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Modeling of Pharmaceutical Biotransformation by Enriched Nitrifying Culture under Different Metabolic Conditions

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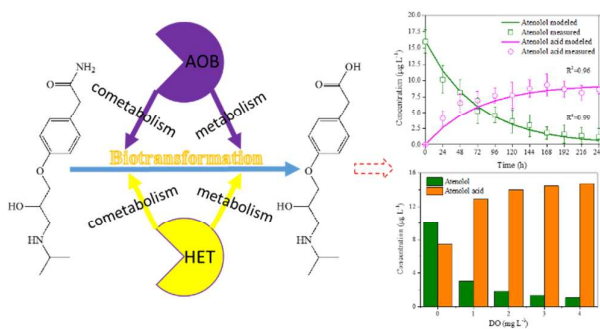
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18 Abstract

19 Pharmaceutical removal could be significantly enhanced through cometabolism during
20 nitrification processes. So far pharmaceutical biotransformation models have not considered
21 the formation of transformation products associated with the metabolic type of
22 microorganisms. Here we reported a comprehensive model to describe and evaluate the
23 biodegradation of pharmaceuticals and the formation of their biotransformation products by
24 enriched nitrifying cultures. The biotransformation of parent compounds was linked to the
25 microbial processes via cometabolism induced by ammonium oxidizing bacteria (AOB)
26 growth, metabolism by AOB, cometabolism by heterotrophs (HET) growth and metabolism
27 by HET in the model framework. The model was calibrated and validated using experimental
28 data from pharmaceuticals biodegradation experiments at realistic levels, taking two
29 pharmaceuticals as examples, i.e., atenolol and acyclovir. Results demonstrated the good
30 prediction performance of the established biotransformation model under different metabolic
31 conditions, as well as the reliability of the established model in predicting different
32 pharmaceuticals biotransformations. The linear positive correlation between ammonia
33 oxidation rate and pharmaceutical degradation rate confirmed the major role of cometabolism
34 induced by AOB in the pharmaceutical removal. Dissolved oxygen was also revealed to be
35 capable of regulating the pharmaceutical biotransformation cometabolically and the substrate
36 competition between ammonium and pharmaceuticals existed especially at high ammonium
37 concentrations.

38

39 **Keywords:** Cometabolism, pharmaceutical, model, ammonia oxidizing bacteria,
40 biotransformation product, substrate competition

41

42 **Introduction**

43 The ubiquitous occurrence and fate of pharmaceuticals in the environment and
44 engineering systems have attracted the concerns of the scientists and the public for decades
45 due to their potential ecotoxic impact on aquatic ecosystems.^{1,2} These organic compounds
46 were present in the wastewater at concentrations ranging from pg L^{-1} to $\mu\text{g L}^{-1}$.^{3,4} As the
47 wastewater treatment plants (WWTPs) were originally designed for chemical oxygen demand
48 and other nutrients removal, the incomplete removal was found for pharmaceuticals in the
49 treatment processes, being a major pathway for pharmaceuticals to enter the environment.⁵

50 Autotrophic biomass (e.g., enriched nitrifying sludge) was capable of transforming the
51 pharmaceuticals cometabolically during the wastewater treatment process and thus the
52 pharmaceutical removal was reported to be positively correlated to nitrification rate.^{6,7}
53 Ammonia oxidizing bacteria (AOB) in the nitrifying biomass could degrade a broad range of
54 substrates including aromatic and aliphatic compounds due to the non-specific enzyme
55 ammonia monooxygenase (AMO).⁸⁻¹⁰ The presence of the growth substrate (i.e. ammonium)
56 was required for cometabolism which should be taken into account when predicting the fate
57 of pharmaceuticals.¹¹ In addition to cometabolism, pharmaceuticals could also be degraded as
58 the energy and carbon source for microorganisms through metabolic biotransformation.¹¹
59 Furthermore, the formed biotransformation products might be more toxic and persistent.¹²
60 Hence the biotransformation products should be considered for a more comprehensive
61 understanding of the fate of pharmaceuticals in the nitrifying activated sludge.

62 Mathematical modeling offers a useful tool and is adopted widely to analyze complicated
63 metabolic pathways. Cometabolic biotransformations were previously modeled through first-
64 order kinetics and mixed order kinetics like Monod expression¹³⁻¹⁵ and have evolved from
65 only considering the cometabolic substrates to incorporating the relationships between
66 cometabolic substrates and growth substrates, such as competitive interaction and toxicity

67 inhibition.¹⁵ However, the previous literature has rarely considered the formation of
68 biotransformation products in the cometabolic biotransformation models for pharmaceuticals.

69 The aim of this work is to develop and test a comprehensive modeling framework to
70 describe the pharmaceuticals biotransformation at realistic levels as well as the formation of
71 their biotransformation products by the enriched nitrifying sludge under different metabolic
72 conditions. Microbial processes contributing to the pharmaceutical biotransformation were
73 considered as follows: growth-linked cometabolism by AOB, metabolic transformation by
74 AOB, growth-linked cometabolism by heterotrophs (HET) and metabolic transformation by
75 HET. To this end, atenolol and acyclovir were selected as the model compounds in this study
76 as they were frequently found in the wastewater with the highest concentrations of 25 and 1.8
77 $\mu\text{g L}^{-1}$, respectively, which have been reported to be increasingly removed under nitrifying
78 conditions.¹⁶⁻¹⁸ It has been reported that they can be biotransformed into atenolol acid and
79 carboxy-acyclovir, respectively.^{18,19} Model calibration and validation were carried out with
80 experimental data using atenolol as parent compounds under different metabolic conditions.
81 Model evaluation was also conducted using the experimental data from acyclovir
82 biotransformation. The effects of dissolved oxygen (DO) and ammonium concentrations on
83 pharmaceutical biotransformation were investigated using the validated model to provide
84 insights into the process dynamics. The reported model in this work is expected to be used as
85 a tool to fully understand the fate of pharmaceuticals associated with different metabolisms
86 by responsible microorganisms in the complicated activated sludge system.

87

88 **Materials and Methods**

89

90 **Model development**

91 A multi-species and multi-substrate model was developed to describe the pharmaceutical
92 biotransformation processes by the enriched nitrifying sludge. This biotransformation model
93 comprehensively considered the consumption of the pharmaceuticals and the formation of
94 transformation products accompanied with the simultaneous nitrification in the enriched
95 nitrifying sludge. It describes the relationships among eight soluble substrates as defined in
96 Table S1 in Supporting Information (SI), i.e., ammonium (S_{NH_4}), nitrite (S_{NO_2}), nitrate (S_{NO_3}),
97 readily biodegradable substrates (S_S), oxygen (S_{O_2}), pharmaceutical (parent compound, PC,
98 S_{PC}), primary biotransformation product (BP, S_{BP}) and other biotransformation products (OP,
99 S_{OP}), and five particulate species, i.e., AOB (X_{AOB}), HET (X_{HET}), NOB (nitrite oxidizing
100 bacteria, X_{NOB}), slowly biodegradable substrates (X_S) and inert biomass (X_I). Nine processes
101 are considered: (1) metabolic transformation of PC by AOB; (2) cometabolic transformation
102 of PC coupled to growth of AOB; (3) endogenous decay of AOB; (4) hydrolysis; (5)
103 metabolic transformation of PC by HET; (6) cometabolic transformation of PC coupled to
104 growth of HET; (7) endogenous decay of HET; (8) growth of NOB; and (9) endogenous
105 decay of NOB. The kinetic expressions and the stoichiometric matrix of the proposed
106 biotransformation model are summarized in Tables S2 and S3 in SI, respectively. The
107 definitions, values, units and sources of all parameters used in the biotransformation model
108 are listed in Table S4 in SI.

109 Pharmaceutical biodegradation was reported to be linked to AOB due to the non-specific
110 enzyme AMO as well as HET, which was not related to the activity of NOB.²⁰ In this model,
111 the microbial growth-linked kinetic expressions (processes 2 and 6 in Table S2 in SI) are
112 described using the Monod equations, which are associated with cometabolic
113 biotransformation of pharmaceuticals.²⁰ The concentration of growth substrates S_{NH_4} and S_S
114 is also involved in the Monod equations. The basis of the cometabolic biotransformation
115 expressions is the concept of transformation coefficient parameters such as AOB growth-

116 linked T_{PC-AOB}^c and HET growth-linked T_{PC-HET}^c . The pharmaceutical biotransformation
117 reactions directly conducted via metabolism by AOB and HET are described by pseudo-first
118 order kinetic expressions (processes 1 and 5 in Table S2 in SI). For each reaction, the rate is
119 expressed by an explicit function of the concentrations of relevant pharmaceuticals in the
120 process. For microbial metabolic biodegradation of PC, the key parameters are biomass
121 normalized PC degradation rate coefficients in the absence of AOB and HET growth, i.e.
122 k_{PC-AOB} and k_{PC-HET} . Processes 1, 2, 5 and 6 together contribute to pharmaceutical
123 biotransformation in the enriched nitrifying sludge.

124 The formation of biotransformation products is modeled using the specific stoichiometry
125 coefficients in processes 1, 2, 5 and 6. The coefficients α_{BP}^m and α_{BP}^c indicate the
126 transformation of PC to BP under metabolism and cometabolism conditions by AOB,
127 respectively. Similarly, the coefficients β_{BP}^m and β_{BP}^c present the transformation of PC to BP
128 under metabolism and cometabolism conditions by HET, respectively.

129

130 **Atenolol and acyclovir biotransformation experiments**

131 Experimental data from our previous biodegradation experiments of atenolol (Case *I*)
132 and acyclovir (Case *II*) under different conditions by an enriched nitrifying sludge were used
133 for model evaluation in this work.^{21,22} The chemicals used in the batch experiments and the
134 enrichment of nitrifying cultures in the sequencing batch reactor (SBR) are described in Text
135 S1 and S2 in SI. Details of the experimental conditions applied in different scenarios are
136 provided in Table S5 in SI. Briefly, 4-L beaker was used as the batch reactor with enriched
137 nitrifying cultures inoculated to degrade parent compounds at an initial $15 \mu\text{g L}^{-1}$. The mixed
138 liquid suspended solid (MLVSS) concentration was kept at approximately 1 g L^{-1} . All the
139 batch experiments were conducted in duplicates. The designs for Experiments 1-3 were same
140 for atenolol (Case *I*) and acyclovir (Case *II*). In Experiment 1, 30 mg L^{-1} allylthiourea (ATU)

141 was added to inhibit nitrifying activities,^{20,23,24} leading to the dominant contribution from
142 HET to pharmaceutical biotransformation.¹¹ Initial ammonium concentration was provided at
143 50 mg-N L⁻¹. No external ammonium was supplied during the entire experimental period
144 (240 h). In Experiment 2, no initial and external ammonium was provided during 240 h. In
145 Experiment 3, constant ammonium concentration was maintained at 50 mg-N L⁻¹ by dosing a
146 mixture of ammonium bicarbonate and potassium bicarbonate as ammonium feeding solution
147 and pH buffer at the same time, which could ensure the cometabolic biotransformation by
148 AOB. The Experiment 4 was exclusively designed for atenolol biotransformation, where
149 constant ammonium concentrations of 25 mg-N L⁻¹ were provided using the dosing method
150 in Experiment 3 during the experimental period. Samples were collected periodically to
151 analyse mixed liquid suspended solid (MLSS) concentration and its volatile fraction (i.e.,
152 MLVSS), NH₄⁺, NO₂⁻, NO₃⁻, atenolol, acyclovir and their biotransformation products
153 atenolol acid and carboxy-acyclovir. The detailed chemical analysis procedures could be
154 found in the previous work.^{21,22,25}

155 The contribution of sorption to removal of atenolol and acyclovir was insignificant based
156 on our previous studies.^{22,25} This is in consistent with low sorption coefficient K_D (0.04) of
157 atenolol and low octanol-water partition coefficient Log K_{OW} (0.16) of atenolol as well as Log
158 K_{OW} (-1.59) of acyclovir.²⁶⁻²⁸ Volatilization was considered negligible given the low values of
159 Henry's law constants for atenolol (1.37×10^{-18} atm m³ mol⁻¹) and acyclovir (3.2×10^{-22} atm m³
160 mol⁻¹).²⁹ Hydrolysis would not contribute to the degradation of atenolol and acyclovir, which
161 was confirmed previously and was in consistent with the absence of their transformation
162 products.^{22,25} Photodegradation was also insignificant considering the turbidity of the sludge
163 and the aluminum foil covering the reactor. Therefore, microbially induced biodegradation
164 should be the main mechanism for pharmaceutical removal in both atenolol and acyclovir
165 biotransformation experiments.

166

167 **Model calibration and validation**

168 The biotransformation model used in this work consists of 9 biochemical processes and
169 27 stoichiometric and kinetic parameters (as shown in Tables S2 and S4 in SI). Most of these
170 parameters were well established in previous literature, therefore the reported values were
171 directly used in this developed model. However, the information on biomass growth-linked
172 PC transformation coefficients T_{PC-AOB}^c and T_{PC-HET}^c and microbial endogenous
173 transformation coefficients k_{PC-AOB} and k_{PC-HET} was limited.²⁰ Considering the key role of
174 cometabolism induced by AOB growth in biotransformation, the maximum specific growth
175 rate of AOB $\mu_{max, AOB}$ was of significance to the developed model. Furthermore, the
176 sensitivity analysis suggested the four key parameters k_{PC-AOB} , k_{PC-HET} , T_{PC-AOB}^c and $\mu_{max, AOB}$
177 are highly sensitive to the biotransformation processes in terms of the experimental
178 measurements (examples shown in Figure S1 in SI). Model calibration was therefore
179 conducted to estimate the values of k_{PC-AOB} , k_{PC-HET} , T_{PC-AOB}^c and $\mu_{max, AOB}$ based on
180 experimental measurements through minimizing the sum of squares of the deviations
181 between the measured and modeled values for the concentrations of parent compounds and
182 biotransformation products under different conditions. In addition, the four stoichiometric
183 coefficients, i.e., α_{BP}^m , α_{BP}^c , β_{BP}^m and β_{BP}^c , for the transformation of PC to BP under metabolism
184 and cometabolism conditions could be determined based on the respective molecular mass
185 and concentrations of BP and PC measured in the experiments.

186 Experimental data from atenolol biotransformation (Case I) of Experiments 1-3 were
187 firstly used for model calibration. Concentrations of ammonium, nitrite, DO, atenolol and
188 atenolol acid from Experiment 1 and Experiment 2 were fitted by model simulations to
189 estimate k_{PC-HET} and k_{PC-AOB} , respectively, whereas concentrations of ammonium, nitrite, DO,

190 atenolol and atenolol acid from Experiment 3 were fitted to estimate $\mu_{max, AOB}$ and T_{PC-AOB}^c ,
191 using the k_{PC-HET} and k_{PC-AOB} values obtained in previous experiments (Experiment 1 and
192 Experiment 2). Model validation was then carried out with the calibrated parameters using
193 the independent experimental data sets from atenolol biotransformation of Experiment 4.²¹
194 Specifically, in Experiment 4, batch experiments with atenolol as the parent compound at an
195 initial concentration of $15 \mu\text{g L}^{-1}$ were conducted using the same enriched nitrifying sludge
196 (i.e., same microbial composition) in the constant presence of ammonium of 25mg-N L^{-1} and
197 at DO of around 2.5mg L^{-1} . There were no significant gaps between batch experiments,
198 leading to insignificant biomass changes. The ammonium and DO concentrations applied
199 were different from of Experiment 3 at ammonium of 50mg-N L^{-1} and DO of 3.0mg L^{-1}
200 (Table S5 in SI). To further verify the validity and applicability of the model, the model was
201 also applied to evaluating the acyclovir biotransformation data from Case II of Experiments
202 1-3. The key model parameters were recalibrated for Case II using the three sets of batch
203 experimental data (Table S5 in SI).

204 The sensitivity analysis, parameter estimation, parameter uncertainty evaluation and
205 model simulations were done through employing a modified version of software AQUASIM
206 2.1d according to Batstone et al.³⁰, with a 95% confidence level for significance testing and
207 parameter uncertainty analysis. The standard errors and 95% confidence intervals of
208 individual parameter estimates were calculated from the mean square fitting errors and the
209 sensitivity of the model to the parameters. Residual sum of squares (RSS) between the
210 objective data and model was used as the objective function.

211

212 **Results**

213

214 **Model calibration with experimental data from atenolol biotransformation**

215 As atenolol acid was the sole biotransformation products with no other products
216 identified in all batch experiments, the dynamics of the substrate S_{OP} was not modeled herein.
217 The model was first calibrated to illustrate the biotransformation of atenolol catalysed solely
218 by HET in Experiment 1 (i.e. with addition of ATU to inhibit the nitrifying activity). Given
219 that no exogenous organic carbon was supplied during culture enrichment and the only
220 organic carbon in the batch experiments was pharmaceuticals, the growth of HET was
221 considered extremely low and the cometabolic transformation rate of pharmaceuticals linked
222 to growth of HET was not modeled with T_{PC-HET}^c omitted for estimation.²⁰ With AOB related
223 parameters k_{PC-AOB} and T_{PC-AOB}^c set to zero, only the parameter k_{PC-HET} was estimated with
224 its best-fit value shown in Table 1 for Experiment 1. The predicted atenolol and atenolol acid
225 concentration profiles with the established model were demonstrated in Figure 1A, along
226 with the measured experimental values. Atenolol experienced a continuous decrease by 94.3%
227 from the beginning to the end of experiments accompanied with a gradual increase of
228 atenolol acid until 168 h and a stable stage until 240 h at a conversion efficiency of 62.6%
229 (Figure 1A), which was well captured by the model predictions.

230 The experimental data obtained from Experiment 2 (i.e., in the absence of ammonium)
231 were used to further calibrate the developed model in terms of atenolol and atenolol acid
232 dynamics. Without the presence of the growth substrate, the ammonium released from cell
233 lysis process during bacterial decay was minor and AOB growth-linked cometabolism would
234 be considered to have negligible contribution to atenolol biotransformation. Therefore, only
235 the metabolic biotransformation by AOB and HET were involved in the biotransformation of
236 atenolol for Experiment 2. The parameter value of k_{PC-HET} obtained in Experiment 1 was
237 used directly without any modification. Another key model parameter k_{PC-AOB} related to AOB
238 metabolism was thus reliably estimated during atenolol biotransformation (value as shown in
239 Table 1). As shown in Figure 1B, although atenolol demonstrated a sharp decrease by 97.4%

240 over the whole experimental period, the production of atenolol acid indicated a lower
241 transformation efficiency in the absence of ammonium (29.1%) compared with the
242 experiments with addition of ATU (see Figure 1A), again well matching the model
243 predictions.

244 In Experiment 3, the presence of ammonium at 50 mg-N L^{-1} was provided constantly to
245 ensure the cometabolic biodegradation of atenolol by both AOB and HET at DO of 3.0 mg L^{-1} .
246 Together with the rest of the parameters involved, the parameter values of k_{PC-HET} and
247 k_{PC-AOB} estimated in the previous two experiments were applied in the biotransformation
248 model. The key parameters related to AOB induced cometabolism, i.e., T_{PC-AOB}^c and $\mu_{max, AOB}$,
249 were then estimated with the optimum values listed in Table 1. Figure S2A in SI showed the
250 well agreement between predicted and measured concentrations of ammonium, nitrite and
251 DO based on the proposed model, supporting the capability of the model to describe the two-
252 step nitrification processes in terms of nitrite accumulation, as well as the suitability of the
253 selected parameters related to DO dynamics for the cometabolic biodegradation processes by
254 the enriched nitrifying culture (i.e., the $K_{O_2, AOB}$ and $K_{O_2, HET}$ values for AOB and HET). It
255 should be noted that the nitrate concentrations were not specifically modeled, which were
256 slightly higher than that in the SBR in all experiments since the biomass in batch experiments
257 was taken directly from SBR with a background nitrate concentration up to 1000 mg L^{-1} . As
258 shown in Figure 1C, concomitant with the gradual decrease of atenolol at a removal
259 efficiency of 88.0%, atenolol acid was formed at an increasing trend with 86.9% conversion
260 efficiency. This was obviously higher than the experiments in the absence of ammonium and
261 with the addition of ATU, indicating a positive role of AOB induced cometabolism in
262 atenolol transformation. The model described these observations reasonably well.

263 Overall, the developed model could satisfactorily capture all dynamics associated with
264 atenolol and atenolol acid in all batch biodegradation experiments under different metabolic

265 conditions. The good agreement between model simulations and measured data in Figure 1
266 supports the capability of the developed model in describing the microbial growth related
267 biotransformation of atenolol in enriched nitrifying cultures. The obtained parameter linked
268 to AOB growth during ammonia oxidation, i.e., AOB-induced cometabolic atenolol
269 transformation coefficient T_{PC-AOB}^c , was estimated at $0.012 \pm 0.000036 \text{ m}^3 \text{ g COD}^{-1}$. It was
270 lower than the reported value of $0.0715 \pm 0.0227 \text{ m}^3 \text{ g COD}^{-1}$ for atenolol biodegradation by
271 an enriched nitrifying sludge.²⁰ The non-growth metabolism by HET and the non-growth
272 metabolism by AOB on atenolol biodegradation also described the experimental data with the
273 addition of ATU and in the absence of ammonium well. The estimated parameters of k_{PC-HET}
274 and k_{PC-AOB} were 0.000180 ± 0.000017 and $0.000140 \pm 0.000012 \text{ m}^3 \text{ g COD}^{-1} \text{ h}^{-1}$, which were
275 lower than but in the same order of magnitude as the literature reported values ($0.00093 \pm$
276 0.00018 and $0.00067 \pm 0.00023 \text{ m}^3 \text{ g COD}^{-1} \text{ h}^{-1}$, respectively).²⁰ The discrepancy in these
277 parameters values could be probably ascribed to the difference in the community structure in
278 the adopted nitrifying cultures or different operating conditions. The model could be
279 potentially applied to a widespread extent despite that the parameter values would vary
280 according to the experimental conditions. As suggested, it was difficult to compare these
281 coefficients (k_{PC-HET} , k_{PC-AOB} and T_{PC-AOB}^c) with other pharmaceuticals as most existing
282 models did not consider the specific biochemical processes.²⁰

283

284 **Model validation with atenolol biotransformation under different conditions**

285 In order to further confirm the validity and reliability of the developed model, model
286 validation was carried out to compare the model simulations to the independent experimental
287 data, which were not used for model calibration. Based on the measured concentrations of
288 atenolol and atenolol acid, the stoichiometric coefficients α_{BP}^c and α_{BP}^m were calculated as 0.58
289 and 0.58, respectively. Applied with previously calibrated parameters in Table 1, the

290 proposed biotransformation model was used to predict dynamics of ammonium, nitrite, DO,
291 atenolol and atenolol acid in the presence of ammonium at a constant concentration of 25 mg-
292 N L⁻¹ and at DO of around 2.5 mg L⁻¹ (significantly different from the ammonium of 50 mg-
293 N L⁻¹ and DO of 3.0 mg L⁻¹ used for model calibration). The model captured the dynamics of
294 ammonium, nitrite and DO, again suggesting the validity of the two-step nitrification model
295 and the suitability of the selected parameters related to DO (see Figure S2B). As shown in
296 Figure 2, atenolol continuously dropped from initial 15 µg L⁻¹ with a final degradation
297 efficiency of 92.9%. The conversion rate of atenolol acid transformed from atenolol was
298 calculated as 57.9%. The model predictions could capture these trends of atenolol
299 degradation and atenolol acid formation very well, which again supports the validity of the
300 developed model for atenolol biotransformation.

301

302 **Model evaluation with experimental data from acyclovir biotransformation**

303 The experimental results obtained with Case II for biotransformation of acyclovir were
304 used to further evaluate the developed model. The developed biotransformation model was
305 recalibrated for acyclovir biodegradation and carboxy-acyclovir formation dynamics under
306 different conditions. Most of the literature reported model parameters were employed at same
307 values as the case of atenolol except the stoichiometry coefficients (α_{BP}^m , α_{BP}^c , β_{BP}^m , β_{BP}^c) for
308 formation of carboxy-acyclovir associated with specific biochemical processes (as shown in
309 Table S4 in SI), which were calculated based on the experimental data. The values for the
310 three key parameters k_{PC-HET} , k_{PC-AOB} and T_{PC-AOB}^c were recalibrated, which were associated
311 with the investigated parent compound. As the enriched nitrifying biomass utilized in the
312 batch biodegradation experiments of acyclovir were same as those in case of atenolol, the
313 maximum growth rate of AOB $\mu_{max, AOB}$ was set to be the same as in case of atenolol during
314 model calibration for acyclovir biotransformation in the presence of ammonium. The

315 obtained parameter values for acyclovir biotransformation were $0.00035 \pm 0.00002 \text{ m}^3 \text{ g}$
316 $\text{COD}^{-1} \text{ h}^{-1}$ (k_{PC-HET}), $0.00005 \pm 0.00003 \text{ m}^3 \text{ g COD}^{-1} \text{ h}^{-1}$ (k_{PC-AOB}) and $0.00093 \pm 0.00049 \text{ m}^3 \text{ g}$
317 COD^{-1} (T_{PC-AOB}^c).

318 The model predictions of acyclovir biotransformation matched the experimental results
319 well under different conditions (Figure 3), further demonstrating the validity of the
320 established model. Parameters values giving the optimum fits with the experimental data
321 were difficult to compare reliably with literature values as this study firstly reported the AOB
322 cometabolic acyclovir transform coefficient T_{PC-AOB}^c . However, compared to other reported
323 compounds, e.g. atenolol,²⁰ it was obvious that parameters k_{PC-AOB} and T_{PC-AOB}^c for acyclovir
324 were lower than those values for atenolol (Table 1), indicating a stronger degradation ability
325 of the AOB culture studied on atenolol than acyclovir. Considering the molecular differences
326 between these two pharmaceuticals, this may imply an affinity property of AOB for different
327 compounds probably due to a preferential substrate selection to AMO active sites.³¹ The
328 parameter k_{PC-HET} for acyclovir was $0.00035 \pm 0.00002 \text{ m}^3 \text{ g COD}^{-1} \text{ h}^{-1}$, which was in the
329 same order of magnitude of the value estimated in this study ($0.000180 \pm 0.000017 \text{ m}^3 \text{ g}$
330 $\text{COD}^{-1} \text{ h}^{-1}$) for atenolol. The conversion efficiencies from acyclovir to carboxy-acyclovir
331 were 83.9%, 43.0% and 29.9% in Experiments 1, 2 and 3, respectively (see Figure 3). These
332 results indicated the importance of metabolism of acyclovir by HET. Oxidation of acyclovir
333 to carboxy-acyclovir might be dominated by unspecific monooxygenase from HET,³² which
334 needs to be confirmed in the further work.

335

336 Discussion

337 In this work, a comprehensive mathematical model is developed to describe the
338 biotransformation of pharmaceuticals and the formation of their products by enriched
339 nitrifying cultures. In the proposed model, processes 1 and 2 (Table S2 in SI) depict the

340 AOB-induced cometabolic and metabolic biotransformation of pharmaceuticals, while
341 processes 5 and 6 (Table S2 in SI) describe the HET-induced cometabolic and metabolic
342 biotransformation of pharmaceuticals, respectively. Sensitivity analysis indicated that four
343 key parameters k_{PC-HET} , k_{PC-AOB} , T_{PC-AOB}^c and $\mu_{max, AOB}$ were critical to the model output and
344 therefore estimated through model calibration. The validity of this biotransformation model is
345 confirmed by independent atenolol biodegradation data and further evaluated by acyclovir
346 biotransformation experiments. Compared to the previous studies where atenolol
347 biodegradation was investigated through experiments and modeling approaches,^{20,21} the
348 proposed model in this work considers the formation of biotransformation products and
349 describes biotransformation of different pharmaceuticals under different metabolic conditions.
350 This microbial processes-linked biotransformation model could enhance our ability to predict
351 the fate of pharmaceuticals and their transformation products during wastewater treatment
352 processes.

353 Since we estimated four model parameters for fitting the experimental data, parameter
354 uniqueness is important, since it is possible that different parameter combinations can give
355 similar simulation accuracy. In our work, we applied a least-squared analysis and evaluated
356 standard errors and 95% confidence intervals of individual parameter estimates. The
357 parameter confidence intervals showed a well-defined range in which the optimum values of
358 parameters reside (Table 1), which indicates good uniqueness of these parameters. In addition
359 to the analysis of the confidence intervals, two other aspects of our experimental design
360 support the uniqueness of the parameter values. First, we used five different experimental
361 parameters (ammonium, nitrite, DO, parent compound, and biotransformation product),
362 which reflect different aspects of the kinetics of the two-step nitrification and pharmaceutical
363 biotransformation by enriched nitrifying culture. Second, we carried out independent
364 experiments to validate the estimated parameters. In particular, the good correspondence for

365 independent experimental data supports the validity of the new model and the uniqueness of
366 the parameters for pharmaceutical biotransformation.

367 The modeling results in this work suggested the cometabolism induced by AOB could
368 play an important role in the pharmaceutical removal in the studied ratio ranges of
369 pharmaceuticals to ammonia for cometabolism. Indeed a positive linear relationship was
370 observed between ammonia oxidation rate and pharmaceutical degradation rates in terms of
371 atenolol and acyclovir based on the validated model (Figure 4A). The atenolol degradation
372 rate increased from 0.012 to 0.16 $\mu\text{g g VSS}^{-1} \text{ h}^{-1}$ while the nitrification rate increased from
373 2.84 to 59.15 $\text{mg NH}_4^+\text{-N g VSS}^{-1} \text{ h}^{-1}$. With respect to acyclovir, the degradation rate changed
374 from 0.014 to 0.10 $\mu\text{g g VSS}^{-1} \text{ h}^{-1}$ whereas the ammonia oxidation rate showed an increase
375 from 2.37 to 36.63 $\text{mg NH}_4^+\text{-N g VSS}^{-1} \text{ h}^{-1}$. Such a positive correlation was also reported in
376 previous literature under certain conditions,^{7,22,25} supporting the notion that majority of
377 atenolol and acyclovir could be cometabolically degraded in the enriched nitrifying cultures.
378 A further assessment on the wide application of the relationship was carried out by
379 simulating the concentration profiles of pharmaceuticals after 240 h. The molar ratios of
380 atenolol to ammonia from 8.42×10^{-7} to 1.91×10^{-5} calculated based on their concentrations
381 was observed to be still within the range for a linearly positive relationship regarding the
382 cometabolic biodegradation of atenolol by the enriched nitrifying cultures used in this work,
383 and the relationship maintained at a same slope (Black solid squares in Figure 4A
384 demonstrated the predicted atenolol degradation rate after 240 h). However, a different slope
385 was found for the relationship between ammonia oxidation rate and the acyclovir degradation
386 rate after 240 h predicted using the developed model (Figure 4B). If the ammonia oxidation
387 rate was higher than the critical value (2.3 $\text{mg NH}_4^+\text{-N g VSS}^{-1} \text{ h}^{-1}$ in this study), the lower
388 slope might indicate a slower increasing trend in acyclovir degradation rate with an
389 increasing ammonia oxidation rate (Figure 4A). Compared with the situation at the lower

390 ammonia oxidation rate, a higher increasing trend in acyclovir degradation rate would arise at
391 higher slope (Figure 4B). The observation that pharmaceutical would not be degraded until
392 the ammonia was depleted³³ revealed a higher pharmaceutical degradation rate at lower
393 ammonia oxidation rate, which supported the findings in this study. Regardless of the
394 different slopes for the relationship, the molar ratios of acyclovir to ammonia ranging from
395 1.62×10^{-11} to 2.26×10^{-5} was obtained to be a valid application range for the cometabolic
396 biodegradation of acyclovir by the enriched nitrifying cultures used in this work.

397 The proposed model framework was expected to be a useful tool to predict the
398 biotransformation of pharmaceuticals and the formation of transformation products under
399 varying conditions, therefore providing the guidance in designing, upgrading and optimizing
400 of the relevant biological wastewater treatment processes. The influence of DO on
401 pharmaceutical biotransformation was investigated by performing model simulations in the
402 enriched nitrifying systems. The pharmaceutical removal efficiencies at 240 h at different DO
403 concentrations ranging from 0 to 4 mg L⁻¹ with ammonium concentration of 50 mg-N L⁻¹ are
404 shown in Figure 5. Overall DO concentration had a positive effect on pharmaceutical
405 removal efficiencies. The concentrations of atenolol and acyclovir decreased rapidly with a
406 prompt increase of atenolol acid and carboxy-acyclovir as DO increased to 1 mg L⁻¹. With
407 DO further increased to 4 mg L⁻¹, a gradual decrease of pharmaceutical concentrations was
408 observed accompanied with a slight increase of their biotransformation products. The
409 degradation efficiencies for atenolol at DO concentrations of 0, 1 and 4 mg L⁻¹ were 44.3%,
410 83.2% and 94.0%, respectively. With regard to acyclovir, its degradation efficiencies were
411 observed to be 36.2%, 81.2% and 87.3%, respectively at DO of 0, 1 and 4 mg L⁻¹. The
412 simulation results revealed that the DO concentration would play an important role in
413 pharmaceutical biotransformation. This was contrary to the previous report that DO in the
414 WWTP had no influence on oxidative biotransformation of selected micropollutants.³⁴ The

415 possible reason could be that the experiments conducted in this study were nitrifying culture
416 based instead of the regular activated sludge in WWTP, suggesting that DO might regulate
417 the pharmaceutical biotransformation cometabolically. It should be noted that the simulation
418 results are to provide insight into the potential impact of DO on pharmaceutical
419 biotransformation by enriched nitrifying culture rather than to accurately predict the reality,
420 which remain to be verified in future work.

421 The growth substrate might also have an impact on the pharmaceutical biotransformation.
422 Different ammonium concentrations ranging from 0 to 100 mg L⁻¹ were applied in the model
423 simulations at different DO concentrations as shown in Figure 6. It was obvious that the
424 degradation efficiencies of studied pharmaceuticals and the formation rates of their
425 transformation products would increase dramatically when ammonium concentrations
426 increase from 0 to 20 mg-N L⁻¹, especially in case of atenolol suggesting the importance of
427 cometabolism on its biotransformation. However, there was no significant enhancement with
428 the increase of ammonium concentrations from 20 to 250 mg-N L⁻¹ (data of 100-250 mg-N L⁻¹
429 were not shown). This was contrary to the previous report where the removal efficiencies of
430 the selected pharmaceuticals were enhanced at higher initial ammonium concentrations.³⁵
431 This could be probably due to the substrate competition between growth substrate
432 (ammonium) and cometabolic substrates (e.g. atenolol or acyclovir). Pharmaceutical levels
433 applied in this study were several orders of magnitude lower than the investigated ammonium
434 concentrations, leading to a competition for AMO active sites and therefore potential
435 decreasing degradation rates at higher ammonium concentrations.^{31,33}

436 In summary, a comprehensive model that considers all microbial processes contributing
437 to pharmaceutical biotransformation as well as the formation of biotransformation products
438 by the enriched nitrifying cultures is developed in this work. The proposed model was
439 successfully calibrated and validated using the biotransformation experiments of atenolol and

440 acyclovir under different metabolic conditions. The linear positive correlation between
441 ammonia oxidation rate and pharmaceutical degradation rate confirmed the major role of
442 cometabolism induced by AOB in the pharmaceutical removal. DO was revealed to be
443 capable of regulating the pharmaceutical biotransformation cometabolically and the substrate
444 competition between ammonium and pharmaceuticals existed at high ammonium
445 concentrations. More verification should be conducted using other pharmaceuticals'
446 biotransformation data for this developed model to facilitate its application as a useful tool in
447 prediction of pharmaceutical fate, especially in the real municipal wastewater systems, where
448 other processes (e.g., the competition between different parent compounds on the enzyme
449 active sites) need to be considered in future work.

450

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455

456 **Supporting Information**

457 Additional texts, tables and figures are shown in Supporting Information.

458

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566

Table and Figure Legends

567

568 **Table 1.** Estimated parameter values for the biotransformation model in this study

569

570 **Figure 1.** Model calibration with experimental data from atenolol biodegradation: (A)

571 Experiment 1, with addition of allylthiourea (ATU); (B) Experiment 2, in the absence of

572 ammonium; and (C) Experiment 3, in the presence of ammonium ($50 \text{ mg NH}_4^+ \text{-N L}^{-1}$).

573

574 **Figure 2.** Model validation results of atenolol biotransformation by the enriched nitrifying

575 culture in the presence of ammonium of 25 mg-N L^{-1} (Experiment 4).

576

577 **Figure 3.** Model evaluation with experimental data from acyclovir biodegradation: (A)

578 Experiment 1, with addition of allylthiourea (ATU), (B) Experiment 2, in the absence of

579 ammonium and (C) Experiment 3, in the presence of ammonium ($50 \text{ mg NH}_4^+ \text{-N L}^{-1}$).

580

581 **Figure 4.** (A) The relationship between ammonia oxidizing rate and the pharmaceutical

582 degradation rates in terms of atenolol and acyclovir (black solid squares indicate the atenolol

583 degradation rates after 240 h); and (B) The relationship between ammonia oxidizing rate and

584 the acyclovir degradation rate after 240 h at a different linear fit slope.

585

586 **Figure 5.** Predicted final concentrations of (A) atenolol and atenolol acid and (B) acyclovir

587 and carboxy-acyclovir at time of 240 h at different concentrations of dissolved oxygen (DO)

588 in the enriched nitrifying culture system.

589

590 **Figure 6.** Predicted concentrations of pharmaceuticals and their transformation products at

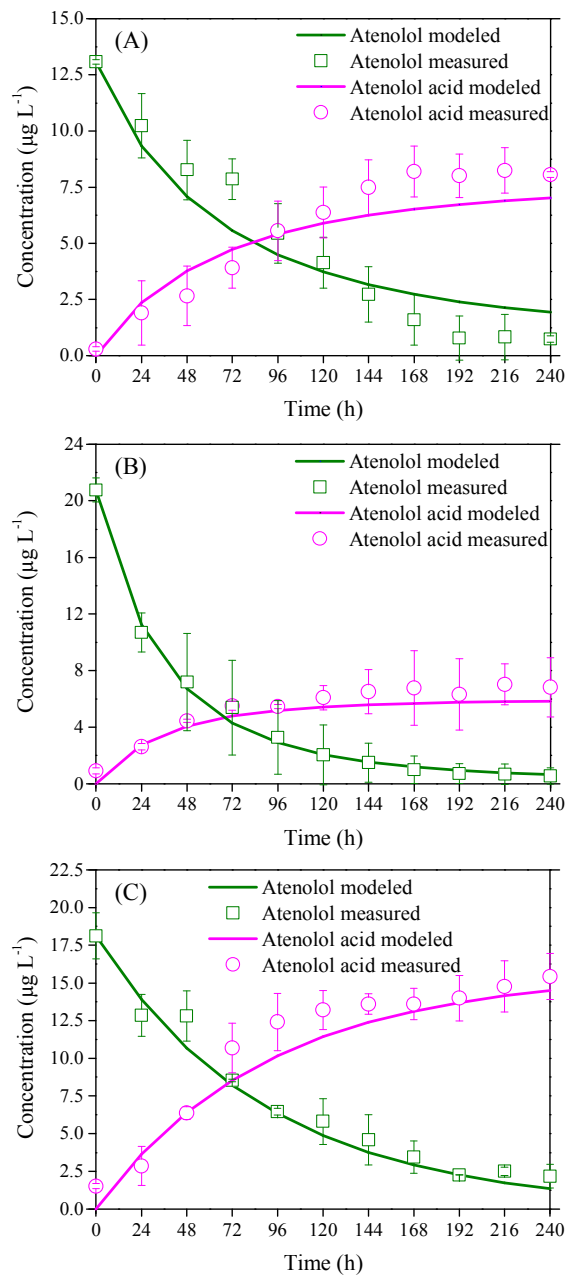
591 time of 240 h at initial concentrations of $15 \mu\text{g L}^{-1}$ with different ammonium concentrations

592 ranging from 0 to 100 mg-N L^{-1} at different DO levels.

593 **Table 1.** Estimated parameter values for the biotransformation model in this study

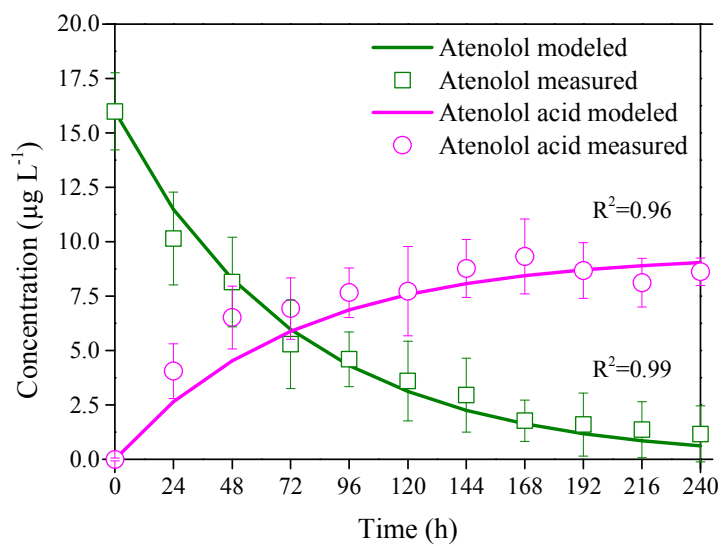
Parameters	Definition	Unit	Estimated	
			atenolol	acyclovir
k_{PC-HET}	Heterotrophs (HET) transformation coefficient	$\text{m}^3 \text{ g COD}^{-1} \text{ h}^{-1}$	0.000180	0.00035 ±
			±	0.00002
			0.000017	
k_{PC-AOB}	Ammonia oxidizing bacteria (AOB) transformation coefficient	$\text{m}^3 \text{ g COD}^{-1} \text{ h}^{-1}$	0.000140	0.00005 ±
			±	0.00003
			0.000012	
T_{PC-AOB}^c	Parent compound biotransformation coefficient rate linked to AOB growth (cometabolism)	$\text{m}^3 \text{ g COD}^{-1}$	0.012 ±	0.00093 ±
			0.000036	0.00049
$\mu_{max, AOB}$	Maximum specific growth rate of AOB	h^{-1}	0.012 ± 0.0023	

594



595

596 **Figure 1.** Model calibration with experimental data from atenolol biodegradation: (A)
597 Experiment 1, with addition of allylthiourea (ATU); (B) Experiment 2, in the absence of
598 ammonium; and (C) Experiment 3, in the presence of ammonium ($50 \text{ mg NH}_4^+ \text{-N L}^{-1}$).

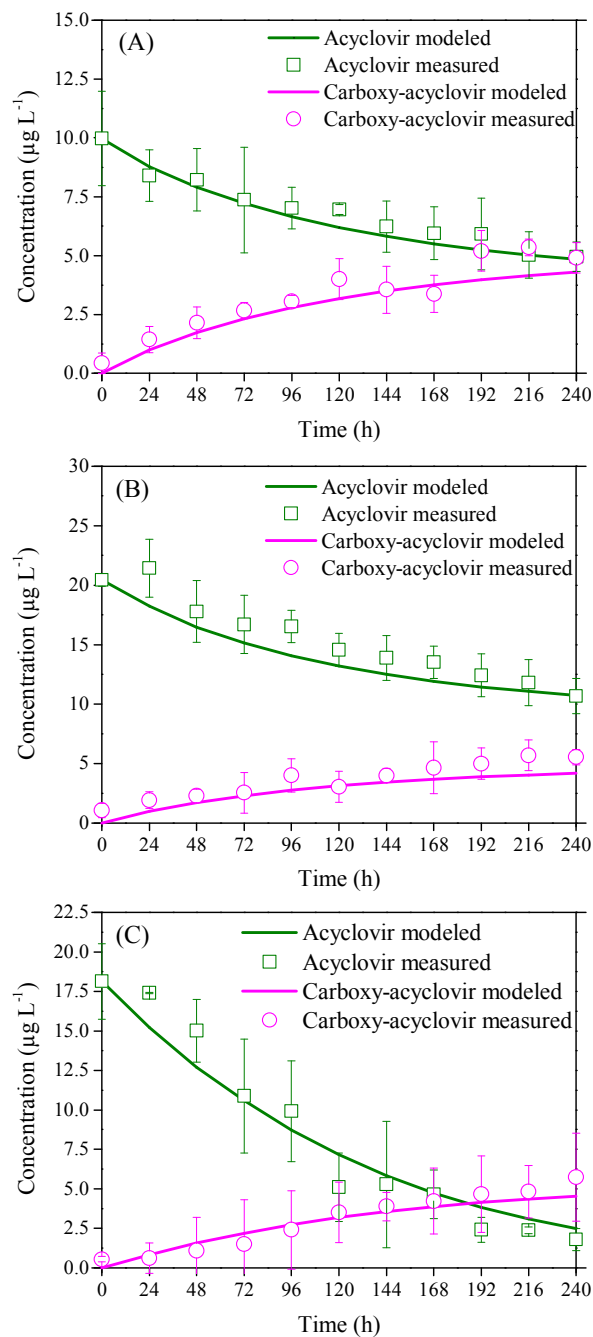


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600

601 **Figure 2.** Model validation results of atenolol biotransformation by the enriched nitrifying602 culture in the presence of ammonium of 25 mg-N L^{-1} (Experiment 4).

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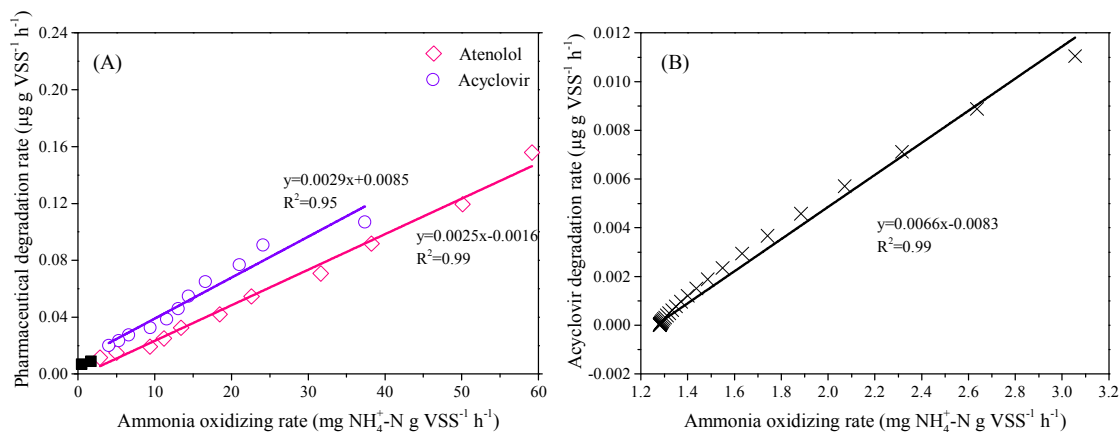
604

605

606 **Figure 3.** Model evaluation with experimental data from acyclovir biodegradation: (A)

607 Experiment 1, with addition of allylthiourea (ATU), (B) Experiment 2, in the absence of

608 ammonium and (C) Experiment 3, in the presence of ammonium ($50 \text{ mg NH}_4^+ \text{-N L}^{-1}$).



609

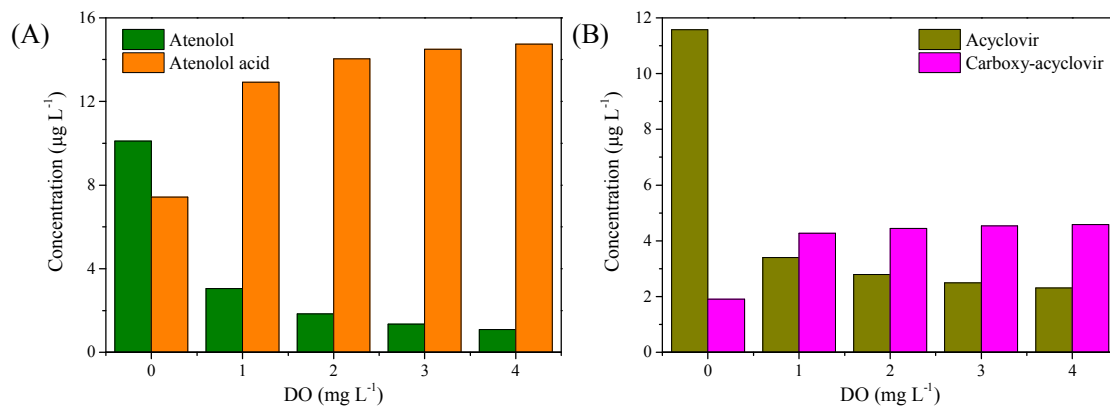
610

Figure 4. (A) The relationship between ammonia oxidizing rate and the pharmaceutical

611 degradation rates in terms of atenolol and acyclovir (black solid squares indicate the atenolol

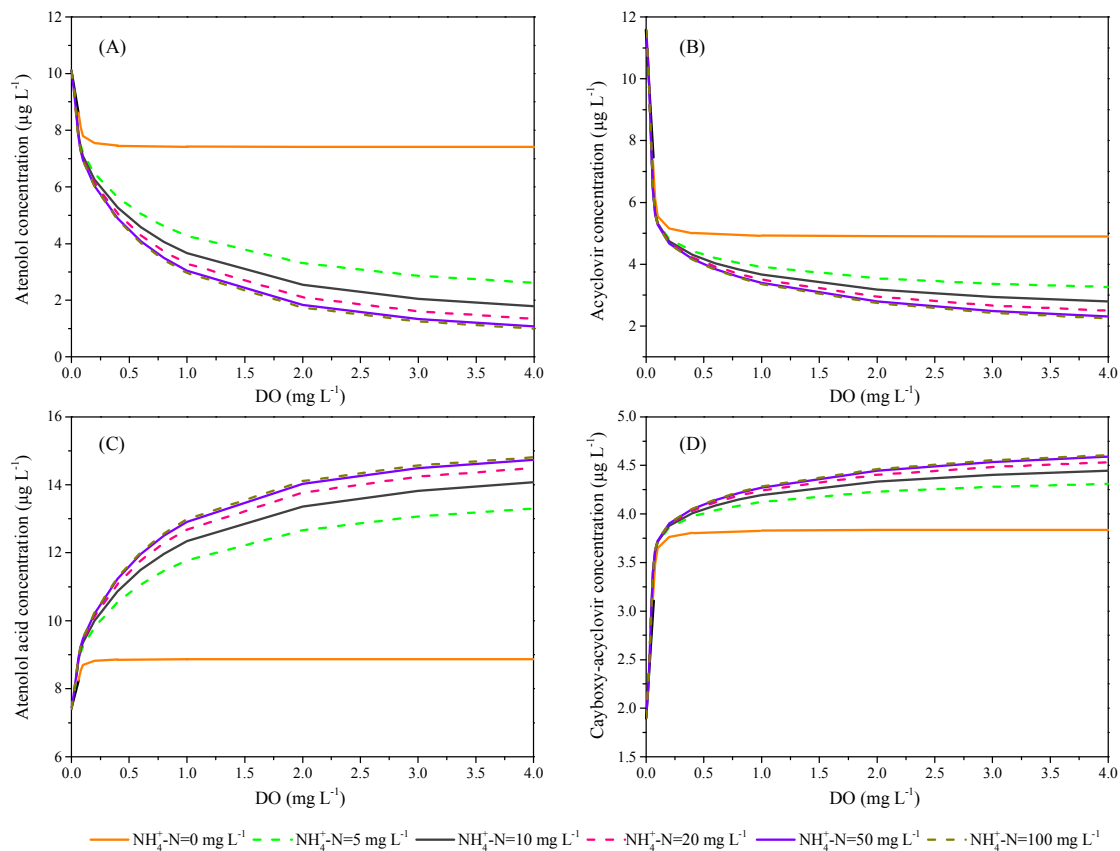
612 degradation rates after 240 h); and (B) The relationship between ammonia oxidizing rate and

613 the acyclovir degradation rate after 240 h at a different linear fit slope.



614

615 **Figure 5.** Predicted final concentrations of (A) atenolol and atenolol acid and (B) acyclovir
616 and carboxy-acyclovir at time of 240 h at different concentrations of dissolved oxygen (DO)
617 in the enriched nitrifying culture system.



618

619 **Figure 6.** Predicted concentrations of pharmaceuticals and their transformation products at
620 time of 240 h at initial concentrations of 15 µg L⁻¹ with different ammonium concentrations
621 ranging from 0 to 100 mg-N L⁻¹ at different DO levels.