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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

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

1. Introduction

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

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

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

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

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

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

2. Material and methods

2.1. Description of treatment systems and operational conditions

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

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

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

2.2. Liquid chromatography – mass spectrometry

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

2.3. Amplicon-based next-generation sequencing

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

2.4. Quantitative PCR (qPCR)

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

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

2.5. Metatranscriptomics

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

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

3. Results & Discussion

3.1. Overall Reactor Performances and OMP removal efficiency

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

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

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

3.2. Effect of OMPs on reactor microbial communities

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

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

3.2.1. Aerobic System

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

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

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

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

3.2.2. Anaerobic system

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

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

Denitrifying and sulfate reducing bacteria were not generally present in the anaerobic sludge (<0.01%); however several methanogenic genera were detected at varying relative abundances. *Methanobacterium*, a hydrogenotrophic methanogen, was determined to be the most abundant archaeal genus in the reactor sludge at an average relative abundance of >1% of total microbial community throughout the experiment. *Methanosaeta* (<0.20%) and *Methanospirillum* (<0.10%) were also consistently detected in the sludge. No significant changes based on OMP presence or phase of operation were observed for any of the methanogens. However, *Smithella propionica*, a syntrophic bacterium known to grow in co-culture with hydrogenotrophic methanogens (Liu et al., 1999), did experience a significant increase in relative abundance from pre-OMP conditions (0.84%) to Phase 1 (2.5%) and Phase 2 (3.3%). This coincided with previously described increases in *Microbacter margulisiae*, a propiogenic bacterium that could have contributed to the rise in *S. propionica* relative abundance by serving as a substrate source. Relative abundances of *S. propionica* in a similar system that was operated without OMP presence were comparable (<0.50%) to those observed in pre-OMP conditions (Harb et al., 2015), suggesting that the presence of OMPs potentially increases syntrophic interactions in anaerobic MBR systems. Other syntrophic bacterial genera also exhibited OMP-related changes in relative abundance (Figure S4). *Syntrophobacter* decreased from >2% in pre-OMP conditions to <0.50% in Phases 1 and 2. Conversely, unclassified Syntrophaceae increased from <1% in pre-OMP conditions to above 3.5%

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

3.3. Changes in biodegradation gene expression

3.3.1. Gene group-based analysis

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

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

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

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

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

For the anaerobic system, total BDG expression also showed no significant changes in relative abundance upon the addition of OMPs in Phase 1, but increased from 1.51% of total transcripts in Phase 1 to 1.81% in Phase 2 (unpaired t -test $P < 0.01$). Average *glx* gene expression in the anaerobic reactor increased between Phase 1 and Phase 2 from 0.05% of total transcripts to 0.15% (unpaired t -test $P = 0.01$), while *ppo* and *carA* gene expression showed no significant changes. Similarly to the aerobic system, the *p450* gene was also the most abundant BDG in the anaerobic MBR system, although with less variability, increasing from 0.55% to 0.65% of total sequences between Phase 1 and Phase 2 (unpaired t -test $P < 0.01$).

A previous study on organic pollutant BDGs in CAS also found the *p450* gene to be the most abundant BDG in the activated sludge (Fang et al., 2013). A similar study on BDGs in freshwater and marine sediments found that entirely different gene groups were dominant in those environments (Fang et al., 2014), suggesting that activated sludge systems likely favor biodegradation via the cytochrome *p450* pathway. The present study substantiates these findings from a gene expression perspective for aerobic and anaerobic sludge. The higher variability of *p450* expression, and of the dominant BDGs in general, in the aerobic system as compared to the anaerobic system could be attributed to the slower growth conditions of the anaerobic sludge, which support general functional 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

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

3.3.2. Gene function-based analysis

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

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

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

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

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

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

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

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

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

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

increase of *sulI* gene concentrations across Phase 1 showed strong correlation with
 sulfamethoxazole concentrations in the reactor effluent for that same timeframe
 (Spearman's rank = 0.82, $P < 0.05$) (Figure S5). For the anaerobic MBR, relative
 abundance of *sulI* gene copies followed a similar trend of increasing across Phase 1,
 peaking and subsequently dropping upon the initiation of Phase 2. *sul2* gene
 concentrations, however, did not follow this trend in either reactor, decreasing
 consistently in the aerobic system from 1.4×10^{-2} copies/16S rRNA copy in pre-OMP
 conditions to below 3.0×10^{-3} copies/16S rRNA copy and showing no significant changes
 in relative abundance in the anaerobic reactor (Figure 4).

The *intl1* gene, which encodes for class 1 integrons, exhibited a similar shift to that of
sulI during Phase 1 with good correlation in both the aerobic (Spearman's rank = 0.90, P
 < 0.05) and anaerobic system (Spearman's rank = 0.83, $P < 0.01$), while also dropping in
 relative abundance at the initiation of Phase 2 (Figure S6). The trimethoprim resistance
 associated *dfrA5* gene showed no significant changes in either reactor system (Figure S7)
 while the *dfrA14* gene was undetected. *sulI*, along with several other sulfamethoxazole
 and trimethoprim resistance genes, has been commonly associated with class 1 integron
 gene cassettes (Hu et al., 2011). Its similarity in gene response to the *intl1* gene during
 Phase 1 and Phase 2 suggest that the majority of *sulI* gene copies in this study were
 indeed part of such an integrated cassette. The differences in gene abundance trends
 between *sulI* and *sul2* implies that the effect of sulfamethoxazole presence and
 concentration on related ARGs may be affected by the genes' association with integrons
 and their subsequent incorporation into specific gene cassettes. Previous studies have

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

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

4. Conclusions

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

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

5. Supplementary Data

Additional information on the synthetic wastewater composition (Table S1), the OMPs spiked in the wastewater feed (Table S2), aerobic system schematic (Figure S1), anaerobic system schematic (Figure S2), chemical skeletal formulas for OMPs (Figure S3), relative abundances of syntrophic bacteria (Figure S4), *sulI* gene and sulfamethoxazole concentrations in aerobic sludge (Figure S5), integrase (*intI1*) gene concentrations (Figure S6), and *dfrA5* gene concentrations (Figure S7) are presented in Appendix A. qPCR copy number determination method, primers used and standard curves are presented in Appendix B.

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

Closest species match	Pre-OMP Avg. (%)	Phase 1 Avg. (%)	Phase 2 Avg. (%)	P-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

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

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

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

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

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

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