Accepted Manuscript

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PII: S0045-6535(16)31728-3

DOI: 10.1016/j.chemosphere.2016.12.014

Reference: CHEM 18469

To appear in: *ECSN*

Received Date: 15 September 2016

Revised Date: 2 December 2016

Accepted Date: 4 December 2016

Please cite this article as: Xu, Y., Yuan, Z., Ni, B.-J., Biotransformation of acyclovir by an enriched nitrifying culture, *Chemosphere* (2017), doi: 10.1016/j.chemosphere.2016.12.014.

Biotransformation of Acyclovir by an Enriched Nitrifying Culture

Yifeng Xu, Zhiguo Yuan, Bing-Jie Ni*

Advanced Water Management Centre, The University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia

*Corresponding author:

Dr. Bing-Jie Ni

Advanced Water Management Centre

The University of Queensland

Australia

Phone: + 61 7 3346 3230

Fax: +61 7 3365 4726

E-mail: b.ni@uq.edu.au

Abstract

This work evaluates the biodegradation of the antiviral drug acyclovir by an enriched nitrifying culture during ammonia oxidation and without the addition of ammonium. The study on kinetics was accompanied with the structural elucidation of biotransformation products through batch biodegradation experiments at two different initial levels of acyclovir (15 mg L^{-1} and 15 μg L^{-1}). The pseudo first order kinetic studies of acyclovir in the presence of ammonium indicated the higher degradation rates under higher ammonia oxidation rates than those constant degradation rates in the absence of ammonium. The positive correlation was found between acyclovir degradation rate and ammonia oxidation rate, confirming the cometabolism of acyclovir by the enriched nitrifying culture in the presence of ammonium. Formation of the product carboxy-acyclovir (P239) indicated the main biotransformation pathway was aerobic oxidation of the terminal hydroxyl group, which was independent on the metabolic type (i.e. cometabolism or metabolism). This enzyme-linked reaction might be catalyzed by monooxygenase from ammonia oxidizing bacteria or heterotrophs. The formation of carboxy-acyclovir was demonstrated to be irrelevant to the acyclovir concentrations applied, indicating the revealed biotransformation pathway might be the dominant removal pathway of acyclovir in wastewater treatment.

Keywords: Biotransformation; nitrification; cometabolism; ammonia oxidizing bacteria; acyclovir; wastewater treatment.

1. Introduction

In recent years, the increasing concerns have been focused on the emerging pharmaceuticals in aquatic environment due to their potential hazardous effects on living organisms (Daughton and Ternes, 1999; Kümmerer, 2009; Sirés and Brillas, 2012). Large amounts of pharmaceuticals were used by human beings or manufactured for veterinary drugs, leading to their widespread occurrence in the wastewater, surface water and ground water (Luo et al., 2014). Wastewater treatment plant (WWTP) was an important pathway for pharmaceuticals entering into the environment (Kosma et al., 2010; Tijani et al., 2013). Inefficient removal efficiencies of these compounds were observed during treatment processes because WWTPs were mainly designed for bulk nutrient removal (Joss et al., 2006; Kosma et al., 2014; Ternes, 1998).

Nitrification process was observed to be able to enhance the removal of pharmaceuticals (Batt et al., 2006; Fernandez-Fontaina et al., 2012). The involved ammonia oxidizing bacteria (AOB) were probably responsible for cometabolic biodegradation of pharmaceuticals due to its non-specific enzyme ammonia monooxygenase (AMO), which was confirmed to degrade a broad range of organic substrates including aliphatic and aromatic compounds (Keener and Arp, 1994; Lauchnor and Semprini, 2013; Rasche et al., 1990; Skotnicka-Pitak et al., 2009). Furthermore, biotransformation products formed during treatment processes may be more persistent and could probably contribute to the overall toxicity (Miao and Metcalfe, 2003; Pérez et al., 2006; Quintana et al., 2005; Ternes et al., 2007). Therefore, the biotransformation products should also be considered in order to get a comprehensive understanding of the behavior and fate of pharmaceuticals in the environment and engineered systems.

As an important antiviral drug, acyclovir has been consumed largely especially for influenza epidemics. Due to their potential ecosystem alterations and the development of viral resistances,

antiviral drugs have recently attracted the interest of research. For example, a substantial removal (98%) of acyclovir was found in the wastewater treatment with the concentration decreasing from 1780 ng L^{-1} to 27 ng L^{-1} (Prasse et al., 2010). Although lab-scale biodegradation of acyclovir was previously studied by the activated sludge from the nitrification zone of a real wastewater treatment plant (Prasse et al., 2011), the effect of metabolic conditions on the formation of biotransformation products and the specific contributions of AOB and heterotrophs to acyclovir removal has not been clearly defined so far.

This study aims to investigate the biodegradation kinetics, products and pathways of acyclovir by an enriched nitrifying culture through batch biodegradation experiments under different metabolic conditions, i.e., with and without the addition of growth substrate, ammonium. The kinetic analysis was accompanied with the structural elucidation of biotransformation products. The initial acyclovir concentration at 15 mg L^{-1} and 15 μg L^{-1} were applied to verify if the biotransformation products and pathways formed under high concentration would occur at environmentally relevant levels.

2. Materials and Methods

2.1 Chemicals

Acyclovir (>98%) was purchased from Thermo Fisher, Australia. Carboxy-acyclovir was provided by Toronto Research Chemicals. Isotope labeled compound acyclovir-d4 was obtained from Santa Cruz Biotechnology. HPLC grade organic solvents (methanol, acetonitrile, hexane and acetone) were supplied by Sigma-Aldrich, Australia. The individual standard stock solution of acyclovir was prepared on a weight basis in methanol at 1 mg mL^{-1} and stored at -20 °C. The

84 calibration curve was obtained by diluting the stock solution appropriately in methanol/water
85 (25:75, v/v). Acyclovir feed solution used in the batch experiments was prepared in Milli-Q
86 water (Millipore, Inc.) at initial concentration of 1 g L^{-1}.
87
88 **2.2 Enriched nitrifying culture**
89 An 8-L lab-scale sequencing batch reactor (SBR) was inoculated with the activated sludge
90 from a domestic wastewater treatment plant in Brisbane, Australia. It was operated with the aim
91 for the enrichment of nitrifying culture (containing AOB and nitrite oxidizing bacteria (NOB) to
92 perform full nitrification) in cycles of 6 h. For each cycle, it consisted of aerobic feeding (260
93 min), aeration (30 min), waste (1 min), settling (60 min) and decanting (9 min). 2 L synthetic
94 wastewater consisting of 1 g L^{-1} NH_4^+-N was fed into the reactor during each feeding period,
95 resulting in a hydraulic retention time (HRT) of 24 h. The solid retention time (SRT) was
96 controlled at around 15 d. Dissolved oxygen (DO) was controlled between 2.5-3.0 mg L^{-1} using
97 programmed logic controllers (PLC) and pH was maintained at the range of 7.5-8.0.
98 The synthetic wastewater for the enriching the nitrifying culture contained per liter (Kuai
99 and Verstraete, 1998): 5.63 g of NH_4HCO_3 (1 g NH_4^+-N), 5.99 g of $NaHCO_3$, 0.064 g of each of
100 KH_2PO_4 and K_2HPO_4 and 2 mL of a trace element solution. The trace element stock solution
101 contained: 1.25 g L^{-1} EDTA, 0.55 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.40 g L^{-1} $CoCl_2 \cdot 6H_2O$, 1.275 g L^{-1}
102 $MnCl_2 \cdot 4H_2O$, 0.40 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.05 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 1.375 g L^{-1} $CaCl_2 \cdot 2H_2O$, 1.25
103 g L^{-1} $FeCl_3 \cdot 6H_2O$ and 44.4 g L^{-1} $MgSO_4 \cdot 7H_2O$.
104 The biodegradation experiments in this study were conducted after more than 1 year of
105 stable reactor operation with the AOB and NOB population accounting for over 80% of the
106 microbial community with almost 100% conversion of NH_4^+ to NO_3^-. The mixed liquor volatile

suspended solids (MLVSS) concentration was stable at 1437.6 ± 112.9 mg L^{-1} (mean and standard errors, respectively, n=10). According to the microbial community analysis with fluorescence in situ hybridization (FISH) (Law et al., 2011), ammonia-oxidizing *beta-proteobacteria* accounted for 46 ± 6% (n=20) of the bacterial populations and the *Nitrospira* genera (nitrite oxidizers) constituted 38 ± 5% (n=20) of the bacterial populations.

2.3 Batch experiments

All batch experiments were conducted in 4 L beakers coupled with PLC controllers. Enriched nitrifying biomass was withdrawn from the SBR during aeration phase when ammonium was almost depleted. The biomass was added into the beaker to obtain the MLVSS concentration of approximately 1000 mg L^{-1} at the beginning of the batch tests. All the batch experiments were divided into two series according to the initial acyclovir concentration. High concentration (15 mg L^{-1}) was selected to identify any possible biotransformation products and elucidate the biotransformation pathways while low concentration (15 μg L^{-1}) was used to study its degradation profile and verify the biotransformation products under environmentally relevant concentration. For each concentration level, different sets of experiments were performed (in duplicates for each experiment) (Table 1). EXP1 was conducted to assess biodegradation of acyclovir in the presence of ammonium. The constant ammonium concentration (50 mg L^{-1}) was provided by automatically adding a mixture of ammonium bicarbonate and sodium bicarbonate, which was controlled by PLC as a pH adjustment process. The adding volume was controlled to be minor, which would not change the total volume significantly. EXP2 was performed in the absence of ammonium during the overall time course. EXP3 was carried out with the initial addition of allylthiourea (ATU), which could inhibit ammonia oxidation probably by chelating

the copper of AMO active site (Ginestet et al., 1998). The control experiments, EXP4 and EXP5, were used to assess the contribution of abiotic degradation and hydrolytic degradation to acyclovir losses using NaN_3 and pure water (without biomass), respectively. NaN_3 was a chemical inhibitor used for the inactivation of microbial activities (Rattier et al., 2014). Aerobic conditions were achieved with controlled air supply to obtain DO concentration of 2.5-3.0 mg L^{-1}. The pH was maintained in the range of 7.5-8.0 during the time course in all tests. Mixed liquor samples were taken periodically and immediately frozen until analysis.

2.4 Sample preparation and chemical analysis

For experiments at initial acyclovir concentration of 15 mg L^{-1}, samples were centrifuged at 12000 g for 5 min without filtration to obtain 1 mL supernatant for further direct structural elucidation of the biotransformation products and quantification. For experiments at initial acyclovir concentration of 15 μg L^{-1}, the samples were concentrated through solid phase extraction (SPE) with vacuum manifold (J. T. Baker, The Netherlands) with the recovery for acyclovir of 87.2 ± 6.4% (n=3, 10 μg L^{-1} added). 50 mL samples were first centrifuged at 14000 rpm for 5 min. The supernatant was flowing through Oasis HLB cartridges (6 mL, 200 mg, Waters, USA) at a rate of 5 mL min^{-1} after conditioned with 10 mL methanol and 10 mL Milli-Q water. Then cartridges were dried under vacuum for 30 min before they were eluted with 10 mL methanol and 10 mL of hexane/acetone (50:50, v/v). The extracted elutes were evaporated to dryness under gentle nitrogen stream. The residue was reconstituted in 250 μL methanol and 750 μL Mmilli-Q water with 20 μL acyclovir-d4 (internal standard) added before further analysis.

The samples were analyzed by the ultra-fast liquid chromatography (UFLC) (Shimadzu, Japan) coupled with a 4000 QTRAP hybrid triple quadruple-linear ion trap mass spectrometer

153 (QqLIT-MS) equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, USA).
154 Chromatographic separation was carried out with the injection volume of 20 μL using an Alltima
155 C18 column (Alltech Associates Inc., USA) at 40 °C. The mobile phase contained (A) H_2O and
156 (B) CH_3CN at a flow rate of 1 mL min^{-1}. The gradient of (B) was conducted as follows: it was
157 linearly increased to 5% B after 0.5 min, further increased to 20% B for 12.5 min, increased to
158 50% B within 5 min, increased to 100% B for 2 min, kept constant for 4 min and finally was
159 decreased to 5% B for 1 min. The total running time including the conditioning of the column to
160 the initial conditions was 27 min. Positive electrospray ionization (ESI+) mode was applied with
161 the corresponding parameters: drying gas temperature of 500 °C, drying gas 50 psi, curtain gas
162 30 psi, spraying gas 50 psi. Tentative structures of biotransformation products were identified
163 using the full scan mode at a declustering potential of 80 V and mass range of 50-300 amu
164 followed by the product ion scan mode (MS^2) and sequential fragmentation using the ion trap.
165 Concentrations of acyclovir and its biotransformation product were analyzed in the multiple
166 reaction monitoring (MRM) mode with two transition ions for confirmation and quantification,
167 respectively. The samples from the experiments at initial 15 mg L^{-1} acyclovir need to be diluted
168 100 times in methanol/Milli-Q water (25:75, v/v) prior to quantification. More detailed
169 information could be obtained in Table S1 in the supporting information (SI).

170 Ammonium (NH_4^+-N) concentrations controlled in the batch biodegradation experiments
171 were measured with a Lachat QuikChem8000 Flow Injection Analyzer (Lachat Instrument,
172 Milwaukee) and were shown in Figure S1 in SI. Nitrite was not accumulated significantly with
173 the concentration lower than 1 mg L^{-1} for the experimental period and same nitrate concentration
174 was observed as the SBR effluent (up to 1000 mg L^{-1}).

175

3. Results

3.1 Control experiments

The sorption ability of acyclovir onto the biomass was considered negligible due to the low value of octanol-water partition coefficient (Log K_{ow}, -1.59) (Kasim et al., 2004), which could also be observed from the control experimental results in this study. Regardless of the initial concentration of acyclovir, both abiotic control (EXP4) and hydrolytic control (EXP5) experiments demonstrated the stability of acyclovir over the time course without any transformation products (Figure S2). Sorption and hydrolysis would not contribute to acyclovir removal. Given that the reactors were covered with aluminum foil from photodegradation, biodegradation by nitrifying biomass was the major pathway for acyclovir removal in all the experiments.

3.2 Acyclovir biodegradation in the presence of ammonium

The removal efficiency, transformation efficiency and degradation constant of acyclovir in all the biodegradation experiments were summarized in Table 2. Figure 1 shows the results from the biodegradation experiments in the presence of ammonium. The decrease of acyclovir and formation of the product were plotted using their respective concentrations normalized to the initial acyclovir concentration. At initial concentration of 15 mg L^{-1}, acyclovir underwent a gradual decrease with approximately 65.1% removal at the end of experiments (Figure 1A). After careful screening in the full scan chromatogram followed by spectrum analysis based on nitrogen rule and the existence of the peak [m+Na], etc, one major biotransformation product P239 was found at retention time of 4.88 min (data not shown) with nominal mass of 239. Its

199 structural elucidation was carried out in the following section 3.5. With the available reference
200 standard (carboxy-acyclovir), it was increased gradually from the beginning of the experiments
201 to 6.95 mg L^{-1} (58.6% of conversion rate) at 240 h.

202 At initial 15 μg L^{-1} concentration, the removal efficiency for acyclovir (88.2%) was higher
203 than that obtained at higher initial level (65.1%) (Figure 1B). The same major product P239 was
204 continuously increased to 5.74 μg L^{-1}. Only 33.0% of the removed parent compound was
205 transformed to P239 while the remaining might be transformed to other minor products or
206 mineralized.

207 For both initial concentration levels, acyclovir biodegradation followed the pseudo first
208 order degradation kinetics (Figure S3). Same as their concentration profiles, acyclovir also
209 showed the higher degradation constant (0.0071 L g_{VSS}^{-1} h^{-1}) at initial 15 μg L^{-1} concentration
210 than 0.0034 L g_{VSS}^{-1} h^{-1} at 15 mg L^{-1} concentration. These degradation constants were lower than
211 the reported value (4.9 L g_{SS}^{-1} d^{-1}) (Prasse et al., 2011), probably due to unaccustomed sludge to
212 acyclovir. Long-term adaption to pharmaceuticals would enhance the degradation ability of the
213 activated sludge (Pomiès et al., 2015). The decreasing ammonia oxidation rate observed during
214 the experimental period (Figure 2A) might be due to the inhibition of acyclovir or its
215 transformation product (Radniechi et al., 2008; Sathyamoorthy et al., 2013), which could lead to
216 decreasing rates of both substrates. NOB has been proved to be not associated with
217 pharmaceutical (e.g., atenolol) degradation in previous work, with AMO as the main responsible
218 of the cometabolism (Fernandez-Fontaina et al., 2012; Sathyamoorthy et al., 2013; Xu et al.,
219 2016). Regardless of initial acyclovir concentration, the positive relationship between acyclovir
220 degradation rate and ammonia oxidation rate suggested the cometabolic biodegradation of
221 acyclovir by AOB in the presence of ammonium (Figure 2B). The cometabolism also applies to

222 higher concentration of non-growth substrate although it was in the range of the growth substrate
223 concentration (Quintana et al., 2005).
224
225 **3.3 Acyclovir biodegradation in the absence of ammonium**
226 Without the presence of growth substrate, acyclovir was removed by enriched nitrifying
227 biomass up to 40.9% with the final concentration being 8.5 mg L^{-1} for the higher initial acyclovir
228 concentration experiments (Figure 3A). Simultaneously, the product P239 showed a rapid
229 growing profile compared with the results from experiments with ammonia oxidation. Its
230 concentration was quantified as 8.7 mg L^{-1} at 240 h. Nearly constant mass balance during the
231 time course indicated that almost all the acyclovir removed in the absence of ammonium was
232 transformed to P239. Furthermore, the mass balance did not show a decreasing trend even after
233 15 d. Thus, P239 might be the only biotransformation product.
234 For the low initial acyclovir experiments, the removal rate of acyclovir only reached up to
235 47.8% without ammonia oxidation, which was significantly lower than that observed in the
236 presence of ammonium (88.2%) (Figure 3B). Regardless of the initial acyclovir concentration,
237 the cometabolism in the obligatory presence of growth substrate played a positive role in
238 degrading acyclovir than the metabolic degradation without ammonia oxidation. The formation
239 of P239 showed a slower increasing trend compared to that observed in the high initial acyclovir
240 concentration experiments.
241 The linear regression on the concentration profiles of acyclovir in Figures S4A and B
242 demonstrated the constant degradation rate of acyclovir in the biodegradation experiments
243 without ammonia oxidation. Acyclovir degradation rates during the higher initial concentration
244 and lower initial concentration experiments were 0.027 mg g_{VSS}^{-1} h^{-1} and 0.043 μg g_{VSS}^{-1} h^{-1},

respectively, which were lower than their corresponding degradation rates (0.051, 0.046, 0.042, 0.038, 0.034, 0.031, 0.028 mg g_{VSS}^{-1} h^{-1} and 0.18, 0.15, 0.12, 0.090, 0.071, 0.056, 0.044 μg g_{VSS}^{-1} h^{-1} at time 0, 24, 48, 72, 96, 120, 144 h) under higher ammonia oxidation rates in the experiments in the presence of ammonium, further confirmed the important role of cometabolic biodegradation by AOB for acyclovir removal.

3.4 Acyclovir biodegradation with ATU inhibition

ATU was added at the beginning of the experiments in order to inhibit the nitrifying activities of AOB, thus likely leading to the exclusive degradation of acyclovir by heterotrophs. As no external organic source was provided in the experiments, acyclovir degradation might attribute to heterotrophic metabolic activity. Figure 4A illustrates that acyclovir experienced a slow gradual decrease with a removal rate of 36.2% over the experimental period at the higher initial concentration, which was slightly lower than 40.9% obtained in the absence of ammonium. P239 was still the only product formed with the concentration increasing to 6.4 mg L^{-1} when nitrifying activities were inhibited. Practically 94.1% of the consumed acyclovir was transformed to P239 and the mass balance leveled off for the overall time course. Therefore, no other major products might be formed by heterotrophs.

As shown in Figure 4B, acyclovir at initial 15 μg L^{-1} also declined gradually with a removal efficiency of 50.3% accompanied by the continuous increase of its product P239 (4.9 μg L^{-1} at 240 h). The mass balance analysis also indicated the constant mass during the experimental period with only one product P239 formed.

The linear regression in Figures S4C and D showed acyclovir degradation constants for experiments at the higher initial concentration and the lower initial concentration were calculated

as 0.02 mg g_{VSS}^{-1} h^{-1} and 0.018 μg g_{VSS}^{-1} h^{-1}, respectively. Compared to the values obtained in the absence of ammonium, heterotrophs played a major contribution to acyclovir degradation under the condition without ammonia oxidation by AOB. The role of AOB and heterotrophs has been investigated in previous studies on pharmaceutical biodegradation (Khunjar et al., 2011; Tran et al., 2013). The fact that the same biotransformation products for 17β-ethinylestradiol were formed by AOB or heterotrophs (Khunjar et al., 2011) was consistent to the observations in this study. The results confirmed that P239 was the major biotransformation product of acyclovir by the enriched culture independent on its initial concentration.

3.5 Structural elucidation of biotransformation product

The full scan chromatogram of the samples indicated the formation of the product P239 during all biodegradation experiments. Its structure was then identified through the analysis and comparison of the product ion (MS^2) spectrum of the biodegradation samples with that of the available standard carboxy-acyclovir. Figure 5 showed the MS^2 spectrum of the molecular ion *m/z* 240, which was the protonated P239. The most abundant fragment ions were *m/z* 152 and 135. The molecular ion *m/z* 240 underwent the loss of 88 Da to produce the fragment ion *m/z* 152. Further fragmentation of *m/z* 152 led to a loss of NH_3 molecule to form *m/z* 135. The similar fragmentation pattern was previously reported in the literature (Prasse et al., 2011). Another minor fragmentation pathway was to form *m/z* 122 with a loss of 44 Da from *m/z* 164, following the loss of 76 Da from molecular ion *m/z* 240. Collision induced dissociation of the molecular ion *m/z* 240 could lead to the third route to obtain the fragment ion *m/z* 61, via the formation of the fragment ion 89. There were no further fragmentation pathways from the lowest *m/z* 61. The same fragment ions and fragment pattern observed in the standard solution further confirmed that

P239 could be assigned to 9-carboxymethoxymethylguaine (carboxy-acyclovir) (Figure S5).

The same fragment ions *m/z* 152 and 135 were also formed in the MS^2 spectrums of the parent compound acyclovir, except *m/z* 164 and 122, which were also reported in previous literature (Prasse et al., 2011). The only difference between acyclovir and P239 was the third pathway forming *m/z* 75 and *m/z* 89, respectively (Figure S6), which was attributed to the oxidation of the hydroxyl group to the carboxy group. This also supported the structural identification of P239 in this work.

4. Discussion

In this work, the biodegradation of the antiviral drug acyclovir by an enriched nitrifying culture was investigated during ammonia oxidation and without the addition of ammonium. Acyclovir degradation rates based on pseudo first order kinetics under higher ammonia oxidation rate in the presence of ammonium were higher than those constant values in the absence of ammonium. The positive correlation observed between acyclovir degradation rate and ammonia oxidation rate further confirmed the cometabolism of acyclovir by the enriched nitrifying culture in the presence of ammonium, which was also supported from the similar relationship for 17α-ethinylestradiol (Yi and Harper, 2007).

Based on the identified product, the main biotransformation pathway was proposed for acyclovir degradation: from acyclovir to carboxy-acyclovir (P239). This reaction was attributed to the oxidation of the terminal hydroxyl group to the carboxy group, which was typically catalyzed by AMO from AOB or ammonia oxidizing archaea (AOA) for most pharmaceuticals including other antiviral drugs (abacavir, emtricitabine, ganciclovir, lamivudine and zidovudine), amide-containing compounds (e.g. propachlor) and tertiary amines such as mianserin (Funke et

al., 2016; Helbling et al., 2010; Men et al., 2016). Although it was also observed in mammalian metabolism of acyclovir (Prasse et al., 2011), the enzyme-induced alcohol oxidation has not been investigated solely for heterotrophs previously. However, the formation of carboxy-acyclovir in experiments with ATU addition in this work indicated that the monooxygenase from heterotrophs could also catalyze the alcohol oxidation of such compound. Although other possibly formed products were not identified in the presence of ammonium, the same biotransformation product carboxy-acyclovir found with ammonia oxidation and with nitrification inhibited in this study was different from previous report on iopromide, where dehydroxylated and carboxylated products were formed, respectively (Batt et al., 2006). It was proposed that oxidation of acyclovir to carboxy-acyclovir might be catalyzed by monooxygenase from either AOB or heterotrophs (Men et al., 2016).

It was also noted that the formation of carboxy-acyclovir was independent on the metabolic type, i.e. regardless of the presence of ammonia oxidation by AOB. This was contradictory to the observation that the generation of 4-chlorobenzoic acid was related to the metabolic type and only produced by microbial hydrolysis of the amide bond of bezafibrate under cometabolism (Quintana et al., 2005). Comparing with acyclovir, the human metabolite carboxy-ibuprofen was not found in biodegradation of ibuprofen following alcohol oxidation (Quintana et al., 2005). The possible reason could be related to the specific structures of the studied pharmaceuticals. The guanine group of acyclovir showed no significant changes during biodegradation with the primary hydroxyl being the only vulnerable group. However, whether acyclovir could be biotransformed to other products is not confirmed in the product identification in this study although the mass balance analysis in the presence of ammonium demonstrated the possible formation of other products. Further work would be required to confirm the thorough

biotransformation pathway of acyclovir.

The biotransformation pathway to carboxy-acyclovir by enriched nitrifying cultures was independent of the initial concentration of acyclovir, i.e. 15 μg L^{-1} or 15 mg L^{-1}. This was in consistent with the previous study on trimethoprim by nitrifying activated sludge (Eichhorn et al., 2005). Two metabolites were formed and the degradation route was independent on the initial concentration of trimethoprim (20 mg L^{-1} or 20 μg L^{-1}). However, biodegradation of trimethoprim was also investigated by nitrifying activated sludge in another recent study (Jewell et al., 2016), resulting in different biotransformation products under different spiked concentration of trimethoprim (500 μg L^{-1} or 5 μg L^{-1}). From the mass balance analysis, carboxy-acyclovir seems to be the only product by heterotrophs either in higher initial concentration or lower initial concentration of acyclovir. There might be other minor biotransformation products formed through cometabolism by enriched nitrifying cultures as carboxy-acyclovir had a low final percentage and the total mass showed a decreasing trend, which was vastly different from those under other conditions (in the absence of ammonium and with addition of ATU), which could not be confirmed yet currently and required future efforts.

5. Conclusion

Biodegradation of acyclovir by an enriched nitrifying culture was investigated during ammonia oxidation and without the presence of ammonium at different initial concentrations of acyclovir in this study. The key conclusions are:

- Biodegradation of acyclovir was positively related to the ammonia oxidation rate, confirmed the key role of cometabolism by AOB in acyclovir removal.
- Carboxy-acyclovir was produced from acyclovir regardless of the presence of ammonium

and thus unaffected by metabolic type.

- The same biotransformation pathway from acyclovir to carboxy-acyclovir was observed at different initial concentrations of acyclovir.
- Alcohol oxidation was the biotransformation reaction catalyzed by non-specific enzyme monooxygenase, probably either from AOB or heterotrophs.

Acknowledgement

This study was supported by the Australian Research Council (ARC) through Discovery Early Career Researcher Award DE130100451. Bing-Jie Ni acknowledges the support of ARC Discovery Project DP130103147 and The University of Queensland Foundation Research Excellence Award.

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Table and figure legends

Table 1. Conditions of conducted batch experiments with acyclovir (same design of key experimental conditions for experiments at initial acyclovir of 15 mg L^{-1} and 15 μg L^{-1})

Table 2. The acyclovir removal efficiency, biotransformation efficiency and degradation constant in the conducted biodegradation experiments (high, initial acyclovir concentration of 15 mg L^{-1}; low, initial acyclovir concentration of 15 μg L^{-1}) with possible mechanisms involved

Figure 1. Concentration profiles of acyclovir and its product normalized to the initial acyclovir of (A) 15 mg L^{-1} and (B) 15 μg L^{-1} in the experiments with ammonia oxidation.

Figure 2. (A) Ammonia oxidation rate during the time course in the acyclovir biodegradation experiments with ammonia oxidation; (B) the relationship between acyclovir degradation rate and ammonia oxidation rate in the presence of ammonium.

Figure 3. Concentration profiles of acyclovir and its product normalized to the initial (A) 15 mg L^{-1} and (B) 15 μg L^{-1} in the experiments without ammonia addition.

Figure 4. Concentration profiles of acyclovir and its product normalized to the initial (A) 15 mg L^{-1} and (B) 15 μg L^{-1} in the experiments with inhibition of ammonia oxidation of AOB by allythiourea (ATU) addition.

Figure 5. The fragmentation pathways of P239 under ESI+ conditions derived from MS^2 experiments in the QqLIT mass spectrometer.

506 **Table 1.** Conditions of conducted batch experiments with acyclovir (same design of key
507 experimental conditions for experiments at initial acyclovir of 15 mg L^{-1} and 15 μg L^{-1})

Experiments	EXP1	EXP2	EXP3	EXP4	EXP5
Initial ammonium (mg L^{-1})	50	0	50	50	50
Ammonium control	Constant	0	Constant	Constant	Constant
Approximate VSS (mg L^{-1})	1000	1000	1000	1000	0
Volume (L)	4	4	4	4	4
ATU (mg L^{-1})	0	0	30	0	0
NaN_3 (mg L^{-1})	0	0	0	500	0

508 **Table 2.** The acyclovir removal efficiency, biotransformation efficiency and degradation
509 constant in the conducted biodegradation experiments (high, initial acyclovir concentration of 15
510 mg L^{-1}; low, initial acyclovir concentration of 15 μg L^{-1}) with possible mechanisms involved

Experiments	EXP1-high	EXP1-low	EXP2-high	EXP2-low	EXP3-high	EXP3-low
Removal efficiency (%)	65.1	88.2	40.9	47.8	36.2	50.3
Biotransformation efficiency (%)	58.6	33.0	~100	72.6	94.1	83.8
Biodegradation constant	0.0034 L g_{VSS}^{-1} h^{-1}	0.0071 L g_{VSS}^{-1} h^{-1}	0.027 mg g_{VSS}^{-1} h^{-1}	0.043 μg g_{VSS}^{-1} h^{-1}	0.02 mg g_{VSS}^{-1} h^{-1}	0.018 μg g_{VSS}^{-1} h^{-1}
Main mechanisms	Cometabolism by AOB		Metabolism by AOB and heterotrophs		Metabolism by heterotrophs	

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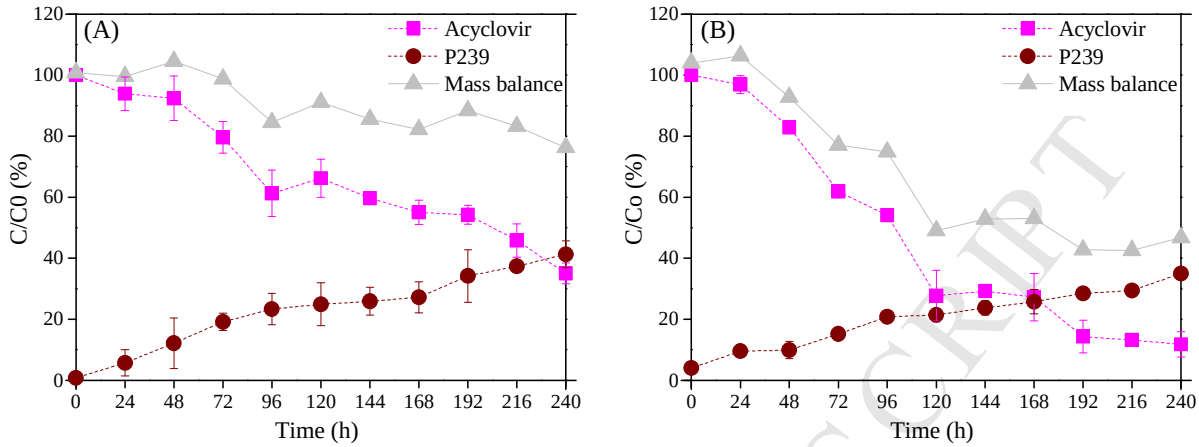

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515 **Figure 1.** Concentration profiles of acyclovir and its product normalized to the initial acyclovir

516 of (A) 15 mg L^{-1} and (B) 15 μg L^{-1} in the experiments with ammonia oxidation.

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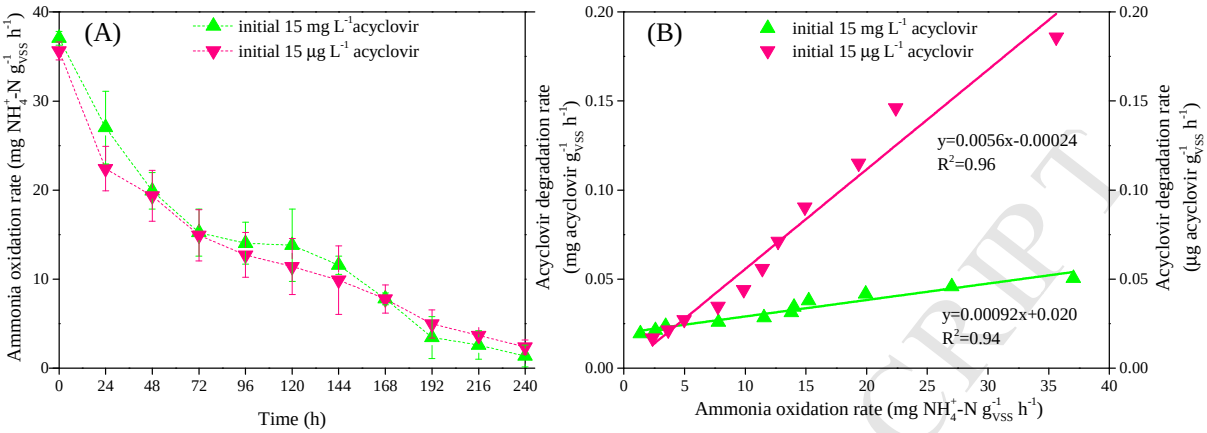

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520 **Figure 2.** (A) Ammonia oxidation rate during the time course in the acyclovir biodegradation
521 experiments with ammonia oxidation; (B) the relationship between acyclovir degradation rate
522 and ammonia oxidation rate in the presence of ammonium.

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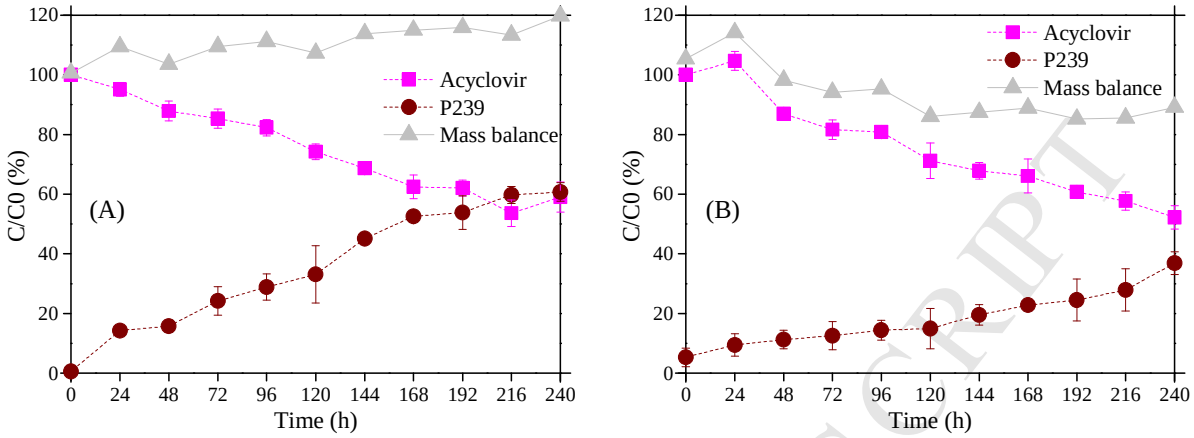

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527 **Figure 3.** Concentration profiles of acyclovir and its product normalized to the initial (A) 15 mg

528 L^{-1} and (B) 15 μg L^{-1} in the experiments without ammonia addition.

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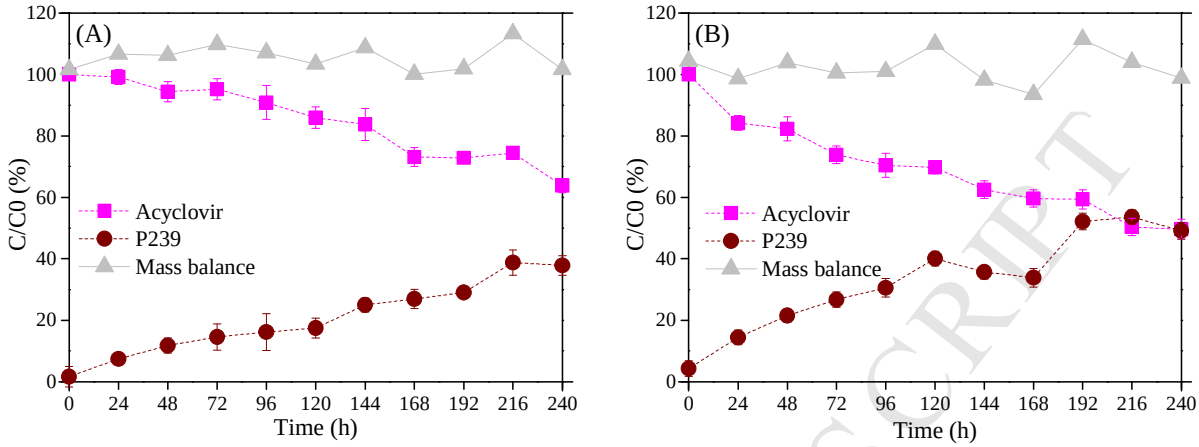

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533 **Figure 4.** Concentration profiles of acyclovir and its product normalized to the initial (A) 15 mg
534 L^{-1} and (B) 15 μg L^{-1} in the experiments with inhibition of ammonia oxidation of AOB by
535 allythiourea (ATU) addition.

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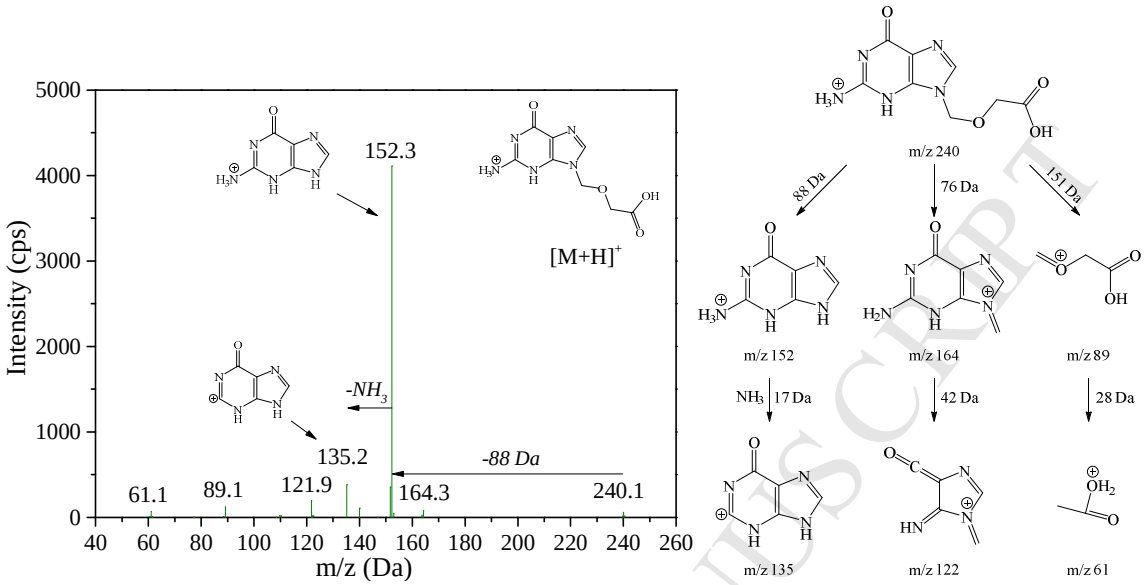

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540 **Figure 5.** The fragmentation pathways of P239 under ESI+ conditions derived from MS^2

541 experiments in the QqLIT mass spectrometer.

Highlights

- Acyclovir biodegradation followed cometabolism by AOB in the presence of ammonium.
- Alcohol oxidation was the main biotransformation pathway producing carboxy-acyclovir.
- Formation of carboxy-acyclovir was independent on the initial concentration of acyclovir.
- Metabolic type has no effect on the biotransformation pathway into carboxy-acyclovir.
- The enzyme-linked oxidation could be catalyzed by AOB or heterotrophs.