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**Organic micropollutants in aerobic and anaerobic membrane bioreactors: Changes
in microbial communities and gene expression**

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1 **Abstract**

2 Organic micro-pollutants (OMPs) are contaminants of emerging concern in wastewater
3 treatment due to the risk of their proliferation into the environment, but their impact on
4 the biological treatment process is not well understood. The purpose of this study is to
5 examine the effects of the presence of OMPs on the core microbial populations of
6 wastewater treatment. Two nanofiltration-coupled membrane bioreactors (aerobic and
7 anaerobic) were subjected to the same operating conditions while treating synthetic
8 municipal wastewater spiked with OMPs. Microbial community dynamics, gene
9 expression levels, and antibiotic resistance genes were analyzed using molecular-based
10 approaches. Results showed that presence of OMPs in the wastewater feed had a clear
11 effect on keystone bacterial populations in both the aerobic and anaerobic sludge while
12 also significantly impacting biodegradation-associated gene expression levels. Finally,
13 multiple antibiotic-type OMPs were found to have higher removal rates in the anaerobic
14 MBR, while associated antibiotic resistance genes were lower.

15

16 **KEYWORDS:** Trace organic compounds; bioreactor; transcriptomics; antibiotic
17 resistance genes; MBR

18

19 **Abbreviations**

20 AOB, ammonia-oxidizing bacteria; ARG, antibiotic resistance gene; BDG,
21 biodegradation gene; CAS, conventional activated sludge; COD, chemical oxygen
22 demand; DEET, diethyltoluamide; HRT, hydraulic retention time; LC-MS/MS, liquid
23 chromatography tandem mass spectrometry; LCFA, long chain fatty acid; MBR,
24 membrane Bioreactor; MG-RAST, metagenomic rapid annotation using subsystems

25 technology; MLSS, mixed liquor suspended solids; mMDS, metric multidimensional
26 scaling; MWCO, molecular weight cutoff; NF, nanofiltration; NOB, nitrite-oxidizing
27 bacteria; OMP, organic micropollutants; OTU, operational taxonomic unit; PAC, power
28 activated carbon; PBS, phosphate buffered saline; pKa, acid dissociation constant; PCA,
29 principal component analysis; qPCR, quantitative polymerase chain reaction; RDP,
30 ribosomal database project; SBR, sequencing batch reactor; TCEP, tris(2-
31 chloroethyl)phosphate; TDCPP, tris(1,3-dichloroisopropyl)phosphate; UF, ultrafiltration

32 **1. Introduction**

33 Recent advances in aerobic membrane bioreactor (MBR) technology have led to the
34 widespread implementation of full-scale MBR systems for municipal wastewater
35 treatment. Anaerobic MBRs have not yet been successfully applied at a large scale for the
36 treatment of low-strength (e.g., domestic) wastewaters, but research interest in the subject
37 remains high (Smith et al., 2012). The advantages of MBRs when compared to
38 conventional wastewater treatment are well established and include small treatment plant
39 footprints and high quality effluent.

40
41 However, an emerging concern surrounding wastewater treatment is the issue of organic
42 micropollutant (OMP) removal rates and their proliferation into the environment (Bolong
43 et al., 2009). OMPs are present in municipal wastewater due to household use of
44 pharmaceuticals, antibiotics, and other personal care products. Combined waste streams
45 that include hospitals and other facilities producing high OMP concentration effluent
46 amplify the potential impact of these compounds (Verlicchi et al., 2015). Many previous

47 studies have addressed removal of OMPs in conventional activated sludge (CAS)
48 treatment systems in comparison with aerobic MBRs (Cirja et al., 2008; De Wever et al.,
49 2007; Radjenović et al., 2009; Zuehlke et al., 2006). Generally, these studies found that
50 removal rates of OMPs were significantly affected by treatment plant operating
51 conditions and that, overall, aerobic MBR systems were more efficient than CAS in the
52 removal of OMPs. The issue of OMPs in anaerobic MBRs has only recently developed as
53 a topic of interest and will become increasingly important as anaerobic MBR technology
54 evolves as a suitable municipal wastewater treatment option (Monsalvo et al., 2014;
55 Wijekoon et al., 2015).

56

57 Despite the essential role that microbes play in both aerobic and anaerobic wastewater
58 treatment, studies characterizing the relationship between OMPs and the microbial
59 communities of those systems have been limited (Fang et al., 2013). This is mainly due to
60 the fact that, at trace levels, these compounds exhibit negligible toxicity or antibacterial
61 effect to change the viability and performance of biological treatment systems
62 (Radjenović et al., 2009). Both aerobic and anaerobic wastewater treatment technologies
63 are generally very robust once acclimatized, therefore a lack of impact by OMPs on
64 overall performance does not necessarily imply a lack of effect on microbial dynamics
65 and their associated gene expressions. Given the inherent differences between the core
66 microbial communities of aerobic and anaerobic reactors, it is likely that those OMP
67 compounds would have unique influences on the microbial community structures and
68 gene expression in each system. Considering the recent increase in aerobic MBRs used
69 for municipal wastewater treatment and a growing interest in anaerobic MBRs for similar

70 applications, there is a need for further understanding of the effect of OMPs on each of
71 the two systems.

72

73 Specifically, among the OMPs that are commonly found in untreated wastewater,
74 antibiotics are of interest due to their potential to facilitate antibiotic resistance gene
75 (ARG) propagation and transfer (Hong et al., 2013). The influence of these OMPs on
76 ARG abundance is especially critical in high concentration microbial environments such
77 as biological wastewater treatment systems and their effluents. Although several recent
78 studies have focused on the fate and persistence of ARGs in CAS and anaerobic digestion
79 systems (Burch et al., 2015; Christgen et al., 2015; Yang et al., 2014a; Zhang et al.,
80 2015), no research to date has compared the effect of specific antibiotic-type OMPs on
81 associated ARGs in aerobic versus anaerobic MBR systems. Furthermore, no studies
82 have been conducted that assess the impact of OMPs on microbial communities and their
83 gene expression in MBR systems. As a result, the present study was designed to examine
84 the effects of the presence and accumulation of various OMPs on the microbial
85 communities in lab scale aerobic and anaerobic MBRs by using high-throughput 16S
86 rRNA gene sequencing, metatranscriptomics, and quantitative PCR to analyze the core
87 microbial communities, gene expression profiles, and ARG abundance, respectively.

88 **2. Material and methods**

89 **2.1. Description of treatment systems and operational conditions**

90 This study compared two different lab-scale wastewater treatment systems; an aerobic
91 sequential batch reactor (SBR) and an anaerobic MBR operated with a side-stream

92 ultrafiltration (UF) membrane. Schematic diagrams of each system are shown in Figures
93 S1 and S2 of the Supplementary Data and have been detailed previously (Wei et al.,
94 2015a; Wei et al., 2015b). Reactors were operated and sampled in 2 primary phases: (1)
95 without any additional membrane separation and (2) with a nanofiltration (NF)
96 membrane downstream of the reactor effluent (flat-sheet DOW NF90, 200-400 Da
97 MWCO). These 2 phases are subsequently referred to as Phase 1 and Phase 2. Phase 1
98 was 55-60 days in duration while Phase 2 was 25-30 days. At the end of Phase 2, a single
99 dose (100 mg/L) of powder activated carbon (PAC) was added to the sludge of each
100 reactor to assess its effect on OMP removal. Operational conditions were maintained
101 based on Phase 2 parameters for 10 days after the addition of PAC. Reactors had working
102 volumes of 2 L and were maintained at pH 7. pH was monitored and controlled
103 continuously by a built-in pH controller using 1M NaOH and 1M HCl. The aerobic and
104 anaerobic reactors were maintained at 20 °C and 35 °C, respectively to represent typical
105 operating conditions for each system. Hydraulic retention times (HRTs) of both systems
106 were set at 12 h during Phase 1. Upon the addition of the NF membrane to both systems
107 in Phase 2, HRTs were increased to 24 h due to trans-membrane flux limitations. To
108 maintain reactor organic loading rates of 0.8 g/L/d throughout operation, influent
109 chemical oxygen demand (COD) was set at 400 mg/L and 800 mg/L for Phases 1 and 2,
110 respectively. The synthetic wastewater was made up of a mix of organic and inorganic
111 compounds and trace metals, as summarized in Table S1. A cocktail of OMP compounds
112 was spiked into the feed synthetic wastewater at individual compound concentrations of
113 10-20 µg/L for Phase 1 (12 h HRT) and 20-40 µg/L for Phase 2 (24 h HRT) to maintain
114 consistent OMP loading rates to each system. Samples were also taken from reactor

115 sludges before the commencement of OMP spiking in Phase 1 and after the addition of
116 PAC at the end of Phase 2. The cocktail of spiked OMPs consisted of a mixture of
117 pharmaceutical compounds, antibiotics, personal care products, and pesticides that are
118 commonly detected in raw wastewater (Teerlink et al., 2012). Those compounds included
119 acetaminophen, amitriptyline, atenolol, atrazine, bezafibrate, bisphenol A, caffeine,
120 carbamazepine, clofibrac acid, dilantin, diclofenac, diethyltoluamide (DEET),
121 diphenhydramine, fluoxetine, gemfibrozil, ibuprofen, iopromide, methylparaben,
122 naproxen, oxybenzone, primidone, propylparaben, sucralose, sulfamethoxazole,
123 trimethoprim, tris(2-chloroethyl)phosphate (TCEP), and tris(1,3-
124 dichloroisopropyl)phosphate (TDCPP). The chemical properties of these OMPs and their
125 skeletal structures are presented in Table S2 and Figure S3, respectively.

126 **2.2. Liquid chromatography – mass spectrometry**

127 OMP compound concentrations were determined by liquid chromatography coupled
128 tandem mass spectrometry (LC-MS/MS). Samples were analyzed using an Agilent
129 Technology 1260 Infinity Liquid Chromatography unit with AB SCIEX QTRAP 5500
130 mass spectrometer (Applied Biosystems) as previously described (Wei et al., 2015a).
131 Isotopically labeled standards of each OMP compound were spiked prior to solid phase
132 extraction. Recoveries of all target compounds were determined to be > 80% after
133 corrections based on isotope recovery rates. Standard deviations for all compounds used
134 in this study were < 20% of detection values.

135 **2.3. Amplicon-based next-generation sequencing**

136 1 mL of sludge from each reactor was sampled every one to two weeks. Samples were
137 pelleted by adding 0.6 mL of 1X PBS solution and centrifuging for 10 min at 9400 g,
138 then subsequently stored at -20 °C. Genomic DNA was extracted from the stored reactor
139 biomass using the UltraClean® Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad,
140 CA) with slight modifications to the protocol by adding lysozyme and achromopeptidase
141 to the lysis buffer (Hong et al., 2011). PCR amplification of the extracted DNA was
142 performed with barcoded forward primer 515F (5'-GTGYCAGCMGCCGCGGTA-3')
143 and reverse primer 909R (5'-CCCCGYCAATTCMTTTRAGT-3') based on thermal
144 cycling conditions previously described (Al-Jassim et al., 2015). PCR amplicons were
145 gel-purified with Wizard SV Gel and PCR Clean-up system (Promega, Madison, WI).
146 Concentrations were determined by Qubit fluorometer (Invitrogen, Carlsbad, CA).
147 Equimolar concentrations of the samples were mixed, and then submitted to KAUST
148 Genomics Core lab for Ion Torrent sequencing (Life Technologies, Carlsbad, CA) on 318
149 chips. Sequences obtained from Ion Torrent platforms were processed and analyzed using
150 the Ribosomal Database Project (RDP) Classifier, operational taxonomic unit (OTU)-
151 based sequence sorting, and Primer E Version 7 Software as described previously (Harb
152 et al., 2015). All Ion Torrent sequencing files were deposited in the Short Read Archive
153 (SRA) of the European Nucleotide Archive (ENA) under study accession number
154 PRJEB12338.

155 **2.4. Quantitative PCR (qPCR)**

156 qPCR was performed to determine the abundances of known antibiotic resistance genes
157 present in the reactor sludge. *sul1* and *sul2* genes were targeted due to their association

158 with sulfamethoxazole resistance while *dfrA5* and *dfrA14* genes were targeted due to their
159 association with trimethoprim resistance. The *intI1* gene, which encodes for the type 1
160 integrase enzyme (Na et al., 2014), is commonly associated with the horizontal
161 propagation of resistance genes and was also targeted. Details of the qPCR protocol,
162 primer sequences, plasmid preparation and standard curves are provided in the
163 Supplementary Data as Appendix B.

164 **2.5. Metatranscriptomics**

165 6 mL of sludge from each reactor was sampled every one to two weeks. Samples were
166 pelleted by adding 4 mL of 1X PBS solution and centrifuging for 10 min at 9400 g, then
167 subsequently stored in 10 mL RNeasy RNA stabilization solution (Qiagen, Valencia,
168 CA) at -20 °C. Biomass was extracted for total RNA using the RNeasy Midi Kit (Qiagen)
169 following the manufacturers' protocol. Total RNA was further enriched for mRNA by
170 removing ribosomal RNA using the Epicentre Ribo-Zero Magnetic rRNA Removal Kit
171 for bacteria (Illumina, Madison, WI). RNA quality control was performed using the 2200
172 TapeStation bioanalyzer (Agilent Technologies, Santa Clara, CA) and concentrations
173 were quantified by qPCR using the Applied Biosystems® 7900HT Fast Real-Time PCR
174 system (Thermo Fisher Scientific, Carlsbad, CA). RNA sequencing was performed on the
175 Illumina HiSeq 2000 platform by the KAUST Genomics Core Lab team using standard
176 protocols. In total, 28 samples were run on two lanes. Read libraries were constructed
177 using the TruSeq Stranded Total RNA Library Preparation Kit (Illumina). Low quality
178 sequence reads (i.e. Phred score <20) were removed and sequence adapters were trimmed
179 from all raw reads. For each sequence file, contigs were assembled from the raw

180 sequences using the Short Oligonucleotide Analysis Package (SOAP) de novo algorithm
181 (Luo et al., 2012). Assembled sequence files were further filtered of contigs shorter than
182 500 bp. Gene sequence contigs were analyzed using the NCBI BLASTX algorithm
183 against an existing biodegradation gene (BDG) database as previously described (Fang et
184 al., 2014). BLASTX results were sorted and filtered based on a minimum e-value of
185 1×10^{-5} . Sequence files were further uploaded to the MG-RAST server and analyzed for
186 functional gene abundance by annotating sequences on the Subsystems database based on
187 an e-value of $< 1 \times 10^{-5}$, minimum identity cutoff of 60%, and minimum alignment length
188 of 30 nt. All transcriptomic sequences are available on the MG-RAST online server under
189 project ID number 13768 and accession numbers 4635665 through 4635692.

190 **3. Results & Discussion**

191 **3.1. Overall Reactor Performances and OMP removal efficiency**

192 Both aerobic and anaerobic reactors showed consistent COD removals from the synthetic
193 municipal wastewater (400–800 mg COD/L) of higher than 90% throughout operation in
194 both Phases 1 and 2. Upon addition of the NF membrane in Phase 2, total COD removal
195 of each system increased to above 98% due to the higher rejection of refractory organics
196 (Wei et al., 2015a; Wei et al., 2015b). Reactor effluent COD concentrations dropped by
197 10–15% for the initial 1–3 days after the addition of PAC in Phase 2 before subsequently
198 returning to pre-PAC levels. This was likely due to the limited sorption capacity of the
199 PAC dosage used (100 mg/L). Biomass concentrations in both reactors were relatively
200 stable at 3–4 g/L MLSS for the aerobic system and 5–7 g/L MLSS for the anaerobic
201 system. Differences in sludge concentrations are known to impact overall sorption

202 capacities, however the relative MLSS stability and lack of sludge wasting (except as
203 necessary for sampling) in each system likely limited its overall effect on removal rates.
204 In the anaerobic MBR, biogas production was stable with over 70% methane content
205 throughout operation and was not affected by OMP presence or accumulation. The
206 consistency in performance of each reactor regardless of the presence of OMPs in the
207 feed or their accumulation within the system (after the addition of NF membrane)
208 reiterates similar findings of previous studies (Monsalvo et al., 2014; Tadkaew et al.,
209 2011). Due to the nature of these compounds in municipal wastewater, OMPs in MBRs
210 typically would not exceed trace level concentrations ($<50 \mu\text{g/L}$) and thus are unlikely to
211 affect the overall efficiency of these treatment systems (i.e., no toxic effect on the
212 microbial communities) (Radjenović et al., 2009).

213

214 Removal rates of individual OMPs varied between the aerobic and anaerobic systems.
215 The addition of the NF membrane in Phase 2 provided a high rejection of all OMP
216 compounds ($>75\%$), which resulted in the decoupling of compound retention rates from
217 the HRT of each reactor. This allowed for the accumulation of OMPs in the system and
218 an increased exposure to the microbial community. Based on these two operational
219 phases, the effect of OMPs on each system was evaluated under both constant
220 concentration ($10\text{-}20 \mu\text{g/L}$, Phase 1) and compound accumulation conditions ($20\text{-}40 \mu\text{g/L}$
221 with NF membrane rejection, Phase 2).

222 3.2. Effect of OMPs on reactor microbial communities

223 Although no significant changes were observed in reactor performances between pre-
224 OMP conditions and the different operational phases, relative abundance of the microbial
225 communities of the aerobic and anaerobic systems were impacted by both OMP
226 introduction (Phase 1) and accumulation (Phase 2). To illustrate these changes, a metric
227 multidimensional scaling plot (mMDS) was generated for each of the two reactors
228 (Figure 1A and 1B). The mMDS plots were generated using bootstrapped averages to
229 ensure the statistical reliability of each sample set (i.e., by evaluating the significance of
230 each sample set within itself by linear regression). This plot utilizes a distance matrix
231 applied to all samples by which each is represented as a point in the two-dimensional
232 space. Samples with higher community similarity are closer in proximity in the mMDS
233 and *vice versa*. The communities of each reactor showed clear clustering based on both
234 phases of operation, as well as strong separation from the sample taken before the
235 addition of OMPs to the synthetic wastewater feed and those taken after the addition of
236 PAC to the reactor biomass. Only one sample point was taken from each reactor prior to
237 the introduction of OMPs to the wastewater in Phase 1. This, along with the lack of
238 control reactor systems, allows for limited interpretation of the microbial community
239 dynamics under OMP-free conditions. Nonetheless, the strong clustering of each
240 operational phase in the mMDS plots implies a clear effect of OMPs on the microbial
241 community structures. Given the clear separation of samples at phase transitions (e.g., the
242 end of Phase 1 and the start of Phase 2), temporal variation did not appear to contribute to
243 the differences between phases as a dominant factor. To further evaluate how these
244 changes in OMP loading affected the communities of each of the systems, the core

245 microorganisms of both the aerobic and anaerobic processes were investigated at the
246 species level.

247 3.2.1. Aerobic System

248 The aerobic sludge experienced significant shifts in microbial community relative
249 abundance between pre-OMP conditions, Phase 1, and Phase 2 of the experiment. Core
250 bacterial genera known to be dominant in CAS processes are well established in the
251 literature (Zhang et al., 2012). Several OTUs closely related to these bacterial groups
252 were significantly affected by the introduction of OMPs to the wastewater feed (Phase 1)
253 and the accumulation of OMPs in the reactor system (Phase 2). Among those bacteria
254 was the species *Lysobacter dokdonensis*, which declined from over 25% relative
255 abundance of the total microbial community in pre-OMP conditions to 9.2% in Phase 1
256 and 2.1% in Phase 2 (unpaired *t*-test $P < 0.01$). Conversely, bacteria that showed
257 significant increases in relative abundance across the phases of operation were
258 *Terrimonas lutea* and *Ferruginibacter alkalilentus* (Table 1A). Bacteria of these genera
259 have been previously linked to membrane fouling in aerobic MBRs (Xiong et al., 2016),
260 suggesting a possible connection between OMP presence in wastewater and increased
261 fouling potential. The increase in relative abundance of *F. alkalilentus* from below 0.40%
262 in pre-OMP conditions to above 6.5% during Phase 2 could potentially be attributed to its
263 alkaliphilic nature, as several previous studies have shown correlations between
264 alkaliphilic bacteria and enhanced biodegradation of organic compounds (Kanekar et al.,
265 1998; Sarethy et al., 2011). Furthermore, the pKa values of the majority of the OMPs
266 used in this study were greater than 4, implying either alkaline or weakly acidic

267 properties (Table S2), which could have contributed to a favorable environment for such
268 bacteria.

269

270 Two bacterial species that are known to be responsible for floc formation in CAS,
271 *Trichococcus flocculiformis* and *Ottowia pentelensis*, increased in relative abundance in
272 the aerobic system upon the introduction of OMPs in Phase 1, but declined significantly
273 during Phase 2 (unpaired *t*-test $P < 0.01$ and $P = 0.06$, respectively). This suggests that
274 the presence of OMPs but not their accumulation in the reactor could have enhanced the
275 presence of these floc-forming bacteria. This is despite the fact that *T. flocculiformis* is a
276 filamentous bacterium that could ultimately contribute to sludge bulking, while *O.*
277 *pentelensis* is a rod-shaped bacterium that would likely be involved in the early stages of
278 floc formation (Felföldi et al., 2011; Scheff et al., 1984). Additionally, OTUs associated
279 with both ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB)
280 indicated a positive reaction to the presence of OMPs. *Nitrosomonas ureae* increased
281 from $<0.01\%$ relative abundance in the pre-OMP community to 0.56% in Phase 1 and
282 1.8% in Phase 2. *Nitrobacter hamburgensis* experienced a similar but less drastic increase
283 in relative abundance between pre-OMP conditions and Phases 1 and 2 (Table 1A). The
284 significant increase in relative abundance of *N. ureae* could be attributed to the
285 capabilities of AOB to oxidize, in addition to ammonia, a large variety of organic
286 compounds such as OMPs (Tran et al., 2013). Approximately half of the OMPs that were
287 used in this study were potential nitrogen sources, containing derived states of ammonia
288 such as amines and amides (Figure S3). Several of these compounds, such as fluoxetine
289 and DEET, were readily biodegradable in the aerobic system (Wei et al., 2015b).

290 Biodegradation of these compounds likely activated similar pathways to those utilized in
291 the oxidation of ammonia and/or nitrite, potentially enriching for AOBs and NOBs.

292 3.2.2. Anaerobic system

293 The different OMP conditions also resulted in significant relative abundance changes of
294 the dominant bacteria of the anaerobic sludge. Bacterial groups that were affected
295 included acetogenic, fermentative, and syntrophic bacteria (Table 1B), all of which are
296 essential to anaerobic digestion. To illustrate, OTUs associated with the acetogenic
297 bacteria *Lutispora thermophila* decreased in relative abundance from over 34% in the
298 pre-OMP phase to 25% in Phase 1 and 12% in Phase 2 (unpaired *t*-test $P < 0.05$). The
299 fermentative bacteria *Petrimonas sulfuriphila* experienced a similar decrease in relative
300 abundance from 15% in pre-OMP conditions to less than 1% in Phase 2. These changes
301 coincided with significant increases in other fermentative bacteria such as *Alkalitalea*
302 *saponilacus*, *Microbacter margulisiae*, and *Prolixibacter bellariivorans*. The OTUs
303 associated with these 3 species made up a combined 6.8% of total sequences prior to
304 OMP addition and increased to a combined 39% of total sequences in Phase 2. The
305 increase of the alkaliphilic *A. saponilacus* further elucidates the potential role of
306 alkaliphilic bacteria in the biodegradation of OMPs previously discussed for the aerobic
307 system, especially considering that all of the compounds determined to be highly
308 biodegradable in the anaerobic system had pKa values of greater than 5 (Wei et al.,
309 2015a). These variations in the fermentative bacterial community and their relation to
310 OMP presence in the wastewater were seen although sulfate reducers, nitrate reducers,

311 and methanogens are regarded as the main anaerobic microbial groups responsible for
312 trace organic compound removal (Wijekoon et al., 2015).

313

314 Denitrifying and sulfate reducing bacteria were not generally present in the anaerobic
315 sludge (<0.01%); however several methanogenic genera were detected at varying relative
316 abundances. *Methanobacterium*, a hydrogenotrophic methanogen, was determined to be
317 the most abundant archaeal genus in the reactor sludge at an average relative abundance
318 of >1% of total microbial community throughout the experiment. *Methanosaeta*
319 (<0.20%) and *Methanospirillum* (<0.10%) were also consistently detected in the sludge.

320 No significant changes based on OMP presence or phase of operation were observed for
321 any of the methanogens. However, *Smithella propionica*, a syntrophic bacterium known
322 to grow in co-culture with hydrogenotrophic methanogens (Liu et al., 1999), did
323 experience a significant increase in relative abundance from pre-OMP conditions (0.84%)
324 to Phase 1 (2.5%) and Phase 2 (3.3%). This coincided with previously described
325 increases in *Microbacter margulisiae*, a propiogenic bacterium that could have
326 contributed to the rise in *S. propionica* relative abundance by serving as a substrate
327 source. Relative abundances of *S. propionica* in a similar system that was operated
328 without OMP presence were comparable (<0.50%) to those observed in pre-OMP
329 conditions (Harb et al., 2015), suggesting that the presence of OMPs potentially increases
330 syntrophic interactions in anaerobic MBR systems. Other syntrophic bacterial genera also
331 exhibited OMP-related changes in relative abundance (Figure S4). *Syntrophobacter*
332 decreased from >2% in pre-OMP conditions to <0.50% in Phases 1 and 2. Conversely,
333 unclassified Syntrophaceae increased from <1% in pre-OMP conditions to above 3.5%

334 and 4.0% relative abundance in Phase 1 and Phase 2, respectively. *Syntrophomonas* was
335 also detected (<0.10%) in the anaerobic sludge but did not show any significant variation.
336 The family Syntrophaceae includes *Smithella* and *Syntrophus*, both of which are known
337 to grow in co-culture with hydrogen-utilizing methanogens, such as *Methanobacterium*.
338 These observations imply that the presence of OMPs at levels found in municipal
339 wastewater would select for specific fermentative and syntrophic bacterial groups without
340 affecting the overall functionality of anaerobic MBR systems, methanogen relative
341 abundance, or biogas production.

342 **3.3. Changes in biodegradation gene expression**

343 **3.3.1. Gene group-based analysis**

344 Recent research has given insight into the importance of biodegradation of OMPs as a
345 primary removal mechanism in both aerobic and anaerobic wastewater treatment systems
346 (Radjenović et al., 2009; Vasiliadou et al., 2013). The present work examined more
347 specifically the expression of genes responsible for biodegradation of organic
348 compounds. Metatranscriptomic data from sludge samples of both reactors were analyzed
349 against a biodegradation gene (BDG) database to determine the abundance of specific
350 genes groups across different phases of operation, and to infer potential links between the
351 upregulation of genes and the biodegradability of specific OMPs. Removal by
352 biodegradation was evaluated for a majority of the OMPs by each system in previously
353 published studies that examined the same aerobic and anaerobic systems (Wei et al.,
354 2015a; Wei et al., 2015b).

355 Results of principal component analyses (PCA) of the most prevalent BDGs (>0.10% of
356 total contigs) in both systems revealed that expression levels of these genes were affected
357 by the introduction of OMPs to the system (Phase 1) and their accumulation (Phase 2)
358 (Figure 1C and 1D). BDGs that showed the strongest correlations (Pearson's coefficient ρ
359 > 0.7) with operational phase in the aerobic system were the *bphA1*, *lip*, *carA*, *glx*, and
360 *p450* genes, while in the anaerobic system the *bphA1*, *xylA* and *glx* genes showed a
361 similar degree of correlation. The dominant BDGs in terms of expression levels (> 0.20%
362 relative abundance) were the same for the aerobic and anaerobic reactors and included
363 *carA*, *glx*, *lip*, *p450*, and *ppo* gene groups. However, these genes reacted differently to the
364 phases of operation in each system, showing high variability in the aerobic versus the
365 anaerobic system (Figure 2).

366

367 In the aerobic system, total BDG transcript relative abundance showed no significant
368 change upon the introduction of OMPs in Phase 1 and decreased between Phase 1 and
369 Phase 2 from 2.6% of total transcripts to 2.0% (unpaired *t*-test $P = 0.01$). The most
370 abundant BDG in the aerobic reactor was the *p450* gene, which increased steadily in
371 relative expression from 0.87% in pre-OMP conditions to 1.5% at the end of Phase 1.
372 This gene decreased again in relative abundance in Phase 2 to an average of 0.80% of
373 total transcripts (Figure 2). Other dominant BDGs that showed similar decreases in
374 relative expression between Phase 1 and Phase 2 in the aerobic system were the *glx* and
375 *ppo* genes, declining from 0.51% and 0.55% relative abundance to 0.20% and 0.25%,
376 respectively. *carA* genes decreased from 0.3% of total sequences in pre-OMP conditions

377 to below 0.10% at the end of Phase 1 and throughout Phase 2 (unpaired t -test $P < 0.05$)

378 while *lip* genes showed a similar but less significant reduction.

379

380 For the anaerobic system, total BDG expression also showed no significant changes in

381 relative abundance upon the addition of OMPs in Phase 1, but increased from 1.51% of

382 total transcripts in Phase 1 to 1.81% in Phase 2 (unpaired t -test $P < 0.01$). Average *glx*

383 gene expression in the anaerobic reactor increased between Phase 1 and Phase 2 from

384 0.05% of total transcripts to 0.15% (unpaired t -test $P = 0.01$), while *ppo* and *carA* gene

385 expression showed no significant changes. Similarly to the aerobic system, the *p450* gene

386 was also the most abundant BDG in the anaerobic MBR system, although with less

387 variability, increasing from 0.55% to 0.65% of total sequences between Phase 1 and

388 Phase 2 (unpaired t -test $P < 0.01$).

389

390 A previous study on organic pollutant BDGs in CAS also found the *p450* gene to be the

391 most abundant BDG in the activated sludge (Fang et al., 2013). A similar study on BDGs

392 in freshwater and marine sediments found that entirely different gene groups were

393 dominant in those environments (Fang et al., 2014), suggesting that activated sludge

394 systems likely favor biodegradation via the cytochrome *p450* pathway. The present study

395 substantiates these findings from a gene expression perspective for aerobic and anaerobic

396 sludge. The higher variability of *p450* expression, and of the dominant BDGs in general,

397 in the aerobic system as compared to the anaerobic system could be attributed to the

398 slower growth conditions of the anaerobic sludge, which support general functional

399 stability even with varying microbial community structure (Fernandez et al., 2000).

400

401 Dissimilarities between the gene abundance response levels of the two systems may have
402 been attributed to differences in enzyme pathway association within each gene group. To
403 investigate, contigs that returned positive matches to BDGs in the database with an
404 identity similarity of >80% were sorted for each reactor system. For example, in the
405 aerobic system, *p450* genes associated with hydroxylase and monooxygenase were most
406 abundant, while ferredoxin reductase-associated *p450* genes were predominant in the
407 anaerobic sludge. *carA* genes also differed between the two systems, with the majority of
408 *carA* genes in the aerobic system being affiliated with carbazole dioxygenase and ring-
409 hydroxylating dioxygenase (RHD) and the majority in the anaerobic system being
410 associated with nitrite reductase and FAD reductase.

411

412 Other gene groups, including *lip*, *ppo*, and *bphA1*, showed continuity between the aerobic
413 and anaerobic system. *lip* genes were associated with peroxidase or catalase enzymes,
414 which are responsible for the polymerization of phenols (Nicell et al., 1993), and showed
415 significantly higher relative abundance in the anaerobic reactor, making up 0.46% of total
416 transcripts as compared to 0.25% in the aerobic sludge (unpaired *t*-test $P < 0.0001$). *ppo*
417 genes in both systems were generally affiliated with laccase and copper oxidases, which
418 are commonly utilized in bioremediation applications due to their ability to also oxidize
419 phenols, such as bisphenol A (Mukherjee et al., 2013; Suresh et al., 2008; Yang et al.,
420 2014b). Bisphenol A and 3 other phenol-containing OMPs were used in this study
421 (Figure S3). The observed *ppo* gene expression potentially played a role in the

422 biodegradation of bisphenol A in the aerobic system, as the compound showed high
423 biological removal (>60%) for the majority of both Phases 1 and 2 (Wei et al., 2015b).

424 3.3.2. Gene function-based analysis

425 Further analysis of the transcripts from the MBR systems using the MG-RAST
426 subsystems database reiterated the positive effect of OMP accumulation in Phase 2 of
427 operation on biodegradation-related gene expression in the anaerobic MBR (Table 2).
428 Gene expression associated with the metabolism of aromatic compounds increased by
429 24% from Phase 1 (1.10% of total genes) to Phase 2 (1.37% of total genes) in the
430 anaerobic reactor (unpaired *t*-test $P < 0.05$), while genes associated with that function
431 showed no significant differences in expression levels in the aerobic reactor (unpaired *t*-
432 test $P > 0.8$). It was determined that genes responsible for n-phenylalkanoic acid
433 degradation, and more specifically those related to the production of long-chain-fatty-
434 acid (LCFA) CoA ligase, were the primary gene groups that significantly increased
435 between Phase 1 and Phase 2 in the anaerobic system, increasing by 39% and 35%,
436 respectively (unpaired *t*-test $P < 0.01$ and $P < 0.05$). Although present in the aerobic
437 sludge, neither of these functional gene groups showed any significant changes across its
438 operational phases.

439
440 Given that n-phenylalkanoic acids are present in a variety of pharmaceutical-type OMPs
441 that can be synthesized both chemically and biologically (Aktürk et al., 2002; Sandoval et
442 al., 2005), the increase in gene expression related to their biodegradation in the anaerobic
443 MBR system in Phase 2 implies the adaptability of the anaerobic system to their presence

444 at increasing concentrations. Furthermore, LCFA CoA ligase (acyl-CoA synthetase) is
445 known to target the carboxyl or hydroxyl groups as an initial step of compound
446 conversion. Two hydroxyl-containing OMPs that initially showed low biodegradability in
447 Phase 1 of operation in the anaerobic system were atenolol and acetaminophen. Both of
448 these compounds' biological removal rates increased to above 50% in Phase 2 (Wei et al.,
449 2015a), further substantiating that the upregulation of genes associated with LCFA CoA
450 ligase would be an advantage in their biodegradation. The above observations, along with
451 the positive correlation of BDGs such as *p450* and *glx* with increased OMP presence,
452 suggest a general trend favoring the anaerobic reactor as compared to the aerobic system
453 in which similar BDG expression either decreased or remained constant across
454 operational phases.

455
456 Gene expression associated with nitrogen metabolism, denitrification, and ammonia
457 assimilation also experienced significant increases between Phase 1 and Phase 2 in the
458 anaerobic system (unpaired *t*-test $P < 0.01$, $P = 0.01$, and $P = 0.02$, respectively),
459 although not in the aerobic system (Table 2). The upregulation of gene expression related
460 to nitrogen metabolism observed in the anaerobic MBR may have been due to several of
461 the OMPs serving as nitrogen sources, as the accumulation of these compounds in Phase
462 2 coincided with higher levels of transcription of these genes. Despite having higher
463 relative abundance in the aerobic system, these functional genes either decreased or
464 showed no changes across the phases of operation. Cytochrome C oxidase gene
465 expression was also found to decrease significantly between Phase 1 and Phase 2 in the
466 aerobic system (unpaired *t*-test $P < 0.01$). Cytochrome C oxidase has been previously

467 identified as a primary component in the electron transport chains of the ammonia and
468 nitrite oxidizing bacteria that increased in the aerobic sludge throughout operation
469 (Berben, 1996; Schmid et al., 2008). These decreases in gene expression imply that
470 although nitrifying bacterial populations increased in relative abundance (Section 3.2.1),
471 the accumulation of OMPs in the system in Phase 2 may have inhibited this associated
472 pathway.

473
474 Increases in transcription between phases in the anaerobic reactor sludge were also
475 observed for genes associated with cytochromes and functionally related electron carriers
476 as well as Ton and Tol transport systems, specifically cytochrome associated ferredoxin
477 reductase and TonB dependent receptors, respectively (unpaired *t*-test $P \leq 0.01$). The
478 association of ferredoxin reductase with the cytochrome *p450* enzyme (Hannemann et al.,
479 2007) further emphasizes the importance of the increase in *p450* gene abundance
480 observed in the anaerobic system between Phases 1 and 2. Additionally, given that TonB-
481 dependent receptors regulate the outer membrane transport of high molecular weight
482 compounds (Noinaj et al., 2010), the upregulation of genes associated with TonB
483 transport systems in the anaerobic MBR sludge during Phase 2 could be related to higher
484 uptake rates of OMPs by the microbial community.

485 **3.4. Implications of OMPs for antibiotic resistance gene fate in MBRs**

486 The presence of antibiotic-type OMPs in wastewater influents stands to potentially
487 impact the persistence of antibiotic resistant genes (ARGs) in aerobic and anaerobic
488 wastewater treatment. This remains an issue of interest due to the potential for horizontal

489 gene transfer within the sludge and ARG proliferation in treated effluents (Michael et al.,
490 2013). With this in mind, several prominent ARGs associated with sulfamethoxazole and
491 trimethoprim resistance were quantified using qPCR and compared to the specific
492 concentrations of these antibiotics in the pre-NF reactor effluents. The removal
493 efficiencies of these two OMPs differed significantly between the aerobic and the
494 anaerobic systems (Figure 3). In general, these antibiotic-type OMPs were more readily
495 biodegraded by the anaerobic MBR. Sulfamethoxazole concentrations in the aerobic
496 reactor effluent were above 3 µg/L at the initiation of Phase 1 and continued to increase
497 until the end of Phase 2. Conversely, in the anaerobic effluent, sulfamethoxazole
498 concentrations were below 1.5 µg/L at the beginning of Phase 1 and steadily decreased to
499 below 0.20 µg/L by the end of Phase 2, showing nearly complete removal. Likewise,
500 trimethoprim concentrations in the aerobic reactor effluent were above 8 µg/L for most of
501 Phase 1 and Phase 2, while in the anaerobic system concentrations were below 0.30 µg/L
502 and decreased to below detectable limits.

503

504 Despite the differences in removal rates of these two antibiotic OMPs, several of their
505 associated ARGs responded similarly to the different phases of operation for both MBR
506 systems. Specifically, the abundances of two ARGs associated with sulfamethoxazole
507 resistance, *sul1* and *sul2*, followed similar trends across Phase 1 and Phase 2 in both
508 reactors. The *sul1* gene increased in relative abundance across the span of Phase 1 in the
509 aerobic MBR system from 1.7×10^{-3} copies per 16S rRNA gene copy to 3.1×10^{-2}
510 copies/16S rRNA copy. *sul1* gene copies subsequently dropped in relative abundance at
511 the initiation of Phase 2 to below 5.0×10^{-3} copies/16S rRNA copy (Figure 4). The

512 increase of *sul1* gene concentrations across Phase 1 showed strong correlation with
513 sulfamethoxazole concentrations in the reactor effluent for that same timeframe
514 (Spearman's rank = 0.82, $P < 0.05$) (Figure S5). For the anaerobic MBR, relative
515 abundance of *sul1* gene copies followed a similar trend of increasing across Phase 1,
516 peaking and subsequently dropping upon the initiation of Phase 2. *sul2* gene
517 concentrations, however, did not follow this trend in either reactor, decreasing
518 consistently in the aerobic system from 1.4×10^{-2} copies/16S rRNA copy in pre-OMP
519 conditions to below 3.0×10^{-3} copies/16S rRNA copy and showing no significant changes
520 in relative abundance in the anaerobic reactor (Figure 4).
521
522 The *intl1* gene, which encodes for class 1 integrons, exhibited a similar shift to that of
523 *sul1* during Phase 1 with good correlation in both the aerobic (Spearman's rank = 0.90, P
524 < 0.05) and anaerobic system (Spearman's rank = 0.83, $P < 0.01$), while also dropping in
525 relative abundance at the initiation of Phase 2 (Figure S6). The trimethoprim resistance
526 associated *dfrA5* gene showed no significant changes in either reactor system (Figure S7)
527 while the *dfrA14* gene was undetected. *sul1*, along with several other sulfamethoxazole
528 and trimethoprim resistance genes, has been commonly associated with class 1 integron
529 gene cassettes (Hu et al., 2011). Its similarity in gene response to the *intl1* gene during
530 Phase 1 and Phase 2 suggest that the majority of *sul1* gene copies in this study were
531 indeed part of such an integrated cassette. The differences in gene abundance trends
532 between *sul1* and *sul2* implies that the effect of sulfamethoxazole presence and
533 concentration on related ARGs may be affected by the genes' association with integrons
534 and their subsequent incorporation into specific gene cassettes. Previous studies have

535 shown that treatment system type and antibiotic presence can significantly affect the
536 proliferation of ARGs in wastewater effluents (Burch et al., 2015; Ghosh et al., 2009;
537 Yang et al., 2014a; Zhang et al., 2015).

538

539 It should be noted that the average and peak *sul1*, *sul2*, *int11*, and *dfrA5* gene relative
540 abundance values were over an order of magnitude lower in the anaerobic reactor sludge
541 as compared to the aerobic system across all phases of operation, with high significance
542 (unpaired *t*-test $P < 0.05$). This suggests that the aerobic reactor system could have a
543 higher potential for horizontal gene transfer than that of the anaerobic system at similar
544 operating conditions and similar OMP and antibiotic exposure levels. These lower ARG
545 concentrations in the anaerobic sludge reflect similar findings to those of a previous
546 study, which found that tetracycline ARGs were lost from bacterial cultures at a more
547 rapid rate under anaerobic conditions than under aerobic conditions (Rysz et al., 2013).
548 These differences in ARG proliferation potential, combined with the significantly higher
549 removal rates of sulfamethoxazole and trimethoprim by the anaerobic system, imply that
550 anaerobic digestion, specifically anaerobic MBRs, could be a viable option for reducing
551 the risk of ARG persistence in the environment.

552 4. Conclusions

553 Although overall reactor performance is not altered by the introduction of OMPs at low
554 concentrations, both aerobic and anaerobic MBR microbial communities are significantly
555 affected both in terms of community structure and biodegradation-associated gene
556 expression. Given the lower ARG prevalence in the anaerobic system, this study

557 reiterates the potential impact of aerobic versus anaerobic-based MBRs on ARG
558 proliferation in their effluents. These findings, in combination with other benefits of the
559 anaerobic technology (i.e., reduced energy and sludge disposal costs), exemplify the
560 advantages of incorporating anaerobic digestion into MBR systems, either in the form of
561 fully anaerobic MBRs or anoxic/oxic MBRs.

562 **5. Supplementary Data**

563 Additional information on the synthetic wastewater composition (Table S1), the OMPs
564 spiked in the wastewater feed (Table S2), aerobic system schematic (Figure S1),
565 anaerobic system schematic (Figure S2), chemical skeletal formulas for OMPs (Figure
566 S3), relative abundances of syntrophic bacteria (Figure S4), *sull* gene and
567 sulfamethoxazole concentrations in aerobic sludge (Figure S5), integrase (*int11*) gene
568 concentrations (Figure S6), and *dfrA5* gene concentrations (Figure S7) are presented in
569 Appendix A. qPCR copy number determination method, primers used and standard
570 curves are presented in Appendix B.

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576 **Table 1.** Reactor based microbial comparison for aerobic and anaerobic reactor systems.
 577 Average relative abundances of core microbial species identified in reactor biomass in
 578 Pre-OMP stage, OMPs in feed wastewater (Phase 1), and NF membrane addition and
 579 OMP accumulation in reactor (Phase 2).

Closest species match	Pre-OMP Avg. (%)	Phase 1 Avg. (%)	Phase 2 Avg. (%)	<i>P</i> -value	Identity Match (%)
(A) Aerobic MBR					
<i>Trichococcus flocculiformis</i>	4.56	23.63	1.20	<0.01	99
<i>Ottowia pentelensis</i>	3.39	10.37	5.73	0.063	97
<i>Lysobacter dokdonensis</i>	25.01	9.17	2.11	<0.01	93
<i>Terrimonas lutea</i>	3.26	3.71	8.06	<0.05	96
<i>Ferruginibacter alkalilentus</i>	0.31	1.21	6.80	<0.05	94
<i>Comamonas terrigena</i>	1.56	0.99	1.76	<0.05	95
<i>Nitrobacter hamburgensis</i>	1.43	1.78	1.74	0.94	98
<i>Nitrosomonas ureae</i>	0.002	0.56	1.79	0.081	96
(B) Anaerobic MBR					
<i>Lutispora thermophila</i>	34.64	25.25	11.82	<0.05	88
<i>Petrimonas sulfuriphila</i>	15.18	6.20	0.93	<0.05	99
<i>Alkalitalea saponilacus</i>	2.32	3.28	14.27	<0.01	88
<i>Microbacter margulisiae</i>	4.24	9.00	14.92	<0.05	86
<i>Prolixibacter bellariivorans</i>	0.28	2.50	9.39	<0.01	86
<i>Cloacibacterium normanense</i>	1.90	3.98	1.08	<0.05	98
<i>Smithella propionica</i>	0.84	2.46	3.32	<0.05	97
<i>Syntrophobacter sulfatireducens</i>	1.34	0.43	0.30	0.075	99

580
581

582 **Table 2.** Functional gene expression level relative abundance (%) as determined by the
 583 MG-RAST Subsystems classification system for dominant genes relevant to OMP
 584 biodegradation by microorganisms in the aerobic and anaerobic reactor systems for
 585 Phases 1 and 2.

Expressed functional gene classification	Aerobic				Anaerobic				Aer.: Ana. P-value
	Pre-OMP (%)	Ph. 1 Avg. (%)	Ph. 2 Avg. (%)	Ph. 1: Ph. 2 P-value	Pre-OMP (%)	Ph. 1 Avg. (%)	Ph. 2 Avg. (%)	Ph. 1: Ph. 2 P-value	
Metabolism of aromatic compounds	1.62	1.67	1.75	0.81	1.33	1.10	1.37	0.04	0.01
n-Phenylalkanoic acid degradation	0.77	0.63	0.55	0.36	0.53	0.53	0.73	<0.01	0.65
Long-chain-fatty-acid-CoA ligase (EC 6.2.1.3)	0.22	0.23	0.22	0.90	0.23	0.30	0.37	0.04	<0.01
Acetyl-CoA Acetyltransferase (EC 2.3.1.9)	0.27	0.20	0.12	0.05	0.20	0.15	0.18	0.24	0.26
Homogentisate degradation (EC 1.13.11.5)	0.23	0.24	0.20	0.24	0.18	0.09	0.07	0.25	<0.01
Nitrogen metabolism	1.63	1.54	1.24	0.05	0.78	0.62	1.01	<0.01	<0.01
Denitrification	0.40	0.43	0.38	0.59	0.02	0.03	0.12	0.01	<0.01
Ammonia assimilation	0.34	0.33	0.35	0.79	0.43	0.21	0.32	0.02	0.76
Cytochromes and functionally related electron carriers	0.42	0.33	0.34	0.87	0.13	0.16	0.33	<0.01	0.02
Bacterial chemotaxis	0.40	0.37	0.59	0.20	0.06	0.10	0.33	<0.01	0.01
Ton and Tol transport	1.34	1.25	1.60	0.10	0.71	0.58	0.74	0.01	<0.01
Electron accepting reactions	1.51	1.35	0.82	<0.01	0.61	0.60	0.77	0.01	<0.01
Terminal cytochrome C oxidases	1.01	0.85	0.39	<0.01	0.06	0.07	0.15	0.01	<0.01

586 **Figure 1.** Microbial community metric multidimensional scaling plot (mMDS) for the
587 (A) aerobic reactor system and (B) anaerobic reactor system under different phases of
588 operation. Principal coordinate analysis (PCA) of dominant biodegradation genes (BDGs)
589 expressed in both the (C) aerobic and (D) anaerobic systems with vectors showing the
590 contributing gene groups with strong correlation to sample layout (Pearson's coefficient p
591 > 0.7).

592
593 **Figure 2.** Relative abundance dynamics of the dominant biodegradation genes (BDGs)
594 ($>0.20\%$ of total contigs) across all phases of operation for both (A) aerobic and (B)
595 anaerobic reactor systems.

596
597 **Figure 3.** OMP concentrations of sulfamethoxazole and trimethoprim in aerobic (left)
598 and anaerobic (right) reactor systems.

599
600 **Figure 4.** *sul1* and *sul2* gene concentrations normalized against 16S rRNA gene in
601 aerobic (left) and anaerobic (right) systems.

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603

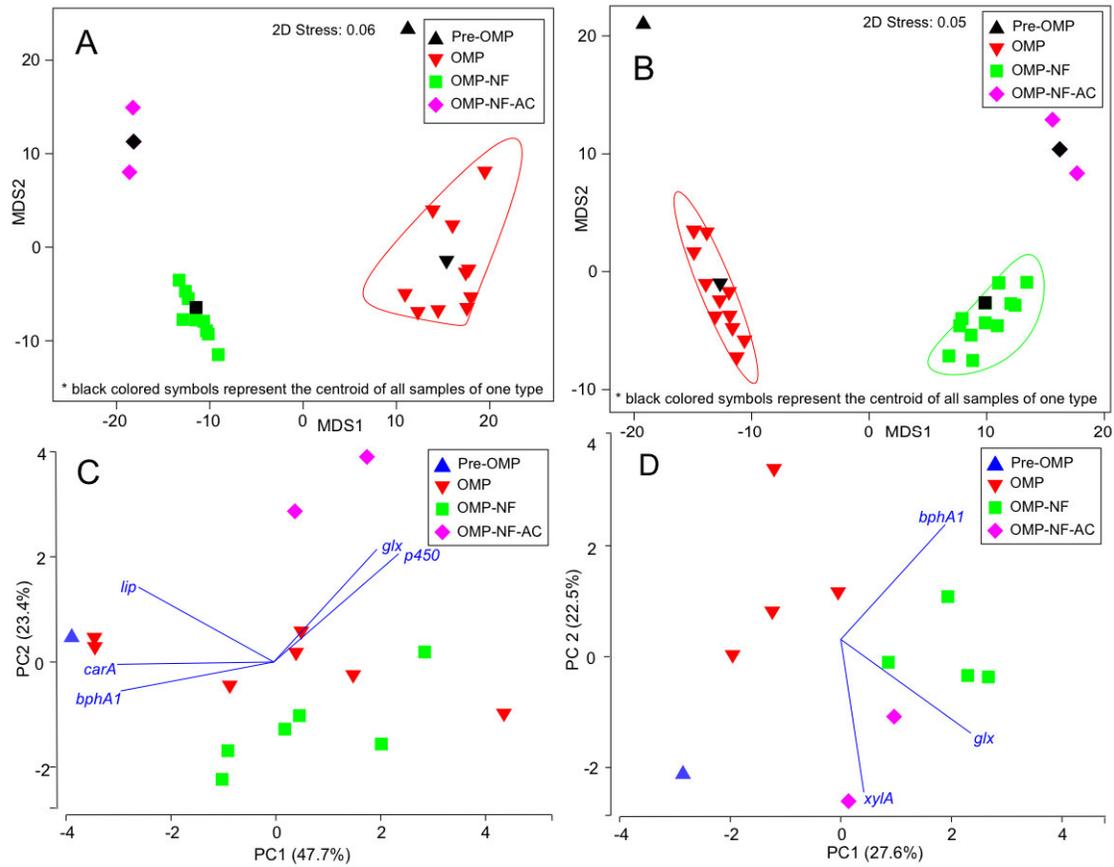
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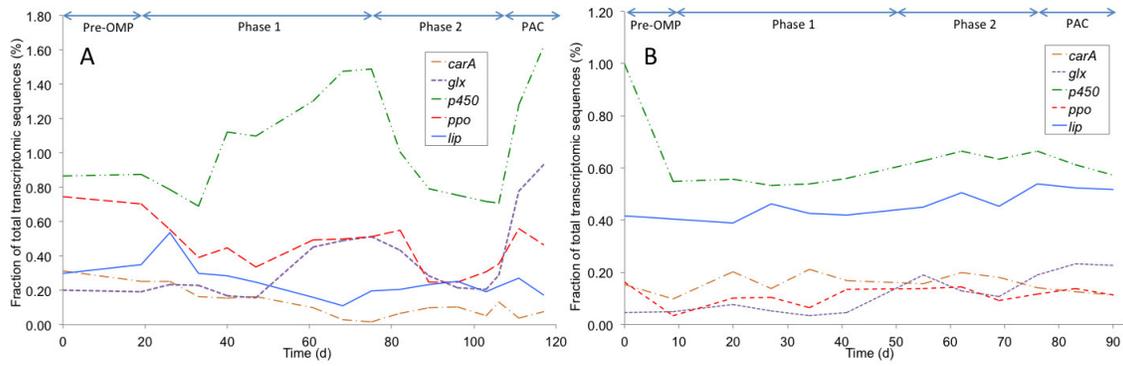
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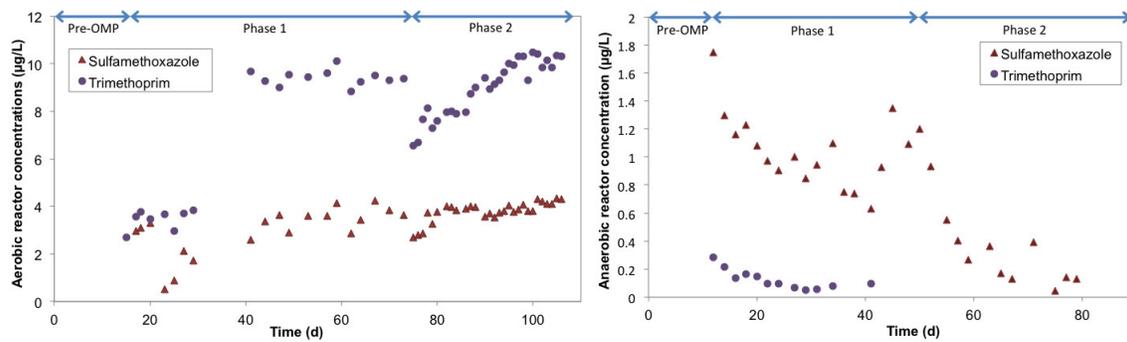


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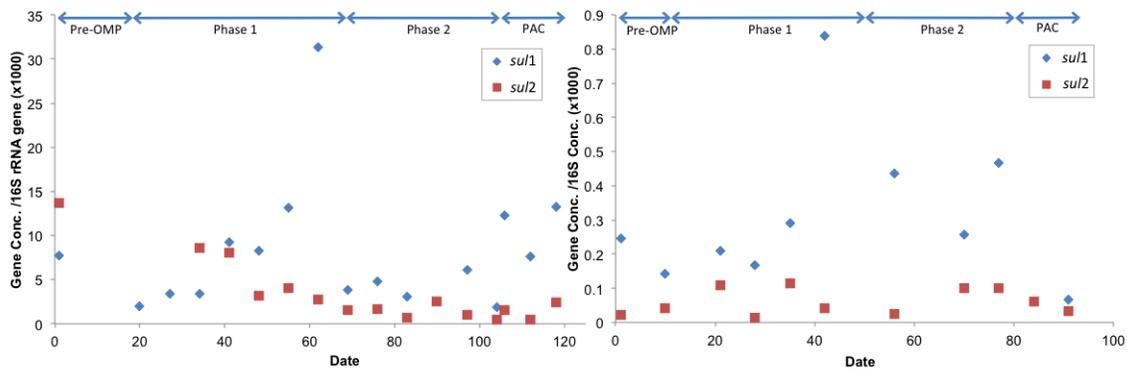
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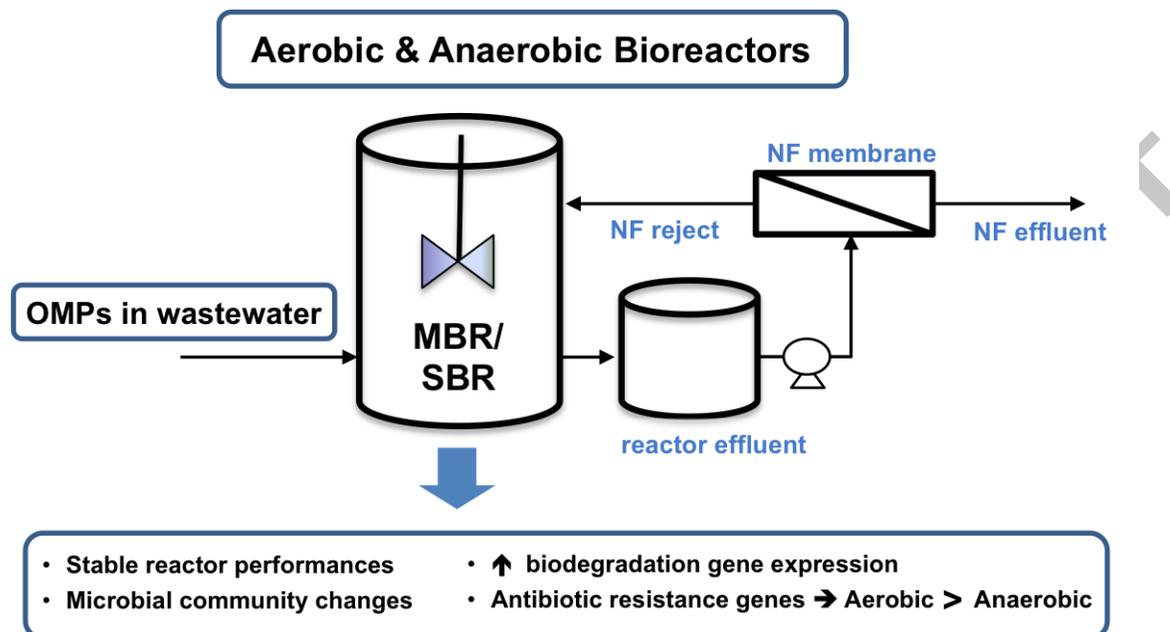
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- Aerobic and anaerobic MBR systems were fed with OMP-spiked wastewater.
 - Key microbial communities in both systems exhibited significant shifts in abundance.
 - BDG expression levels showed links with specific OMP presence in both MBRs.
 - Antibiotic-type OMP removal efficiency was enhanced in the anaerobic system.
 - ARGs generally exhibited higher abundances in the aerobic sludge.

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