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Degradation Kinetics and Mechanism of Antibiotic Ceftiofur in Recycled Water Derived from a Beef Farm

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Supporting Information

ABSTRACT: Ceftiofur is a third-generation cephalosporin antibiotic that has been widely used to treat bacterial infections in concentrated animal feeding operations (CAFOs). Land application of CAFO waste may lead to the loading of ceftiofur residues and its metabolites to the environment. To understand the potential contamination of the antibiotic in the environment, the degradation kinetics and mechanisms of ceftiofur in solutions blended with and without the recycled water derived from a beef farm were investigated. The transformation of ceftiofur in aqueous solutions in the presence of the CAFO recycled water was the combined process of hydrolysis and biodegradation. The total degradation rates of ceftiofur at 15 °C, 25 °C, 35 °C, and 45 °C varied from $0.4-2.8 \times 10^{-3}$, $1.4-4.4 \times 10^{-3}$, $6.3-11 \times 10^{-3}$, and $11-17 \times 10^{-3}$ h^{-1}, respectively, in aqueous solutions blended with 1 to 5% CAFO recycled water. Hydrolysis of ceftiofur increased with incubation temperature from 15 to 45 °C. The biodegradation rates of ceftiofur were also temperature-dependent and increased with the application amounts of the recycled CAFO water. Cef-aldehyde and desfuroylceftiofur (DFC) were identified as the main biodegradation and hydrolysis products, respectively. This result suggests that the primary biodegradation mechanism of ceftiofur was the cleavage of the β-lactam ring, while hydrolytic cleavage occurred at the thioester bond. Unlike DFC and ceftiofur, cef-aldehyde does not contain a β-lactam ring and has less antimicrobial activity, indicating that the biodegradation of ceftiofur in animal wastewater may mitigate the potentially adverse impact of the antibiotic to the environment.

KEYWORDS: ceftiofur, concentrated animal feeding operations, recycled water, biodegradation, hydrolysis

INTRODUCTION

Veterinary pharmaceuticals are widely used in concentrated animal feeding operations (CAFOs) for growth-improvement and disease control. Antibiotics are a major group of veterinary medicines, accounting for more than 70% of all consumed pharmaceuticals.[1] It has been reported that 30 to 90% of the administered antibiotics can be excreted unmetabolized in animal manure and urine.[2] The analysis of CAFO wastewater has also shown that many antibiotic residues are present at μg/L to mg/L levels.[3,4] In the United States, an estimated 335 million metric tons of animal wastes are produced annually from large confined-animal farms.[5] These animal wastes, including manure and wastewater, are usually land-applied in nearby fields as a valuable fertilizer and/or soil amendment. However, this agricultural practice leads to a transfer of active pharmaceutical residues from animal waste to soil, which may subsequently enter into the surrounding aquatic environment by leaching and runoff.

The potential human and environmental health risks posed by residual antibiotics and their metabolites in surface and groundwater are receiving growing attention because of increased antibiotic resistance of microorganisms in the environment.[6] For example, multiple antibiotic resistance genes have been detected in lagoon water and groundwater underlying CAFOs.[7−9] These antibiotic resistance genes may be transferred to animals and humans through drinking water and food chains, resulting in decreased susceptibility to antibiotics and diminished success in subsequent treatments.[10,11] Therefore, it is critical to better understand the fate of antibiotic residues in CAFO waste disposal systems and to develop proper waste handling processes that cost-effectively minimize the loading of antibiotics and their metabolites to the environment.

Ceftiofur is a semisynthetic β-lactam antibiotic that has been commonly used for the treatment of respiratory diseases in cattle and other animals since 1987.[12,13] The metabolism of ceftiofur following intramuscular administration in various animal species has been well documented.[13−17] Ceftiofur can be rapidly metabolized in vivo to furoic acid and desfuroylceftiofur (DFC) (Figure 1), the latter having antibacterial activity similar to that of the parent drug. DFC as a principal metabolite can reversibly bind to proteins in plasma and tissues to form a variety of

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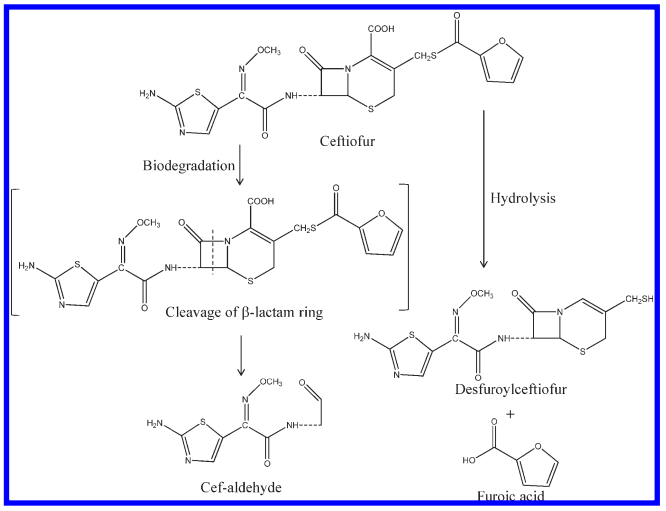

Figure 1. Hydrolysis and biodegradation pathways of ceftiofur.

conjugates with glutathione and cysteine.[14,18] DFC and its derivatives can often be detected in the plasma, tissue, and urine of cattle, swine, and rats, but the parent drug is rarely found in animal bodies one to two days after intramuscular injection.[13,14,18]

In addition to being metabolized, a large portion of administrated cefitiofur is excreted in the urine and feces of various animal species following intramuscular treatment. For example, previous metabolism studies in swine and rats showed that more than 60% of the dose of cefitiofur is excreted in urine and that around 10% of the dose is found in feces.[14,18] This result suggests that most unmetabolized antibiotics exist in CAFO waste disposal systems with animal manure and wastewater, and will eventually enter the environment after land application of animal waste. Biodegradation and chemical hydrolysis are two primary transformation mechanisms of cefitiofur in the environment.[19,20] It has been reported that the aerobic degradation half-lives of sodium ceftiofur in three soils collected from different states in the U.S. range from 22 to 49 days.[19] Addition of cattle feces could enhance the degradation rates of cefitiofur, which suggests that microorganisms play an important role in controlling the environmental fate of cefitiofur.[19] More recently, some strains of bacteria from bovine fecal microflora were isolated that could cleave the β-lactam ring and convert cefitiofur to inactive products.[21] To date, very few studies have investigated the fate of cefitiofur in CAFO waste disposal systems, and the effect of various factors on this antibiotic degradation has not yet been well documented.

The primary objective of this study was to investigate the degradation kinetics and mechanisms of cefitiofur in the recycled water collected from a beef farm and to evaluate the effects of incubation conditions, including temperature and initial matrix concentrations, on the degradation rates of this antibiotic. The recycled water was derived from the beef farm animal wastewater, indicating that it had high microbial activity conducive to the biodegradation of cefitiofur.

MATERIALS AND METHODS

Chemicals and Recycled Water. The ceftiofur standard (>98%) was purchased from Sigma Aldrich (St. Louis, MO). Stock solutions of this antibiotic were prepared in 1:1 acetonitrile−water as needed. Fresh working standards (1.0−95.0 μmol/L) were prepared daily from the stock solution. Acetonitrile (HPLC grade) and formic acid (ACS certified) were obtained from Fisher Scientific (Fair Lawn, NJ). Deionized water (>17.6 MΩ-cm) was supplied by a Labconco Water Pro Plus system (Kansas City, MO). All other chemicals were used as received. All glassware used in this study were baked overnight at 450 °C.

The recycled water was collected from a University of Illinois beef farm located in Champaign, IL. This beef farm has more than 1,000 cattle including approximately 100 to 150 breeding cattle. Large volumes of water are used daily to flush the beef farm barns, which generates animal manure-containing wastewater. Most of the wastewater is recycled after treatment in settling pits, solid separators, and aerobic settling tanks. A diagram of the waste disposal system is provided in the Supporting Information (Figure S1). The treated water is usually recycled to wash barns or blended with surface or groundwater for agricultural irrigation. The recycled water used in the study was taken from the outlet adjacent to the settling tank at about 15 cm below the surface using a self-made collector. The sample was stored in a 4-L solvent bottle and immediately transported to the laboratory in an ice-filled cooler. The physical−chemical parameters and main composition of the collected recycled water are summarized in Table S1 (Supporting Information). A preliminary laboratory analysis revealed that the concentrations of ceftiofur in all collected water samples were less than the detection limit of the method described below (10 μg/L).

Degradation Experiments. The effects of temperature and addition of varying amounts of recycled water on the degradation kinetics of ceftiofur were determined using a laboratory incubation method. Briefly, 0, 1, 3, and 5% (by volume) of recycled water was added to deionized water and mixed thoroughly, yielding incubation solutions with total suspended solid concentrations of 0, 0.11, 0.33, and 0.55 g/L, respectively. Amber bottles (250 mL) with Teflon lined caps served as reactors and were filled with incubation solutions. Kinetic experiments were initiated by spiking stock ceftiofur solution into the reactor bottles. The initial concentration of ceftiofur was 19.1 μmol/L. All reactors were vigorously shaken and then incubated in the dark at different temperatures (15 °C, 25 °C, 35 °C, and 45 °C). A control experiment was concurrently performed in sterile solutions blended with recycled water, which were autoclaved twice at 120 °C before spiking stock ceftiofur solutions. Additionally, ceftiofur solutions without any additions of the recycled water were also conducted to monitor hydrolysis at different temperatures. All experiments were carried out in triplicate.

At regular time intervals, 0.75 mL aliquots of incubation solution were withdrawn from each reaction bottle, transferred into centrifuge vials, and mixed with 0.75 mL of acetonitrile. Samples were immediately centrifuged at 11,000 rpm for 10 min and then filtered through a 0.45-μm membrane (Iso-Disc, Supelco, Bellefonte, PA) using a syringe. Preliminary experiments revealed that the addition of acetonitrile and centrifugation effectively quenched ceftiofur biodegradation. All samples were stored at 4 °C and analyzed within a week using high pressure liquid chromatography/photodiode array detection (HPLC/PDA) and liquid chromatography/mass spectrometry (LC/MS). Preliminary experiments showed that ceftiofur concentrations were stable at this temperature and time period. The recovery of ceftiofur ranged from 95 to 99% in aqueous solutions blended with 0 to 5% recycled water derived from the beef farm.

HPLC and LC/MS Analysis. Concentrations of ceftiofur in the extracts were analyzed using a Waters 2695 Separations Module HPLC equipped with a Waters 996 PDA detector. Separation was performed using a Hypersil C18 column (250 mm × 4.6 mm i.d.; particle size, 3 μm; Keystone Scientific, Bellefonte, PA). The mobile phase consisted of acetonitrile/water (60:40, v/v), the flow rate was 0.8 mL/min, and the detector wavelength was 290 nm. Under these conditions, the retention time for ceftiofur was 15.2 min.

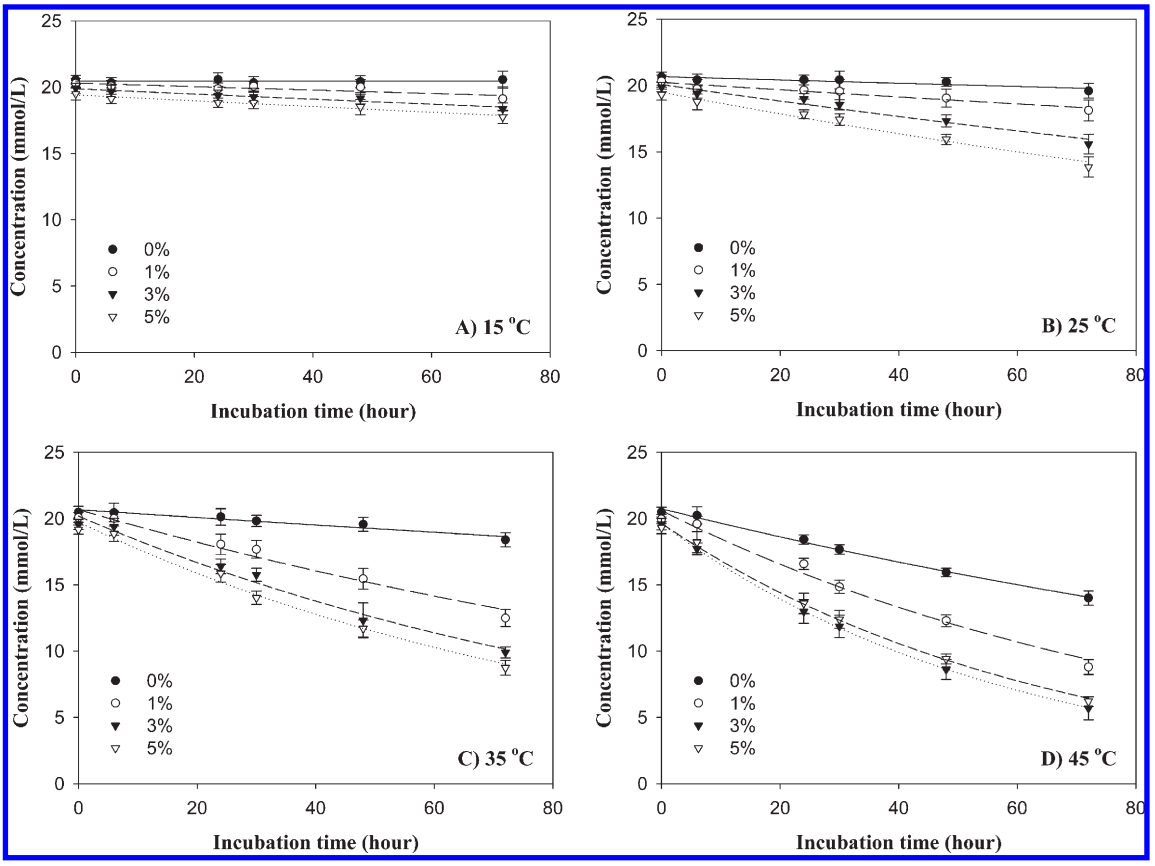

Figure 2. Degradation of ceftiofur in solutions blended with 0, 1, 3, and 5% recycled water derived from the beef farm at different temperatures: (A) 15 °C; (B) 25 °C; (C) 35 °C; and (D) 45 °C. Standard deviation of triplicate samples is shown as error bars.

To identify the degradation products of ceftiofur, a LC/MS equipped with an electrospray ionization source (Quattro Macro, QA1140, Waters, Milford, MA) was used. The chromatographic conditions were the same as those described above. After LC separation, a flow splitter (Micro splitter P451, Upchurch Scientific, Oak Harbor, WA, U.S.) was used to achieve a flow rate of 0.2 mL/min for MS. LC/MS total ion chromatograms were recorded between 100 and 900 m/z at a rate of 2 scans per second. The electrospray source parameters were optimized by infusion of ceftiofur standard solutions. Both positive and negative ionization modes were operated to obtain mass spectra for the identification of degradation products of ceftiofur in different time series samples. The electrospray conditions were capillary voltage 3500 V for positive mode and 3000 V for negative mode; sample cone voltage at 30 V; source temperature at 120 °C; and nitrogen desolvation gas flow of 450 L/min at 350 °C.

RESULTS AND DISCUSSION

Degradation Kinetics of Ceftiofur in Recycled Water Derived from a Beef Farm. Time courses for the degradation of ceftiofur in aqueous solutions blended with the recycled water derived from the beef farm are shown in Figure 2 along with its hydrolysis at different temperatures. The decline of ceftiofur in the solutions mixed with the recycled water was much more rapid compared to the corresponding sterile solutions, indicating that biodegradation could dominate the dissipation of this antibiotic in the aquatic environment containing CAFO wastewater. Biodegradation rates usually depend on the activity of microorganisms in the wastewater, including active bacteria and fungi numbers and their growth rates. In this experiment, the dissipation of ceftiofur in nonsterile aqueous solutions in the presence of CAFO wastewater was a combined process of two concurrent transformations: abiotic degradation and biodegradation. Example time courses for the disappearance of ceftiofur in the sterile aqueous solutions containing 0, 1, 3, and 5% of beef farm recycled water at 35 °C showed no significant difference (Figure S2, Supporting Information). This result suggests that the abiotic degradation of ceftiofur in the solutions blended with the recycled water is mainly due to hydrolysis and that other chemical transformation mechanisms, such as complexation with metal ions, are negligible.

Generally, the kinetics of hydrolysis or biodegradation in the environment can be represented by a simple first-order model.[22−24] Therefore, the degradation rate of ceftiofur in the presence of CAFO wastewater would be the sum of two concurrent first-order reactions:

$$dC/dt = -k_1C - k_2C = -kC \quad (1)$$

$$k = k_1 + k_2 \quad (2)$$

Upon rearrangement and integration, eq 1 becomes

$$\ln C = -(k_1 + k_2)t + \ln C_0 = -kt + \ln C_0 \quad (3)$$

where k (h^{-1}) is the total degradation rate constant at a fixed incubation temperature, k_1 (h^{-1}) is the hydrolysis rate constant, k_2 (h^{-1}) is the biodegradation rate constant, C_0 (μmol/L) is the initial concentration of ceftiofur, and C (μmol/L) is the concentration of

Table 1. Hydrolysis and Total Degradation Rate Constants ($\times 10^{-3}$ h^{-1}) in Solutions Blended with Recycled Water Derived from the Beef Farm under Different Incubation Temperatures[a]

		total degradation rate		
temperature (°C)	hydrolysis	1% recycled water	3% recycled water	5% recycled water
15	0.1 ± 0.1 (0.67)	0.4 ± 0.2 (0.83)	1.1 ± 0.1 (0.99)	2.8 ± 0.1 (0.93)
25	0.3 ± 0.1 (0.79)	1.4 ± 0.2 (0.97)	3.2 ± 0.3 (0.98)	4.4 ± 0.3 (0.99)
35	1.4 ± 0.1 (0.97	6.3 ± 0.6 (0.99)	9.6 ± 0.7 (0.96)	11.0 ± 0.7 (0.99)
45	5.4 ± 0.1 (0.99)	11.0 ± 0.7 (0.98)	17.0 ± 0.1 (0.99)	16.0 ± 0.4 (0.95)

[a] Regression coefficients (r) are showed in parentheses.

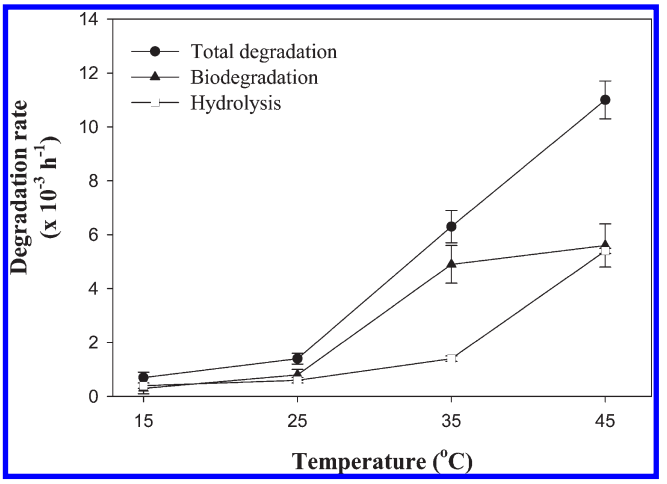

Figure 3. Variation of total degradation, hydrolysis, and biodegradation rate constants of ceftiofur vs incubation temperatures in solutions blended with 1% recycled water derived from the beef farm.

ceftiofur at time t. If there is only hydrolysis, eq 3 can be written as follows:

$$\ln C = -k_1 t + \ln C_0 \quad (4)$$

Values of the rate constants (k and k_1) can be calculated from the slope of semilogarithmic plots of concentration versus time.

In this study, the hydrolysis rate constants of ceftiofur (k_1) in water solutions and its total degradation rate constants (k) in the solutions blended with CAFO recycled water were well-described by eqs 4 and 3, respectively (Figure 2). Hydrolysis and total degradation rate constants of ceftiofur under different incubation temperatures are listed in Table 1. With increasing temperature, the total degradation of ceftiofur in solutions containing the recycled water from the beef farm consistently increased. The total degradation half-lives of ceftiofur at 15 °C, 25 °C, 35 °C, and 45 °C varied from 24−41 days, 6.6−21 days, 2.6−4.6 days, and 1.7−2.6 days, respectively, in aqueous solutions blended with 1 to 5% CAFO recycled water.

Effect of Temperature on Hydrolysis and Biodegradation of Ceftiofur. Hydrolysis of ceftiofur and its biodegradation in aqueous solutions blended with the CAFO recycled water derived from the beef farm were assessed under different incubation temperatures. The hydrolysis rates of ceftiofur increased with increasing temperature in solutions without the addition of CAFO wastewater (Table 1). For instance, hydrolysis rates increased from 0.1 × 10^{-3} to 5.4 × 10^{-3} h^{-1} as the temperature increased from 15 to 45 °C. The effect of temperature on hydrolysis can generally be represented by the Arrhenius equation:

$$\ln k_1 = -E_a/RT + \ln A \quad (5)$$

where A is the pre-exponential factor; E_a (J/mol) is the activation energy, R (J/K·mol) is the universal gas constant, and T is the absolute temperature (K). The calculated E_a as obtained from fitting the hydrolysis rate constants to the Arrhenius equation is about 103 kJ/mol (r = 0.996), indicating that an increase of 10 °C accelerates the hydrolysis rate by about 4.1 times at typical environmental temperatures.[22] In this experiment, each 10 °C increase in temperature increased the average rate of ceftiofur hydrolysis by 3.8 times.

The biodegradation rate constant (k_2) of ceftiofur can be calculated by subtracting the hydrolysis rate constant (k_1) from the total degradation rate constant (k) according to eq 2. The effect of temperature on the rates of hydrolysis and biodegradation for solutions blended with 1% recycled water derived from the beef farm is shown in Figure 3. Biodegradation of ceftiofur accelerated as the incubation temperature increased from 15 to 45 °C. However, the rate of increase for biodegradation is different from hydrolysis (Figure 3). The hydrolysis rate consistently increased with temperature, while ceftiofur biodegradation increased rapidly when temperature increased from 15 to 35 °C, but then only increased slightly between 35 and 45 °C. This indicates that the optimum temperature for ceftiofur biodegradation was between 35 and 45 °C, which is the most suitable temperature for the growth of active microorganisms in various environmental media. Similar temperature effects on the biodegradation kinetics of testosterone were observed for swine manure-borne bacteria.[25]

The difference in temperature effects on hydrolysis and biodegradation was also reflected by the relative contribution of these two degradation mechanisms to total ceftiofur dissipation (Figure 3). At 35 °C, biodegradation accounted for about 78% of the overall ceftiofur degradation, and hydrolysis contributed around 22%. It appears that biodegradation is the predominant pathway for ceftiofur degradation at that incubation temperature. This biodegradation contribution, however, diminished as temperature increased further. In contrast, the relative contribution of hydrolysis increased from 22 to 49% when the temperature increased from 35 to 45 °C, suggesting that hydrolysis plays a much greater role in ceftiofur degradation at higher incubation temperatures.

Effect of Application Amounts of Recycled Water on Ceftiofur Degradation. The addition of the CAFO recycled water inoculated the aqueous ceftiofur solutions with a large population of microorganisms, nutrients, and other compositions, which

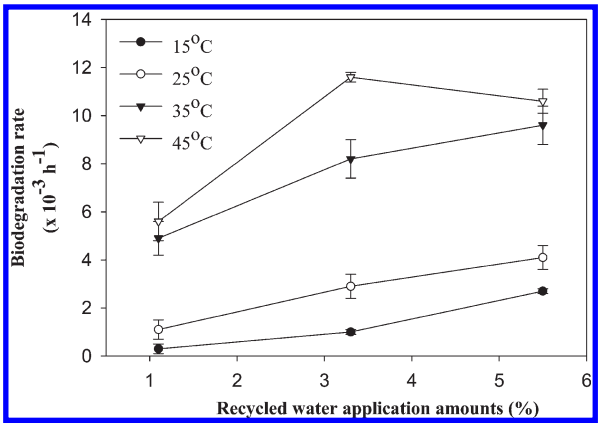

Figure 4. Effect of the application amounts of recycled water derived from the beef farm on the biodegradation rates of ceftiofur under different incubation temperatures.

subsequently resulted in biologic and chemical degradations. A similar disappearance of ceftiofur in the sterile solutions containing different amounts of recycled water (Figure S2, Supporting Information) indicates that the abiotic components (e.g., metal ions) with varying concentrations would not have remarkable effects on the degradation kinetics of the antibiotic.

The effect of application amounts of the recycled water on cefitiofur biodegradation is shown in Figure 4. The biodegradation rates of ceftiofur were significantly enhanced as the application amounts of recycled water increased except at 45 °C, where the biodegradation rate in the solution blended with 3% CAFO recycled water was slightly higher than that in the solution mixed with 5% recycled water. These results suggest that the increase in the application amounts of recycled water introduced more active microorganisms with the result that ceftiofur biodegradation was increased. For instance, at 35 °C, the biodegradation rate of ceftiofur in the solution blended with 1% recycled water was $4.9 \times 10^{-3}\ h^{-1}$, and the rates were enhanced 1.7 and 2.0 times when the incubated solutions were mixed with 3 and 5% recycled water, respectively.

A previous study also revealed that addition of sediment or activated sludge could improve the biodegradation potential of veterinary antibiotics in surface water due to the introduction of active microorganisms.[26] Similarly, ceftiofur biodegradation in this study was attributed to the variety of microorganisms existing in the beef farm recycled water. Also, the effect of the application amounts of the recycled water on ceftiofur biodegradation was more significant at low incubation temperatures. The biodegradation rates of ceftiofur were enhanced 9.0, 3.7, 2.0, and 1.9 times at 15 °C, 25 °C, 35 °C, and 45 °C, respectively, as the application rates of the recycled water were increased from 1% to 5% (Figure 4).

Degradation Mechanism and Pathway. To identify major degradation products of ceftiofur in the aqueous solutions blended with the CAFO recycled water, the extracted samples were analyzed by LC/MS. The peak of the parent compound ceftiofur, with a retention time of 15.2 min (Figure S3-A, Supporting Information), was confirmed by molecular ions of $[M + H]^+$ at 523.9 and $[M + K]^+$ at 561.9, as well as one collision-induced dissociation (CID) fragment at 241.1 (Figure S4-A, Supporting Information), which is consistent with an authentic standard and the previous report.[17] One major biodegradation product, cef-aldehyde, with a retention time at 7.9 min (Figure S3-B, Supporting Information), was detected in the aqueous solution spiked with the CAFO recycled water. Cef-aldehyde was confirmed by its mass spectrum with $[M + H]^+$ at 243.1 and a CID fragment at 126.0 (Figure S4-B, Supporting Information). It appears that cef-aldehyde was formed through breaking the β-lactam ring of ceftiofur (Figure 1), suggesting that the main biodegradation pathway of ceftiofur in this experimental system was the cleavage of its β-lactam ring.

A previous report showed that the β-lactamases of some isolated bacterial strains could cleave the β-lactam ring and inactivate ceftiofur, but that study did not identify any likely metabolites, presumably because they were quickly converted to nondetectable products.[21] To our knowledge, our study is the first reported occurrence of cef-aldehyde as a major metabolite of ceftiofur in aqueous solutions blended with animal wastewater. Cef-aldehyde could further decompose and would disappear in several days under appropriate incubation temperatures.

Additionally, one major hydrolysis product, DFC, was found in the hydrolysis experiments with a retention time at 10.4 min (Figure S3-C, Supporting Information). DFC was confirmed by its major ions of $[M + H]^+$ 430.0, $[M + K]^+$ 467.9, and the same CID fragment 241.1 (Figure S4-C, Supporting Information) as that of ceftiofur (Figure S4-A, Supporting Information). This indicated that DFC shares the same structure with its parent compound including one cephalosporin ring connected to one thiazole ring (Figure 1). A similar mass spectrum of DFC was also reported in various previous studies.[13,18,27] The formation of DFC involved cleavage of the thioester bond of ceftiofur (Figure 1), and the primary hydrolysis pathway is consistent with that proposed by Koshy et al.[27] DFC is believed to have antimicrobial activity similar to that of its parent compound ceftiofur since it still retains the β-lactam ring.[13,15,16] In contrast, cef-aldehyde formed from the cleavage of the ceftiofur β-lactam ring has less antimicrobial activity, which indicates that the biodegradation of ceftiofur to cef-aldehyde may mitigate the potentially adverse impact of this antibiotic in the environment.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Schematic diagram showing the beef farm waste disposal system, reconstructed ion chromatograms of ceftiofur after biodegradation and hydrolysis, electrospray LC/MS mass spectra, and physio-chemical parameters of recycled water. This material is available free of charge via the Internet at http://pubs.acs.org.

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