Chemosphere 170 (2017) 25–32

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Biotransformation of acyclovir by an enriched nitrifying culture

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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.

ARTICLE INFO

Article history:
Received 15 September 2016
Received in revised form
2 December 2016
Accepted 4 December 2016
Available online 7 December 2016

Handling Editor: Klaus Kümmerer

Keywords:
Biotransformation
Nitrification
Cometabolism
Ammonia oxidizing bacteria
Acyclovir
Wastewater treatment

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.

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

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http://dx.doi.org/10.1016/j.chemosphere.2016.12.014
0045-6535/

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 calibration curve was obtained by diluting the stock solution appropriately in methanol/water (25:75, v/v). Acyclovir feed solution used in the batch experiments was prepared in Milli-Q water (Millipore, Inc.) at initial concentration of 1 g L^{-1}.

2.2. Enriched nitrifying culture

An 8-L lab-scale sequencing batch reactor (SBR) was inoculated with the activated sludge from a domestic wastewater treatment plant in Brisbane, Australia. It was operated with the aim for the enrichment of nitrifying culture (containing AOB and nitrite oxidizing bacteria (NOB) to perform full nitrification) in cycles of 6 h. For each cycle, it consisted of aerobic feeding (260 min), aeration (30 min), waste (1 min), settling (60 min) and decanting (9 min). 2 L synthetic wastewater consisting of 1 g L^{-1} NH_4^+-N was fed into the reactor during each feeding period, resulting in a hydraulic retention time (HRT) of 24 h. The solid retention time (SRT) was controlled at around 15 d. Dissolved oxygen (DO) was controlled between 2.5 and 3.0 mg L^{-1} using programmed logic controllers (PLC) and pH was maintained at the range of 7.5–8.0.

The synthetic wastewater for the enriching the nitrifying culture contained per liter (Kuai 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 KH_2PO_4 and K_2HPO_4 and 2 mL of a trace element solution. The trace element stock solution 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} $MnCl_2 \cdot 4H_2O$, 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 g L^{-1} $FeCl_3 \cdot 6H_2O$ and 44.4 g L^{-1} $MgSO_4 \cdot 7H_2O$.

The biodegradation experiments in this study were conducted after more than 1 year of stable reactor operation with the AOB and NOB population accounting for over 80% of the 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.

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}).

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