Identification of recalcitrant compounds in a pilot-scale AB system: an Adsorption (A) stage followed by a Biological (B) stage to treat municipal wastewater.

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Abstract

This manuscript presents a comparison of the A-stage and B-stage sludges in terms of anaerobic biodegradability and low molecular weight compounds present in the supernatant using Gas Chromatography-Mass Spectrometry (GC-MS). The GC-MS analysis of A-stage and B-stage supernatants identified respectively 43 and 19 organic compounds consisting mainly of aromatics (27.9% and 21%), alcohols (25.6% and 15%) and acids (30.2% and 15%). The methane potential was found to be 349±1 mL CH_4/g VS and 238±12 mL CH_4/g VS, respectively. After anaerobic digestion of these sludges, a greater proportion of aromatics (42% and 58%) and a lower proportion of acids (10% and 10%) and alcohols (16% and 10%) was observed.

Keywords AB process, anaerobic biodegradability, dissolved organic compounds, Soluble Microbial Products (SMP), sewage sludge, GC-MS

1. Introduction

In the recent years, research efforts aiming to improve energy efficiency of wastewater treatment processes in large centralized wastewater treatment plants (WWTPs) have increased. Concerns over global warming impacts, energy sustainability, and biosolids generation are among several key drivers towards the establishment of more energy-efficient WWTPs (Chai et al., 2015). The biosolids management system is cost-intensive as it typically accounts for 25-60% of the total operational costs of conventional activated sludge (CAS)-based WWTPs (Canales et al., 1994; Verstraete & Vlaeminck, 2011). Innovative design and treatment strategies, therefore, are required to achieve more cost-effective and energy self-sufficient WWTPs by minimizing energy consumption while increasing its recovery.

An approach towards an energy-neutral, if not -positive, wastewater treatment process is to recover the potential energy available in raw municipal wastewaters (Shizas & Bagley, 2004). A well-structured strategy deploying a two-stage process, the so-called AB process, has been suggested for the recovery of caloric energy content from sewage organics (Böhnke, 1977; Meerburg et al., 2015; Versprille et al., 1984). The first stage is an extremely high loaded biosorption stage (A-stage), which is subsequently followed by a low loaded biological stage (B-stage) to ensure the removal of dissolved organics and ammonia. The A-stage treatment at the entry of WWTP allows biological concentration of sewage with minimum oxidation of organics to CO_2, and consequently producing a concentrated sludge stream to be channeled to the anaerobic digester. The entrapped organics (chemical energy) can then be recovered through an efficient conversion to biogas without significant energy losses (Verstraete et al., 2009). A characteristic feature of the A-stage reactor is operation with high food to microorganisms (F/M) ratios, short hydraulic retention times (HRTs), and short solid retention times (SRTs), to achieve high reduction rate of sewage organics (Boehnke et al., 1997). Indeed, the treatment with short SRT has been demonstrated to significantly improve the biodegradability of sludge in the downstream anaerobic digester (Ge et al., 2013). The separation of excess sludge in the A-stage can be achieved through an intermediate clarifier (henceforth referred to as 'A-stage clarifier') or dynamic membrane filtration unit (Ersahin et al., 2012; Roest et al., 2012).

During the biosorption process, the A-sludge retains particulate and colloidal organic substances within the biomass matrix, and therefore leaving mainly dissolved organics in the effluents. This would mean reduced aeration energy requirement and lower sludge production in the following B-

stage (Versprille et al., 1984), and therefore may lead to considerable energy savings and overall reduction in biosolids generation. There is currently little information available regarding the biodegradability of the excess sludge and the types of dissolved organics leaving the A and B stages. Effluents from biological processes contain a wide range of complex organic compounds, including soluble microbial products (SMP) and extracellular polymeric substances (EPS), released during bacterial metabolism in mixed culture in bioreactors. Generally, in order to evaluate the performance of biological wastewater treatment processes, only the common generic parameters are measured. These include measures such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), mixed liquor volatile suspended solids, and total organic carbon (TOC), which are done according to Standard Methods from the American Public Health Association (APHA) (Eaton and Franson, 2005). It is important to clearly identify the primary components of SMPs and ECPs in order to understand the fundamental mechanisms of biological activity that create these compounds, and how to reduce these compounds in the effluent. Preliminary results from Aquino (2004) on the identification of SMPs using GC-MS surprisingly revealed long chain alkenes and alkanes, as well as some aromatic compounds such as phthalates in significant concentration (low mg/L). Shen et al. (2012) showed that the concentration of SMPs in wastewater treatment plants ranged roughly from 5 to 25 mg TOC/L, with the major component being polysaccharides (ca. 3–18 mg/L) followed by humic substances (ca. 2–10 mg/L); while the protein concentration was relatively low (<5 mg/L). The SMPs presented a broad molecular weight distribution from smaller than 1 kDa to over 100 kDa. In addition, these compounds constitute the main foulants in membrane bioreactors which are being used more widely around the world (Mei et al., 2014).

Thus so far, there is virtually no report on the A-sludge's biodegradability and its comparison with the B-sludge, a more conventional type of sludge, and the type of organics and their concentration in each stage.

In this study, gas chromatography coupled with mass spectrometry (GC-MS) was used to identify recalcitrant low molecular weight (MW) organics (<580 Da) that were not adsorbed in the A-stage and appeared in the influent to the B-stage. Moreover, the recalcitrant compounds and soluble microbial products (SMPs) produced in the B-stage were also identified and compared with those in the A-stage. These are the compounds that are most likely to foul the membrane when MBRs are used in the B-stage, and that could also appear in the final effluent. There is therefore interest to shed more light on these compounds, in particular from an AB process treating combined industrial municipal wastewaters.

2. Material and methods

2.1 Reactors configuration and operating conditions

A pilot unit was operated with an AB process to treat real municipal wastewater from households and small businesses in Singapore. The pilot plant was run in a continuous flow mode with an average wastewater flow of 1000 m^3/d. It consisted of an equalization tank, 2 coarse (5 mm) rotary drum screen units, a high-rate A-stage contact tank, a primary/A-stage clarifier, 2 fine (2 mm) rotary drum screen units, and an ultrafiltration membrane bioreactor (MBR) system which comprised 5 biological tanks (2 anoxic tanks and 3 aerobic tanks), 1 membrane tank and 1 deoxygenation tank. A simplified schematic diagram of the pilot plant is shown in Figure 1. The raw influent consisted of a mixture of incoming municipal wastewaters and dewatered digested

sludge and was drawn through submersible pumps operating in constant flowrate mode. Initial screening was subsequently performed through 5 mm perforated screen units followed by a screw conveyor type grit removal system. The A-stage was designed with an SRT of 0.5 d (calculated over the entire contact tank and clarifier) and a total HRT of 2 h, consisting of 0.5 h and 1.5 h for the contact tank and clarifier, respectively. To protect the downstream MBR process, 2 mm fine screens were provided for the removal of smaller solid particles. The following B-stage was operated with a 5-h HRT in the Modified Ludzack – Ettinger (MLE) configuration with a step-feed of 50% influent to the first anoxic zone and the other 50% to the second anoxic zone. A target SRT of 5 d was set in order to maintain the slow-growing nitrifying organisms for N removal. Dissolved oxygen (DO) concentrations were maintained at 0.3 and 1 mg O_2/L in the corresponding contact tank and aerobic tanks.

2.2 Physicochemical analyses

Sludge samples were taken from the pilot plant on 26th March 2015. Physico-chemical parameters such as Total Solids (TS), Volatile Solids (VS), Total Suspended Solids (TSS), Volatile Suspended Solids (VSS) and COD concentrations were immediately analyzed in accordance with Standard Methods for the Examination of Water and Wastewater (APHA, 1995). Calorific value was determined using an oxygen bomb calorimeter (IKA, Malaysia) to measure the energy content in the sludge. The calorimeter unit consisted of a stainless steel bomb, a water jacket, an ignition unit, a thermometer, and a mechanical stirrer. Internal volume of the stainless steel bomb was approximately 350 mL and the volume of water jacket surrounding the bomb was 2 L. The

mechanical stirrer was used to keep the water jacket uniformly mixed. After centrifugation, the biomass pellet was frozen at -20°C and subsequently freeze-dried at 0.01 mbar vacuum and -45°C overnight. Next, the dried samples were crushed into powder, weighed and combusted using high pressure oxygen (30 bar) in bomb calorimeter. The temperature rise in the water jacket during combustion was used to calculate the energy content of sludge samples. The heat capacity of the bomb was determined using benzoic acid as a standard (Shizas & Bagley, 2004).

2.3 Liquid-Liquid extraction

Liquid-liquid extraction was performed on 100 mL of filtered supernatant (<0.45 m) using 70 mL Dichloromethane (GC-MS grade, Merck). This solvent was chosen because it has been used by other researchers for SMP analysis on GC-MS (Wu & Zhou, 2010). All glassware was washed with acetone prior to the procedure. A blank containing only distilled water was run along as control. Mixing was provided for 3 minutes by manually inverting the extraction funnel and separation of the 2 phases was then allowed for 5 minutes. Traces of water were removed by mixing the solvent phase with 2 spoons of Na_2SO_4. Solvent evaporation was then carried out at 50°C under vacuum until 1 mL of solvent phase was obtained.

2.4 Gas Chromatography – Mass Spectrometry

The samples (injection volume: 1 µL) were then analyzed using a Shimadzu gas chromatograph equipped with an autosampler and a QP2010Ultra mass spectrometry detector (Shimadzu, Japan). The analytes were separated using an Rtx® -5MS column of 30m x 0.25 mm with a film thickness

of 0.25 μm. The temperature program of the GC-MS oven was: 50°C, hold 7 min, rate 7°C min^{-1} to 325°C, hold 14 min. Helium was used as a carrier gas at a column flowrate of 1 mL/min. The injector temperature was set at 280°C (splitless injection mode), and the MS was operated in the electron impact ionisation mode (70 eV). The transfer line and ion source temperatures were 280 and 230°C, respectively. Scan runs were made with a range from *m/z* 30 to 580. The chromatographic peaks were identified either by direct analysis of the mass spectrum or/and comparison with the NIST11 library (National Institute of Standards and Technology, Gaithersburg, MD, USA, http://www.nist.gov/srd/mslist.htm). The retention indexes were calculated by the library according to alkanes standards retention times (Trzcinski & Stuckey, 2010). Quantification was done separately for each unknown compound using the alkane with the closest retention time.

2.4 Biochemical Methane Potential

Biochemical methane potential (BMP) of the A-stage and B-stage sludges was determined in batch assays using an Automatic Methane Potential Test System (AMPTS II, Bioprocess Control, Sweden). The assay was performed to examine the biodegradability of substrate subjected to the anaerobic incubation through the measurement of its cumulative methane production. The AMPTS reactor was seeded with anaerobic sludge which was collected from a mesophilic digester at Ulu Pandan Water Reclamation Plant in Singapore. The assay was conducted at 35°C for approximately 28 days. Prior to the assay, the inoculum was degassed at 35°C for one week to remove the residual carbon source. Biomedium containing nutrients and vitamin was prepared in

accordance with Owen et al. (1979). 200 mL of inoculum, 100 mL of substrate, and 50 mL of biomedium were added to each reactor which was subsequently flushed with nitrogen gas at 5 psi for approximately 5 min. Batch reactor without substrate addition was used as negative control and its methane production was subtracted from the methane production in the test bottles. All assays were performed in duplicate. The composition of biogas was analyzed with gas chromatography as previously reported (Tian et al., 2014). The percentage of biodegradability was calculated through stoichiometric conversion of CH_4 production from organic degradation as described in Speece (1996). Sample preparation of the anaerobically digested sludge prior to GC-MS analysis was done as described above. SMPs from the anaerobic inoculum used in the AMPTS were also analyzed following the same procedure and is referred to as "AMPTS control" in results and discussion.

3. Results and discussion

3.1 Physicochemical characteristics

Table 1 shows the properties of A-stage and B-stage sludge collected in this study. The physical properties were very similar and both sludges had similar organic content (VS/TS ratio). Although the TCOD was around 4-6 g/L in both sludges, the B-stage had a significantly lower SCOD (38 mg/L) compared to the A-stage (153 mg/L). This is due to the biodegradation of dissolved organics in the membrane bioreactor. The calorific value was higher in the A-stage sludge due to the concentration of carbon including dissolved organics, and possibly cellulose and lignin from raw sewage.

3.2 GC-MS analysis of recalcitrant compounds and SMPs in AB process

144 peaks appeared on the chromatograph from the A-stage sludge supernatant (Supplementary material), but only 43 (30%) could be identified with a match percentage greater than 80% (Figure 2 top left). Their concentration was not higher than 5 µg/L, except for a few acid compounds detected at a higher concentration such as dodecanoic (11.2 µg/L), hexadecanoic (28.5 µg/L), oleic (21.1 µg/L) and octadecanoic acids (20.5 µg/L) (Table 2). Long chain fatty acids (LCFA) originate from the degradation of fats, oils and grease present in raw sewage. LCFAs could have been taken up by Poly-phosphate accumulating microorganisms (PAO) in the B stage.

It has been recently reported that LCFA can be used as sole carbon source for EBPR and were found to enhance PAO activity (Tayà et al., 2015). It is also possible that some of the compounds detected in this study by GC-MS were inhibitory or toxic to PAOs which can explain why the Bio-P removal was not stable according to Qing (2015).

Aromatic compounds were found in the low MW range (<150 Da) as well as in the high MW range (>300 Da) and bis(2-ethylhexyl) isophthalate was the largest aromatic compound in this sample with a MW of 390 Da. Overall, it was found that the compounds were mainly aromatic (27.9%), alcohols (25.6%) or acids (30.2%) (Figure 2 top right). The other compounds were alkanes, amines and ester, but in much smaller proportions.

The total number of peaks was significantly greater in the A-stage supernatant (144) compared to the B-stage supernatant (84) (Figure 2 bottom left). This is consistent with the A-stage chromatograph that shows more peaks compared to the B-stage (Supplementary material). The B-stage chromatogram also displayed a flatter baseline which is an indication that it had fewer peaks.

Similarly, the number of identified peaks with a match percentage greater than 80% was higher in the A-stage with 43 peaks versus 19 peaks in the B-stage supernatant. However, the B-stage supernatant was less characterized than the A-stage supernatant with 23% of the peaks being identified versus 30% for the A-stage supernatant.

The A-stage supernatant contained high molecular weight (MW) compounds with Retention Index (RI) greater than 3000 and the greatest molecular weight was 534 Da for 9-Octadecenoic acid (Z)-, octadecyl ester. In contrast, the B-stage supernatant did not contain any compounds with RI greater than 3000 indicating that high MW compounds from the A-stage were hydrolyzed. This is relevant since membrane modules (ultrafiltration) are submerged in the B-stage membrane tank and the type of organics, their concentration and molecular weight will affect the fouling because they are the same size as the pore diameter (Mei et al., 2014). From this study, there were clear differences between the A-stage and B stage in terms of number of compounds, the type of organics, their concentration and molecular weight. The A-stage is a rapid physical separation step and the compounds detected in the A-stage supernatant are therefore very likely to be recalcitrant from raw sewage. In contrast, the B-stage is a biological step and soluble microbial products are more likely to be dominant in that sample.

Zhou et al. (2009b) investigated SMPs in the effluent of a bench scale aerobic sequencing batch reactor treating distillery wastewater and found only 13 components by GC-MS whereas in this study 19 were found in the B-stage supernatant; They found that alkanes and esters such as heneicosane (19.8%), hexadecanoic acid, butyl ester (18.4%) and tetratetracontane (10.4%) were in significant percentage of the total compounds. Alkanes such as octacosane (3.3%), hentriacontane (2.4%), dotriacontane (2.4%) and acids such hexadecanoic acid, trimethylsilyl ester (1.2%) and acetic acid, octadecyl ester (3.8%) were also found but in lower proportions. Alkanes were the

most common compounds which were found in the effluent of a SAMBR-treated solid waste leachate (Trzcinski & Stuckey, 2010) and UASB effluent (Zhou et al., 2009a). These long chain carbohydrates (or alkanes) and esters are frequently found in the biological treatment effluent and are known to be the main components of SMP in aerobic reactors (Janga et al., 2007; Liang et al., 2007). In this study, aromatic, alcohols and acids were more dominant presumably due to the more complex raw wastewater and also because of the short SRT applied in the pilot plant. It is known that the accumulation of SMPs becomes more pronounced at short SRTs (Liang et al., 2007) .

Overall, there was a radical shift of compounds between the A-stage and B-stage. In fact, the B-stage supernatant consisted of completely different compounds, except three: flutolanil (a common pesticide), triacetine and n-Nonadecanol-1, and their concentrations decreased compared to the A-stage supernatant, showing that indeed some compounds could be biodegraded in the process or removed through adsorption to the B-stage sludge. The new compounds in B-stage were either SMPs or biodegradation end-products of residual COD in the soluble phase.

The B-stage supernatant contained very diverse compounds such as aromatics (21%), alcohols (15%), acids (15%), alkanes (10%), alkenes (15%), aldehydes (10%), amide (5%) and ester (5%) as shown in Figure 2 (bottom, right).

3.3 Anaerobic Biodegradability

The cumulative methane production is shown in Figure 3 where it can be seen that 349±1 mL CH_4/g VS and 238±12 mL CH_4/g VS were produced from the A-stage and B-stage sludges, respectively, showing the greater biodegradability (+47%) of the A-stage sludge. From the COD

mass balance and considering the theoretical COD equivalence of 395 mL CH_4 per gram COD (Speece, 1996), it was derived that 53% and 42% of the COD in A-stage and B-stage sludges were converted to methane gas, respectively.

Moreover, the respective methane content in the biogas were 64% and 54% showing the higher energy content of the biogas obtained from the A-stage sludge. This is consistent with the calorific value given in Table 1 which confirms that the A-stage yielded sludge with a greater carbon content and biodegradability potential compared to the more conventional aerated waste activated sludge. This indicates the capacity of the AB system to rapidly capture the carbon from raw sewage and channel it to the existing anaerobic digester to increase energy production.

3.4 GC-MS analysis of recalcitrant compounds and SMPs after anaerobic digestion (AD)

After anaerobic digestion (AD) tests, SMPs and recalcitrant compounds in the supernatant of the digested sludges were also analyzed using GC-MS. A few peaks (identified by ** in Supplementary Table S2) were also found in the inoculum used in the anaerobic biodegradability test, for instance p-cresol which was detected in relatively high concentration. It was found that the number of peaks decreased from 144 to 124 in the digested A-stage sludge (Figure 4 top left). This shows that some compounds were anaerobically degraded to methane, CO_2 or converted to new biomass while new molecules appeared as end-product of the anaerobic process or SMPs produced by anaerobic metabolism. Among these 124 peaks, only 31 (or 25%) were identified and only 6 were in common before and after the anaerobic biodegradability test (identified by *** in Supplementary Table S2). These compounds originated therefore from the raw wastewater and not

296 from the anaerobic metabolism. One of them was oleic acid and its concentration had decreased
297 from 21.14 µg/L before AD to 1.4 µg/L after AD. However, the concentration of some of these
298 increased through the anaerobic digestion test which could be the result of biological degradation
299 of colloids and large molecules in the sludge sample.
300
301 The number of low molecular weight compounds (with RI lower than 1200) was 3 before AD
302 (Table 2), and this increased to 10 after AD (Supplementary Table S2) showing that high
303 molecular weight compounds were hydrolyzed to low molecular weights compounds during
304 anaerobic digestion tests. The number of compounds with RI>3000 (chain with more than 30
305 Carbons) was 5 before AD and 4 after AD. In both B-stage supernatants (before AD in Table S1
306 and after AD in Table S3) no compounds with RI>3000 was found showing a different molecular
307 weight distribution than in A-stage.
308 It was observed that the distribution of compounds also changed with a significantly greater
309 proportion of aromatic compounds: 42% after AD versus 28% before AD. This is because
310 aromatic compounds are generally more recalcitrant and therefore represent a major fraction of
311 residual compounds after AD. All the aromatic compounds detected after AD were smaller than
312 206 Da which is different than before AD where they were found in the low (<150 Da) and high
313 ranges of MW (>300 Da). From the results of the A-stage sludge, it can be added that aromatic
314 biodegradation end-products and SMPs were all smaller then about 200 Da (aromatics are shown
315 with † in Supplementary S2). Alcohols and acids were secondary compounds with 16% and 10%
316 of the total number of compounds, respectively (Figure 4 top right). These proportions were 25.6%
317 and 30% in the sample before AD (Figure 2 top right).

In conclusion, there were fewer compounds after AD with a higher proportion of aromatics and a lower proportion of acids and alcohols.

In the B-stage supernatant the total number of compounds decreased from 84 to 76 before and after AD, respectively, while the number of identified peaks remained 19 (Figure 4 bottom left). When comparing before and after AD, only 2 compounds (2,4,7,9-Tetramethyl-5-decyn-4,7-diol and propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester) were common in both samples indicating that there was a radical shift of compounds during anaerobic digestion of the B-stage sludge. The number of compounds with RI lower than 1200 was 2 before AD and became 10 after AD showing that hydrolysis of larger molecular weight compounds was taking place during the BMP tests (Supplementary materials Tables S1 and S3).

The proportion of various compounds significantly changed during the AD process. The percentage of aromatic compounds increased to 58 % while the percentage of alcohols and acids decreased to 10% each (Figure 4 bottom right). The further stabilization in the B stage due to the process configuration was confirmed with a lower number of compounds compared to the A-stage supernatant (19 versus 31) and also by a higher degree of aromaticity: 58% versus 42%. This was expected since the SRT is longer in the B-stage (5 days) than in the A-stage (0.5 days) and retention of bacteria by the membrane in the B-stage can also contribute to a better biodegradation of SMPs. The role of the A-stage is also to provide protection to the B-stage and buffer any organic shock that may occur. The higher number of compounds in the A-stage compared to the B-stage showed that indeed the process configuration allowed for fewer contaminants ending up in the B-stage. This provides protection for the biological process in the B-stage as fewer toxic or inhibitory compounds were detected.

In this study aromatics were detected in both the aerobic sludges (from A-stage and B-stage) and the anaerobically digested sludges, but the degree of aromaticity was greater in the anaerobically digested sludge.

The concentrations were typically less than 5 µg/L which is too low to explain the residual SCOD given in Table 1: 153 mg/L and 38 mg/L in the A-stage and B-stage sludge, respectively.

This is because the use of GC-MS is limited to the identification of non-polar, volatile and thermostable compounds and many peaks in the chromatograms could not be identified.

Techniques such as LC-MS or Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI-ToF-MS) would certainly shed more light on the nature of the high MW compounds that were not detected and could explain the residual COD in the effluent.

4. Conclusions

This study showed that the supernatant of both A-stage and B-stages sludges contained aromatics (27.9% and 21.1% of identified compounds), long chain alkanes (7% and 10.5%), alcohols (25.6% and 15.8%), acids (30.2% and 15.8%) and esters (2.3% and 5.3%). More methane could be produced from the A-stage sludge (349±1 mL CH_4/g VS) compared to the B-stage sludge (238±12 mL CH_4/g VS). After anaerobic digestion of these sludges, the total number of compounds detected by GC-MS was lower, and there was a greater proportion of aromatic compounds (42% and 58%).

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