

1 Title:

2 **Characterization of soluble microbial products (SMPs) in a membrane bioreactor (MBR)**
3 **treating municipal wastewater containing pharmaceutical compounds**
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49 **Characterization of soluble microbial products (SMPs) in a membrane bioreactor**
50 **(MBR) treating synthetic wastewater containing pharmaceutical compounds**

51
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62

63 **Abstract**

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

82

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

85

86 **1. Introduction**

87

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

95 Soluble microbial products (SMP) are organic compounds biologically derived from wastewater
96 treatment processes (Rosenberger et al., 2006; Drews et al., 2007; Liang et al., 2007). Previous studies
97 have reported considerable variability in the production of SMPs in response to environmental
98 stresses imposed on the microorganisms, such as the presence of toxic compounds (Avella et al., 2010;
99 Han et al., 2013; Wu et al., 2015). Han (2013) investigated the effects of continuous Zn (II) exposure
100 on SMP production, and found that the SMP content in the activated sludge increased slightly at
101 below 400 mg/L of Zn (II), but rose sharply at 600 and 800 mg/L Zn. Wang and Zhang (2010)
102 characterized SMPs under stressed conditions and revealed that microorganisms exposed to 50 ppm
103 CrCl₃ increased their generation of low MW hydrophilic protein-like materials. In a recent study on
104 the effect of continuous Ni(II) exposure on the organic degradation and SMP formation in anaerobic
105 reactors, Wu et al. (2015) indicated that more protein than polysaccharide was produced, suggesting
106 the prominent function of protein when reacting to the negative effect of toxic metals. Although a
107 number of studies on the characteristics and fate of SMPs in different bioreactors for the treatment of
108 municipal and industrial wastewater and landfill leachate have been carried out (Trzcinski and

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

111 To date, research on SMP production and characterisation has been limited to studies focused on
112 several major components such as proteins, polysaccharides, humic substances, and fulvic acid
113 (Barker and Stuckey, 1999; Dignac et al., 2000), and their precise composition remains unclear (Liang
114 et al., 2007). Furthermore, SMPs have a broad spectrum of molecular weights (MW) ranging from
115 greater than 100 kDa to less than 1 kDa (Shin and Kang, 2003; Jarusutthirak and Amy, 2006), and
116 low-MW SMPs are commonly predominant in secondary wastewater effluents (Aquino and Stuckey,
117 2002). In a previous study on the identification of primary compounds using gas chromatography -
118 mass spectrometry (GC-MS), Aquino and Stuckey (2004) detected long-chain alkenes and alkanes, as
119 well as some aromatic compounds in significant concentrations (low mg/L). More recently, Trzcinski
120 and Stuckey (2009) demonstrated that a number of aliphatic molecules were degraded in the
121 submerged anaerobic MBR, while some aromatic recalcitrants such as Bis (2-ethylhexy) phthalate
122 were retained in the MBR permeate. Nevertheless, only a few researchers have focused on the
123 chemical identification of low-MW SMPs using sophisticated instruments, e.g., GC-MS (Kunacheva
124 and Stuckey, 2014). Therefore, in order to better understand the fundamental mechanisms of secretion,
125 fate and biodegradability of individual SMPs in biological wastewater treatment processes, as well as
126 how to reduce the levels of these compounds in the effluent, more work needs to be done to
127 specifically identify SMP composition and characteristics.

128 Many reported studies indicated that membrane bioreactors (MBRs) are more effective than
129 conventional activated sludge (CAS) for the removal of pharmaceuticals, due to long sludge retention
130 times (SRTs), high mixed liquor concentrations, minimal sludge production, and high biomass
131 diversity (Joss et al., 2005; Kümmerer, 2009). SMP/extracellular polymeric substance (EPS) which
132 accumulate in MBR systems have been shown to be a consequence of high membrane rejection and
133 low biodegradability. Their formation, composition and behaviour may become even more complex
134 in MBR systems compared to conventional CAS due to the MBRs retaining biomass at high cell
135 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

152

153 Eight pharmaceuticals (carbamazepine, ibuprofen, naproxen, diclofenac, caffeine, ketoprofen,
154 salicylic acid, and clofibrac 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

163 fiber ultrafiltration (UF) membrane (ZeeWeed 500, GE Singapore), made of polyvinylidene fluoride,
164 was submerged inside the aerobic compartment, and its effective membrane surface area was 565 cm²
165 with a nominal pore size of 0.04 μm. To control the MBR process, 3 min of filtration followed by 1
166 min of relaxation was achieved using fully automated SCADA software (IFIX).

167 The MBRs were inoculated with biomass obtained from Ulu Pandan Wastewater Reclamation
168 Plant (WRP), Singapore. Synthetic wastewater was used in this study to simulate domestic sewage,
169 and its chemical composition is given in Table 1. The influent for MBR_{control} and MBR_{pharma} was
170 prepared in two 70-L glass tanks (maintained at 4°C). The selected pharmaceuticals were spiked into
171 the influent of the MBR_{pharma} resulting in a final concentration of 25 μg/L for each pharmaceutical.
172 The concentration of mixed liquor suspended solid (MLSS) in the aeration tank was maintained at
173 around 3-6 g/L with an average sludge retention time (SRT) of 25 d for each MBR. The hydraulic
174 retention time (HRT) was approximately 10 h, and a permeate flux of 13 - 15 L/m² h (LMH) was
175 maintained. Level sensors were installed in the two MBRs to control the feeding of influents and
176 production of membrane permeates. Both MBRs were fitted with a gas diffuser located on the bottom
177 of the aeration tank to maintain the dissolved oxygen (DO) concentration in the sludge at about 3-4
178 mg/L for biological oxidation and to achieve membrane scouring. The TMP was monitored
179 automatically using a digital pressure gauge (Ashcroft). General parameters, such as membrane flux,
180 pH, DO, and temperature were automatically recorded using a data logger. After 60 days of
181 acclimatisation, the activated sludge in both MBRs reached a steady state; thereafter, the two MBRs
182 were operated continuously for a period of 6 months.

183 .

184 2.3. Analytical methods

185

186 2.3.1 Detection of water quality parameters and pharmaceutical concentrations

187

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

190 concentration. The measurement of MLSS, mixed liquor volatile suspended solids (MLVSS),
191 chemical oxygen demand (COD), and ammonium ($\text{NH}_4^+\text{-N}$) was in accordance with Standard
192 Methods (APHA, 2005).

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

200

201 2.3.2 SMP and EPS extraction

202

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

210

211 2.3.3 SMP molecular weight (MW) distribution

212

213 A 10 mL sample was first centrifuged at 10,000 rpm for 10 min and then filtered with a 0.22 mm
214 PTFE syringe filter (SLFG013NK, Millipore, Millex-FG). A high performance size exclusion
215 chromatograph (HP-SEC) (Agilent Technologies, 1260 LC system) equipped with the PL Aquagel-
216 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):

$$221 \text{Log (MW)} = 9.8823 - 0.6748 (\text{Rt}) \quad \text{Eq. (1)}$$

222

223 2.3.4 Three-dimensional fluorescence excitation emission matrix (EEM)

224

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.

231

232 2.3.5 Gas chromatography - mass spectrometry (GC-MS)

233

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 Na₂SO₄. The
242 solvent was evaporated was at 50°C under vacuum until 1 mL of solvent remained.

243 The samples were then analyzed using a gas chromatograph (5890 Series) equipped with a
244 QP2010Ultra Mass Spectrometry Detector (Shimadzu, Japan). The analytes were separated using an
245 Rtx-5MS column (30 m x 0.25 mm with a film thickness of 0.25 μm). The GC_MS oven temperature
246 program was: 50 $^{\circ}\text{C}$, hold 7 min, rate 7 $^{\circ}\text{C min}^{-1}$ and then thereafter increased to 325 $^{\circ}\text{C}$ and hold 14
247 min. Helium was the carrier gas at a flowrate of 1 mL/min. The injector temperature was set at 270 $^{\circ}\text{C}$,
248 and the MS was operated in the electron impact ionisation mode (70 eV). The transfer line and ion
249 source temperatures were 290 and 220 $^{\circ}\text{C}$, respectively. Scan runs were made with a range from m/z
250 30 to 580. The chromatograms were analysed using the NIST11 library (National Institute of
251 Standards and Technology, Gaithersburg, MD, USA, <http://www.nist.gov/srd/mslist.htm>), and a
252 match percentage was obtained by comparing the mass spectrum of a peak with that of a known
253 compound from the library. The retention indexes were calculated by the library according to alkanes
254 standards retention times (Trzcinski and Stuckey, 2010). Quantification was done separately for each
255 unknown compound using the alkane with the closest retention time.

256

257 3. Results and discussion

258

259 3.1. Treatment performance of MBR systems

260

261 Basic performance parameters including MLVSS, MLSS, DCOD, and $\text{NH}_4^+\text{-N}$ are summarized in
262 Table 2. The average MLSS concentrations ranged from 4.1-4.9 g/L, while the values for MLVSS
263 were 3.8-4.5 g/L. The SCOD concentration in the effluent of the $\text{MBR}_{\text{control}}$ and $\text{MBR}_{\text{pharma}}$ was 10.9
264 and 14.3 mg/L, resulting in high removals of 97.8% and 97.1%, respectively, indicating the efficiency
265 of MBRs in wastewater treatment. With respect to $\text{NH}_4^+\text{-N}$, average removal efficiencies of 95.8% in
266 $\text{MBR}_{\text{pharma}}$ and 95.0% in $\text{MBR}_{\text{control}}$ were observed. The high rate of nitrification achieved may be due
267 to the effective retention of slow growing nitrifying microorganisms by the membrane, which cannot
268 be achieved by gravity clarification in CAS systems (Chang et al., 2002).

269 Figure 2 shows the removal efficiencies of selected pharmaceuticals in the MBR mixed liquor and
270 effluent. As expected, no significant removal of recalcitrant carbamazepine was observed (5.4% and
271 9.6% for the aerobic stage and MBR permeate, respectively), implying its persistence in CAS and
272 membrane filtration processes. In contrast, all the highly biodegradable compounds such as caffeine,
273 ibuprofen and salicylic acid, exhibited high removal rates in both the aerobic stage (99%, 91.4% and
274 92.2%, respectively) and MBR permeate (99.5%, 98.1% and 94.5%, respectively). This finding is
275 consistent with previous studies (Kim et al., 2007; Miège et al., 2009; Radjenović et al., 2009),
276 implying that biodegradation was the main removal mechanism for hydrophilic pharmaceuticals.
277 Although ketoprofen can serve as a sole substrate for microbial growth, and is considered
278 biodegradable (Quintana et al., 2005), relatively low removal was obtained in the aerobic stage
279 (55.7%), but this improved significantly after UF membrane filtration (78.4%). In particular, the
280 removal of clofibric acid and diclofenac was significantly more efficient due to membrane filtration
281 (61.8% and 75.2%, respectively) than biodegradation in the aerobic stage (33.4% and 43.1%,
282 respectively).

283

284 3.2. Profiles of proteins and carbohydrates

285

286 Proteins and carbohydrates are usually found to be the primary components of SMPs in activated
287 sludge (Kunacheva and Stuckey, 2014; Sheng et al., 2010). The variations in protein and carbohydrate
288 concentrations are shown Figure 3. The average protein and polysaccharide concentrations were
289 3.95 ± 0.37 mg/L and $11.79 \text{ mg/L} \pm 2.44$ mg/L [$n = 24$] in the aerobic MBR stage, while in the MBR
290 effluent were 1.13 ± 0.18 mg/L and 4.09 ± 0.38 mg/L, respectively. This finding agrees with Juang et al.
291 (2013) who investigated the effects of SMP on MBR fouling potential, and reported that the average
292 protein and carbohydrate concentrations were 2.32 and 12.07 mg/L in the MBR supernatant, while in
293 the MBR effluent were 1.14 and 7.39 mg/L, respectively. Moreover, a higher average concentration
294 ($P < 0.05$) of protein (1.46 mg/L) and polysaccharide (4.68 mg/L) was observed in the MBR_{pharma}
295 effluent compared to that in the MBR_{control} (1.03 mg/L for protein; 4.34 mg/L for polysaccharides),

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

300 Production and consumption of soluble organics are dynamic processes, thus the concentrations
301 measured at any point in time present a momentary equilibrium, which can easily be disturbed and
302 shifted by changes in the environment (Rosenberger et al., 2006). Substrate utilization, biomass decay,
303 and EPS hydrolysis are believed to be the major processes contributing to SMP formation (Fenu et al.,
304 2010). It is assumed that the analysed organics analysed were part of the bacterial EPS that was
305 transferred into the liquid phase of the activated sludge, and thus form the soluble EPS or SMP by a
306 variety of different mechanisms. Considering the fact that proteins and carbohydrates are the
307 dominant constituents of cell walls (Pérez Silva et al., 2009), introduction of pharmaceuticals may
308 disturb cellular function and damage cell membranes, and inevitably lead to cell lysis and an increase
309 in SMP concentration. In addition, EPS/SMPs play a key role in protecting the inner microorganisms
310 against environmental stress (Sheng et al., 2010). In the presence of toxic substances, microbial cells
311 in activated sludge and biofilms utilized the substrate to generate more EPS, which act as a diffusional
312 barrier between the cell wall and extreme environments to protect the cells from the harsh
313 environment.

314

315 3.3. Molecular weight (MW) distribution of SMPs

316

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

322 As shown in Figure 4, although the location of the major peaks was similar, the relative intensities
323 of the major peaks in the two MBRs were different. Compared to MBR_{control}, a significant increase in
324 the intensities of macro- (13,091 kDa and 1,587 kDa) and intermediate-MW (189 kDa) compounds in
325 the anoxic MBR_{pharma} was observed, implying that the presence of pharmaceuticals enhanced the
326 accumulation of high- and intermediate fractions in the MBR_{pharma} during start-up stage. A similar
327 result was also found by Aquino and Stuckey (2004) who revealed the presence of toxic compounds
328 (Cr and CHCl₃) caused a higher accumulation of SMPs with high-MWs. Likewise, Avella et al.
329 (2010) indicated that the presence of a cytostatic drug (cyclophosphamide) caused a significant
330 increase in SMPs in MBR supernatants. The presence of toxic compounds (e.g., pharmaceuticals)
331 tends to cause cell lysis and release intracellular high MW SMPs in response to environmental stress
332 (Aquino and Stuckey, 2004). Hence, EPS in a non-hydrolysed form may also constitute part of the
333 high MW SMP (Aquino and Stuckey, 2004). In addition, although many SMPs produced during
334 biological treatment were degraded, high MW compounds exposed to a toxic environment were likely
335 to be degraded more slowly and result in an increase in SMPs (Chen et al., 2014).

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

344

345 3.4. Excitation emission matrix (EEM) fluorescence contours

346

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

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

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

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

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

390

391 3.5. Identification and characterization of SMPs using GC-MS

392

393 3.5.1 SMPs in the aerobic stage

394

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

403 esters such as heneicosane (19.8%), hexadecanoic acid, butyl ester (18.4%) and tetratetracontane
404 (10.4%) were a significant percentage of the total compounds present. In particular, these long-chain
405 carbohydrates (or alkanes) and esters are frequently reported in biological treatment effluent, and are
406 known to be the main components of low-MW SMPs in aerobic reactors (Janga et al., 2007; Liang et
407 al., 2007).

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

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

430 SMPs generated in the treatment of pharmaceutical wastewater. In addition, the presence of
431 pharmaceuticals also influenced the dominant types of compounds present in wastewater. Alkanes
432 were the most common SMP (51%) in the MBR_{control}, while aromatics were the most dominant SMPs
433 (40%) in the MBR_{pharma}. This suggests that more refractory SMPs are produced in the presence of
434 pharmaceuticals, because the aromatic compounds are generally more recalcitrant and therefore
435 represent a major fraction of residual compounds in the MBR_{pharma}.

436

437 3.5.2 SMPs in the MBR effluent

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

452 A significant decrease in aromatics (from 16 to 7) in the MBR_{pharma} permeate was observed,
453 compared to the MBR_{pharma} aerobic mixed liquor. Aromatic SMPs such as benzoic acid, dl-alanyl-l-
454 leucine, glycyl-L-proline, formamide, (2-acetylphenyl)-, and 1h-1-benzazepine, 2,3,4,5-tetrahydro-,
455 were only detected in the aerobic stage and disappeared in the permeate. This finding is different from
456 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

483 In the present study, the accumulation, composition, and characteristics of SMPs was examined in
484 an MBR treating wastewater containing pharmaceutical compounds. The exposure of biomass to
485 pharmaceutical compounds increased the production of SMPs, and a higher average concentration ($P >$
486 0.05) of protein (1.46 mg/L) and polysaccharides (4.68 mg/L) was observed in the MBR_{pharma} effluent
487 compared to that in the MBR_{control} (1.03 mg/L for protein; 4.34 mg/L for polysaccharides). HPLC-
488 SEC analysis revealed that the presence of pharmaceuticals enhanced the accumulation of high- and
489 intermediate MW fractions in the MBR_{pharma}. Measurements of EEM fluorescence spectra indicated
490 that exposure to pharmaceuticals seemed to stimulate the production of tyrosine and tryptophan
491 containing solutes. GC-MS analysis revealed that there were clear differences in the SMPs between
492 the MBR_{control} and MBR_{pharma} in terms of the number of compounds, predominant types of organics,
493 their concentration and molecular weight, biodegradability and recalcitrance, implying that the
494 presence of pharmaceutical compounds have caused a radical shift in the SMPs produced.

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