**Impact of microbial physiology and microbial community structure on
pharmaceutical fate driven by dissolved oxygen concentration in nitrifying
bioreactors**

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Abstract

Operation at low dissolved oxygen (DO) concentrations (< 1 mg/L) in wastewater treatment could save utilities significantly by reducing aeration energy costs. However, few studies have evaluated the impact of low DO on pharmaceutical biotransformations during treatment. DO concentration can impact pharmaceutical biotransformation rates during wastewater treatment both directly and indirectly: directly by acting as a limiting substrate that slows the activity of the microorganisms involved in biotransformation; and indirectly by shaping the microbial community and selecting for a community that performs pharmaceutical biotransformation faster (or slower). In this study, nitrifying bioreactors were operated at low (~ 0.3 mg/L) and high (> 4 mg/L) DO concentrations to understand how DO growth conditions impacted microbial community structure. Short-term batch experiments using the biomass from the parent reactors were performed under low and high DO conditions to understand how DO concentration impacts microbial physiology. Although the low DO parent biomass had a lower specific activity with respect to ammonia oxidation than the high DO parent reactor biomass, it had faster biotransformation rates of ibuprofen, sulfamethoxazole, 17α -ethinylestradiol, acetaminophen, and atenolol in high DO batch conditions. This was likely because the low DO reactor had a 2x higher biomass concentration, was enriched for ammonia oxidizers (4x higher concentration), and harbored a more diverse microbial community (3x more unique taxa) as compared to the high DO parent reactor. Overall, the results show that there can be indirect benefits from low DO operation over high DO operation that support pharmaceutical biotransformation during wastewater treatment.

Keywords

Dissolved oxygen; Pharmaceuticals; Biotransformation; Nitrification; Wastewater treatment

1. Introduction

Energy conservation is an increasingly desirable goal in wastewater treatment given rising concerns over fossil fuel energy resources and climate change. The most energy intensive process in activated sludge wastewater treatment is aeration, which makes up 45 - 75% of a conventional wastewater treatment plant's (WWTP's) total energy costs (Rosso et al., 2008). Therefore, one strategy for implementing more sustainable wastewater treatment involves a reduction in energy consumption by moving towards treatment that minimizes aeration (Flores-Alsina et al., 2011; Leu et al., 2009; Rosso et al., 2008). Conventional nitrifying activated sludge WWTPs typically operate with bulk liquid dissolved oxygen (DO) concentrations of greater than 2 mg/L to ensure complete nitrification and stable nitrifying populations (WEF, 2008), which results in a substantial energy demand for aeration. There is mounting evidence that stable carbon removal and nitrification can occur at low DO concentrations (<1 mg/L) (Jimenez et al., 2011; Schuyler et al., 2009), suggesting that substantial energy and cost savings are possible by reducing DO levels. Advances in sensor technology and aeration control in recent years have demonstrated that strategies such as ammonia-based aeration control can result in operation at DO concentrations between 0.5 and 1.0 mg/L (Rieger et al., 2014; Uprety et al., 2015). Low DO treatment also can enable simultaneous nitrification and denitrification, resulting in supplemental carbon savings in addition to aeration energy savings (Daigger and Littleton, 2000; Jimenez et al., 2011).

Notable fundamental research on low DO wastewater treatment has focused on nitrification (Arnaldos et al., 2013; Bellucci et al., 2011; Liu et al., 2013; Park and Noguera, 2004); nitrifiers grow relatively slowly as compared to heterotrophs due to their chemolithoautotrophic metabolism and inferior oxygen scavenging ability. Selecting for nitrifiers that are adapted to low DO environments and taking advantage of improvements in mass transfer efficiencies during low DO operation can result in at least 20% less energy for aeration (Arnaldos and Pagilla, 2014). As we move toward low DO treatment processes, there is a need to better understand of how low DO environments affect the function and structure of nitrifying wastewater communities to ensure stable performance without compromising treatment outcomes.

Ammonia oxidizers, which perform the first step of nitrification using the enzyme ammonia monooxygenase (AMO), may also play a major role in pharmaceutical biotransformation during wastewater treatment. AMO can catalyze the oxidation of a relatively wide range of substrates (Arp et al., 2002). Several studies have shown that pharmaceutical loss, and specifically estrogen transformation, is enhanced in nitrifying activated sludge systems (Suarez et al., 2010; Tran et al., 2009; Vader et al., 2000), and have implicated the involvement of AMO. Further, pure culture work has shown the ability of AMO to biotransform compounds such as natural and synthetic estrogens, as well as bisphenol A (Khunjar et al., 2011; Shi et al., 2004; Sun et al., 2012). Many enzymes besides AMO also act as primary and/or secondary catalysts of oxidation reactions. WWTPs with long solids retention times (SRTs) that support the growth of nitrifiers also support the growth and activity of other slow growing heterotrophs that may be involved in pharmaceutical biotransformations (Vuono et al, 2016). Thus, while greater nitrifying activity may be indicative of environments capable of enhanced

83 biotransformation, other microbial processes may be responsible for those biotransformations,
84 many of which are poorly understood and not well-characterized.

85 Low DO treatment can impact the rate and extent of pharmaceutical biotransformation by
86 1) slowing the activity of the microorganisms involved in biotransformation because DO acts as
87 a limiting substrate in respiration or catabolic reactions; and/or 2) selecting for a community that
88 is more (or less) efficient at biotransformation. The objective of this work was to determine the
89 impact of low DO treatment on pharmaceutical biotransformations by nitrifying communities,
90 and specifically to discern between effects of DO concentration on microbial physiology
91 (activity) and on microbial community structure (selection). This work focused on nitrifiers as
92 they are thought to be particularly sensitive to DO limitation and because nitrifiers have been
93 implicated in the biotransformation of many pharmaceuticals. Parent chemostat reactors were
94 used to grow nitrifying enrichment cultures under low (~ 0.3 mg/L) and high (> 4 mg/L) DO
95 concentrations to understand how DO growth conditions impact microbial community structure.
96 Short-term batch experiments using the biomass from the parent reactors were performed under
97 low and high DO conditions to understand how DO concentration impacts microbial physiology.
98 This experimental design allowed us to distinguish between long-term (microbial community
99 structure) and short-term (physiologic) effects of DO on pharmaceutical biotransformations.
100 Allylthiourea-inhibited batch experiments were also conducted to assess the link between
101 nitrification and pharmaceutical biotransformations. This research will contribute to
102 improvements in the design and operation of WWTPs using low DO-adapted metabolisms in
103 wastewater treatment and a better understanding of the impact that energy efficient treatment
104 strategies on pharmaceutical removal.

2. Materials and methods

2.1 Parent reactors and water quality analyses

Two parent chemostat reactors, a high DO and a low DO reactor, were operated for over a year. Each reactor consisted of a 19 L plastic container with a liquid volume of 12 L. The residence time in each reactor was 20 days, and this was achieved by intermittently feeding 25 mL of influent and wasting 25 mL every hour. The SRT was selected to support the growth of slow-growing microorganisms and ensure that nitrifiers, particularly in the low DO reactor, were not washed out. The influent to the reactors was a synthetic medium containing 195 mg-N/L as ammonia-N plus trace nutrients (influent details provided in Table A1, Appendix A). pH was controlled via base addition (30 g/L sodium bicarbonate) to maintain the pH at approximately 7.5. The high DO parent reactor was aerated constantly to maintain a DO concentration above 4 mg/L. The DO concentration in the low DO parent reactor was controlled using real-time feedback from an optical DO probe (Orion RDO® Pro, In Situ Inc.). The DO probe triggered the aerator to turn on when the DO dropped below the setpoint of 0.2 mg/L, which resulted in a DO concentration that fluctuated between 0.2 and 0.4 mg/L. The reactors were operated at room temperature (typically between 21 and 24 °C). Both reactors were seeded with sludge from the Ann Arbor, MI WWTP, which uses an anaerobic-aerobic process to achieve biological phosphorus removal and nitrification. The Ann Arbor WWTP has an SRT of approximately 9 days and operates at a DO concentration of 2-3 mg/L in the aerobic basins. Samples were collected from the parent reactors one or two times per week and analyzed for soluble nitrogen species concentrations (ammonia-N, nitrite-N, and nitrate-N) according to Standard Methods (methods 4500F-NH₃, 4500B-NO₂⁻, and 4110; APHA et al., 2005). Samples were filtered through 0.45 µm nitrocellulose filters and stored at 4°C until analysis (within 48 hours of

sampling for ammonia- and nitrite-N analyses, and within one week of sampling for nitrate analyses).

2.2 Pharmaceutical selection

The pharmaceuticals selected for investigation included: acetaminophen, acetylsulfamethoxazole, atenolol, caffeine, carbamazepine, 17 α -ethinylestradiol (EE2), glyburide, ibuprofen, naproxen, sulfamethoxazole, trimethoprim, and venlafaxine (Table 1). These compounds were selected based on their prevalence in WWTP influents and effluents, structural characteristics, and reported low sorption to biomass such that biotransformation is likely the primary mechanism responsible for loss during treatment.

2.3 Pharmaceutical biotransformation batch experiments

Batch experiments were performed using the biomass taken from the low and high DO parent reactors (after over a year of operation) to determine the extent and rate of pharmaceutical biotransformation by each parent community. Batch experiments were performed for each parent biomass at two different DO concentrations: high DO (constantly aerated) and low DO (DO maintained between 0.2 and 0.4 mg/L). DO control in the low DO batch reactors during the batch experiments was performed by sparging with a mixture of nitrogen gas and air. Optical DO probes (WTW, Weilheim, Germany) were used to monitor the DO concentration throughout the batch experiments. Biomass from the parent reactors was concentrated and washed prior to initiating the batch experiments. For each batch experiment, 500 mL of the mixed liquor from a parent reactor was centrifuged (5 min at 5,500 rpm, 20 °C) to pellet, and then washed three times with N-free media. The pelleted and washed biomass was then re-suspended in N-free media to make a final volume of 200 mL.

A mixture of the pharmaceuticals dissolved in methanol was added to 500 mL glass flasks (250 μ L of a 1 mg/L mixture) and allowed to evaporate until dry in a fume hood. After evaporation was complete, 50 mL of a phosphate buffered media containing 150 mg-N/L as ammonia-N was added to each flask such that after the 200 mL of washed biomass was added to the flask the initial concentration of each compound, ammonia, and phosphate buffer were 1 μ g/L, 30 mg-N/L, and 0.05 M, respectively. The flask with 50 mL of buffered nitrogen media was placed on a stir plate for 1 hour to allow the dried compounds to re-dissolve in the media prior to adding the washed biomass and initiating the biotransformation experiment. Two batch reactors were aerated constantly (high DO batch conditions), two reactors were maintained at low DO concentrations (as described above), two additional constantly-aerated reactors were supplemented with 10 mg/L of allylthiourea (ATU) (Khunjar et al., 2011), an ammonia oxidation inhibitor, to understand the role of nitrifiers in pharmaceutical biotransformation, and a final constantly-aerated batch reactor consisted of biomass inactivated with sodium azide (0.1% w/v; Batt et al., 2007). Complete ammonia oxidation inhibition using ATU was confirmed by measuring the concentration of ammonia-N across the batch experiments (Figure A1, Appendix A).

2.4 Samples collection and analysis from biotransformation experiments

Samples were collected from each batch reactor at approximately $t = 2$ min, 2.5 h, 5 h, 8.5 h, 12 h, 1 d, 2 d, and 3 d. To sample, 10 mL was collected from each flask using a graduated glass pipette, the sample was spiked with 100 μ L of 50 μ g/L stock containing a mixture of deuterated pharmaceutical analogs (Toronto Research Chemicals, Toronto, ON, Canada), filtered through a 0.3 glass fiber filter (Sterlitech Inc., Kent, Washington), and stored at 4°C until analysis. Ammonia-N, nitrite-N, and nitrate-N concentrations were determined in all samples as

described above. Pharmaceutical concentrations were determined in samples corresponding to time points of 2 min, 5 h, 12 h, 1 d, 2 d, and 3 d via on-line solid phase extraction, liquid chromatography, and mass spectrometry (described below).

Biomass samples for microbial community analysis were collected from the parent reactors on the day that biomass was taken from them for the biotransformation batch experiments. Briefly, 50 mL samples of mixed liquor were collected from each parent reactor, biomass was pelleted via centrifugation at 6,200 x g for 5 minutes at 4°C and the supernatant was discarded. The biomass pellet was transferred to a 2 mL microcentrifuge tube, re-pelleted, decanted, and the biomass was stored at -80°C until DNA was extracted. Total and volatile suspended solids (TSS/VSS) concentrations of the parent reactors were also determined according to Standard Methods (method 2540; APHA et al., 2005) on the day of the batch experiments.

2.5 Pharmaceutical quantification

Pharmaceuticals were quantified via on-line pre-concentration followed by high performance liquid chromatography (HPLC) and high resolution mass spectrometry (HRMS). Matrix-matched external calibration curves containing a mixture of the target compounds and deuterated analogs were used for quantification. On-line pre-concentration of the compounds was performed using the Equan™ system (Thermo Fisher Scientific, Grand Island, New York) (system details are provided in Fayad et al. 2013). The on-line pre-concentration was performed using a Hypersil Gold aQ trapping column (20 x 2.1 mm, 12 µM particle size; Thermo Fisher Scientific) and chromatographic separation was done with an Accucore aQ column (50 x 2.1 mm, 2.6 µm particle size; Thermo Fisher Scientific). A 500 µL sample was injected onto the trapping column. A mobile phase containing water with 0.1% formic acid and methanol with

0.1% formic acid was applied via gradient flow to elute all compounds with minimal overlap. Total run time for one sample was 16 minutes at a flow rate of 1 mL/min on the trapping column and 0.175-0.250 mL/min on the analytical column. Complete method details including gradient flow rates are provided in Appendix A.

Ionization of the compounds was achieved by positive electron spray ionization (ESI) for: atenolol, 17 α -ethinylestradiol (EE2), sulfamethoxazole, trimethoprim, venlafaxine, acetyl-sulfamethoxazole, caffeine, carbamazepine, acetaminophen, and glyburide; negative ESI was used for: ibuprofen and naproxen. The following source parameters were used: capillary temperature of 250 °C, auxiliary gas heater temperature of 275 °C, a spray voltage of 3.5 kV, sheath gas flow rate of 30 arbitrary units, auxiliary gas flow rate of 20 arbitrary units, and sweep gas flow rate of 1 arbitrary unit. In both positive and negative mode, a full scan ranging from 150 to 750 m/z was performed at a resolution of 70,000 and target automatic gain control (AGC) of 1 x 10⁻⁶. All data were collected and processed using the Thermo TraceFinder Software Version 3.2 (Thermo Fisher Scientific).

2.6 Molecular analyses

DNA was extracted from the frozen biomass samples collected from the parent reactor on the day of the biotransformation batch experiments, as described above. DNA extraction involved three bead beating steps (Mini-Beadbeater-96, BioSpec Products, Bartlesville, OK) with 0.1 mm diameter zirconium beads in lysis buffer, followed by proteinase K digestion, and automated extraction using the Maxwell 16 Blood LEV kit according to the manufacturer's instructions (Promega, Madison, WI). Extracted DNA quality was verified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and DNA quantity was determined using the QuantiFluor dsDNA kit (Promega, Madison, WI). DNA extracts were stored at -20 °C

until they were submitted for sequencing or analyzed by quantitative polymerase chain reaction (qPCR).

Polymerase chain reaction (PCR), multiplexing, and sequencing was performed by the Center for Microbial Systems (University of Michigan, Ann Arbor, MI) using the Illumina MiSeq platform (San Diego, CA) targeting the V4 region of the 16S rRNA gene (Caporaso et al., 2011). The resulting sequences were processed and analyzed using Mothur (version 1.33.3) (Schloss et al., 2009) following the MiSeq standard operating procedure. Operational taxonomic units (OTUs) were defined based on 97% sequence similarity. Sequence classification was performed using the Ribosomal Database Project (Cole et al., 2009) and the Basic Local Alignment Search Tool (BLAST; NCBI, Bethesda, MD). Sequences were deposited on NCBI (accession number [SRP076048](#)).

The abundance of ammonia oxidizers was quantified in the parent reactor biomass samples via qPCR. Primers targeting the bacterial ammonia monooxygenase gene (*amoA*) were used (*amoA*-1F (5'-GGGGTTTCTACTGGTGGT-3') and *amoA*-2R (5'-CCCCTCKGSAAAGCCTTCTTC-3'); Rotthauwe et al., 1997). qPCR standards were prepared using PCR-amplified *amoA* products from pooled DNA extracts from the parent reactors (detailed in Appendix A) (He and McMahon, 2011; Sonthiphand et al., 2013). qPCR was performed on a Mastercycler Realplex ep (Eppendorf, Hamburg, Germany). 20 µL qPCR reaction volumes consisted of 10 µL of 2x EvaGreen Fast Plus qPCR Master Mix (Biotium, Hayward, CA), 0.2 µL each of 50 µM forward and reverse primer (final concentration of 50 µM), 8.6 µL of nuclease free water, and 1 µL of DNA template. All samples were run in triplicate at three different dilutions: 5x, 50x, and 500x, and each plate contained no template

controls. Parent reactor biomass samples were also collected prior to the batch experiments and screened for the presence of AOA via and qPCR using primers targeting the archaeal ammonia monooxygenase gene (A26F (5' -GACTACATMTTCTAYACWGAYTGGGC-3') and A416R (5' -GGKGTCA TRTATGG WGGYAA YGTTGG-3') (Park et al., 2008)). No amplification of the target gene was detected in samples from either reactor, indicating that AOB were the dominate ammonia oxidizing microorganisms in the reactors.

3. Results and Discussion

3.1 Reactor performance and microbial community characteristics of parent reactors

Complete and stable nitrification was achieved in both parent reactors. The low and high DO parent reactors achieved ammonia removal efficiencies of $99.0 \pm 2.3\%$ and $99.9 \pm 0.1\%$, respectively, over the 6 months prior to when the batch experiments were performed. Further, no buildup of nitrite was observed in either reactor, demonstrating complete nitrification. Effluent total inorganic nitrogen (sum of ammonia, nitrite, and nitrate-N) concentrations were slightly lower in the low DO than the high DO parent reactor, with average total inorganic nitrogen (TIN) removal efficiencies of 11% and 2%, respectively. Nitrogen assimilation by biomass for growth likely accounted for all of the N-removal in the high DO reactor, while a small amount of N-removal in the low DO reactor may have been due to denitrification (accounting for approximately 7.5% of the 11% of the TIN removal).

While the low and high DO parent reactors achieved similar effluent quality and performance with respect to nitrification efficiency, there were observable differences in biomass concentration between the reactors. The suspended solids concentration in the low DO parent reactor was significantly higher than the high DO parent reactor (shaded bars in Figure 1),

despite being loaded with the same amount of electron donor (195 mg ammonia-N/L/day). qPCR targeting the *amoA* gene was used to quantify the abundance of AOB in the parent reactors. The results indicated that the low DO parent reactor had significantly more ammonia oxidizers than the high DO parent reactor (open bars in Figure 1). Further, the ratio of ammonia oxidizers to total biomass concentration as VSS (ratio of the open bar to shaded bar values for each reactor in Figure 1) was more than two times greater in the low than the high DO parent reactor. Therefore, low DO growth conditions not only resulted in greater biomass concentrations and a greater abundance of ammonia oxidizers, but also an enriched ammonia oxidizer population. Previous studies have found that nitrifiers grown under low DO conditions had greater observed yields than nitrifiers grown under high DO conditions (Bellucci et al., 2011; Liu and Wang, 2013). The observed yield coefficient is determined by the true growth yield, endogenous decay rate, and the sludge age (Grady et al., 2011). The endogenous decay coefficient is a lumped parameter that accounts for loss in cell mass due to oxidation of internal storage products for energy required for cell maintenance and death and predation by other organisms (Lawrence and McCarty, 1970). A reduced endogenous decay rate in low DO environments is consistent with the greater observed yield in the low versus high DO parent reactor. It is also consistent with previous research that showed increased observed yields and reduced specific sludge degradation rates under low DO conditions compared to fully aerobic conditions (Habermacher et al., 2015). One explanation for why this happens is that low DO inhibits select protozoan predators (Madoni, 1994), which reduces decay rate and increases biomass concentration.

The batch experiments were performed by taking the same volume of mixed liquor from each of the reactors, and this resulted in the batch experiments with the low DO parent reactor biomass having greater total biomass concentrations and a greater abundance of ammonia

oxidizers than those performed using the high DO parent reactor biomass. Ammonia oxidation rates in the batch experiments were significantly different under low and high DO batch conditions, and between the low and high DO parent reactors (Figure 2). Faster ammonia oxidation rates were observed in the high DO batch conditions, regardless of parent reactor biomass, illustrating the direct impact of DO as a limiting substrate. Under low DO batch conditions, the low DO parent biomass performed ammonia oxidation slightly faster than the high DO parent biomass. However, when normalized to *amoA* copy concentration, the rates were not significantly different between the batch experiments (unpaired t-test, $p = 0.65$). Under high DO batch conditions, the ammonia oxidation rates normalized to *amoA* copy concentration for the low DO parent biomass were significantly slower than the high DO parent biomass (unpaired t-test, $p = 0.025$). Thus, while the low DO parent reactor harbored a more abundant population of ammonia oxidizers, they were slower at performing ammonia oxidation under high DO conditions. Furthermore, this demonstrated that the low DO parent reactor biomass was not able to adapt well to high DO conditions over the three-day batch experiments. Trade-offs between substrate affinity and maximum substrate utilization rates have been demonstrated in pure cultures (Elbing et al., 2004; Wirtz, 2002) and this biochemical trade-off is theorized to be a driver of microbial diversification (Gudelj et al., 2007). Our results indicate that low DO growth conditions may select for ammonia oxidizers with high oxygen affinities, i.e. “K” strategists, with high substrate affinities and low maximum activity, as opposed to “r” strategists. Even though the normalized rates were different between the low and high DO parent reactor biomasses, the raw rates were not significantly different (unpaired t-test, $p = 0.30$). The increased abundance of ammonia oxidizers due to low DO growth conditions compensated for the slower per organism rates of ammonia oxidation.

In addition to characterizing the impact of DO concentration on the abundance of microorganisms and ammonia oxidizers, amplicon sequencing of the 16S rRNA gene was performed to understand community composition differences between the low and high DO parent reactors. The richness (number of unique OTUs), inverse Simpson and Shannon diversity indices were calculated at equal sequencing depths for the low and high DO parent reactor communities (Table 2). These three diversity indices vary in their weighting of OTU abundance (richness does not account for abundance, whereas the Shannon index weights abundance more heavily than the inverse Simpson index) and all show that the low DO parent reactor supported the growth of a much more diverse community (larger value for all indices) than the high DO reactor (Table 2). Rarefaction curves depicting the number of unique OTUs from the samples collected from each parent reactor show that there were significantly more unique OTUs in the low than the high DO reactor (Figure 3). While the microbial community as a whole was more diverse in the low DO reactor, the dominant AOB and NOB in the reactors were similar (AOB: *Nitrosomonas*-type; NOB: *Nitrospira*-type) based on OTU groupings with a 97% similarity threshold (Table A4, Appendix A).

3.2 Direct impact of DO concentration on pharmaceutical transformations

Only a subset of the compounds biotransformed during the batch experiments. No significant biotransformation of trimethoprim, venlafaxine, acetyl-sulfamethoxazole, caffeine, carbamazepine, glyburide, sucralose, and erythromycin was observed by either the low or high DO parent communities. For the compounds for which we observed significant biotransformation (ibuprofen, sulfamethoxazole, 17 α -ethinylestradiol, acetaminophen, and atenolol), both DO concentration during the batch experiment and the DO concentration of the parent reactor impacted the degree of biotransformation.

For ibuprofen, sulfamethoxazole, 17 α -ethinylestradiol, and acetaminophen, greater loss of the parent compound was observed in high versus low DO batch conditions (Figure 4). Atenolol was the one exception – greater loss was observed under low DO than high DO batch conditions (Figure 4). The first-order rate constant describing the biotransformation rate of each of the compounds varied significantly between compounds (Table 3). Ibuprofen biotransformation occurred most rapidly, with a half-life of 2-6 hours in high DO batch conditions. Conversely, complete loss of the other compounds was not observed over the 3-day batch experiments, and the biotransformation rate constants were significantly lower (Table 3). These results are consistent with previous studies that report the relative biodegradability of these compounds in wastewater systems (Joss et al., 2006; Yu et al., 2006).

While complete and stable nitrification is possible at considerably lower bulk liquid DO concentrations than typically used in conventional activated sludge treatment systems, these results (and other previous studies, e.g. Jimenez et al., 2011) indicate that low DO slows the rate of ammonia oxidation. Our results show that this also reduces the rate of trace chemical transformations. If DO is a limiting substrate, it will slow the activity of aerobic microorganisms and impact their kinetics, resulting in reduced rates of biotransformation of primary and cometabolic substrates. These results suggest that DO concentration will impact the degree of transformation of these compounds during treatment and that the oxygen half-saturation constant (K_{O_2}) of the microbial groups involved in biotransformation is an important parameter to predict the impact of DO concentration on the rate of pharmaceutical biotransformation. More research is needed to determine oxygen half-saturation constants (K_{O_2}) for pharmaceuticals in order to accurately model their fate in WWTPs and determine appropriate operational targets for DO concentration that do not negatively impact pharmaceutical removal. It is also important to note

that we only tracked the disappearance of the parent compound in this study. DO may impact the types of biotransformations that occur, and transformation products may vary in their stability and bioactivity in the environment. Further research is needed to understand how DO impacts the types of transformation products that are formed during treatment and assess their relative toxicity.

Atenolol was the only compound for which we observed a faster biotransformation rate under low than high DO batch conditions. Other studies have reported that atenolol undergoes an initial amide-hydrolysis and is converted to atenolol-acid in wastewater treatment systems (Barbieri et al., 2012b; Kern et al., 2010). Hydrolysis reactions, unlike hydroxylation reactions, do not use oxygen as a substrate in catalysis. Faster hydrolysis rates in low DO conditions indicate that oxygen is not a limiting substrate in this reaction and might be enhanced as low DO environments can enable the growth of both aerobic and anoxic organisms. Previous studies have observed atenolol loss in anoxic environments during wastewater treatment (Carucci et al., 2006; Park et al., 2009; Stadler et al., 2015). Our previous study on sequencing batch reactors treating real municipal wastewater observed greater loss of atenolol in high than low DO conditions (Stadler et al., 2015). Another study of atenolol fate in different redox conditions in aquifer material found that removal occurred fastest in the most reducing conditions considered (sulphate reducing conditions; Barbieri et al., 2011). The collective results suggest that we do not fully understand the factors that control atenolol transformation and there may be a complex relationship between redox condition, microbial activity, and transformation rate.

3.3 Links between nitrification and pharmaceutical biotransformations

Batch experiments were performed using allylthiourea, a specific inhibitor of ammonia oxidation, to understand the relationship between nitrification and pharmaceutical

biotransformations. Allylthiourea inhibits ammonia oxidation and acts by binding to the copper center of ammonia monooxygenase (Bédard and Knowles, 1989). As expected, no ammonia loss was observed in batch experiments with allylthiourea (Figure A1, Appendix A). Batch experiment results showed that the biotransformation of certain compounds (sulfamethoxazole, 17 α -ethinylestradiol, and acetaminophen) was linked to nitrification, as the extent and rate of transformation of these compounds were reduced in the presence of allylthiourea (Figure 4). Allylthiourea did not cease the transformation of these compounds, which indicates that both heterotrophs and nitrifiers were involved in their biotransformation in the uninhibited batches. For ibuprofen, the presence of the inhibitor had no significant effect on the rate of transformation. However, for atenolol, the presence of the inhibitor resulted in an increase in the rate of biotransformation, indicating that reduced competition with nitrifiers is a possible explanation for the observed greater atenolol transformation.

The link between enhanced transformation of sulfamethoxazole, 17 α -ethinylestradiol, and acetaminophen and nitrification could be due to several different factors: first, ammonia oxidizers may be directly involved in the transformation of these compounds. Ammonia monooxygenase is a non-specific enzyme that is capable of oxidizing alternative substrates (Hyman et al., 1994; Keener and Arp, 1994; Roh et al., 2009). Previous studies have demonstrated the ability of ammonia oxidizers to transform EE2 using pure cultures and ATU-inhibited experiments (Khunjar et al., 2011; Shi et al., 2004; Yi and Harper, 2007) and thus are consistent with our findings. For sulfamethoxazole, a previous study found that heterotrophic activity was primarily responsible for sulfamethoxazole biotransformation and lower biotransformation rates were observed in nitrifying conditions (Fernandez-Fontaina et al., 2016). While we observed reduced biodegradation in the batches with allylthiourea, biotransformation

401 did not cease, and thus supporting that both nitrifiers and heterotrophs contributed to
402 biotransformation. Similarly for acetaminophen, biotransformation was reduced but did not cease
403 in the presence of allylthiourea, indicating the involvement of both nitrifiers and heterotrophs.
404 These results are consistent with previous studies implicating the involvement of ammonia
405 oxidizers in the biotransformation of acetaminophen in WWTPs (Chiron et al., 2010; Rattier et
406 al., 2014).

407 Another possible explanation for the link between nitrification and enhanced
408 pharmaceutical biotransformation is that nitrite oxidizers are involved in biotransformation. Few
409 studies have directly investigated the role of the nitrite oxidizers in pharmaceutical
410 biotransformations, and their contribution is often overlooked as compared to ammonia
411 oxidizers. Inhibition of ammonia oxidation with allylthiourea also ceases the production of
412 nitrite, and therefore decreases the activity of nitrite oxidizers. A final possible explanation of the
413 link between transformation and nitrification is that the products of nitrification (nitrite and
414 nitrate) may be involved in abiotic transformations of these compounds. Previous studies have
415 observed the biologically mediated abiotic transformation of sulfamethoxazole with nitrite under
416 anoxic conditions (Barbieri et al., 2012a; Nödler et al., 2012). Another study demonstrated that
417 EE2 underwent abiotic nitration (addition of one or more nitro groups on the aromatic ring of
418 EE2) in the presence of high nitrite concentrations (10 - 500 mg-NO₂⁻-N/L), and that this
419 reaction was enhanced at low pH (Gaulke et al., 2008). In the case of acetaminophen, ammonia
420 monooxygenase was found to play a key role in the bio-nitritation of acetaminophen (Chiron et
421 al., 2010). In our experiments, nitrite concentrations were always significantly lower than those
422 reported in these studies (below 2.5 mg-NO₂⁻-N/L) and the pH was near neutral; therefore,

nitrite-mediated abiotic transformations are unlikely to be a primary mechanism of transformation.

3.4 Indirect impacts of DO concentration on pharmaceutical transformations

By comparing biotransformation rates between experiments using biomass from the low versus high DO parent reactor, the indirect impacts of DO on pharmaceutical biotransformation via changes in microbial community structure were identified. For all of the compounds studied, greater loss of the parent compound (Figure 4) and faster biotransformation rates (Table A3, Appendix A) were observed in batch experiments using low DO parent reactor biomass than high DO parent biomass (under equivalent batch DO conditions). These results suggest that DO growth condition impacts the microbial community, and that selection in turn impacts the potential for pharmaceutical biotransformations.

One selection impact of long-term low DO growth conditions was reduced specific activity with respect to ammonia oxidation rates in high DO batch conditions. Despite this, the observed specific nitrification rates were comparable between the low and high DO parent biomass under high DO batch conditions because of the increased abundance of ammonia oxidizers in the low DO parent reactor. Similarly, for ibuprofen, sulfamethoxazole, and acetaminophen, the faster observed biotransformation rates under high DO batch conditions can be explained by the increased biomass concentration in the low DO parent reactor, as the VSS-normalized biotransformation rate constants were comparable between the low and high DO parent biomass (Table 4-3). However, for EE2 and atenolol, the specific (VSS-normalized) biotransformation rate constants were greater for the biomass from the low DO parent than the high DO parent reactor. This indicates that low DO conditions selected for a community with

greater potential for faster specific biotransformation rates compared to long-term high DO growth conditions.

Despite identifying similar dominant AOB and NOB genera (*Nitrosomonas*-type and *Nitrospira*-type, respectively) in the low and high DO parent reactors, the communities functioned differently based on specific nitrification rates and certain pharmaceutical biotransformation rates. One possible explanation for the differences in specific activity could be microdiversity (Gruber-Dorninger et al., 2015). The length and region of the 16S rRNA gene that was sequenced only provides resolution at the genus level, and thus diversity within *Nitrosomonas* and *Nitrospira*-type groupings was not discernable with the methods applied. Sequencing of the ammonia monooxygenase gene would likely better capture diversity within the ammonia oxidizing populations. A number of studies on low DO nitrification systems have reported conflicting results with respect to how DO shifts (or does not shift) nitrifying microbial communities. Several studies have hypothesized that low DO environments select for nitrifier lineages with high oxygen affinities (Gieseke et al., 2001; Park and Noguera, 2004). The results, however, have been conflicting in terms of the specific lineages of AOBs identified in low DO environments, with several studies identifying AOB belonging to the *Nitrosomonas* genera (Arnaldos et al., 2013; Bellucci et al., 2011; Park and Noguera, 2004) and others finding that *Nitrosospira* (Li et al., 2007) dominated in low DO conditions. Further, the recent discovery of organisms within the genus of *Nitrospira* that completely oxidize ammonia to nitrate (comammox) has altered our understanding of the aerobic nitrification process (Daims et al., 2015; van Kessel et al., 2015). Low DO conditions have been shown to favor the growth of *Nitrospira* sp. over *Nitrobacter* sp. performing nitrite oxidation (Daims et al., 2001; Schramm et al., 1998), and thus could also possibly favor *Nitrospira* that perform complete ammonia

oxidation. It is not yet known how low DO conditions influence the abundance and activity of comammox and whether comammox bacteria are involved in pharmaceutical biotransformation.

An alternative hypothesis for the impact of oxygen limitation on ammonia oxidizing communities is that the community undergoes a physiological, as opposed to structural, adaptation to low DO conditions. Kowalchuk et al. (1998) found no correlation between differences in ammonia oxidizer community structure and oxygen availability in 5 different soil/sediments, and therefore suggested that physiological differences may outplay phylogenetic groupings. In a study by Arnaldos et al. (2013), enhanced expression of a particular heme protein and increased specific oxygen uptake rates were observed in a nitrifying enrichment culture adapted to low DO conditions in comparison to a high DO enrichment. In response to oxygen limitation, microorganisms ramp up oxygen delivery machinery such as heme proteins that transport oxygen and key enzymes that use oxygen such as terminal oxidases and oxygenases, potentially resulting in differences in specific oxidation rates.

Beyond nitrifiers, our results indicated that different DO concentrations in the parent reactors resulted in distinct microbial communities and greater microbial biodiversity in the low DO parent reactor. An additional limiting substrate (in this case, oxygen) can result in greater diversity according to resource competition theory because competition drives diversification (Huston, 1994). Biodiversity is generally believed to positively associate with productivity (Cardinale et al., 2012), but the relationship between biodiversity and function in WWTPs is not well understood. One study of 10 full-scale wastewater treatment systems observed a significant positive association between biodiversity and the collective rate of multiple pharmaceutical biotransformations (Johnson et al., 2014). Conversely, another study of estrogen fate in lab-scale bioreactors found conflicting results with respect to the removal of endocrine disruptors; they

observed that a decrease in diversity correlated with an increase in the removal of endocrine disruptors (estrone (E1), 17 β -estradiol (E2) and 17 α -ethinylestradiol (EE2)) (Pholchan et al., 2013). This discrepancy may be due to the choice of compounds and experimental design of the studies. If a decrease in diversity lead to an increase in the abundance of specific (and potentially rare) taxa involved in biotransformation, there would be no observable association between transformation rates and biodiversity. Or if a compound's biotransformation is catalyzed by many species in the community, there would not be an association between transformation and biodiversity because the community is functionally redundant. However if a compound's biotransformation is performed by rare taxa in a community, and reductions in diversity do not result in the proliferation of those taxa, then we would expect to observe an association between transformation rates and biodiversity. Our results support the notion that increased biodiversity may have contributed to the greater collective biotransformation rates observed in the low DO than the high DO parent community.

4. Summary and potential applications

DO concentration can impact the rate of pharmaceutical biotransformation during wastewater treatment. The results of this study illustrate a nuanced relationship between DO concentration and pharmaceutical biotransformation rates, as DO impacts both microbial physiology and microbial community structure. Short term batch experiments showed that high DO conditions resulted in faster biotransformation rates for ibuprofen, sulfamethoxazole, EE2, and acetaminophen. Atenolol was the one exception for which faster biotransformation occurred in low DO batch conditions. Long-term low DO growth conditions also selected for a community that had faster net biotransformation rates, but lower specific activity with respect to ammonia oxidation. Long-term low DO growth conditions resulted in a greater biomass

concentration, an enrichment of ammonia oxidizers, and increased microbial diversity compared to high DO growth conditions.

Knowledge of oxygen half saturation constants for pharmaceuticals will allow utilities to poise the DO concentration such that it does not drastically impact biotransformation rates, while still saving on aeration energy and taking advantage of other benefits of low DO treatment (e.g. reduced decay and increased microbial diversity). This work and other studies (Liu and Wang, 2013) suggest that despite lower specific rates, reduced decay in low DO conditions that results in an enrichment of ammonia oxidizers can result in comparable nitrification performance to a high DO system. Higher biomass concentrations that could result from long term low DO conditions could entail greater sludge handling requirements in a full-scale treatment system. If the sludge could be used to generate biogas via anaerobic digestion, benefits from greater sludge production could be realized. An energy balance accounting for aeration energy savings and energy associated with sludge handling should be performed to determine net energy benefits (or losses) of implementing a low DO treatment process. This research also supports a positive association between microbial biodiversity and pharmaceutical biotransformation. DO concentration is one operational parameter that may influence microbial diversity in a treatment system, but there are likely many others, such as solids retention time, substrate quantity and quality, temperature, salinity, and physical configuration. Designing treatment systems that harbor diverse microbial communities may be one way to enhance pharmaceutical biotransformation during treatment. Cycling between low and high DO environments could be one strategy for both enhancing pharmaceutical biotransformation by supporting a diverse microbial community while also providing non oxygen-limiting conditions to enable maximum biotransformation rates.

5. Conclusions

- DO concentration impacts microbial physiology and microbial community structure, and both of those in turn impact pharmaceutical biotransformation rates.
- Despite lower specific ammonia oxidation rates, greater biomass concentrations and an enrichment of the ammonia oxidizer population in the low DO system resulted in higher pharmaceutical biotransformation rates and nitrification rates comparable to the high DO system. This indicates that low DO treatment has both benefits due to pharmaceutical transformation and drawbacks with respect to ammonia oxidation. Further research is needed to determine kinetic parameters, such as oxygen half saturation constants for pharmaceutical biotransformations, to predict and model the impact of DO on biotransformation rates.
- Long-term low DO conditions supported the growth of a more diverse microbial community than high DO conditions. Designing treatment systems that harbor diverse microbial communities may be one way to enhance pharmaceutical biotransformation during treatment. More research is needed to understand its relative importance in driving biodiversity in wastewater treatment systems.
- Given the noted impacts of long-term low DO conditions on the microbial community function, operational strategies should be investigated that use both low DO to shape the microbial community and high DO to enable non-DO-limited biotransformation rates.

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Table 1

Table 1. Compounds selected for investigation

Chemical	Structure	Chemical Formula and Molecular Weight	Use
Acetaminophen		$C_8H_9NO_2$ 151.16 g/mol	Analgesic and antipyretic
Acetyl-sulfamethoxazole		$C_{12}H_{13}N_3O_4S$ 295.31 g/mol	Antibiotic TP
Atenolol		$C_{14}H_{22}N_2O_3$ 266.34 g/mol	Beta-blocker
Caffeine		$C_8H_{10}N_4O_2$ 194.19 g/mol	Stimulant
Carbamazepine		$C_{15}H_{12}N_2O$ 236.27 g/mol	Anticonvulsant
17 α -Ethinylestradiol		$C_{20}H_{24}O_2$ 296.40 g/mol	Synthetic hormone
Glyburide		$C_{23}H_{28}ClN_3O_5S$ 494.00 g/mol	Antidiabetic
Ibuprofen		$C_{13}H_{18}O_2$ 206.29 g/mol	Nonsteroidal anti-inflammatory drug
Naproxen		$C_{14}H_{14}O_3$ 230.26 g/mol	Nonsteroidal anti-inflammatory drug
Sulfamethoxazole		$C_{10}H_{11}N_3O_3S$ 253.28 g/mol	Antibiotic
Trimethoprim		$C_{14}H_{18}N_4O_3$ 290.32 g/mol	Antibiotic
Venlafaxine		$C_{17}H_{27}NO_2$ 277.40 g/mol	Antidepressant

Table 2

Table 2. Richness and diversity indices of the low and high DO parent reactor communities

	Operational taxonomic unit (OTU) richness	Inverse Simpson diversity	Shannon diversity
Low DO	1,140	59	5.2
High DO	350	21	3.8

Table 3

Table 3. Observed specific kinetic biotransformation constants (normalized by VSS concentration) for ibuprofen, EE2, sulfamethoxazole, acetaminophen, and atenolol. No biotransformation rate is reported for EE2 for low DO batch conditions because no significant biotransformation occurred.

	k_{obs} [L mg ⁻¹ day ⁻¹]				
	Ibuprofen	EE2	Sulfamethoxazole	Acetaminophen	Atenolol
Low DO parent & low DO batch	2.4E-02 ± 1.3E-03	N.A.	2.8E-03 ± 1.2E-03	2.9E-03 ± 1.6E-03	3.8E-03 ± 2.4E-04
Low DO parent & high DO batch	1.1 E-01 ± 6.4E-03	2.1E-02 ± 2.2E-03	3.3E-03 ± 2.1E-04	2.4E-02 ± 7.7E-03	1.9E-03 ± 2.0E-04
High DO parent & low DO batch	1.4E-02 ± 6.0E-03	N.A.	2.2E-03 ± 8.1E-05	7.0E-03 ± 3.8E-03	2.9E-03 ± 3.7E-04
High DO parent & high DO batch	1.0E-01 ± 3.0E-02	1.3E-02 ± 4.1E-03	3.1E-03 ± 9.5E-04	2.1E-02 ± 6.2E-03	8.3E-04 ± 2.8E-04

Figure 1

Figure 1. Volatile suspended solids (VSS) concentration (shaded bar) and *amoA* copies per milliliter (open bars) in the low and high DO parent reactors. Error bars represent the standard deviation from the mean of technical triplicates.

Figure 2

Figure 2. Ammonia oxidation rates in the batch experiments with biomass from each parent reactor. Non-normalized ammonia oxidation rates are shown in blue bars (A) and ammonia oxidation rates normalized to *amoA* copies per mL are shown in orange bars (B). Bars represent the average of duplicates and the dots represent the individual measurements.

Figure 3

Figure 3. Rarefaction curves of the microbial communities sampled from the low DO and high DO parent reactors.

Figure 4

Figure 4. Percent loss of ibuprofen, sulfamethoxazole, 17 α -ethinylestradiol (EE2), acetaminophen, and atenolol in the low (red) and high (blue) DO parent communities. The solid markers represent low DO batch conditions (0.2-0.3 mg/L), and the open markers represent high DO batch conditions (constantly aerated, DO > 4 mg/L). Black outlined markers represent inhibited batch conditions. Azide-inhibited controls are not shown for EE2 and acetaminophen because of abiotic reactions with azide contributed to losses of these compounds (details in Appendix A). ATU = allylthiourea.

Graphical abstract

Direct effects

e.g. oxygen is the limiting substrate and slows the activity of the microbes involved in biotransformation

Indirect effects

e.g. oxygen concentration selects for a microbial community that is more (or less) efficient at degrading micropollutants