Title:

Characterization of soluble microbial products (SMPs) in a membrane bioreactor (MBR) treating municipal wastewater containing pharmaceutical compounds

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Characterization of soluble microbial products (SMPs) in a membrane bioreactor (MBR) treating synthetic wastewater containing pharmaceutical compounds

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Abstract

This study investigated the behaviour and characteristics of soluble microbial products (SMP) in two anoxic-aerobic membrane bioreactors (MBRs): $MBR_{control}$ and MBR_{pharma}, for treating municipal wastewater. Both protein and polysaccharides measured exhibited higher concentrations in the MBR_{pharma} than the $MBR_{control}$. Molecular weight (MW) distribution analysis revealed that the presence of pharmaceuticals enhanced the accumulation of SMPs with macro- (13,091 kDa and 1,587 kDa) and intermediate-MW (189 kDa) compounds in the anoxic MBR_{pharma}, while a substantial decrease was observed in both MBR effluents. Excitation emission matrix (EEM) fluorescence contours indicated that the exposure to pharmaceuticals seemed to stimulate the production of aromatic proteins containing tyrosine (10.1-32.6%) and tryptophan (14.7-43.1%), compared to $MBR_{control}$ (9.9-29.1% for tyrosine; 11.8-42.5% for tryptophan). Gas chromatography - mass spectrometry (GC-MS) analysis revealed aromatics, long-chain alkanes and esters were the predominant SMPs in the MBRs. More peaks were present in the aerobic MBR_{pharma} (196) than anoxic MBR_{pharma} (133). The SMPs identified exhibited both biodegradability and recalcitrance in the MBR treatment processes. Only 8 compounds in the MBR_{pharma} were the same as in the $MBR_{control}$. Alkanes were the most dominant SMPs (51%) in the $MBR_{control}$, while aromatics were dominant (40%) in the MBR_{pharma}. A significant decrease in aromatics (from 16 to 7) in the MBR_{pharma} permeate was observed, compared to the aerobic MBR_{pharma}. Approximately 21% of compounds in the aerobic $MBR_{control}$ were rejected by membrane filtration, while this increased to 28% in the $MBR_{pharma.}$.

Keywords: Soluble microbial products (SMP); pharmaceutical compounds; membrane bioreactor (MBR); anoxic-aerobic; wastewater treatment.

1. Introduction

Pharmaceutical and personal care products (PPCPs) are considered as "emerging contaminants" and many of them are frequently detected in wastewater treatment plant (WWTP) effluents, and surface water and drinking water due to their hydrophilic character and persistence in the aquatic environment (Verlicchi and Zambello, 2015). Although the presence of these compounds in the environment corresponds to low concentration levels (from parts per trillion to parts per billion), their continuous release from WWTPs may pose a potential long-term threat to aquatic and terrestrial ecosystems (Carballa et al., 2004; Kimura et al., 2007).

Soluble microbial products (SMP) are organic compounds biologically derived from wastewater treatment processes (Rosenberger et al., 2006; Drews et al., 2007; Liang et al., 2007). Previous studies have reported considerable variability in the production of SMPs in response to environmental stresses imposed on the microorganisms, such as the presence of toxic compounds (Avella et al., 2010; Han et al., 2013; Wu et al., 2015). Han (2013) investigated the effects of continuous Zn (II) exposure on SMP production, and found that the SMP content in the activated sludge increased slightly at below 400 mg/L of Zn (II), but rose sharply at 600 and 800 mg/L Zn. Wang and Zhang (2010) characterized SMPs under stressed conditions and revealed that microorganisms exposed to 50 ppm $CrCl_3$ increased their generation of low MW hydrophilic protein-like materials. In a recent study on the effect of continuous Ni(II) exposure on the organic degradation and SMP formation in anaerobic reactors, Wu et al. (2015) indicated that more protein than polysaccharide was produced, suggesting the prominent function of protein when reacting to the negative effect of toxic metals. Although a number of studies on the characteristics and fate of SMPs in different bioreactors for the treatment of municipal and industrial wastewater and landfill leachate have been carried out (Trzcinski and

Stuckey, 2010; Wu and Zhou, 2010; Juang et al., 2013), little information is available with regards to the effects of pharmaceuticals on SMP formation and characterization.

To date, research on SMP production and characterisation has been limited to studies focused on several major components such as proteins, polysaccharides, humic substances, and fulvic acid (Barker and Stuckey, 1999; Dignac et al., 2000), and their precise composition remains unclear (Liang et al., 2007). Furthermore, SMPs have a broad spectrum of molecular weights (MW) ranging from greater than 100 kDa to less than 1 kDa (Shin and Kang, 2003; Jarusutthirak and Amy, 2006), and low-MW SMPs are commonly predominant in secondary wastewater effluents (Aquino and Stuckey, 2002). In a previous study on the identification of primary compounds using gas chromatography - mass spectrometry (GC-MS), Aquino and Stuckey (2004) detected long-chain alkenes and alkanes, as well as some aromatic compounds in significant concentrations (low mg/L). More recently, Trzcinski and Stuckey (2009) demonstrated that a number of aliphatic molecules were degraded in the submerged anaerobic MBR, while some aromatic recalcitrants such as Bis (2-ethylhexy) phthalate were retained in the MBR permeate. Nevertheless, only a few researchers have focused on the chemical identification of low-MW SMPs using sophisticated instruments, e.g., GC-MS (Kunacheva and Stuckey, 2014). Therefore, in order to better understand the fundamental mechanisms of secretion, fate and biodegradability of individual SMPs in biological wastewater treatment processes, as well as how to reduce the levels of these compounds in the effluent, more work needs to be done to specifically identify SMP composition and characteristics.

Many reported studies indicated that membrane bioreactors (MBRs) are more effective than conventional activated sludge (CAS) for the removal of pharmaceuticals, due to long sludge retention times (SRTs), high mixed liquor concentrations, minimal sludge production, and high biomass diversity (Joss et al., 2005; Kümmerer, 2009). SMP/extracellular polymeric substance (EPS) which accumulate in MBR systems have been shown to be a consequence of high membrane rejection and low biodegradability. Their formation, composition and behaviour may become even more complex in MBR systems compared to conventional CAS due to the MBRs retaining biomass at high cell retention times (Wang and Waite, 2009; Shen et al., 2010). Furthermore, under environmental stress,

136 the cells may produce more EPS and SMPs as a result of metabolic changes in order to survive,
137 possibly even resulting in cell rupture (Aquino and Stuckey, 2004). Therefore, changes in SMP
138 quantity and composition may reveal the response and resistance of activated sludge in an MBR to the
139 exposure to pharmaceuticals. However, changes in SMP concentration and composition have rarely
140 been examined, and a greater understanding of the role SMPs plays in the resistance of CAS to
141 pharmaceutical exposure is needed.
142 In this study, the occurrence and characteristics of SMPs in MBRs treating municipal wastewater
143 containing pharmaceutical compounds was investigated. The main objectives were to i) characterize
144 the SMP MW distribution using high performance liquid chromatography (HPLC) - size exclusion
145 chromatography (SEC); ii) investigate the chemical composition of SMPs using three-dimensional
146 fluorescence excitation emission matrix (EEM); and iii) identify low-MW SMPs in the biological
147 treatment processes using GC-MS.
148
149 **2. Materials and methodologies**
150
151 2.1. Pharmaceuticals
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153 Eight pharmaceuticals (carbamazepine, ibuprofen, naproxen, diclofenac, caffeine, ketoprofen,
154 salicylic acid, and clofibric acid) were selected because they are frequently detected in the aquatic
155 environment (Verlicchi and Zambello, 2015). They were purchased from Sigma-Aldrich (Singapore)
156 with purity > 99%, and their chemical structures and physicochemical properties are given in
157 Supplementary Table 1.
158
159 2.2. Lab-scale MBR
160
161 Two identical lab-scale MBR systems, i.e., $MBR_{control}$ and MBR_{pharma}, consisting of an anoxic
162 compartment (3 L) and an aeration compartment (7 L), were operated in parallel (Figure 1). A hollow

fiber ultrafiltration (UF) membrane (ZeeWeed 500, GE Singapore), made of polyvinylidene fluoride, was submerged inside the aerobic compartment, and its effective membrane surface area was 565 cm^2 with a nominal pore size of 0.04 μm. To control the MBR process, 3 min of filtration followed by 1 min of relaxation was achieved using fully automated SCADA software (IFIX).

The MBRs were inoculated with biomass obtained from Ulu Pandan Wastewater Reclamation Plant (WRP), Singapore. Synthetic wastewater was used in this study to simulate domestic sewage, and its chemical composition is given in Table 1. The influent for $MBR_{control}$ and MBR_{pharma} was prepared in two 70-L glass tanks (maintained at 4°C).The selected pharmaceuticals were spiked into the influent of the MBR_{pharma} resulting in a final concentration of 25 μg/L for each pharmaceutical. The concentration of mixed liquor suspended solid (MLSS) in the aeration tank was maintained at around 3-6 g/L with an average sludge retention time (SRT) of 25 d for each MBR. The hydraulic retention time (HRT) was approximately 10 h, and a permeate flux of 13 - 15 L/m^2 h (LMH) was maintained. Level sensors were installed in the two MBRs to control the feeding of influents and production of membrane permeates. Both MBRs were fitted with a gas diffuser located on the bottom of the aeration tank to maintain the dissolved oxygen (DO) concentration in the sludge at about 3-4 mg/L for biological oxidation and to achieve membrane scouring. The TMP was monitored automatically using a digital pressure gauge (Ashcroft). General parameters, such as membrane flux, pH, DO, and temperature were automatically recorded using a data logger. After 60 days of acclimatisation, the activated sludge in both MBRs reached a steady state; thereafter, the two MBRs were operated continuously for a period of 6 months.

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2.3. Analytical methods

2.3.1 Detection of water quality parameters and pharmaceutical concentrations

Influents, anoxic mixed liquors, aerobic mixed liquors, and membrane effluents were collected twice a week from the two MBRs for measurement of conventional parameters and pharmaceutical

concentration. The measurement of MLSS, mixed liquor volatile suspended solids (MLVSS), chemical oxygen demand (COD), and ammonium (NH_4^+-N) was in accordance with Standard Methods (APHA, 2005).

Prior to the determination of pharmaceutical concentrations, solid phase extraction (SPE) was conducted using Oasis HLB cartridges (Waters, Milford, MA, USA). The target pharmaceuticals were analyzed using an ultra performance liquid chromatography - tandem mass spectrometry system (LCMS - 8030, Shimadzu, Japan) in both negative and positive ion mode. Chromatographic separation was achieved with a Gemini-NX C18 column (110 Å, 75 x 2.0mm; 3 µm particle size) and a C18 guard column, both supplied by Merck (Singapore). Separation and detection of the analytes followed a procedure based on a modification of the methods described by Ternes et al. (2005).

2.3.2 SMP and EPS extraction

The extraction of SMPs and bound EPS followed the procedure described by Sponza (2002). Sludge was harvested following centrifugation at 12,000 g for 15 min; the resulting supernatant represented the SMPs. Next, the dewatered sludge pellet was washed with saline water (0.9% NaCl solution) twice prior to extraction. The mixed liquor was then subjected to sonication at 20k Hz for 2 min, and centrifuged at 12,000 g for 15 min. The phenol-sulfuric acid method (Dubois et al., 1956) and the Lowry method (Lowry et al., 1951) were used for determination of the concentrations of polysaccharides and proteins, respectively.

2.3.3 SMP molecular weight (MW) distribution

A 10 mL sample was first centrifuged at 10,000 rpm for 10 min and then filtered with a 0.22 mm PTFE syringe filter (SLFG013NK, Millipore, Millex-FG). A high performance size exclusion chromatograph (HP-SEC) (Agilent Technologies, 1260 LC system) equipped with the PL Aquagel-OH 8 lm MIXED-M column was used for the MW distribution analysis; Milli-Q water was used as

217 the mobile phase at a flow rate of 1 mL min^{-1}. Polyethylene glycols (PEGs) and polyethylene oxide
218 standards with molecular weights of 500 kDa, 70 kDa, 4 kDa, 600 Da and 106 Da were used for the
219 calibration. MW was calculated according to the calibration curve and a linear relationship was
220 derived between the log of MW (Da) and retention time (Rt: min) as shown in Eq. (1):
221 $\text{Log (MW)} = 9.8823 - 0.6748 \text{ (Rt)}$ Eq. (1)
222
223 2.3.4 Three-dimensional fluorescence excitation emission matrix (EEM)
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225 Three-dimensional EEM fluorescence spectra were measured using a luminescence spectrometry
226 (Perkin Elmer LS55 Fluorescence Spectrometer). The spectrometer slits were set at 10 nm for both
227 excitation and emission and excitation wavelengths were increased from 220 nm to 600 nm in 10 nm
228 steps; for each excitation wavelength the emission was detected from 300 nm to 550 nm in 10 nm
229 steps. The software FL Winlab Version 4.00.03 (Perkin Elmer) was employed for handling the EEM
230 data, which were plotted as elliptically shaped contours.
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232 2.3.5 Gas chromatography - mass spectrometry (GC-MS)
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234 In the present study, identification of SMPs was carried out using GC-MS, which allows for the
235 detection of non-polar, volatile and thermo-stable low-MW (< 500 Da) compounds. Prior to the GC-
236 MS analysis, liquid-liquid extraction was performed on a 100 mL filtered supernatant (< 0.45 μm)
237 using 70 mL dichloromethane (GC-MS grade, Merck) (Wu and Zhou, 2010), this solvent was selected
238 because it had been used by previous researchers for SMP analysis using GC-MS (Wu and Zhou,
239 2010). All glassware was washed with acetone prior to the procedure. Mixing was for 3 minutes by
240 manually inverting the extraction funnel and separation of the 2 phases occurred over 5 minutes.
241 Traces of water were removed by mixing the solvent phase with 2 spoons (5 mL) of Na_2SO_4. The
242 solvent was evaporated was at 50°C under vacuum until 1 mL of solvent remained.

The samples were then analyzed using a gas chromatograph (5890 Series) equipped with a QP2010Ultra Mass Spectrometry Detector (Shimadzu, Japan). The analytes were separated using an Rtx-5MS column (30 m x 0.25 mm with a film thickness of 0.25 µm). The GC_MS oven temperature program was: 50 °C, hold 7 min, rate 7 ºC min^{-1} and then thereafter increased to 325°C and hold 14 min. Helium was the carrier gas at a flowrate of 1 mL/min. The injector temperature was set at 270°C, and the MS was operated in the electron impact ionisation mode (70 eV). The transfer line and ion source temperatures were 290 and 220 °C, respectively. Scan runs were made with a range from *m/z* 30 to 580. The chromatograms were analysed using the NIST11 library (National Institute of Standards and Technology, Gaithersburg, MD, USA, http://www.nist.gov/srd/mslist.htm), and a match percentage was obtained by comparing the mass spectrum of a peak with that of a known compound from the library. The retention indexes were calculated by the library according to alkanes standards retention times (Trzcinski and Stuckey, 2010). Quantification was done separately for each unknown compound using the alkane with the closest retention time.

3. Results and discussion

3.1. Treatment performance of MBR systems

Basic performance parameters including MLVSS, MLSS, DCOD, and NH_4^+-N are summarized in Table 2. The average MLSS concentrations ranged from 4.1-4.9 g/L, while the values for MLVSS were 3.8-4.5 g/L. The SCOD concentration in the effluent of the $MBR_{control}$ and MBR_{pharma} was 10.9 and 14.3 mg/L, resulting in high removals of 97.8% and 97.1%, respectively, indicating the efficiency of MBRs in wastewater treatment. With respect to NH_4^+-N, average removal efficiencies of 95.8% in MBR_{pharma} and 95.0% in $MBR_{control}$ were observed. The high rate of nitrification achieved may be due to the effective retention of slow growing nitrifying microorganisms by the membrane, which cannot be achieved by gravity clarification in CAS systems (Chang et al., 2002).

Figure 2 shows the removal efficiencies of selected pharmaceuticals in the MBR mixed liquor and effluent. As expected, no significant removal of recalcitrant carbamazepine was observed (5.4% and 9.6% for the aerobic stage and MBR permeate, respectively), implying its persistence in CAS and membrane filtration processes. In contrast, all the highly biodegradable compounds such as caffeine, ibuprofen and salicylic acid, exhibited high removal rates in both the aerobic stage (99%, 91.4% and 92.2%, respectively) and MBR permeate (99.5%, 98.1% and 94.5%, respectively). This finding is consistent with previous studies (Kim et al., 2007; Miège et al., 2009; Radjenović et al., 2009), implying that biodegradation was the main removal mechanism for hydrophilic pharmaceuticals. Although ketoprofen can serve as a sole substrate for microbial growth, and is considered biodegradable (Quintana et al., 2005), relatively low removal was obtained in the aerobic stage (55.7%), but this improved significantly after UF membrane filtration (78.4%). In particular, the removal of clofibric acid and diclofenac was significantly more efficient due to membrane filtration (61.8% and 75.2%, respectively) than biodegradation in the aerobic stage (33.4% and 43.1%, respectively).

3.2. Profiles of proteins and carbohydrates

Proteins and carbohydrates are usually found to be the primary components of SMPs in activated sludge (Kunacheva and Stuckey, 2014; Sheng et al., 2010). The variations in protein and carbohydrate concentrations are shown Figure 3. The average protein and polysaccharide concentrations were 3.95±0.37 mg/L and 11.79 mg/L±2.44 mg/L [n = 24] in the aerobic MBR stage, while in the MBR effluent were1.13±0.18 mg/L and 4.09±0.38 mg/L, respectively. This finding agrees with Juang et al. (2013) who investigated the effects of SMP on MBR fouling potential, and reported that the average protein and carbohydrate concentrations were 2.32 and 12.07 mg/L in the MBR supernatant, while in the MBR effluent were 1.14 and 7.39 mg/L, respectively. Moreover, a higher average concentration ($P < 0.05$) of protein (1.46 mg/L) and polysaccharide (4.68 mg/L) was observed in the MBR_{pharma} effluent compared to that in the $MBR_{control}$ (1.03 mg/L for protein; 4.34 mg/L for polysaccharides),

implying that the exposure of biomass to pharmaceutical compounds increased the production of SMPs. Similarly, Aquino and Stuckey (2004) investigated SMP formation in anaerobic chemostats in the presence of toxic compounds, and reported that with chloroform the normalized accumulation of SMPs increased from 2% to 8%, whereas with Cr, the normalized ratio reached as high as 20%.

Production and consumption of soluble organics are dynamic processes, thus the concentrations measured at any point in time present a momentary equilibrium, which can easily be disturbed and shifted by changes in the environment (Rosenberger et al., 2006). Substrate utilization, biomass decay, and EPS hydrolysis are believed to be the major processes contributing to SMP formation (Fenu et al., 2010). It is assumed that the analysed organics analysed were part of the bacterial EPS that was transferred into the liquid phase of the activated sludge, and thus form the soluble EPS or SMP by a variety of different mechanisms. Considering the fact that proteins and carbohydrates are the dominant constituents of cell walls (Pérez Silva et al., 2009), introduction of pharmaceuticals may disturb cellular function and damage cell membranes, and inevitably lead to cell lysis and an increase in SMP concentration. In addition, EPS/SMPs play a key role in protecting the inner microorganisms against environmental stress (Sheng et al., 2010). In the presence of toxic substances, microbial cells in activated sludge and biofilms utilized the substrate to generate more EPS, which act as a diffusional barrier between the cell wall and extreme environments to protect the cells from the harsh environment.

3.3. Molecular weight (MW) distribution of SMPs

The MW distribution of SMP was identified using LC-SEC. Five peaks representing different MW fractions were identified (Figure 4) in both the $MBR_{control}$ and MBR_{pharma}. Peak 1 (13,091 kDa), and Peak 2 (1,587 kDa) demonstrates the presence of high-MW (> 500 kDa) SMPs. Peak 3 (189 kDa) and Peak 4 (53 kDa) indicate intermediate-MW fractions (500 kDa < MW <1 kDa), while Peak 5 (71 Da) indicates the low-MW fraction (MW < 1 kDa).

As shown in Figure 4, although the location of the major peaks was similar, the relative intensities of the major peaks in the two MBRs were different. Compared to $MBR_{control,}$ a significant increase in the intensities of macro- (13,091 kDa and 1,587 kDa) and intermediate-MW (189 kDa) compounds in the anoxic MBR_{pharma} was observed, implying that the presence of pharmaceuticals enhanced the accumulation of high- and intermediate fractions in the MBR_{pharma} during start-up stage. A similar result was also found by Aquino and Stuckey (2004) who revealed the presence of toxic compounds (Cr and $CHCl_3$) caused a higher accumulation of SMPs with high-MWs. Likewise, Avella et al. (2010) indicated that the presence of a cytostatic drug (cyclophosphamide) caused a significant increase in SMPs in MBR supernatants. The presence of toxic compounds (e.g., pharmaceuticals) tends to cause cell lysis and release intracellular high MW SMPs in response to environmental stress (Aquino and Stuckey, 2004). Hence, EPS in a non-hydrolysed form may also constitute part of the high MW SMP (Aquino and Stuckey, 2004). In addition, although many SMPs produced during biological treatment were degraded, high MW compounds exposed to a toxic environment were likely to be degraded more slowly and result in an increase in SMPs (Chen et al., 2014).

In addition, a substantial decrease in the concentrations of macro- (13,091 kDa and 1,587 kDa) and intermediate-MW compounds (189 kDa and 53 kDa) was observed in the MBR effluent, regardless of the presence of pharmaceuticals. This finding clearly shows that membrane filtration rejected an important high MW fraction of the soluble macromolecules in the reactor's bulk solution. The intermediate-MW fraction of 53 kDa only appeared in the anoxic supernatant, and was not found in MBR aerobic supernatant and effluent, regardless of the presence of pharmaceuticals. This observation suggests that the high- and intermediate- MW species might break down into simpler low- MW solutes.

3.4. Excitation emission matrix (EEM) fluorescence contours

Measurements of EEM fluorescence spectra were carried out to help analyse SMP composition, and the results are shown in Figure 5. In this present study, five peaks were readily identified in

different treatment units of both MBRs. The first dominant peak was at the excitation/emission wavelengths (Ex/Em) of 220/320 nm (Peak A), and is related to aromatic proteins (tyrosine). The second main peak was at the Ex/Em of 225/362 nm (Peak B), and is associated with aromatic proteins (tryptophan). The other two peaks were located at Ex/Em of 230/426 nm (Peak C) and 285/394 (Peak D), are associated with fulvic acid-like and humic acid-like solutes. The last peak (Peak E) at the Ex/Em of 270/360 nm (Peak E) was described as a tryptophan protein-like solute.

In addition, the location of Peak A was red-shifted (15 nm) along the emission axis in the effluent, while Peak B demonstrated a blue-shift (5 nm) in the effluent, compared to those in the anoxic mixed liquor. A red shift is related to the presence of carbonyl containing substituents, hydroxyl, alkoxyl, amino groups and carboxyl constituents (Wang et al., 2009), while a blue shift is associated with the decomposition of condensed aromatic moieties, and the breakup of large molecules into smaller fragments, such as a decrease in the number of aromatic rings, a reduction of conjugated bonds in a chain structure, a conversion of a linear ring to a non-linear system, or an elimination of particular functional groups including carbonyl, hydroxyl and amine (Coble, 1996). As mentioned above, the shift in wavelength indicated that the oxidation stage and properties of organic matter were different during the biological treatment processes.

In order to further examine the compositional changes of SMPs with the exposure to pharmaceuticals, a fluorescence regional integration (FRI) analysis (Chen et al., 2003; Wang and Zhang, 2010; Chen et al., 2014) was also conducted and is shown in Figure 6. Regions I, II, III and IV represent the tyrosine, tyrosine-like protein, tryptophan and tryptophan-like proteins, respectively. Regions V and VI represent fulvic acid-like and humic acid-like substances. It can be seen that the SMPs were dominated by fluorescence in Regions I, III, V and VI. Regions I and III accounted for more than 45.1% , whereas Regions II and IV accounted for less than 15.5%, implying that the majority of proteins in the SMPs were both tyrosine and tryptophan over other types of proteins containing amino acids such as leucine, alanine, glycine, lysine, proline, serine, and threonine etc. Amino acid composition of proteins is often used to describe protein sequences and to design predictive algorithms (e.g., the tendency of proteins to crystallize), and the percentage of occurrence

of specific amino acids in proteins depends on the protein dimensions (Carugo, 2008). It is also worth noting that down the treatment process from influent to effluent, a significant increase in Regions I and III was observed, implying that these aromatic amino acids were the most difficult to break down. Furthermore, each region exhibited different trends to the exposure of pharmaceuticals, and seemed to stimulate the production of SMPs in Regions I and III, resulting in an increase in amino acids such as tyrosine (10.1-32.6%) and tryptophan (14.7-43.1%), compared to $MBR_{control}$ (9.9-29.1% for tyrosine; 11.8-42.5% for tryptophan). It has been well documented that protein plays a significant role in microorganisms' adaptation to the presence of toxic compounds, the mechanisms of which include sequestrating the metal through binding and mitigating the toxicity by enzymatic detoxification (Bruins et al., 2000). The protein production in the activated sludge was probably enhanced under pharmaceutical exposure, implying the important role proteins play in cell adaption to pharmaceutical toxicity. In contrast, Regions V and VI decreased under the exposure to pharmaceuticals, and the fulvic acid-like substances reduced from 19.7-35.0% to 17.8-29.5% , while the humic acid-like substances reduced from 11.7-12.9% to 9.9-11.0%.

3.5. Identification and characterization of SMPs using GC-MS

3.5.1 SMPs in the aerobic stage

Compared to SMPs generated in the anoxic MBR_{pharma} (133) (Supplementary Figure 1), the number of compounds increased to 196 in the aerobic MBR_{pharma}, and 40 compounds (20%) were identified with a match percentage greater than 80% (Figure 7a). Increasing SMP formation down the biological treatment processes might be due to the higher biomass concentrations in the aerobic stage (Table 2) and the greater growth rate of microorganism leading to higher substrate utilization. The predominant SMPs were aromatics accounting for 39%, followed by esters (17%), alkanes (14%) and alcohol (14%) (Figure 8a). This result was consistent with Zhou et al. (2009) who investigated SMPs in the effluent of a sequencing batch reactor treating distillery wastewater, and found that alkanes and

esters such as heneicosane (19.8%), hexadecanoic acid, butyl ester (18.4%) and tetratetracontane (10.4%) were a significant percentage of the total compounds present. In particular, these long-chain carbohydrates (or alkanes) and esters are frequently reported in biological treatment effluent, and are known to be the main components of low-MW SMPs in aerobic reactors (Janga et al., 2007; Liang et al., 2007).

During biological treatment, both biodegradable and refractory organic compounds are released into the system associated with the lysis of cells. In the present study, the majority of compounds, such as small organic acids (Heptanoic acid, Octanoic acid,), alcohols (n-Pentadecanol, 1-Decanol, 2-methyl-), short-chain alkanes (2-Dodecene, (Z)-), which were present in the anoxic liquor, could not be detected in the aerobic liquor (Supplementary Table 1 and Table 3). This finding indicated that these simple compounds might have been easily biodegraded in the aerobic processes. In contrast, nearly a quarter of SMPs, due to their chemical structure, e.g., substituted ring compounds, cross-linked cell wall fragments, were present in both the anoxic and aerobic liquors, implying that these refractory compounds were not easily biodegraded under any form of metabolism. Most of these compounds were aromatics, such as benzoic acid, 3-methyl-, hydrocinnamic acid, N-Methyl-1H-benzimidazol-2-amine, etc.

In the aerobic stage of the $MBR_{control}$, 41 peaks (18%) were identified with a match percentage greater than 80%, while 165 peaks (72%) were unidentified (Figure 7b). Among the dominant compounds identified were alkanes (51%), aromatics (20%) and esters (17%) (Figure 8b). Only 8 compounds in the MBR_{pharma} (e.g., benzoic acid, dodecanoic acid, 2-butenoic acid, 2-propenylidene ester, etc.,) were the same as in the $MBR_{control}$, and this implies that the presence of pharmaceutical compounds resulted in a shift in SMP production and their properties (Table 3 and 4). Moreover, certain aromatics (e.g., 3(2H)-pyridazinone, 6-chloro-), esters (e.g., tricosyl pentafluoropropionate), alkanes (e.g., propane, 1,1,2,3-tetrachloro-) and ketones (e.g., 2-propanone, 1,1,3,3-tetrachloro-), could only be detected in the aerobic MBR_{pharma} and not in the $MBR_{control}$. This finding indicates that these compounds may possibly only be generated during the biological treatment of wastewater containing pharmaceuticals, although no references can be found on the formation and composition of

SMPs generated in the treatment of pharmaceutical wastewater. In addition, the presence of pharmaceuticals also influenced the dominant types of compounds present in wastewater. Alkanes were the most common SMP (51%) in the $MBR_{control}$, while aromatics were the most dominant SMPs (40%) in the MBR_{pharma}. This suggests that more refractory SMPs are produced in the presence of pharmaceuticals, because the aromatic compounds are generally more recalcitrant and therefore represent a major fraction of residual compounds in the MBR_{pharma}.

3.5.2 SMPs in the MBR effluent

Fewer compounds with a match percentage greater than 80% (23) were detected in MBR_{pharma} effluent than those (40) in the MBR_{pharma} aerobic stage (Figure 7c). The dominant compounds were aromatics (30%), alkanes (22%) and esters (22%) (Figure 8c). The number of esters decreased (from 9 to 5) in the MBR_{pharma} permeate compared to the MBR_{pharma} aerobic stage, and a similar decreasing trend could also be found with alkanes (Table 3 and 5). Furthermore, all the compounds detected in the MBR_{pharma} permeate were smaller than 394 Da, while this value was 578 Da in the aerobic $MBR_{pharma.}$ The rejection of these higher MW compounds may be due to the formation of a tighter gel layer on the membrane surface, as well as the interactions between microorganisms and the compounds (e.g., SMP and organic substances such as colloids) that contributed to the formation of the gel layer (Jarusutthirak and Amy, 2006; Rosenberger et al., 2006). Moreover, it can be seen that out of the 23 compounds identified in the MBR_{pharma} permeate, 13 compounds were found in the aerobic liquor. This finding indicates that the SMPs in the aerobic stage and MBR permeate were more or less similar.

A significant decrease in aromatics (from 16 to 7) in the MBR_{pharma} permeate was observed, compared to the MBR_{pharma} aerobic mixed liquor. Aromatic SMPs such as benzoic acid, dl-alanyl-l-leucine, glycyl-L-proline, formamide, (2-acetylphenyl)-, and 1h-1-benzazepine, 2,3,4,5-tetrahydro-, were only detected in the aerobic stage and disappeared in the permeate. This finding is different from Liang et al. (2007) who investigated SMPs in an MBR operated at different SRTs, and reported that

457 the percentage of aromatic compounds in the total SMPs increased after passing through the
458 membrane. This discrepancy might be due to the different pore sizes of the membranes used in the
459 two studies (MF with 0.4 μm versus UF with 0.04 μm in this study), and low pressure microfiltration
460 (MF) MBRs may have lower SMP rejection rates (Juang et al., 2013). In contrast, other recalcitrant
461 aromatics such as 3(2h)-pyridazinone, 6-chloro-, phenol, 2-chloro-5-methyl-, benzoic acid,
462 hydrocinnamic acid, 1h-indol-4-ol, and n-methyl-1h-benzimidazol-2-amine, which were found in the
463 aerobic stage, were still present in the MBR permeate and clearly difficult to remove through
464 membrane rejection. Although these recalcitrant aromatic compounds shared the same characteristics
465 as the selected pharmaceuticals, such as a 6 carbon ring fused to a 5 carbon ring, or rings containing
466 nitrogen or oxygen with a double bond, there was no strong evidence from the literature to conclude
467 that these compounds were the degradation by-products of the selected pharmaceuticals.
468 The total number of peaks found in the $MBR_{control}$ effluent was 181(Figure 7d), and alkane was the
469 dominant compound in the $MBR_{control}$ effluent (Figure 8d). Table 6 shows the compounds detected in
470 the $MBR_{control}$ effluent. Approximately 21% of the compounds in the aerobic $MBR_{control}$ were rejected
471 by the membrane, while this number was lower than the MBR_{pharma} (28%). Avella et al. (2010)
472 reported that membranes could reject up to 95% of proteins and up to 68% of polysaccharides in the
473 MBR_{pharma}, while the values were 98% and 92% in the $MBR_{control}$, respectively. Previous studies have
474 identified the EPS/SMP as one of the most significant factors responsible for membrane fouling
475 (Jarusutthirak and Amy, 2006; Janga et al., 2007), and cake resistance was found to be strongly
476 related to SMP content in the supernatant (Meng et al., 2009). Indeed, the accumulation of EPS in the
477 MBR mix liquor would have facilitated the formation of an EPS fouling gel layer on the membrane
478 surface and eventually lead to pore blocking. Therefore, an increase in the EPS/SMP when cultures
479 are exposed to pharmaceuticals would inevitably result in increasing membrane fouling.
480
481 **4. Conclusions**
482

In the present study, the accumulation, composition, and characteristics of SMPs was examined in an MBR treating wastewater containing pharmaceutical compounds. The exposure of biomass to pharmaceutical compounds increased the production of SMPs, and a higher average concentration ($P > 0.05$) of protein (1.46 mg/L) and polysaccharides (4.68 mg/L) was observed in the MBR_{pharma} effluent compared to that in the $MBR_{control}$ (1.03 mg/L for protein; 4.34 mg/L for polysaccharides). HPLC-SEC analysis revealed that the presence of pharmaceuticals enhanced the accumulation of high- and intermediate MW fractions in the MBR_{pharma}. Measurements of EEM fluorescence spectra indicated that exposure to pharmaceuticals seemed to stimulate the production of tyrosine and tryptophan containing solutes. GC-MS analysis revealed that there were clear differences in the SMPs between the $MBR_{control}$ and MBR_{pharma} in terms of the number of compounds, predominant types of organics, their concentration and molecular weight, biodegradability and recalcitrance, implying that the presence of pharmaceutical compounds have caused a radical shift in the SMPs produced.

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