity and 50% to heat (Cho et al., 2014).

2.6. Anaerobic kinetics models

A modified Gompertz equation was used to model the methane production during the BMP assay (Zwietering et al., 1990; Yue et al., 2008). The modified Gompertz equation is as shown in Eq. 5:

$$P = P_{\max} \exp\left\{-\exp\left[\frac{R_{\max}}{P_{\max}}e(\lambda - t) + 1\right]\right\} \quad (5)$$

Where P (mL) is the cumulative methane produced from the sludge at time t, P_{max} (mL) is the maximum cumulative methane produced from the sludge, R_{max} (mL CH₄ d⁻¹) is the maximum methane production rate during the BMP assay and λ (d) is the lag phase time of the methane production.

The data points of cumulative methane produced (P) were fitted to the anaerobic digestion time (t) using the Origin software (OriginLab, USA) according to Eq. 5. The $P_{\max}$, $R_{\max}$ and λ for each sludge were then obtained for comparison.

3. Results and Discussion

3.1. Individual pre-treatments

3.1.1. COD and biopolymers solubilization

As shown in Table 1, soluble proteins and carbohydrates only represented a small fraction of the SCOD in the raw sludge, indicating this initial SCOD did not originate from the degradation of microbial cells. This raw SCOD might be attributed to the soluble lipids contained in the PS, inorganic interferences (such as ammonium which contributes to COD) and/or refractory organics which were not degraded during the activated sludge process and remained in the WAS fraction. The $SCOD_+$ increase was different during ultrasonication and ozonation. For ultrasonicated sludge, $SCOD_+$ increased linearly with specific energy input as shown in Fig. 1a (TS: around 17 g L⁻¹). The $SCOD_+$ was 3,450 mg L⁻¹ after 21 kJ g⁻¹ TS ultrasonication and values did not plateau. However, for ozonated sludge, $SCOD_+$ increased markedly to around 2,400 mg L⁻¹ at an ozone dosage of 0.02 g O₃ g⁻¹ TS and then plateaued at around 3,700 mg L⁻¹ at higher ozone dosages as shown in Fig. 1b. Proteins and carbohydrates were responsible for around 80% of the COD solubilization measured for both pre-treatments. The equivalent COD of the solubilized proteins and carbohydrates after 21 kJ g⁻¹ TS ultrasonication were 2,370 mg L⁻¹ and 450 mg L⁻¹, respectively. The equivalent COD of

the solubilized proteins and carbohydrates were 2,500 mg L⁻¹ and 560 mg L⁻¹ after 0.08 g O₃ g⁻¹ TS ozonation, respectively. These are consistent with the past results, showing that ultrasound and ozone solubilized the extra-cellular and intra-cellular biopolymers in the sludge (Wang et al., 2006; Zhang et al., 2009) .

3.1.2. *Change in pH*

In addition, ultrasound and ozone resulted in different changes in pH value. The sludge pH remained relatively constant at around 5.9 with increasing specific energy input as shown in Fig. 1c. However, the sludge pH decreased obviously with increasing ozone dosage. The sludge pH dropped from 5.9 to 5.2 after 0.04 g O₃ g⁻¹ TS ozonation. These results were consistent with past results and indicated that ultrasound and ozone did not disrupt the sludge in the same way (Bougrier et al., 2006). The pH drop during ozonation was due to the formation of acidic compounds. Yasui and Miyaji (1992) observed the formation of carboxylic acids during human waste ozonation. Bougrier et al. (2006) suggested that the pH decrease was due to the formation of volatile fatty acids from the degradation of lipid compounds in sludge. Yan et al. (2009) found that the lactic acid concentration increased after ozonation due to the oxidation of the soluble macromolecules. This difference in the products of the pre-treatment process used would have impact on subsequent anaerobic degradation.

3.1.3. *Molecular weight distribution*

In order to illustrate the difference in the solubilized substances between ultrasonication and ozonation, the MW distribution chromatograms of the raw sludge, the ultrasonicated sludge and ozonated sludge are compared in Fig. 2a and 2b. No peak with

the same retention time was detected in ultrasonicated and ozonated sludge, indicating solubilization of different compounds in both pre-treatments. High MW compounds ($R_t < 6$ min) were found to be the main solubilized compounds in both pre-treatments as shown in both UV and RI signals. The corresponding MWs of these compounds were over 500 kDa according to the retention time of the largest standard polymer (500 kDa) shown as dash line. Such high MW compounds were reported as cell fragments and extracellular polymers in WAS with MW as high as 10^5 kDa (Pavoni et al., 1972; Namkung and Rittmann, 1986; Schiener et al., 1998; Aquino et al., 2006). It should be noted that RI is a universal detector which detects most of the eluted substances if these are present in sufficient concentrations, while the UV 254 nm detector provides good sensitivity towards aromatic compounds (Trzcinski et al., 2011). For both ultrasonicated and ozonated samples, the detected peaks had similar retention times in both the UV and RI detector signals as shown in Fig. 2a and 2b, suggesting most of the solubilized high MW compounds were possibly aromatic compounds. Yang et al. (2013) reported that tryptophan proteins were the main solubilization products after ultrasonication while humic acids and fulvic acids were the primary solubilization products after ozonation.

In addition, low MW compounds ($8 \text{ min} < R_t < 13.5 \text{ min}$) were also released in both pre-treated sludge but not as obvious as the high MW ones. MWs of these polymers were lower than 27 kDa according to Eq. 4. Peaks with retention time longer than 13.5 min were also detected. These peaks stand for compounds which are smaller than 106 Da (retention time of the standard polymer is shown as the dash line). UV response of such compounds was lower in ozonated sludge than in raw sludge as shown in Fig 2a. This decrease in UV response of the simple organic compounds was because of the

mineralization effect induced by ozone (Weemaes et al., 2000; Ahn et al., 2002). Simple organic compounds were oxidized into carbon dioxide.

3.2. Combined pre-treatment

3.2.1. Sequence determination

In order to investigate the interaction between ultrasound and ozone, various sequences were tested. The SCOD₊ changes in each sequence are shown in Fig. 3a and 3b (TS: around 17 g L⁻¹). The maximum SCOD₊ values obtained in the ULS-Ozone and Ozone-ULS sequences were around 4,000 and 3,800 mg L⁻¹, respectively showing an improved COD solubilization compared to each individual treatment. Such SCOD concentration increase was slightly higher than the maximum SCOD increase (3,300 mg L⁻¹) reported in a previous study treating WAS with combined ultrasonication and ozonation process (Xu et al., 2010). Although WAS has been shown to be more susceptible to ultrasonic pre-treatment than mixtures of PS and WAS, a higher solubilization was observed in this study in comparison to Xu et al. (2010). This is because the mixed sludge used in this study had a higher TS concentration (around 17 g L⁻¹) than the WAS (around 10 g L⁻¹) used in the previous work (Xu et al., 2010). Therefore, more solids were available for solubilization.

Although the SCOD₊ values were similar between the two selected sequences in most of the combination conditions, the Ozone-ULS sequence did not appear to be substantially advantageous because the SCOD₊ increase was marginal during the subsequent ultrasonication step, especially at high prior ozone dosage (e.g. 0.12 g O₃ g⁻¹ TS) as shown in Fig. 3b. This is because ozone significantly solubilized the solids in

sludge and fewer solids were available for the ultrasonic mechanical disruption.

Therefore, the ULS-Ozone sequence was chosen for subsequent experiments.

3.2.2. *Sludge solubilization*

Prior ultrasonication did not enhance the COD solubilization induced by ozone. The COD solubilized by ozone decreased with the prior ultrasonication energy as shown in Fig. 4a. For example, the SCOD₊ induced by 0.048 g O₃ g⁻¹ TS ozonation was 2,600 mg L⁻¹ for raw sludge but was only 1,500 mg L⁻¹ and 400 mg L⁻¹ for sludge which had been pre-ultrasonicated at 9 kJ g⁻¹ TS and 21 kJ g⁻¹ TS, respectively. This is because changes in SCOD concentration can be due to organic solubilization but also degradation of organics to oxidized species such as CO₂. Previous works have shown that mineralization and degradation of the soluble organics due to ozone oxidation resulted in SCOD concentration decrease (Ahn et al., 2002; Erden and Filibeli, 2011).

Foladori et al. (2010) suggested that the VSS could be used as an alternative to represent the particulate organics in sludge sample. The VSS solubilization induced by ozone for both raw and pre-ultrasonicated sludge is shown in Fig. 4b. In contrast with the SCOD results, a greater VSS solubilization due to ozonation was obtained when the specific energy input was lower than 12 kJ g⁻¹ TS. The highest VSS solubilization induced by ozone was 41.3% when 0.048 g O₃ g⁻¹ TS ozonation was applied after 9 kJ g⁻¹ TS ultrasonication. The same ozone dosage applied without prior ultrasonication only induced 21.1% VSS solubilization. This implied that ultrasound made the organic solids easily disrupted by ozone. Agglomerations of particulates would have become smaller in size after ultrasonic dispersion resulting in a higher specific surface area, and therefore

affording a greater probability of contact with ozone. This is important because the half-life of ozone is only 30 min, and is likely much lower in practice as it may react with none target materials before it has the chance to do so with the target organic solids. Besides, smaller particles are more readily ozonated than bigger ones because of a lower mass transfer resistance (Cesbron et al., 2003). Similar synergistic solids solubilization was reported in a previous study when WAS was pre-treated. Yang et al. (2013) reported that combined ultrasonication and ozonation pre-treatment solubilized 6.1% more solids than ultrasound and ozone were applied individually. Results from this work confirmed the positive interaction between ultrasound and ozone pre-treatments also applied on a mixed PS and WAS sample.

VSS solubilization induced by ozone started to decrease when specific energy was higher than $12 \text{ kJ g}^{-1} \text{ TS}$. This was possibly due to the reaction between ozone and solubilized organics released by the prior ultrasound. Cesbron et al. (2003) showed that the soluble and particulate organics competed for ozone in a sludge ozonation system. There have more organics released after high energy ultrasonication ($> 12 \text{ kJ g}^{-1} \text{ TS}$). The reaction between soluble organics and ozone became significant and less ozone was therefore available for the organic solids. This negated the positive effect of the prior ultrasonic dispersion resulting in the decrease of VSS solubilization induced by ozone.

3.2.3. *Molecular weight distribution*

In order to evaluate the size of the solubilization products by ozone when sludge was pre-ultrasonicated, the MW chromatograms of the ultrasonicated sludge and the ultrasonicated sludge with subsequent ozonation are shown in Fig. 5a and 5b. Organic

solids were mainly solubilized in the form of low MW components ($8 \text{ min} < R_t < 13.5 \text{ min}$) as shown by the arrows in Fig. 5b. In addition, the response increase in high MW compounds was not as marked as was observed when sludge was only ozonated (as shown in Fig. 2b).

Some of the high MW components released by ultrasound were found to be very sensitive to ozone attack. The peak which represented high MW components ($R_t: 4 \text{ min}$) disappeared after ozonation was applied as shown by an arrow in Fig. 5a. This suggested prior ultrasound released more organics which could be ozone scavengers. Consequently, the reaction between these soluble organics and ozone became more pronounced compared to the situation when ozone was directly applied to the raw sludge. It should be borne in mind that organic solids solubilization by ozone was also enhanced in ultrasonicated sludge ($\leq 12 \text{ kJ g}^{-1} \text{ TS}$) as shown in Fig. 4b. This meant that the prior ultrasonication step enabled better utilization of ozone by the soluble and particulate organics in sludge. This is important because utilization of the applied ozone had significant influence on performance of a sludge ozonation system (Chu et al., 2008; Manterola et al., 2008).

Despite the synergistic effects caused by the combined treatment, the potential of refractory compounds formation should not be neglected. Yang et al. (2013) observed solubilization of humic acid-like substances when ozone was used to pre-treat WAS sample. Such solubilization was enhanced when the ultrasonication and ozonation pre-treatment were combined simultaneously (Yang et al., 2013). Therefore, it is reasonable to hypothesize such formation of humic acid-like substances may also happen when

ultrasonication and ozonation were applied sequentially. Macromolecules containing humic acids (such as extracellular polymeric substances) were first mechanically released due to the ultrasonication and then chemically degraded to humic acid-like substances and other compounds due to the ozone oxidation. This might also contribute to the degradation of high MW components (Rt: 4 min) as shown in Fig. 5b. In addition, solubilization of the PS fraction may also contribute to such formation of refractory products. For example, residual toilet paper fibres or lint commonly found in PS contains lignin which could be degraded into humic acids due to the ultrasonication-ozonation pre-treatment.

3.3. Anaerobic digestion

Change of the anaerobic biodegradability of the raw sludge, ozonated sludge, ultrasonicated sludge, and ULS-Ozonated sludge during the BMP assay is shown in Fig. 6. Biodegradability of the ultrasonicated sludge increased in the first 4 d of the anaerobic digestion. However, a lag phase was observed for both ozonated sludge and ULS-Ozonated sludge in the first 4 d of the anaerobic digestion. Their corresponding biodegradabilities in the first 4 d were lower than the raw sludge. This was because the oxidized species generated by ozone had inhibited the methanogens as these are known to thrive at very low redox potentials (Weemaes et al., 2000). After d 7, most of the oxidized species were reduced via acidification and no further inhibition effect was observed. The biodegradability increased much faster in the ozonated and the ULS-Ozonated sludge compared to the raw and ultrasonicated sludges. For all the tested

samples, the increase in biodegradability became insignificant after 15 d of anaerobic digestion and reached plateau values after 22 d.

The combined ULS-ozonation pre-treatment resulted in an ultimate biodegradability of 265 mL CH₄ g⁻¹ COD_{added} which was 34.7% more compared to the raw sludge (196 mL CH₄ g⁻¹ COD_{added}). However, the sum of ultimate biodegradability increase (BI) was only 27.7% when ultrasonication (9.9%) and ozonation (17.8%) had been applied at the same conditions. Therefore, 25.7% increase in BI [Calculation demonstration: (34.7% - 27.7%)/27.7% × 100%] could be obtained when ultrasonication and ozonation were sequentially combined. One possibility of this synergistic increase is the synergistic VSS solubilization as mentioned in Section 3.2.2. Besides, the MW reduction in the sequential combined treatment may also contribute to the synergistic biodegradability increase. Eskicioglu et al. (2006) indicated that the macromolecules with MW higher than 300 kDa were mostly complex cell fragments and humic acids which were harder to be anaerobically biodegraded. In this work, the soluble high MW components (MW > 500 kDa) released by ultrasound were effectively degraded by ozone and most of the organic solids were solubilized to low MW components (MW < 27 kDa) which benefited the subsequent anaerobic digestion.

However, it should be noted that the increases in methane production were only 10.9%, 6.6% and 15.4% after the ultrasonication, ozonation and ultrasonication-ozonation pre-treatment, respectively. These figures were lower than the increases in the anaerobic biodegradability for the ozonated and ULS-Ozonated sludge. This was related to the degradation of sludge caused by the ozonation process (as indicated by the decrease in

substrate TCOD concentration shown in Section 2.5). Although anaerobic biodegradability was improved by oxidizing the hardly degradable components in sludge, some biodegradable organics were also lost during the ozonation. The energy balance was calculated as introduced in Section 2.5 and detailed calculation is shown in supplementary material Table S1. The energy balances were 93.8, -42, -386, and -523.8 kJ, for one litre of the raw sludge, ultrasonicated, ozonated sludge and ULS-Ozonated sludge, respectively. This indicated the tested pre-treatment conditions were not economically feasible, especially when ozone was used. Reduction of ozone dosage would not only save some energy but also reduce the loss of biodegradable organics during ozonation. It should also be noted that the laboratory scale ultrasonication and ozonation processes could not be operated at equally high intensity and efficiency as operated in a full scale plant (Pérez-Elvira et al., 2009). These aforementioned factors could be responsible for the negative energy balance obtained in this study.

The authors had tried to compare the anaerobic digestion results of this work with previous studies. However, only one report on the methane production increasing after combined ultrasonication and ozonation pre-treatment could be identified. Consequently, the results from this study were not only compared with studies using the same pre-treatment method but also with those using other pre-treatment methods such as shown in Table 2. Xu et al. (2010) showed anaerobic biodegradability increased by 93%, 100% and 75% after 30, 45 and 120 min of simultaneous combined ultrasonication and ozonation pre-treatment. In addition, their results also suggested over dosage of ozone could have negative effect on methane production. This highlighted the importance of identifying the appropriate ozone dosage for the combined ultrasonication-ozonation pre-treatment

process. Seng et al. (2010) suggested methane production after ultrasonication pre-treatment could be further enhanced by alkaline pre-treatment. Although the increases due to ozone (from 10.9% to 15.4%) and alkaline (from 12.8% to 17.3%) pre-treatments were similar, the ozone pre-treatment does not add dissolved solids to the sludge unlike the alkaline pre-treatment (e.g. sodium ions). In the literature review, the steam-explosion pre-treatment was noted to have very good performance as shown in Table 2. Steam-explosion pre-treatment not only imposes mechanical shear effect on the sludge from the moisture expansion but also thermally and chemically disintegrates the sludge with the high temperature steam (Zhao et al., 2013). The steam-explosion pre-treatment resulted in higher methane production increase (+52% and +100%) than that induced by the combined ultrasonication-ozonation pre-treatment (+15.4%) as reported in this study. However, Dereix et al. (2006) also indicated the extra energy recovered could not compensate for the energy cost of the pre-treatment process. This indicated both the ultrasonication-ozonation and steam-explosion pre-treatments required further studies if a positive energy balance is to be achieved.

3.4. Anaerobic kinetics analysis

The impact of each pre-treatment process on the methane production kinetics was analyzed. The fitted curves are as shown in supplementary materials Fig. S2 and the kinetic parameters obtained are summarized in Table 3. The regression coefficients (R^2) were all higher than 95%, indicating the modified Gompertz model was suitable for the kinetic prediction in this instance. The predicted $P_{\max}$ was in good agreement with the methane production results as mentioned in Section 3.3. Negative λ values were obtained

for the raw and ultrasonicated sludge. This suggested the anaerobic digestion had not been inhibited. These negative λ values were omitted because these are only mathematically possible but not possible in reality (Nevot et al., 2007). Due to the inhibition effect of ozone as mentioned in Section 3.3, the ozonated and ULS-Ozonated sludge yielded positive λ values of 1.75 and 1.72, respectively. The $R_{\max}$ of raw sludge was improved from 3.53 to 4.32 and 4.21 mL CH₄ d⁻¹ after the ultrasonication and ozonation pre-treatment, respectively. Ozonation following the ultrasonication process further increased the $R_{\max}$ to 4.54 mL CH₄ d⁻¹. This is because some organics in the ULS-Ozonated sludge were converted to other easily biodegradable substances (e.g. VFAs) instead of methane during the inhibition period. Once the methanogens were acclimated, these easily biodegradable organics were rapidly converted to methane which contributed to the higher methane production rate.

4. Conclusions

Ultrasound and ozone were found to disrupt the sludge differently and induce synergistic effects when sequentially combined. The ULS-Ozone sequence was shown to be advantageous compared to the Ozone-ULS sequence. The ULS-Ozone sequence pre-treatment had worked as follows:

- Prior ultrasonication helped disperse the biological flocs and enhanced the reaction between ozone and the organic solids.
- The resulting organic solids were then solubilized by ozone in the form of low MW organics (MW < 27 kDa).
- The concentration increase of high MW compounds (MW > 500 kDa) during the

ozonation had then also not been significant.

- Ozone utilization by the ultrasonicated sludge was better compared to non ultrasonicated sludge. Some of the high MW compounds solubilized by ultrasound were likely ozone scavengers and hence effectively degraded by ozone.
- Synergistic sludge biodegradability increase was observed due to the aforementioned synergistic actions.
- The maximum methane production rate for the ultrasonicated sludge was further improved due to the subsequent ozonation process.

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589

590 List of Tables:

591 **Table 1 Characteristics of raw sludge**

Parameter	Value range
Total solids (g L ⁻¹)	16.2-17.2
Volatile solids (g L ⁻¹)	12.6-13.4
Total suspended solids (g L ⁻¹)	15.5-15.9
Volatile suspended solids (g L ⁻¹)	12.4-13.3
Total COD (mg L ⁻¹)	16,800-25,000
Soluble COD (mg L ⁻¹)	700-1,200
Equivalent COD of soluble proteins (mg L ⁻¹)	<100
Equivalent COD of soluble carbohydrates (mg L ⁻¹)	<50

592

593 **Table 2 Summary of the anaerobic digestion results from this work and previous studies**

References	Pre-treatment conditions	Anaerobic digestion conditions	Methane production increase
This study	ultrasonication (9 kJ g ⁻¹ TS)	Batch, 30 d	+10.9%
(PS/WAS)	ultrasonication (9 kJ g ⁻¹ TS)/ozonation (0.036 g O ₃ g ⁻¹ TS)		+15.4%
Dereix et al. (2006)	Steam-explosion, 220 °C, 2.07 MPa	Batch, 65 d	+52%
(TWAS/biosolids)	Steam-explosion, 260 °C, 4.14 MPa		+100%
Seng et al. (2010)	ultrasonication (3.8 kJ g ⁻¹ TS)	Semi-continuous, SRT ^a : 25 d	+12.8%
(WAS)	ultrasonication (3.8 kJ g ⁻¹ TS)/alkaline (0.01 g NaOH g ⁻¹ TS)		+17.3%
Xu et al. (2010)	ultrasonication/ozonation (30 min)	Batch, 14 d	+93% ^b

(WAS)	ultrasonication/ozonation (45 min)	+106% ^b
	ultrasonication/ozonation (120 min)	+75% ^b

^a Solids retention time

^b Increase based on anaerobic biodegradability (i.e. mL CH₄ g⁻¹ COD_{added})

Table 3 Summary of the methane production kinetics analysis

Sludge	P _{max} (mL CH ₄)	R _{max} (mL CH ₄ d ⁻¹)	λ (d)	R ² (%)
Raw	33.3	3.53	-	96.7
Ultrasonicated	36.5	4.32	-	97.2
Ozonated	36.1	4.21	1.75	99.5
ULS-Ozonated	39.5	4.54	1.72	99.5

Table S1 Energy balance analysis as in one litre of raw and pre-treated sludges

Sludge	Methane produced (L)	Energy recovered as electricity ^a (kJ)	Energy recovered as waste heat ^a (kJ)	Energy input for the pre-treatment (kJ)	Energy balance (kJ)
		A	B	C	D = C – A – B
Raw	3.45	35.2	58.6	-	93.8
Ultrasonicated	3.83	39	65	146	-42
Ozonated	3.68	37.5	62.5	486	-386
ULS-Ozonated	3.98	40.6	67.6	632	-523.8

^a Calorific value of methane is 33.94 kJ L⁻¹ and 30% of the combustion heat was assumed to be recovered as electricity and 50% of the combustion heat was assumed to be recovered as waste heat

607 List of Figures:

Figure 1 (a) Increase of COD, equivalent COD of proteins and carbohydrates in soluble phase with specific energy input in individual ultrasonication (b) Increase of COD, equivalent COD of proteins and carbohydrates in soluble phase with ozone dosage in individual ozonation (c) Change in pH with specific energy input and ozone dosage (SCOD₊: the SCOD increase induced by pre-treatment)

Figure 2 MW distribution chromatograms of the soluble substances in raw sludge, ultrasonicated sludge (15 kJ g⁻¹ TS) and ozonated sludge (0.06 g O₃ g⁻¹ TS) in (a) UV signal (254 nm) (b) RI signal

Figure 3 (a) Change in SCOD₊ with ozone dosage for raw and ultrasonicated (at various specific ultrasonic energy inputs) in sequence of ULS-ozone (b) Change in SCOD₊ with specific energy input for raw and ozonated sludge (at various ozone dosages) in sequence of Ozone-ULS (SCOD₊: the SCOD increase induced by pre-treatment)

Figure 4(a) SCOD₊ induced by different ozone dosages for raw sludge and ultrasonicated sludge (at various specific energy inputs) (b) VSS solubilization induced by different ozone dosages for raw sludge and ultrasonicated sludge (at various specific energy inputs)

Figure 5 MW distribution chromatograms of the soluble substances in ultrasonicated sludge (9 kJ g⁻¹ TS) and ultrasonicated sludge (9 kJ g⁻¹ TS) with various subsequent ozone dosages in (a) UV signal (254 nm) (b) RI signal

Figure 6 Results of BMP assays for raw sludge, ozonated sludge, ultrasonicated sludge and ULS-Ozonated sludge

Figure S2 Modelling of the methane production of (a) raw sludge (b) ultrasonicated sludge (c) ozonated sludge (d) ULS-Ozonated sludge with the modified Gompertz equation