FINAL REPORT

Elucidation of the Rates and Extents of Pharmaceutical Biotransformation during Nitrification

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Project USGS 2011MA291B
Massachusetts Water Resources Research Center
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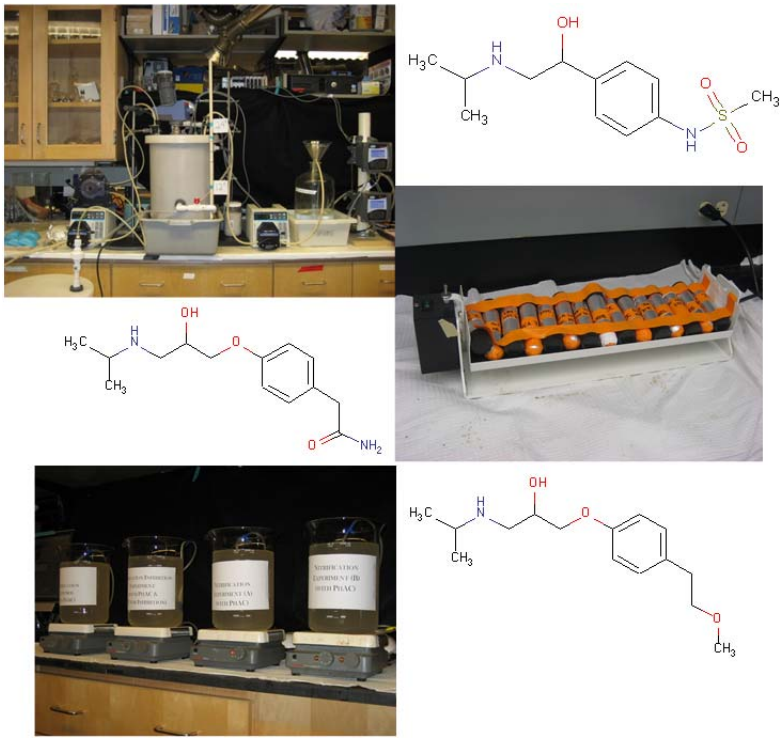
May 2013



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Problem and Research Objectives:

Reduction of nutrient discharges and, more generally, management of the nitrogen cycle are challenges currently faced by the Nation's community of water professionals (NAE, 2008). In the Northeast United States, impacts of excess nutrients on water quality in the Long Island Sound and Narraganset Bay have resulted in the promulgation of stringent limits on nutrient discharges within the States of Connecticut and Rhode Island, respectively. Within the Commonwealth of Massachusetts the Department of Environmental Protection (MADEP) has indicated that the development of total maximum daily loads for nutrients and the management of nutrient discharges are among its priorities for the next two decades (MADEP, 2008b). In fact, MADEP is evaluating options for stringent nitrogen standards total nitrogen (TN) < 5-8 mg/L) for wastewater treatment plants within the Connecticut River watershed, the Blackstone River watershed, and the Ten Mile River watershed (MADEP, 2008a).

Overlain in both space and time with the challenges related to nutrient control is the emerging challenge of understanding and mitigating the influence of microconstituents on environmental health (Schwarzenbach et al., 2006). The occurrence of microconstituents in the environment is now receiving significant attention across the engineering, science, and lay communities (e.g., Daughton and Ternes, 2000; Kolpin et al., 2002; Associated-Press, 2008). In its landmark national reconnaissance, the United States Geological Survey (USGS) established the presence of microconstituents in surface water bodies across the country including several water bodies located within the Commonwealth of Massachusetts (Kolpin et al., 2002). A more recent USGS project on Cape Cod detected 43 microconstituents among 14 sampling sites that included wastewater influents and drinking water supplies (Zimmerman, 2005).

Pharmaceutically active compounds (PhACs) are particularly concerning as microconstituents because the explosion of development and use of these chemicals over the last 30 years, and a growing body of evidence that suggests: (i) PhACs are neither fully removed nor fully transformed in conventional wastewater treatment plants (Heberer, 2002; Ternes et al., 2004; Stephenson and Oppenheimer, 2007); and (ii) chronic exposure, even at concentrations on the order of ng/L, may have adverse effects on ecosystems, such as impaired embryo development and modification of feeding behavior (Cleuvers, 2003; Kostich and

Lazorchak, 2008; Quinn et al., 2009). Recent research suggests that PhACs may be better removed where wastewater treatment was designed to meet stringent regulations on nitrogen discharge (Clara et al., 2005; Joss et al., 2005; Kimura et al., 2005). Unfortunately, however, the vast majority of studies examining the fate of pharmaceuticals through the wastewater treatment process focus on the disappearance of the parent compound. Only a few studies have attempted to elucidate the biochemical processes responsible for PhAC degradation and the biodegradation products formed by these processes (Zwiener et al., 2002). Thus, there is a need for mechanistic research to elucidate the processes that degrade or remove pharmaceuticals during nutrient removal.

The overall objective of the project was to elucidate the attenuation potential and rates of selected pharmaceuticals by nitrifying bacteria. This objective was achieved using a combination of laboratory scale experiments and mathematical modeling. Batch experiments were used to evaluate sorption and biodegradation of selected PhACs during nitrification. The batch experiments were conducted using a mixed biomass consortium from a nitrification enrichment culture. Where biodegradation of the PhACs was observed, mathematical modeling was used to: (i) evaluate the rate of PhAC degradation; and (ii) link the degradation rate to models of ammonia oxidizing bacteria (AOB) growth.

Methodology:

Materials

Pharmaceuticals selected for this research were purchased from Sigma Aldrich (Saint Louis, MO) and included atenolol (ATN), metoprolol (MET) and sotalol (SOT). Purified water (resistivity ≥ 18.2 mΩ/cm and total organic carbon (TOC) ≤8 ppb) was obtained from a MilliQ Gradient A-10 station (Millipore Inc.). Unless otherwise specified, all chemicals were purchased from Fisher Scientific and Acros Organics.

Nitrification Enrichment Consortium

A sequencing batch reactor (SBR) was used to enrich sludge collected from a municipal wastewater treatment facility in Massachusetts. Seed biomass was collected from the second

stage of a two stage facility (stage 1- BOD removal followed by clarification, stage 2- nitrification with clarification). The nitrification enrichment SBR was generally operated on a 8-h cycle (90 min fill, 315 min react (aerobic), 60 min settle, 15 min decant) with pH between 7.5 and 8.0 and DO between 2.5 and 3.0 mg/L. The feed solution to the SBR comprised ammonium sulfate, potassium dihydrogen phosphate and nutrients to promote the growth of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). No exogenous carbon was added to the SBR.

Analytical Methods

ATN, MET and SOT were quantified with fluorescence detection subsequent to separation on an Agilent Series 1100 HPLC equipped with a Kinetix C-18 column (Phenomenex, 2.1 mm x 150 mm, 100 Å). Quantification of ATN was based on FLD excitation wavelength (λ_{EX}) of 235 nm and emission wavelength (λ_{EM}) of 314 nm. For MET and SOT, λ_{EX} / λ_{EM} were 228/324 nm and 235/319 nm, respectively. Method detection limits for ATN, MET and SOT (in picograms on column) were 100, 150 and 150, respectively. Ammonia nitrogen concentrations (S_{NH}) were measured using a colorimetric assay: HACH method 10031 with UV absorbance at 655 nm measured using a Perkin Elmer lambda 25 UV/VIS spectrophotometer. Concentrations of nitrite (S_{NO2}) and nitrate (S_{NO3}) were quantified using Dionex ICS 2000 Ion Chromatograph. Total suspended solids (TSS) and volatile suspended solids (VSS) were measured using methods 2540D and 2540E of Standards Methods, respectively. DNA was extracted from the frozen biomass samples prepared from the batch experiments using MOBio Powersoil isolation kits (MOBIO, Carlsbard, CA) and stored at -80 ^{o}C until needed for further analysis. DNA concentration and quality were measured using a using nanodrop lite UV spectrophotometer (Thermofisher Scientific). qPCR was used to estimate the abundance of total bacteria (EUB), ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). AOB abundance was measured using the ammonia monooxygenase gene subunit A (amoA). Abundance of both Nitrospira (NOB-Ns) and Nitrobacter (NOB-Nb) were measured by targeting the 16s rRNA gene (NOB-Ns; NOB-Nb). EUB abundance was measured using 16s rRNA gene targeted primers (see Table 1 for details).

Table 1. Primers and conversions used in qPCR analyses.

Target	Primer Information				Cell Gene Copies Information		Cell Mass Information	
	Primer ID	Sequence (5'-3')	Pos.	Primer Sequence Reference	$C_{CELL-GENE-COPIES}$ (copies/cell)	Reference	$M_{BACTERIAL-CELL}$ (g-VSS/cell)	Reference
EUB	1055f	ATGGCTGTCGTCAGCT	1055-1070	Ferris et al. (1996)	4.2	Klappenbach et al. (2001); Graham et al. (2007)	$2.8x10^{-13}$	Ahn et al. (2008)
	1392r	ACGGGCGGTGTGTAC	1392-1406					
AOB amoA	amoA-1F	GGGGTTTCTACTGGTGGT	332-339	Rotthauwe et al. (1997)	2.5	Norton et al. (2002)	$1.6x10^{-13}$	Farges et al. (2012)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC	802-822					
NOB-Ns	NTSPAf	CGCAACCCCTGCTTTCAGT	1081–1099	Kindaichi et al. (2006)	1	Graham et al. (2007)	$1.4x10^{-13}$	Farges et al. (2012)
	NTSPAr	CGTTATCCTGGGCAGTCCTT	1128–1147					
NOB-Nb	1198f	ACCCCTAGCAAATCTCAAAAAACCG	1198-1223	Graham et al. (2007)	1	Starkenburg et al. (2006)	$1.4x10^{-13}$	Farges et al. (2012)
	1423r	CTTCACCCCAGTCGCTGACC	1423-1443					

In addition to providing estimates of gene copy concentrations, qPCR data were used to estimate biomass (total bacteria, AOB and NOB) concentrations (in mg COD/L) using Equation 1 with conversion factors for each consortium as shown in Table 1.

$$X_{BIOMASS}\left(\frac{g-COD}{L}\right)=\left[\frac{\left[C_{SAMPLE-GENE-COPIES}\left(\frac{copies}{L}\right)\right]}{\left[C_{CELL-GENE-COPIES}\left(\frac{copies}{cell}\right)\right]}\left(\frac{cells}{L}\right)M_{BACTERIAL-CELL}\left(\frac{g-VSS}{cell}\right)\right]\left[1.42\left(\frac{g-COD}{g-VSS}\right)\right] \quad (1)$$

Experimental evaluation of pharmaceutical sorption

Pharmaceutical sorption was evaluated using batch experiments setup in 30 ml foil covered glass vials closed with Teflon-lined caps. Vials contained mixed liquor from the nitrification SBR and one pharmaceutical at initial concentrations ranging from 0.5 to 50 µg/L. Sorption of the pharmaceutical at each concentration was assessed in triplicate. Homogenous samples are collected every six to eight hours. Samples are centrifuged and the pharmaceutical concentration in the aqueous phase was measured. The sorbed pharmaceutical concentration ($\mu g \cdot g\text{-}SS^{-1}$) was calculated. Equilibrium was considered to have been achieved when the measured aqueous PhAC concentration of three successive samples are the same. Positive controls are included to assess pharmaceutical sorption to the glass vial. Sorption isotherms are developed using the equilibrium sorption data; the sorption coefficient (K_D) was calculated for each pharmaceutical.

Predictive models for pharmaceutical sorption during biological wastewater treatment

Reported values for distribution coefficients (K_D) describing PhAC sorption during biological wastewater treatment were compiled from peer-reviewed studies (total of 388 K_D values for 66 PhACs from 12 studies). The ability of single parameter models based on octanol-water partitioning coefficients (K_{OW}) of the PhACs (Eq. 2) was examined. The single parameter model evaluated was extended to include models based on the apparent partition coefficients (K_D) (i.e., K_{OW} corrected to the experimental pH).

$$\log K_{D,PhAC}=\alpha \log K_{OW,PhAC}+\beta \quad (2)$$

We also evaluated two separate polyparameter predictive modeling approaches for PhAC sorption: (i) Linear Free Energy Relationship (LFER) employing predictors developed by Abraham (1993) and (ii) quantitative structural activity relationship (QSAR) models of the form shown in Eq. 3 utilizing PhAC chemometric properties which are typically available early in the drug development/design process. We found that LFER models were not robust enough to describe PhAC sorption (Sathyamoorthy and Ramsburg, 2013).

$$\begin{aligned}\log K_{D,PhAC} = \chi &+ a\left[\log K_{OW,PhAC} \text{ or} \log D_{PhAC}\right] + b\left[\left(\log MW \text{ or} \log MV\right)\right] \\ &+ c\left[\log(vdWSA)\right] + d\left[\log(TPSA)\right] + e\left(nAroC\right) + f\left(Pi.Energy\right) \\ &+ g\left(nHBD\right) + h\left(nHBA\right) + i\left(nRB\right) \\ &+ j\left(Dom.Species\right) + k\left(\alpha_{+}\right) + l\left(\alpha_{-}\right)\end{aligned} \quad (3)$$

Polyparameter QSARs of increasing complexity were systematically developed by addition of a new predictor to the previously best model until the addition of another predictor was not statistically significant (i.e., $p > 0.05$). A leading coefficient (χ) was included in models evaluated – omission of the leading coefficient would imply that the sorption mechanism can be entirely described by the predictor variables, which has limited physical meaning. For each model, the statistical significance of predictors was evaluated at $p < 0.05$, residuals were checked for homoscedasticity, and multicollinearity between predictors was evaluated.

Models were developed and evaluated using Minitab 16.1.1, and assessed using a suite of statistics. The ability of each model to capture the variance in the data set used to develop the model was evaluated using the correlation coefficient (R^2) and adjusted-R^2 (adj-R^2). The predictive capability of models was assessed through predicted-R^2 (pred-R^2) and Nash-Sutcliffe Efficiency (NSE) (Nash and Sutcliffe, 1970). Unlike R^2 which describes the goodness of correlation, pred-R^2 is a goodness of prediction statistic based upon the prediction residuals of sum squares (Myers et al., 2010). The NSE ranges from $-\infty$ to 1 and is typically greater than 0. Negative NSE values are possible and indicate that the mean of the measured K_D values from the data set was a better predictor than the predictive model. Strong predictive capability is generally characterized by pred-$R^2 > 0.7$ and NSE > 0.7 (McCuen et al., 2006).

Experimental evaluation of pharmaceutical biodegradation

A series of batch experiments was conducted to evaluate the biodegradation of the three, selected beta blockers (ATN, MET, SOT) by a nitrification activated sludge system. Selection of these three beta blockers permitted assessment of biodegradation within a family of pharmaceuticals that differ by one-to-two functional groups. Biomass for all experiments was taken from a nitrification enrichment sequencing batch reactor (Nit-SBR) maintained in the PI's laboratory. The Nit-SBR was continuously operated with a feed with ammonia and without the any exogenous organic carbon. Our experimental protocol included controls (in the absence of pharmaceutical) for nitrification (i.e., ammonia + nitrite oxidation) and nitrite oxidation. These controls characterize the microbial consortia obtained from our nitrification sequencing batch reactor before each experiment. Nitrification experiments that contain pharmaceutical were conducted in duplicate. Controls were also included to evaluate pharmaceutical degradation when nitrification was inhibited using allylthiourea (ATU). Time course samples are collected to quantify pertinent solutes during each experiment (i.e., each set of four reactors - two experimental replicates and two controls).

Modeling of pharmaceutical degradation and ammonia oxidation

PhAC biodegradation was modeled using two approaches: a pseudo first order model based on reactor total biomass concentration as measured using VSS (Eq. 4) and a consortium level cometabolic model that incorporates the relevant modules from the Activated Sludge Model framework (Henze et al., 2000) with nitrification modeled as a two-step process (Chandran and Smets, 2000; Hiatt and Grady, 2008). The pseudo first order approach (Eq. 4) is frequently used to model microconstituent degradation despite its lack of mechanistic or process significance (Urase and Kikuta, 2005; Joss et al., 2006; Fernandez-Fontaina et al., 2012; Helbling et al., 2012). Although such a formulation is convenient, it is of limited value when comparing systems with different design or operating conditions. The principal shortfall of this approach is that it does not link PhAC degradation to a specific process occurring within the mixed culture.

$$\frac{dS_{PhAC}}{dt} = -(k_{BIO} X_{TOT}) S_{PhAC} \quad (4)$$

To address this shortcoming, a consortium level model was developed. Existing approaches for cometabolic biodegradation modeling (Criddle, 1993; Alvarez-Cohen and Speitel, 2001) were adapted to integrate PhAC biodegradation into the ASM framework. Three PhAC biodegradation scenarios were explored using the consortium level model as shown in Eq. 5: (i) cometabolic biodegradation linked to ammonia oxidizing bacteria (AOB) growth; (ii) biodegradation by AOB in the absence of growth; and (iii) biodegradation due to heterotrophs (HET) present in the mixed culture.

$$\frac{dS_{PhAC}}{dt} = -\left\{ \begin{array}{l} \left[\left\{ \left[T_{PhAC-AOB} \mu_{AOB} \right] + \left[k_{PhAC-AOB} \right] \right\} X_{AOB} \right] + \\ \left[\left\{ \alpha_{PhAC-HET} \right\} X_{HET} \right] \end{array} \right\} S_{PhAC} \quad (5)$$

Here $T_{PhAC-AOB}$ is a PhAC transformation coefficient linked to AOB growth [$L^3 M_{COD}^{-1}$], μ_{AOB} is the AOB growth rate [T^{-1}], $k_{PhAC-AOB}$ is a biomass normalized PhAC degradation rate coefficient in the absence of AOB growth [$L^3 M_{COD}^{-1} T^{-1}$] and X_{AOB} is the AOB concentration [$M_{COD} 3^{L-1}$]. PhAC degradation is linked to X_{HET} using a single biomass normalized PhAC degradation rate coefficient $\alpha_{PhAC-HET}$ [$L^3 M_{COD}^{-1} T^{-1}$] because heterotroph growth was not modeled (i.e., the analogous transformation capacity was not evaluated for heterotrophs). It is important to note that while this research places emphasis on evaluating biodegradation by nitrifying organisms, the model framework proposed here is flexible and readily adapted to other consortia and processes. For instance, as data related to role of heterotrophs in biodegradation of those PhACs evaluated in this research, it may be possible to replace $\alpha_{PhAC-HET}$ with more explicit parameters linked to growth as done herein for AOB.

Principal Findings and Significance:

Sorption of pharmaceuticals during biological wastewater treatment

Evaluation of the sorption of the three beta-blockers (ATN, MET, and SOT) during nitrification in batch experiments suggested that sorption holds limited potential as an attenuation mechanism for these pharmaceuticals. Of the three beta-blockers, only MET sorbed to the inactivated nitrification SBR mixed liquor to an extent that permitted calculation

of a statistically non-zero distribution coefficient (K_D). The measured sorption coefficient for MET was highly dependent on experimental conditions. Two separate experiments produced K_D values of 0.26 ± 0.03 and 0.09 ± 0.01 L/g-SS.

Based upon these results we undertook a more significant assessment of pharmaceutical sorption during biological wastewater treatment. The assessment examined all available (published) data for sorption of pharmaceuticals during biological wastewater treatment - a total of 309 measured K_D values for 65 pharmaceuticals. Principal findings are reported here. Full details from this research are available in Sathyamoorthy and Ramsburg (2013). One of the aspects we evaluated was the role of experimental protocols (i.e., experiment type and biomass inactivation method) on measurements of K_D values. While the data are limited, our meta-analysis suggests the experiment type (batch or continuous flow) and inactivation method (chemical, physical, or no inactivation) (see Figure 1 and Figure 2) does not explain the large variation in measured K_D values. Therefore, large ranges in the reported values for K_D are unrelated to differences in experimental conditions. Rather, they are related to variations in the interaction between the pharmaceutical and the biosolids surface.

Conventional wisdom suggests that the hydrophobic interactions dominate the sorption of organic chemicals to biomass. It is also common to assume equilibrium and apply a linear isotherm to describe the sorption. The combination of these assumptions has led many researchers to attempt to correlate pharmaceutical sorption (described using the K_D) to the octanol-water distribution coefficient for the pharmaceutical (K_{ow}) (Stevens-Garmon et al., 2011; Hyland et al., 2012). Results from our research suggest that one parameter models based on octanol-water partitioning (even when $\log K_{OW}$ was corrected to the experimental pH conditions, i.e., log D) are generally ineffective at describing sorption of negatively-charged, uncharged, and positively-charged PhACs during biological treatment (Figure 3).

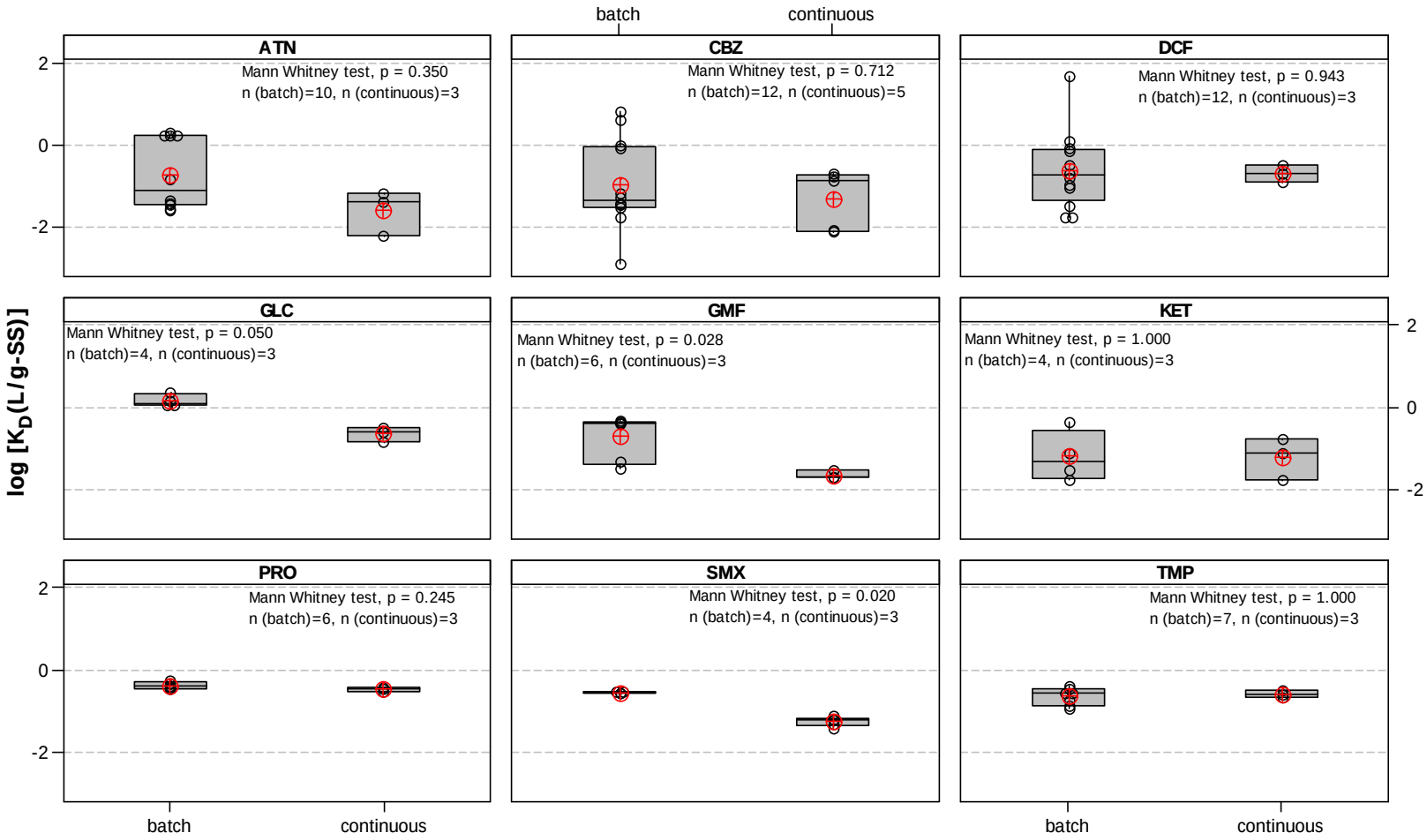


Figure 1. Comparison of measured sorption coefficients for atenolol (ATN), carbamazepine (CBZ), diclofenac (DCF), glibenclamide (GLC), gemfibrozil (GMF), ketoprofen (KET), propranolol (PRO), sulfamethaxazole (SMX) and trimethoprim (TMP) from batch and continuous experiments. Individual data points shown using small black circles; horizontal line indicates median; mean indicated by large red circle with cross-hairs. Box extents indicate 25th (Q1) and 75th (Q3) percentile with whiskers extending to upper limit [Q3 + 1.5(Q3-Q1)] and lower limit [Q1 - 1.5(Q3-Q1)]. Also shown are p-value of one-tailed Mann Whitney test and number of data points from batch [n (batch)] and continuous [n (continuous)] experiments.

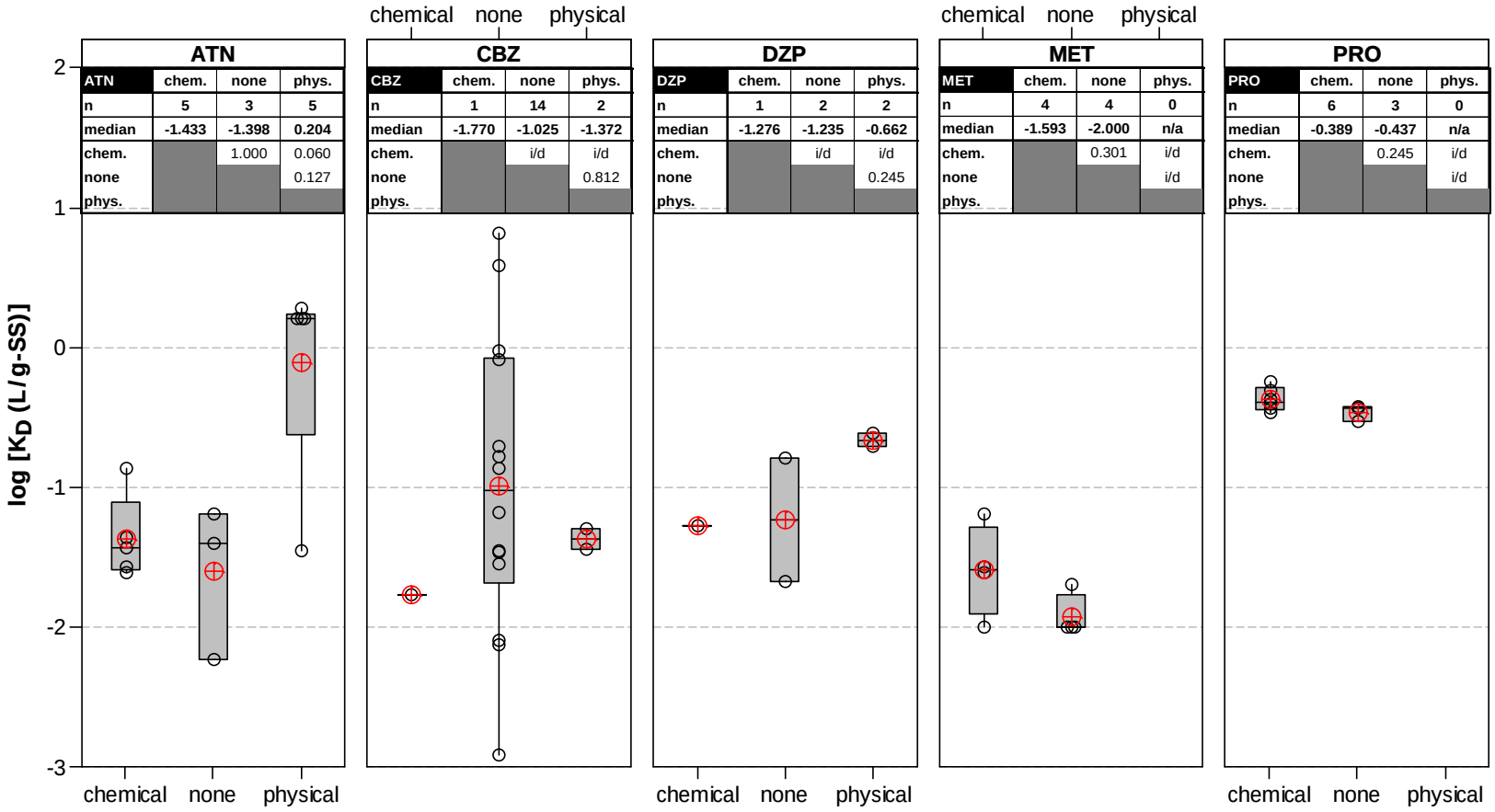


Figure 2. Measured Sorption Coefficients for atenolol (far left), carbamazepine, diazepam (middle), metoprolol and propranolol (far right) from batch and continuous experiments using chemical inactivation (e.g., NaN3) no biomass inactivation, and physical inactivation (e.g., lyophylization). Individual data points shown using small black circles; horizontal line indicates median; mean indicated by large red circle with cross-hairs. Box extents indicate 25th (Q1) and 75th (Q3) percentile with whiskers extending to upper limit [Q3 + 1.5(Q3-Q1)] and lower limit [Q1 - 1.5(Q3-Q1)]. Also shown are number of data points (n), median log[K_D(L/g-SS)] and p-value of one-tailed Mann Whitney test evaluating differences between inactivation methods (note: n/a = not applicable, i/d = insufficient data available for statistical evaluation).

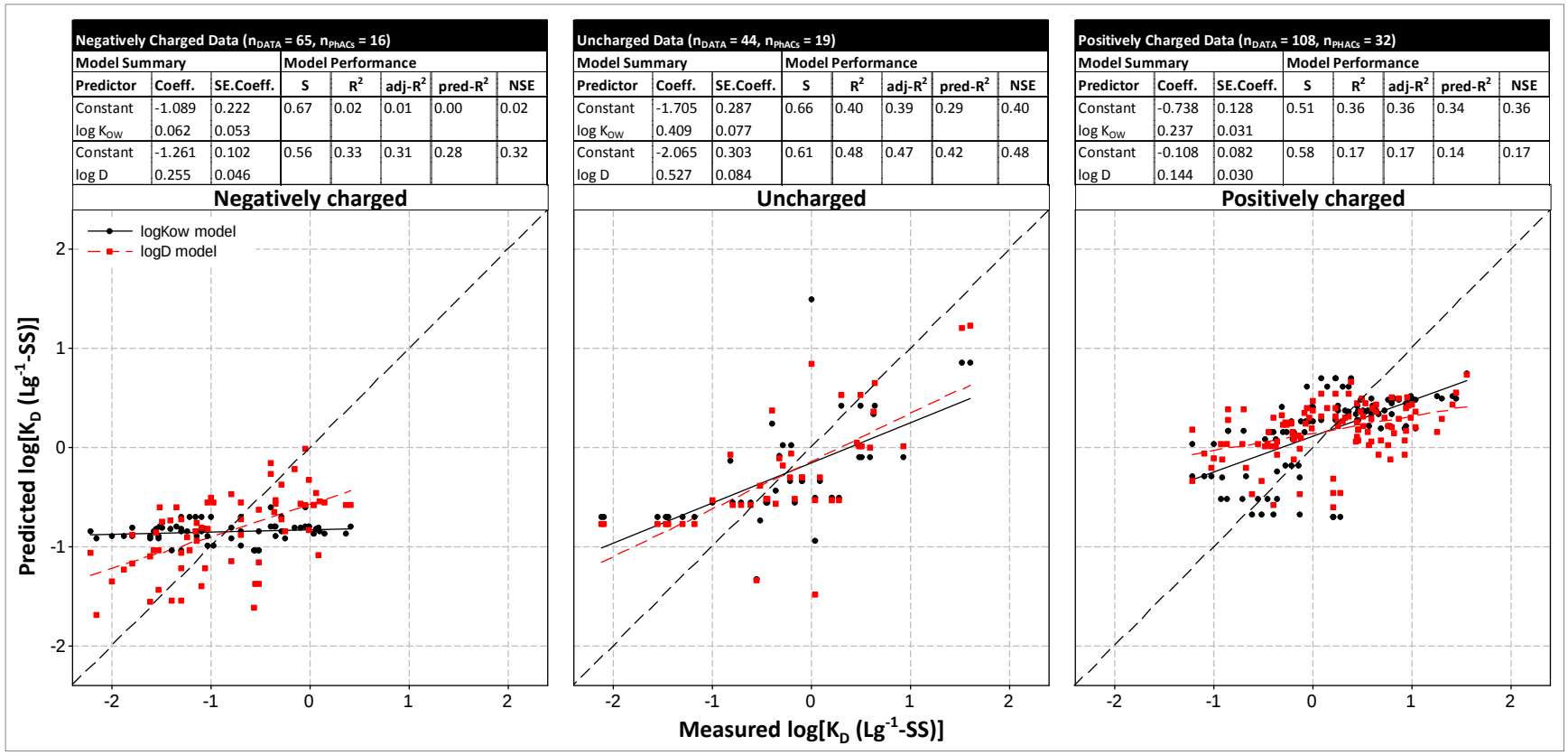


Figure 3. Reported log K_D values with predictions using one-parameter models based on log K_{OW} (black) and log D (red) for negatively charged (left), uncharged PhACs (middle) and positively charged PhACs (right). Model coefficients and performance is shown in the overlying tables.

Polyparameter quantitative structural activity relationship (QSAR) models were explored as an alternative means of predicting the observed sorption extents. The QSAR models employed a suite of molecular descriptors that are readily available during drug design and development process. The predictor variables included molecular weight (MW), molecular volume (MV), aromaticity, number of rotatable bonds (n.RB), hydrogen bonding capacity (hydrogen bond donors- nHBD and acceptors- nHBA) and polar surface area (PSA). Models of increasing complexity were systematically developed by adding one of the aforementioned predictors to the best model of with a given number of predictor variables. The performance of each model was evaluated using two main statistics – adjusted r-square (adj-R^2) and predicted r-square (pred-R^2). As noted in the methodology section, model residuals were checked for homoscedasticity and multicollinearity between model variables was evaluated. The polyparameter QSAR models developed in this research provide a significant improvement in the ability to predict K_D values (see Figure 4 and Table 2 for model details). The plateau in predictive capability at approximately 50% - 60% (Figure 4), however, suggests that while the best polyparameter QSAR models offer improvement over previously established correlations, none can be characterized as having strong predictive power. Importantly, QSAR models with a higher degree of predictive capability (pred $R^2 > 0.80$) can be developed for scenarios where the uncharged species is greater than 85% of the total PhAC mass present in a system. But, restrictions on the fraction of uncharged species degrade model utility and practicability, especially in the case of acidic PhACs. For example, only 12 of the 66 PhACs tested to date would meet this threshold under normal treatment conditions. We hypothesize that the performance plateau results from only including solute-based descriptors, and suggest future research focus on characterization of the sorbent surface to better characterize the mechanistic interactions between sorption sites on biosolids and pharmaceuticals (Sathyamoorthy and Ramsburg, 2013).

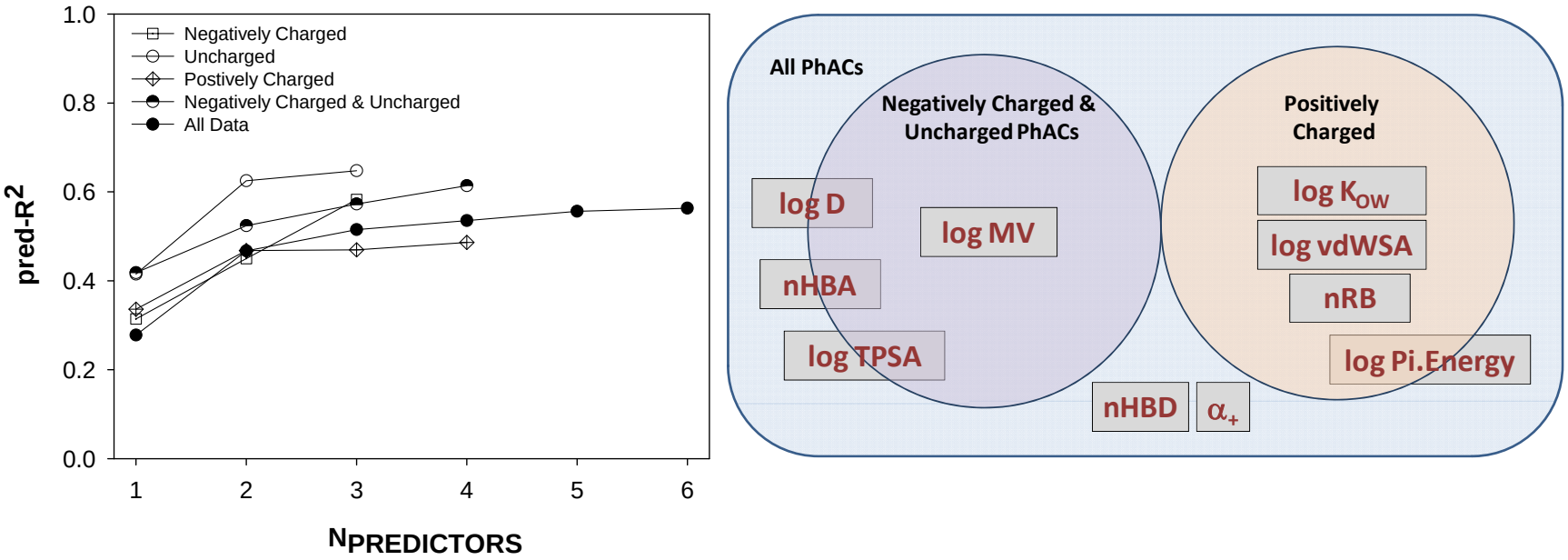


Figure 4. Left: Predictive capability (pred-R^2) of polyparameter QSAR models with increasing number of statistically significant predictors. Note that correlation for uncharged PhACs can be improved when the fraction of uncharged mass is > 85% (see discussion in text). Right: Predictors which are significant in predictive models for sorption of negatively charged/unchanged pharmaceuticals, positively charged pharmaceuticals and all pharmaceuticals. Model details and summary statistics are provided in Table 2.

Table 2. Summary of best fit polyparameter QSAR models developed to describe the sorption of pharmaceuticals to suspended solids biological treatment.

$N_{PRED.}$	Model Summary: $\log[K_D(Lg^{-1}\text{-}SS)]$ =	Model Performance				
		S	R^2	adj-R^2	pred-R^2	NSE
Uncharged PhACs (n_{DATA} = 44; n_{PhACs} = 19)						
3	QSAR Model: [-3.12±0.29] + [(0.63±0.07)log D] + [(0.30±0.06)nHBA] + [(-0.07±0.03)nRB]	0.45	0.73	0.71	0.65	0.73
Negatively Charged PhACs (n_{DATA} = 65; n_{PhACs} = 16)						
3	[5.88±1.69] + [(0.37±0.05)logD] + [(0.30±0.05)nHBA] + [(- 3.56±0.78)logMV]	0.44	0.60	0.58	0.56	0.61
Positively Charged PhACs (n_{DATA} = 108; n_{PhACs} = 32)						
4	(7.65±2.24) + [(0.34±0.04)]log(K_{OW})] + [(1.65±0.31)]log(PiEnergy)] + [(-4.34±0.94)]log(vdWSA)] + [(0.05±0.02)]log(nRB)]	0.44	0.54	0.52	0.49	0.54
Models for Grouped PhACs						
Negatively Charged and Uncharged PhACs (n_{DATA} = 109; n_{PhACs} = 16)						
4	[4.54±1.36) + [(0.39±0.04)logD] + [(0.32±0.04)nHBA] + [(-2.41±0.59)logMV] + [(-0.86±0.25)log(TPSA)]	0.48	0.64	0.63	0.61	0.64
All PhACs (n_{DATA} = 217; n_{PhACs} = 54)						
6	(-1.74±0.46) + [(0.22±0.03)logD] + [(0.92±0.10)α_+] + [(0.99±0.28)log(Pi.Energy)] + [(-0.85±0.17)log(TPSA)] + [(0.14±0.05)nHBD] + [(0.08±0.03)nHBA]	0.53	0.59	0.58	0.56	0.59

Parameter values are reported with the standard error of the estimate. See Table 1 for definition of the predictors.

Biodegradation of beta-blockers during nitrification

Several studies have reported that WWTPs operated at long solids retention times (SRTs ≥ 8-10 days) demonstrate improved removal of PhACs (Kreuzinger et al., 2004; Clara et al., 2005; Joss et al., 2006), yet it remains unclear if this observation is related to the presence of slow growing bacteria (e.g., nitrifying bacteria) or an increase in the microbial diversity (Shi et al., 2004; Batt et al., 2006; Reif et al., 2008; Tran et al., 2009; Suarez et al., 2010; Falas et al., 2012; Fernandez-Fontaina et al., 2012). Thus, the role of nitrification processes in the biodegradation of three beta blockers – atenolol (ATN), metoprolol (MET) and sotalol (SOT) was evaluated (see Table 3 for the structure and properties of each pharmaceutical). Full details of this research are available in a forthcoming manuscript (Sathyamoorthy et al., in preparation). Focus in this report is placed on the key findings.

Results related to characterization of biomass in batch experiments

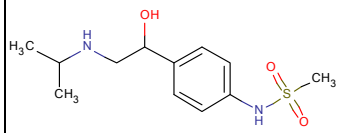
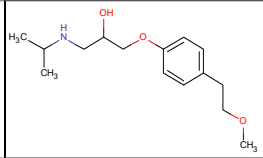
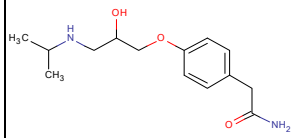
The qPCR in this research targeted the amoA gene of AOB and the 16s rRNA gene of EUB, NOB-Ns and NOB-Nb using a composite DNA sample from each reactor. Results from these analyses indicate that AOB are the dominant nitrifying consortium in these samples, making up between ~75% and 85% of the nitrifying population (i.e., AOB + NOB). This is within the range noted in previous studies of nitrifying populations (Li et al., 2006). Nitrobacter are dominant NOB effectively accounting for the remainder of the nitrifying population. Nitrospira NOB account for less than 0.1% of the nitrifying population. The negligible fraction of Nitrospira results from the high ammonia concentrations used in the nitrification enrichment SBR which was the seed biomass source for these experiments. High ammonia levels result in high nitrite concentrations during the SBR cycle which favors Nitrobacter over Nitrospira (Schramm et al., 2000).

Results related to pharmaceutical biodegradation

Results indicate that ATN was degraded during nitrification whereas no degradation was observed for MET or SOT (see Figures 5, 6 and 7). Interestingly, atenolol biodegradation was also noted in the nitrification inhibition control (275 μM ATU for nitrification inhibition). The

extent of ATN biodegradation in the experimental reactors was ~80% compared to ~30% in the nitrification inhibition control. The extent of ATN degradation in a follow up experiment conducted to evaluate biodegradation of ATN during nitrite oxidation was comparable to the nitrification inhibition control (~28%). Collectively, these data suggest that although ATN was biodegraded by non-nitrifying bacteria present in the culture (presumably heterotrophs), nitrifying bacteria had a substantial role in ATN degradation. Furthermore, this research demonstrates that not all pharmaceuticals within the same compound or therapeutic class are biodegraded by the same group of bacteria.

Table 3: Properties of pharmaceuticals selected for this research with reported concentrations in environmental systems

		SOT	MET	ATN
Basic Parameters	**Formula**	$C_{12}H_{20}N_2O_3S$	$C_{16}H_{21}NO_2$	$C_{14}H_{22}N_2O_3$
	MW (g/mol)	272.4	259.3	266.3
	Structure			
Partitioning	**Log K_{OW}**	0.24	3.48	0.16
	pK_A	8.35, 9.98	9.7	9.6
Geometry & Stereochemistry	**TPSA (A^2)**	78.4	50.7	84.6
	%.Aro.C	50%	40%	43%
	No. Rot.Bonds	6	6	8
	H-bond Don.	3	2	3
	Acc.	5	4	4
	VdW SA (A^2)	430.76	474.69	440.41
Environmental concentration	**WWTP influent (ug/L)**		0.21-0.25 (Siemens et al., 2008) 1.80-2.60 (Siemens et al., 2008)	-- (Miege et al., 2008) 2.3(Ternes et al., 2007)
	Prim. Effluent (ng/L)	180-567 (Lee et al., 2007)	214-664 (Lee et al., 2007)	1,180-2,210 (Lee et al., 2007)
	WWTP Effluent (ng/L)	162-429 (Lee et al., 2007)	177-402 (Lee et al., 2007)	642-1,680 (Lee et al., 2007)

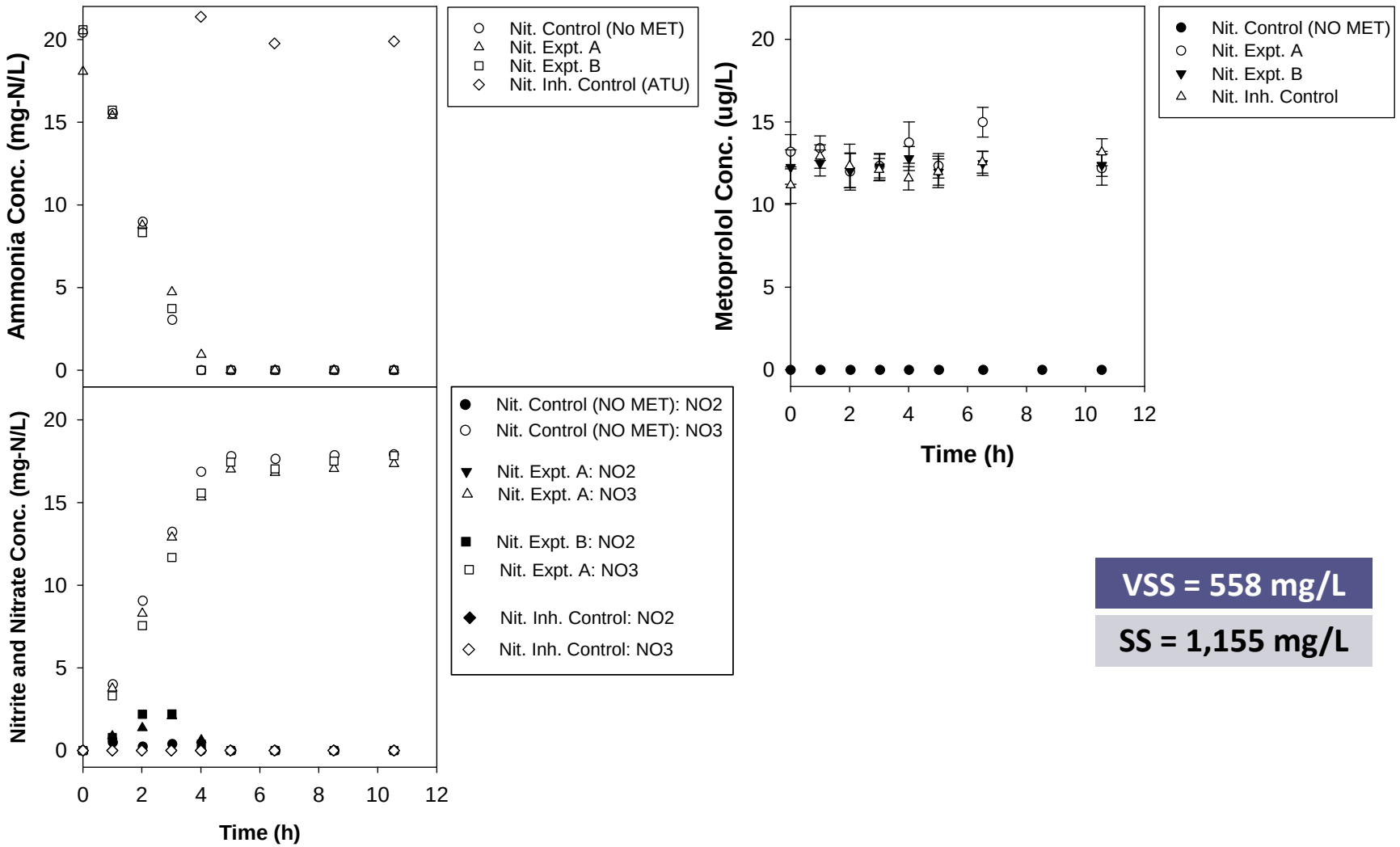


Figure 5. Results from experiment evaluating biodegradation of metoprolol during nitrification: concentration in the aqueous phase of ammonia (top left panel), nitrite and nitrate (bottom left panel) and metoprolol (top right panel). Each plot contains data from four reactors: one nitrification control reactor which has no metoprolol (Nit.Control (No MET)), two experimental reactors (Nit.Expt. A and Nit.Expt. B) and one nitrification inhibition control reactor (Nit.Inh.Control) where ATU is used to inhibit nitrification. Also shown in the bottom right are VSS and SS for reactors.

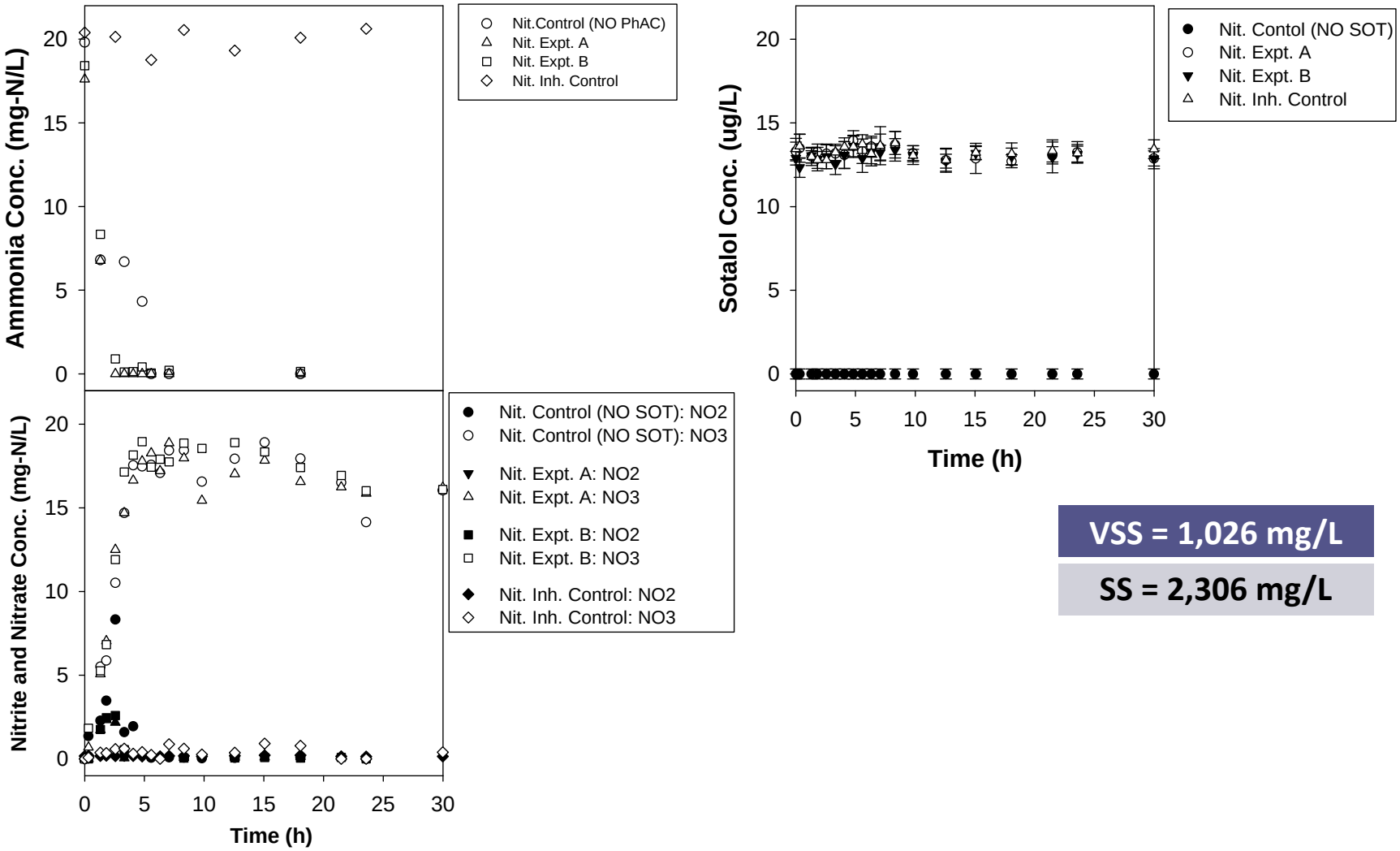


Figure 6. Results from experiment evaluating biodegradation of sotalol during nitrification: concentration in the aqueous phase of ammonia (top left panel), nitrite and nitrate (bottom left panel) and sotalol (top right panel). Each plot contains data from four reactors: one nitrification control reactor which has no sotalol (Nit.Control (No SOT)), two experimental reactors (Nit.Expt. A and Nit.Expt. B) and one nitrification inhibition control reactor (Nit.Inh.Control) where ATU is used to inhibit nitrification. Also shown in the bottom right are VSS and SS for reactors.

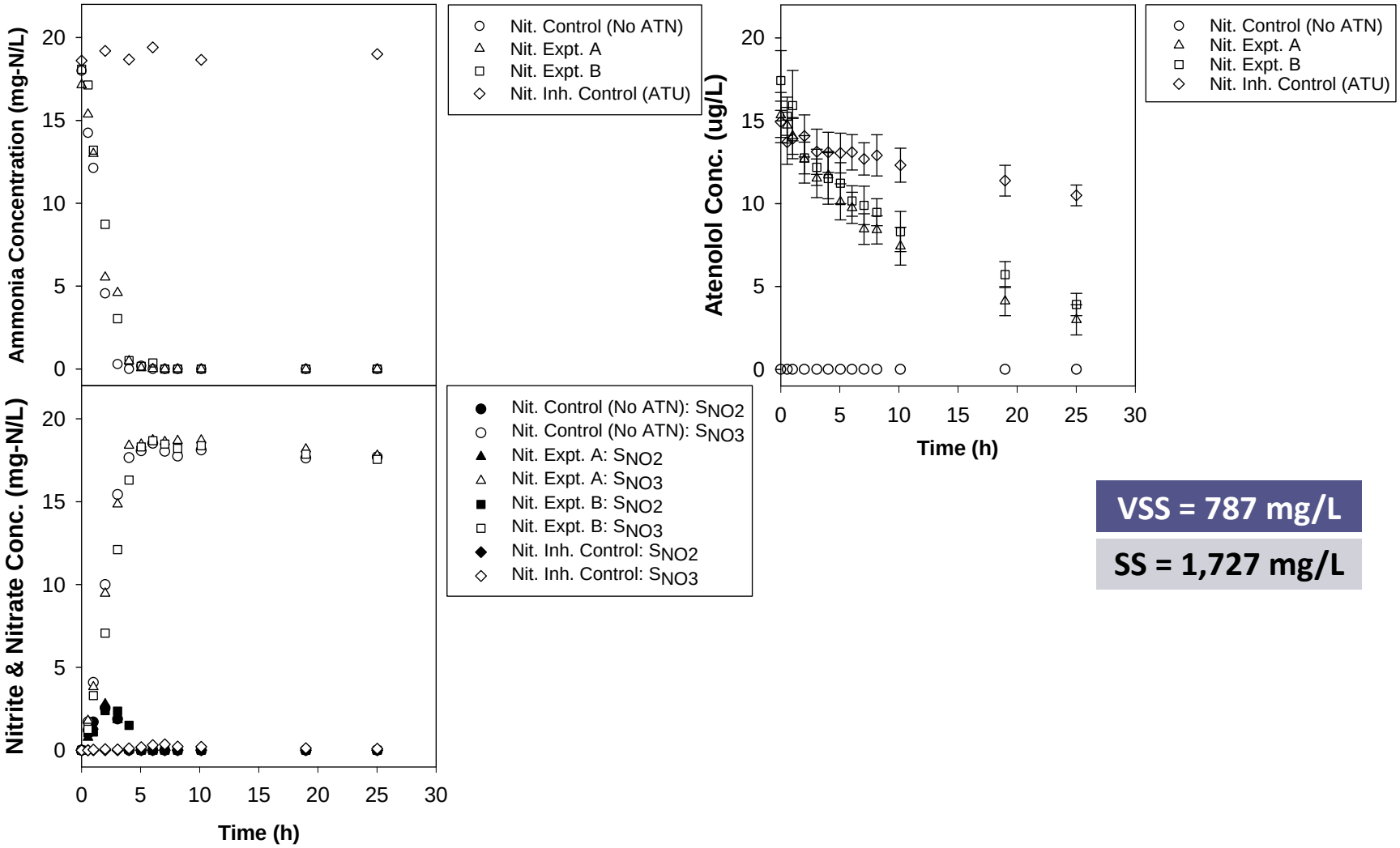
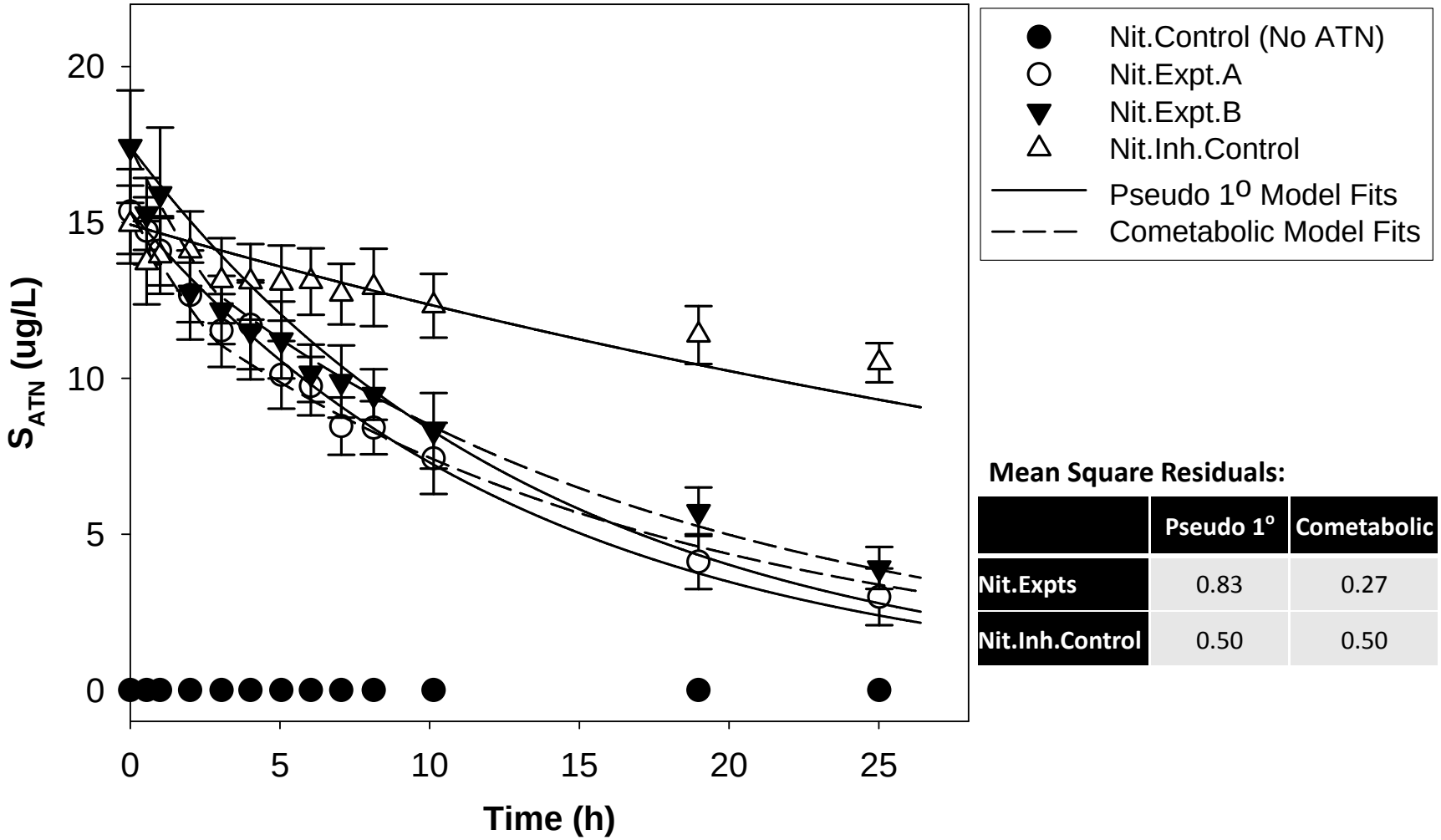


Figure 7. Results from experiment evaluating biodegradation of atenolol during nitrification: concentration in the aqueous phase of ammonia (top left panel), nitrite and nitrate (bottom left panel) and atenolol (top right panel). Each plot contains data from four reactors: one nitrification control reactor which has no atenolol (Nit.Control (No ATN)), two experimental reactors (Nit.Expt. A and Nit.Expt. B) and one nitrification inhibition control reactor (Nit.Inh.Control) where ATU is used to inhibit nitrification. Also shown in the bottom right are VSS and SS for reactors.

The pseudo-first-order biodegradation rate coefficient for ATN fit using data from replicate experimental reactors ($k_{BIO,NIT}$) was 2.39 ± 0.21 L.g-VSS^{-1}.d^{-1}. The analogous rate coefficient using data from the Nit.Inh.Control ($k_{BIO,NIT.INH.}$) was 0.56 ± 0.10 L.g-VSS^{-1}.d^{-1} (see Figure 8 for model fits). The biodegradation rate of ATN under nitrification conditions was approximately four times greater than when nitrification was inhibited using ATU. This was consistent with the hypothesis that the activity of nitrifying bacteria controls the degradation of ATN in this nitrification enrichment culture. The $k_{BIO,NIT}$ values for ATN determined in this research are comparable to those reported by Maurer et al. (2007) (0.98 L.g-SS^{-1}.d^{-1} in batch experiments using biomass from an MBR operated at 20 d SRT) and Wick et al. (2009) (1.90 and 1.10 L.g-SS^{-1}.d^{-1} in batch experiments using sludge from a suspended growth system operated at 18 d). Neither Maurer *et al.* nor Wick *et al.*, however, report nitrogen concentration data which prohibits elucidation of any link between nitrification processes and PhAC biodegradation within their experiments. Both studies also reported attenuation of MET and SOT as resulting from nitrification though this was not observed in our experiments (Figures 5 and 6).

A coupled nitrification cometabolic PhAC degradation model was used to evaluate the role of ammonia oxidizing bacteria in ATN degradation noted in the replicate nitrification experiments and the nitrification inhibition control. Using the data from Nit.Inh.Control, $\alpha_{ATN\text{-}HET}$ was estimated to be 12.6 ± 2.50 L·g-COD^{-1}·d^{-1}. This estimate was utilized to model ATN biodegradation in the replicate experimental reactors and estimate values for the transformation capacity of ATN by AOB (i.e., $T_{ATN\text{-}AOB}$) and the rate associated with ATN biodegradation by AOB in the absence of growth through $k_{ATN\text{-}AOB}$. The best fit values determined for $T_{ATN\text{-}AOB}$ and $k_{ATN\text{-}AOB}$ were to be 71.5 ± 22.7 L·g-COD^{-1} and 16.1 ± 5.58 L·g-COD^{-1}·d^{-1}, respectively. Shown in Figure 8 is a comparison of the model fits and experimental data for the two replicate experiments and the nitrification inhibition control.



Mean Square Residuals:

	Pseudo 1^{o}	Cometabolic
Nit.Expts	0.83	0.27
Nit.Inh.Control	0.50	0.50

Figure 8. ATN concentration in batch experiments evaluating ATN degradation during nitrification. Results from Nit.Expt.A Nit.Expt.B and Nit.Inh.Control are shown with model simulations a using pseudo-first-order model with $k_{BIOL,NIT.}$ = 2.39 and $k_{BIOL,NITINH..}$ 0.56 L.g-VSS^{-1}·d^{-1} and cometabolic model with for $T_{PhAC\text{-}AOB}$ = 0.060 ± 0.017 L mg-COD^{-1} and $k_{PhAC\text{-}AOB}$ = 0.017 ± 0.004 L mg-COD^{-1} d^{-1}. Also shown is a comparison of the mean square residuals for each model based on the experimental data (Nit.Expts.) and inhibition control data (Nit.Inh.Control).

To our knowledge, this research is the first to report transformation coefficients for cometabolic biodegradation of any PhAC by nitrifying communities. Consequently, no existing data are available to compare with the results from this research. However, there is a significant body of knowledge related to cometabolic biodegradation processes in environmental systems (Chang et al., 1993; Criddle, 1993; Alvarez-Cohen and Speitel, 2001) and nitrifying communities more specifically (Ely et al., 1997; Kocamemi and Cecen, 2005, 2010b, a). The $T_{ATN\text{-}AOB}$ value obtained herein (71.5 ± 22.7 $L \cdot g\text{-}COD^{-1}$) is similar to those reported for TCE at concentrations below 350 $\mu g L^{-1}$ (~50 $L \cdot g\text{-}COD^{-1}$, Kocamemi and Cecen, 2010a). Note here that Kocamemi and Cecen (2010a) estimated the transformation capacities they report in their Table 2 by taking the slope of the line formed between the origin and the highest reported degradation rate shown in their Figure 1. In fact, there is a theoretical basis for and evidence of a non-zero intercept based upon cometabolic degradation when there is no growth (e.g., Ely et al. 1997). Thus, the data from Kocamemi and Cecen (2010a) were refit to produce both a slope (indicative of $T_{ATN\text{-}AOB}$) and intercept (indicative of $k_{ATN\text{-}AOB}$). Our estimates of the $T_{TCE\text{-}AOB}$ based upon the data from Kocamemi and Cecen (2010a) assume a yield coefficient for ammonia oxidation of 0.15 $mg\text{-}COD \cdot mg\text{-}N^{-1}$.

Results related to nitrification

Ammonia, nitrite and nitrate concentrations during the batch nitrification experiments with ATN, MET and SOT are shown in the left panels of Figures 5, 6 and 7. Complete nitrification was achieved in all control and replicate reactors for each experiment. No accumulation of nitrite was observed in any of the experiments and the highest nitrite concentration observed was less than 5 mg-N/L which is below levels where nitration of PhACs is considered relevant (Gaulke et al., 2008). Successful inhibition of nitrification was achieved with ATU addition to the inhibition control reactors in each experiment as demonstrated through no production of either nitrite or nitrate during the course of the experiment.

The discrepancy between measured and modeled concentrations of ammonia, nitrite and nitrate concentrations in the ATN experiment were found to be larger at low ammonia concentrations. That is to say, in the case of ATN, the model was unable to satisfactorily predict

the nitrification process when the ammonia concentration was at or below the half saturation value. Interestingly, the same effect of the PhAC on ammonia oxidation rates at low ammonia concentration was not observed in predictions from MET or SOT experiments. The predictive capability for the nitrification process was significantly improved when the nitrogen specie data were refit assuming competitive inhibition of AOB growth by ATN (Bailey J.E and Ollis D.F, 1986). These data suggest that ATN may competitively inhibit ammonia oxidation in these batch experiments. The inhibition constant $K_{I,ATN-AOB}$ was determined to be 1.84 ± 0.39 μgL^{-1}. This suggests that the presence of ATN, at levels consistent with those found in wastewater treatment facilities, in the range of 0.2 to 2.0 μgL^{-1} (Lee et al., 2007; Wick et al., 2009; Jelic et al., 2012) may reduce the growth rate of AOB.

Summary and Implications

The goal of this research was to evaluate the role of sorption and degradation of PhACs in wastewater treatment facilities aimed at meeting stringent nutrient standards. Sorption was evaluated using both batch experiments with specific PhACs and predictive modeling that is more generally applicable. The role of nitrifying bacteria and nitrification processes in PhAC biodegradation was evaluated using a nitrification enrichment culture.

Our experiments indicate that only MET appreciably sorbed to the biosolids in the nitrifying enrichment culture. Broader evaluation of PhAC sorption, across a range of processes and unit operations, using existing values of the PhAC distribution coefficient suggests that the conventional use of single-parameter models based on octanol-water partition coefficients has limited predictive capability. To overcome this limitation, polyparameter QSAR models were developed using chemometric properties of PhACs. These polyparameter models suggest that the single best predictor for PhAC sorption is the charge of the dominant species. Other important predictors include molecular weight (MW), molecular volume (MV), aromaticity, number of rotatable bonds (n.RB), hydrogen bonding capacity (hydrogen bond donors- nHBD and acceptors- nHBA) and polar surface area (PSA). While results indicate that the polyparameter models developed herein significantly enhance predicative capability, the best models can only explain approximately 60% of the variance in the available PhAC sorption data.

More research is therefore required to assess the role that biosolids surface properties have in PhAC sorption.

The relevance of sorption as an attenuation mechanism is illustrated in Figure 9 which shows the fraction of PhAC mass that is associated with biosolids for various distribution coefficients and biomass concentrations. For a conventional activated sludge (CAS) system operating at a typical mixed liquor suspended solids (MLSS) concentration of 3,000 mg L^{-1}, PhACs with a K_D equal to 0.37 L g^{-1} SS will be evenly distributed between the biosolid and aqueous phases. For a membrane bioreactor (MBR) operating at 10,000 - 11,000 mg L^{-1} MLSS the same distribution occurs for at much lower values of K_D (0.10 L g^{-1} SS).

Laboratory experiments were coupled with mathematical modeling to evaluate the biodegradation of the beta blockers ATN, MET and SOT. Results indicate that only ATN was readily degraded by the nitrification enrichment culture used herein. Thus, care should be taken to avoid assuming that the occurrence of nitrification in WWTPs operated at long solids retention times leads to greater biodegradation of PhACs due to a greater presence of nitrifying organisms. It remains an open question; however, if the greater biodiversity associated with longer solids retention times can be relied upon to aid degradation of PhACs. Certainly, additional research is warranted to evaluate the biodegradation of those beta blockers studied here by microbial consortia that are more indicative of wastewater treatment facilities.

Results from the biodegradation experiments conducted with ATN indicate that ATN degradation resulted from ammonia oxidation. In fact, the ATN results suggest that the role of ammonia oxidizing bacteria in PhAC biodegradation may be more relevant than previously estimated. It is conventionally assumed that the role of nitrifying bacteria in PhAC biodegradation is limited by the fact that these organisms represent only a small fraction of the biomass in a wastewater treatment plant. Our research suggests that even when AOB make up 5% of the total biomass in a WWTP reactor, they contribute between 7% - 17% to the biodegradation rate of ATN (Figure 10). That is to say, their contribution outweighs their proportion in the biomass. Additional research is necessary to evaluate the extent to which this observation holds true for other PhACs co-metabolically degraded by the biochemical processes responsible for nitrification.

ATN degradation was accurately described through the use of a coupled nitrification-cometabolic PhAC biodegradation model. The model represents a novel use of an integrated cometabolic biodegradation module within the ASM model framework. This approach is particularly relevant considering the widespread utility of the ASM modeling framework in industrial WWTP process simulators (e.g., Biowin, GPSx, etc.). Consortium level assessments of PhAC biodegradation offer increased sophistication and greater generalizability over pseudo first order biodegradation rate coefficients which offer no mechanistic insight. Additional research is therefore warranted to elucidate cometabolic transformation capacities for a host of PhACs undergoing degradation by heterotrophs and nitrifiers. Development of a suite of transformation capacities for a number of PhACs under a wide range of conditions will provide the foundation necessary for the development of models which can predict PhAC fate in wastewater treatment facilities based upon chemometric properties.

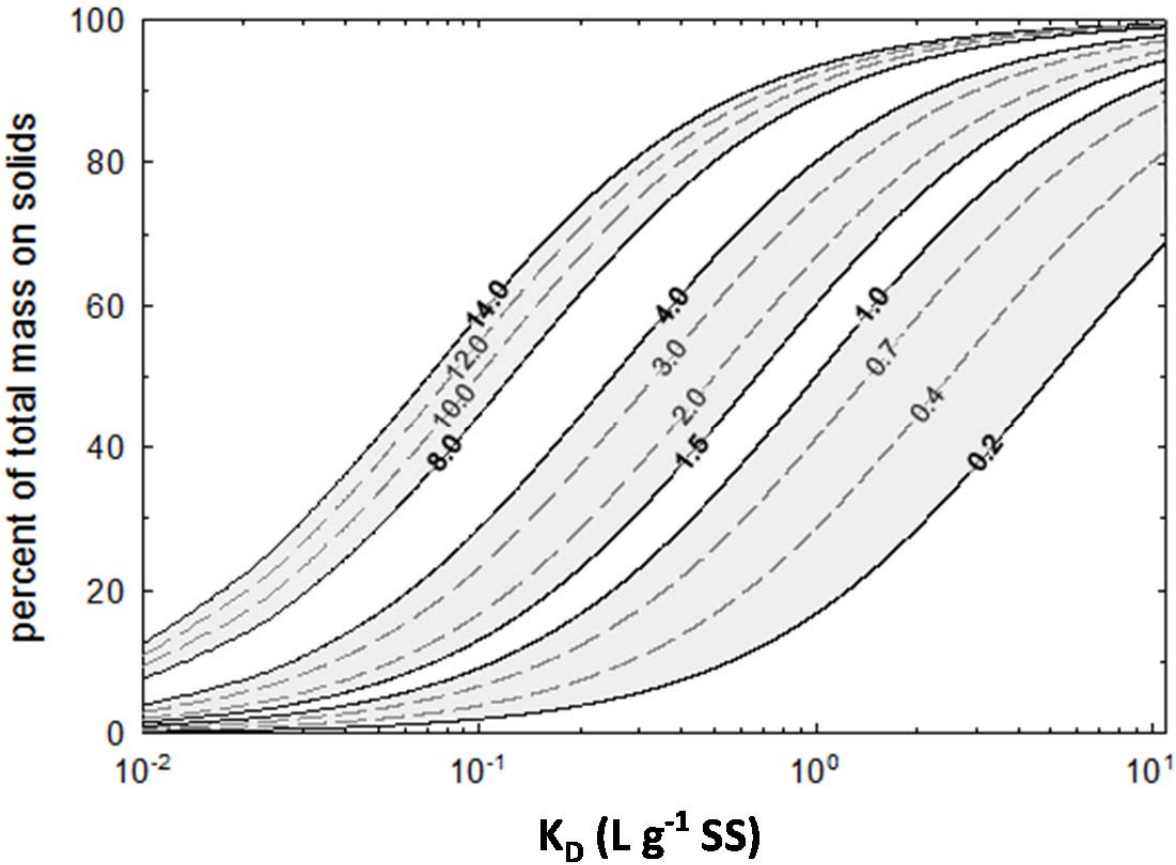


Figure 9. Fraction of PhAC sorbed to mixed liquor solids for PhACs with K_D values ranging from 0.01 to 10 L g^{-1} SS. Lines are shown for different reactor mixed liquor concentrations (indicated on the plot in g L^{-1}). Three data bands are shown for (from left to right): membrane bioreactors (MLSS = 8.0 – 14.0 g L^{-1}), suspended growth/conventional activated sludge systems (MLSS = 1.5 – 4.0 g L^{-1}) and lab scale systems (MLSS = 0.2 - 1.0 g L^{-1}).

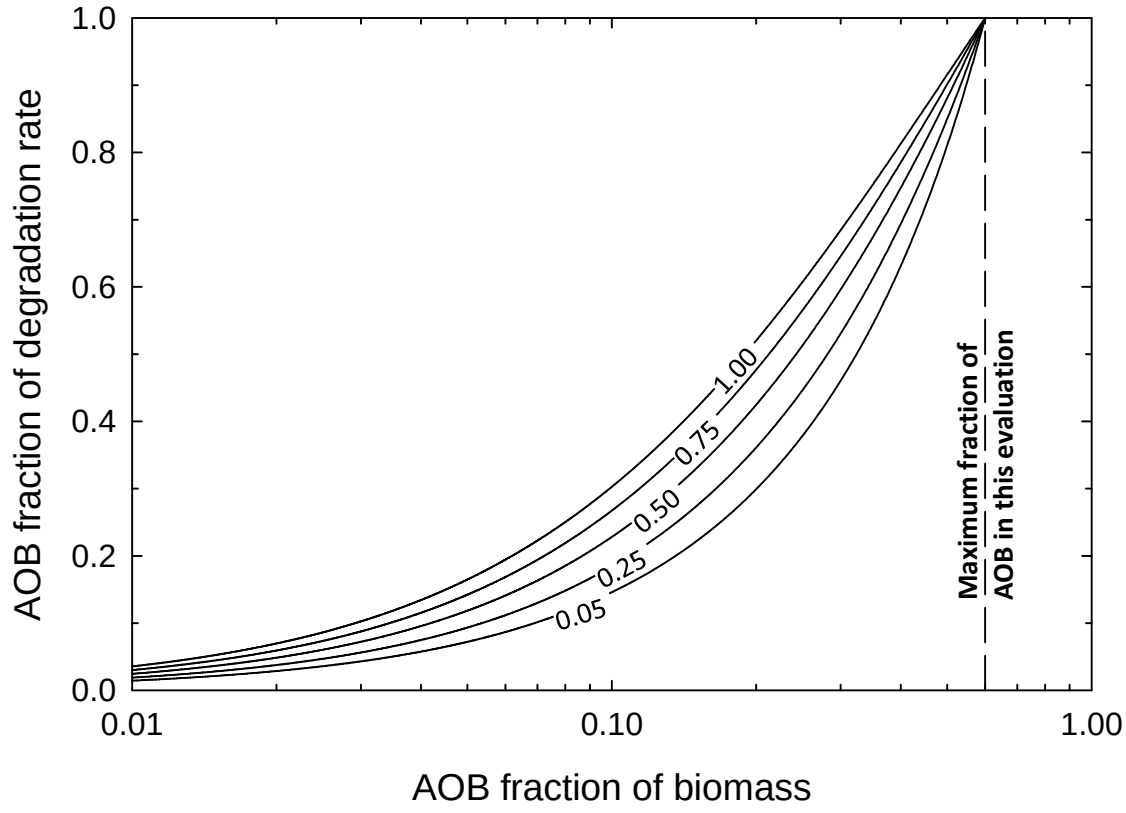


Figure 10. Fractional contribution of AOB to the rate of ATN biodegradation. Each curve represents a fraction of the maximum specific growth rate of AOB on ammonia (taken here to be 0.5 d^{-1}). Note that the plot assumes that AOB comprise 60% of nitrifiers which is, therefore, the maximum fraction of the biomass that AOB can represent (vertical line).

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Student Support

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Notable Achievements and Awards

None to report

Follow-on Funding

None to report

Publications and Conference Presentations:

a. Articles in Refereed Scientific Journals

Sathyamoorthy S. and Ramsburg C.A., Assessment of Quantitative Structural Property Relationships for Prediction of Pharmaceutical Sorption during Biological Wastewater Treatment, *Chemosphere*, In Press (DOI: http://dx.doi.org/10.1016/j.chemosphere.2013.01.061)

Sathyamoorthy, S., Chandran K. and Ramsburg C.A., Degradation of Beta Blockers during Ammonia Oxidation, *In Preparation.*

b. Book Chapter

None to report

c. Dissertations

Sathyamoorthy, S., A Laboratory and Modeling Investigation of Pharmaceutical Attenuation through Biodegradation and Sorption in Single Sludge Nitrification Systems, Anticipated completion date: August 2013

d. Water Resources Research Institute Reports

None to report

e. Conference Proceedings

Sathyamoorthy S. and Ramsburg C.A., Degradation of selected pharmaceuticals during nitrification, in Proceedings of: WEFTEC 2012, WEF, New Orleans, LA, 2012

f. Other Publications

None to report