공학박사학위논문

Fate and long-term effects of chlortetracycline antibiotics on anaerobic digestion of swine manure

돈분의 혐기성 소화 중
클로르테트라싸이클린 항생제의 거동과 장기 영향

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서울대학교 대학원

건설환경공학부

이 창 민

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이 논문을 공학박사 학위논문으로 제출함

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Fate and long-term effects of chlortetracycline antibiotics on anaerobic digestion of swine manure

by

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of the requirements for the degree of

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ABSTRACT

Fate and long-term effects of chlortetracycline antibiotics on anaerobic digestion of swine manure

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The growth promotion effect of veterinary antibiotics on livestock enabled a factory farming system. It brought on the overuse of veterinary antibiotics in the livestock industry. The consumption of antibiotics for livestock increased from only hundreds of tons in 1950 to 63,200 tons in 2010. Since veterinary antibiotics are excreted with the livestock manure after the administration, the overuse of veterinary antibiotics has led to continuous contamination of the aquatic and terrestrial environment via the livestock manure. In the environment, veterinary antibiotics caused many problems such as microbial toxicity, the prevalence of antibiotic-

resistant genes and bacteria, the emergence of super-bacteria, and exposure of veterinary antibiotics to humans.

Anaerobic digestion has been getting more attention as a sustainable treatment method to treat organic wastes such as livestock manure and food waste in recent decades. At the same time, there has been a growing concern that anaerobic digestion systems treating livestock manure will be the reservoirs supplying veterinary antibiotics to the environment. The fate of veterinary antibiotics in AD will change and affect the environmental concentration and hazardous in the environmental metrics. In addition, even after excretion with livestock manure, veterinary antibiotics maintain their antimicrobial activity. Since the AD process is a series of microbial metabolizations, the antimicrobial effect can undermine the performance of the AD system. Understanding veterinary antibiotics' fate and effects on anaerobic digestion are necessary to prevent the prevalence of veterinary antibiotics in the environment and mitigate the anthropogenic environmental problems.

In this regard, this study aimed to evaluate the fate and long-term effects of chlortetracycline on the AD of swine manure (SM). Chlortetracycline (CTC) was selected as representative veterinary antibiotics due to its highest usage, excretion rate, and microbial toxicity. Specific objectives were to predict environmental concentrations of chlortetracycline in swine manure and soil for evaluation of the ecological risk of the chlortetracycline, to evaluate the transformation of chlortetracycline in anaerobic digestion treating swine manure by developing a

mathematical model, and to elucidate long-term effects of chlortetracycline on the performance and microbial community of the anaerobic digestion of swine manure.

In order to predict concentrations of CTC generated from slurry pit and evaluate its ecological risk in soil, slurry pit farm practices and their uncertainties were considered based on the European Medicine Agency guidelines. Sensitivity analysis was conducted on the exposure estimation of CTC in soil employing the Monte Carlo simulation. Transformation of CTC in swine manure and anaerobic digestate was investigated in batch test. Mass balance-based ordinary differential equations were established by assuming their reversible and irreversible 1st order kinetics reactions. Simulation was conducted to predict CTC and transformation products in CSTR treating swine manure by using estimated kinetic constants and the estimated concentrations of CTC and transformation in swine manure from slurry pit. Two continuous-stirred tank reactors treating SM w/ and w/o CTC spiking (3 mg/L) were operated for 900 days to assess the long-term effects of CTC on the anaerobic digestion. The test concentration was determined based on the predicted environmental concentration of CTC in swine manure from slurry pit. Performance amd stability parameters such as methane generation, organic removal, volatile fatty acid concentrations, ammonia concentration, alkalinity, etc. were monitored. Microbial diversity and community were evaluated to elucidate the long-term inhibition.

The predicted environmental concentrations of CTC in the slurry pit and soil were in a range of 0.54 - 5.64 mg/kg$_{manure}$ and 3.42 - 67.59 μg/kg$_{soil}$, respectively,

for a 90% confidence level. The predicted ranges included the measured values reported in previous studies. The probability of risk quotient was estimated at 9.3% based on the Monte Carlo simulation. If anaerobic digestion is applied before fertilization/composing, the probability of ecological risk is significantly reduced to 0.6 %. The four most influential factors on the exposure to CTC in soil were identified as nitrogen in fertilizer/compost, daily cleaning water usage, the ratio of sick pigs requiring antibiotics, and pit emptying cycles.

In the swine manure and its anaerobic digestate, epimer, isomer, and epi-isomer of CTC were transformed from the CTC. They accounted for 60 - 93 % (w/w) of the residual total CTCs. The CTC is expected to be transported by solid-phase of anaerobic digestate (> 70 % by wt.) while most of ECTC, EICTC, and ICTC remained in the liquid phase (> 60 % by wt.). A series of ordinary differential equations based on the 1st order kinetics demonstrated the kinetics of degradation and transformation in the swine manure and its anaerobic digestate. The simulation included observed concentration of CTC from full-scale biogas plants treating swine manure.

At around day 300 after the CSTR operation, total chemical oxygen demand reduction and methane generation decreased in the test reactor due to the reduced methane generation rate and mineralization ratio of the SM. The methane generation was not recovered during 300 days even after the CTC exposure was stopped. The inhibition is caused by a reduction of microbial diversity and change of the microbial community to an inefficient state in terms of the AD performance.

The concentration of CTC in slurry pit and manure compost-applied soil can be efficiently estimated by considering uncertainties of field conditions. The ecological risk of CTC in the soil is probable and affected by slurry pit farm practices. It is difficult to reduce the risk under 5% by controlling the farm practices. AD of swine manure is recommended not only for organic waste treatment but also for the ecological risk reduction under 0.5%. Transformation of CTC during the AD implies that the environmental risk based on the concentration of CTC can be underestimated due to unnoticed reversible transformation from ECTC to CTC and the expression of ARGs by ICTC. The suggested model can be utilized for the prediction of the concentrations of CTC and the transformation products in a CSTR treating swine manure. Batch test for estimating transformation kinetic constants is recommended to be conducted without degradation until the equilibrium achieved. It is hard to predict the inhibition by using physicochemical indicators of the AD system and recover from the inhibition within 300 days. Continuous exposure to CTC for at least 100 days needs to be avoided for the sustainable management of AD plants treating SM.

Keywords: Anaerobic digestion; Fate; Livestock manure; Long-term effect; Veterinary antibiotics

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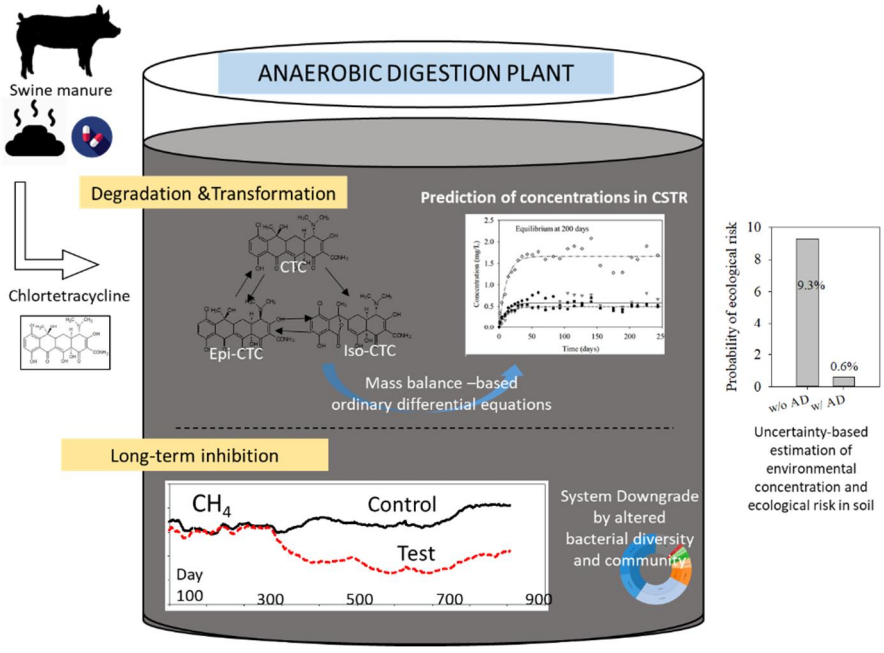
Graphical Abstract



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CHAPTER 1
INTRODUCTION

1.1 Background

In 1948, Thomas Jukes, who was a researcher of a pharmaceutical company Lederle, announced for the first time that a veterinary antibiotic can promote the growth of chicken. The antibiotics was chlortetracycline produced from *Streptomyces aureofaciens*. Since pharmaceutical companies in the United States and Europe recognized the growth promotion effect is valid not only for chicken but also for swine and cattle, many types of veterinary antibiotics such as oxytetracycline, streptomycin, bambermycin, efrotomycin were utilized for the purpose. The utilization of veterinary antibiotics for growth promotion enabled a factory farming system called as concentrated animal feeding operation (CAFO). Livestock can be raised in a feeding environment designed to reduce unit cost for meat production and convenience of the breeding without consideration on genetic diversity, hygiene, and their nature. Under this circumstance, animals are stressed out and prone to disease because of weakened immune systems. The veterinary antibiotics could prevent the problems and boost meat production at the same time. As a result, the production of veterinary antibiotics increased drastically. The consumption of antibiotics for livestock increased from only hundreds of tons in 1950 to 63,200 tons in 2010. This is twice the amount of antibiotics used for humans every year.

Veterinary antibiotics are excreted with the livestock manure after the administration. The overuse of veterinary antibiotics has led to continuous contamination of the aquatic and terrestrial environment via livestock manure. Since most livestock manure is utilized for soil fertilizer, the soil is contaminated with veterinary antibiotics. In case of rain events, the veterinary antibiotics in the soil can be flowed into the surface and groundwater by runoff. Finally, the veterinary antibiotics in the environment caused many problems such as microbial toxicity, prevalence of antibiotic-resistant genes and bacteria (ARGs and ARB), emergence of super bacteria, and exposure of veterinary antibiotics to humans (Bruhn, 2003; Chopra, I. and Roberts, 2001; Kümmerer, 2009; Phillips et al., 2004; Sarmah et al., 2006; Van Boeckel et al., 2015; Xie et al., 2010). For these reasons, E.U. members banned the use of veterinary antibiotics for growth production in 2006 based on the precautionary principle. However, the largest consumers of antibiotics such as the United States, Brazil, and China still allow the use for the livestock industry by providing voluntary guidelines. The increase rate of usage is highest in developing countries where the consumption of meat increases with the increase of population. The usage of veterinary antibiotics was projected to increase to 105,600 tons by 2030 (Van Boeckel et al., 2015).

Anaerobic digestion (AD) is an engineered biological process for treating organic wastes such as sewage sludge, livestock manure, and food waste. Since anaerobes utilize organic wastes as carbon sources and produce methane and carbon dioxide as final products in the process, AD has been getting more attention as a

sustainable approach to managing organic wastes in recent decades. At the same time, there has been a growing concern that AD systems treating livestock manure will be the reservoirs supplying the veterinary antibiotics to the environment because AD is primarily applied before composting and fertilization. During the AD of livestock manure, the veterinary antibiotics are degraded, adsorbed, and transformed into their isomers. The fate of veterinary antibiotics in AD will change and affect the environmental concentration and hazardous in the environmental media. In addition, even after excretion with livestock manure, veterinary antibiotics maintain their antimicrobial activity. Since the AD process is a series of microbial metabolizations, the antimicrobial effect can undermine the performance of the AD system (i.e. reduction of organic material and production of methane). Understanding veterinary antibiotics' fate and effects in anaerobic digestion is a prerequisite step to prevent, mitigate and manage the prevalence of tetracyclines in the environment.

1.2 Research scope and objectives

In this thesis, swine manure was chosen for the target livestock manure. Chlortetracycline was selected as representative tetracycline antibiotics. Due to the highest usage, excretion rate, and microbial toxicity, it has been considered to have the worst negative impact on the environment (Daghrir and Drogui, 2013; Kim et al., 2011; Park and Choi, 2008).

A primary objective of this study is to evaluate the fate and long-term effects of chlortetracycline on the anaerobic digestion of swine manure.

The specific objectives of this study are:

1) Predicting environmental concentration of chlortetracycline in swine manure and soil for evaluation of the ecological risk of the chlortetracycline
2) Evaluating transformation of chlortetracycline in anaerobic digestion treating swine manure by developing a mathematical model
3) Elucidating long-term effects of chlortetracycline on the performance and microbial community of the anaerobic digestion of swine manure

1.3 Dissertation structure

This dissertation is comprised of 6 chapters (Figure 1.1). As described above, chapter 1 included the background, objectives, and dissertation structure of this dissertation. Literature review on occurrence and transport of veterinary antibiotics to environment, human and environmental risk of veterinary antibiotics, and anaerobic digestion of livestock manure containing antibiotics were described in chapter 2. In chapter 3, chlortetracycline concentrations in swine manure and soil were estimated and its risk probability in soil was assessed by considering uncertainties in farming practices and environmental transport. Chapter 4 demonstrated transformation products of chlortetracycline in swine manure and its anaerobic digestate and a prediction model for the transformation products in the anaerobic digestion. Long-term effects of chlortetracycline on the performance of the anaerobic digestion of swine manure were evaluated and elucidated with the microbial community in chapter 5.

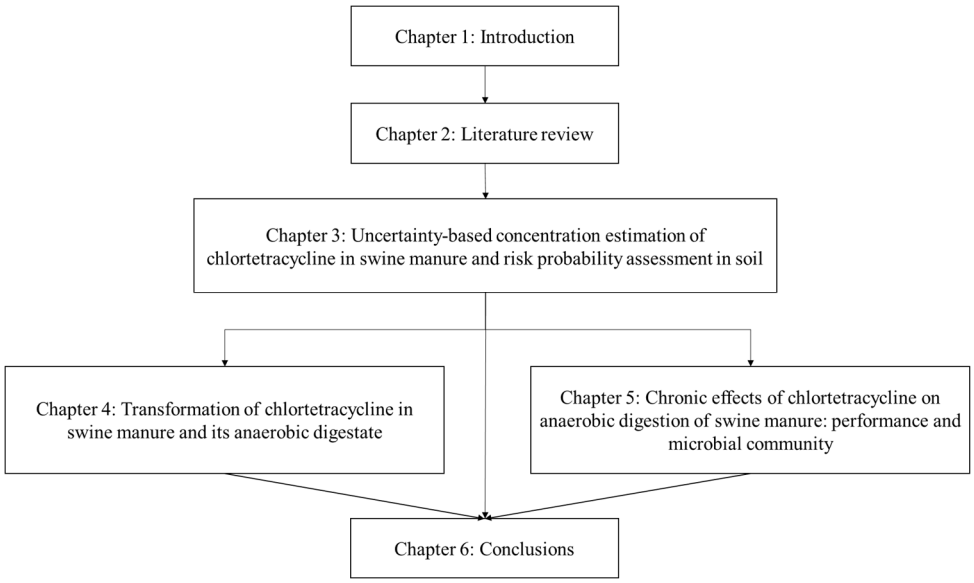

Figure 1. 1 Structure of dissertation

References

Bruhn, S.T., 2003. Pharmaceutical antibiotic compounds in soils: A review. J. Plant Nutr. Soil Sci. 166, 145–167. https://doi.org/10.1002/jpln.200390023

Chopra, I. and Roberts, M., 2001. Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance. Microbiol. Mol. Biol. Rev. 65, 232–260. https://doi.org/10.1128/MMBR.65.2.232

Daghrir, R., Drogui, P., 2013. Tetracycline antibiotics in the environment: A review. Environ. Chem. Lett. 11, 209–227. https://doi.org/10.1007/s10311-013-0404-8

Kim, K.R., Owens, G., Kwon, S.I., So, K.H., Lee, D.B., Ok, Y.S., 2011. Occurrence and environmental fate of veterinary antibiotics in the terrestrial environment. Water. Air. Soil Pollut. 214, 163–174. https://doi.org/10.1007/s11270-010-0412-2

Kümmerer, K., 2009. Antibiotics in the aquatic environment - A review - Part I. Chemosphere 75, 417–434. https://doi.org/10.1016/j.chemosphere.2008.11.086

Park, S., Choi, K., 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology 17, 526–538. https://doi.org/10.1007/s10646-008-0209-x

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C.,

Preston, R., Waddell, J., 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother. 53, 28–52. https://doi.org/10.1093/jac/dkg483

Sarmah, A.K., Meyer, M.T., Boxall, A.B.A., 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65, 725–759. https://doi.org/10.1016/j.chemosphere.2006.03.026

Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A., Laxminarayan, R., 2015. Global trends in antimicrobial use in food animals. Proc. Natl. Acad. Sci. 112, 5649–5654. https://doi.org/10.1073/pnas.1503141112

Xie, X., Zhou, Q., He, Z., Bao, Y., 2010. Physiological and potential genetic toxicity of chlortetracycline as an emerging pollutant in wheat (Triticum aestivum L.). Environ. Toxicol. Chem. 29, 922–928. https://doi.org/10.1002/etc.79

CHAPTER 2
LITERATURE REVIEW

2.1 Occurrence and transport of veterinary antibiotics to environment

2.1.1 Use of veterinary antibiotics in the livestock industry

In 2010, global antibiotic usage was estimated at 63,000 tons (Van Boeckel et al., 2015). It was twice higher than that used for humans (30,000 tons). The largest consumer of antibiotics for food animals was China (15,000 tons). U.S.A, Brazil, Germany, and India followed the heavy veterinary antibiotics consumers in order. China, U.S.A, Brazil, and India were expected to be the largest consumers still in 2030. The global consumption was projected at 105,000 tons. China, U.S.A, and Brazil accounted for 50 % of the future global consumption. Those were expected to be around 33,000, 10,000, 8,000 tons, respectively. This is because they not only produce the most food animals but also consume meats. The increase rate of usage was forecasted to be highest in developing countries such as Indonesia, Myanmar, Nigeria, Peru, and Philippines where consumption of meat increases with the increase of population. By 2030, they were expected to use 2 – 3 times more veterinary antibiotics than that in 2010 (Van Boeckel et al., 2015).

Among legally approved seven antibiotics (i.e. aminoglycosides, cephalosporins, lincosamides, Macrolides, penicillins, sulfas, and tetracyclines) in the U.S.A., tetracyclines were the most sold drug class. The amount was 4,117 tons accounting for 67 % (w/w) of the total sales (Fig. 2-1). 50 % and 42 % of the tetracyclines were sold for swine and cattle, respectively. In the Republic of Korea, 651 tons of veterinary antibiotics were sold for cattle, swine, and chicken. The most sold veterinary antibiotic was penicillin. It was 185 tons and 28 % of the total sales. Tetracycline was the second-most sold veterinary antibiotics (87 tons, 13 % by wt.). Different from the usage pattern of the U.S.A, 72 % of the tetracyclines were purchased for swine while 20 % and 7 % were for chicken and cattle.

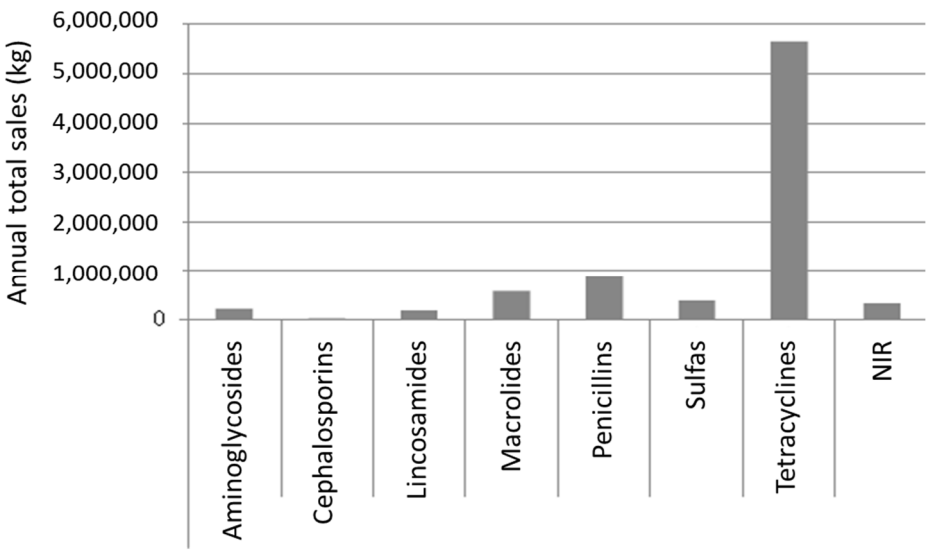

Figure 2.1 Domestic sales of approved 8 veterinary antibiotics (aminoglycosides, cephalosporins, lincosamides, macrolides, penicillins, sulfas, tetracyclines, and not independently reported (NIR)) in U.S.A (FDA, 2020)

2.1.2 Occurrences and transport of veterinary antibiotics to the environment

Administered veterinary antibiotics are not fully metabolized in the animal's body and excreted with manure. The excretion rate is known as 30 to 70 % by wt. for tetracycline (Arikan et al., 2007; Capleton et al., 2006; Dewey et al., 1999). Since most livestock manure is utilized for compost and/or liquid fertilizer, the manure containing the veterinary antibiotics is distributed to the terrestrial environment directly or as compost/fertilizer. In the soil, the veterinary antibiotics can flow into surface water by rain run-off, food chain by absorption to plants, groundwater by leaching. Also, those in the surface water can pass through drinking water treatment plants and finally be included in drinking water (Frade et al., 2014; Kim et al., 2011) (Fig. 2-2).

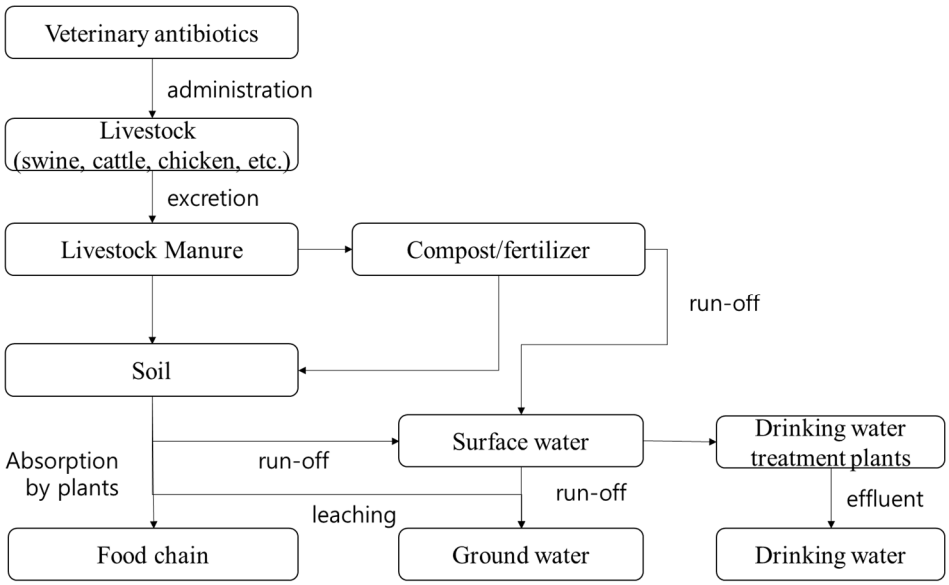

Figure 2.2 Occurrences and transport pathways of human and veterinary antibiotics

2.1.3 Presence of tetracyclines in the environment

Studies analyzing and estimating concentrations of tetracycline have reported a wide range of concentrations in the livestock manure and environment. In this dissertation, the concentrations in livestock manure and terrestrial environments were discussed. Tetracyclines were reported to be in a range of several micrograms per liter to tens of milligrams per liter in the livestock manure (Campagnolo et al., 2002). The concentration varied by sampling points, types of livestock manure, and storage periods in biosolids (Chenxi et al., 2008). When chlortetracycline was orally administered to cattle according to standard for the usage, the cattle manure contained 10 – 12 mg/L of chlortetracycline (Arikan, 2008; Ince et al., 2013). The concentration in livestock manure was estimated to be 1.5 mg/L in the Republic of Korea and 6 mg/L in the U.S.A by considering the usage, excretion rate, and manure production (Huang et al., 2011; Kwon and Kim, 2015). Swine manure is gathered into a slurry pit which is located under the swine farm's floor. In the slurry pit, the concentrations of tetracyclines were measured as a maximum of 56 mg/kg_{maure} (Carballo et al., 2016; Martínez-Carballo et al., 2007; Zhang et al., 2015; Zhao et al., 2010; Zhou et al., 2013). Weighted average concentration considering the number of measured samples corresponded to 2.12 mg/kg_{maure}.

In the soil, 7 – 199 $\mu g/kg_{soil}$ of tetracyclines have been reported (Aust et al., 2008; Halling-Sørensen et al., 1998; Hamscher et al., 2002). It should be noted out that the results were only cases showing the presence of tetracyclines in the soil and

tetracyclines were under detection limits in many cases. Many factors affect the residual tetracyclines in the soil. For example, the amount of fertilizer/compost, their frequency of distribution, rain events, sunlight, and characteristics of soil can affect the degree of transport and reactions such as degradation and sorption.

Table 2.1 Measured concentrations of chlortetracycline, oxytetracycline, and tetracycline in manure from the slurry pit

Literatures	Types of TC (CTC, OTC, TC)	Average (mg/kg_{manure})	Minimum – Maximum (mg/kg_{manure})	Number of samples	Note
Carballo et al., 2016	CTC	0.08 ± 0.19	0.00 - 0.56	8	a farm with 2,000 - 3,000 pigs in Spain
	OTC	0.28 ± 0.29	0.00 - 0.76	8	
Zhao et al., 2010	CTC	1.15 ± 4.43	0.16 - 21.06	26	31 farms with 500 - 5,000 pigs in China
	OTC	2.69 ± 17.00	0.15 - 59.06	26	
Li et al., 2013	TC	5.29	0.32 - 30.55	18	18 farms with 100 - 10,000 pigs in China
	OTC	11.81	0.73 - 56.81	18	
	CTC	3.19	0.68 - 22.34	18	
Zhang et al., 2015	TC	0.60	0.02 - 31.00	7	9 farms in China
	OTC	1.10	0.02 - 43.43	7	
	CTC	1.04	0.02 - 215	7	
Martínez-Carballo et al., 2007	CTC	-	0.10 - 46.00	17	Farms with 400 - 1,000 pigs in Austria
	TC	-	0.36 - 23.00	22	
Weighted Average		2.12			

2.1.4 Chemistry of tetracycline antibiotics

Tetracycline has a chemical structure in which various functional groups are attached with four linearly connected carbon rings as a body. It is classified into different types of tetracyclines according to the substitution of functional groups. In order to pose antibiotic activity, it is necessary to maintain a linearly connected carbon ring, a three-dimensional arrangement of 4a, 12a (A-B ring connection) positions, and a keto-enol system at positions 11, 12, and 12a (Chopra, I. and Roberts, 2001; Daghrir and Drogui, 2013). The tetracyclines inhibit metabolism concerning protein synthesis by binding to ribosomal acceptor (A) site and prohibiting aminoacyl-tRNA from attached to the site The chlortetracycline used in this study is one of the tetracycline families in which a hydrogen at carbon no. 7 (C7) is substituted with a chlorine ion (Cl) (Fig. 2.3). If the hydrogen is substituted with hydroxide, it is oxytetracycline.

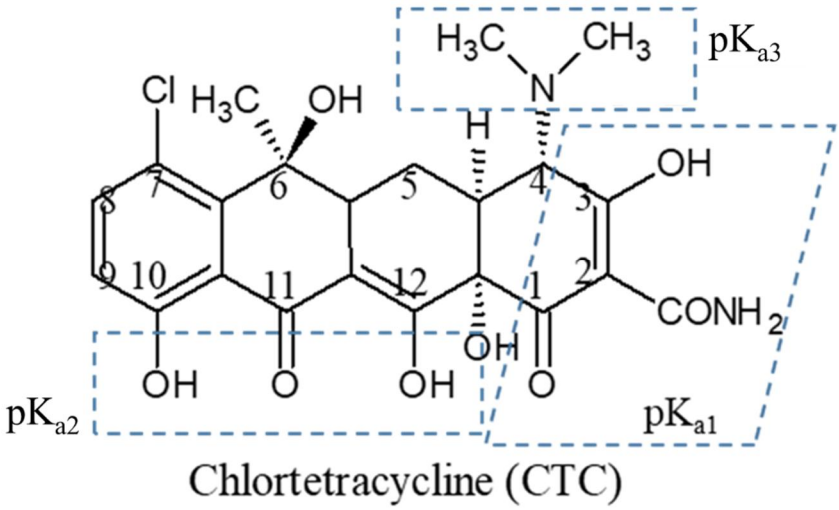

Figure 2.1 Chemical structure of chlortetracycline

The molecular formula and chemical properties of tetracyclines, oxytetracycline, and chlortetracycline were listed in Table 2-2. The tetracyclines have amphiphilic characteristic (i.e. hydrophilic and hydrophobic), but more hydrophilic (solubility: 0.008 to 0.062 mol/L; octanol-water distribution coefficient ($\log K_{OW}$): -1.25 to -1.12). Those have extremely low Henry's constant (3.35×10^{-24} to 3.91×10^{-26} atm*m^3/mol) indicating that rarely volatilized from liquid and solid-state. Due to the three ionizable functional groups at C1-3, C4, and C10-12, those have three pKa values. Thus, those represent various net charges in aqueous conditions. Under pH 3, monovalent cation governs. A zwitterion is the most common chemical form at neutral pH. Over pH 8, their net charge poses mono- and di-valent anionic characteristics (Chopra and Roberts, 2001; Daghrir and Drogui, 2013; Gaugain et al., 2015).

In the environment, tetracyclines are electrostatically stabilized by chelating with divalent cations such as Mg^{2+}, Cu^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, and $Co2^{+}$. Metal chelation means that the positive electric charge of metal ion combines with two or more negative charges of electron donor (i.e. tetracyclines) by forming separate covalent bonds. The chelation occurs at the tricarbonylamide group of C1-3, dimethylamino group of C4, phenolic diketone group of C10-12 (Fig. 2.3).

Table 2.2 Chemical properties of tetracyclines (mofified from Daghrir and Drogui (2013))

Molecule	Molecular formula	Molecular weight (g/mol)	Solubility (mol/L)	Log K_{OW}	Henry's law constant (atm* m^3/mol)	pK_{a1}	pK_{a2}	pK_{a3}
Chlortetracycline	$C_{22}H_{24}C_{l2}N_2O_8$	478.8	0.008	-0.62	3.45E-24	3.3 ± 0.3	7.6 ± 0.0	9.3 ± 0.3
Tetracycline	$C_{22}H_{25}C_lN_2O_8$	480.9	0.041	-1.25	-	3.2 ± 0.3	7.8 ± 0.1	9.6 ± 0.3
Oxytetracycline	$C_{22}H_{25}C_lN_2O_9$	496.9	0.062	-1.12	3.91E-26	3.2 ± 0.3	7.5 ± 0.0	8.9 ± 0.3

2.2 Environmental toxicity and risk of tetracycline antibiotics

Various types of toxicity tests have been performed for the tetracycline antibiotics (Table 2-3). Tetracyclines exhibited comparable or higher toxicity than other veterinary antibiotics. Acute toxicity of tetracyclines for *L. gibba* (7 days, ASTM G-3. E1415-91) represented that 10% effective concentration (EC_{10}) of chlortetracycline was 0.069 mg/L and half-maximal effective concentration (EC_{50}) of oxytetracycline was 1.152 mg/L. The EC_{10} of amoxicillin and EC_{50} of sulfathiazole, which are the major veterinary antibiotics, were 1.000 mg/L and 3.552 mg/L, respectively (Brain et al., 2004). For the V. *fischeri*, D. *magna*, M. *macrocopa*, and O. *latipes* (48 h, US EPA-821-R-02-012), the EC_{50} of chlortetracycline and oxytetracycline were 0.05 and 0.17 mg/L (Park and Choi, 2008). Those of sulfamethoxazole, sulfathiazole, and amoxicillin were 0.03, 0.1, and 0.0037 mg/L, respectively. Epimer and isomer of tetracyclines showed less toxicity than that of parent compounds while anhydrate-form products exhibited comparable or higher toxicity depending on target organisms (Halling-Sørensen et al., 2002). Chlortetracycline can inhibit the growth of plants in terms of germination, mitosis, chromosomal aberration, and sister chromatid exchange (Xie et al., 2010). Its minimum inhibitory concentrations (> 5 mg/L) were much higher than the measured and predicted environmental concentrations implying that the probability of inhibition would be quite low.

Based on environmental concentrations and toxicity of tetracyclines, their environmental risk has been assessed in various ways. Tetracyclines likely pose risk in the terrestrial environment. Menz et al. (2015) prioritized potential environmental risks of 15 veterinary antibiotics in Germany by using usage pattern-based exposure screening. For the average and worst scenario, the concentrations of tetracycline and chlortetracycline were expected to represent the highest concentrations in soil. Oxytetracycline was located in the middle group. Similarly, Park and Choi, (2008) evaluated the hazard of 11 types of veterinary antibiotics on aquatic environments by assessing their acute toxicities and using their reported environmental concentrations. Hazard quotients were calculated according to the ratio of measured environmental concentrations to predicted no effective concentrations (PNEC). Chlortetracycline was expected as the highest hazard compound (hazard quotients = 13.8). Oxytetracycline was also expected to show hazardousness in the aquatic environment (hazard quotients = 2). Among the test antibiotics, sulfamethoxazole represented a similar hazard quotient with that of chlortetracycline (hazard quotients = 13.4). The others showed less hazardousness, which was in a range of 0.012 – 1.62.

Table 2.3 Toxicity of tetracycline and their epimers and isomers

Targets	Methods	Criteria	Results	References
Activated sludge bacteria	Viable plat counting (48 h, ISO 15522)	EC_{50}[a] (mg/L)	TC = 0.08; ATC = 0.03; ETC = 0.14; CTC = 0.03; ACTC = 0.24; ICTC = 36.3; ECTC = 1.65; EACTC =0.25; OTC = 0.08	Halling-Sørensen et al., 2002
Soil bacteria (17 Gram-negative strains and 2 Gram-positive strains)	Viable plat counting (48 h, ISO 15522)	MIC[b] (mg/L)	For 15 strains of sensitive Pseudomonads, TC = 2.0; ATC = 12.0; ETC = 12.0; CTC = 0.5; ACTC = 32.0; ICTC = 32.0; ECTC = 16.0; OTC = 1.0 For *Agrobacterium* sp., *Moraxella* sp. and *Bacillus* sp. TC = 0.25; ATC = 0.25; ETC = 0.5; CTC = 0.25; ACTC = 0.25; ICTC = > 32.0; ECTC = 0.75; OTC = 0.25	Halling-Sørensen et al., 2002
V. *fischeri*, D. *magna*, M. *macrocopa*, O. *latipes*	48 h, US EPA-821-R-02-012	EC_{50} /LOEC[c]	Sulfamethoxazole = 0.03; Sulfathiazole = 0.1; Amoxicillin = 0.0037; Ampicillin = 163; CTC = 0.05; OTC = 0.17	Park and Choi, 2008
L. *gibba*	7 d, ASTM (G-3. E1415-91)	EC_{50} (mg/L)	EC50 for Sulfathiazole = 3.552 and OTC = 1.152; EC10 for CTC = 0.069 and amoxicillin = > 1.000	Brain et al., 2004

Wheat (*Tritium aestivum* L.)	Germination, Miotic activity, chromosomal aberration assay, and sister chromatid exchange (24, 48, 72 h)	MIC (mg/L)	For CTC, Germination: 25 mg/L; Mitotic index: 25 mg/L; Chomosomal aberration (CA) frequency: 10 mg/L; sister chromatid exchange: 5 mg/L	Xie et al., 2010
Chlorella vulgaris	Algal growth	EC_{50} (mg/L)	TC = 7.73; ATC = 5.96; ETC = 8.42	Xu et al., 2019
Chlorella vulgaris	Cell permeability	MIC (mg/L)	TC = 5; ATC = 10; ETC = 10	Xu et al., 2019

[a] Effective concentration; [b] Minimum inhibitory concentration; [c] Lowest observed effective concentration

2.3 Anaerobic digestion of livestock manure containing antibiotics

2.3.1 Process of anaerobic digestion

Anaerobic digestion is a series of metabolizations during which anaerobes use organic matter as an electric donor and acceptor. The organic matter is converted into methane, carbon dioxide, and water through hydrolysis, acidogenesis, acetogenesis, and methanogenesis. In the industrial and environmental area, anaerobic digestion is utilized primarily for reducing biodegradable organic matter such as food waste, livestock manure, and sewage sludge, and secondarily for producing profitable organic acid, and biogas (Carlsson et al., 2012; Carrere et al., 2016; Chernicharo et al., 2015; Curry and Pillay, 2012; Rajagopal et al., 2013a). Figure 2.4 shows the main microbial groups involved in hydrolysis, acidogenesis, acetogenesis, and methanogenesis and their substrates.

During the hydrolysis process, particulate organic matter such as carbohydrates, proteins, and fats are decomposed into water-soluble compounds such as polysaccharides, amino acids, and fatty acids, and dimers. The hydrolytic bacteria cause hydrolysis on the surface of particulate organic matter by producing extracellular amylases, proteases, and lipases. The hydrolysis of particulate matter can occur by physicochemical reactions. Hydrolysis of a non-decomposable polymer

material such as cellulose can be a late-limiting step for the AD. In acidogenesis, acidogenic bacteria convert the soluble organic materials into organic acids (e.g. formic, acetic, propionic, and butyric acid), alcohol (methanol, ethanol), aldehyde, carbon dioxide, and hydrogen. Amino acids and peptides, which are energy sources of anaerobic microorganisms, can be produced through protein decomposition. acidogenic microbes generally consist of facultative bacteria and obligatory bacteria. Hydrogen and acetic acid are generated through the decomposition of the soluble organic matter by fermentative bacteria and decomposition of the organic acid by acetogenic bacteria in the process of acetogenesis. Since about 70 % (based on the unit electron) of methane is produced by the conversion of acetic acid, acetogenesis is the most important step in determining the amount of biogas. The process of methane generation (methanogenesis) is a process in which acetic acid, hydrogen, carbon dioxide, methanol, and methylamine are converted into methane and carbon dioxide by methanogenic bacteria and archaea. About 70% of methane is generated from acetic acid (acetotrophic methanogenesis), and about 30% of methane is generated from hydrogen and carbon dioxide (hydrogenotrophic methanogenesis). Although the proportion of methane generation by the hydrogenotrophic methanogenesis is smaller than that by acetotrophic methanogenesis, the hydrogenotrophic methanogenesis is necessary for maintaining the activity and growth of acidogenic bacteria (Khadaroo et al., 2019; Okolie, Jude and Adekunle, Kayode, 2015; Regueiro et al., 2012).

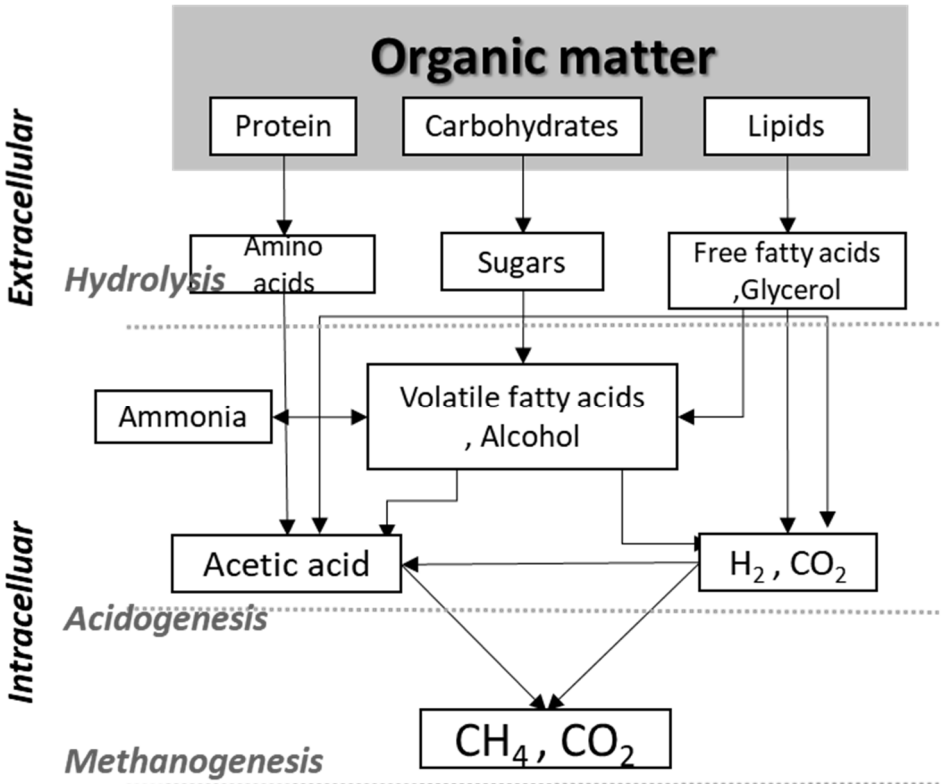

Figure 2.2 Process of anaerobic digestion: hydrolysis, acidogenesis, and methanogenesis

2.3.2 Fate of tetracyclines during anaerobic digestion

In environmental conditions, reactions such as degradation, adsorption, photolysis, and transformation have been known to affect the fate of tetracyclines (Cycoń et al., 2019; Daghrir and Drogui, 2013; Kümmerer, 2009). Degradation and adsorption were considered major reactions to the fate of tetracyclines during AD. Recently, a transformation of tetracyclines to epimers and isomers has been getting attention because they still pose antimicrobial activities of tetracyclines. Photolysis was not considered a reaction that occurred in the anaerobic digestion. Since the anaerobes grow well in dark condition, AD plants are designed to be in dark condition. In this chapter, degradation, adsorption, and transformation of tetracyclines were reviewed. As a result, it could be found that there remained several ambiguities in the literature regarding the transformation of tetracyclines. Transformation products and their kinetics in the livestock manure and anaerobic digestion were little documented. The identification of transformation products and kinetics are necessary for the accurate estimation of environmental exposure concentrations.

2.3.2.1 Degradation

Degradation of tetracyclines represents a conversion of a tetracycline compound into lower molecular weight of compounds. Most of the present studies have evaluated the degradation based on the decrease of parent compound's concentrations (Stone et al., 2009; Wu et al., 2009). Some papers distinguished the concentration decrease with adsorption (Kim et al., 2005; Li and Zhang, 2013; Wu et al., 2009); however, the decrease of concentration by transformation products has rarely been considered. Since epimers and isomers have the same molecular weight as their parent compound, transformation should not be considered as the degradation (Table 2.4). Álvarez et al. (2010), Arikan, (2008), and Arikan et al. (2006) evaluated the concentration of epimer and isomer with chlortetracycline and oxytetracycline. By considering the sum of concentrations, they assessed that 30 – 60 % of the initial amount of the parent compounds were changed to the transformation products not degraded to lower molecular weight compounds. Based on the sum of concentrations, the degradation kinetics of total concentration could be interpreted by using the 1st order kinetics. The degradation kinetics include the transformation kinetics of each compound. If the epimerization and isomerization can be distinguished, the degradation constants of each compound will be estimated and prediction of the parent compound, its epimer, and isomer will be possible.

Table 2.4 Summary of biodegradation test for tetracyclines in previous studies

Matrix (TS % by wet wt.)	Experimental conditions	Observed 1st order degradation coefficients (day^{-1})	Note	References
Cattle manure (4%)	– Batch, 35°C – 2 and 12 mg-CTC/L	0.016 (2 mg-CTC/L) 0.038 (12 mg-CTC/L)	– Buffer-extractable concentration – Based on the sum of CTC, ECTC, and ICTC	Arikan, 2008
Cattle manure (5%)	– Batch, anaerobic, 35°C, 60 days – 10 - 60 mg-OTC/L	0.012	– Buffer extractable concentration – OTC, and isomers	Arikan et al., 2006
Aerobically digested biosolid	– Dark, Aerobic, 20 - 25°C – 0.1 mg-TC/L	0.086	– No concentration changes after partial degradation – No consideration on isomers	Wu et al., 2009
Swine manure (3.3 %)	– Batch, anaerobic, 10 – 20°C 216 days – 28 mg-CTC/L	0.012	– Buffer extractable concentration – No concentration changes after partial degradation – No consideration on isomers	Stone et al., 2009

Swine manure (3 %)	– Batch, anaerobic, 10 – 20°C 216 days – 0, 20, 100, 200 mg/L (OTC:CTC = 1:1)	0.184, 0.216, 0.169 (CTC) 0.052, 0.045, 0.058 (OTC)	– No concentration changes after partial degradation in the 100, 200 mg/L assays – Based on sum of isomers	Álvarez et al., 2010
Cattle manure (5 %)	– Batch, anaerobic 35°C – 50 - 200 mg-OTC/L	0.013 – 0.033	– Buffer extractable concentration – OTC, isomers	Coban et al., 2016

2.3.2.2 Adsorption

The adsorption of tetracyclines can be elucidated by two electrostatic mechanisms (C. Lee et al., 2020a; Pollard and Morra, 2017). Those are cation bridging and cation exchange. The cation bridging occurs by polyvalent cations which connect negatively charged surfaces of livestock manure and tetracyclines. Cation exchange is caused by the substitution of monovalent cations (e.g., Na^+, K^+) bonded to the negative charge of livestock surface to a positively charged functional group (i.e. amine group) of tetracyclines (Fig. 2.5). Change of pH affects electrostatic adsorption. Since tetracyclines have the three functional groups and corresponding three pKa, the charges of each functional group vary depending on the pH (Lü et al., 2012). According to Lee et al. (2020), the linear distribution coefficients of chlortetracycline in the cattle manure decreased with the increase of pH because the proportion of negatively charged chlortetracycline linearly increased with the pH increased in a range of pH 7 – 8.

Existing studies have indicated that the linear distribution coefficients of tetracyclines for biosolids such as livestock manure, sewage sludge, and anaerobic digestate were in a range of 2 – 79 L/g. It means that more than 90 % (w/w) of tetracycline remains in solid-phase when assuming 50 g/L of livestock manure (5 % of total solid by wet wt.). As like degradation reaction, most studies evaluated the degree of adsorption according to the decrease of parent compound's concentration and did not consider the transformation. In this case, the degree of adsorption might

be overestimated.

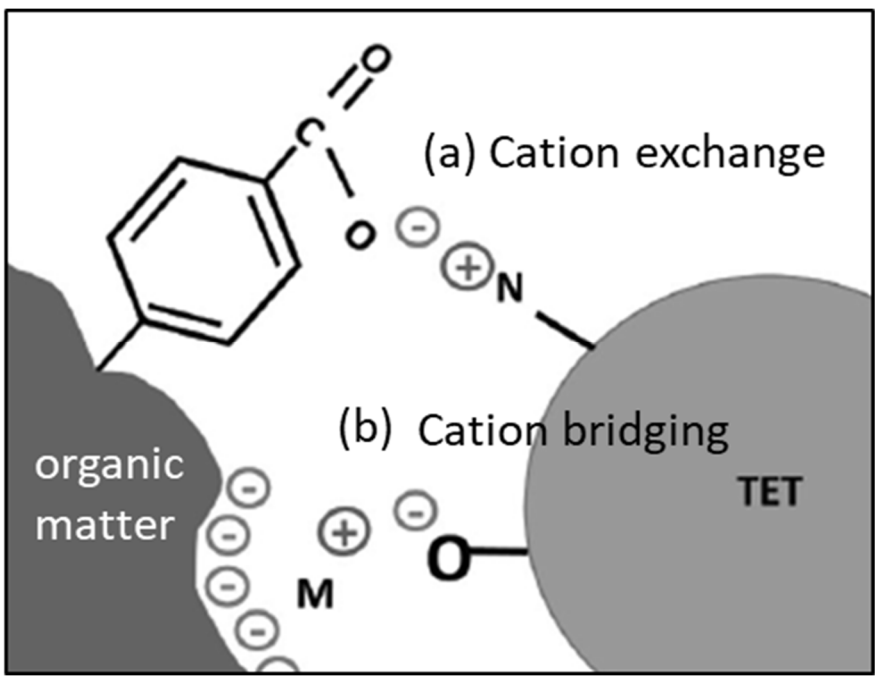

Figure 2.5 Sorption mechanisms of tetracyclines on organic matter; (a) cation exchange, (b) cation bridging (modified from Pollard and Morra, 2017)

Table 2.5 Summary of sorption tests for tetracycline in previous studies

Sorbents	Experimental conditions	Linear distribution coefficients (L/g)	Notes	References
Activated sludge	– Dark, Aerobic, 25 ℃ – Initial concentration: 0.1 mg-TC/L	4 – 8	– Hydrolysis, volatilization by aeration: not happened – Divalent cation concentration ↑, K_D↓ – No change of sorption capacity in neutral pH – No isomer analysis	Li and Zhang, 2013
Activated sludge	– Batch, pH 7.2 by phosphate buffer – 1.0, 3.6 g-solid/L	8.4, 22.6	– Hysteresis on protein s and metal on activated sludge – No isomer analysis	Kim et al., 2005
Swine manure	– Dark, Aerobic, 20 ℃ – Initial concentration: 0.1 - 6 mg-TC/L	63 - 79	– K_{oc} estimated from K_{ow} << K_{oc} estimated from actual K_D – sorption by hydrophobicity and by metal complexation, hydrogen bonding – No isomer analysis	Loke et al., 2002
Aerobically digested biosolid	– Dark, Aerobic, 23 ℃ – 0.1 - 1 mg-TC/L,	7.3	– Kinetic: Freundlich, linear – No isomer analysis	Wu et al., 2009

	1 g-solid/L				
Dried anaerobic digestate	– 12.5 g-solid /L, pH 6, 32 °C – 0.2 – 4 mg-TC/L, 48 h	2 – 8.7	– Exponential relationship between K_D and concentration of Al^{3+} and Fe^{3+}		D'Angelo, 2017
Sludge-derived sorbent	– 1 g-solid/L, 0.7 g-TC/L	Langmuir capacity: 512 – 672 mg/g	– Kinetic: Pseudo first order		Ocampo-Pérez et al., 2012

2.3.2.3 Transformation

TC can exist as various transformation products by undergoing intramolecular changes. There are 12 types of transformation products with five chiral centers. Since Doerschuk et al. (1955) first discovered the existence of an epimer of TC in 1995, pharmacologists have mainly considered epimers, isomers, epi-isomers, and anhydrous products as the transformation production focusing on the changes in aqueous conditions (Doerschuk et al., 1955; HUSSAR et al., 1968; McCormick et al., 1957a, 1956; Remmers et al., 1963; Sokoloski et al., 1977; Yuen and Sokoloski, 1977). 4-Epi-tetracycline (ETC) is a diastereomer produced by the reversible change in bonding angle of the C4 dimethyl amino group. Cleavage of the C6 hydroxyl group irreversibly leads to the formation of iso-tetracycline (ITC) which is a structural isomer (Huang and Chen, 2009). 4-Epi-iso-tetracycline is the epimer form of ITC (Fig. 2.6). Anhydrous tetracycline (ATC) is formed when H_2O is removed from tetracycline (McCormick et al., 1957b). These transformation products maintain antimicrobial activities of the parent compound (i.e. tetracycline). ETC can reversibly be transformed into TC in mild acidic conditions (pH 4 – 6) (Zhao et al., 2020a). The European Union set maximum residual limits in food-producing animals for chlortetracycline (CTC) and 4-epi-chlortetracycline (Kennedy et al., 1998). ITC can bind to Tet Repressor, which regulates the expression of the cell membrane to efflux tetracycline resistance, and cause expression of ARGs (Volkers et al., 2011). ATC showed toxicity equal to or less than that of TC (visible

plate counting, 48 h) (Halling-Sørensen et al., 2002). In this regard, there has recently been a great interest in the transformation products of TC from tissues, processed meat, and environmental media (e.g. manure slurry and terrestrial/aquatic systems) (Álvarez et al., 2010; Arikan, 2008; Blanchflower et al., 1997; Cessna et al., 2020; Chen and Huang, 2011, 2010; Dzomba, 2016; Fritz and Zuo, 2007; Gaugain et al., 2015; Grote et al., 2004; Huang and Chen, 2009; Kennedy et al., 1998; Kennedy, 2008; Loftin et al., 2008; Qiao et al., 2012; Shelver and Varel, 2012; Singh, 2016; Søeborg et al., 2004; Spielmeyer et al., 2016; Zhao et al., 2020b). From the first literature for the discovery of epimer to the recent study on the transformation of tetracycline in the environmental media, studies in which test matrix and relative proportion of TC and its transformation products can be determined were shown in Table 2.6.

Understanding their fate in environmental pathways is a prerequisite step to prevent, mitigate and manage the prevalence of tetracyclines and transformation products. The occurrences of the transformation products in manure, soil, and water matrix were the major research focus, whereas information regarding transformation kinetics and equilibrium is still largely lacking in available literature (Table 2.6). Since the epimerization and isomerization happen simultaneously and affect each other, all transformation products generated in a certain matrix should be identified and interpreted together to figure out transformation kinetics and equilibrium. Transformation products were measured selectively in some studies (Álvarez et al., 2010; Loftin et al., 2008; Shelver and Varel, 2012; Spielmeyer et al., 2016). These

might make it difficult to assess the fate of transformation products of tetracycline. The kinetics of the transformation can be evaluated by ordinary differential equations. Yuen and Sokoloski (1977) suggested an analytical solution for concentrations of TC, ETC, ATC, and Epi-ATC in pH 1.5 of phosphoric acid solution. However, to the best of my knowledge, assessment of transformation kinetics in livestock manure has little been documented.

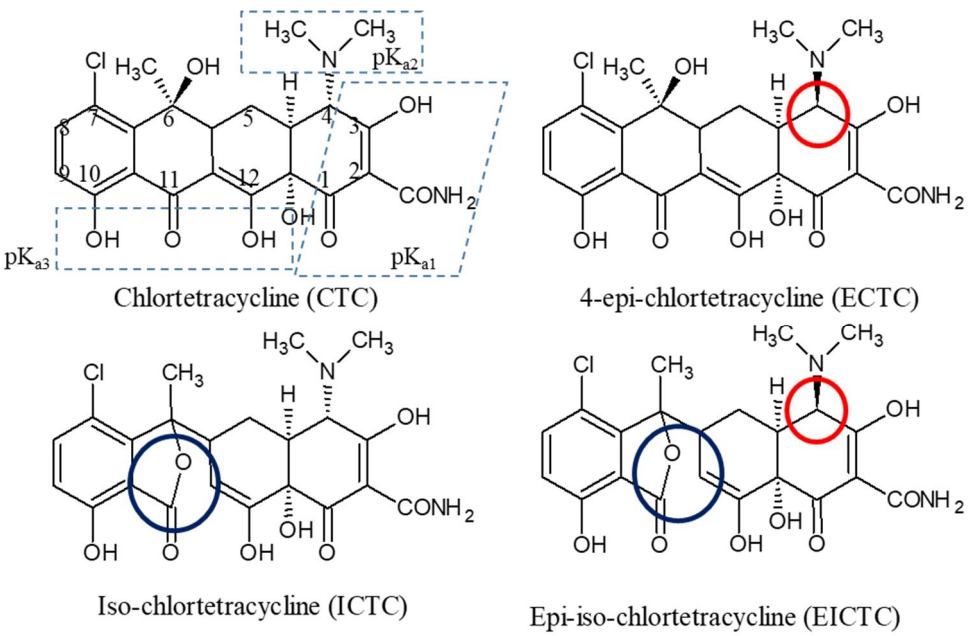

Figure 2.6 Chemical structures of epimer and isomer of chlortetracycline

Table 2.6 Literatures on transformation of tetracycline family in various conditions

Types	Experimental condition	Time	Results (by wt.)	Note	References
TC ETC	25°C; 1 M NaH_2PO_4 in 2:1 methanol-water; pH 4.6	24 h	TC:ETC = 1.5:1	– ETC was first identified	Doerschuk et al., 1955
TC CTC ICTC	Various combinations of Solvent (DI water/Methanol), buffer (NaH_2PO_4/HOAc/N_3PO_4), and temperature	Maximum 114 h	ETC 7 % (pH 3.5 DI water) Epimerization for TC, CTC, ICTC 0 – 60 %		McCormick et al., 1957
TC ETC	(1) 0.01, 0.10, 1.00 M phosphate buffer at pH 4; 23 °C (2) 0.01, 0.10, 1.00 M citrate buffer at pH 4, 23 °C (3) 0.01 M phosphate buffer at pH 4; 4, 23, 37, 42, 56 °C (4) DI water (5) pH 2.4, 3.2, 4.0, 5.0, 6.0 in 0.1 M phosphate buffer at pH 4; 23 °C	8.5 h	ETC 45, 55, 37, 33 and 20 % at pH 2.4, 3.2, 4.0, 5.0, 6.0, respectively (experimental condition (5))	– 1st order epimerization kinetic was first identified and the rate constant was evaluated – As phosphate and citrate buffer concentration increased, epimerization rate constant increased (70 times larger than D.I water condition) – The rates at pH 3 – 5 were the fastest	Remmers et al., 1963

TC CTC	pH 4.0, 5.0, and 6.0 with 0.1 and 1.0 M CH_3COONa For each pH condition, 30, 37, 50 °C were tested, respectively. $CuCl_2$, $CaCl_2$ (metal:TC = 1:5 – 5:1) (0.1 N H_2SO_4 for spectrophotometric assay)		(Part of results) ETC 34% at pH 4 ETC 25% at pH 5 k = 0.04 and 0.33 h^{-1} for 0.1 and 1 M buffer, respectively (pH 4, 37 °C)	– 1st order epimerization rates were evaluated – no effect by Ca – Cu promoted degradation than epimerization and CTC were unstable in solution (Those might be because isomerization was not considered yet)	HUSSAR et al., 1968
TC ETC Anhydro-TC Epi-anhydro TC	pH 1.5 and 1 M phosphate solution at 60, 70, 75, and 80 °C	Maximum 4 h	Anhydro-TC 55 % and Epi-anhydro 45 % at 75 °C	– Analytical solutions for reversible epimerization and irreversible anhydration kinetics of TC were suggested and the rate constants were evaluated	Yuen and Sokoloski, 1977
CTC ECTC ECTC EICTC	Hen's eggs collected on the fifth day of CTC administration from CTC-administrated hen (300 mg CTC per kg of body weight for five days)	-	CTC 15 – 17 % ICTC 44 – 50 % ECTC 5.5 – 6.7 % EICTC 27 – 32 %	– The existence of epi-iso-CTC in the eggs was identified by analyzing HPLC-MS results	Kennedy et al., 1998

CTC ICTC ECTC ACTC EACTC	Matrix: Soil interstitial water and DI water Conditions: pH 3 – 9; Temperature = 6 and 20 °C Light/darkness; aerobic/anaerobic (total 80 combinations for each chemical)	0, 1, 3, 7, 21, 42, and 60 days	(20 °C, dark, anaerobic, DI water half-life (day)) CTC: 20 days at pH 5.6 and 2.5 days at pH 8.5; ICTC: 50 days at pH 5.6 and 100 days at pH 8.5; ECTC: 30 days at pH 5.6 and 5 days at pH 8.5; Anh-CTC: 0.5 day at pH 5.6 and 18 days at pH 8.5	– Comparison of half-lives based on parent compound (w/o consideration on epimerization products) – No effect by aerobic/anaerobic – Light: k_d↑ – Temperature ↑: k_d↑ – k_d in soil matrix > k_d in D.I water	Søeborg et al., 2004

CTC, ICTC, ECTC, anhydro-CTC	Matrix: Manure slurry (urine and feces), blood, swine carcass (Slaughter-plasma, liver, kidney, muscle, and bone), manure Medication: 100 mg-CTC/kg twice daily for 10 days (total 45 g) and 11 days of interruption due to animal welfare regulation	-	In swine carcass and blood (mg/kg) CTC: 46 – 77; ECTC: 19 – 44; ICTC: 2 – 18; EICTC: 0 – 14 In manure slurry (20 days-average) (mg/kg) CTC: 59; ECTC: 29;; ICTC: 12; EICTC: -	–	During 8 months storage of manure slurry, the concentration of CTC and ECTC decreased 80 and 70 % (w/w), respectively, but concentrations of metabolites (ICTC and EICTC) were increased	Grote et al., 2004*
TC, ETC, oxytetracycline (OTC)	Matrix: 14 market milk samples	-	TC: 44 µg/L (one cases) ETC: 18 – 65 µg/L (12 cases) OTC: 13 – 106 µg/L (14 cases)	–	ETC was detected in most cases but TC was detected in only one case (Detection limit = 2 µg/L)	Fritz and Zuo, 2007*
CTC, ECTC, ICTC	Matrix: cattle manure excreted after medication of CTC for 5 days (22 mg/kg body mass) Condition: anaerobic condition	Sampling day: 0, 12, 23, and 33	CTC: 48 – 16 % (w/w) ECTC: 33 – 29 % (w/w) ICTC: 19 –	–	Concentrations of CTC and ECTC decreased while the concentration of ICTC increased during the anaerobic degradation of cattle manure	Arikan, 2008*

		days after finishing medication	54 % (w/w)		
CTC TC	Matrix: 0.015, 0.05, and 0.084 mg/L Na_2HPO_4 Conditions: pH 2, 5, 7, 9, and 11 Temperature: 7, 22, and 35 °C	Every day for 3 weeks	CTC: $k_d > 1.15$ h ($t_{1/2} < 6$ h) at 35 °C and pH 7 TC: $k_d = 0.01$ h ($t_{1/2} = 63$ h) at 35 °C and pH 7	– Pseudo-first order kinetic constants and half-lives based on each parent compound – No effect by ionic strength – Epimers and isomers were not evaluated	Loftin et al., 2008
TC, ETC, OTC, CTC	Matrix: 1.78 g Al_2O_3/L (Aluminum oxide) Conditions: pH 5; Temperature 22 °C; 40 μM of TCs	0, 3, 6, 12, and 24 h	TC: 0.015 h^{-1} ($t_{1/2} = 46$ h) CTC: 0.018 h^{-1} ($t_{1/2} = 39$ h) OTC: 0.006 h^{-1} ($t_{1/2} = 116$ h)	– Pseudo-first order kinetic constants based on each parent compound – Transformation of TCs on the surface of Al_2O_3 was suggested based on a comparison of adsorption and transformation kinetics – The closer pH of a solution is to pH 7, the higher k_d was observed – TC was dehydrated by Al_2O_3, but CTC was isomerized by Al_2O_3	Chen and Huang, 2010

CTC OTC	Matrix: swine manure (0.8 g volatile suspended solids/L) + anaerobic granular biomass (2 g volatile suspended solids /L) Condition: 35 °C and anaerobic; 10, 50, and 200 mg/L of CTC and OTC in the same assay, respectively	0, 7, 14, and 21 day	EOTC 5 – 10 % (w/w) CTC: 53 – 68 % (w/w) ECTC: 32 – 47 % (w/w) k_d of CTC: 0.17 – 0.22 d^{-1} k_d of OTC: 0.045 – 0.058 d^{-1}	– Isomers were not analyzed – Decrease of 40 % OTC and 60 % CTC for the first hour might have been caused by isomerization of each parent compound	Álvarez et al., 2010*
CTC ECTC ICTC	Matrix: Swine manure (6% TS by wet wt.) excreted between 21 and 28 days after initiation of CTC treatment (55 mg/kg body mass) Conditions: 22 °C (pH 6.2 and 7.3), 38 °C (pH 6.1 – 6.3), and 55 °C (pH 6.2 – 6.6)	0, 1, 3, 7, 14, 21, and 25 (or 28) days	CTC: $t_{1/2}$ = 16 h at pH 7 and 22 °C; 6 h at pH 7 and 35 °C Ratio of CTC to ICTC: 0.73 – 0.99 (by w/w)	– Although ECTC rarely existed, the increased amount of ICTC exceeded the decreased amount of CTC detected. It may imply that other metabolites were converted into ICTC	Shelver and Varel, 2012*

TC CTC OTC	Matrix: anaerobic digestate of swine manure, cattle manure, and Maize Conditions: 40 ℃; pH 8.1; 5 mg-TC/kg, 5 mg-OTC/kg, 5, 50, and 200 mg-CTC/kg	0, 1, 3, 7, 14, 21, and 35 (or 42) days	After 35 days, 70, 52, 89 % of TC, OTC, and CTC were decreased, respectively The ratio of CTC to ICTC: 2:8 ~ 1:9 after 21 days	– Regardless of autoclaving, the decrease in CTC concentration was the same. It means that abiotic factors led to the elimination – Transformation products of TC and OTC were not analyzed – ECTC was not analyzed for CTC assay		Spielmeyer et al., 2016*

*When values in graphs were not demonstrated by authors, the values were read by eye based on tick interval

2.3.2 Effects of tetracyclines on anaerobic digestion

The antimicrobial mechanisms can be classified into two types: bactericidal and bacteriostatic (Fig. 2.7). Bactericidal antibiotics inhibit synthesis of the cell wall and kill microorganisms. Amoxicillin and penicillin are bactericidal antibiotics. Bacteriostatic antibiotics impede the synthesis of protein and folate required for the growth of microorganisms. Tetracyclines, sulfonamides, and macrolides are included in bacteriostatic antibiotics.

Existing studies have reported that the anaerobic digestion of organic waste can be inhibited by the presence of veterinary antibiotics (Álvarez et al., 2010; Arikan et al., 2006; Aydin et al., 2015b, 2015a; Carey et al., 2016; Cetecioglu et al., 2013, 2012; Coban et al., 2016; Fountoulakis et al., 2008; Gartiser et al., 2007; Ince et al., 2013; Stone et al., 2009; Tian et al., 2018; Xiong et al., 2017). Tetracyclines inhibited the performance of anaerobic digestion for livestock manure in terms of removal of organic matter and production of biogas generation. The test concentrations, inhibition effects, and experimental conditions were listed according to the types of tetracyclines in Table 2.5. The high concentrations over the environmental concentrations of tetracyclines and short-term experiments to evaluate their inhibition effects have been the major research focus; however, whether the probable concentrations of VAs that may be present in manure affect its AD treatment have gotten less attention. If the microbial community in an AD system is adapted to the bacteriostatic effect, the community will diverge into

different phases compared to that without tetracycline's pressure. In that case, the inhibition effects concerning digestion performances would be different from those observed from high concentrations and short-term exposure.

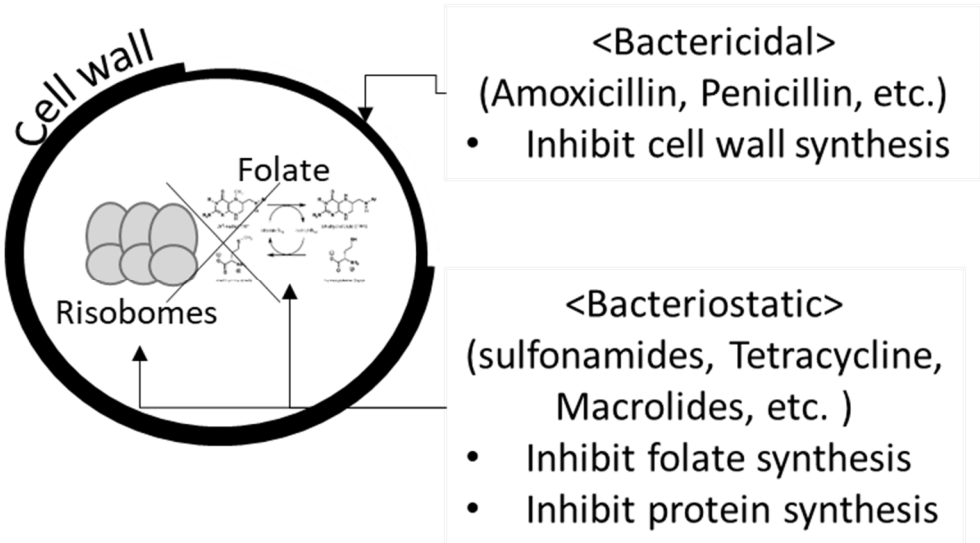

Figure 2.7 Mode of actions of veterinary antibiotics

Table 2.7 Summary of inhibitory effects of tetracyclines on anaerobic digestion

Antibiotics	Test concentration	Effect			Note	References
	mg/L	Reduction of CH_4 production (%) compared to control	COD	VFA		
Chlortetracycline	5	20	N.A.	C2 and C4 < 10 %	Substrate: VFAs, Batch, 12 h	Sanz et al., (1996)
	27 (initial) 11.6 (final)	28	SCOD 33 % ↑	C1 – 4 100 % ↑	Substrate: swine manure Batch, 125 d, TS 33 g/L, 10 – 20 ℃	Stone et al., (2009)
	40	50	N.A.	C2 and C4 < 10%	Substrate: VFAs, Batch, 12 h	Sanz et al., (1996)
	4.9, 10.4, 43.4	10, 20, 50	N.A.	N.A.	ISO/DIS 13641, 100 mg total organic carbon /L, Batch, 60 days, TS 1 g/L	Gartiser et al., (2007)
	10, 50, 100	45, 56, 64	N.A.	N.A.	Substrate: swine manure Batch, VSS 11 g/L, 21 days	Álvarez et al., (2010)
	2 (50 days) – 4 (50 days) mg/L	From 50 to 100 days, 50 %	Increase after 90 days	Accumulation in propionic acid, 200 mg/L	Substrate: saccharose 2,500 mg COD/L Sequencing batch, VSS 6.4 g/L, 100 days	Liu et al., (2021)
	4 (50 days) – 8 (50 days)	From 30 to 100 days, 71 %	Increase after 50	Accumulation in propionic acid,	Substrate: saccharose 2,500 mg COD/L	Liu et al., (2021)

	mg/L		days	600 mg/L	Sequencing batch, VSS 6.4 g/L, 100 days	
Oxytetracycline	10 (initial) 4.37 (final)	27	N.A.	N.A.	Substrate: cattle manure Batch, 65 days, TS 50 g/L	Arikan et al., (2006)
	4 10	60 50	N.A.	N.A.	Substrate: cattle manure Batch, 30 days, TS 5 g/L	Ince et al., (2013)
	125, 250 (estimated)	-	N.A.	N.A.	Substrate: swine manure Batch, 10 days, TS 9.7 g/L	Lallai et al., (2002)
	40 (50 days) – 200 (40 days) – 1,000 (100 days)mg/L	75% by 1,000 mg/L	N.A.	Accumulation 50 to 150 mg/L	Substrate: a mixture of primary and secondary sludge CSTR, SRT 20 days	Tian et al., (2018)
Tetracycline	1.7 (12 days) - 5.7 (23 days) - 8.5 (28 days)	10 % 19 % 62% (failed)	- - Fail	Accumulation in acetate and propionic acid	Substrate: VFAs (4.4 g/L) Sequencing batch (24 h), 160 days	Cetecioglu et al., (2013)
	2.1, 5.6, 37.3	10, 20, 50	N.A.	N.A.	ISO/DIS 13641, 100 mg total organic carbon /L, Batch, 60 days, TS 1 g/L	Gartiser et al., (2007)
	200, 400, 800	7, 80, 85	N.A.	Accumulation 2,000 – 4000 mg/L	Substrate: sewage sludge, corn straw Batch, 10 days, VS 20 g/L, 55 ℃	Hou et al., (2016)

References

Álvarez, J.A., Otero, L., Lema, J.M., Omil, F., 2010. The effect and fate of antibiotics during the anaerobic digestion of pig manure. Bioresour. Technol. 101, 8581–8586. https://doi.org/10.1016/j.biortech.2010.06.075

Arikan, O.A., 2008. Degradation and metabolization of chlortetracycline during the anaerobic digestion of manure from medicated calves. J. Hazard. Mater. 158, 485–490. https://doi.org/10.1016/j.jhazmat.2008.01.096

Arikan, O.A., Sikora, L.J., Mulbry, W., Khan, S.U., Foster, G.D., 2007. Composting rapidly reduces levels of extractable oxytetracycline in manure from therapeutically treated beef calves. Bioresour. Technol. 98, 169–176. https://doi.org/10.1016/j.biortech.2005.10.041

Arikan, O.A., Sikora, L.J., Mulbry, W., Khan, S.U., Rice, C., Foster, G.D., 2006. The fate and effect of oxytetracycline during the anaerobic digestion of manure from therapeutically treated calves. Process Biochem. 41, 1637–1643. https://doi.org/10.1016/j.procbio.2006.03.010

Aust, M.-O., Godlinski, F., Travis, G.R., Hao, X., McAllister, T.A., Leinweber, P., Thiele-Bruhn, S., 2008. Distribution of sulfamethazine, chlortetracycline and tylosin in manure and soil of Canadian feedlots after subtherapeutic use in cattle. Environ. Pollut. 156, 1243–1251.

https://doi.org/10.1016/J.ENVPOL.2008.03.011

Blanchflower, W.J., McCracken, R.J., Haggan, A.S., Kennedy, D.G., 1997. Confirmatory assay for the determination of tetracycline, oxytetracycline, chlortetracycline and its isomers in muscle and kidney using liquid chromatography-mass spectrometry. J. Chromatogr. B Biomed. Appl. 692, 351–360. https://doi.org/10.1016/S0378-4347(96)00524-5

Brain, R.A., Johnson, D.J., Richards, S.M., Sanderson, H., Sibley, P.K., Solomon, K.R., 2004. Effects of 25 pharmaceutical compounds to Lemna gibba using a seven-day static-renewal test. Environ. Toxicol. Chem. 23, 371–382. https://doi.org/10.1897/02-576

Campagnolo, E.R., Johnson, K.R., Karpati, A., Rubin, C.S., Kolpin, D.W., Meyer, M.T., Esteban, J.E., Currier, R.W., Smith, K., Thu, K.M., McGeehin, M., 2002. Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. Sci. Total Environ. 299, 89–95. https://doi.org/10.1016/S0048-9697(02)00233-4

Capleton, A.C., Courage, C., Rumsby, P., Holmes, P., Stutt, E., Boxall, A.B.A., Levy, L.S., 2006. Prioritising veterinary medicines according to their potential indirect human exposure and toxicity profile. Toxicol. Lett. 163, 213–223. https://doi.org/10.1016/j.toxlet.2005.10.023

Carballo, M., Aguayo, S., González, M., Esperon, F., Torre, A. de la, 2016.

Environmental Assessment of Tetracycline's Residues Detected in Pig Slurry and Poultry Manure. J. Environ. Prot. (Irvine,. Calif). 07, 82–92. https://doi.org/10.4236/jep.2016.71008

Carlsson, M., Lagerkvist, A., Morgan-Sagastume, F., 2012. The effects of substrate pre-treatment on anaerobic digestion systems: A review. Waste Manag. 32, 1634–1650. https://doi.org/10.1016/J.WASMAN.2012.04.016

Carrere, H., Antonopoulou, G., Affes, R., Passos, F., Battimelli, A., Lyberatos, G., Ferrer, I., 2016. Review of feedstock pretreatment strategies for improved anaerobic digestion: From lab-scale research to full-scale application. Bioresour. Technol. 199, 386–397. https://doi.org/10.1016/J.BIORTECH.2015.09.007

Cessna, A.J., Kuchta, S.L., Bailey, J., Waiser, M., Tumber, V., 2020. Isomerization of chlortetracycline in prairie wetland water. J. Environ. Qual. 49, 1435–1444. https://doi.org/10.1002/jeq2.20079

Cetecioglu, Z., Ince, B., Gros, M., Rodriguez-Mozaz, S., Barceló, D., Orhon, D., Ince, O., 2013. Chronic impact of tetracycline on the biodegradation of an organic substrate mixture under anaerobic conditions. Water Res. 47, 2959–2969. https://doi.org/10.1016/j.watres.2013.02.053

Chen, W.R., Huang, C.H., 2011. Transformation kinetics and pathways of tetracycline antibiotics with manganese oxide. Environ. Pollut. 159, 1092–

1100. https://doi.org/10.1016/j.envpol.2011.02.027

Chen, W.R., Huang, C.H., 2010. Adsorption and transformation of tetracycline antibiotics with aluminum oxide. Chemosphere 79, 779–785. https://doi.org/10.1016/j.chemosphere.2010.03.020

Chenxi, W., Spongberg, A.L., Witter, J.D., 2008. Determination of the persistence of pharmaceuticals in biosolids using liquid-chromatography tandem mass spectrometry. Chemosphere 73, 511–518. https://doi.org/10.1016/j.chemosphere.2008.06.026

Chernicharo, C.A.L., van Lier, J.B., Noyola, A., Bressani Ribeiro, T., 2015. Anaerobic sewage treatment: state of the art, constraints and challenges. Rev. Environ. Sci. Biotechnol. 14, 649–679. https://doi.org/10.1007/s11157-015-9377-3

Chopra, I. and Roberts, M., 2001. Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance. Microbiol. Mol. Biol. Rev. 65, 232–260. https://doi.org/10.1128/MMBR.65.2.232

Curry, N., Pillay, P., 2012. Biogas prediction and design of a food waste to energy system for the urban environment. Renew. Energy 41, 200–209. https://doi.org/10.1016/j.renene.2011.10.019

Cycoń, M., Mrozik, A., Piotrowska-Seget, Z., 2019. Antibiotics in the soil

environment—degradation and their impact on microbial activity and diversity. Front. Microbiol. https://doi.org/10.3389/fmicb.2019.00338

D'Angelo, E., 2017. Sorption-desorption equilibrium and diffusion of tetracycline in poultry litter and municipal biosolids soil amendments. Chemosphere 188, 494–501. https://doi.org/10.1016/j.chemosphere.2017.09.015

Daghrir, R., Drogui, P., 2013. Tetracycline antibiotics in the environment: A review. Environ. Chem. Lett. 11, 209–227. https://doi.org/10.1007/s10311-013-0404-8

Dewey, C.E., Cox, B.D., Straw, B.E., Bush, E.J., Hurd, S., 1999. Use of antimicrobials in swine feeds in the United States. Swine Heal. Prod. 7, 19–25.

Doerschuk, A.P., Bitler, B.A., McCormick, J.R.D., 1955. Reversible isomerizations in the tetracycline family. J. Am. Chem. Soc. 77, 4687. https://doi.org/10.1021/ja01622a085

Dzomba, P., 2016. University of Zimbabwe Speciation and Persistence of Tetracycline Antibiotics in the Aquatic Environment : Characterization in terms of a Linear Rate Model.

FDA, U.S., 2020. 2019 Summary report on antimicrobials sold or distributed for use in food-producing animals.

Frade, V.M.F., Dias, M., Teixeira, A.C.S.C., Palma, M.S.A., 2014. Environmental

contamination by fluoroquinolones. Brazilian J. Pharm. Sci. 50, 41–54. https://doi.org/10.1590/S1984-82502011000100004

Fritz, J.W., Zuo, Y., 2007. Simultaneous determination of tetracycline, oxytetracycline, and 4-epitetracycline in milk by high-performance liquid chromatography. Food Chem. 105, 1297–1301. https://doi.org/10.1016/j.foodchem.2007.03.047

Gartiser, S., Urich, E., Alexy, R., Kümmerer, K., 2007. Anaerobic inhibition and biodegradation of antibiotics in ISO test schemes. Chemosphere 66, 1839–1848. https://doi.org/10.1016/j.chemosphere.2006.08.040

Gaugain, M., Gautier, S., Bourcier, S., Jacques, A.M., Laurentie, M., Abjean, J.P., Hurtaud-Pessel, D., Verdon, E., 2015. 6-Iso-chlortetracycline or keto form of chlortetracycline? Need for clarification for relevant monitoring of chlortetracycline residues in food. Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 32, 1105–1115. https://doi.org/10.1080/19440049.2015.1036381

Grote, M., Vockel, A., Schwarze, D., Mehlich, A., Freitag, M., 2004. Fate of antibiotics in food chain and environment originating from pigfattening (part 1). Fresenius Environ. Bull. 13, 1216–1224.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P., Ingerslev, F., Holten Lutzheft,

H., Jorgensen, S., 1998. Occurence, Fate and Effects pf PHarmaceutical Substrances in the Environment. Chemosphere 36, 357–393.

Halling-Sørensen, B., Sengeløv, G., Tjørnelund, J., 2002. Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria, including selected tetracycline-resistant bacteria. Arch. Environ. Contam. Toxicol. 42, 263–271. https://doi.org/10.1007/s00244-001-0017-2

Hamscher, G., Sczesny, S., Höper, H., Nau, H., 2002. Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Anal. Chem. 74, 1509–1518. https://doi.org/10.1021/ac015588m

Hou, G., Hao, X., Zhang, R., Wang, J., Liu, R., Liu, C., 2016. Tetracycline removal and effect on the formation and degradation of extracellular polymeric substances and volatile fatty acids in the process of hydrogen fermentation. Bioresour. Technol. 212, 20–25. https://doi.org/10.1016/j.biortech.2016.03.156

Huang, C.-H., Chen, W.-R., 2009. Transformation of tetracyclines mediated by Mn(II) and Cu(II) ions in the presence of oxygen. Environ. Sci. Technol. 43, 401–407. https://doi.org/10.1021/es802295r

Huang, C.H., Renew, J.E., Smeby, K.L., Pinkston, K., Sedlak, D.L., 2011. Assessment of Potential Antibiotic Contaminants in water and preliminary

occurrence analysis, Journal of Contemporary Water Research and Education.

HUSSAR, D.A., NIEBERGALL, P.J., SUGITA, E.T., DOLUISIO, J.T., 1968. Aspects of the epimerization of certain tetracycline derivatives. J. Pharm. Pharmacol. 20, 539–546. https://doi.org/10.1111/j.2042-7158.1968.tb09802.x

Ince, B., Coban, H., Turker, G., Ertekin, E., Ince, O., 2013. Effect of oxytetracycline on biogas production and active microbial populations during batch anaerobic digestion of cow manure. Bioprocess Biosyst. Eng. 36, 541–546. https://doi.org/10.1007/s00449-012-0809-y

Kennedy, D.G., McCracken, R.J., Carey, M.P., Blanchflower, W.J., Hewitt, S.A., 1998. Iso- and epi-iso-chlortetracycline are the principal metabolites of chlortetracycline in the hen's egg. J. Chromatogr. A 812, 327–337. https://doi.org/10.1016/S0021-9673(98)00280-5

Kennedy, F., 2008. Tetracycline antibiotic distribution and transformation in aquatic systems.

Khadaroo, S.N.B.A., Poh, P.E., Gouwanda, D., Grassia, P., 2019. Applicability of various pretreatment techniques to enhance the anaerobic digestion of Palm oil Mill effluent (POME): A review. J. Environ. Chem. Eng. 7, 103310. https://doi.org/10.1016/j.jece.2019.103310

Kim, K.R., Owens, G., Kwon, S.I., So, K.H., Lee, D.B., Ok, Y.S., 2011. Occurrence and environmental fate of veterinary antibiotics in the terrestrial environment.

Water. Air. Soil Pollut. 214, 163–174. https://doi.org/10.1007/s11270-010-0412-2

Kim, S., Eichhorn, P., Jensen, J.N., Weber, A.S., Aga, D.S., 2005. Removal of antibiotics in wastewater: Effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. Environ. Sci. Technol. 39, 5816–5823. https://doi.org/10.1021/es050006u

Kümmerer, K., 2009. Antibiotics in the aquatic environment - A review - Part I. Chemosphere 75, 417–434. https://doi.org/10.1016/j.chemosphere.2008.11.086

Kwon, A.-Y., Kim, S.C., 2015. Estimating Predicted Environmental Concentration of Veterinary Antibiotics in Manure and Soil. Korean J. Soil Sci. Fertil. 48, 100–104. https://doi.org/10.7745/kjssf.2015.48.2.100

Lallai, A., Mura, G., Onnis, N., 2002. The efects of certain antibiotics on biogas production in the naerobic digestion. Bioresour. Technol. 82, 205–208.

Lee, C., Jeong, S., Ju, M., Kim, J.Y., 2020. Fate of chlortetracycline antibiotics during anaerobic degradation of cattle manure. J. Hazard. Mater. 386, 121894. https://doi.org/10.1016/J.JHAZMAT.2019.121894

Li, B., Zhang, T., 2013. Removal mechanisms and kinetics of trace tetracycline by two types of activated sludge treating freshwater sewage and saline sewage. Environ. Sci. Pollut. Res. 20, 3024–3033. https://doi.org/10.1007/s11356-012-

1213-5

Li, Y.X., Zhang, X.L., Li, W., Lu, X.F., Liu, B., Wang, J., 2013. The residues and environmental risks of multiple veterinary antibiotics in animal faeces. Environ. Monit. Assess. 185, 2211–2220. https://doi.org/10.1007/s10661-012-2702-1

Liu, Y., Li, X., Tan, Z., Yang, C., 2021. Inhibition of tetracycline on anaerobic digestion of swine wastewater. Bioresour. Technol. 334, 125253. https://doi.org/10.1016/j.biortech.2021.125253

Loftin, K.A., Adams, C.D., Meyer, M.T., Surampalli, R., 2008. Effects of Ionic Strength, Temperature, and pH on Degradation of Selected Antibiotics. J. Environ. Qual. 37, 378. https://doi.org/10.2134/jeq2007.0230

Lü, G., Wu, L., Wang, Xiaolong, Liao, L., Wang, Xiaoyu, 2012. Adsorption of chlortetracycline from water by rectories. Chinese J. Chem. Eng. 20, 1003–1007. https://doi.org/10.1016/S1004-9541(12)60429-7

Martínez-Carballo, E., González-Barreiro, C., Scharf, S., Gans, O., 2007. Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. Environ. Pollut. 148, 570–579. https://doi.org/10.1016/J.ENVPOL.2006.11.035

McCormick, J.R.D., Fox, S.M., Smith, L.L., Bitler, B.A., Reichenthal, J., Origoni, V.E., Muller, W.H., Winterbottom, R., Doerschuk, A.P., 1957a. Studies of the Reversible Epimerization Occurring in the Tetracycline Family. The

Preparation, Properties and Proof of Structure of Some 4-epi-Tetracyclines. J. Am. Chem. Soc. 79, 2849–2858. https://doi.org/10.1021/ja01568a050

McCormick, J.R.D., Fox, S.M., Smith, L.L., Bitler, B.A., Reichenthal, J., Origoni, V.E., Muller, W.H., Winterbottom, R., Doerschuk, A.P., 1957b. Studies of the Reversible Epimerization Occurring in the Tetracycline Family. The Preparation, Properties and Proof of Structure of Some 4-epi-Tetracyclines. J. Am. Chem. Soc. 79, 2849–2858. https://doi.org/10.1021/ja01568a050

McCormick, J.R.D., Fox, S.M., Smith, L.L., Bitler, B.A., Reichenthal, J., Origoni, V.E., Muller, W.H., Winterbottom, R., Doerschuk, A.P., 1956. On the nature of the reversible isomerizations occurring in the tetracycline family. J. Am. Chem. Soc. 78, 3547–3548. https://doi.org/10.1021/ja01595a084

Menz, J., Schneider, M., Kümmerer, K., 2015. Usage pattern-based exposure screening as a simple tool for the regional priority-setting in environmental risk assessment of veterinary antibiotics: A case study of north-western Germany. Chemosphere 127, 42–48. https://doi.org/10.1016/j.chemosphere.2014.12.091

Ocampo-Pérez, R., Rivera-Utrilla, J., Gómez-Pacheco, C., Sánchez-Polo, M., López-Peñalver, J.J., 2012. Kinetic study of tetracycline adsorption on sludge-derived adsorbents in aqueous phase. Chem. Eng. J. 213, 88–96. https://doi.org/10.1016/j.cej.2012.09.072

Okolie, Jude, A., Adekunle, Kayode, F., 2015. A Review of Biochemical Process of

Anaerobic Digestion. Adv. Biosci. Biotechnol. 6, 205–212. https://doi.org/10.4236/abb.2015.63020

Park, S., Choi, K., 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology 17, 526–538. https://doi.org/10.1007/s10646-008-0209-x

Pollard, A.T., Morra, M.J., 2017. Fate of tetracycline antibiotics in dairy manure-amended soils. Environ. Rev. 26, 102–112. https://doi.org/10.1139/er-2017-0041

Qiao, M., Chen, W., Su, J., Zhang, B., Zhang, C., 2012. Fate of tetracyclines in swine manure of three selected swine farms in China. J. Environ. Sci. (China) 24, 1047–1052. https://doi.org/10.1016/S1001-0742(11)60890-5

Rajagopal, R., Massé, D.I., Singh, G., 2013. A critical review on inhibition of anaerobic digestion process by excess ammonia. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2013.06.030

Regueiro, L., Veiga, P., Figueroa, M., Alonso-Gutierrez, J., Stams, A.J.M., Lema, J.M., Carballa, M., 2012. Relationship between microbial activity and microbial community structure in six full-scale anaerobic digesters. Microbiol. Res. 167, 581–589. https://doi.org/10.1016/j.micres.2012.06.002

Remmers, E.G., Sieger, G.M., Doerschuk, A.P., 1963. Some observations on the kinetics of the C·4 epimerization of tetracycline. J. Pharm. Sci. 52, 752–756.

https://doi.org/10.1002/jps.2600520809

Sanz, J.L., Rodríguez, N., Amils, R., 1996. The action of antibiotics on the anaerobic digestion process. Appl. Microbiol. Biotechnol. 46, 587–592. https://doi.org/10.1007/s002530050865

Shelver, W.L., Varel, V.H., 2012. Development of a UHPLC-MS/MS method for the measurement of chlortetracycline degradation in swine manure. Anal. Bioanal. Chem. 402, 1931–1939. https://doi.org/10.1007/s00216-011-5637-4

Singh, A.K., 2016. Distribution, Sorption and Desorption of Tylosin, Chlortetracycline and their Metabolites in Pig Manure. J. Agric. Stud. 4, 65. https://doi.org/10.5296/jas.v4i4.10128

Søeborg, T., Ingerslev, F., Halling-Sørensen, B., 2004. Chemical stability of chlortetracycline and chlortetracycline degradation products and epimers in soil interstitial water. Chemosphere 57, 1515–1524. https://doi.org/10.1016/j.chemosphere.2004.09.020

Sokoloski, T.D., Mitscher, L.A., Yuen, P.H., Juvarkar, J. V., Hoener, B., 1977. Rate and proposed mechanism of anhydrotetracycline epimerization in acid solution. J. Pharm. Sci. 66, 1159–1165. https://doi.org/10.1002/jps.2600660829

Spielmeyer, A., Stahl, F., Petri, M.S., Zerr, W., Brunn, H., Hamscher, G., 2016. Transformation of Sulfonamides and Tetracyclines during Anaerobic Fermentation of Liquid Manure. J. Environ. Qual. 46, 160.

https://doi.org/10.2134/jeq2016.04.0152

Stone, J.J., Clay, S.A., Zhu, Z., Wong, K.L., Porath, L.R., Spellman, G.M., 2009. Effect of antimicrobial compounds tylosin and chlortetracycline during batch anaerobic swine manure digestion. Water Res. 43, 4740–4750. https://doi.org/10.1016/j.watres.2009.08.005

Tian, Z., Zhang, Y., Yang, M., 2018. Chronic impacts of oxytetracycline on mesophilic anaerobic digestion of excess sludge: Inhibition of hydrolytic acidification and enrichment of antibiotic resistome. Environ. Pollut. 238, 1017–1026. https://doi.org/10.1016/j.envpol.2018.02.023

Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A., Laxminarayan, R., 2015. Global trends in antimicrobial use in food animals. Proc. Natl. Acad. Sci. 112, 5649–5654. https://doi.org/10.1073/pnas.1503141112

Volkers, G., Petruschka, L., Hinrichs, W., 2011. Recognition of drug degradation products by target proteins: Isotetracycline binding to tet repressor. J. Med. Chem. 54, 5108–5115. https://doi.org/10.1021/jm200332e

Wu, C., Spongberg, A.L., Witter, J.D., 2009. Sorption and biodegradation of selected antibiotics in biosolids. J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng. 44, 454–461. https://doi.org/10.1080/10934520902719779

Xie, X., Zhou, Q., He, Z., Bao, Y., 2010. Physiological and potential genetic toxicity

of chlortetracycline as an emerging pollutant in wheat (Triticum aestivum L.). Environ. Toxicol. Chem. 29, 922–928. https://doi.org/10.1002/etc.79

Xu, D., Xiao, Y., Pan, H., Mei, Y., 2019. Toxic effects of tetracycline and its degradation products on freshwater green algae. Ecotoxicol. Environ. Saf. 174, 43–47. https://doi.org/10.1016/j.ecoenv.2019.02.063

Yuen, P.H., Sokoloski, T.D., 1977. Kinetics of concomitant degradation of tetracycline to epitetracycline, anhydrotetracycline, and epianhydrotetracycline in acid phosphate solution. J. Pharm. Sci. 66, 1648–1650. https://doi.org/10.1002/jps.2600661143

Zhang, H., Luo, Y., Wu, L., Huang, Y., Christie, P., 2015. Residues and potential ecological risks of veterinary antibiotics in manures and composts associated with protected vegetable farming. Environ. Sci. Pollut. Res. 22, 5908–5918. https://doi.org/10.1007/s11356-014-3731-9

Zhao, F., Zhang, D., Xu, C., Liu, J., Shen, C., 2020a. The enhanced degradation and detoxification of chlortetracycline by Chlamydomonas reinhardtii. Ecotoxicol. Environ. Saf. 196, 110552. https://doi.org/10.1016/j.ecoenv.2020.110552

Zhao, F., Zhang, D., Xu, C., Liu, J., Shen, C., 2020b. The enhanced degradation and detoxification of chlortetracycline by Chlamydomonas reinhardtii. Ecotoxicol. Environ. Saf. 196, 110552. https://doi.org/10.1016/j.ecoenv.2020.110552

Zhao, L., Dong, Y.H., Wang, H., 2010. Residues of veterinary antibiotics in manures

from feedlot livestock in eight provinces of China. Sci. Total Environ. 408, 1069–1075. https://doi.org/10.1016/j.scitotenv.2009.11.014

Zhou, L.J., Ying, G.G., Liu, S., Zhang, R.Q., Lai, H.J., Chen, Z.F., Pan, C.G., 2013. Excretion masses and environmental occurrence of antibiotics in typical swine and dairy cattle farms in China. Sci. Total Environ. 444, 183–195. https://doi.org/10.1016/j.scitotenv.2012.11.087

CHAPTER 3
UNCERTAINTY-BASED CONCENTRATION ESTIMATION OF CHLORTETRACYCLINE IN SWINE MANURE AND RISK PROBABILITY ASSESSMENT IN SOIL[1]

3.1 Introduction

Livestock manure can contain veterinary antibiotics (VAs) administrated for livestock disease treatment. Aquatic and terrestrial environments are exposed to the excreted VAs with the use of livestock manure as liquid fertilizer and compost. Thus, it causes various environmental problems. The presence of VAs in the environment can decrease the diversity of soil microbial ecosystems by their direct toxicity on the soil microorganisms and their metabolism (Gelband et al., 2015; Romanelli et al., 2015). This also increases dependence on VAs by increasing antibiotic-resistant genes and bacteria. (Chopra and Roberts, 2001; Van Boeckel et al., 2015). In 2010, the amount of VAs used around the world was twice as high as the amount of antibiotics used for humans (Gelband et al., 2015). Thus, it is

[1] A significant portion of this chapter was published in the following article: Lee, C., An, J., Lee, Y. S., Choi, K., & Kim, J. Y. (2021). Uncertainty-based concentration estimation of chlortetracycline antibiotics in swine farms and risk probability assessment for agricultural application of manure. Journal of Hazardous Materials, 402, 123763.

necessary to primarily manage VAs before exposure to the aquatic environment.

Evaluation of the environmental concentration of VAs at the point of exposure constitutes the primary step for ecological risk assessment (European medicines agency, 2016) with toxicity tests on target organisms. The evaluations are performed in two ways, by measuring or by predicting the environmental concentration of VAs in the soil and water environment. The former is a method of quantitatively and qualitatively analyzing the VAs in environmental samples by using analytical instruments. This requires labor and capital costs to establish reliable analytical methodologies for the target sample matrix and VAs (Aga et al., 2016). However, the latter is a method involving calculation of the environmental concentration of VAs by considering factors such as dosage, excretion rate, and generation of livestock manure. This method is applicable to situations in which the quantitative and qualitative measurements of VAs are not practical. For example, these situations include but not limited to the cases requiring assessment of a wide area and/or for multiple types of VAs, and those related to or influenced by many environmental factors. Various exposure scenarios can also be developed and assessed for prediction purposes. Additionally, given that most of VAs usage is limited to livestock (e.g., pigs, cows, and chickens) and that livestock farms manage excreted manure, it is easy to predict concentrations of VAs from livestock by considering factors related to exposure pathways.

The prediction methodology that is typically used corresponds to guidelines of the European Medicines Agency (EMA) (European Medicines Agency, 2016).

This method was designed to adopt a decision on the use of VAs for livestock farming based on the prediction of the concentration of VAs in the environment and evaluation of the consequent ecological risks. Release estimation and environmental distribution are mainly considered to calculate the predicted environmental concentration (PEC). Many researchers used the EMA guidelines to calculate the PEC of VAs (Kullik and Belknap, 2017; Spaepen et al., 1997) and evaluate ecological risks with the results of toxicity tests (Di Guardo and Finizio, 2017; Menz et al., 2015). However, to the best of our knowledge, there is a paucity of studies that consider the release estimation and environmental distribution factors related to farm practices and the associated uncertainties. Huang et al. (2011) tried to estimate concentrations of tetracyclines, macrolides, sulfonamides, etc., in cattle and swine manure by considering dosage, excretion rate, and wastewater generated in a confined animal feeding operation. The concentrations in the cattle and swine manure were estimated in a range of 730 – 4,000 μg/kg and 6 – 133 mg/kg, respectively. Factors related to the generation and management of livestock manure (e.g. bodyweight of livestock, production of livestock manure, degradation, amount of liquid fertilizer, and/or compost) are expected to imply high uncertainties (Huang et al., 2011; Kelly et al., 2003). Thus, by reflecting on the uncertainties, the probabilities of the prediction and the sensitive factors affecting these can be evaluated and compared with each other. Furthermore, the results of the sensitivity analysis can be used in the decision-making process to identify the exposure pathway wherein risk management is primarily required. Additionally, consideration of farm

practice factors reduces the possible deviation from uniform model simulations and enables specific risk management with the results of the sensitivity analysis. For example, pigs are raised on a grid-type structure termed the slurry pit, and excreted swine manure is stored in a manure container located under the slurry pit (Huang et al., 2011). In the slurry pit system, swine manure containing VAs can be diluted by water to clean livestock farms and swine manure.

Chlortetracycline (CTC) is considered as the most harmful VA to the environment among all broad-spectrum VAs (Daghrir and Drogui, 2013). Broad-spectrum VAs corresponds to antibiotics that are used to treat a wide array of disease-causing bacteria including gram-positive and -negative. CTC has been the most consumed VA worldwide given its ease of use, low price, prevention and treatment effects, and growth promotion (Kim et al., 2011). Furthermore, CTC represents a high excretion rate (70 % by wt.) and long half-life (30 – 80 days) when compared to other broad-spectrum VAs such as erythromycin and clarithromycin. Thus, it is highly likely that CTC persists in terrestrial and aquatic systems for a long time with relatively high concentrations (Chenxi et al., 2008; Kumar et al., 2005). In addition, the acute toxicity of CTC was reported tens or hundred times greater than those of other antibiotics such as ampicillin and amoxicillin (V. *fischeri* in 15 mins) (Park and Choi, 2008), and trimethoprim (M. *aeruginosa* and S. *capricornutum* in 7 days) (Halling-Sørensen et al., 1998).

Hence, the objectives of this study were to estimate the PEC of CTC in a slurry pit and soil by considering the uncertainty of slurry pit farm practices, to

evaluate ecological risks of CTC on soil microorganisms, and to suggest factors affecting risks based on the sensitivity analysis. In order to achieve the objectives, scenarios wherein CTC is exposed to soil were established and evaluated by assuming a slurry pit-based pig farm. CTC was used as a representative VA of tetracycline families including tetracycline (TC), oxytetracycline (OTC), and CTC. Uncertainties of each factor were assessed via Monte Carlo simulations, and risks were characterized based on the EMA guidelines.

3.2 Materials and methods

3.2.1 Scenario and equations for prediction of concentration of CTC

The estimation of PEC_{soil} of CTC was conducted according to the following six steps: 1) excreted swine manure after administration of CTC; 2) swine manure in the slurry pit; 3) swine manure in the storage tank; 4) swine manure after anaerobic digestion; 5) swine manure after fertilization/composting; and 6) soil fertilized by swine manure-based fertilizer/compost. The PECs of CTC for each step were estimated by considering factors affecting the PECs and their uncertainties. Anaerobic digestion (AD) is an optional biological treatment process in which anaerobes degrade organics in swine manure and generate biogas mainly consisting of methane and carbon dioxide. Anaerobic digestion is not an obligatory process prior to fertilization/composting, and thus the PEC_{soil} was estimated based on two scenarios, namely Scenario w/o AD and w/ AD.

Excretion period of CTCs after administration, daily cleaning water usage, and pit emptying cycles were considered as farm practice factors in addition to the factors included in model equations of the EMA. In order to consider the uncertainties of each factor, their probability distributions were assumed as a point distribution when values of the factor were fixed (e.g., standard methodology for the use of VAs), normal distribution when statistics and/or experimental data are

available (e.g., bodyweight of swine manure), and uniform distribution when statistics and/or experimental data are not available but only a few values were reported. Equations, factors, and corresponding assumptions that are used are listed in Table 3.1.

Table 3.1 Models and input parameters to calculate PEC_{soil} of CTC

Parameters (unit)	Meaning	Equations or input values	Distribution	References
PEC_{manure} (mg/kg_{manure})	$\frac{D \times BW \times P \times ER}{Ef} \times \frac{1}{Ex}$			
D (mg/kg_{BW}/day)	Daily dose of the antibiotics	30	Point	Grote et al., 2004; Stone et al., 2009; Winckler and Grafe, 2001
BW (kg/head)	Body weight of swine	80 ± 10	Normal	USDA, 2015
P (day)	Administration period	5	point	Arikan, 2008
ER (unitless)	Excretion ratio	0.6 ± 0.1	Normal	Kwon and Kim, 2015; Sarmah et al., 2006
Ef (kg_{manure}/head/day)	Daily excretion of manure	4.2 – 8.6	Uniform	Jørgensen et al., 2013; Schiavon et al., 2016; KMOE, 2015
Ex (day)	Excretion period	8 - 15	Uniform	Grote et al., 2004; Stone et al., 2009; Winckler and Grafe, 2001
PEC_{pit} (mg/kg_{manure})	$PEC_{manure} \times \frac{Ex}{Cp} \times \frac{Ef}{(Ef + CW)} \times PSR$			
Cp (day)	Pit emptying cycle	16 – 50	Uniform	Petersen et al., 2016
CW (kg/day/head)	Daily cleaning water usage	4.4 - 50	Uniform	Huang, C. H., Renew, J. E., Smeby, K. L., Pinkston, K., and Sedlak, 2011; Lee et al., 2015
PSR (Unitless)	Ratio of pigs with disease	0.1 – 0.4	Uniform	USDA, 2015
PEC_{tank} (mg/kg)	$PEC_{manure\_pit} \times e^{-k_{d\_tank} * St}$			
k_{d_tank}	1st order	0.009	Point	Chenxi et al.,

(day^{-1})	degradation rate in biosolid			2008
St (day)	Storage period	30 - 120	Uniform	
PEC_{AD} (mg/kg)	$\frac{\sum_{t=1}^{\tau} PEC_{tank} \times VR \times (\frac{1}{1+k_d\tau} - \frac{1}{1+k_d\tau} e^{-(\frac{1+k_d*\tau}{\tau})*t}))}{\tau}$			For Scenario w/AD
VR (Unitless, w/w)	mixing ratio of manure to co-digestion substrate	0.6 – 0.9	Uniform	Astals et al., 2012; Riaño et al., 2011
k_{d_ad} (day^{-1})	1st order biodegradation rate in anaerobic digestion	0.01 – 0.46	Uniform	Álvarez et al., 2010; Arikan, 2008; Lee et al., 2020
τ_{AD} (day)	Hydraulic retention time	20 - 25	Uniform	Wilkie, 2005
t (day)	Operation period	1 - τ_{AD}	Point	
PEC_{comp} (mg/kg)	$Scenario\ w/o\ AD: PEC_{tank} \times e^{-(k_{d\_comp} \times \tau_{comp})}$			$Scenario\ w/\ AD: PEC_{AD} \times e^{-(k_{d\_comp} \times \tau_{comp})}$
k_{d_comp} (day^{-1})	1st order biodegradation rate in fertilization/composting	0.026	point	Kwon, 2011
τ_{comp} (day)	Composting time	15-20	Uniform	Liu et al., 2015
PEC_{soil} ($\mu g/kg_{soil}$)	$\frac{PEC_{comp} \times NS}{BD \times PD \times N} \times \frac{1\ ha}{10{,}000\ m^2} \times \frac{1{,}000\ ug}{mg}$			
BD (kg/m^3)	Bulk density of dry soil	1,500	Point	EMA, 2016
PD (m)	Penetration depth into soil	0.05	Point	EMA, 2016
N ($kg\ N/kg_{comp}$)	Nitrogen contents in fertilizer/compost	0.003 – 0.021		Won et al., 2018; Zhu, 2007, 2006
NS (kg N/ha)	Nitrogen spreading limit	170		EMA, 2016

3.2.1.1 PEC of CTC in swine manure

The PEC_{manure} (mg/kg_{manure}) represents the average concentration of CTC in swine manure generated after the use of CTC (Eq. 1). Given that some of the administrated VAs are excreted with manure over a period of time, the administration period of VAs and number of days that VAs are excreted were considered. It is known that CTC is excreted with manure for approximately 10 days after administration. For example, by assuming that CTC was administrated for five days, swine manure can contain CTC until day 15 from the first day of CTC administration (Grote et al., 2004; Winckler and Grafe, 2001).

$$\boldsymbol{PEC_{manure}} = \frac{\boldsymbol{D \times BW \times P \times ER}}{\boldsymbol{Ef}} \times \frac{\boldsymbol{1}}{\boldsymbol{Ex}}$$

Eq. 1

where PEC_{manure}: concentration of CTC in manure (mg/kg_{manure}); D: daily dose of CTC (mg/kg_{BW}/day); BW: body weight of swine (kg/head); P: administration period (day); ER: excretion rate of administered CTC through swine manure (unitless); Ef: Daily generation of swine manure (kg_{manure}/head/day); and Ex: Period during which CTC is excreted with swine manure after the administration

3.2.1.2 PEC of CTC in the slurry pit

PEC_{pit} (mg/kg_{manure}) denotes the concentration of CTC in swine manure from the slurry pit. Swine manure was assumed to be stored in the slurry pit immediately after the generation. Thus, swine manure containing CTC can be diluted by swine manure in the container under the slurry pit and water used for cleaning the farm (Huang et al., 2011). In addition, since the slurry pit is emptied and cleaned, the degree of dilution of CTC is expected to vary based on the time the swine manure is stored in the slurry pit prior to emptying the swine manure. For example, if CTC is excreted for 10 days and the slurry pit is emptied on the 7th day after administration of CTC, then the average amount of CTC in the swine manure corresponds to 70% of the total amount of CTC to be excreted by assuming a uniform excretion of CTC with the manure. Based on the PEC_{manure}, PEC_{pit} was calculated based on dilution by daily cleaning water usage, pit emptying cycle, and the ratio of pigs in treatment as shown in Eq. 2.

$$\boldsymbol{PEC_{pit} = PEC_{manure} \times \frac{Ex}{Cp} \times \frac{Ef}{(Ef+CW)} \times PSR}$$

Eq. 2

where PEC_{pit}: concentration of CTC in a slurry pit (mg/kg); C_p: pit emptying cycle (day); CW: daily cleaning water usage (kg/day/head); and PSR: Ratio of pigs requiring antibiotics (unitless)

3.2.1.3 PEC of CTC in a storage tank

Storage tank denotes the space in which the swine manure from the slurry pit is stored prior to anaerobic digestion or fertilization/composting. The PEC_{tank} was estimated based on an assumption that CTC is degraded with the 1st order kinetic model. Thus, the storage period and 1st order degradation rate of CTC in swine manure are considered in the estimation (Eq. 3) as follows:

$$\boldsymbol{PEC_{tank} = PEC_{pit} \times e^{-(k_{d\_tank} \times St)}}$$

Eq. 3

where PEC_{tank}: concentration of CTC in storage tank (mg/kg_{manure}); k_{d_tank}: 1st order degradation rate in storage tank (day^{-1}); and St: storage period (day)

3.2.1.4 PEC of CTC in the effluent of the anaerobic digester

PEC_{AD} (mg/kg) that denotes the concentration of CTC in the effluent of an anaerobic digester is estimated based on the 1st order degradation kinetic model and single-stage and continuously-stirred tank reactor (CSTR). An analytical solution considering the 1st order degradation kinetic model and hydraulic retention time was used to obtain PEC_{AD} by assuming ideal CSTR conditions (i.e. complete mixing and steady-state conditions). Additionally, the mixing ratio of swine manure to the co-digestion substrate is considered to represent dilution by co-digestion of swine

manure with other organic waste as follows (Eq. 4):

$$\boldsymbol{PEC_{AD}} = \frac{\sum_{t=1}^{\tau_{AD}} \boldsymbol{PEC_{tank}} \times \boldsymbol{VR} \times \left(\frac{1}{1+k_d\tau_{AD}} - \frac{1}{1+k_d\tau_{AD}} e^{-\left(\frac{1+kd*\tau_{AD}}{\tau}\right)*t}\right)}{\tau_{AD}}$$

Eq. 4

where PEC_{AD}: concentration of CTC in the effluent of the anaerobic digester; VR: mixing ratio of manure to the co-digestion substrate (unitless, w/w); k_d: 1st order biodegradation rate (day^{-1}); $\tau_{AD:}$ hydraulic retention time (day); and t: operation period (day)

3.2.1.5 PEC of CTC in fertilizer/compost

The PEC_{comp} is also estimated based on 1st order degradation kinetic model during the fertilization/composting of swine manure. Hence, the duration of fertilization/composting (τ_{comp}, day) and 1st order biodegradation of CTC are used to represent PEC_{comp} assuming batch condition (Eq. 5).

$$\boldsymbol{PEC_{comp}} = \boldsymbol{PEC_{tank}}\ (\boldsymbol{Scenario\ w/o\ AD})\boldsymbol{or}\ \boldsymbol{PEC_{AD}}(\boldsymbol{Scenario\ w/\ AD}) \times e^{-(k_{d\_comp} \times \tau_{comp})}$$

Eq. 5

where PEC_{comp}: concentration of CTC in fertilizer/compost (mg/kg_{comp}); k_{d_comp}: 1st order biodegradation rate in fertilization/composting (day^{-1}); and τ_{comp}:

fertilization/composting period (day)

3.2.1.6 PEC of CTC in soil

Based on the EMA guidelines, PEC_{soil} ($\mu g/kg_{soil}$) denotes the concentration of CTC in 5-cm topsoil. For the PEC_{soil}, use of fertilizer/compost based on nitrogen spreading limitation, bulk density of dry soil, penetration depth into the soil, and nitrogen contents in fertilizer/compost are considered in Eq. 6 as follows:

$$\boldsymbol{PEC_{soil}} = \frac{\boldsymbol{PEC_{comp} \times NS}}{\boldsymbol{BD \times PD \times N}} \times \frac{\boldsymbol{1\ ha}}{\boldsymbol{10{,}000\ m^2}} \times \frac{\boldsymbol{1{,}000\ ug}}{\boldsymbol{mg}}$$

Eq. 6

where PEC_{soil}: concentration of CTC in soil ($\mu g/kg_{soil}$); BD: bulk density of dry soil (kg/m^3); PD: penetration depth into soil (m); N: Nitrogen content in fertilizer/compost (kg_N/kg_{comp}); and NS: nitrogen spreading limit (kg_N/ha)

The equations, input variables, and assumptions of distributions above are summarized in Table 3.1.

3.2.2 Characterization of ecological risk

In order to assess the probability of ecological risk of CTC in soil, a risk

quotient (RQ, unitless) was used. According to the US EPA, the definition of the risk quotient (RQ) is 'ratio of a point estimate of exposure by a point estimate of effects'. The RQ can be calculated based on the ratio of PEC_{soil} and predicted no-effect concentration (PNEC) by considering the results of the toxicity test (TOX) and assessment factor (AF) as shown in Eq. 7 and 8. The values of AF corresponded to 1,000 for TOX obtained from the acute toxicity test or 100 for that obtained from the chronic toxicity test (EMA, 2016). If the value of RQ exceeds one, the ecological risk of CTC is probable. In this study, the ecological risk was represented as the probability of RQ exceeding one and not an absolute value by considering the uncertainties of PEC_{soil}. Probabilistically estimated RQ represents the probability for CTC to expose ecological risk in soil based on the scenarios.

$$\mathrm{RQ} = \frac{PEC_{soil}}{PNEC} \qquad \text{Eq. 7}$$

$$\mathrm{PNEC} = \frac{TOX}{AF} \qquad \text{Eq. 8}$$

The TOX was determined to be 50 mg/kg_{soil} for median effective concentration on microbial activity, which corresponds to the lowest concentration among values affecting microbial activity, diversity of the microbial community, bacterial growth, nitrification, and iron reduction (Grenni et al., 2018; Li et al., 2013; Thiele-Bruhn and Beck, 2005). The value corresponded to the result of an acute toxicity test, and thus an AF of 1,000 is used. Finally, the PNEC was determined as

50 $\mu g/kg_{soil}$ based on the values of TOX and AF.

3.2.3 Uncertainty analysis and sensitivity analysis

The uncertainty analysis was conducted using the Monte Carlo simulation. Monte Carlo simulation was performed by using Microsoft Excel-based Crystal ball 12.1 Software (Oracle Co., U.S.A). The distribution of PEC_{pit} and PEC_{soil} were obtained via 1,000,000 iterations of random sampling by assuming the types of the probability distribution for each variable as presented in Table 3.1.

Sensitivity analysis was also performed using the Monte Carlo simulation. The contribution of each variable to the variance of the prediction was represented as a percentage. The contribution to the variance was calculated via squaring rank correlation coefficients of each input variable and normalizing them to 100%. According to the Crystal ball 12.1, the Monte Carlo simulation performed at least 10,000 times ensures appropriate accuracy of the contribution to variance.

3.3 Results and discussion

3.3.1 Predicted CTC concentration in swine manure from the slurry pit

Cumulative probability was plotted against PEC_{pit} (Fig. 3.1). The average PEC_{pit} values for Scenario w/o AD and w/ AD were 2.20 $\pm$ 1.77 and 2.20 $\pm$ 1.76 mg/kg_{manure}, respectively. The values for the 5^{th} percentile and 95^{th} percentile were in the range of 0.54 - 5.64 and 0.54 - 5.66 mg/kg_{manure}, respectively (Table 3.2). Given that PEC_{pit} was not related to the anaerobic digestion step, it was reasonable that the same average and range of values were obtained in both scenarios.

Furthermore, the results were compared with those of previous studies that measured the concentration of CTC in swine manure captured from a slurry pit and that of OTC and TC (Carballo et al., 2016; Jacobsen and Halling-Sørensen, 2006; Li et al., 2013; Martínez-Carballo et al., 2007; Zhang et al., 2015; Zhao et al., 2010). Thus, the PEC_{pit} of this study and measured concentrations were considerably similar. Zhao et al. (2010) obtained 26 swine manure samples from slurry pits in 31 farms in China, which raised 500 to 5,500 pigs and measured concentrations of CTC and OTC in the manure. The average concentrations of CTC and OTC were 1.15 $\pm$ 4.43 and 2.69 $\pm$ 17.00 mg/kg_{manure}, respectively, and were in the range of 0.16 - 21.06 and 0.15 - 59.06 mg/kg_{manure}, respectively. Additionally, Martínez-Carballo et al. (2007) reported that the ranges of concentrations of CTC and TC in swine manure from the

slurry pit corresponded to 0.1 - 46.0 and 0.4 - 23.0 mg/kg_{manure}, respectively. In the study, 17 samples for CTC and 22 samples for TC were obtained from slurry pits in a farm in Austria where 400 - 1,000 pigs were raised (Table 3.3). A weighted average of the measured concentration corresponding to 2.12 mg/kg_{manure} was obtained in studies that described the mean value and number of samples (Table 3.3) by assuming that the concentration of CTC in the slurry pit was normally distributed. The result was in agreement with the PEC_{pit} of this study (2.20 mg/kg_{manure}). This revealed that the concentration of CTC in a slurry pit could be effectively estimated as a form of the probability distribution by considering uncertainties of slurry pit farm practices.

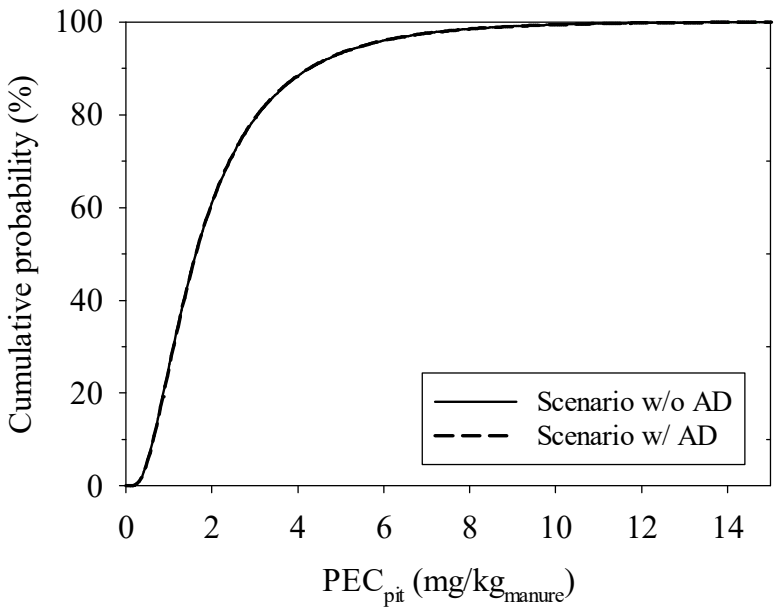

Figure 3.1 Cumulative probability plotted against the concentration of CTC in swine manure from slurry pit. Probability is obtained from the Monte Carlo simulation with 1,000,000 iterations

Table 3.2 Summary of statistics for PEC_{pit}, $PEC_{soil,}$ and RQ

Scenario w/o AD						
Classification	Mean	std	Min	5^{th} percentile	95^{th} percentile	Max
PEC_{pit} (mg/kg_{manure})	2.20	1.77	0.14	0.54	5.64	20.90
PEC_{soil} ($\mu g/kg_{soil}$)	22.03	25.78	0.68	3.42	67.59	668.72
RQ	0.44	0.52	0.01	0.07	1.35	13.37
P(RQ > 1)	9.3%					
Scenario w/ AD						
Classification	Mean	std	Min	5^{th} percentile	95^{th} percentile	Max
PEC_{pit} (mg/kg_{manure})	2.20	1.76	0.09	0.54	5.66	24.61
PEC_{soil} ($\mu g/kg_{soil}$)	7.84	6.47	0.32	1.11	22.21	107.97
RQ	0.15	0.17	0.00	0.02	0.44	4.03
P(RQ > 1)	0.6%					

Table 3.3 Previously reported measured concentrations of CTC, OTC, and TC in manure from the slurry pit

Literatures	Types of TC (CTC, OTC, TC)	Average (mg/kg_{manure})	Minimum – Maximum (mg/kg_{manure})	Number of samples	Note
Carballo et al., 2016	CTC	0.08 ± 0.19	0.00 - 0.56	8	a farm with 2,000 - 3,000 pigs in Spain
	OTC	0.28 ± 0.29	0.00 - 0.76	8	
Zhao et al., 2010	CTC	1.15 ± 4.43	0.16 - 21.06	26	31 farms with 500 - 5,000 pigs in China
	OTC	2.69 ± 17.00	0.15 - 59.06	26	
Li et al., 2013	TC	5.29	0.32 - 30.55	18	18 farms with 100 - 10,000 pigs in China
	OTC	11.81	0.73 - 56.81	18	
	CTC	3.19	0.68 - 22.34	18	
Zhang et al., 2015	TC	0.60	0.02 - 31.00	7	9 farms in China
	OTC	1.10	0.02 - 43.43	7	
	CTC	1.04	0.02 - 215	7	
Martínez-Carballo et al., 2007	CTC	-	0.10 - 46.00	17	Farms with 400 - 1,000 pigs in Austria
	TC	-	0.36 - 23.00	22	
Weighted Average		2.12			

3.3.2 Predicted CTC concentration in the soil

Cumulative probability was plotted against the values of PEC_{soil} ($\mu g/kg_{soil}$) (Fig. 3.2). In Scenario w/o AD, the average PEC_{soil} was 22.03 $\pm$ 25.78 $\mu g/kg_{soil}$ and the values for the 5th percentile and 95th percentile were in the range of 3.42 - 67.59 $\mu g/kg_{soil}$. In Scenario w/ AD, the average and range of PEC_{soil} were 7.84 $\pm$ 6.47 and 1.11 - 22.21 $\mu g/kg_{soil}$, respectively (Table 3.2). The anaerobic digestion step reduced CTC by 65% (w/w) compared to the average values of the two scenarios and it reduced CTC by 67% (w/w) when compared to the 95th percentile values.

These findings agree with those in a study conducted by Hamscher et al. (2002). The study measured concentrations of CTC and TC within a 30-cm soil layer that was fertilized by swine manure containing 0.1 mg-CTC/kg and 4 mg-TC/kg for more than 12 months by 30 - 50 m^3/ha per year. The measured concentrations of CTC and TC were 43 - 198 μg-TC/kg_{soil} and 4.6 - 7.1 μg-CTC/kg_{soil}, respectively. The range of measured concentrations of TC was broader than the PEC_{soil} of this study. It is noteworthy out that the concentration of TC in swine manure and the amount of swine manure usage were larger than those of this study (approximately 2 mg-CTC/kg_{manure} and approximately 20 m^3/ha per year). The concentration of VAs in the soil is expected to vary significantly, not only because of the amount of fertilizer/compost, but also because many factors cannot be included in the uncertainties such as the number of instances of compost distribution per year, climate (e.g. rainfall and sunlight), and characteristics of soil (e.g., moisture content,

organic contents, and pH).

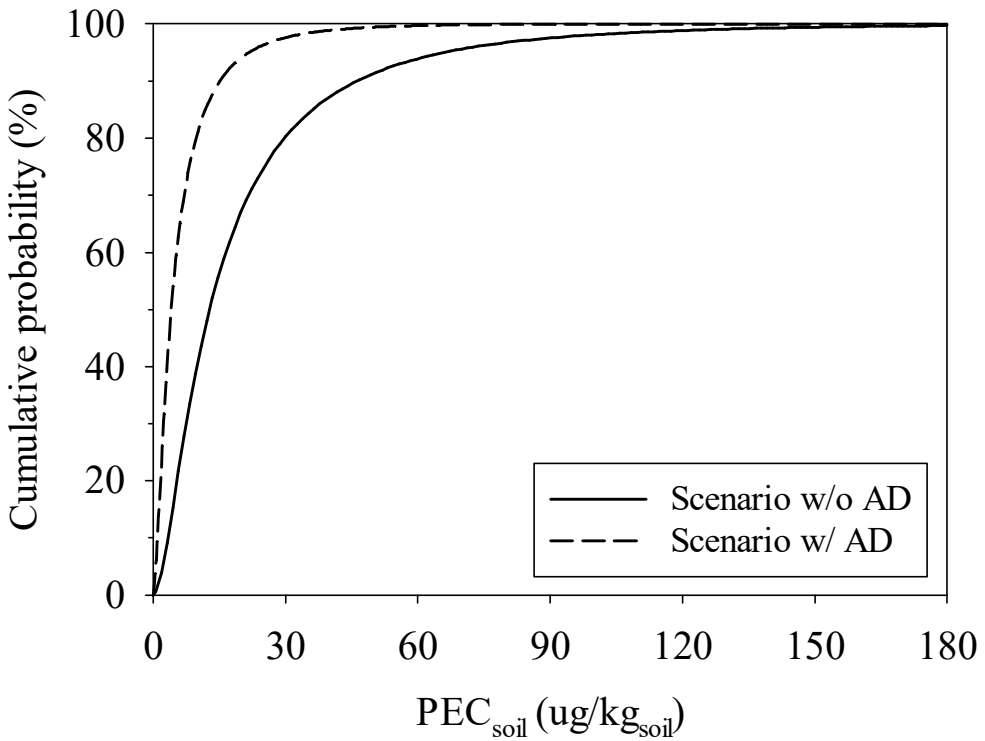

Figure 3.2 Cumulative probability plotted against the concentration of CTC in soil. The probability was obtained from the Monte Carlo simulation with 1,000,000 iterations

3.3.3 Ecological risk of CTC in soil

In order to assess the ecological risk of CTC in the soil environment, the probability of RQ (=PEC_{soil}/PNEC) exceeding one was evaluated. The PNEC (=TOX/AF) was determined as 50 μg/kg_{soil} by considering 50 mg/kg_{soil} of TOX for median effective concentration on microbial activity and an AF of 1,000.

The cumulative probability was plotted against RQ by considering the probability distribution of PEC_{soil} (Fig. 3.3). Based on the result of Scenario w/o AD (black dot in Fig. 3.3), ecological risk of CTC for nitrification bacteria in soil possibly existed (P(RQ > 1) = 9.3%). Based on the result of Scenario w/ AD (white dot in Fig. 3.3), the ecological risk of CTC for Scenario w/ AD was significantly lower than that for Scenario w/o AD (P(RQ > 1) = 0.6%). Since the RQ was assessed based on the PEC_{soil} of CTC, the decrease of CTC concentration during AD significantly decreased the risk (i.e. value of RQ). The average of estimated RQ for Scenarios w/o and w/ AD corresponded to 0.44 ± 0.52 (5^{th} percentile: 0.07, 95^{th} percentile: 1.35) and 0.15 ± 0.17 (5^{th} percentile: 0.02, 95^{th} percentile: 0.44), respectively (Table 3.2). The results indicated that AD has a practical effect on the elimination of the ecological risk of CTC in soil.

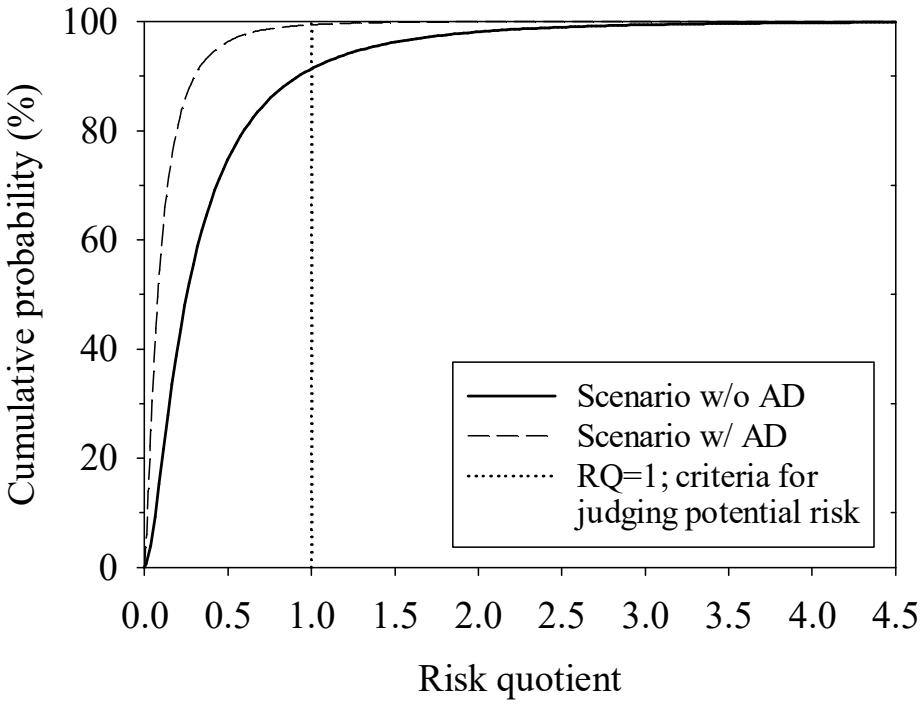

Figure 3.3 Cumulative probability plotted against risk quotient (RQ) of CTC in soil. Probabilities exceeding one correspond to 9.3 and 0.6% for Scenario w/o and w/ AD, respectively. The probability was obtained from the Monte Carlo simulation with 1,000,000 iterations

3.3.4 Sensitive parameters on the PEC_{soil}

The effect of input variables on the increase or decrease of RQ was represented as the contribution of each variable to the variance of the prediction. The top eight input variables with absolute high sensitivity were shown in Fig. 3.4. Positive values indicate that the variable is related to an increase in the RQ, and vice-versa. Larger absolute values denote greater sensitivity. Thus, the top seven input variables corresponded the same in Scenario w/o and w/ AD. They included nitrogen content in fertilizer/compost, daily cleaning water usage, the ratio of sick pigs requiring CTC, pit emptying cycle, storage period, excretion rate, and bodyweight of pigs in the order of the absolute value. The only difference was that the 8th most sensitive input variable was daily manure generation in Scenario w/o AD and the 1st order degradation coefficient of anaerobic digestion in Scenario w/ AD. The other input variables exhibited extremely low sensitivity with a contribution of less than 0.1% (data not shown). Nitrogen content in fertilizer/compost, daily cleaning water usage, pit emptying cycle, and storage period exhibited 'negative' contributions indicating that an increase of each factor would decrease the CTC exposure concentration and risk. On the other hand, the ratio of sick pigs requiring CTC, excretion rate, and bodyweight of pigs showed a 'positive' contribution indicating that their increases would increase the CTC exposure concentration and risk. All signs of direction shown for each factor were confirmed correct. Since the amount of fertilizer/compost usage was determined according to total nitrogen limitation in

soil, the increase of nitrogen content decreased the fertilizer/compost usage and decreased the PEC_{soil}. Increased daily cleaning water usage would dilute the CTC concentration and hence decrease the PEC. In addition, the increased excretion rate of CTC would increase PEC_{soil}. In addition to factors that are expected to naturally affect the RQ (e.g., the storage period and excretion rate of CTC), daily cleaning water usage, the ratio of sick pigs, and pit emptying cycle associated with farm practices were the most important factors affecting RQ of CTC in soil. The result indirectly confirmed that the prediction of Huang et al. (2011) that cleaning water use is an important factor in determining the concentration of VAs to be released into nature.

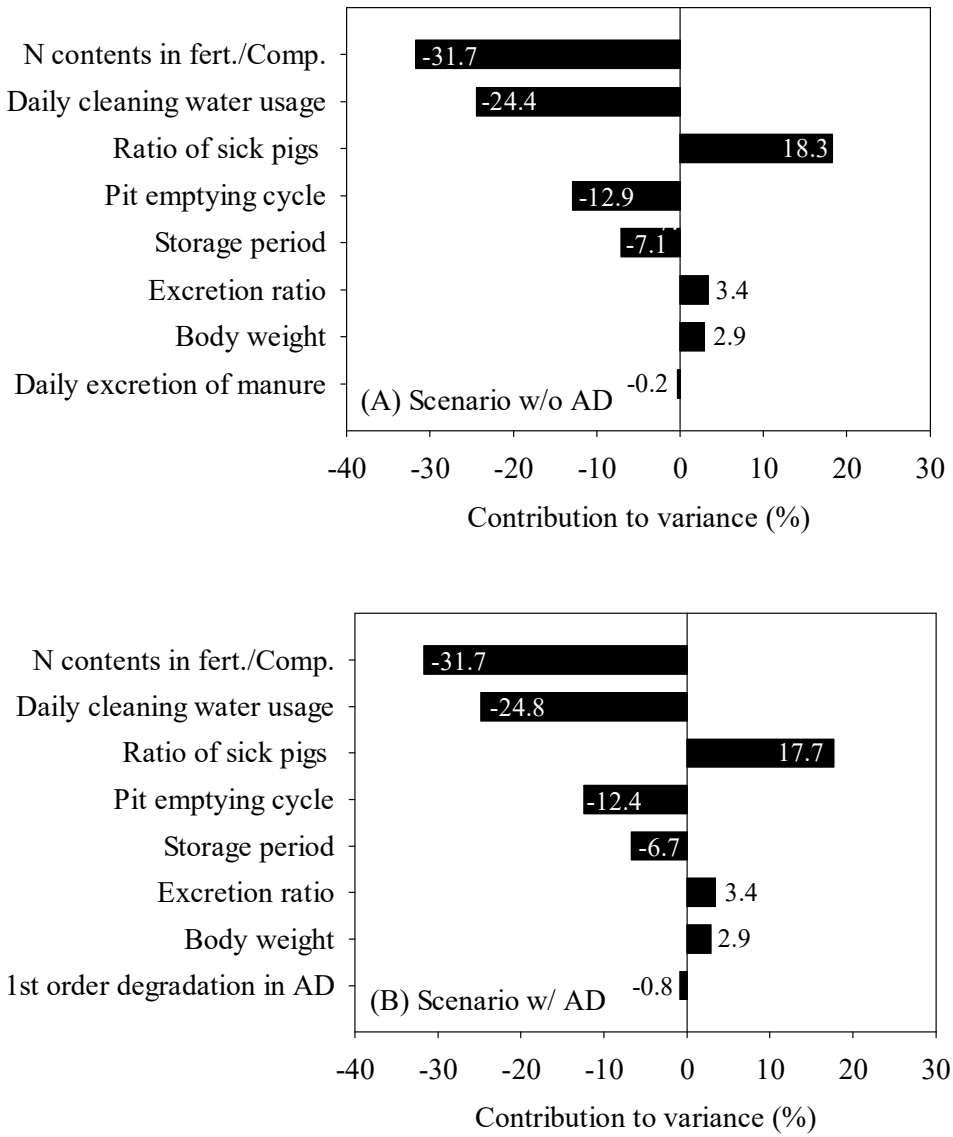

Figure 3.4 Results of sensitivity analysis: contributions of each parameter on RQ; (A) Scenario w/o AD, (B) Scenario w/ AD (N: nitrogen, fert.: fertilizer, comp.: compost, AD: anaerobic digestion)

The seven most sensitive variables can be primarily considered to manage the CTC risk in soil. Among the variables, the storage period is the only variable that can be used for mitigation and management of the risk. Nitrogen content in fertilizer/compost, the ratio of sick pigs requiring CTC, excretion rate, and bodyweight of pigs cannot be controlled. Daily cleaning water usage and pit emptying cycle are not appropriate from a common-sense point of view for controlling the risk. Daily cleaning water usage and pit emptying cycle are affected by all pigs in the farm and farmers would not precisely measure and control those variables for the management of the risk.

The probability of the risk quotient to be over one was simulated depending on the storage period (Fig. 3.5). In the case of scenario 1 (w/o AD), the probability was 25 % when the storage period is zero. It was decreased exponentially decreased to 3 % when the storage period is 120 days (probability = 25.188 * exp(-0.0142*day), R^2 = 0.9853). The probability for scenario 2 (w/ AD) was expected to be 3 % when the storage period is zero and decreased to 0.1 % for day 120 (probability = 3.235 * exp(-0.0249*day), R^2 = 0.9955). For the risk probability of scenario 1 to be less than 10, 5, and 1 %, the storage period should be 65, 113, and 227 days, respectively. For the scenario 2, 47, 75, and 139 days of storage are required to make the risk 1, 0.5, and 0.1 %, respectively. There is currently no standard for the probability of being at risk. The risk exists for the scenario 1 even if the storage periods are set to be 60 –

120 days.

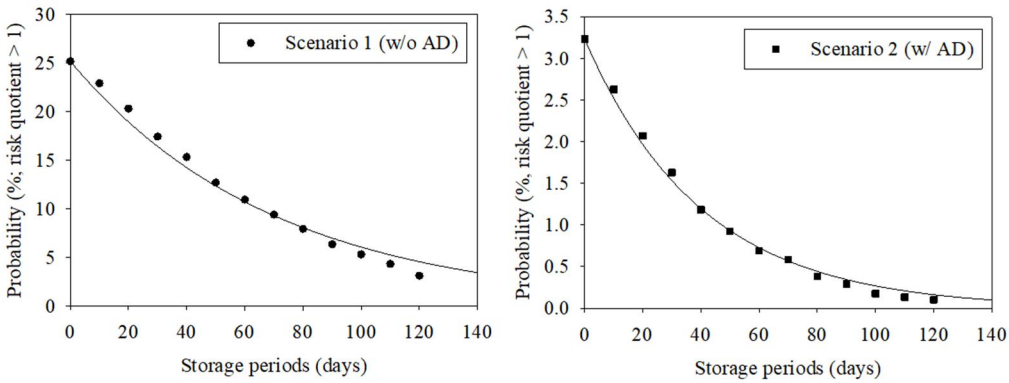

Figure 3.5 Probability of risk quotient to be over one for Scenario 1 (left) and 2 (right)

3.3.5 Limitations

Given the paucity of data, the distributions of some variables were assumed to be uniform. For example, the pit emptying cycle (C_p) and clean water volume (CW) are likely to exist as a normal distribution as pig farming is systemized. However, given the lack of statistical data, minimum-maximum values of available data were assumed for a uniform distribution. In addition, the 1st order biodegradation rates in fertilization/compost for Scenario w/o and w/ AD were assumed to be identical. The biodegradation rate of CTC during fertilization/compost after AD may be different from that w/o AD. Therefore, collection and investigation of insufficient statistics should be performed in conjunction with the development of

systemized risk management methodologies for VAs.

In addition, the ecological risk of CTC on soil microorganisms was evaluated assuming a single farm that manages swine manure with a slurry pit. However, there are cases in which various types of livestock manure, such as cattle manure, swine manure, and poultry manure from many farms, are mixed and managed for AD. In these cases, the farm-specific probability of PEC_{pit} should be reflected together. In addition, the reduction of solid contents during AD was not considered. Volatile solid is reduced during the AD because the organic fraction of swine manure is converted into biogas consisting of methane and carbon dioxide. However, considering that the mass of effluent is mostly liquid, the volatile solid reduction can be negligible for the estimation of PEC_{comp}.

Moreover, given that VAs are mostly distributed to the solid phase of the manure due to their electrostatic characteristics and the liquid/solid content of manure is separated for fertilization/composting, respectively, there must exist uncertainties in phase distribution and various scenarios of liquid/solid separation (Singh, 2016). According to the EMA guidelines, phase distributions of VAs and liquid/solid separation of livestock manure are not considered yet for the estimation of PEC_{manure} and PEC_{soil}. Since the phase distributions and liquid/solid separation directly affect bioavailability and toxicity (Zhang et al., 2014a), consideration of those factors should be prerequisite steps for the development of the estimation and assessment.

Although CTC is considered as a VA to cause the most harmful effect on

the environment, many types of broad-spectrum VAs are consumed for livestock disease treatment. Thus, the ecological risk of other VAs on the soil environment is certain to exist. In other words, although the probability of RQ by CTC in soil exceeding one was less than 10%, the probability of RQ exceeding one is likely to increase due to the presence of other VAs. Nevertheless, the possibility that the probability of RQ exceeding one increases due to the presence of other VAs is expected to be low because broad-spectrum VAs are not used in duplicate, and the amount of veterinary medicines (e.g. vaccines) other than the broad-spectrum VAs is significantly lower than that of broad-spectrum VAs.

3.4 Summary

Consideration of livestock farming practices is necessary for the reliable prediction of veterinary antibiotics concentrations in livestock manure and soil and the characterization of their ecological risks. The predicted environmental concentrations of CTC in the slurry pit and soil were in a range of 0.54 - 5.64 mg/kg_{manure} and 3.42 - 67.59 $\mu g/kg_{soil}$, respectively, for a 90% confidence level. The predicted ranges included the measured values reported in previous studies. The probability of risk quotient (RQ) exceeding one was estimated at 9.3% based on the Monte Carlo simulation. The four most influential factors on the exposure to CTC in soil were identified as nitrogen in fertilizer/compost, daily cleaning water usage, the ratio of sick pigs requiring antibiotics, and pit emptying cycles. For the risk probability of scenario 1 to be less than 10, 5, and 1 %, the storage period should be 65, 113, and 227 days, respectively. For the scenario 2, 47, 75, and 139 days of storage are required to make the risk 1, 0.5, and 0.1 %, respectively. The risk exists for the scenario 1 even if the storage periods are set to be 60 – 120 days. The results indicate that the ecological risk of CTC in the soil is possible and can be significantly affected by slurry pit farm practices. The suggested scenario and uncertainties for evaluating the ecological risk of CTC can be applied to 'Guidance for developing ecological soil screening levels' of US EPA. US EPA requires five assessment factors for evaluating the quality of information obtained from external sources: Soundness,

Applicability and Utility, Clarity and Completeness, and Uncertainty and Variability, and Evaluation and Review (67 FR 57225, 2002).

References

Aga, D. S., Lenczewski, M., Snow, D., Muurinen, J., Sallach, J. B., & Wallace, J. S. (2016). Challenges in the Measurement of Antibiotics and in Evaluating Their Impacts in Agroecosystems: A Critical Review. *Journal of Environment Quality*, *45*(2), 407. https://doi.org/10.2134/jeq2015.07.0393

Álvarez, J. A., Otero, L., Lema, J. M., & Omil, F. (2010). The effect and fate of antibiotics during the anaerobic digestion of pig manure. *Bioresource Technology*, *101*(22), 8581–8586. https://doi.org/10.1016/j.biortech.2010.06.075

Arikan, O. A. (2008). Degradation and metabolization of chlortetracycline during the anaerobic digestion of manure from medicated calves. *Journal of Hazardous Materials*, *158*(2–3), 485–490. https://doi.org/10.1016/j.jhazmat.2008.01.096

Astals, S., Nolla-Ardèvol, V., & Mata-Alvarez, J. (2012). Anaerobic co-digestion of pig manure and crude glycerol at mesophilic conditions: Biogas and digestate. *Bioresource Technology*, *110*, 63–70. https://doi.org/10.1016/j.biortech.2012.01.080

Carballo, M., Aguayo, S., González, M., Esperon, F., & Torre, A. de la. (2016). Environmental Assessment of Tetracycline's Residues Detected in Pig Slurry and Poultry Manure. *Journal of Environmental Protection*, *07*(01), 82–92.

https://doi.org/10.4236/jep.2016.71008

Chenxi, W., Spongberg, A. L., & Witter, J. D. (2008). Determination of the persistence of pharmaceuticals in biosolids using liquid-chromatography tandem mass spectrometry. *Chemosphere*, *73*(4), 511–518. https://doi.org/10.1016/j.chemosphere.2008.06.026

Chopra, I. and Roberts, M. (2001). Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews*, *65*(2), 232–260. https://doi.org/10.1128/MMBR.65.2.232

Daghrir, R., & Drogui, P. (2013). Tetracycline antibiotics in the environment: A review. *Environmental Chemistry Letters*, *11*(3), 209–227. https://doi.org/10.1007/s10311-013-0404-8

Di Guardo, A., & Finizio, A. (2017). Sustainable use of veterinary pharmaceuticals on the territory (Sust-PHarm): Linking available database of manure management and environmental fate models. *Science of the Total Environment*, *575*, 1014–1026. https://doi.org/10.1016/j.scitotenv.2016.09.168

Gelband, H., Miller-Petrie, M., Pant, S., Gandra, S., Levinson, J., Barter, D., White, A., & Laxminarayan, R. (2015). State of the World ' S Antibiotics. *Center for Disease Dynamics, Economics & Policy*.

Grenni, P., Ancona, V., & Barra Caracciolo, A. (2018). Ecological effects of antibiotics on natural ecosystems: A review. *Microchemical Journal*, *136*, 25–39. https://doi.org/10.1016/j.microc.2017.02.006

Grote, M., Vockel, A., Schwarze, D., Mehlich, A., & Freitag, M. (2004). Fate of antibiotics in food chain and environment originating from pigfattening (part 1). *Fresenius Environmental Bulletin*, *13*(11 B), 1216–1224.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P., Ingerslev, F., Holten Lutzheft, H., & Jorgensen, S. (1998). Occurence, Fate and Effects pf PHarmaceutical Substrances in the Environment. *Chemosphere*, *36*(2), 357–393.

Hamscher, G., Sczesny, S., Höper, H., & Nau, H. (2002). Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Analytical Chemistry*, *74*(7), 1509–1518. https://doi.org/10.1021/ac015588m

Huang, C. H., Renew, J. E., Smeby, K. L., Pinkston, K., and Sedlak, D. L. (2011). Assessment of Potential Antibiotic Contaminants in water and preliminary occurrence analysis. In *Journal of Contemporary Water Research and Education* (Vol. 120, Issue 4).

Jacobsen, A. M., & Halling-Sørensen, B. (2006). Multi-component analysis of tetracyclines, sulfonamides and tylosin in swine manure by liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, *384*(5), 1164–1174. https://doi.org/10.1007/s00216-005-0261-9

Jørgensen, H., Prapaspongsa, T., Vu, V. T. K., & Poulsen, H. D. (2013). Models to quantify excretion of dry matter, nitrogen, phosphorus and carbon in growing pigs fed regional diets. *Journal of Animal Science and Biotechnology*, *4*(1), 42. https://doi.org/10.1186/2049-1891-4-42

Kelly, L. A., Taylor, M. A., & Wooldridge, M. J. A. (2003). Estimating the predicted environmental concentration of the residues of veterinary medicines: Should uncertainty and variability be ignored? *Risk Analysis*, *23*(3), 489–496. https://doi.org/10.1111/1539-6924.00329

Kim, K. R., Owens, G., Kwon, S. I., So, K. H., Lee, D. B., & Ok, Y. S. (2011). Occurrence and environmental fate of veterinary antibiotics in the terrestrial environment. *Water, Air, and Soil Pollution*, *214*(1–4), 163–174. https://doi.org/10.1007/s11270-010-0412-2

Kullik, S. A., & Belknap, A. M. (2017). Flexing the PECs: Predicting environmental concentrations of veterinary drugs in Canadian agricultural soils. *Integrated Environmental Assessment and Management*, *13*(2), 331–341. https://doi.org/10.1002/ieam.1833

Kumar, K., C. Gupta, S., Chander, Y., & Singh, A. K. (2005). Antibiotic Use in Agriculture and Its Impact on the Terrestrial Environment. *Advances in Agronomy*, *87*(05), 1–54. https://doi.org/10.1016/S0065-2113(05)87001-4

Kwon, A.-Y., & Kim, S. C. (2015). Estimating Predicted Environmental Concentration of Veterinary Antibiotics in Manure and Soil. *Korean Journal of Soil Science and Fertilizer*, *48*(2), 100–104. https://doi.org/10.7745/kjssf.2015.48.2.100

Lee, C., Jeong, S., Ju, M., & Kim, J. Y. (2020). Fate of chlortetracycline antibiotics during anaerobic degradation of cattle manure. *Journal of Hazardous Materials*, *386*, 121894. https://doi.org/10.1016/J.JHAZMAT.2019.121894

Lee, S.-H., Choi, J.-Y., Yoo, S.-H., Kim, Y. D., & Shin, A. (2015). Estimation of Water Footprint for Livestock Products in Korea. *Journal of The Korean Society of Agricultural Engineers*, *57*(2), 85–92. https://doi.org/10.5389/ksae.2015.57.2.085

Li, Y. X., Zhang, X. L., Li, W., Lu, X. F., Liu, B., & Wang, J. (2013). The residues and environmental risks of multiple veterinary antibiotics in animal faeces. *Environmental Monitoring and Assessment*, *185*(3), 2211–2220. https://doi.org/10.1007/s10661-012-2702-1

Liu, B., Li, Y., Zhang, X., Feng, C., Gao, M., & Shen, Q. (2015). Effects of composting process on the dissipation of extractable sulfonamides in swine manure. *Bioresource Technology*, *175*, 284–290. https://doi.org/10.1016/j.biortech.2014.10.098

Martínez-Carballo, E., González-Barreiro, C., Scharf, S., & Gans, O. (2007). Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. *Environmental Pollution*, *148*(2), 570–579. https://doi.org/10.1016/J.ENVPOL.2006.11.035

Menz, J., Schneider, M., & Kümmerer, K. (2015). Usage pattern-based exposure screening as a simple tool for the regional priority-setting in environmental risk assessment of veterinary antibiotics: A case study of north-western Germany. *Chemosphere*, *127*, 42–48. https://doi.org/10.1016/j.chemosphere.2014.12.091

Paepen, K. A. R. I. S., Eemput, L. E. O. J. J. V. A. N. L., Islocki, P. E. G. W., & Erschueren, C. H. V. (1997). *Hazard / Risk Assessment A UNIFORM*

PROCEDURE TO ESTIMATE THE PREDICTED ENVIRONMENTAL CONCENTRATION OF THE RESIDUES OF VETERINARY MEDICINES IN SOIL. *16*(9), 1977–1982.

Park, S., & Choi, K. (2008). Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology*, *17*(6), 526–538. https://doi.org/10.1007/s10646-008-0209-x

Petersen, S. O., Olsen, A. B., Elsgaard, L., Triolo, J. M., & Sommer, S. G. (2016). *Estimation of Methane Emissions from Slurry Pits below Pig and Cattle Confinements*. https://doi.org/10.1371/journal.pone.0160968

Riaño, B., Molinuevo, B., & García-González, M. C. (2011). Potential for methane production from anaerobic co-digestion of swine manure with winery wastewater. *Bioresource Technology*, *102*(5), 4131–4136. https://doi.org/10.1016/j.biortech.2010.12.077

Romanelli, C, Cooper, D, Campbell-Lendrum, D, Maiero, M, Karesh, W B., Hunter, D, Golden, C, D. (2015). Connecting Global Priorities: Biodiversity and Human Health, a State of Knowledge Review. *World Health Organization and Secretariat for the Convention on Biological Diversity*, 360pp. https://doi.org/10.13140/RG.2.1.3679.6565

Sarmah, A. K., Meyer, M. T., & Boxall, A. B. A. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*, *65*(5), 725–759. https://doi.org/10.1016/j.chemosphere.2006.03.026

Schiavon, S., Dal Maso, M., Cattani, M., & Tagliapietra, F. (2016). *Italian Journal of Animal Science A simplified approach to calculate slurry production of growing pigs at farm level*. https://doi.org/10.4081/ijas.2009.431

Singh, A. K. (2016). Distribution, Sorption and Desorption of Tylosin, Chlortetracycline and their Metabolites in Pig Manure. *Journal of Agricultural Studies*, *4*(4), 65. https://doi.org/10.5296/jas.v4i4.10128

Stone, J. J., Clay, S. A., Zhu, Z., Wong, K. L., Porath, L. R., & Spellman, G. M. (2009). Effect of antimicrobial compounds tylosin and chlortetracycline during batch anaerobic swine manure digestion. *Water Research*, *43*(18), 4740–4750. https://doi.org/10.1016/j.watres.2009.08.005

Thiele-Bruhn, S., & Beck, I.-C. (2005). Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. *Chemosphere*, *59*(4), 457–465. https://doi.org/10.1016/J.CHEMOSPHERE.2005.01.023

United states department of agriculture. (2015). Swine 2012 Part II. *United States Department of Agriculture*.

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A., & Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences*, *112*(18), 5649–5654. https://doi.org/10.1073/pnas.1503141112

Wilkie, A. (2005). Anaerobic digestion of dairy manure: Design and process considerations. *Dairy Manure Management: Treatment, Handling, …*, 301–312. https://biogas.ifas.ufl.edu/Publs/NRAES176-p301-312-Mar2005.pdf

Winckler, C., & Grafe, A. (2001). Use of veterinary drugs in intensive animal production: Evidence for persistence of tetracycline in pig slurry. *Journal of Soils and Sediments*, *1*(2), 66–70. https://doi.org/10.1007/BF02987711

Won, S., You, B.-G., Shim, S., Ahmed, N., Choi, Y.-S., & Ra, C. (2018). Nutrient variations from swine manure to agricultural land. *Asian-Australas J Anim Sci*, *31*(5), 763–772. https://doi.org/10.5713/ajas.17.0634

Zhang, H., Luo, Y., Wu, L., Huang, Y., & Christie, P. (2015). Residues and potential ecological risks of veterinary antibiotics in manures and composts associated with protected vegetable farming. *Environmental Science and Pollution Research*, *22*(8), 5908–5918. https://doi.org/10.1007/s11356-014-3731-9

Zhang, Y., Boyd, S. A., Teppen, B. J., Tiedje, J. M., & Li, H. (2014). Role of tetracycline speciation in the bioavailability to escherichia coli for uptake and expression of antibiotic resistance. *Environmental Science and Technology*, *48*(9), 4893–4900. https://doi.org/10.1021/es5003428

Zhao, L., Dong, Y. H., & Wang, H. (2010). Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. *Science of the Total Environment*, *408*(5), 1069–1075. https://doi.org/10.1016/j.scitotenv.2009.11.014

Zhu, N. (2006). Composting of high moisture content swine manure with corncob in a pilot-scale aerated static bin system. *Bioresource Technology*, *97*(15), 1870–1875. https://doi.org/10.1016/J.BIORTECH.2005.08.011

Zhu, N. (2007). Effect of low initial C/N ratio on aerobic composting of swine

manure with rice straw. *Bioresource Technology*, *98*(1), 9–13. https://doi.org/10.1016/J.BIORTECH.2005.12.003

CHAPTER 4
TRANSFORMATION OF CHLORTETRACYCLINE IN SWINE MANURE AND ITS ANAEROBIC DIGESTATE

4.1 Introduction

Veterinary antibiotics have been used not only for treating and preventing disease but also for promoting the growth of livestock. Some countries such as EU members, the Republic of Korea, and Mexico banned the use for growth promotion; however, most countries still allow the use of veterinary antibiotics as food additives by voluntary guidelines or without regulations. Due to the absence of regulations, the consumption of veterinary antibiotics is steadily increasing with the increase of meat consumption and a shift from extensive to intensive farming over the world (Gelband et al., 2015). The usage in the livestock industry in 2030 was expected to increase by 60 % (105,600 tons) compared to that in 2010.

Tetracycline (TC) is the most commonly used veterinary antibiotics due to its low price and broad-spectrum antibiotic effect. TC accounted for 67 % of total sales (6,189,260 kg) among seven veterinary antibiotics (i.e. aminoglycosides, amphenicols, cephalosporins, fluoroquinolones, lincosamides, macrolides, penicillins, sulfas, and tetracyclines) approved in the United States in 2019 (FDA,

2020). Since TC is not fully metabolized by livestock, it can be excreted with livestock manure and milk (Arikan, 2008; Fritz and Zuo, 2007). Around 20 – 70 % of the administered TC is excreted within 15 days (Arikan, 2008; Grote et al., 2004; Kwon and Kim, 2015; Stone et al., 2009; Winckler and Grafe, 2001). In chapter 3, it was estimated that 5^{th}- 95^{th} percentile concentrations of CTC in swine manure and soil are in a range of 0.5 to 5.6 mg/kg and 3.4 to 67.6 μg/kg, respectively, by considering uncertainties of the administration and farming conditions(Lee et al., 2021)(Lee et al., 2021). The ubiquitousness of TC can result in the prevalence of antibiotics resistance genes (ARGs) and bacteria (ARB). It treats the sustainability and resilience of microbial communities in the environment (Leng et al., 2016; Ramanan et al., 2021; Van Boeckel et al., 2014).

TC can exist as various transformation products by undergoing intramolecular changes. There are 12 types of transformation products with five chiral centers. Since Doerschuk et al. (1955) first discovered the existence of an epimer of TC in 1995, pharmacologists have mainly considered epimers, isomers, epi-isomers, and anhydrous products as the transformation production focusing on the changes in aqueous conditions (Doerschuk et al., 1955; HUSSAR et al., 1968; McCormick et al., 1957a, 1956; Remmers et al., 1963; Sokoloski et al., 1977; Yuen and Sokoloski, 1977). 4-Epi-tetracycline (ETC) is a diastereomer produced by the reversible change in bonding angle of the C4 dimethyl amino group. Cleavage of the C6 hydroxyl group irreversibly leads to the formation of iso-tetracycline (ITC) which is a structural isomer (Huang and Chen, 2009). 4-Epi-iso-tetracycline is the epimer

form of ITC. The anhydrous tetracycline (ATC) is formed when H_2O is removed from tetracycline (McCormick et al., 1957b). These transformation products maintain antimicrobial activities of the parent compound (i.e. tetracycline). ETC can reversibly be transformed into TC in mild acidic conditions (pH 4 – 6) (Zhao et al., 2020a). The European Union set maximum residual limits in food-producing animals for chlortetracycline (CTC) and 4-epi-chlortetracycline (Kennedy et al., 1998). ITC can bind to Tet Repressor, which regulates the expression of the cell membrane to efflux tetracycline resistance, and cause expression of ARGs (Volkers et al., 2011). ATC showed toxicity equal to or less than that of TC (visible plate counting, 48 h) (Halling-Sørensen et al., 2002). In this regard, there has recently been a great interest in the transformation products of TC from tissues, processed meat, and environmental media (e.g. manure slurry and terrestrial/aquatic systems) (Álvarez et al., 2010; Arikan, 2008; Blanchflower et al., 1997; Cessna et al., 2020; Chen and Huang, 2011, 2010; Dzomba, 2016; Fritz and Zuo, 2007; Gaugain et al., 2015; Grote et al., 2004; Huang and Chen, 2009; Kennedy et al., 1998; Kennedy, 2008; Loftin et al., 2008; Qiao et al., 2012; Shelver and Varel, 2012; Singh, 2016; Søeborg et al., 2004; Spielmeyer et al., 2016; Zhao et al., 2020b). From the first literature for the discovery of epimer to the recent study on the transformation of tetracycline in the environmental media, studies in which test matrix and relative proportion of TC and its transformation products can be determined were shown in Table 2.4.

Understanding their fate in environmental pathways is a prerequisite step to prevent, mitigate and manage the prevalence of tetracyclines and transformation

products. The occurrences of the transformation products in manure, soil, and water matrix were the major research focus, whereas information regarding transformation kinetics and equilibrium is still largely lacking in available literature (Table 2.4). Since the epimerization and isomerization happen simultaneously and affect each other, all transformation products generated in a certain matrix should be identified and interpreted together to figure out transformation kinetics and equilibrium. Transformation products were measured selectively in some studies (Álvarez et al., 2010; Loftin et al., 2008; Shelver and Varel, 2012; Spielmeyer et al., 2016). These might make it difficult to assess the fate of transformation products of tetracycline. The kinetics of the transformation can be evaluated by ordinary differential equations. Yuen and Sokoloski (1977) suggested an analytical solution for concentrations of TC, ETC, ATC, and Epi-ATC in pH 1.5 of phosphoric acid solution. However, to the best of our knowledge, assessment of transformation kinetics in livestock manure has little been documented.

In this regard, this study aimed to investigate the transformation of CTC in swine manure and anaerobic digestate, which are the primary matrices containing veterinary antibiotics, to assess the transformation kinetics in the matrices, and to predict their exposure concentration through anaerobic digestion of swine manure. 4-epi-chlortetracycline (ECTC), 6-iso-chlortetracycline (ICTC), Epi-ICTC (EICTC), Anhydrous-CTC (ACTC) were evaluated as the transformation products.

4.2 Materials and methods

4.2.1 Chemicals and sludge

A diluted stock solution of chlortetracycline hydrochloride (CAS no. 64-72-2, Sigma-Aldrich, USA), 4-epi-chlortetracycline hydrochloride (CAS no. 101342-45-4, Carbosynth, UK), iso-chlortetracycline (CAS no. 101342-45-4, Carbosynth, UK), and anhydro-chlortetracycline hydrochloride (CAS no. 65490-24-6, Carbosynth, UK) were used as standard samples for the quantification and qualification using high-pressure liquid chromatography (HPLC) equipped with sing quadrupole mass spectrometry (MS).

Swine manure was captured from a composting facility located in the Republic of Korea and kept at a -20 ℃ refrigerator before use. Anaerobic digestate of swine manure was obtained from a lab-scale continuously-stirred tank reactor treating swine manure for three years (Organic loading rate = 2.1 g-VS/L/day; Total volume = 10 L, working volume 8 L, and hydraulic retention time = 20 days).

4.2.2 Transformation tests

Totally five media were applied for the transformation tests: D.I water (DI); Swine manure (SM); anaerobic digestate of swine manure (AD); autoclave-treated anaerobic digestate (AAD); 0.45 μm-filtered and autoclave-treated anaerobic digestate (FAAD). In addition to swine manure and its anaerobic digestate, the DI assay was set for the control assay. AAD assay was established for abiotic control but methane was generated from the assay. Accordingly, only effects of matrix characteristics on the transformation was discussed. FAAD assay was intended to identify whether solid content affects transformation. Each assay was set by adding 175 mL of each medium to a 265 mL serum bottle. Autoclaving was conducted at 120 ℃ and 0.192 MPa for 30 minutes. The test concentration of CTC was 10 mg-CTC/L. It was estimated to make the concentrations of transformation products over detection limits by considering expected transformation rate and dilution during extraction. The pH of each assay was adjusted to 6.9 – 7.2 using HCl and NaOH before injecting a CTC stock into each assay. The experiment was initiated after the injection. All assays were purged with N_2 gas, closed by rubber stoppers and aluminum caps, and stored in a dark constant temperature agitator. Based on a preliminary test on equilibrium time, samples for analysis of CTC, its transformation products, and change of solution chemistry were collected after 4, 12, 21, 38, 112, 180, and 480 hours from each assay. At the time of each sampling, 4 mL of sample was obtained and stored in a 15 mL polypropylene tube at -20 ℃ refrigerator under

darkroom conditions until analysis. The assays were closed and stored by the above same manner. It has been confirmed that the polyethylene tubes are suitable for the experiment because CTC was not adsorbed or decomposed in the polyethylene tubes at -20 ℃ and dark conditions (Ciarlone et al., 1990; Shelver and Varel, 2012; Søeborg et al., 2004). All analyses were carried out within 10 days of sampling.

4.2.3 Quantification and qualification of CTC

As performed by Álvarez et al. (2010), Arikan (2008), and Lee et al. (2020), CTC, ECTC, and ICTC, and ACTC in the samples obtained on each sampling day were extracted by a series of ultrasonic solvent extraction (USE) and solid-phase extraction (SPE) and measured by using the HPLC-MS (Agilent 1260, Agilent Technologies, Inc., USA). More details on the USE, SPE, and HPLC-MS analysis were followed by the author's previous study (Lee et al., 2020). When D.I water was used for USE, the concentrations were considered as water-soluble concentrations. When 0.1 M Na_2-EDTA buffer was applied to USE, those were regarded as buffer-soluble concentrations. The difference between the buffer- and water-soluble concentration was utilized for the evaluation of mass fraction in solid and liquid phases.

It was confirmed that peaks of the ECTC, ICTC, and ACTC standards can be qualitatively separated with that of CTC using the HPLC and quantified using the MS with SIM mode at *m/z* 479. Inevitable epimerization and isomerization occurred during the time for the HPLC-MS analysis (Grote et al., 2004). In other words, additional peaks appeared in addition to the targeted standard's peak. It was around 1 – 15 % (w/w) of the targeted compound (data not shown). EICTC which was not used for making calibration curves was identified from the ICTC standard. A peak having the same retention time with ECTC was generated from the ICTC standard. Since ICTC is irreversibly transformed from CTC and can be reversibly transformed

into EICTC, the peak was considered not as ECTC but as EICTC (Kennedy et al., 1998). It was proved by identifying daughter ion generation from HPLC-MS/MS. ECTC generated fragment ions at *m/z* 479, 444, and 462 but EICTC produced the fragment ions at *m/z* 479, and 462 which are the same fragment ions observed from ICTC (Fig. 4.2).

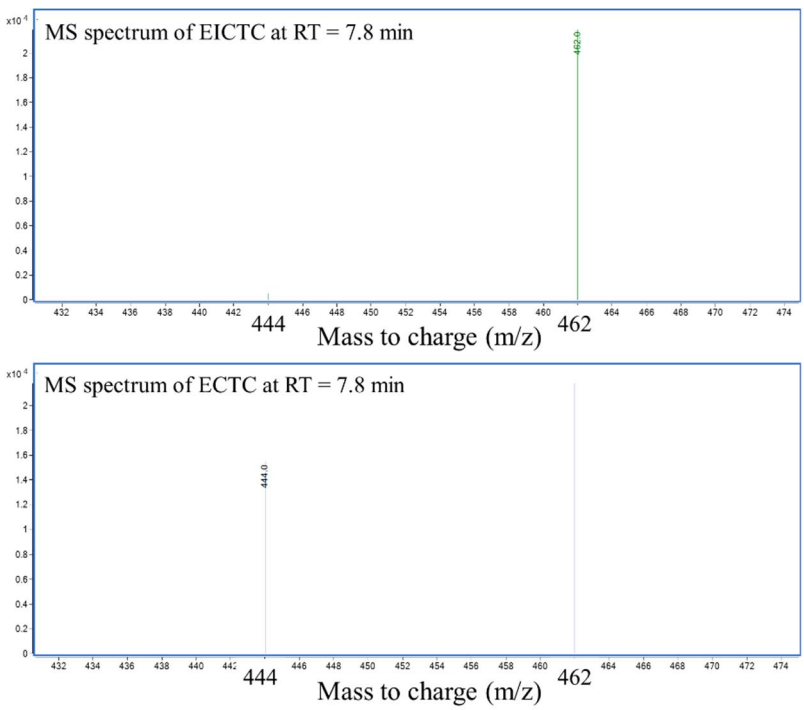

Figure 4.1 MS spectrum of ECTC and EICTC obtained from ICTC standard

The sum of the peak area of the target compound and the transformation products were used to obtain a calibration curve. The calibration curve for each standard represented linearity with $R^2 > 0.98$. The low- (0.05 – 0.50 mg/L for CTC;

0.05 – 0.30 mg/L for ECTC and ICTC) and high-range (0.25 – 1.00 mg/L for CTC; 0.30 – 1.00 mg/L for ECTC and ICTC) calibration curves were prepared for each compound and used to quantify water-soluble and buffer-soluble concentrations included in each range. Limit of quantification (LOQ) was determined as 0.05 mg/L according to the lowest concentration that is distinguishable from noise by 99% of confidence level (10 times of signal to noise).

Table 4.1 Summary of HPLC-MS analysis for quantification of CTC, ECTC, ICTC, and ACTC

Compounds	Retention time (mins)	m/z	Low-range calibration curve (mg/L)	High-range calibration curve (mg/L)	LOQ (mg/L)
CTC	8.5	479	0.05 – 0.50	0.25 – 1.00	0.05
ECTC	7.8	479	0.05 – 0.30	0.30 – 1.00	0.05
ICTC	8.2	479	0.05 – 0.30	0.30 – 1.00	0.05
ACTC	12.2	461	0.05 – 0.30	0.30 – 1.00	0.05

4.2.4 Kinetic analysis

Based on the identification of transformation products, simultaneous linear ordinary differential equations were established by considering CTC, ECTC+ EICTC, and ICTC and assuming 1st order kinetics of epimerization and isomerization (Eq. 4.1 – 4.3). It has been expected that the degradation, epimerization, and isomerization of tetracyclines followed the 1st order kinetic model (Álvarez et al., 2010; Yuen and Sokoloski, 1977). ECTC, ICTC, and EICTC were

identified in all assays but not ACTC. EICTC could not be quantified because its peak was overlapped with that of ECTC and there is a lack of commercially available EICTC standard. ICTC represented the same peak area to concentration relationship with ECTC. The slopes of the low-range calibration curve for ICTC and ECTC were statistically the same (421,217 ± 54,863 and 401,139 ± 12,840 area/mg/L, respectively). When ICTC was transformed into EICTC, the sum of two peaks was maintained (Fig. 4.2). These indicate that EICTC had the same HPLC-MS peak area to concentration relationship with ICTC because epimerization occurs according to a 1 to 1 molar ratio. Thus, the concentration measured by using the low-range calibration curve for ECTC was regarded as the sum of ECTC and EICTC concentrations.

The EICTC could not be separately quantified in this study. It is simply possible if its peak is separated from that of ECTC but the separation conditions (types of eluent, flow rate, type of column, etc.) did not provide the separated ECTC and EICTC peaks. The EICTC also can be quantified by using the differences in the generation of daughter ions. ECTC generated fragment ions at *m/z* 479, 444, and 462 but EICTC produced the fragment ions at *m/z* 479, and 462 which are the same fragment ions observed from ICTC (Fig. 4.2). The concentration of ECTC+EICTC and ECTC can be measured at m/z 479 and 444, respectively and the difference is the concentration of EICTC. However, the m/z 444 of ECTC was not uniformly generated from the ECTC standard solution.

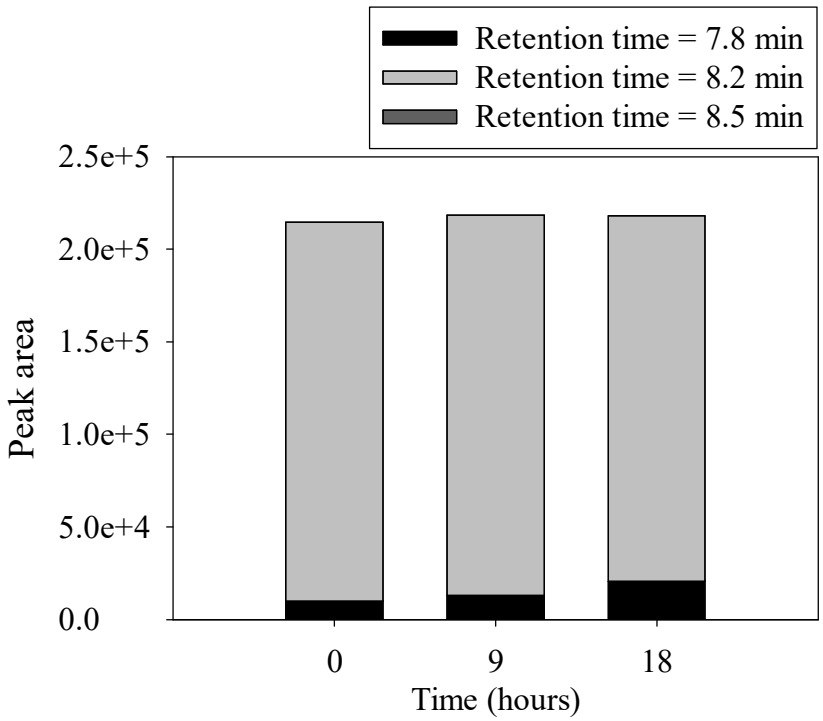

Figure 4.2 Change in HPLC-MS peak area of ICTC in pH 4.5

The below simultaneous linear ordinary differential equations can be solved by the characteristic equation. The eq. 4.1 – 4.3 have an solution because the number of equations equals to the number of variables and equations are independent each other (i.e. non-singular matrix). In addition, an initial condition was provided for the batch test. The analytical solutions for CTC, ECTC+EICTC, and ICTC were obtained via a series of Python codes. The codes can be provided by the author upon request.

Differential evolution, which is a population-based metaheuristic search algorithm for nonlinear and non-differentiable continuous functions, was applied to

estimate the kinetic constants. Global minimum for the error function using innumerable population of kinetic constants can be found effectively because the differential evolution iteratively improves a candidate solution by making mutations and retaining the better candidate solutions that represent a lower error (Bilal et al., 2020; Georgioudakis and Plevris, 2020; Storn and Price, 1997). The minimum and maximum values for each constant were conservatively set as 0.000001 and 3 h^{-1}, respectively, based on estimated values in previous studies (Table 2.4). Error function was set as sum of squares of deviation. The differential evolution starts with a random initial candidate. It can generate a different result in every simulation for given simulation parameters (e.g. evolution strategy, population size, mutation constant). Each simulation for parameter estimation was conducted ten times to identifiy whether the simulation represents the same results for each differential evolution and global minimum was found.

Since totally eight parameters need to be estimated simultaneously, differential evolution may require heavy iteration and/or it would be difficult to set candidate solutions. The equilibrium between the buffer-extractable concentrations of CTC, ECTC+EICTC, and ICTC was used to reduce the number of parameters. Two relationships (i.e. CTC vs ECTC+EICTC and CTC vs ICTC (or ECTC+EICTC vs ICTC)) can be obtained from observed equilibrium (Eq. 4.4 and 4.5) and the three degradation kinetic constants (i.e. $k_{d,c}$, $k_{d,e}$, $k_{d,i}$) were assumed to be zero when the sum of concentrations of CTC and transformation products was constant. Thus, only three constants were required to be estimated by fitting the analytical solutions to

observed results among the five kinetic constants (i.e. k_{tce}, k_{tci}, k_{tec}, k_{tei}, and k_{tie}) (Eq. 4.6 – 4.8)

The buffer-extractable concentrations of CTC, ECTC+EICTC, and ICTC from the batch tests were used to identify whether the batch mode model demonstrates the epimerization and isomerization well. Additionally, buffer-extractable concentrations of CTC, ECTC+EICTC, and ICTC were obtained from a 10 L of lab-scale continuous-stirred tank reactor (CSTR) for 250 days to calibrate the coefficients (Fig. 5.1). Analytical solutions for CSTR-mode were calculated by using Eq. 4.9 – 4.11. The CSTR treated the swine manure with 2.5 g-VS/L/day of organic loading rate. The swine manure was spiked with CTC and the concentration in influent was 3 mg-CTC. The effective volume was 8 L and its hydraulic retention time was 20 days.

The batch-mode analytical solutions were verified using three cases prior to fitting to the experimental results. All kinetic constants were 10^{-9} h^{-1} for Case 1. Epimerization kinetic constants ($k_{t,ce}$ and $k_{t,ie}$) were assumed much higher (10^{-2} h^{-1}) than isomerization kinetic constants ($k_{t,ci}$ and $k_{t,ei}$) (10^{-9} h^{-1}) for Case 2 and vice versa for Case 3. For all cases, initial concentrations of CTC, ECTC+EICTC, and ICTC were set as 10, 6, and 3 mg/L, respectively. The concentrations of CTC, ECTC+EICTC, and ICTC were simulated for 500 hours.

Batch mode

$$\frac{dC_{ctc}}{dt} = -\left(k_{d,c} + k_{t,ce} + k_{t,ci}\right) * C_{ctc}(t) + k_{t,ec} * C_{ectc+eictc}(t)$$

Equation 4.1

$$\frac{dC_{ectc+eictc}}{dt} = -\left(k_{d,e} + k_{t,ec} + k_{t,ei}\right) * C_{ectc+eictc}(t) + k_{t,ce} * C_{ctc}(t) + k_{t,ie} * C_{ictc}(t)$$

Equation 4.2

$$\frac{dC_{ictc}}{dt} = -(k_{d,i} + k_{t,ie}) * C_{ictc}(t) + k_{t,ci} * C_{ctc}(t) + k_{t,ei} * C_{ectc+eictc}(t)$$

Equation 4.3

If equilibrium is observed and total concentration is conserved,

$$k_{t,ec} = \frac{C_{ctc,eq} * (k_{t,ce} + k_{t,ci})}{C_{ectc+eictc,eq}}$$

Equation 4.4

$$k_{t,ei} = \frac{(k_{t,ce} * C_{ctc,eq} + k_{t,ie} * C_{ictc,eq})}{C_{ectc+eictc,eq}} - \frac{C_{ctc,eq} * (k_{t,ce} + k_{t,ci})}{C_{ectc+eictc,eq}}$$

Equation 4.5

$$\frac{dC_{ctc}}{dt} = -\left(k_{t,ce} + k_{t,ci}\right) * C_{ctc}(t) + \frac{C_{ctc,eq} * (k_{t,ce} + k_{t,ci})}{C_{ectc+eictc,eq}} * C_{ectc+eictc}(t)$$

Equation 4.6

$$\frac{dC_{ectc+eictc}}{dt} = -\left(\frac{(k_{t,ce} * C_{ctc,eq} + k_{t,ie} * C_{ictc,eq})}{C_{ectc+eictc,eq}}\right) * C_{ectc+eictc}(t) + k_{t,ce} * C_{ctc}(t) + k_{t,ie} * C_{ictc}(t)$$

Equation 4.7

$$\frac{dC_{ictc}}{dt} = -(k_{t,ie}) * C_{ictc}(t) + k_{t,ci} * C_{ctc}(t) + \left(\frac{(k_{t,ce} * C_{ctc,eq} + k_{t,ie} * C_{ictc,eq})}{C_{ectc+eictc,eq}} - \frac{C_{ctc,eq} * (k_{t,ce} + k_{t,ci})}{C_{ectc+eictc,eq}}\right) * C_{ectc+eictc}(t)$$

Equation 4.8

CSTR mode

$$\frac{dC_{ctc}}{dt} = \frac{C_{in,c}}{\theta} - \left(\frac{1}{\theta} + k_{d,c} + k_{t,ce} + k_{t,ci}\right) * C_{ctc}(t) + k_{t,ec} * C_{ectc+eictc}(t)$$

Equation 4.9

$$\frac{dC_{ectc+eictc}}{dt} = \frac{C_{in,e}}{\theta} - \left(\frac{1}{\theta} + k_{d,e} + k_{t,ec} + k_{t,ei}\right) * C_{ectc+eictc}(t) + k_{t,ce} * C_{ctc}(t) + k_{t,ie} * C_{ictc}(t)$$ Equation 4.10

$$\frac{dC_{ictc}}{dt} = \frac{C_{in,i}}{\theta} - (\frac{1}{\theta} + k_{d,i} + k_{t,ie}) * C_{ictc}(t) + k_{t,ci} * C_{ctc}(t) + k_{t,ei} * C_{ectc+eictc}(t)$$

Equation 4.11

where, C_{α} = Concentration of α (mg/L); $C_{in,\alpha}$ = concentration of α in influent of CSTR; $C_{\alpha,eq}$ = Concentration of α at equilibrium (mg/L); θ = hydraulic retention time (h); $k_{d\alpha}$ = degradation rate constant of α (h^{-1}); $k_{t,\alpha\beta}$ = epimerization or isomerization rate constant of α to β (h^{-1}); α and β = CTC or ECTC+EICTC or ICTC;

4.2.5 Analytical methods

As physicochemical parameters of matrices, pH, proximate analysis (water, volatile solid, and fixed solid contents), total and soluble chemical oxygen demand (TCOD and SCOD), the concentrations of NH_4^+, PO_4^{3-}, and divalent cations (Ca^{2+} Mg^{2+} Fe^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}) were analyzed and listed in Table 4.2. The CODs, NH_4^+, and PO_4^{3-} were measured using waster analysis kits (Humas, Republic of Korea) based on EPA 410.4, 350.1, and 365.1, respectively. The divalent metals (Ca^{2+} Mg^{2+} Fe^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}) were analyzed by using an inductively coupled plasma equipped with optical emission spectroscopy (icap 7400 duo icp-oes system, Thermo Scientific, U.S.A).

Table 4.2 Physicochemical properties of each assay

	D.I water (DI)	Swine manure (SM)	Anaerobic digestate (AD)	Autoclaved anaerobic digestate (AAD)	Filtered and autoclaved anaerobic digestate (FAAD)	Unit
pH	6.9 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	-
Water content	100.0 ± 0.0	94.3 ± 0.2	95.2 ± 0.6	96.5 ± 0.1	99.6 ± 0.0	wt. % (wet basis)
Volatile solid	-	3.7 ± 0.2	2.7 ± 0.6	1.9 ± 0.2	0.2 ± 0.0	
Fixed solid	-	2.0 ± 0.0	2.1 ± 0.2	1.5 ± 0.3	0.2 ± 0.0	
TCOD	-	42.0 ± 2.0	22.0 ± 2.0	19.0 ± 1.7	1.4 ± 0.2	g/L
SCOD	-	3.6 ± 0.2	1.9 ± 0.8	4.5 ± 1.0	1.2 ± 0.2	
NH_4^+	-	1,093 ± 82	1,289 ± 75	810 ± 73	387 ± 46	mg/L
PO_4^{3-}	-	856 ± 18	751 ± 24	620 ± 27	51 ± 7	
Ca^{2+}	-	62.20 ± 15.98	12.44 ± 1.98	96.20 ± 20.20	28.46 ± 2.31	
Mg^{2+}	-	13.80 ± 7.90	13.26 ± 3.51	52.94 ± 16.54	39.93 ± 2.93	
Fe^{2+}	-	1.28 ± 0.24	3.21 ± 0.84	3.89 ± 0.60	1.06 ± 0.08	
Mn^{2+}	-	0.13 ± 0.09	0.05 ± 0.01	0.53 ± 0.09	0.03 ± 0.00	
Zn^{2+}	-	0.17 ± 0.02	0.35 ± 0.03	0.79 ± 0.24	0.41 ± 0.04	
Cu^{2+}	-	0.13 ± 0.03	0.17 ± 0.05	0.25 ± 0.01	0.30 ± 0.05	

4.2.6 Statistical analysis

All assays and analyses were conducted in triplicate. Statistical differences between two data groups were evaluated according to the Student's *t*-test with a 5% significance level. The statistical tests were performed by using SigmaPlot 12.0 Software (Systat Software, Inc., USA). In order to explore factors affecting the transformation, Pearson and Spearman correlation analyses were conducted by using the relative ratio of CTC, ECTC+EICTC, and ICTC and the physicochemical properties. The bivariate analyses were performed using SPSS 26.0 software (SPSS Inc., Chicago, USA).

4.3 Results and discussion

4.3.1 Equilibrium between transformation products

The water- and buffer-soluble concentration of CTC, ECTC+EICTC, and ICTC in each assay were shown in Fig. 4.4. In order to verify whether the USE and SPE processes caused chemical degradation and transformation, water- and buffer-soluble concentrations in D.I water assay were compared. The sum of water-soluble concentrations and that of buffer-soluble concentrations at each sample point were statistically the same ($p < 0.05$) implying that no chemical degradation occurred via the extraction processes. When compared the concentrations of CTC, ECTC+EICTC, and ICTC, respectively, the water- and buffer-soluble concentrations of some samples represented statistical differences. The differences were average 0.46 mg/L (minimum 0.2 mg/L and maximum 0.8 mg/L) but did not affect trends of changes of CTC, ECTC+EICTC, and ICTC concentrations. It also can be due to the inevitable transformation during the time for HPLC-MS analysis as observed from standard solutions.

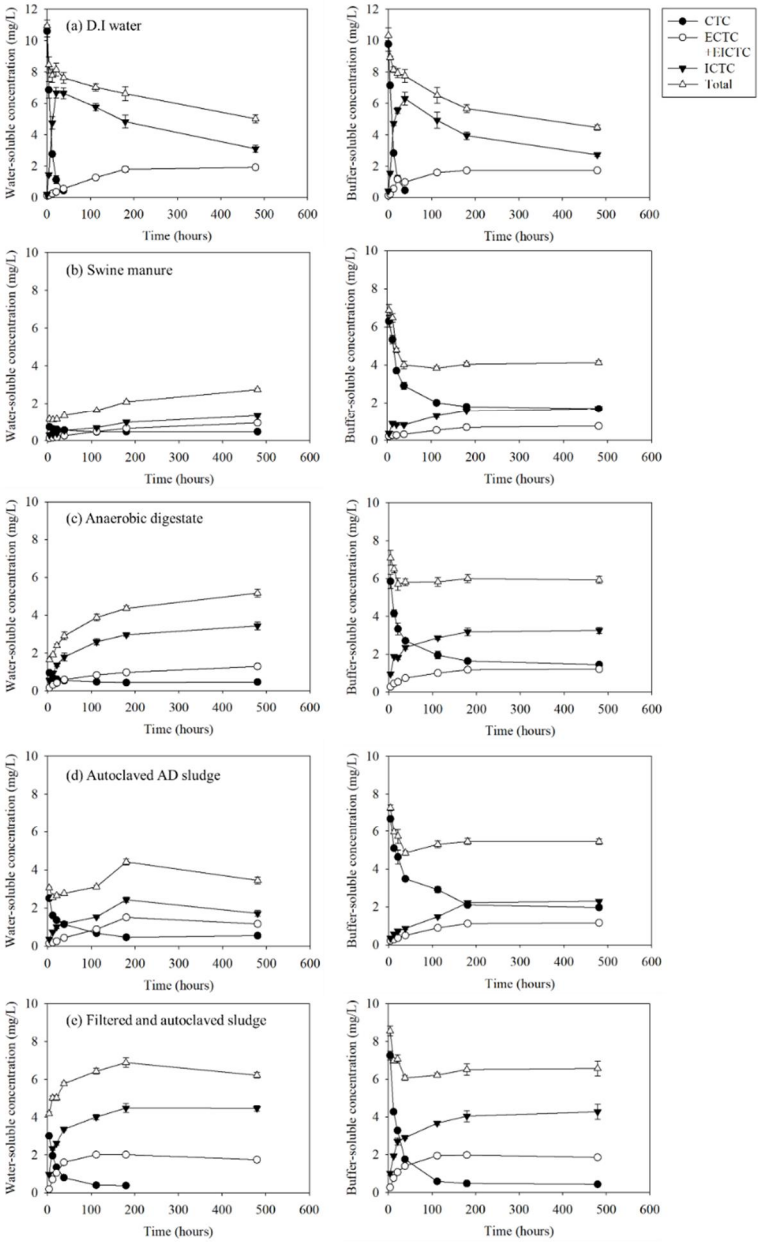

Figure 4.3 Water- (left column) and buffer-soluble (right column) concentration of CTC, ECTC+EICTC, and ICTC in (a) DI water, (b) Swine manure, (c) anaerobic digestate of swine manure, (d) the autoclave-treated anaerobic digestate, and (e) the autoclave-treated and 0.45 μm-filtered anaerobic digestate

The equilibriums between CTC, ECTC+EICTC, and ICTC in the SM, AD, AAD, and FAAD assays were achieved after 180 hours (Fig. 4.5). The ratios for each assay observed during 480 hours were described in Appendix S4.1. CTC rarely remained in DI and FAAD assays where solid contents were removed. In the SM, AD, and AAD assays, CTC was left in a range of 25 – 41 % (by wt.). The percentages of ECTC+EICTC and ICTC were 18 – 39 and 42 – 65 % (by wt.), respectively (Fig. 4.6). Adsorption of CTC onto the surface of solid-phase prevented the epimerization and isomerization because functional groups of CTC where the epimerization and isomerization occurs are fixed through the adsorption. CTC is adsorbed by electrostatic interactions such as cation bridging and cation exchange (Pollard and Morra, 2017; Pulicharla et al., 2017). Cation bridging is caused by multivalent cations that can bind with negatively charged functional groups of biosolids and CTC together. The tricarbonyl (pK_a = 3.3) and dimethyl amino group (pK_a = 7.5) groups of CTC can represent negative charges in neutral pH (6 - 8). The C11 of the phenolic β-diketone group is connected to the C6 of CTC when the isomerization occurs. Cation exchange happens when a positive amine group (C4) or phenolic β-diketone group (C11) of CTC is exchanged with monovalent cations and bonded to the negatively charged surface of biosolids. The C4 is the position where the epimerization occurs. These findings are in line with previous studies analyzing TC's transformation products in livestock manure and soil. CTC remained with ECTC and ICTC in the presence of biosolids (C. Lee et al., 2020b; Shelver and Varel, 2012; Spielmeyer et al., 2016) while most of CTC were transformed into ECTC and ICTC

in aqueous conditions without solids (Chen and Huang, 2011; Søeborg et al., 2004).

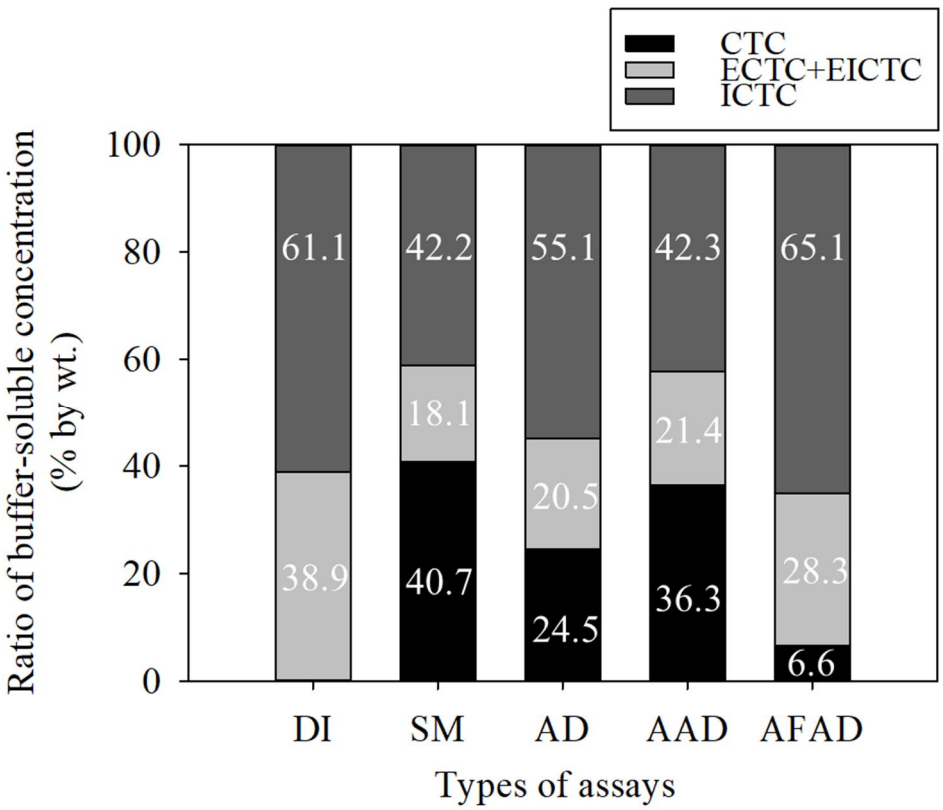

Figure 4.4 Ratio between buffer-concentrations of CTC, ECTC, and ICTC in DI water (DI), swine manure (SM), anaerobic digestate of swine manure (AD), the autoclave-treated anaerobic digestate (ADD), and the autoclave-treated and 0.45 μm-filtered anaerobic digestate (AFAD) assays at 480 hours

Parameters such as pH, phosphate, and divalent cations in each assay can affect the degree of epimerization and isomerization in an aqueous solution. The AAD assay represented higher divalent cations and SCOD, and lower NH_4^+ and PO_4^{3-} than those of the AD assay due to the autoclaving. The SM assay had higher

SCOD and PO_4^{3-} and lower NH_4^+ than those of the AD assay (Table 4.2). Pearson's coefficients indicated that total solid had a positive correlation with the ratio of CTC in the equilibrium state. According to Remmers et al. (1963), phosphate buffer boosted the epimerization of TC in aqueous conditions at pH 4 but it was the opposite in this study. Given that the epimerization rate was fast at pH 3 – 5 regardless of buffer addition, the neutral pH and presence of solid contents of this study contributed to the differences in the epimerization. Among divalent cations, Cu^{2+} was in positive relationship with the ECTC ($p < 0.05$) (Table 4.3). Hussar et al. (1968) reported that Cu^{2+} promoted degradation of CTC in pH 4 – 6. Since the study did not analyze the concentration of ICTC, it could not be confirmed whether the CTC was degraded into smaller molecular weight compounds or transformed into epimers and isomers. There were limitations on comparing the epimerization and isomerization phenomena with existing studies. The most common limitation was that some studies evaluated epimer or isomer of TCs and loss in the CTC could not be identified as transformation or degradation. In other cases, equilibrium was not confirmed and physicochemical properties such as phosphate concentration and divalent cations were not provided.

Table 4.3 Pearson's correlation coefficients between CTC, ECTC+EICTC, and ICTC and properties of solution chemistry

Compounds	CTC		ECTC+EICTC		ICTC	
Parameters	coefficients	*p*-value	coefficients	*p*-value	coefficients	*p*-value
pH	-0.107	0.893	0.251	0.749	0.071	0.929
TS	0.886*	0.042	-.991*	0.009	-0.769	0.231
TNH_3	0.632	0.368	-0.878	0.122	-0.503	0.497
PO_4^{3-}	0.885	0.115	-.994*	0.006	-0.798	0.202
TCOD	0.878	0.122	-0.941	0.059	-0.797	0.203
SCOD	0.888	0.112	-0.645	0.355	-.952*	0.048
Cu^{2+}	-0.715	0.285	0.912*	0.038	0.588	0.412
Zn^{2+}	0.029	0.971	0.237	0.763	-0.164	0.836
Ca^{2+}	0.664	0.336	-0.316	0.684	-0.781	0.219
Mg^{2+}	-0.188	0.812	0.529	0.471	0.022	0.978
Mn^{2+}	0.551	0.449	-0.243	0.757	-0.671	0.329
Fe^{2+}	0.373	0.627	-0.336	0.664	-0.402	0.598

*: $p < 0.05$

3.3.2 Phase distribution of epimer and isomer of CTC

Mass fractions of CTC, ECTC+EICTC, and ICTC in solid and liquid phases were evaluated. Most of CTC remained in the solid phase while ECTC+EICTC and ICTC were in the liquid phase. It was expected that CTC and its transformation products show similar mass fractions since those have similar chemical structures. For the SM, AD, and AAD assays, the solid mass fractions for CTC, ECTC+EICTC, and ICTC were 70 – 80, 17 – 27, and 22 – 40 % (w/w), respectively (Fig. 4.5). Since the water-soluble concentrations of ICTC in the FAAD assay were statistically the same or higher than the buffer-soluble concentrations, the solid mass fraction could not be calculated. This phenomenon that tetracyclines represent different phase distribution was also observed in studies that analyzed the parent compound and its transformation products together and their phase distribution using buffer and water (Lee et al., 2020). CTC is electrostatically adsorbed to the surface of livestock manure. Na_2-EDTA buffer extracts the adsorbed CTC by chelating with metal ions bonded with CTC. Chelation means that the positive electric charge of a compound combines with two or more negative charges of electron donor (i.e. tetracyclines) by forming separate covalent bonds. If the chelation of ECTC and ICTC with metal ions is stronger than CTC, they can be less detached by the EDTA. Bonding constants of CTC, ECTC, and ICTC with metal ions were not available in the existing literature.

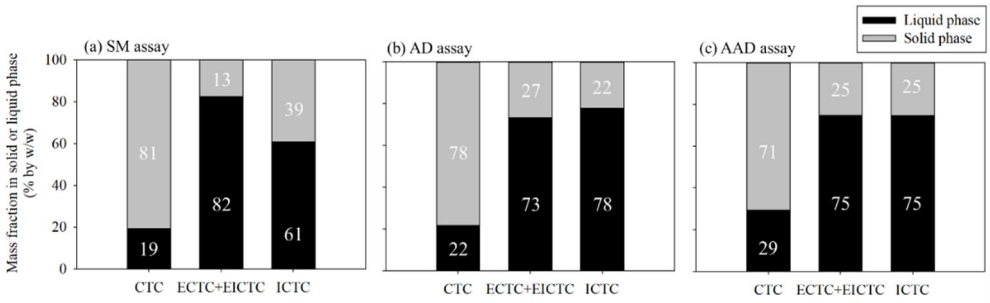

Figure 4.5 Mass fractions of CTC, ECTC+EICTC, and ICTC in solid and liquid phases: (a) SM assay, (b) AD assay, and (c) AAD assay

3.3.3 Kinetics of epimerization and isomerization

The total buffer extractable concentrations in the SM, AD, AAD, and FAAD assays were maintained after 38 hours except for the DI assay. Both water- and buffer-soluble concentrations of DI assay were linearly decreased from 38 to 480 hours following exponential decrease (Fig. 4.3). These results are in line with studies describing the change of TC concentrations in livestock manure (Arikan, 2008; Shelver and Varel, 2012; Spielmeyer et al., 2016). TCs did not fully disappear in the presence of solids such as biomass, and livestock manure, while continuously decreasing in aqueous solution. Solids may provide protection from photolysis and biodegradation of TCs (Li and Hu, 2016). It also could be due to a decrease of the Na_2-EDTA buffer-extractable portion from the matrices. The buffer detaches TCs electrostatically attached to the surface of biosolids by chelating with TCs (Pulicharla et al., 2017). With the increase of time, TCs are possibly more sequestrated into the biosolids (Gonzalez-Gil et al., 2018; Plósz et al., 2012). Accordingly, the kinetic analysis was performed for the period during which the CTC concentrations were maintained in the SM, AD, AAD, and FAAD assays (i.e. 38 – 480 hours). The degradation rate coefficients (k_d) were assumed to be zero.

The analytical solutions were verified by confirming the facts that those operated well as the three cases (Fig. 4.6). In case 1, the initial concentrations were maintained because the transformation kinetic constants were close to zero. CTC and ICTC were transformed into ECTC and EICTC in case 2. In case 3, concentrations

of CTC and ECTC+EICTC converged into zero and ICTC increased as they decreased (Fig. 4.6).

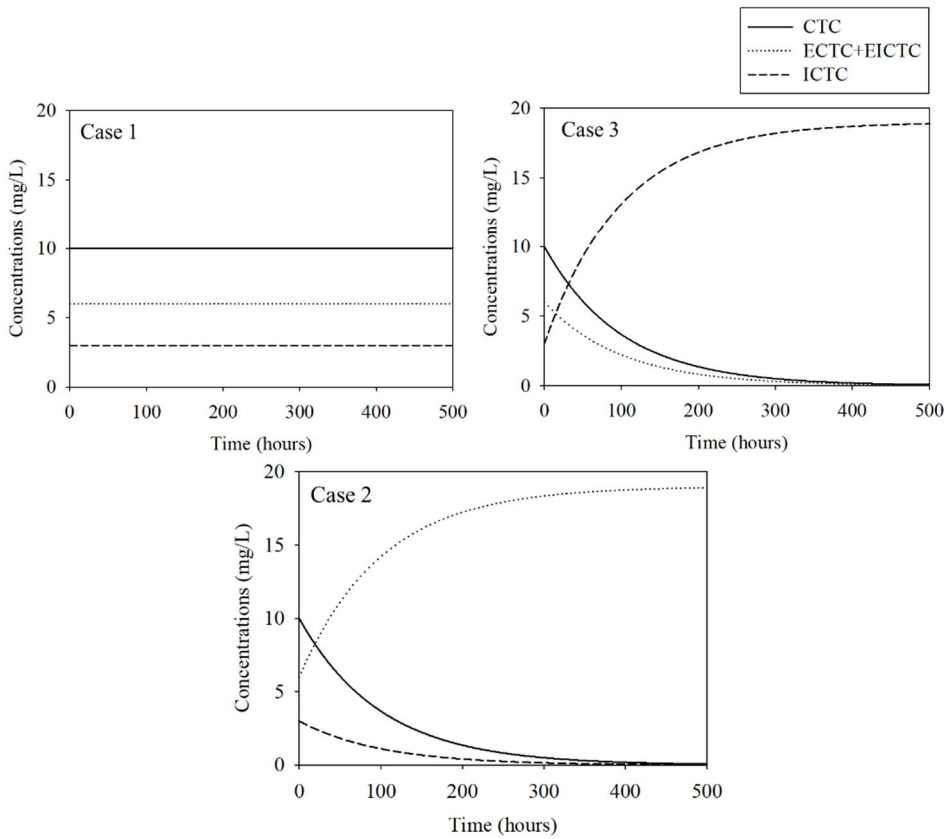

Figure 4.6 Simulation results for the verification of the analytical solutions. Case 1: All kinetic constants were 10^{-9} h^{-1}; Case 2: Epimerization kinetic constants ($k_{t,ce}$ and $k_{t,ie}$) were assumed much higher (10^{-2} h^{-1}) than isomerization kinetic constants ($k_{t,ci}$ and $k_{t,ei}$) (10^{-9} h^{-1}) and vice versa for Case 3. Initial concentrations of CTC, ECTC+EICTC, and ICTC were set as 10, 6, and 3 mg/L, respectively

The experimental and fitting results for the SM, AD, AAD, and FAAD assays were described in Fig. 4.7. The analytical solutions demonstrated the changes of buffer-soluble concentrations and the equilibrium states well. It was fitted well for not only exponential changes but also point of inflection observed at FAAD assay. It was first identified that the epimerization and isomerization of CTC in swine manure and its anaerobic digestate can be demonstrated by using ordinary differential equations assuming first-order kinetics and differential evolution. The kinetic constants were in a range of 0.014 – 0.608 h^{-1} except for $k_{t,ci}$ for the AAD assay. Above 0.832 of correlation coefficients were achieved except ECTC+EICTC and ICTC of FAAD (Table 4.4). The transformation constants were in agreement with those of previous studies. Since the epimerization and isomerization have never been distinguished and interpreted simultaneously, there were no comparable constants in the present studies. Instead, pseudo-1^{st} order decay rates of CTC were compared. The pseudo-1^{st} order decay rates of CTC for SM, AD, AAD, and FAAD were 0.040, 0.046, 0.034, and 0.055, respectively (R^2 = 0.949 – 0.989). In livestock manure, the decay rates were 0.038 h^{-1} (cattle manure, 47 g-TS/L, pH 7.6, and 35 °C) (Arikan, 2008), 0.043 h^{-1} (swine manure, 60 g-TS/L, pH 7, and 22 °C) (Shelver and Varel, 2012), and 0.22 h^{-1} (2.8 g-TS/L, pH 7, and 35 °C) (Álvarez et al., 2010). It is noteworthy that the 47 g-TS/L of cattle manure and the 60 g-TS/L of swine manure represented closely similar decay rates with those of this study. The TSs of this study were 34 – 57 g-TS/L except for the filtered test assay (i.e. FAAD assay). This supports the aforementioned explanation that the solid contents contributed to the

degree of transformations.

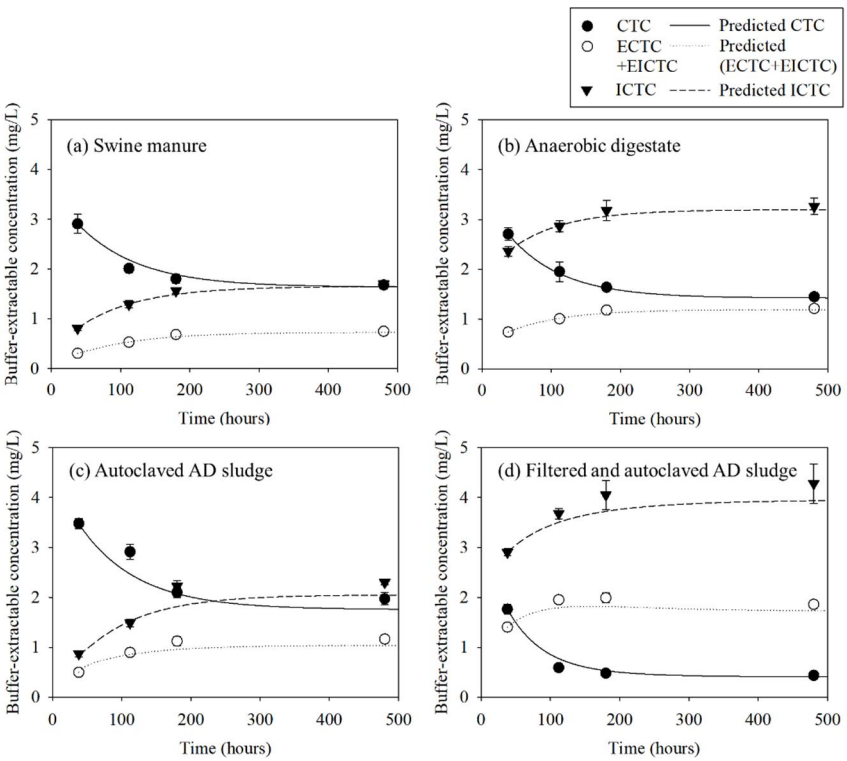

Figure 4.7 Experimental (dots) and fitted (lines) concentrations of CTC, ECTC+EICTC, and ICTC for (a) swine manure, (b) anaerobic digestate, (c) autoclaved anaerobic digestate, and (d) filtered and autoclaved anaerobic digestate

Table 4.4 Estimated kinetic constants of epimerization and isomerization and correlation coefficients for CTC, ECTC+EICTC, and ICTC for each assay

Unit: day^{-1}	k_{tce}	k_{tec}	k_{tei}	k_{tie}	k_{tci}	R^2
Swine manure (SM)	0.0216 ± 0.0002	0.3451 ± 0.0000	0.5301 ± 0.0049	0.3628 ± 0.0023	0.1314 ± 0.0002	CTC: 0.958 ECTC+EICTC: 0.979 ICTC: 0.984
Anaerobic digestate (AD)	0.0087 ± 0.0006	0.2626 ± 0.0000	0.6081 ± 0.0066	0.3197 ± 0.0027	0.2107 ± 0.0006	CTC: 0.998 ECTC+EICTC: 0.969 ICTC: 0.965
Autoclaved AD sludge (AAD)	0.0077± 0.0000	0.0139 ± 0.0000	0.0694 ± 0.0000	0.0356 ± 0.0000	0.0006 ± 0.0000	CTC: 0.843 ECTC+EICTC: 0.833 ICTC: 0.832
Filtered and autoclaved AD sludge (FAAD)	0.2114 ± 0.0000	0.0978 ± 0.0000	0.0988 ± 0.0000	0.0640 ± 0.0000	0.2070 ± 0.0000	CTC: 0.970 ECTC+EICTC: 0.674 ICTC: 0.767

The degradation and transformation coefficients were calibrated by using the CSTR data. Initial concentrations in the CSTR were set as zero because the concentrations in the swine manure were under detection limits (data not shown). Given that steady was achieved, the concentration ratios at each point can be used for the boundary conditions. The equations 4.4, 4.5, and 4.6 were applied to the CSTR data to calibrate degradation and transformation constants (Table 4.5). When assuming steady state at day 200, the R^2 values of the CTC, ECTC+EICTC, and ICTC were the highest. In order to compare the results, two cases with boundary conditions assuming steady state at day 80 and 200 were depicted in Fig. 4.8. The estimated coefficients were listed in Table 4.5. After day 60, the average

concentration of the sum of CTCs was 1.65 ± 0.22 mg/L. Both cases represented similar concentrations with the average, 1.57 and 1.66 mg/L. Since the case assuming equilibrium at day 200 fitted close to the average of each concentration (i.e. middle of the concentration fluctuation), its R^2 value was the highest (R^2 = 0.493 for CTC; 0.620 for ECTC+EICTC; 0.606 for ICTC). Transformation rate constants estimated from CSTR (Table 4.5 (b)) were one to three digits higher than those from batch-mode (Table 4.4). It should be noted out that steady state time was not considered to estimate kinetic constants with the CSTR model (eq. 4.4–4.6). The observed equilibrium time was 7.5 days (180 hours) for the batch tests (Fig. 4.7). A simulation assuming batch condition and the transformation constants estimated from CSTR (Table 4.5 (b)) showed that steady state was instantly achieved not during 7.5 days as observed in the batch test (Fig. 4.9). It represents that equilibrium time was not considered in the CSTR model. Transformation kinetics constants were estimated by assuming batch condition, equilibrium time at 7.5 days, and equilibrium ratio from day 200 of CSTR data. The digits of constants (Table 4.5 (c)) were closely similar to those from the batch test (Table 4.4 (AD)).

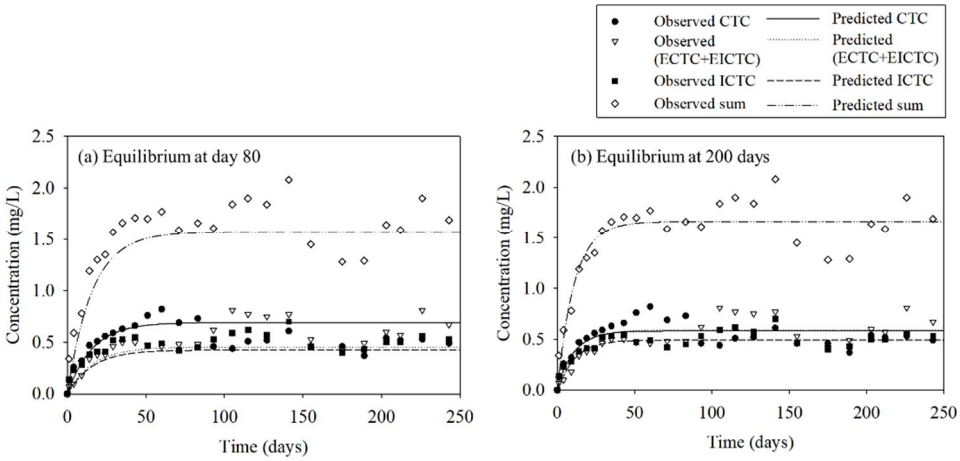

Figure 4.8 Calibration of CSTR-mode model using CSTR data with boundary condition assuming equilibrium at (a) day 80 and (b) day 200

Table 4.5 Estimated degradation, epimerization, and isomerization kinetic coefficients in a CSTR treating swine manure with boundary condition assuming equilibrium at (a) day 80, (b) day200, and (c) at day 200 with equilibrium time at 7.5 day

Unit: day^{-1}	k_{dc}	k_{de}	k_{di}	k_{tce}	k_{tci}	k_{tie}	k_{tec}	k_{tei}
(a) equilibrium at day 80	0.0051	0.0251	0.0770	26.6141	9.6108	48.5560	55.0921	30.9048
	R^2 = 0.005 for CTC; 0.283 for ECTC+EICTC; 0.056 for ICTC							
(b) equilibrium at day 200 w/o equilibrium time	0.0222	0.0666	0.0297	1.3040	0.7961	8.5274	1.8901	6.3897
	R^2 = 0.493 for CTC; 0.620 for ECTC+EICTC; 0.606 for ICTC							
(c) equilibrium at day 200 w/ equilibrium time	0.0032	0.0002	0.0006	0.0038	0.0101	1.2232	0.0125	1.0102
	R^2 = 0.306 for CTC; 0.667 for ECTC+EICTC; 0.464 for ICTC							

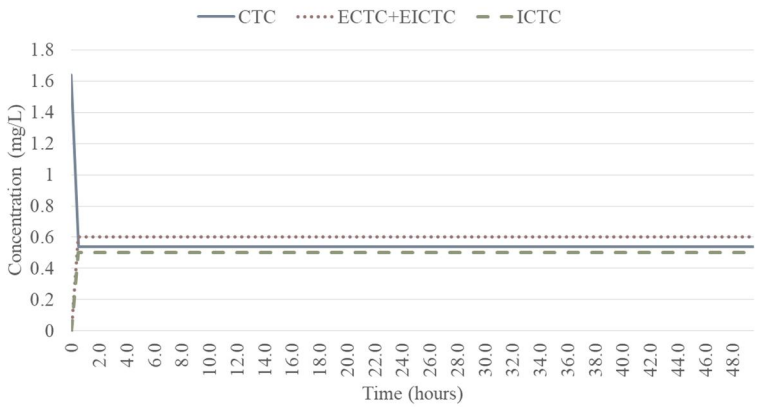

Figure 4.9 A simulation assuming batch condition and the transformation constants estimated from the CSTR data and model (Table 4.5 (b))

Transformation constants changed according to equilibrium time (Table 4.6). When evaluating the concentration of CTC with its transformation products in a CSTR, if the HRT of the CSTR is shorter than steady state time, the kinetic constants should be evaluated through batch experiments and applied to the CSTR model. If the HRT is longer than steady state time, estimation of the transformation kinetic constant is unnecessary. It is required to evaluate equilibrium ratios.

The degradation kinetic constants of CTC, ECTC+EICTC, and ICTC in a CSTR system could be distinguished for the first time by analyzing epimerization and isomerization together (Table 4.5). The degree of degradation rate constants was similar to each other. It is quite an acceptable result because the more having similar chemical structures, the more sharing physicochemical properties (HUSSAR et al., 1968). Since the degradation kinetic constants also change with transformation kinetic constants, batch experiments are needed to get those constants separately or

together.

Table 4.6 Transformation kinetic constants depending on equilibrium time in batch reactor

Equilibrium time	k_{tce} (h^{-1})	k_{tec} (h^{-1})	k_{tei} (h^{-1})	k_{tie} (h^{-1})	k_{tci} (h^{-1})
Day 1	0.0673	0.1208	0.8930	1.1439	0.0669
Day 7	0.0084	0.0211	1.0760	1.3074	0.0151
Day 20	0.0031	0.0064	0.0121	0.0188	0.0040
Day 30	0.0021	0.0045	0.1654	0.2015	0.0029
Day 50	0.0012	0.0026	0.0116	0.0157	0.0017

4.3.4 Estimation of CTC and transformation products in a CSTR

4.3.4.1 Sensitivity tests on HRTs and kinetic constants

The sensitivity of each model parameter on the concentrations in a CSTR (i.e. HRTs, and kinetic parameters) was tested prior to the estimation of concentrations of CTC, ECTC+EICTC, and ICTC. The sensitivity of an input variable on each concentration was calculated according to the percentage of simulation value to reference value minus 100 (%). The estimated concentration in swine manure was 2.20 mg-CTC/L when the uncertainty of farming environments and pattern of manure production are considered. Considering the equilibrium ratio observed in the SM assay (Fig. 4.4), the initial concentrations of CTC, ECTC+EICTC, and ICTC were set as 0.90, 0.38, and 0.93 mg/L from the mean concentration. The kinetic constants obtained from Table 4.5 (b) were used as reference constants. The reference HRT was 20 days. For HRTs, 10, 20, and 30 days were tested. For each constant, -40, -20, +20, and +40 % of variation were tested. The cases for the sensitivity test were summarized in Table 4.7.

Table 4.7 Cases for the sensitivity tests of HRTs, and kinetic parameters on concentrations of CTC, ECTC+EICTC, and ICTC in a CSTR (Variable for each case was underlined)

Cases	kdc	kde	kdi	ktce	ktci	ktie	ktec	ktei	HRT
1	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	10
2	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	30
3	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	20
4	0.0005	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	20
5	0.0007	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	20
6	0.0011	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	20
7	0.0013	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	20
8	0.0009	0.0016	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	20
9	0.0009	0.0022	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	20
10	0.0009	0.0033	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	20
11	0.0009	0.0038	0.0012	0.0543	0.0331	0.3553	0.0787	0.2662	20
12	0.0009	0.0027	0.0007	0.0543	0.0331	0.3553	0.0787	0.2662	20
13	0.0009	0.0027	0.0009	0.0543	0.0331	0.3553	0.0787	0.2662	20
14	0.0009	0.0027	0.0014	0.0543	0.0331	0.3553	0.0787	0.2662	20
15	0.0009	0.0027	0.0017	0.0543	0.0331	0.3553	0.0787	0.2662	20
16	0.0009	0.0027	0.0012	0.0326	0.0331	0.3553	0.0787	0.2662	20
17	0.0009	0.0027	0.0012	0.0434	0.0331	0.3553	0.0787	0.2662	20
18	0.0009	0.0027	0.0012	0.0652	0.0331	0.3553	0.0787	0.2662	20
19	0.0009	0.0027	0.0012	0.0760	0.0331	0.3553	0.0787	0.2662	20
20	0.0009	0.0027	0.0012	0.0543	0.0199	0.3553	0.0787	0.2662	20
21	0.0009	0.0027	0.0012	0.0543	0.0265	0.3553	0.0787	0.2662	20
22	0.0009	0.0027	0.0012	0.0543	0.0398	0.3553	0.0787	0.2662	20
23	0.0009	0.0027	0.0012	0.0543	0.0464	0.3553	0.0787	0.2662	20
24	0.0009	0.0027	0.0012	0.0543	0.0331	0.2131	0.0787	0.2662	20
25	0.0009	0.0027	0.0012	0.0543	0.0331	0.2842	0.0787	0.2662	20
26	0.0009	0.0027	0.0012	0.0543	0.0331	0.4263	0.0787	0.2662	20
27	0.0009	0.0027	0.0012	0.0543	0.0331	0.4974	0.0787	0.2662	20
28	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.0472	0.2662	20
29	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.0630	0.2662	20
30	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.0945	0.2662	20
31	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.1102	0.2662	20
32	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.1597	20
33	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.2129	20
34	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.3194	20
35	0.0009	0.0027	0.0012	0.0543	0.0331	0.3553	0.0787	0.3727	20

The sensitivity was high when the parameter is directly correlated to the concentration (Fig. 4.10). For example, the variation of ICTC showed the highest negative relationship with k_{tie} because ICTC is only reversibly transformed into EICTC (Fig 4.10 (c)). For the variation of CTC, k_{tec} and k_{tie} showed positive gradients meaning that the variation increases with the parameters. Since CTC can only be transformed from ECTC, the sign of gradients was correctly demonstrated (Fig. 4.10 (a)). The sensitivities of degradation parameters were lower than those of transformation parameters. It means that the transformations are the governing kinetics for determining the concentrations of CTC in the anaerobic digestate. Given the transformation kinetics constants varied depending on the characteristic of the matrix by 10 times, it may be one of the reasons for the high concentration variations in parent compounds (i.e. tetracycline, oxytetracycline, and chlortetracycline) observed from the manure (Table 2.1).

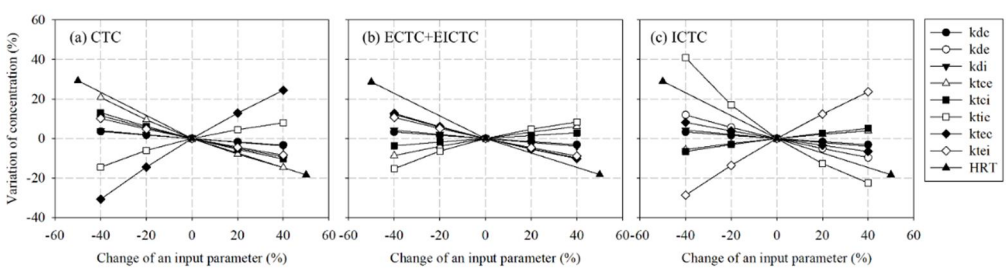

Figure 4.10 Sensitivity of input parameters on the variation of CTC, ECTC+EICTC, and ICTC concentrations

4.3.3.2 Application of the model

The concentrations of CTC, ECTC+EICTC, and ICTC in an AD plant were predicted depending on HRTs and their initial concentrations. The estimated concentration of CTC in swine manure was 2.20 mg-CTC/L. The 5^{th} and 95^{th} percentile concentrations were 0.54 and 5.65 mg/L, respectively (Table 3.2). Considering the equilibrium ratio observed in the SM assay (Fig. 4.4), the initial concentrations of CTC, ECTC+EICTC, and ICTC were set as 1) 0.22, 0.10, and 0.22 mg/L from the 5^{th} percentile concentration, 2) 0.90, 0.38, and 0.93 mg/L from the mean concentration, 3) 2.26, 1.01, and 2.29 from the 95^{th} percentile concentration, respectively. HRT was assumed to be 10, 20, and 30 days, which are the general operating ranges for anaerobic digester treating livestock wastewater (Lee et al., 2021; Rajagopal et al., 2013a; Rowse, 2011) (Table 4.8). The kinetic constants calibrated by table 4.5 (c) were used. The simulation period was set to 1 year (365 days) in consideration of biogas plants operated on an annual basis.

Table 4.8 Cases for simulation of CTC, ECTC+EICTC, and ICTC in a CSTR treating swine manure

Cases	CTC (mg/L)	ECTC+EICTC (mg/L)	ICTC (mg/L)	HRTs (days)
1	0.22	0.10	0.22	10
2	0.22	0.10	0.22	20
3	0.22	0.10	0.22	30
4	0.90	0.38	0.93	10
5	0.90	0.38	0.93	20
6	0.90	0.38	0.93	30
7	2.26	1.01	2.29	10
8	2.26	1.01	2.29	20
9	2.26	1.01	2.29	30

The concentrations reached a plateau after 4 HRTs (40 – 120 days) (data not shown). The concentrations of CTC, ECTC+EICTC, and ICTC were in a range of 0.08 – 1.30, 0.10 – 1.50, and 0.08 – 1.25 mg/L, respectively (Fig. 4.11). The results are in agreement with previous studies that investigated the concentrations of CTC from full-scale anaerobic digestion plants treating swine manure. Assuming total solid is to be the same as 50 g/L (i.e. 5 % by wet wt.), 0.18 mg/L of CTC was detected from a 5,000 m^3 biogas plant treating swine manure and sewage sludge with 80 days of HRT (Gros et al., 2019). Among 15 plants treating swine manure in Germany, two plants discharged digestate containing 0.4 and 0.7 mg/L, respectively (Astrid et al., 2014). Although ECTC and ICTC were not analyzed simultaneously with CTC, it is expected that similar concentrations of ECTC and ICTC existed together with CTC.

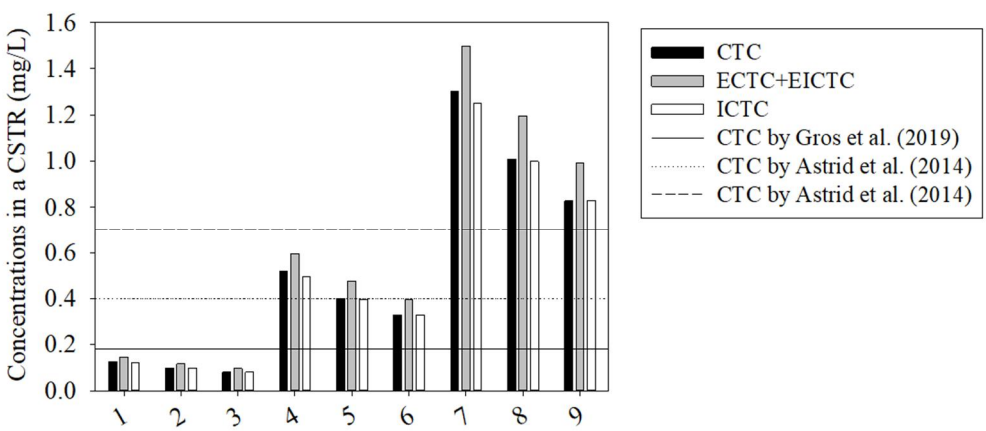

Figure 4.11 Simulation of CTC, ECTC+EICTC, and ICTC concentrations in a CSTR depending on initial concentration and HRT

4.4 Summary

In the swine manure and its anaerobic digestate, ECTC, EICTC, and ICTC are transformed from CTC. They accounted for 60 - 93 % (w/w) of the residual total CTCs. The CTC is expected to be transported by solid-phase of anaerobic digestate (> 70 % by wt.) while most ECTC, EICTC, and ICTC remain in the liquid phase (> 60 % by wt.). The kinetics of degradation and transformation in the swine manure and its anaerobic digestate can be demonstrated by using ordinary differential equations based on the 1st order kinetics. The model can be utilized for the prediction of the concentrations of CTC, ECTC+EICTC, and ICTC in a CSTR treating swine manure. When evaluating the concentration of CTC with its transformation products in a CSTR, if the HRT of the CSTR is shorter than equilibrium time, the kinetic constants should be evaluated through batch experiments. If the HRT is longer than equilibrium time, estimation of the transformation kinetic constant is unnecessary. It is required to evaluate equilibrium ratios. It is recommended for the batch test that equilibrium is achieved and degradation is controlled for more exact estimation of parameters.

References

Álvarez, J. A., Otero, L., Lema, J. M., & Omil, F. (2010). The effect and fate of antibiotics during the anaerobic digestion of pig manure. *Bioresource Technology*, *101*(22), 8581–8586. https://doi.org/10.1016/j.biortech.2010.06.075

Arikan, O. A. (2008). Degradation and metabolization of chlortetracycline during the anaerobic digestion of manure from medicated calves. *Journal of Hazardous Materials*, *158*(2–3), 485–490. https://doi.org/10.1016/j.jhazmat.2008.01.096

Bilal, Pant, M., Zaheer, H., Garcia-Hernandez, L., & Abraham, A. (2020). Differential Evolution: A review of more than two decades of research. *Engineering Applications of Artificial Intelligence*, *90*, 103479. https://doi.org/10.1016/J.ENGAPPAI.2020.103479

Blanchflower, W. J., McCracken, R. J., Haggan, A. S., & Kennedy, D. G. (1997). Confirmatory assay for the determination of tetracycline, oxytetracycline, chlortetracycline and its isomers in muscle and kidney using liquid chromatography-mass spectrometry. *Journal of Chromatography B: Biomedical Applications*, *692*(2), 351–360. https://doi.org/10.1016/S0378-4347(96)00524-5

Cessna, A. J., Kuchta, S. L., Bailey, J., Waiser, M., & Tumber, V. (2020).

Isomerization of chlortetracycline in prairie wetland water. *Journal of Environmental Quality*, *49*(5), 1435–1444. https://doi.org/10.1002/jeq2.20079

Chen, W. R., & Huang, C. H. (2010). Adsorption and transformation of tetracycline antibiotics with aluminum oxide. *Chemosphere*, *79*(8), 779–785. https://doi.org/10.1016/j.chemosphere.2010.03.020

Chen, W. R., & Huang, C. H. (2011). Transformation kinetics and pathways of tetracycline antibiotics with manganese oxide. *Environmental Pollution*, *159*(5), 1092–1100. https://doi.org/10.1016/j.envpol.2011.02.027

Ciarlone, A. E., Fry, B. W., & Ziemer, D. M. (1990). Some observations on the adsorption of tetracyclines to glass and plastic labware. *Microchemical Journal*, *42*(2), 250–255. https://doi.org/10.1016/0026-265X(90)90050-F

Doerschuk, A. P., Bitler, B. A., & McCormick, J. R. D. (1955). Reversible isomerizations in the tetracycline family. *Journal of the American Chemical Society*, *77*(17), 4687. https://doi.org/10.1021/ja01622a085

Dzomba, P. (2016). *University of Zimbabwe Speciation and Persistence of Tetracycline Antibiotics in the Aquatic Environment : Characterization in terms of a Linear Rate Model*. *September*.

FDA, U. S. (2020). *2019 Summary report on antimicrobials sold or distributed for use in food-producing animals*.

Fritz, J. W., & Zuo, Y. (2007). Simultaneous determination of tetracycline, oxytetracycline, and 4-epitetracycline in milk by high-performance liquid chromatography. *Food Chemistry*, *105*(3), 1297–1301.

https://doi.org/10.1016/j.foodchem.2007.03.047

Gaugain, M., Gautier, S., Bourcier, S., Jacques, A. M., Laurentie, M., Abjean, J. P., Hurtaud-Pessel, D., & Verdon, E. (2015). 6-Iso-chlortetracycline or keto form of chlortetracycline? Need for clarification for relevant monitoring of chlortetracycline residues in food. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *32*(7), 1105–1115. https://doi.org/10.1080/19440049.2015.1036381

Gelband, H., Miller-Petrie, M., Pant, S., Gandra, S., Levinson, J., Barter, D., White, A., & Laxminarayan, R. (2015). State of the World ' S Antibiotics. *Center for Disease Dynamics, Economics & Policy*.

Georgioudakis, M., & Plevris, V. (2020). A Comparative Study of Differential Evolution Variants in Constrained Structural Optimization. *Frontiers in Built Environment*, *0*, 102. https://doi.org/10.3389/FBUIL.2020.00102

Gonzalez-Gil, L., Mauricio-Iglesias, M., Carballa, M., & Lema, J. M. (2018). Why are organic micropollutants not fully biotransformed? A mechanistic modelling approach to anaerobic systems. *Water Research*, *142*, 115–128. https://doi.org/10.1016/j.watres.2018.05.032

Grote, M., Vockel, A., Schwarze, D., Mehlich, A., & Freitag, M. (2004). Fate of antibiotics in food chain and environment originating from pigfattening (part 1). *Fresenius Environmental Bulletin*, *13*(11 B), 1216–1224.

Halling-Sørensen, B., Sengeløv, G., & Tjørnelund, J. (2002). Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant

bacteria, including selected tetracycline-resistant bacteria. *Archives of Environmental Contamination and Toxicology*, *42*(3), 263–271. https://doi.org/10.1007/s00244-001-0017-2

Huang, C.-H., & Chen, W.-R. (2009). Transformation of tetracyclines mediated by Mn(II) and Cu(II) ions in the presence of oxygen. *Environmental Science and Technology*, *43*(2), 401–407. https://doi.org/10.1021/es802295r

HUSSAR, D. A., NIEBERGALL, P. J., SUGITA, E. T., & DOLUISIO, J. T. (1968). Aspects of the epimerization of certain tetracycline derivatives. *Journal of Pharmacy and Pharmacology*, *20*(7), 539–546. https://doi.org/10.1111/j.2042-7158.1968.tb09802.x

Kennedy, D. G., McCracken, R. J., Carey, M. P., Blanchflower, W. J., & Hewitt, S. A. (1998). Iso- and epi-iso-chlortetracycline are the principal metabolites of chlortetracycline in the hen's egg. *Journal of Chromatography A*, *812*(1–2), 327–337. https://doi.org/10.1016/S0021-9673(98)00280-5

Kennedy, F. (2008). *Tetracycline antibiotic distribution and transformation in aquatic systems*.

Kwon, A.-Y., & Kim, S. C. (2015). Estimating Predicted Environmental Concentration of Veterinary Antibiotics in Manure and Soil. *Korean Journal of Soil Science and Fertilizer*, *48*(2), 100–104. https://doi.org/10.7745/kjssf.2015.48.2.100

Lee, C., An, J., Lee, Y. S., Choi, K., & Kim, J. Y. (2021). Uncertainty-based concentration estimation of chlortetracycline antibiotics in swine farms and

risk probability assessment for agricultural application of manure. *Journal of Hazardous Materials*, *402*, 123763. https://doi.org/10.1016/j.jhazmat.2020.123763

Lee, C., Jeong, S., Ju, M., & Kim, J. Y. (2020). Fate of chlortetracycline antibiotics during anaerobic degradation of cattle manure. *Journal of Hazardous Materials*, *386*, 121894. https://doi.org/10.1016/J.JHAZMAT.2019.121894

Leng, Y., Bao, J., Chang, G., Zheng, H., Li, X., Du, J., Snow, D., & Li, X. (2016). Biotransformation of tetracycline by a novel bacterial strain Stenotrophomonas maltophilia DT1. *Journal of Hazardous Materials*, *318*, 125–133. https://doi.org/10.1016/j.jhazmat.2016.06.053

Li, S., & Hu, J. (2016). Photolytic and photocatalytic degradation of tetracycline: Effect of humic acid on degradation kinetics and mechanisms. *Journal of Hazardous Materials*, *318*, 134–144. https://doi.org/10.1016/J.JHAZMAT.2016.05.100

Loftin, K. A., Adams, C. D., Meyer, M. T., & Surampalli, R. (2008). Effects of Ionic Strength, Temperature, and pH on Degradation of Selected Antibiotics. *Journal of Environment Quality*, *37*(2), 378. https://doi.org/10.2134/jeq2007.0230

McCormick, J. R. D., Fox, S. M., Smith, L. L., Bitler, B. A., Reichenthal, J., Origoni, V. E., Muller, W. H., Winterbottom, R., & Doerschuk, A. P. (1956). On the nature of the reversible isomerizations occurring in the tetracycline family. *Journal of the American Chemical Society*, *78*(14), 3547–3548. https://doi.org/10.1021/ja01595a084

McCormick, J. R. D., Fox, S. M., Smith, L. L., Bitler, B. A., Reichenthal, J., Origoni, V. E., Muller, W. H., Winterbottom, R., & Doerschuk, A. P. (1957a). Studies of the Reversible Epimerization Occurring in the Tetracycline Family. The Preparation, Properties and Proof of Structure of Some 4-epi-Tetracyclines. *Journal of the American Chemical Society*, *79*(11), 2849–2858. https://doi.org/10.1021/ja01568a050

McCormick, J. R. D., Fox, S. M., Smith, L. L., Bitler, B. A., Reichenthal, J., Origoni, V. E., Muller, W. H., Winterbottom, R., & Doerschuk, A. P. (1957b). Studies of the Reversible Epimerization Occurring in the Tetracycline Family. The Preparation, Properties and Proof of Structure of Some 4-epi-Tetracyclines. *Journal of the American Chemical Society*, *79*(11), 2849–2858. https://doi.org/10.1021/ja01568a050

Plósz, B. G., Langford, K. H., & Thomas, K. V. (2012). An activated sludge modeling framework for xenobiotic trace chemicals (ASM-X): Assessment of diclofenac and carbamazepine. *Biotechnology and Bioengineering*, *109*(11), 2757–2769. https://doi.org/10.1002/bit.24553

Pollard, A. T., & Morra, M. J. (2017). Fate of tetracycline antibiotics in dairy manure-amended soils. *Environmental Reviews*, *26*(1), 102–112. https://doi.org/10.1139/er-2017-0041

Pulicharla, R., Hegde, K., Brar, S. K., & Surampalli, R. Y. (2017). Tetracyclines metal complexation: Significance and fate of mutual existence in the environment. *Environmental Pollution*, *221*, 1–14.

https://doi.org/10.1016/j.envpol.2016.12.017

Qiao, M., Chen, W., Su, J., Zhang, B., & Zhang, C. (2012). Fate of tetracyclines in swine manure of three selected swine farms in China. *Journal of Environmental Sciences (China)*, *24*(6), 1047–1052. https://doi.org/10.1016/S1001-0742(11)60890-5

Rajagopal, R., Massé, D. I., & Singh, G. (2013). A critical review on inhibition of anaerobic digestion process by excess ammonia. In *Bioresource Technology* (Vol. 143, pp. 632–641). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2013.06.030

Ramanan, L., Priscilla, M., Deolalikar, A., Desiraju, K., Pittet, D., Stephen, T., & Mary E., W. (2021). The State of the World's Antibiotics in 2021: A global analysis of antimicrobial resistance and its drivers. In *The Center for Disease Dynamics, Economics & Policy*. https://resistancemap.cddep.org/

Remmers, E. G., Sieger, G. M., & Doerschuk, A. P. (1963). Some observations on the kinetics of the C·4 epimerization of tetracycline. *Journal of Pharmaceutical Sciences*, *52*(8), 752–756. https://doi.org/10.1002/jps.2600520809

Rowse, L. E. (2011). *Design of Small Scale Anaerobic Digesters for Application in Rural Developing Countries*. 1–125. http://scholarcommons.usf.edu/cgi/viewcontent.cgi?article=4519&context=etd

Shelver, W. L., & Varel, V. H. (2012). Development of a UHPLC-MS/MS method for the measurement of chlortetracycline degradation in swine manure.

Analytical and Bioanalytical Chemistry, *402*(5), 1931–1939. https://doi.org/10.1007/s00216-011-5637-4

Singh, A. K. (2016). Distribution, Sorption and Desorption of Tylosin, Chlortetracycline and their Metabolites in Pig Manure. *Journal of Agricultural Studies*, *4*(4), 65. https://doi.org/10.5296/jas.v4i4.10128

Søeborg, T., Ingerslev, F., & Halling-Sørensen, B. (2004). Chemical stability of chlortetracycline and chlortetracycline degradation products and epimers in soil interstitial water. *Chemosphere*, *57*(10), 1515–1524. https://doi.org/10.1016/j.chemosphere.2004.09.020

Sokoloski, T. D., Mitscher, L. A., Yuen, P. H., Juvarkar, J. V., & Hoener, B. (1977). Rate and proposed mechanism of anhydrotetracycline epimerization in acid solution. *Journal of Pharmaceutical Sciences*, *66*(8), 1159–1165. https://doi.org/10.1002/jps.2600660829

Spielmeyer, A., Stahl, F., Petri, M. S., Zerr, W., Brunn, H., & Hamscher, G. (2016). Transformation of Sulfonamides and Tetracyclines during Anaerobic Fermentation of Liquid Manure. *Journal of Environment Quality*, *46*(1), 160. https://doi.org/10.2134/jeq2016.04.0152

Stone, J. J., Clay, S. A., Zhu, Z., Wong, K. L., Porath, L. R., & Spellman, G. M. (2009). Effect of antimicrobial compounds tylosin and chlortetracycline during batch anaerobic swine manure digestion. *Water Research*, *43*(18), 4740–4750. https://doi.org/10.1016/j.watres.2009.08.005

Storn, R., & Price, K. (1997). Differential Evolution – A Simple and Efficient

Heuristic for global Optimization over Continuous Spaces. *Journal of Global Optimization 1997 11:4*, *11*(4), 341–359. https://doi.org/10.1023/A:1008202821328

Van Boeckel, T. P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B. T., Levin, S. A., & Laxminarayan, R. (2014). Global antibiotic consumption 2000 to 2010: An analysis of national pharmaceutical sales data. *The Lancet Infectious Diseases*, *14*(8), 742–750. https://doi.org/10.1016/S1473-3099(14)70780-7

Volkers, G., Petruschka, L., & Hinrichs, W. (2011). Recognition of drug degradation products by target proteins: Isotetracycline binding to tet repressor. *Journal of Medicinal Chemistry*, *54*(14), 5108–5115. https://doi.org/10.1021/jm200332e

Winckler, C., & Grafe, A. (2001). Use of veterinary drugs in intensive animal production: Evidence for persistence of tetracycline in pig slurry. *Journal of Soils and Sediments*, *1*(2), 66–70. https://doi.org/10.1007/BF02987711

Yuen, P. H., & Sokoloski, T. D. (1977). Kinetics of concomitant degradation of tetracycline to epitetracycline, anhydrotetracycline, and epianhydrotetracycline in acid phosphate solution. *Journal of Pharmaceutical Sciences*, *66*(11), 1648–1650. https://doi.org/10.1002/jps.2600661143

Zhao, F., Zhang, D., Xu, C., Liu, J., & Shen, C. (2020a). The enhanced degradation and detoxification of chlortetracycline by Chlamydomonas reinhardtii. *Ecotoxicology and Environmental Safety*, *196*(March), 110552. https://doi.org/10.1016/j.ecoenv.2020.110552

Zhao, F., Zhang, D., Xu, C., Liu, J., & Shen, C. (2020b). The enhanced degradation

and detoxification of chlortetracycline by Chlamydomonas reinhardtii. *Ecotoxicology and Environmental Safety*, *196*, 110552. https://doi.org/10.1016/j.ecoenv.2020.110552

CHAPTER 5
LONG-TERM EFFECTS OF CHLORTETRACYCLINE ANTIBIOTICS ON ANAEROBIC DIGESTION OF SWINE MANURE

5.1 Introduction

The amount of veterinary antibiotics (VAs) used for livestock farming is steadily increasing worldwide due to the increase of livestock numbers and a shift of livestock industries to concentrated animal feeding operations (CAFOs). The consumption of VAs, which stood at 63,200 tons as of 2010, is expected to reach 105,600 tons by 2030. By 2014, the amount of VA usage was greatest in China, the United States, and Brazil, and the growth rate of VA consumption was highest in countries such as Indonesia, Malaysia, and Nigeria, where meat consumption is increasing along with population growth (Gelband et al., 2015; Van Boeckel et al., 2014).

VAs are excreted through livestock manure following administration. The excretion rates vary from 30 to 70 % (w/w) (Arikan et al., 2007; Dewey et al., 1999). Since most of the livestock manure is distributed in the terrestrial environment as fertilizer/compost, concerns about microbial toxicity and antimicrobial resistance have been increased. These problems would occur along with the release pathways

of the VAs to nature (Daghrir and Drogui, 2013; Halling-Sørensen et al., 1998; Jia et al., 2017; Sarmah et al., 2006). There has been a growing interest in the effects of VAs on anaerobic digestion (AD) for livestock manure treatment. AD is an engineered system in which methane energy is generated as organic matter is degraded through anaerobic microbial metabolism. Since the antimicrobial activity of the VAs is maintained even after excretion (Arikan, 2008; Arikan et al., 2006; Stone et al., 2009), it may undermine the sustainability of AD processes, i.e., organic waste reduction and methane recovery. VAs can limit the growth of microorganisms by inhibiting their protein synthesis (bacteriostatic effect) and/or kill anaerobes by inhibiting cell wall synthesis (bactericidal effect). Accordingly, the microbial community in the AD system can be gradually changed (Cheng et al., 2018).

Many studies have evaluated the inhibitory effects of VA on the performance and stability of AD treatment of organic waste, such as livestock manure, sewage sludge, and synthetic wastewater (Álvarez et al., 2010; Arikan et al., 2006; Aydin et al., 2015b, 2015a; Carey et al., 2016; Cetecioglu et al., 2013, 2012; Coban et al., 2016; Fountoulakis et al., 2008; Gartiser et al., 2007; Ince et al., 2013; Stone et al., 2009; Tian et al., 2018; Xiong et al., 2017). Although the effects vary with the types of VAs, their modes of action, test concentrations, and AD conditions, manure excreted after VA administration results in lower biogas generation than control manure (Arikan, 2008; Arikan et al., 2006; Stone et al., 2009). Reductions in organic removal rates and microbial community shifts have been observed (Carey et al., 2016; Cetecioglu et al., 2013, 2012; Coban et al., 2016; Tian et al., 2018; Xiong

et al., 2017). Furthermore, the possible combined effects of VAs on anaerobes have been explored (Aydin et al., 2015b, 2015a). Cheng et al. (2018) summarized these studies on the effects of VAs and concluded that VAs could have a negative impact on the AD of swine manure. However, there remain several ambiguities in literatures as to whether the probable concentrations of VAs that may be present in manure affect its AD treatment. The experimental conditions in previous studies were mostly focused on tens or hundreds of mg/L of VAs that are probabilistically impossible to be in manure and wastewater on a standard administration basis and short-term exposure. VAs are usually present at low concentrations up to several mg/L in livestock manure (Carballo et al., 2016; Li et al., 2013; Martínez-Carballo et al., 2007; Zhang et al., 2015; Zhao et al., 2010). In chapter 3, it was revealed that 5^{th} - 95^{th} percentile concentrations of CTC in swine manure can be in a range of 0.5 to 5.6 mg/kg due to administration standards and dilution by manure that does not contain VAs (Lee et al., 2021). Most AD plants treating livestock manure are operated continuously for extended periods of over a year and are expected to be continuously exposed to VAs in livestock manure. Even low concentrations of VAs in livestock manure could affect the AD by long-term exposure. Pattern of the effects also may be different from those caused by acute exposure and/or dozens and hundreds mg/L of VAs (Liu et al., 2021). Recovery of AD performance after inhibitions caused by VAs can be an important issue for engineers and practitioners involved in sustainable operations and management of the AD system. To the best of our knowledge, information regarding long-term effects of Vas on AD and recovery

from the effects is still largely lacking in available literature.

In this regard, this study aimed to assess long-term effects of CTC on AD of swine manure containing a probably low concentration of chlortetracycline (CTC), which is one of the major broad-spectrum VAs, and to elucidate the long-term inhibitory effects based on AD performance and microbial community. Since the inhibition happened by continuous exposure to CTC, the performance recovery from the inhibition were explored. To this end, the organic removal, methane production and stability parameters were monitored during the 900 day-operation of two lab-scale anaerobic continuous-stirred tank reactors treating swine manure. Changes in the microbial community and its statistical relationship with environmental variables were investigated.

5.2 Materials and methods

5.2.1 Substrate, inoculum, and chlortetracycline

Swine manure was obtained from a storage tank at a composting facility in the Republic of Korea. The swine manure was homogeneously mixed and stored at -20 °C before use. Anaerobically digested sludge used for inoculum was collected from a lab-scale digester treating the same swine manure at 2.5 g-VS/L/day of organic loading rate (OLR) for more than 200 days. The physicochemical characteristics of the inoculum and swine manure were summarized in Table 5.1.

A stock solution of chlortetracycline hydrochloride (CAS no. 64-72-2, Sigma-Aldrich, St Louis, MI, USA) was injected to produce 3 mg CTC/L in the influent of a continuous-stirred tank reactor. In addition, 4-epichlortetracycline hydrochloride (ECTC; CAS no. 101342-45-4, Biosynth Carbosynth, Berkshire, UK) and Iso-chlortetracycline (ICTC; CAS no. 514-53-4, Biosynth Carbosynth), which are stereo and structural isomers of CTC, respectively, were used as standard chemicals to quantify the transformation of CTC into ECTC and ICTC.

Table 5.1 Physicochemical characteristics of the inoculum and the swine manure

	Units	Inoculum	Swine manure
pH	-	7.2	7.5
Water content	% by wet wt.	95.8 ± 0.3	93.7 ± 0.0
Volatile solid (VS)		3.2 ± 0.3	4.6 ± 0.0
Fixed solid (FS)		1.0 ± 0.0	1.7 ± 0.0
TCOD	mg/L	-	49,421 ± 11
SCOD	mg/L		2,500 ± 179
NH_3	mg/L	-	861 ± 12
C	% by dry wt.	32.5	30.4
H		4.6	4.5
O		30.5	19.0
N		2.4	2.5
S		0.6	0.3

5.2.2 CSTR operation

Two CSTRs with 10 and 8 L of total and working volume were operated in a mesophilic (35 ± 1 °C) constant-temperature room for 900 days. After removing residual organic matter in the inoculum anaerobically, the two CSTRs were daily fed with the same volume of swine manure. Before the feeding, the same volume of digestate was removed from each CSTR. The target OLR (2.3 g-VS/L/day) was achieved in a stepwise manner (1.6, 1.9, and 2.3 g-VS/L/day). The final HRT for the 2.3 g-VS/L/day of OLR was 20 days. CSTR 1 was the control reactor used to treat swine manure without CTC spiking. As a test reactor, CSTR 2 was continuously fed with swine manure spiked with CTC from the day 0 to 585.

The test concentration of CTC in the influent of CSTR 2 was set at 3 mg/L according to measured concentrations in literatures and probabilistically estimated concentrations with uncertainties in field conditions in chapter 3. Several recent studies measured concentrations of tetracyclines in swine manure from slurry pits where excreted manure is mixed regardless of VA contamination (Carballo et al., 2016; Li et al., 2013; Martínez-Carballo et al., 2007; Zhang et al., 2015; Zhao et al., 2010). Weighted average of the reported concentrations was calculated as 2.1 mg/kg_{manure} by assuming that the concentrations were distributed normally (Table 3.3). As a result, the inhibition of CTC on methane generation was observed from day 300. In order to identify the performance recovery, CTC exposure to CSTR 2 was ceased from day 585.

5.2.3 Analytical methods

5.2.3.1 Chlortetracycline analysis

The concentrations of CTC and its transformation products (i.e. ECTC, and ICTC) in the CSTR 2 were measured by using a high-pressure liquid chromatography connected with a single quadrupole mass spectrometry (Agilent 1260, Agilent Technologies, Inc., USA). As demonstrated by Lee et al. (2020), a series of ultrasonic solvent extraction (USE) and solid-phase extractions (SPEs) were applied to the digestate of CSTR 2 to remove impurities and elute CTC, ECTC, and ICTC. The methodological detection limits were 0.05 mg/L. The concentration was determined to be water-soluble or buffer-extractable depending on whether the extraction solution used was water or Na_2-EDTA, respectively. The water-soluble concentration was used as the concentration of CTC, ECTC, and ICTC in the liquid phase. The solid phase concentrations were calculated by dividing the difference between the buffer-extractable and water-soluble concentrations by the total solid content of the CSTR 2.

5.2.3.2 Anaerobic digestion performance and microbial taxonomic profiling

In order to evaluate AD performance, methane generation and organic matter removal were monitored. The biogas volume was measured daily using a

tedlar bag attached to each CSTR, and its composition (i.e., CH_4, and CO_2) and nitrogen content were analyzed using a gas chromatograph (GC) connected to a thermal conductivity detector (Young Lin Instrument Co. Ltd., Anyang, Republic of Korea). The chemical oxygen demand (Chromium) was measured using a Water Test Kit (Humas Co. Ltd., Daejeon, Republic of Korea) manufactured based on EPA 410.4. The organic removal was evaluated according to the total chemical oxygen demand (TCOD) removal ratio.

DNA for profiling the microbial taxonomy was extracted from the digestate of both CSTRs collected on day 90, 188, 500, 750 and 808 including seed sludge. Inhibition to methane generation of the test reactor was observed after day 300. The exposure to CTC was ceased for the test reactor since day 600. The DNA extraction was performed using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). Detailed methodologies for targeting primers and determining taxonomic ranks were described in the Appendix S5.1.

5.2.3.3 Stability parameters

As stability parameters, pH, alkalinity, and volatile fatty acids (VFAs) concentrations were evaluated. The concentration of total ammonia was assessed together with the above parameters because a high concentration of ammonia can have a negative impact on the AD of swine manure (Chen et al., 2008; Colón et al., 2015). The alkalinity (mg $CaCO_3$/L) was determined according to the Nordmann

method (Nordmann, 1977). VFAs in the liquid phase were analyzed using a GC equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA, USA). The total ammonia concentration was measured using a Water Test Kit based on EPA 350.2 (Nessler method) (Humas Co. Ltd., Daejun, Republic of Korea).

5.2.4 Statistical analysis

Statistical differences between the AD performance of CSTR 1 and CSTR 2 were determined using a *t*-test with a 95 % confidence level. In order to specify the time when the performance and stability parameters of CSTR 2 started to be different with those of CSTR 1, the moving averages of each data set (e.g., methane generation, TCOD removal rate, SCOD concentration, pH, alkalinity, ammonia concentration) in the range of one to five hydraulic retention times (HRTs) for each sampling day were calculated and compared. The statistical differences in the moving averages were evaluated using the Scipy package in Python. The time was conservatively determined when the statistical differences were maintained for more than a period of two HRTs.

Multivariate analysis was conducted to distinguish similarity between microbial samples (Principal component analysis, PCA) and to find relationship between microbial community and environmental variables (Redundancy analysis, RDA) by using CANOCO 4.5 (Centre for Biometry, Wageningen, Netherlands). Prior to RDA, detrended correspondence analysis (DCA) for bacterial or archaeal

counts of samples was conducted to determine linear or unimodal response model for the response data. The lengths of gradient were below 1.5 for bacterial phylum and archaeal genus samples. It means that RDA using linear response model is more suitable to ordinate the microbial counts to the environmental variables (Lepš and Šmilauer, 2003). All environmental variables were made unitless by Min-Max normalization for the multivariate analyses. Furthermore, Spearman rank correlation coefficients (SRCC) were calculated using SPSS Statistics 26 (IBM, USA) to support positive or negative bivariate relationship between the microbial community and environmental variables.

5.3 Results and discussion

5.3.1 Fate of chlortetracycline

Figure 5.1 showed the observed buffer-extractable concentration and predicted CTC concentration assuming ideal CSTR conditions with non-degradation (the prediction equation (Eq. 5.1).

$$C_{out} = \frac{C_{in}}{1+k_d\tau} * \left(1 - e^{-\left(\frac{1+k_d\tau}{\tau} * t\right)}\right) + C_0 * e^{-\left(\frac{1+k_d\tau}{\tau} * t\right)}$$

Equation 5.1

Where, C_{out} = concentration of a material in the effluent; C_{in} concentration of a material in the influent; k_d = first-order degradation constant; τ = hydraulic retention time; C_0 = initial concentration of a material in the reactor

From day 150, when the target OLR (2.3 g-VS/L/day) was reached and maintained for more than two HRTs, to day 580, the average of the sum of CTC, ECTC, and ICTC (total CTC) was 1.46 ± 0.37 mg/L. Degradability was calculated according to the ratio of the average concentration of the sum of CTC, ECTC, and ICTC (total CTC) to the predicted concentration. The degradability of CTC was 51 ± 12 % (w/w). This is in agreement with previous studies that evaluated the

degradation of CTC together with ECTC and ICTC. Lee et al. (2020) and Arikan (2008) reported that the degradability of total CTC was 20–40 % and 75 %, respectively, during 30 days of the anaerobic degradation of cattle manure. CTC, ECTC, and ICTC were in the ranges of 0.36–0.99, 0.19–0.80, and 0.12–0.60 mg/L for the same period. Since the degree of transformation can be affected by various solution conditions such as pH, temperature, cation presence, ionic strength, etc. (Chen and Huang, 2011; Loftin et al., 2008; McCormick et al., 1957; Søeborg et al., 2004), it could not be elucidated under the current experimental conditions. After CTC exposure ceased on the day 585, the total CTC concentration in the CSTR 2 decreased to zero by day 750.

The water-soluble concentrations of CTC, ECTC, and ICTC were 0.09 ± 0.03, 0.16 ± 0.07, and 0.11 ± 0.07 mg/L, respectively. The corresponding phase distribution coefficients were 0.17 ± 0.16, 0.08 ± 0.04, 0.05 ± 0.04 L/g, respectively. The phase distribution coefficient (L/g solid) was obtained by dividing the concentrations in the solid phase (mg/L) by those in the liquid phase and the total solids (g solid/L). It was assumed that CTC and its transformation products were in equilibrium in the liquid and solid phases because the phase distribution kinetic constant of CTC for sewage sludge is tens to hundreds of times larger than the biodegradation kinetic constant of CTC (Ocampo-Pérez et al., 2012).

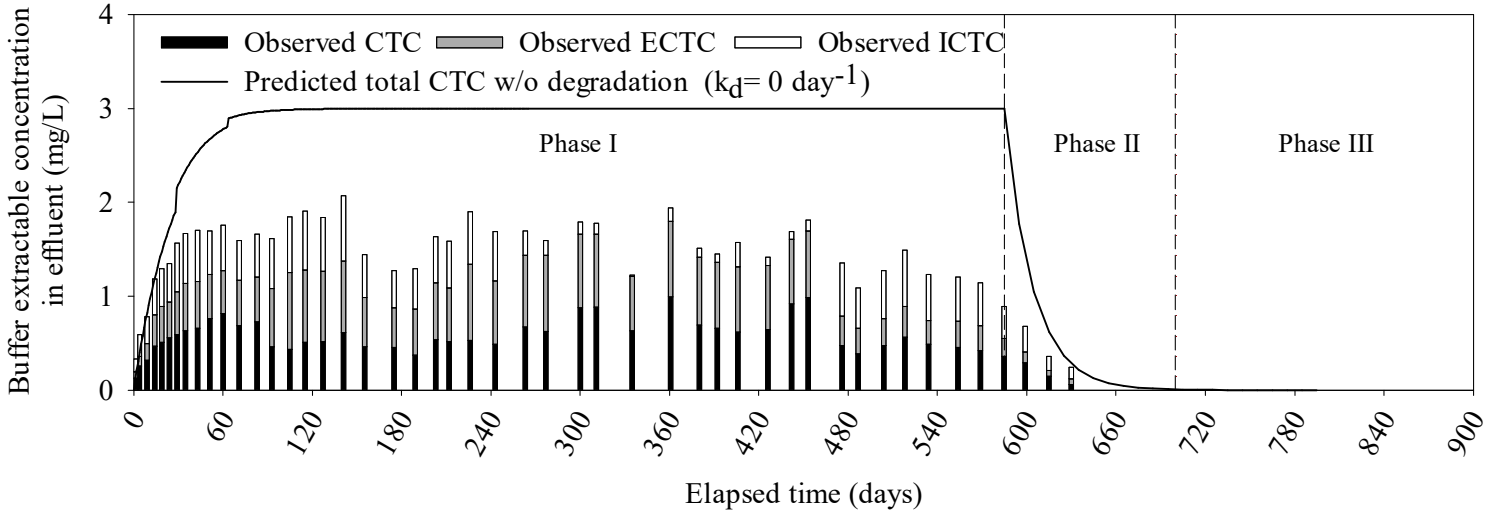

Figure 5.1 Buffer-extractable concentrations of CTC, ECTC, and ICTC in CSTR 2 (Phase I = 3 mg/L of CTC was spiked to swine manure for CSTR 2 and 1.46 ± 0.37 mg/L of CTC was in CSTR 2 during day120-580; Phase II = The CTC spiking was ceased and the concentration decreased to zero; Phase III = CTC concentration in CSTR 2 was maintained at zero)

5.3.2 Anaerobic digestion of swine manure

5.3.2.1 Methane production

During the acclimation to OLR, specific methane yields (SMY) of CSTRs 1 and 2 fluctuated and remained at an average of 104 ± 21 and 93 ± 25 mL/g-VS, respectively, except around day 130 when bottom impurities from the pig slurry pit were introduced to both reactors (Fig. 5.2). The average values of SMY for the two reactors were statistically same until 300 days; 100 ± 24 mL/g-VS for CSTR 1 and 97 ± 28 mL/g-VS for CSTR 2, respectively. These values are relatively low level compared to those reported in the existing literature. The specific methane yield of swine manure varied from 60 to 400 mL-CH_4/g-VS_{added} (Cárdenas-Cleves et al., 2018; Chae et al., 2008; Lee et al., 2018; Lymperatou et al., 2021). The facility where the swine manure was captured filters raw swine manure to make liquid fertilizer and compost separately. The swine manure used in this study was the filtered one. Soluble organic matter and easily biodegradable particles in the raw swine manure was partially lost during the separation. Accordingly, the volatile solid ratio of coarse fiber that can be hardly degraded increased. The biochemical methane potential of the swine manure used for the operation of CSTRs was (data not shown). It means that the low SMY is not due to mechanistic problems in the CSTRs.

The SMY of CSTR 2 decreased after day 300. Until the cessation of CTC exposure on day 585, the SMY of CSTR 2 continuously decreased to 55 ± 17 mL/g-

VS, approximately 53 % that of CSTR 1 (104 ± 17 mL/g-VS) (reduced by 47 % v/v) as of the latest five HRTs (from day 485 to 585) (Fig. 5.2). In the Chapter 4, AD reduced environmental risk of CTC in soil significantly because concentration of CTC is reduced during the AD. Nevertheless, methane generation was reduced. It clearly shows that inhibition can be caused by continuous exposure to 3 mg/L of CTC even if the concentration is decreased half (1.5 mg/L in the CSTR).

The degree of inhibition was similar to previous studies investigating the short-term effect of 20–200 mg/L of CTC on methane production in swine and cattle manure (Álvarez et al., 2010; C. Lee et al., 2020b; Stone et al., 2009). One-to-one mixtures of oxytetracycline (OTC) and CTC, at 20, 100, and 200 mg/L, resulted in a 45, 57, and 64 % (v/v) decrease, respectively, in methane generation during 30 days of batch AD of swine manure (Álvarez et al., 2010). When cattle manure was spiked with CTC at 25, 50, and 100 mg/L, methane generation was reduced by 9, 20, and 20 % (v/v), respectively (Lee et al., 2020b). In the study, 10 mg/L of CTC did not cause inhibition during 30 days of batch AD of cattle manure.

Continuous exposure to tetracycline negatively impacted biogas generation in an anaerobic sequencing batch reactors treating synthetic soluble wastewater (Cetecioglu et al., 2013; Liu et al., 2021). The anaerobic sequencing batch reactors were fed with 1.65 – 8.5 mg/L of tetracycline for maximum 170 days. Biogas generation was reduced by 10 - 45 % (v/v) by the stepwise increase of tetracycline concentrations. The time taken for biogas generation to be reduced by 40 % (v/v) (60 days) was shorter than that in the present study (300 days). This might be because

the average test concentration was twice as high as in the current study. In addition, since a mixtures of soluble organic substrates such as glucose, saccharose, acetic, butyric, and propionic acids were used, tetracycline's bioavailability was not hindered by sorption to substrates (Gonzalez-Gil et al., 2018). The use of soluble organic acids could boost the bioavailability of tetracycline. The neutralization of zwitterionic tetracycline by electrostatic interactions with organic acids can increase its penetration into the cell membrane (Chen et al., 2015; Zhang et al., 2014b). Furthermore, the anaerobes were continuously exposed to tetracycline in a sequential batch reactor during the test. In contrast, the current study used swine manure containing 6.3 % of total solids, and the solid retention time was 20 days.

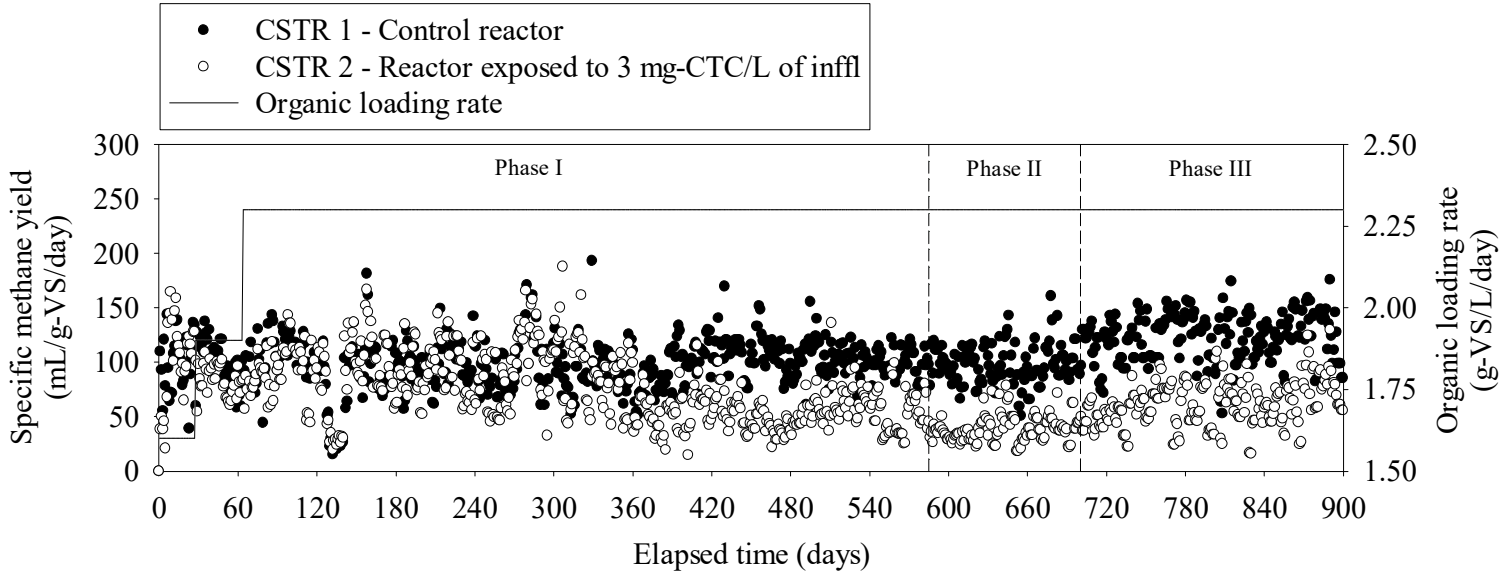

Figure 5.2 Specific methane yields of CSTR 1 (control reactor) and CSTR 2 (test reactor) (Phase I = 3 mg/L of CTC was spiked to swine manure for CSTR 2 and 1.46 ± 0.37 mg/L of CTC was in CSTR 2 during day120-580; Phase II = The CTC spiking was ceased and the concentration decreased to zero; Phase III = CTC concentration in CSTR 2 was maintained at zero)

In order to observe additional methane generation under batch conditions, 100 mL of digestate collected on day 517 from CSTRs 1 and 2 was transferred to two 265 mL serum bottles to observe methane generation under batch conditions (Fig. 5.3), respectively. For the 100 days of additional anaerobic digestion, the digestate from CSTR 2 showed higher cumulative methane generation than that of CSTR 1. The cumulative methane production of CSTRs 1 and 2 were 147 $\pm$ 12 mL (1,470 mL/L) and 172 $\pm$ 13 mL (1,720 mL/L), respectively. The residual organics after 20 days of digestion in CSTR was higher in the CSTR 2 due to the inhibition.

In addition, the total methane generation from continuous and batch reactors were 6,233 mL/$L_{reactor}$ for CSTR 1 and 4,763 mL/$L_{reactor}$ for CSTR 2. This means that swine manure was less digested through the microorganisms in CSTR 2 than those in CSTR1. The methane generation rate of the first-order kinetic model (day^{-1}) in the digestate of CSTR 1 was higher than that of CSTR 2; 0.083 $\pm$ 0.005 day^{-1} and 0.076 $\pm$ 0.004 day^{-1}, respectively. No lag phase was observed, and the maximum methane potential of each model was fixed as the observed value (Table 5.2). Therefore, long-term exposure to CTC seems to impair the methane production performance of CSTR 2 by lowering the methane conversion ratio of the substrate and the rate of methane generation.

In order to assess potential recovery from the inhibition caused by CTC, swine manure was not spiked with CTC from day 585 for CSTR 2. Although CSTR 2 was operated under the same conditions as CSTR 1 for over 300 days (i.e. 15 HRTs), the SMY of CSTR 2 did not recover and was consistently lower than that of CSTR

1. From days 800 to 900, the SMY of CSTR 1 was 122 ± 24 mL/g-VS/L/day, while that of CSTR 2 remained at 67 ± 21 mL/g-VS/L/day (Fig. 2). This clearly shows that the inhibition caused by long-term exposure to low CTC concentrations was irreversible from an anaerobic digestion scale point of view.

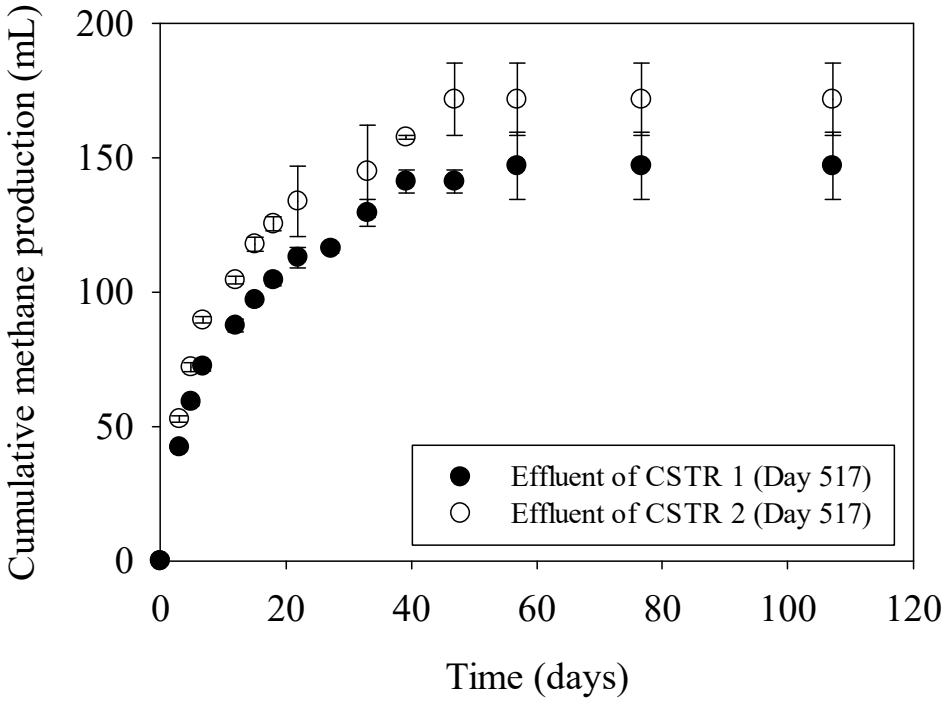

Figure 5.3 Cumulative methane production of effluent obtained at day 517

Table 5.2 Kinetic analysis on the methane generation

	1st order kinetic model		modified Gompertz equation			observed
CSTR	k (day^{-1})	R^2	R (mL-CH_4/day)	λ (day)	R^2	Maximum CH_4 production (mL)
CSTR 1	0.083 ± 0.005	0.9663	9.054 ± 1.623	4.21e-8	0.9004	147 ± 12
CSTR 2	0.076 ± 0.004	0.9719	7.015 ± 1.110	1.31e-10	0.9087	172 ± 13

5.3.2.2 Removal of COD and VS reduction

The changes in the removal ratio of total COD (% by w/w), VS reduction (% w/w), and concentration of soluble COD (mg/L) were represented in Fig. 5.4 using the moving averages of five HRTs data. Similar to the time when methane generation decreased (day 300), day 244 was identified as the time when the TCOD removal ratio and VS reduction of CSTR 2 was significantly lower than those of CSTR 1. It is natural that the inhibition time to TCOD and VS reduction and that of methane generation were similar because methane generation is the result of mineralization of organic matter (i.e., swine manure). By day 585, when CTC exposure ceased, the TCOD removal ratio of CSTR 1 was 37-55 %, whereas that of CSTR 2 was 30-50 % (w/w) (Fig. 5.4). For the VS reduction, the statistical inhibition was intermittently observed due to large measurement errors in VS. The period showing statistical difference was 54% of the total period after the inhibition observed. During 450-650 days, the VS reduction in the CSTR 1 was in a range of 24 – 31 % (w/w) while that of the CSTR 2 was 16-27 % (w/w). During 500-550 and 650-700 days, the VS reductions were the statistically same in both CSTR 1 and 2.

In addition to the mineralization of swine manure, the solubilization of swine manure was suppressed by long-term exposure to CTC. From day 300, the soluble COD in CSTR 1 was maintained at a concentration higher than in CSTR 2 (Fig. 5.4). The moving averages for CSTRs 1 and 2 from days 350 to 550 were in the range of 2,500–4,100 and 1,800–3,500 mg/L, respectively. The inhibitory effect

of CTC on hydrolysis is a widely accepted phenomenon in several studies. It is related to CTC's mode of action. CTC inhibits the production of enzymes and bacterial growth by reversibly binding to 16s rRNA and inhibiting metabolic protein synthesis (Chopra et al., 2001). Since extracellular enzymes hydrolyze organic materials, inhibition can be observed as a decrease in the rate of methane generation and a decrease in SCOD concentration, where hydrolysis is the rate-limiting step (Álvarez et al., 2010; Coban et al., 2016; Stone et al., 2009). All the evidences taken together indicate that long-term exposure to 3 mg-CTC/L in swine manure and 1–2 mg-CTC/L in CSTR can cause a significant inhibitory effect on the AD of swine manure. The degree of inhibition were similar with inhibition caused by 20 mg/L concentration of CTC and oxytetracycline (Álvarez et al., 2010). After CTC exposure ceased the TCOD removal efficiency and SCOD concentration of CSTR 2 were not statistically different with that of CSTR 1. The TCOD removal ratio of CSTR 1 was 40 $\pm$ 6 %, and that of CSTR 2 remained at 38 $\pm$ 8 % (w/w) as of day 800-900. The SCOD concentrations of CSTR 1 and 2 were 1,620 $\pm$ 320 and 1,560 $\pm$ 226 mg/L, respectively, as of the day 810.

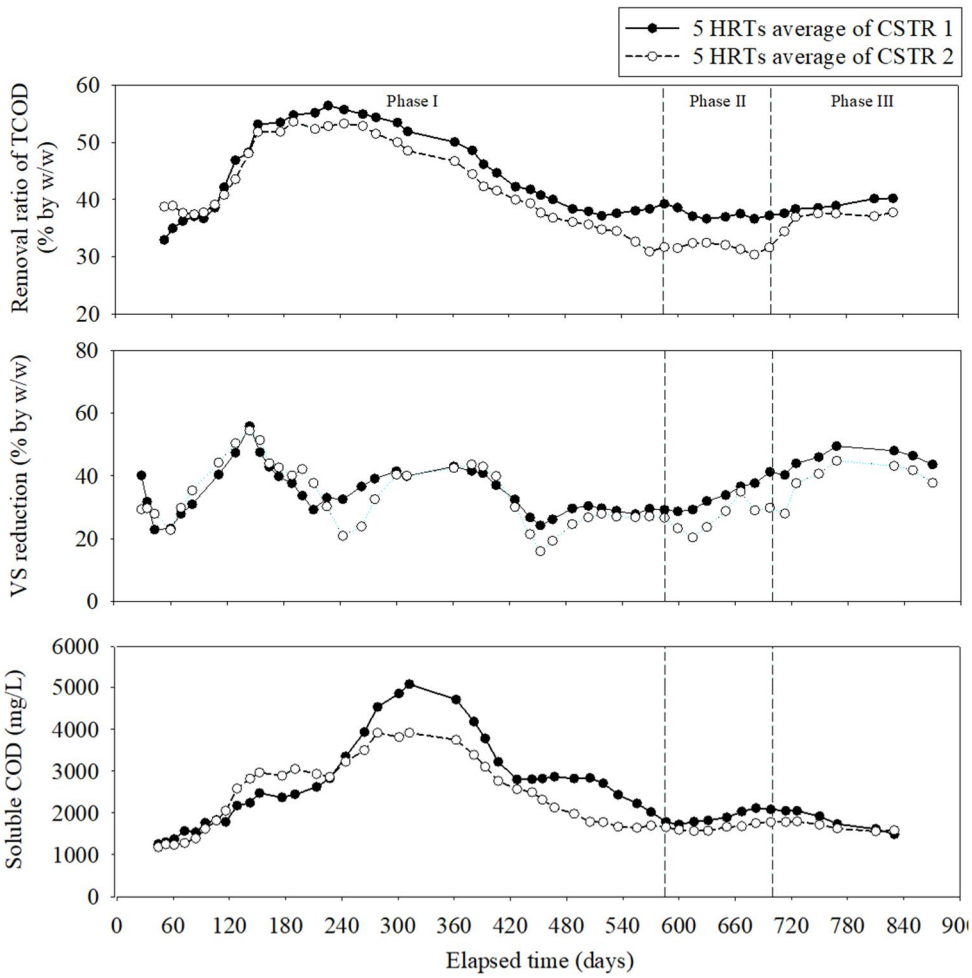

Figure 5.4 TCOD reduction, SCOD concentration, and VS reduction changes of CSTR 1 and 2 (Each point on the lines is the moving average of data before and after 2.5 HRTs of the point) (Phase I = 3 mg/L of CTC was spiked to swine manure for CSTR 2 and 1.46 ± 0.37 mg/L of CTC was in CSTR 2 during day120-580; Phase II = The CTC spiking was ceased and the concentration decreased to zero; Phase III = CTC concentration in CSTR 2 was maintained at zero)

5.3.2.3 Stability parameters

The pH, NH_3, and alkalinity in CSTR 2 were not different from those of CSTR 1 (Fig. 5.5). VFAs in both CSTRs were different; however, not in inhibition level.

The pH was stably maintained at around 7.2 in both reactors after a slight decrease from initial operation (day 0–20) (Fig. 5.5 (a)). The total ammonia concentration in both reactors was 500 mg/L on day 0 and gradually increased to 1,500 mg/L by day 300. The concentration decreased to approximately 500-600 mg/L (Fig. 5.5 (b)). Some studies have considered the inhibition threshold of total ammonia to be 800–1,400 mg/L (González-Fernández and García-Encina, 2009) or more than 3,000 mg/L (Angelidaki and Ahring, 1994, 1993; Massé et al., 2003; Nakakubo et al., 2008). Thus, the decrease in the TCOD reduction ratio and SCOD accumulation in both reactors observed around day 300 might be due to an increase in the total ammonia concentration. However, since there was no difference in ammonia concentration in CSTRs 1 and 2 during the experiment, it was considered that ammonia did not caused the AD inhibitions in CSTR 2.

VFAs were accumulated up to 250 mg/L in CSTR 2 when inhibition occurred (day 400 – 600 day); however, the VFAs concentration in CSTR 2 did not reach inhibition level because methane generation and TCOD removal rate of CSTR 1 did not decrease when the tVFA in CSTR 1 increased to 370 mg/L in the present study (Fig. 5.5 (d)). Accordingly, the alkalinity, which were in a range of 8,000 –

15,000 mg-$CaCO_3$/L in both reactors, did not decrease enough to lead pH drops (Fig. 5.5 (c)). Even when CTC exposure ceased, the VFAs in CSTR 2 did not accumulate compared to control. CTC inhibition can cause accumulation of specific VFAs such as propionic acid and butyric acid (Aydin et al., 2015b; Cetecioglu et al., 2013; Wang et al., 2017); however, Acetate was the most abundant VFA in both reactor during the experiments (Appendix S5.2).

Two independent possible explanations for the patterns of inhibitions are; first, biodegradation of swine manure by microorganisms in CSTR 2 was reduced due to the inhibition of hydrolysis as described above. Second, hydrolyzed swine manure was not fully converted to VFAs, but stored as byproducts. The latter scenario has been suggested by previous studies that observed a higher degree of inhibition on methane generation than on organic matter reduction (called stoichiometric disturbance) (Cetecioglu et al., 2012; Cetecioglu et al., 2013). Although methane production was reduced by 10–18 % (v/v) due to tetracycline exposure, a soluble organic substrate mixture consisting of starch, glucose, and acetate, butyric, and propionic acids was completely removed in the liquid phase (Cetecioglu et al., 2013). Cetecioglu et al. (2013) explained that this stoichiometric disturbance might be partly due to CTC's uncompetitive inhibition characteristics. Unlike competitive or non-competitive inhibition, which leads to kinetic impact affecting substrate utilization rate, uncompetitive inhibition affects the utilization ratio of a substrate by binding the enzyme-substrate complex (Cetecioglu et al., 2012; Fountoulakis et al., 2008). In other words, CTC hinders specific metabolic steps of

methanogenesis and enhances substrate conversion to storage products such as trehalose and glutamate (Shimada et al., 2007). Nevertheless, there is still no evidence of an increase in storage products due to the inhibition caused by CTC. In the current study, trehalose and glutamate, which are major reserve polymers of carbohydrates for anaerobic microorganisms (Shimada et al., 2007), were analyzed to identify whether the storage substances exist differently in CSTR 1 and CSTR 2 through characterization of the 500 MHz nuclear magnetic resonance (NMR) spectra for ^{13}C. However, NMR spectra of trehalose and glutamate were not identified in both reactors comparing with their standard NMR spectra (Appendix S5.3). To obtain samples for the NMR analysis, cell lysis on sample obtained from day 500 was conducted by applying RIPA buffer (25 % dilution by v/v) and ultra-sonication to the centrifuge-separated solid phase of digestate.

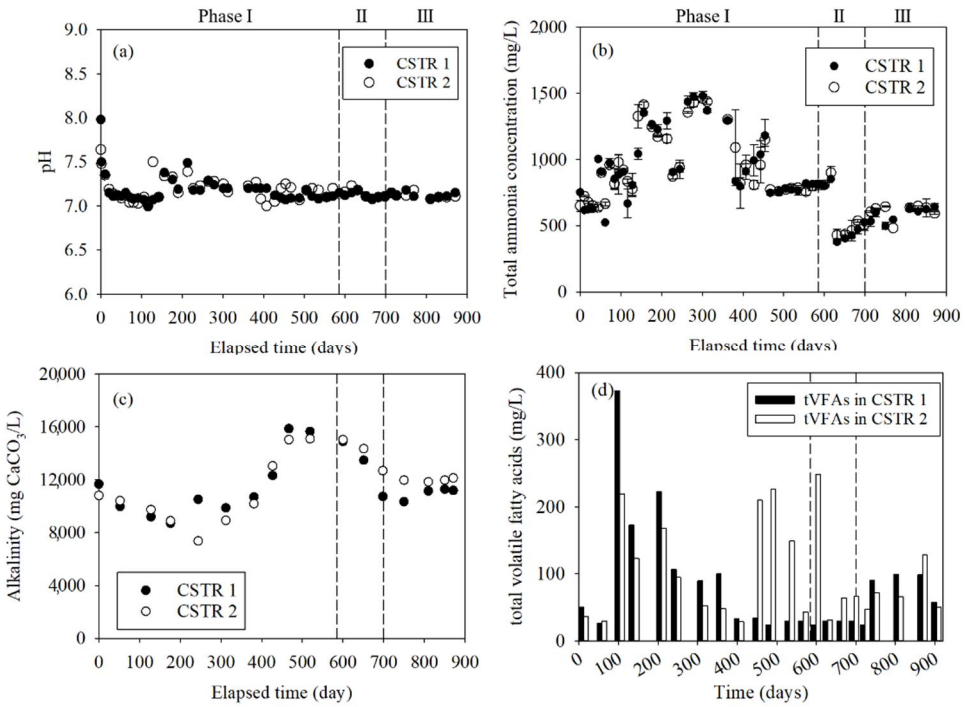

Figure 5.5 Stability parameters of CSTR 1 and 2 ((a) pH, (b) total ammonia concentration, (c) alkalinity, and (d) total volatile fatty acids) (Phase I = 3 mg/L of CTC was spiked to swine manure for CSTR 2 and 1.46 ± 0.37 mg/L of CTC was in CSTR 2 during day120-580; Phase II = The CTC spiking was ceased and the concentration decreased to zero; Phase III = CTC concentration in CSTR 2 was maintained at zero)

In addition to the long-term inhibitions on the performance of CSTR 2, CTCs in the CSTR 2 may have exhibited acute effects on the performance of CSTR 2. The relative ratios between CTC, ECTC+EICTC, and ICTC were different during the operation of CSTR 2. As performance parameters, VS reduction, TCOD removal, tVFA, propionic acid, and acetic acid concentrations and specific methane generation were depicted according to the CTC, ECTC+EICTC, ICTC, and their

summation, respectively (Appendix S5.4). The concentration changes of CTCs did not show noticeable negative relationship with the performance parameters. These results clearly show that the performance deterioration in CSTR 2 were caused by the long-term exposure to CTC although the spiked CTC concentration was decreased by degradation and transformation during AD.

5.3.3 Microbial community

5.3.3.1 Microbial diversity

Good's coverage of the library of all samples exceeded 99 % and rarefaction curves reached plateau (data not shown), indicating that results from all samples represented each microbial community in a specific niche.

Given that microbial diversity implies the evenness and richness of microbial species in a community, the Shannon index has more weight on species richness, and the Simpson index has more focus on evenness. A higher Shannon index represents higher diversity, whereas the Simpson index represents the opposite (Kim et al., 2017). Before the inhibition happened (day 0–250), the bacterial diversity indices (i.e. Shannon and Simpson indices) of CSTR 2 were similar to those of CSTR 1. After the inhibition, the bacterial diversity of CSTR 2 decreased with long-term exposure even though CTC exposure ceased (i.e. the Shannon indices of CSTR 2 were lower than that of CSTR 1 and the Simpson indices of CSTR 2 were the opposite) (Table 3). For the archaeal community, the Shannon and Simpson indices of CSTR 1 and 2 did not represent noticeable trend during the experiment (Table 5.3).

Table 5.3 Diversity parameters of the control (CSTR 1) and test reactors (CSTR 2)

Bacterial community	OTUs		Shannon index		Simpson index	
	Normalized by 28,000 read count					
	CSTR 1	CSTR 2	CSTR 1	CSTR 2	CSTR 1	CSTR 2
Day 0	771	771	3.95	3.95	0.06	0.06
Day 90	652	639	3.55	3.51*	0.08	0.08
Day 188	877	806	3.72	3.38*	0.10	0.14*
Day 500	716	806	3.48	3.32*	0.11	0.15*
Day 750	906	925	4.16	3.99*	0.04	0.06*
Day 808	1,073	1,001	3.58	3.47*	0.08	0.12*
Archaeal community	OTUs		Shannon index		Simpson index	
	Normalized by 35,000 read count					
	CSTR 1	CSTR 2	CSTR 1	CSTR 2	CSTR 1	CSTR 2
Day 0	106	106	1.93	1.93	0.26	0.26
Day 90	86	58	1.36	1.06*	0.41	0.54*
Day 188	243	260	1.46	2.12*	0.38	0.20*
Day 500	300	191	2.14	2.21*	0.16	0.16
Day 750	152	131	2.21	2.31*	0.19	0.14*
Day 808	136	140	2.39	2.28*	0.13	0.16*

*: statistical different CSTR 1 ($p < 0.05$)

5.3.3.2 Taxonomic profiles and principal component analysis of the microbial communities

In order to identify differences in taxonomic profiles between samples, principal component analysis was conducted by using bacterial and archaeal counts in phylum and genus level, respectively. The non-normalized counts and relative abundances of bacterial (phylum level) and archaeal (genus level) communities were provided in Appendix S5.5. The results of PCA analysis were explained by maximum variation of 64.9 % (first axis) and 19.8 % (second axis) for bacterial and maximum variation of 38.5 % (first axis) and 23.4 % (second axis) for archaea (Fig. 5.6). Dominant bacterial communities were changed with time and represented similar profiles for 90, 188, and 500 days. *Firmicutes* and *Bacteroidetes* phyla were dominant in CSTR 1 and 2, respectively, because these constitute the major microbes in pig intestines (Briones et al., 2014). With increase of *Bacteroidetes* (control reactor, R1) and *Chloroflexi* (test reactor, R2), both reactors showed differences for 750- and 808-day samples. With the results of diversity indices, it should be noted out that the ratios of *Firmicutes* and *Bacteroidetes i*n CSTR 1 were lower than those in CSTR 2. *Firmicutes* and *Bacteroidetes* are known to primarily contribute to hydrolysis and acidogenesis (Briones et al., 2014; Iannotti et al., 1982; Ma et al., 2019; Sun et al., 2015; Tuan et al., 2014). The ratios of *Firmicutes* to *Bacteroidetes* in CSTR 1 were 2.6, 0.8 and 1.7 for day 500, 750 and 808, whereas those in CSTR 2 were 3.8, 0.9, and 3.0, respectively. The ratio is known to be associated with not only the health of

the microbiota in the human gut (Magne et al., 2020; Mariat et al., 2009; Vaiserman et al., 2020) but also digestion performance (Briones et al., 2014; Regueiro et al., 2012). According to Briones et al. (2014), a *Bacteroidetes*-dominated community (i.e., low *Firmicutes*/*Bacteroidetes* ratio) with a high-fiber substrate produced more VFAs and methane than a *Firmicutes*-dominated community (i.e. high *Firmicutes*/*Bacteroidetes* ratio). A study by Regueiro et al. (2012), analyzing the relationship between microbial activity and community in six full-scale AD plants, revealed that a higher hydrolytic activity was observed in AD plants with more *Bacteroidetes* (i.e., low *Firmicutes*/*Bacteroidetes* ratio). These results are in line with the results of the present study that valid reads for *Bacteroidetes* in CSTR 1 was higher than that in CSTR 2. After the inhibition happened, the *Bacteroidetes* in CSTR 1 were 9,036, 16,722, and 13,652, whereas that in CSTR 2 were 6,583, 12,707, and 10,832 for day 500, 750, and 808, respectively. Similarly, 0.1–4 mg/L of erythromycin and tetracycline in an anaerobic sequencing batch reactor, decreased *Bacteroidetes* over time but *Firmicutes* was stable for approximately 400 days (Aydin et al., 2016).

Archaeal class in CSTR 1 and CSTR 2 were different except samples obtain at day 90. After day 188, the relative abundances of *Bathyarchaeota* and *Euryarchaeota* were in a range of 45 – 75 and 25 – 55 % in the CSTR 1, respectively. In CSTR 2, those were 50 – 60 and 40 – 50 %, respectively (Appendix S5.5). Previous studies observed that 17 and 20 mg /L CTC could reduce abundance of acetoclastic methanogens and increase abundance of hydrogenotrophic methanogens

(Wang et al., 2018; Xiong et al., 2017); however, CTC did not increase the abundance of hydrogenotrophic methanogens such as *Methanomassiliicoccales* and *Methanomicrobiales* in the current study. The total reads and relative abundance of *Methnomassilliicoccales* and *methanomicrobiales*, which are class-level hydrogenotrophic methanogens, were higher in CSTR 1 than CSTR 2 and acetoclastic methanogens were abundant in the both reactors regardless of CTC inhibition (Table S4). The reasons might because archaea is more resistant to VAs such as TCs inhibiting protein synthesis than bacteria due to impermeability of the cytoplasmic membrane and/or heterogeneous protein compositions (Cheng et al., 2018; Hilpert et al., 1981). The fact that the test concentration (3 mg/L in swine manure and 1–2 mg/L in CSTR) of this study was over five times lower than that of previous studies can be the reason for the different result with the previous studies.

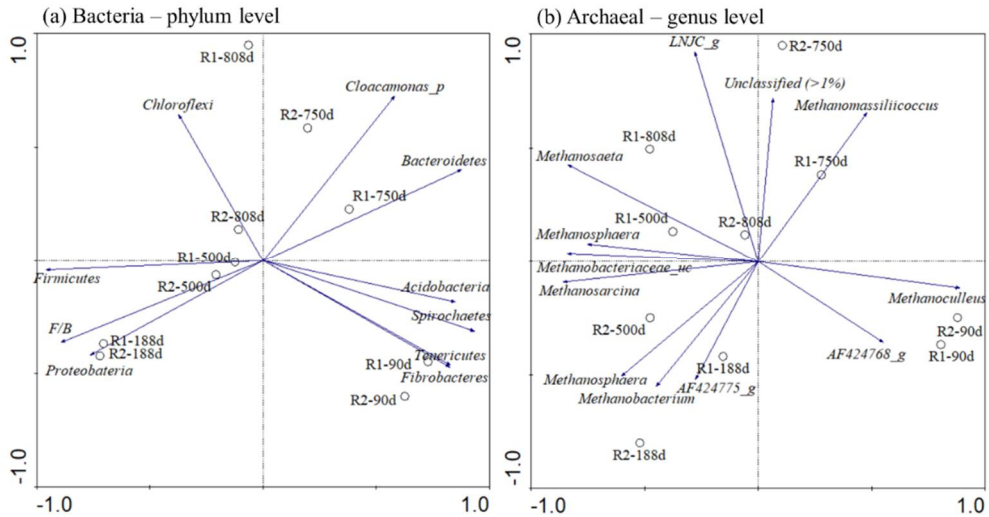

Figure 5.6 Principal component analysis: (a) Bacteria – phylum level; (b) Archaeal – genus level

5.3.4 Bivariate and redundancy analysis between microbial community and environmental factors

The results of SRCC and probability level (i.e. *p*-value) were listed in the Appendix S5.6. CTC exposure was only negatively correlated with CH_4 (SRCC = -0.79) and TCOD removal (SRCC = -0.59) ($p < 0.05$). Bacteria and archaea did not show any positive or negative correlation with CTC exposure periods but represented some relationships with VFAs and TNH_3. Acetic acid showed positive relationship with archaeal genus *AF424768_g* (SRCC = 0.83), and negative relationship with *LNJC_G* (SRCC = -0.65) and *Methanosarcina* (SRCC = -0.71). Propionic acid was positively correlated with *Acidobacteria* (SRCC = 0.69) and archaeal genus *AF424768_g* (SRCC = 0.69) while negatively correlated with *Methanosarcina* (SRCC = -0.89) and *Methanosphaera* (SRCC = -0.70). Butyric acid was positively correlated with not only bacterial phyla *Bacteroidetes* (SRCC = 0.83), *Cloacamonas_p* (SRCC = 0.75), and *Acidobacteria* (SRCC = 0.65), but also archaeal genus *Methanoculleus* (SRCC = 0.65), and *Methanomassiliicoccus* (SRCC = 0.72) ($p < 0.05$). TNH_3 represented negative correlation with *Cloacamonas_p* and *LNJC_g*. These results agreed with the results of PCA (Fig. 5.6) that differences in bacterial community were in relation to the abundance of *Bacteroidetes and Cloacamonas_p*. The differences in archaeal community were distinguished by *Methanosarcina, Methanomassiliicoccus and LNJC_g* after inhibition happened.

The results of RDA analysis were shown in Fig. 5.7 by control/test reactor

and bacteria/archaea. For CSTR 1, the first and the second canonical axes represented 62 % and 19 % of variation for bacteria, and 60 % and 22 % of variation for archaea, respectively. For CSTR 2, the first and the second canonical axes represented 66 % and 22 % of variation for bacteria, and 58 % and 26 % of variation for archaea in CSTR 2, respectively. As consistent with the result of SRCC, the triplot analysis suggested that microbial taxon was not correlated with CTC exposure period but correlated with VFAs (Fig. 5.7 (b) and (d)). For example, butyric acid was positively correlated with *Tenericutes, Fibrobacteres, Methanoculleus,* and *Methanomassillicoccus* in the control reactor while *Cloacomonas_p, LNJC_g*, and *Methanomassillicoccus* showed the positive relationship in the test reactor (Fig. 5.7). Different microbial communities were associated with production of the VFAs in both reactors. It may result in the performance differences without failure of the test reactor.

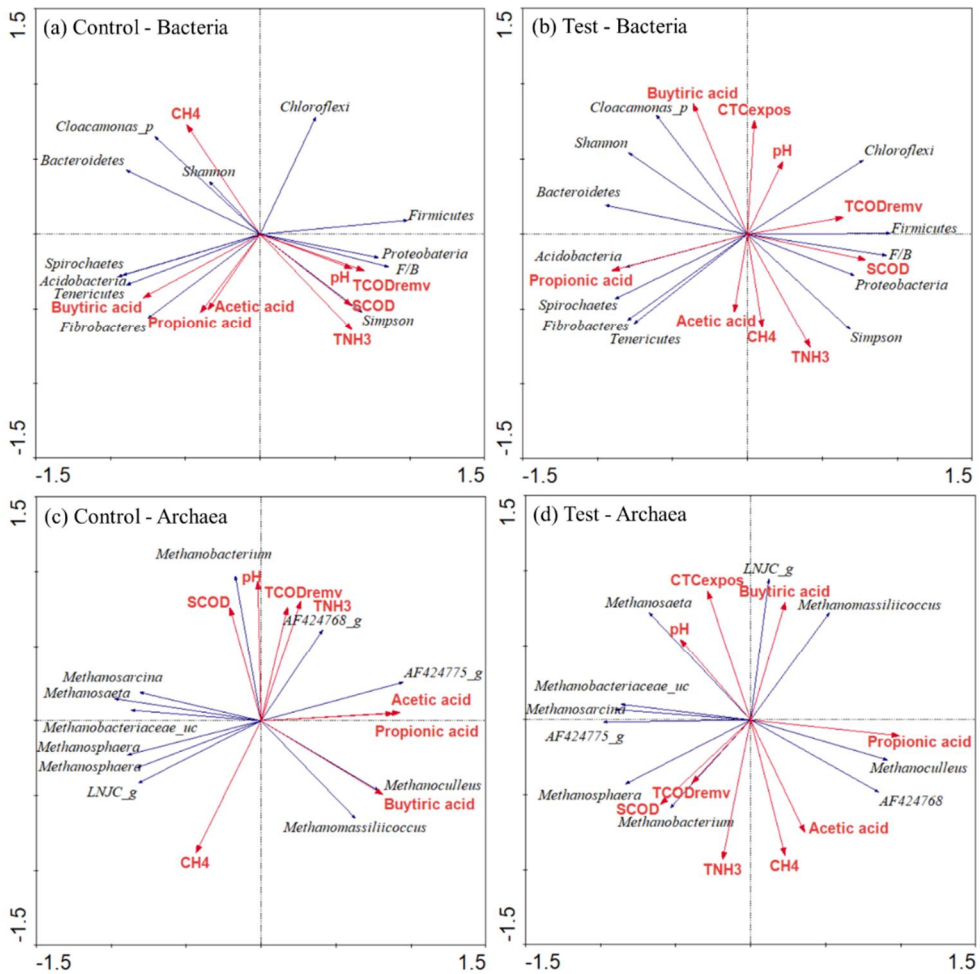

Figure 5.7 Redundancy analysis between microbial community and environmental parameters: (a) control reactor - bacteria; (b) test reactor - bacteria; (c) control reactor – archaea; (d) test reactor - archaea

5.4 Summary

This chapter aimed to investigate long-term inhibition of chlortetracycline (CTC) antibiotics to anaerobic digestion (AD) of swine manure (SM) and recovery from the inhibition. Two continuous-stirred tank reactors treating SM w/wo CTC spiking (3 mg/L) were operated for 900 days. At around day 300, chemical oxygen demand reduction, volatile solid reduction and methane generation decreased in the test reactor due to reduced methane generation rate and mineralization ratio of the SM. The methane generation was not recovered during 300 days even after the CTC exposure was stopped. In addition, the concentration changes of CTCs did not show noticeable negative relationship with the performance parameters. These result clearly show that inhibition can be caused by continuous exposure to 3 mg/L of CTC even if the concentration is decreased half (1.5 mg/L in the CSTR) and correspondingly its environmental risk in soil is reduced significantly. Redundancy analysis using microbial profiles and environmental variables revealed that reduced bacterial diversity and changed balance in microbial species were related to the performance deterioration. Since it is hard to recover from the inhibition and difficult to predict using physicochemical indicators, continuous exposure to CTC needs to be avoided for the sustainable management of AD plants treating SM.

References

Álvarez, J. A., Otero, L., Lema, J. M., & Omil, F. (2010). The effect and fate of antibiotics during the anaerobic digestion of pig manure. *Bioresource Technology*, *101*(22), 8581–8586. https://doi.org/10.1016/j.biortech.2010.06.075

Angelidaki, I., & Ahring, B. K. (1993). Thermophilic anaerobic digestion of livestock waste: the effect of ammonia. *Applied Microbiology and Biotechnology*, *38*(4), 560–564. https://doi.org/10.1007/BF00242955

Angelidaki, I., & Ahring, B. K. (1994). Anaerobic thermophilic digestion of manure at different ammonia loads: Effect of temperature. *Water Research*, *28*(3), 727–731. https://doi.org/10.1016/0043-1354(94)90153-8

Arikan, O. A. (2008). Degradation and metabolization of chlortetracycline during the anaerobic digestion of manure from medicated calves. *Journal of Hazardous Materials*, *158*(2–3), 485–490. https://doi.org/10.1016/j.jhazmat.2008.01.096

Arikan, O. A., Sikora, L. J., Mulbry, W., Khan, S. U., & Foster, G. D. (2007). Composting rapidly reduces levels of extractable oxytetracycline in manure from therapeutically treated beef calves. *Bioresource Technology*, *98*(1), 169–176. https://doi.org/10.1016/j.biortech.2005.10.041

Arikan, O. A., Sikora, L. J., Mulbry, W., Khan, S. U., Rice, C., & Foster, G. D. (2006).

The fate and effect of oxytetracycline during the anaerobic digestion of manure from therapeutically treated calves. *Process Biochemistry*, *41*(7), 1637–1643. https://doi.org/10.1016/j.procbio.2006.03.010

Aydin, S., Cetecioglu, Z., Arikan, O., Ince, B., Ozbayram, E. G., & Ince, O. (2015). Inhibitory effects of antibiotic combinations on syntrophic bacteria, homoacetogens and methanogens. *Chemosphere*, *120*, 515–520. https://doi.org/10.1016/j.chemosphere.2014.09.045

Aydin, S., Ince, B., Cetecioglu, Z., Arikan, O., Ozbayram, E. G., Shahi, A., & Ince, O. (2015). Combined effect of erythromycin, tetracycline and sulfamethoxazole on performance of anaerobic sequencing batch reactors. *Bioresource Technology*, *186*, 207–214. https://doi.org/10.1016/j.biortech.2015.03.043

Aydin, S., Ince, B., & Ince, O. (2016). Assessment of anaerobic bacterial diversity and its effects on anaerobic system stability and the occurrence of antibiotic resistance genes. *Bioresource Technology*, *207*, 332–338. https://doi.org/10.1016/j.biortech.2016.01.080

Berendsen, B. J. A., Lahr, J., Nibbeling, C., Jansen, L. J. M., Bongers, I. E. A., Wipfler, E. L., & van de Schans, M. G. M. (2018). The persistence of a broad range of antibiotics during calve, pig and broiler manure storage. *Chemosphere*, *204*, 267–276. https://doi.org/10.1016/j.chemosphere.2018.04.042

Briones, A., Coats, E., & Brinkman, C. (2014). Should We Build “Obese” or “Lean” Anaerobic Digesters? *PLoS ONE*, *9*(5), e97252.

https://doi.org/10.1371/journal.pone.0097252

Carballo, M., Aguayo, S., González, M., Esperon, F., & Torre, A. de la. (2016). Environmental Assessment of Tetracycline's Residues Detected in Pig Slurry and Poultry Manure. *Journal of Environmental Protection*, *07*(01), 82–92. https://doi.org/10.4236/jep.2016.71008

Carey, D. E., Zitomer, D. H., Kappell, A. D., Choi, M. J., Hristova, K. R., & McNamara, P. J. (2016). Chronic exposure to triclosan sustains microbial community shifts and alters antibiotic resistance gene levels in anaerobic digesters. *Environmental Science: Processes and Impacts*, *18*(8), 1060–1067. https://doi.org/10.1039/c6em00282j

Cetecioglu, Z., Ince, B., Gros, M., Rodriguez-Mozaz, S., Barceló, D., Orhon, D., & Ince, O. (2013). Chronic impact of tetracycline on the biodegradation of an organic substrate mixture under anaerobic conditions. *Water Research*, *47*(9), 2959–2969. https://doi.org/10.1016/j.watres.2013.02.053

Cetecioglu, Zeynep, Ince, B., Orhon, D., & Ince, O. (2012). Acute inhibitory impact of antimicrobials on acetoclastic methanogenic activity. *Bioresource Technology*, *114*, 109–116. https://doi.org/10.1016/j.biortech.2012.03.020

Chen, W. R., & Huang, C. H. (2011). Transformation kinetics and pathways of tetracycline antibiotics with manganese oxide. *Environmental Pollution*, *159*(5), 1092–1100. https://doi.org/10.1016/j.envpol.2011.02.027

Chen, Y., Cheng, J. J., & Creamer, K. S. (2008). Inhibition of anaerobic digestion process: A review. *Bioresource Technology*, *99*(10), 4044–4064.

https://doi.org/10.1016/j.biortech.2007.01.057

Chen, Z., Zhang, Y., Gao, Y., Boyd, S. A., Zhu, D., & Li, H. (2015). Influence of Dissolved Organic Matter on Tetracycline Bioavailability to an Antibiotic-Resistant Bacterium. *Environmental Science and Technology*, *49*(18), 10903–10910. https://doi.org/10.1021/acs.est.5b02158

Cheng, D. L., Ngo, H. H., Guo, W. S., Chang, S. W., Nguyen, D. D., Kumar, S. M., Du, B., Wei, Q., & Wei, D. (2018). Problematic effects of antibiotics on anaerobic treatment of swine wastewater. In *Bioresource Technology* (Vol. 263). https://doi.org/10.1016/j.biortech.2018.05.010

Chopra, I. and Roberts, M. (2001). Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews*, *65*(2), 232–260. https://doi.org/10.1128/MMBR.65.2.232

Coban, H., Ertekin, E., Ince, O., Turker, G., Akyol, Ç., & Ince, B. (2016). Degradation of oxytetracycline and its impacts on biogas-producing microbial community structure. *Bioprocess and Biosystems Engineering*, *39*(7), 1051–1060. https://doi.org/10.1007/s00449-016-1583-z

Colón, J., Forbis-Stokes, A. A., & Deshusses, M. A. (2015). Anaerobic digestion of undiluted simulant human excreta for sanitation and energy recovery in less-developed countries. *Energy for Sustainable Development*, *29*, 57–64. https://doi.org/10.1016/J.ESD.2015.09.005

Daghrir, R., & Drogui, P. (2013). Tetracycline antibiotics in the environment: A

review. *Environmental Chemistry Letters*, *11*(3), 209–227. https://doi.org/10.1007/s10311-013-0404-8

Dewey, C. E., Cox, B. D., Straw, B. E., Bush, E. J., & Hurd, S. (1999). Use of antimicrobials in swine feeds in the United States. *Swine Health and Production*, *7*(1), 19–25. http://www.aasp.org/shap.html

Elmund, G. K., Morrison, S. M., Grant, D. W., & Nevins, M. P. (1971). Role of excreted chlortetracycline in modifying the decomposition process in feedlot waste. *Bulletin of Environmental Contamination and Toxicology*, *6*(2), 129–132. https://doi.org/10.1007/BF01540093

Fountoulakis, M. S., Stamatelatou, K., & Lyberatos, G. (2008). The effect of pharmaceuticals on the kinetics of methanogenesis and acetogenesis. *Bioresource Technology*, *99*(15), 7083–7090. https://doi.org/10.1016/j.biortech.2008.01.008

Gartiser, S., Urich, E., Alexy, R., & Kümmerer, K. (2007). Anaerobic inhibition and biodegradation of antibiotics in ISO test schemes. *Chemosphere*, *66*(10), 1839–1848. https://doi.org/10.1016/j.chemosphere.2006.08.040

Gelband, H., Miller-Petrie, M., Pant, S., Gandra, S., Levinson, J., Barter, D., White, A., & Laxminarayan, R. (2015). State of the World ' S Antibiotics. *Center for Disease Dynamics, Economics & Policy*.

González-Fernández, C., & García-Encina, P. A. (2009). Impact of substrate to inoculum ratio in anaerobic digestion of swine slurry. *Biomass and Bioenergy*, *33*(8), 1065–1069. https://doi.org/10.1016/j.biombioe.2009.03.008

Gonzalez-Gil, L., Mauricio-Iglesias, M., Carballa, M., & Lema, J. M. (2018). Why are organic micropollutants not fully biotransformed? A mechanistic modelling approach to anaerobic systems. *Water Research*, *142*, 115–128. https://doi.org/10.1016/j.watres.2018.05.032

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P., Ingerslev, F., Holten Lutzheft, H., & Jorgensen, S. (1998). Occurence, Fate and Effects pf PHarmaceutical Substrances in the Environment. *Chemosphere*, *36*(2), 357–393.

Hilpert, R., Winter, J., Hammes, W., & Kandler, O. (1981). The sensitivity of archaebacteria to antibiotics. *Zentralblatt Fur Bakteriologie.Allgemeine Angewandte Und Okologische Microbiologie Abt.1 Orig.C Hyg.*, *2*(1), 11–20. https://doi.org/10.1016/S0721-9571(81)80014-2

Hou, G., Hao, X., Zhang, R., Wang, J., Liu, R., & Liu, C. (2016). Tetracycline removal and effect on the formation and degradation of extracellular polymeric substances and volatile fatty acids in the process of hydrogen fermentation. *Bioresource Technology*, *212*, 20–25. https://doi.org/10.1016/j.biortech.2016.03.156

Huang, C.-H., & Chen, W.-R. (2009). Transformation of tetracyclines mediated by Mn(II) and Cu(II) ions in the presence of oxygen. *Environmental Science and Technology*, *43*(2), 401–407. https://doi.org/10.1021/es802295r

Iannotti, E. L., Fischer, J. R., & Sievers, D. M. (1982). Characterization of Bacteria from a Swine Manure Digester. *Applied and Environmental Microbiology*, *43*(1).

Ince, B., Coban, H., Turker, G., Ertekin, E., & Ince, O. (2013). Effect of oxytetracycline on biogas production and active microbial populations during batch anaerobic digestion of cow manure. *Bioprocess and Biosystems Engineering*, *36*(5), 541–546. https://doi.org/10.1007/s00449-012-0809-y

Jia, S., Zhang, X. X., Miao, Y., Zhao, Y., Ye, L., Li, B., & Zhang, T. (2017). Fate of antibiotic resistance genes and their associations with bacterial community in livestock breeding wastewater and its receiving river water. *Water Research*, *124*, 259–268. https://doi.org/10.1016/j.watres.2017.07.061

Kennedy, D. G., McCracken, R. J., Carey, M. P., Blanchflower, W. J., & Hewitt, S. A. (1998). Iso- and epi-iso-chlortetracycline are the principal metabolites of chlortetracycline in the hen's egg. *Journal of Chromatography A*, *812*(1–2), 327–337. https://doi.org/10.1016/S0021-9673(98)00280-5

Kim, B. R., Shin, J., Guevarra, R. B., Lee, J. H., Kim, D. W., Seol, K. H., Lee, J. H., Kim, H. B., & Isaacson, R. E. (2017). Deciphering diversity indices for a better understanding of microbial communities. *Journal of Microbiology and Biotechnology*, *27*(12), 2089–2093. https://doi.org/10.4014/jmb.1709.09027

Lee, C., Jeong, S., Ju, M., & Kim, J. Y. (2020a). Fate of chlortetracycline antibiotics during anaerobic degradation of cattle manure. *Journal of Hazardous Materials*, *386*, 121894. https://doi.org/10.1016/J.JHAZMAT.2019.121894

Lee, C., Jeong, S., Ju, M., & Kim, J. Y. (2020b). Fate of chlortetracycline antibiotics during anaerobic degradation of cattle manure. *Journal of Hazardous Materials*, *386*, 121894. https://doi.org/10.1016/J.JHAZMAT.2019.121894

Li, Y. X., Zhang, X. L., Li, W., Lu, X. F., Liu, B., & Wang, J. (2013). The residues and environmental risks of multiple veterinary antibiotics in animal faeces. *Environmental Monitoring and Assessment*, *185*(3), 2211–2220. https://doi.org/10.1007/s10661-012-2702-1

Loftin, K. A., Adams, C. D., Meyer, M. T., & Surampalli, R. (2008). Effects of Ionic Strength, Temperature, and pH on Degradation of Selected Antibiotics. *Journal of Environment Quality*, *37*(2), 378. https://doi.org/10.2134/jeq2007.0230

Ma, X., Jiang, T., Chang, J., Tang, Q., Luo, T., & Cui, Z. (2019). Effect of Substrate to Inoculum Ratio on Biogas Production and Microbial Community During Hemi-Solid-State Batch Anaerobic Co-digestion of Rape Straw and Dairy Manure. In *Applied Biochemistry and Biotechnology* (Vol. 189, Issue 3, pp. 884–902). Humana Press Inc. https://doi.org/10.1007/s12010-019-03035-9

Magne, F., Gotteland, M., Gauthier, L., Zazueta, A., Pesoa, S., Navarrete, P., & Balamurugan, R. (2020). The Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients? *Nutrients*, *12*(5), 1474. https://doi.org/10.3390/nu12051474

Mariat, D., Firmesse, O., Levenez, F., Guimarães, V. D., Sokol, H., Doré, J., Corthier, G., & Furet, J. P. (2009). The firmicutes/bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiology*, *9*(1), 123. https://doi.org/10.1186/1471-2180-9-123

Martínez-Carballo, E., González-Barreiro, C., Scharf, S., & Gans, O. (2007). Environmental monitoring study of selected veterinary antibiotics in animal

manure and soils in Austria. *Environmental Pollution*, *148*(2), 570–579. https://doi.org/10.1016/J.ENVPOL.2006.11.035

Massé, D. I., Masse, L., & Croteau, F. (2003). The effect of temperature fluctuations on psychrophilic anaerobic sequencing batch reactors treating swine manure. *Bioresource Technology*, *89*(1), 57–62. https://doi.org/10.1016/S0960-8524(03)00009-9

McCormick, J. R. D., Fox, S. M., Smith, L. L., Bitler, B. A., Reichenthal, J., Origoni, V. E., Muller, W. H., Winterbottom, R., & Doerschuk, A. P. (1957). Studies of the Reversible Epimerization Occurring in the Tetracycline Family. The Preparation, Properties and Proof of Structure of Some 4-epi-Tetracyclines. *Journal of the American Chemical Society*, *79*(11), 2849–2858. https://doi.org/10.1021/ja01568a050

Nakakubo, R., Møller, H. B., Nielsen, A. M., & Matsuda, J. (2008). Ammonia inhibition of methanogenesis and identification of process indicators during anaerobic digestion. *Environmental Engineering Science*, *25*(10), 1487–1496. https://doi.org/10.1089/ees.2007.0282

Ocampo-Pérez, R., Rivera-Utrilla, J., Gómez-Pacheco, C., Sánchez-Polo, M., & López-Peñalver, J. J. (2012). Kinetic study of tetracycline adsorption on sludge-derived adsorbents in aqueous phase. *Chemical Engineering Journal*, *213*, 88–96. https://doi.org/10.1016/j.cej.2012.09.072

Rajagopal, R., Massé, D. I., & Singh, G. (2013). A critical review on inhibition of anaerobic digestion process by excess ammonia. *Bioresource Technology*, *143*,

632–641. https://doi.org/10.1016/j.biortech.2013.06.030

Regueiro, L., Veiga, P., Figueroa, M., Alonso-Gutierrez, J., Stams, A. J. M., Lema, J. M., & Carballa, M. (2012). Relationship between microbial activity and microbial community structure in six full-scale anaerobic digesters. *Microbiological Research*, *167*(10), 581–589. https://doi.org/10.1016/j.micres.2012.06.002

Renew, J. E., Smeby, K. L., Pinkston, K., & Sedlak, D. L. (2001). Assessment of Potential Antibiotic Contaminants in. *Journal of Contemporary Water Research and Education*, *120*(1), 30–40.

Sanz, J. L., Rodríguez, N., & Amils, R. (1996). The action of antibiotics on the anaerobic digestion process. *Applied Microbiology and Biotechnology*, *46*(5–6), 587–592. https://doi.org/10.1007/s002530050865

Sarmah, A. K., Meyer, M. T., & Boxall, A. B. A. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*, *65*(5), 725–759. https://doi.org/10.1016/j.chemosphere.2006.03.026

Shimada, T., Zilles, J., Raskin, L., & Morgenroth, E. (2007). Carbohydrate storage in anaerobic sequencing batch reactors. *Water Research*, *41*(20), 4721–4729. https://doi.org/10.1016/j.watres.2007.06.052

Søeborg, T., Ingerslev, F., & Halling-Sørensen, B. (2004). Chemical stability of chlortetracycline and chlortetracycline degradation products and epimers in soil interstitial water. *Chemosphere*, *57*(10), 1515–1524.

https://doi.org/10.1016/j.chemosphere.2004.09.020

Stone, J. J., Clay, S. A., Zhu, Z., Wong, K. L., Porath, L. R., & Spellman, G. M. (2009). Effect of antimicrobial compounds tylosin and chlortetracycline during batch anaerobic swine manure digestion. *Water Research*, *43*(18), 4740–4750. https://doi.org/10.1016/j.watres.2009.08.005

Sun, L., Pope, P. B., Eijsink, V. G. H., & Schnürer, A. (2015). Characterization of microbial community structure during continuous anaerobic digestion of straw and cow manure. *Microbial Biotechnology*, *8*(5), 815–827. https://doi.org/10.1111/1751-7915.12298

Tian, Z., Zhang, Y., & Yang, M. (2018). Chronic impacts of oxytetracycline on mesophilic anaerobic digestion of excess sludge: Inhibition of hydrolytic acidification and enrichment of antibiotic resistome. *Environmental Pollution*, *238*, 1017–1026. https://doi.org/10.1016/j.envpol.2018.02.023

Tuan, N. N., Chang, Y. C., Yu, C. P., & Huang, S. L. (2014). Multiple approaches to characterize the microbial community in a thermophilic anaerobic digester running on swine manure: A case study. *Microbiological Research*, *169*(9–10), 717–724. https://doi.org/10.1016/j.micres.2014.02.003

Vaiserman, A., Romanenko, M., Piven, L., Moseiko, V., Lushchak, O., Kryzhanovska, N., Guryanov, V., & Koliada, A. (2020). Differences in the gut Firmicutes to Bacteroidetes ratio across age groups in healthy Ukrainian population. *BMC Microbiology*, *20*(1), 221. https://doi.org/10.1186/s12866-020-01903-7

Van Boeckel, T. P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B. T., Levin, S. A., & Laxminarayan, R. (2014). Global antibiotic consumption 2000 to 2010: An analysis of national pharmaceutical sales data. *The Lancet Infectious Diseases*, *14*(8), 742–750. https://doi.org/10.1016/S1473-3099(14)70780-7

Wang, Q., & Yates, S. R. (2008). Laboratory study of oxytetracycline degradation kinetics in animal manure and soil. *Journal of Agricultural and Food Chemistry*, *56*(5), 1683–1688. https://doi.org/10.1021/jf072927p

Wang, R., Chen, M., Feng, F., Zhang, J., Sui, Q., Tong, J., Wei, Y., & Wei, D. (2017). Effects of chlortetracycline and copper on tetracyclines and copper resistance genes and microbial community during swine manure anaerobic digestion. *Bioresource Technology*, *238*. https://doi.org/10.1016/j.biortech.2017.03.134

Wang, R., Zhang, J., Liu, J., Yu, D., Zhong, H., Wang, Y., Chen, M., Tong, J., & Wei, Y. (2018). Effects of chlortetracycline, Cu and their combination on the performance and microbial community dynamics in swine manure anaerobic digestion. *Journal of Environmental Sciences*, *67*, 206–215. https://doi.org/10.1016/J.JES.2017.08.023

Xiong, Y., Harb, M., & Hong, P. Y. (2017). Performance and microbial community variations of anaerobic digesters under increasing tetracycline concentrations. *Applied Microbiology and Biotechnology*, *101*(13), 5505–5517. https://doi.org/10.1007/s00253-017-8253-1

Zhang, H., Luo, Y., Wu, L., Huang, Y., & Christie, P. (2015). Residues and potential ecological risks of veterinary antibiotics in manures and composts associated

with protected vegetable farming. *Environmental Science and Pollution Research*, *22*(8), 5908–5918. https://doi.org/10.1007/s11356-014-3731-9

Zhang, Y., Boyd, S. A., Teppen, B. J., Tiedje, J. M., & Li, H. (2014). Organic acids enhance bioavailability of tetracycline in water to Escherichia coli for uptake and expression of antibiotic resistance. *Water Research*, *65*, 98–106. https://doi.org/10.1016/j.watres.2014.07.021

Zhao, L., Dong, Y. H., & Wang, H. (2010). Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. *Science of the Total Environment*, *408*(5), 1069–1075. https://doi.org/10.1016/j.scitotenv.2009.11.014

CHAPTER 6
CONCLUSIONS

A primary objective of this study is to evaluate fate and long-term effects of chlortetracycline on anaerobic digestion of swine manure. The detailed summary of results is given in each chapter. The conclusions corresponding to the three specific objectives proposed at 1. Introduction are presented herein.

(1) The concentration of CTC in slurry pit and soil can be effectively estimated as a form of the probability distribution by considering uncertainties of farm practices. The predicted environmental concentrations of CTC in the slurry pit and soil were in a range of 0.54 - 5.64 mg/kg_{manure} and 3.42 - 67.59 $\mu g/kg_{soil}$, respectively, for a 90% confidence level. Based on the probability of PEC_{soil}, potential ecological risk on soil microbes is expected to exist for CTC ($P(RQ > 1) = 9.3\%$). If anaerobic digestion is applied before fertilization/composing, the probability of ecological risk is significantly reduced ($P(RQ > 1) = 0.6\%$). Daily cleaning water usage, pit emptying cycles, and storage periods associated with slurry pit-based farm practices were identified as drivers of the ecological risk. It is difficult to reduce the risk under 5% by controlling the farm practices. AD of swine manure is recommended not only for organic waste treatment but also for the ecological risk reduction under 0.5%.The suggested scenario and

uncertainties for evaluating ecological risk of CTC can be applied to 'Guidance for developing ecological soil screening levels' of US EPA. US EPA requires five assessment factors for evaluating the quality of information obtained from external sources: Soundness, Applicability and Utility, Clarity and Completeness, and Uncertainty and Variability, and Evaluation and Review (67 FR 57225, 2002).

(2) In the swine manure and its anaerobic digestate, ECTC, EICTC, and ICTC are transformed from CTC. They accounted for 60 - 93 % (w/w) of the residual total CTCs. The CTC is expected to be transported by solid phase of anaerobic digestate (> 70 % by wt.) while most of ECTC, EICTC, and ICTC is remained in the liquid phase (> 60 % by wt.). The kinetics of degradation and transformation can be demonstrated by using ordinary differential equations based on the 1st order kinetics. The model can be utilized for the prediction of the concentrations of CTC, ECTC+EICTC, and ICTC in a CSTR treating swine manure. When evaluating the concentration of CTC with its transformation products in a CSTR, if HRT of the CSTR is shorter than equilibrium time, the kinetic constants should be evaluated through batch experiments and applied to the CSTR model. If the HRT is longer than equilibrium time, estimation of kinetic constant is unnecessary. It is required to evaluate equilibrium ratios. Since the degradation kinetic constants also changes with transformation kinetic

constants, batch experiments is needed to get those constants separately or together.

(3) Exposure to 3 mg/L of CTC for more than one year can downgrade the organic removal and methane production performances of AD treating swine manure even if the concentration is decreased half (1.5 mg/L in the CSTR) and correspondingly its environmental risk in soil is reduced significantly. Typical AD system failures such as VFA accumulation and pH drops would not occur because the inhibition is caused by reduction of microbial diversity and change of the microbial community to inefficient state in terms of AD performances. It is hard to recover from the inhibition for 300 days w/o the exposure and difficult to predict using physicochemical indicators of the AD system. Continuous exposure to CTC for at least 100 days needs to be avoided for the sustainable management of AD plants treating SM.

국문초록

돈분의 혐기성 소화 중
클로르테트라싸이클린 항생제의 거동과 장기 영향

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가축용 항생제에 의한 가축 생장 촉진 효과가 알려지면서 공장식 사육 시스템이 축산 업계에 일반화되었다. 이로 인해 가축용 항생제가 남용되고 있다. 1950 년 수백 톤에 불과하던 항생제 소비량은 2010 년 약 63,000 톤으로 증가했다. 가축에게 투여된 항생제 중 일부는 분뇨를 통해 배출된다. 가축용 항생제가 남용됨에 따라 수계와 육상 환경이 분뇨로 배출된 항생제에 의해 지속적으로 오염되고 있다. 가축용 항생제는 자연계 내에서 미생물 독성, 항생제 내성균 및 내성 유전자 발현 등의 문제를 야기하는 것으로 알려져 있다.

혐기성 소화는 축산 폐수와 같은 유기성 폐기물들을 처리하기 위한 지속 가능한 방법으로 최근 수십 년간 주목 받아왔다. 이와 동시에

가축 분뇨를 처리하는 혐기성 소화조가 가축용 항생제를 자연계에 배출하는 공급원이 될 수 있다는 우려도 제기되었다. 혐기성 소화를 통한 가축용 항생제의 거동에 따라 가축용 항생제의 환경 농도와 환경 매질에서의 위해성이 바뀔 수 있다. 또한 가축 분뇨 내의 가축용 항생제는 여전히 항생 능력을 유지한다. 혐기성 소화는 일련의 생물학적 처리 과정으로 이루어지므로, 혐기성 소화조 내의 가축용 항생제가 소화 시스템에 영향을 미칠 수 있다. 따라서 환경 내 가축용 항생제가 만연하는 것을 방지하고, 인간에 의해 발생한 항생제 문제들을 적절히 관리하기 위해서는 혐기성 소화 중 가축용 항생제의 거동과 영향을 이애할 필요가 있다.

본 연구의 목적은 돈분의 혐기성 소화 중 가축용 항생제 클로르테트라싸이클린 (Chlortetracycline, CTC)의 거동과 영향을 평가하는 것이다. CTC는 사용량과 배출 비율, 독성을 고려해 대표 가축용 항생제로 선택했다. 세부 목적은 토양 내 CTC의 환경 위해성 평가를 위한 분뇨 및 토양 내 CTC 농도를 예측하고, 혐기성 소화 중 CTC의 이성질체화를 평가하는 것이다. 그리고 CTC가 장기적으로 돈분의 혐기성 소화에 미치는 영향을 분석하고자 했다.

가축 사육환경과 항생제 사용 패턴의 불확실성을 반영해 분뇨와 토양 내 CTC의 농도를 예측했다. 그리고 몬테카를로 시뮬레이션을

수행해 각 인자들의 민감도를 평가했다. 분뇨와 토양 내 CTC 예상 농도 범위는 각각 0.54 – 5.65 mg/kg_{manure}, 3.42 – 67.59 $\mu g/kg_{soil}$ 였다. 이는 기존 문헌에서 보고한 측정 농도를 포함하는 범위다. 혐기성 소화를 적용하지 않은 경우 토양 내에서 CTC에 의한 위해도가 발생할 확률은 약 9.3 % 였으며, 혐기성 소화를 적용한 경우 이 확률은 0.6 %로 낮아졌다. 이는 혐기성 소화 중 CTC 농도 감소로 인해 토양 내 CTC의 위해성이 상당히 낮아 진다는 것을 정량적으로 나타낸다. 토양 내 CTC 농도에 가장 큰 영향을 미치는 인자는 퇴비 내 질소 함량, 축사 세척 수 부피, 축사 청소 주기였다. 가축용 항생제의 자연계 노출을 관리하기 위한 체계적인 방법론 수립시 가축 사육 환경을 고려해야한다. 본 연구에서 제시한 영향인자와 인자들의 불확실성은 US EPA의 'Guidance for devoping ecological soil screening levels' 와 같이 평가 인자의 불확실성을 요구하는 방법론에 적용될 수 있다.

돈분과 돈분의 혐기성 소화액 내에서 CTC는 epimer, isomer 및 epi-isomer로 이성질체화되었다. 이 이성질체들은 CTC와 이성질체 농도 합의 60 – 93 %를 차지했다. CTC 보다 많은 양의 이성질체가 존재한다는 것은 CTC만 고려한 위해성이 과소평가되었음을 의미한다. Epimer는 가역적으로 모화합물인 CTC로 변환될 수 있으며, isomer는 환경 내에서 항생제 내성 유전자 발현을 야기하기 때문이다. CTC는 70 % (by wt.)

이상이 소화액 중 고상을 통해 배출될 것으로 예상되며, 이성질체들은 60 % 이상이 액상에 잔존할 것으로 예상된다. CTC는 이성질체에 비해 독성이 매우 높기 때문에 혐기성 소화액의 고상을 우선적으로 관리해야 한다. 1차 동역학을 가정한 미분방정식을 통해 돈분과 소화액 내에서 CTC의 이성질체화 동역학을 분석할 수 있었다. 이 모델은 돈분을 처리하는 연속식 혐기성 소화조 내의 이성질체화 농도를 평가하는데 사용될 수 있다. 연속식 돈분 혐기성 소화조 내 CTC와 이성질체 농도를 평가할 때, 소화조의 수리학적 체류시간이 이성질체화 평형시간보다 짧은 경우 회분식 실험을 통해 이성질체화 동역학 상수를 평가하고 연속식 모델에 적용해야 한다. 수리학적 체류시간이 평형시간 보다 긴 경우, 동역학 상수 분석은 불필요하며, 이성질체 평형 비율 평가가 필요하다.

CTC를 3 mg/L로 투여한 돈분과 그렇지 않은 돈분을 각각 처리하는 2 개의 연속식 반응조를 약 900 일간 운영했다. 혐기성 소화 중 CTC 농도 감소 (소화조 내 약 1.5 mg/L 수준)로 인해 토양 내 CTC의 위해성이 낮아지지만, 낮아진 농도 수준으로도 장기간 노출될 경우 혐기성 소화가 저해될 수 있다. 300 일 경화 후 실험군의 유기물 감소율과 메탄 생산량이 감소하기 시작했다. 이는 메탄 발생속도가 감소하고, 돈분의 메탄 전환율이 낮아졌기 때문이다. CTC 노출을

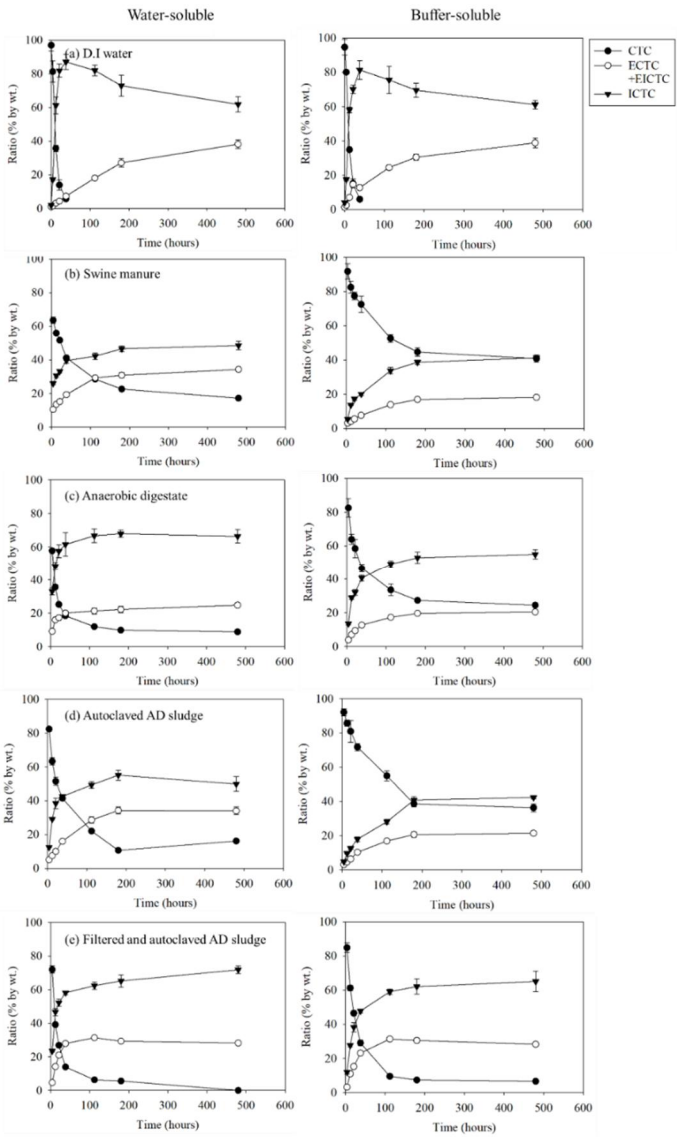
중지한지 300 일 이후까지도 실험군의 소화 성능이 회복되지 않았다. 미생물 군집과 소화조 환경인자를 사용해 중복성 분석을 수행한 결과, 미생물 다양성 감소와 유기산 생성균 군집 변화가 저해 영향과 관련이 있었다. CTC 장기 노출에 의한 저해 현상은 물리화학적 성상 인자들로 예측하기 어렵고 회복가능성이 낮다. 따라서 돈분을 처리하는 혐기성 소화조가 CTC에 지속적으로 노출되는 것을 사전에 방지해야한다.

주요어: 가축 분뇨; 가축용 항생제; 농도 예측; 이성질체화; 테트라싸이클린; 혐기성 소화; 저해 영향

학 번: 2017-31421

APPENDIX

S4.1 Water- and buffer-soluble concentration ratio (% by wt.) of CTC, ECTC, and ICTC in (a) DI water, (b) Swine manure, (c) anaerobic digestate of swine manure, (d) the autoclave-treated anaerobic digestate, and (e) the 0.45 μm-filtered and autoclave-treated anaerobic digestate



S5.1. Microbial taxonomic profiling

DNA was extracted from the digestate of both CSTRs collected on day 90, 188, 500, 750 and 808 including inoculum sludge. DNA extraction was conducted using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). The primers targeting the V3–V4 regions of the 16S rRNA were used to amplify the extracted DNA. The primer sequences used were 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3) for bacteria and A519F (5′-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-CAGCCGCCGCGGTAA-3′) and A958R (5′-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-YCCGGCGTTGAMTCCAATT-3′) for archaea. The process described by Lee et al. (2020) was used for DNA amplification. The DNA extraction procedure was conducted at ChunLab, Inc. (Seoul, Republic of Korea) using an Illumina MiSeq Sequencing System (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

Each sequence read was assigned to determine taxonomic ranks by the following similarities; species ($x \geq 97$ %), genus ($97 > x \geq 94$ %), family ($94 > x \geq 90$ %), order ($90 > x \geq 85$ %), class ($85 > x \geq 80$ %), and phylum ($80 > x \geq 75$ %) using the EzBioCloud 16S rRNA database. Operational taxonomic units (OTUs) were based on a 97 % identity cut-off. Shannon and Simpson's indices which are

indices of microbial community diversity, were calculated using ChunLab's MiSeq pipeline method. The relative abundance of bacteria and archaea was determined by the percentage of the total number of sequences in sample (%). EzBioCloud 16S-based MTP of ChunLab's bioinformatics cloud platform were utilized for the above analytics.

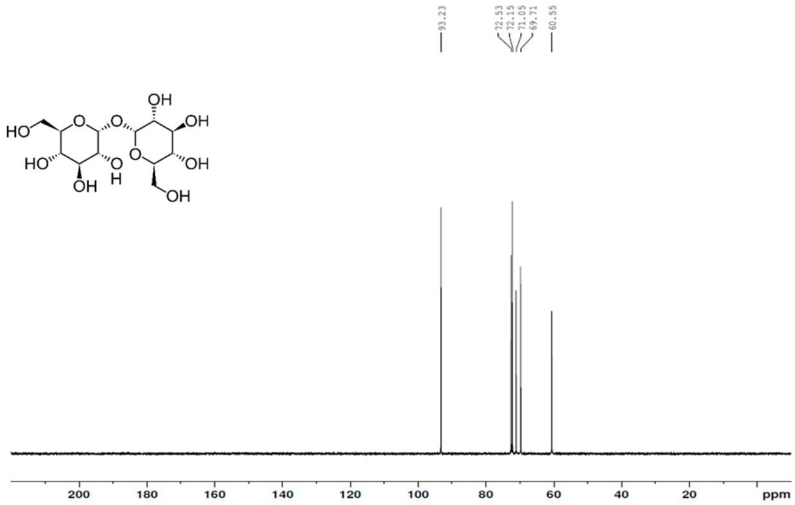
S5.2. Compositions of VFAs in CSTR 1 and 2 (unit: mg/L)

Day	Total VSAs	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Caproic acid
CSTR 1								
0	50.6	37.2	13.3	-	-	-	-	30.2
46	26.4	26.4	-	-	-	-	-	27.9
91	373.3	227.0	32.6	30.0	38.8	44.9	-	65.0
127	172.9	79.3	22.2	21.1	17.6	32.7	-	32.5
196	222.6	159.7	20.7	-	17.6	24.5	-	62.7
233	106.9	106.9	-	-	-	-	-	32.5
282	122.8	80.5	17.8	-	-	24.5	-	27.9
300	90.1	90.1	-	-	-	-	-	60.4
347	99.9	57.6	17.8	-	-	24.5	-	72.0
392	33.6	33.6	-	-	-	-	-	72.0
439	34.0	19.2	14.8	-	-	-	-	-
473	24.0	24.0	-	-	-	-	-	-
520	30.0	30.0	-	-	-	-	-	-
555	30.0	30.0	-	-	-	-	-	-
586	24.0	24.0	-	-	-	-	-	-
616	30.0	30.0	-	-	-	-	-	-
652	30.0	30.0	-	-	-	-	-	-
683	30.0	30.0	-	-	-	-	-	-
711	24.0	24.0	-	-	-	-	-	-
735	90.8	69.2	-	-	5.3	10.2	6.1	20.9
754	160.0	106.9	14.8	7.0	8.8	12.3	10.2	13.9
795	99.7	60.1	7.4	7.0	8.8	8.2	8.2	23.2
815	97.3	72.1	4.4	5.3	5.3	6.1	4.1	20.9
835	120.2	84.1	7.4	5.3	7.0	10.2	6.1	23.2
856	98.8	72.1	5.9	5.3	5.3	6.1	4.1	13.9
891	57.7	36.0	5.9	-	3.5	6.1	6.1	11.6
Day		Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Caproic acid
CSTR 2								
0	36.0	36.0	-	-	-	-	-	-
46	30.0	30.0	-	-	-	-	-	-
91	219.6		16.3	-	8.8	14.3	-	-

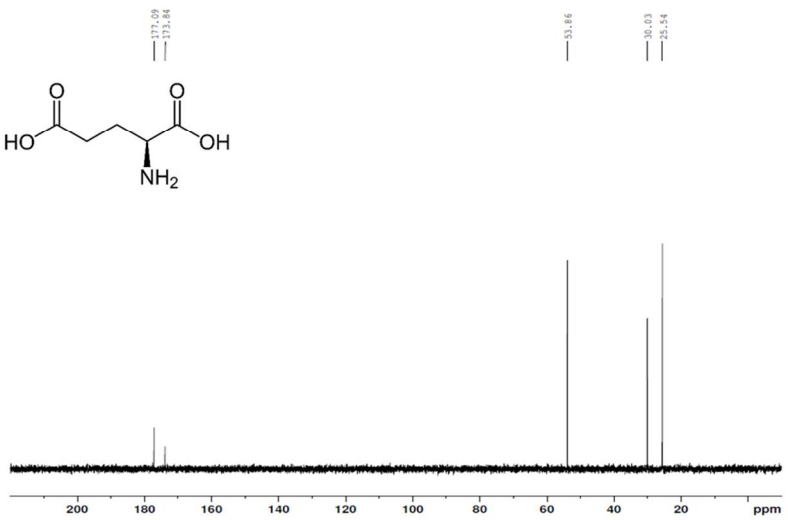
		180.2						
127	123.0	106.9	5.9	-	-	10.2	-	-
196	168.1	168.1	-	-	-	-	-	-
233	94.9	94.9	-	-	-	-	-	-
282	93.7	93.7	-	-	-	-	-	-
300	52.8	52.8	-	-	-	-	-	-
347	48.0	48.0	-	-	-	-	-	-
392	28.8	28.8	-	-	-	-	-	-
439	210.0	66.1	43.0	-	45.8	55.2	-	-
473	226.6	49.2	37.0	-	42.3	49.0	49.0	116.2
520	149.1	50.4	-	-	-	49.0	49.6	55.8
555	43.2	43.2	-	-	-	-	-	55.8
586	248.4	67.3	37.0	44.1	-	51.1	49.0	55.8
616	31.2	31.2	-	-	-	-	-	-
652	64.1	49.2	14.8	-	-	-	-	-
683	66.5	51.6	14.8	-	-	-	-	-
711	47.2	32.4	14.8	-	-	-	-	-
735	71.8	48.0	4.4	3.5	3.5	6.1	6.1	20.9
754	104.5	78.1	7.4	5.3	3.5	6.1	4.1	18.6
795	66.2	48.0	4.4	-	3.5	6.1	4.1	7.0
815	67.7	48.0	5.9	-	3.5	4.1	6.1	11.6
835	126.3	96.1	7.4	5.3	5.3	8.2	4.1	20.9
856	128.1	96.1	7.4	5.3	7.0	8.2	4.1	18.6
891	50.7	36.0	3.0	-	3.5	4.1	4.1	11.6

S5.3. Identification of storage products of carbohydrates in microorganisms of CSTR 1 and 2

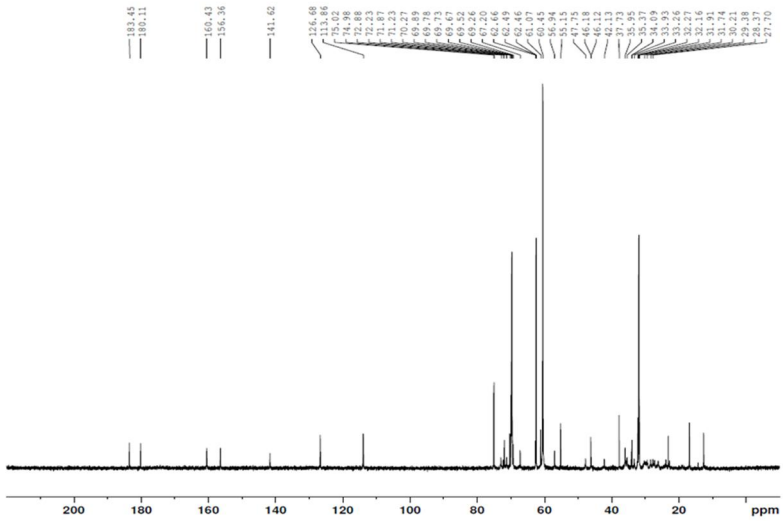
(a) Trehalose



(b) Glutamate



(c) CSTR 1



(d) CSTR 2

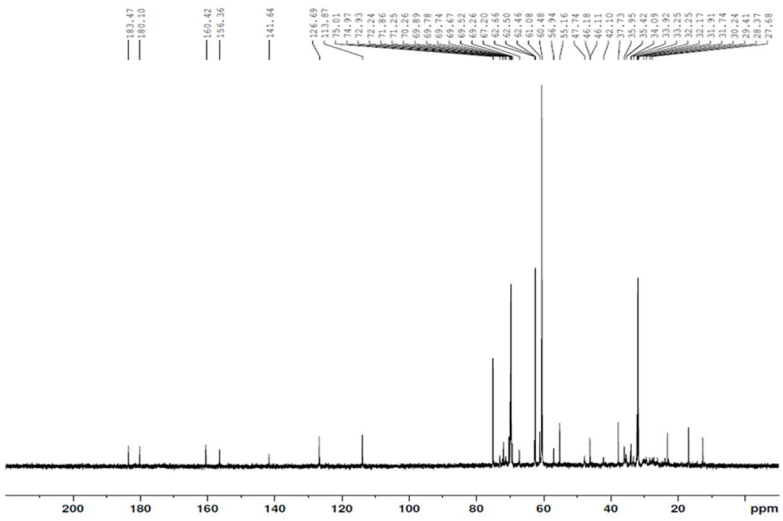
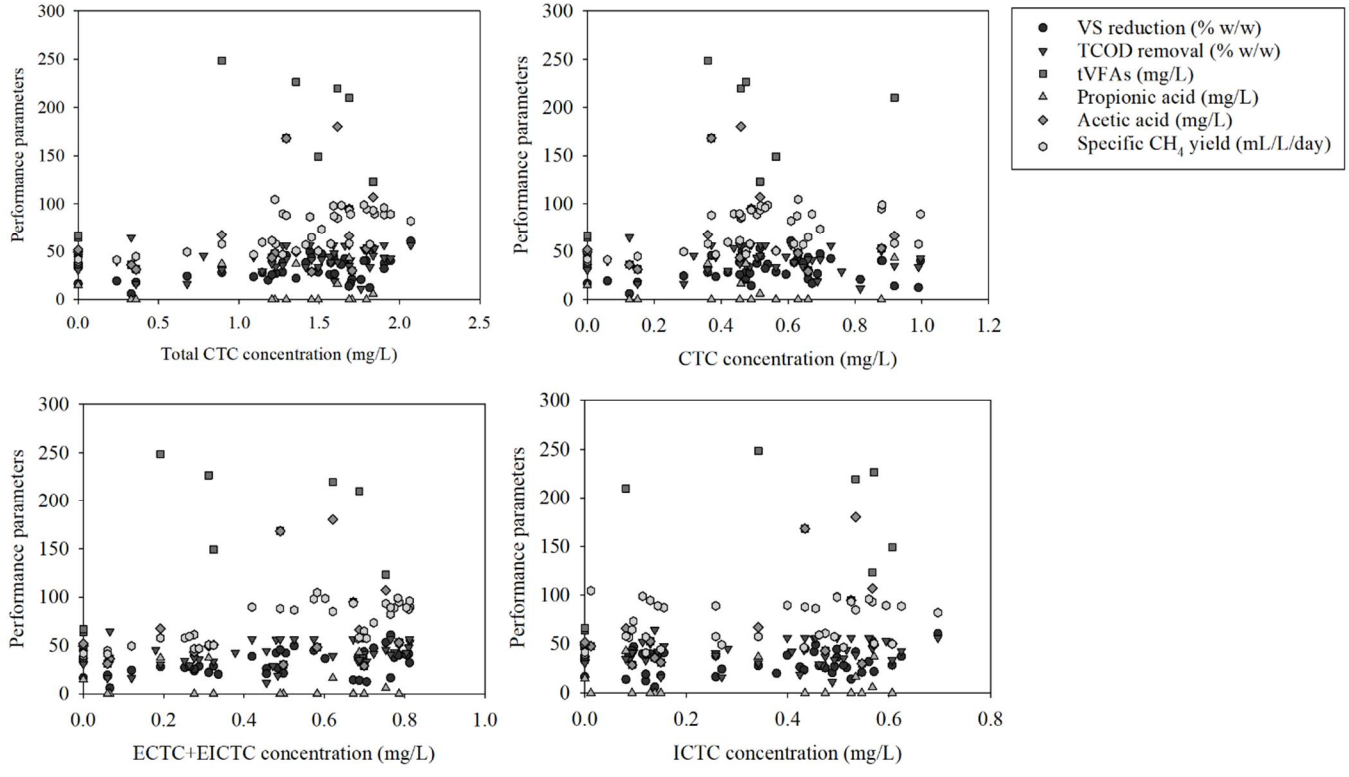


Figure S2 NMR spectra of trehalose (a), Glutamate (b), Day 500 digestate in CSTR 1 (c), and CSTR 2 (d)

S5.4 Performance parameters (VS reduction, TCOD removal, tVFAs, propionic acid, acetic acid and specific methane yield) versus CTC, ECTC+EICTC, ICTC concentrations and their sum



S5.5 The taxon counts and relative abundance of phylum for bacterial and class for archaea communities. Taxon less than 1% in both reactor were considered as etc.

CSTR 1	Counts						Ratio					
Bacteria	Day 0	Day 90	Day 188	Day 500	Day 750	Day 808	Day 0	Day 90	Day 188	Day 500	Day 750	Day 808
Total	29,407	30,036	57,571	37,522	41,104	48,136						
Firmicutes	8,828	5,715	37,695	23,188	13,233	22,683	30.0	19.0	65.5	61.8	32.2	47.1
Bacteroidetes	5,262	14,051	3,479	9,036	16,722	13,652	17.9	46.8	6.0	24.1	40.7	28.4
Cloacamonas_p	7,276	2,857	82	966	3,481	4,191	24.7	9.5	0.1	2.6	8.5	8.7
Proteobacteria	6,215	1,573	12,769	2,065	2,000	2,936	21.1	5.2	22.2	5.5	4.9	6.1
Chloroflexi	9	30	892	190	456	2,053	0.0	0.1	1.5	0.5	1.1	4.3
Spirochaetes	698	1,812	51	426	1,171	404	2.4	6.0	0.1	1.1	2.8	0.8
Fibrobacteres	14	1,807	12	305	365	176	0.0	6.0	0.0	0.8	0.9	0.4
Tenericutes	711	1,079	128	189	782	103	2.4	3.6	0.2	0.5	1.9	0.2
Acidobacteria	0	380	30	44	233	83	0.0	1.3	0.1	0.1	0.6	0.2
Etc.	394	732	2,433	1,113	2,661	1,855	1.3	2.4	4.2	3.0	6.5	3.9
Archaea	Day 0	Day 90	Day 188	Day 500	Day 750	Day 808	Day 0	Day 90	Day 188	Day 500	Day 750	Day 808
Total	35,154	57,613	84,224	54,676	56,499	67,235						
AF424768_o	95	51,598	63,119	27,394	27,968	30,548	0.3	89.6	74.9	50.1	49.5	45.4
Methanosarcinales	16,600	110	9,132	12,287	5,180	14,153	47.2	0.2	10.8	22.5	9.2	21.1
Methanobacteriales	1,382	353	9,890	8,022	1,862	13,193	3.9	0.6	11.7	14.7	3.3	19.6
LNJC_o	84	111	123	2,042	1,388	2,108	0.2	0.2	0.1	3.7	2.5	3.1

AB213092_o	0	243	185	1,399	17,176	0	0.0	0.4	0.2	2.6	30.4	0.0
Methanomassiliicoccales	14,072	2,923	1,423	1,292	1,790	2,081	40.0	5.1	1.7	2.4	3.2	3.1
Methanomicrobiales	2,918	1,843	77	193	339	358	8.3	3.2	0.1	0.4	0.6	0.5
FJ902712_o	0	2	24	99	282	0	0.0	0.0	0.0	0.2	0.5	0.0
Etc.	3	430	251	1,948	514	4,794	0.0	0.7	0.3	3.6	0.9	7.1
CSTR 2	Counts						Ratio					
Bacteria	Day 0	Day 90	Day 188	Day 500	Day 750	Day 808	Day 0	Day 90	Day 188	Day 500	Day 750	Day 808
Total	29,407	28,397	59,957	36,332	34,457	53,202						
Firmicutes	8,828	3,798	42,108	24,914	11,114	32,820	30.0	13.4	70.2	68.6	32.3	61.7
Bacteroidetes	5,262	13,325	3,726	6,583	12,707	10,832	17.9	46.9	6.2	18.1	36.9	20.4
Cloacamonas_p	7,276	1,829	67	914	5,590	2,317	24.7	6.4	0.1	2.5	16.2	4.4
Proteobacteria	6,215	3,389	11,682	1,825	1,421	3,785	21.1	11.9	19.5	5.0	4.1	7.1
Chloroflexi	9	54	573	221	379	400	0.0	0.2	1.0	0.6	1.1	0.8
Spirochaetes	698	2,079	35	371	712	473	2.4	7.3	0.1	1.0	2.1	0.9
Fibrobacteres	14	1,480	29	239	279	286	0.0	5.2	0.0	0.7	0.8	0.5
Tenericutes	711	1,527	102	242	276	125	2.4	5.4	0.2	0.7	0.8	0.2
Acidobacteria	0	190	4	18	84	145	0.0	0.7	0.0	0.0	0.2	0.3
Etc.	394	726	1,631	1,005	1,895	2,019	1.3	2.6	2.7	2.8	5.5	3.8
Archaea	Day 0	Day 90	Day 188	Day 500	Day 750	Day 808	Day 0	Day 90	Day 188	Day 500	Day 750	Day 808
Total	35,154	55,248	63,134	49,043	62,058	52,851						
AF424768_o	95	51,253	37,848	24,150	30,844	31,620	0.3	92.8	59.9	49.2	49.7	59.8
Methanosarcinales	16,600	145	7,187	10,216	9,424	5,774	47.2	0.3	11.4	20.8	15.2	10.9
Methanobacteriales	1,382	84	16,892	11,417	3,205	7,197	3.9	0.2	26.8	23.3	5.2	13.6

LNJC_o	84	150	89	901	3,177	1,039	0.2	0.3	0.1	1.8	5.1	2.0
AB213092_o	0	0	0	0	27	1,976	0.0	0.0	0.0	0.0	0.0	3.7
Methanomassiliicoccales	14,072	1,913	605	823	4,467	1,266	40.0	3.5	1.0	1.7	7.2	2.4
Methanomicrobiales	2,918	1,703	130	79	571	103	8.3	3.1	0.2	0.2	0.9	0.2
FJ902712_o	0	0	333	5	9,618	1,229	0.0	0.0	0.5	0.0	15.5	2.3
Etc.	3	0	50	1,452	725	2,647	0.0	0.0	0.1	3.0	1.2	5.0

3

Appendix S5.6. The taxon counts and relative abundance of phylum for bacterial and class for archaea communities. Taxon less than 1% in both reactor were considered as etc.

Bacteria – Phylum	Firmicutes	Bacteroidetes	Cloacamonas_p	Proteobacteria	Chloroflexi	Spirochaetes	Fibrobacteres	Tenericutes	Acidobacteria	CTCexpos	pH	SCOD	TNH3	TVFAs	Acetic	Propionic	Buytiric	CH4	TCODremv
Firmicutes	1.0																		
Bacteroidetes	**-0.79**	1.00																	
Cloacamonas_p	**-0.67**	**0.81**	1.00																
Proteobacteria	**0.64**	-0.54	-0.62	1.00															
Chloroflexi	0.60	-0.25	-0.04	0.53	1.00														
Spirochaetes	**-0.90**	**0.79**	0.61	-0.49	**-0.65**	1.00													
Fibrobacteres	**-0.78**	**0.72**	0.41	-0.50	**-0.79**	**0.92**	1.00												
Tenericutes	**-0.84**	0.55	0.33	-0.58	**-0.75**	**0.84**	**0.79**	1.00											
Acidobacteria	**-0.78**	**0.87**	**0.67**	-0.41	-0.44	**0.93**	**0.84**	**0.70**	1.00										
CTCexpos	0.16	-0.29	0.01	-0.09	-0.10	-0.05	-0.14	-0.11	-0.19	1.00									
pH	0.31	-0.51	-0.36	0.03	0.10	-0.31	-0.32	0.08	-0.40	0.00	1.00								
SCOD	0.44	-0.48	**-0.64**	0.16	0.08	-0.49	-0.27	-0.16	-0.52	-0.34	0.62	1.00							
TNH3	0.36	-0.58	**-0.84**	0.61	-0.07	-0.35	-0.24	-0.10	-0.37	-0.19	0.11	0.43	1.00						
TVFAs	-0.25	0.16	-0.07	0.18	-0.04	0.25	0.13	0.33	0.35	-0.30	-0.09	0.09	0.52	1.00					
Acetic	-0.33	0.25	-0.04	0.13	-0.14	0.33	0.24	0.37	0.38	-0.21	-0.23	0.05	0.48	**0.96**	1.00				
Propionic	-0.51	0.41	0.29	-0.06	-0.20	0.58	0.40	0.60	**0.69**	-0.32	-0.05	-0.25	0.17	**0.80**	**0.71**	1.00			
Buytiric	-0.57	**0.83**	**0.75**	-0.61	-0.03	0.48	0.41	0.36	**0.66**	-0.40	-0.35	-0.22	-0.50	0.32	0.32	0.45	1.00		
CH4	-0.20	0.53	0.25	0.07	0.21	0.14	0.22	-0.08	0.28	**-0.79**	-0.37	0.14	-0.14	0.10	0.14	0.04	0.51	1.00	
TCODremv	0.33	-0.12	-0.19	0.36	0.47	-0.36	-0.34	-0.35	-0.12	**-0.59**	0.07	0.51	0.36	0.58	0.47	0.30	0.29	0.47	1.00

(Continued)

Archaea – Genus	AF424768_g	AF424775_g	LNJC_g	M_bacteriaceae_uc	M_bacterium	M_culleus	M_massiliicoccus	M_saeta	M_sarcina	M_sphaera	CTCexpos	pH	SCOD	TNH3	TVFAs	Acetic	Propionic	Buytiric	CH4	TCODremv
AF424768_g	1.00																			
AF424775_g	-0.08	1.00																		
LNJC_g	-0.52	-0.61	1.00																	
M_bacteriaceae_uc	-0.13	0.31	-0.21	1.00																
M_bacterium	-0.37	0.04	0.25	0.51	1.00															
M_culleus	0.26	-0.31	0.16	**-0.88**	**-0.69**	1.00														
M_massiliicoccus	0.36	-0.37	0.41	**-0.75**	-0.38	**0.78**	1.00													
M_saeta	-0.31	-0.19	0.59	0.38	**0.73**	-0.31	0.01	1.00												
M_sarcina	-0.56	0.12	0.25	0.54	**0.69**	-0.55	-0.54	**0.73**	1.00											
M_sphaera	-0.30	-0.19	0.32	0.36	**0.68**	-0.35	-0.31	**0.64**	**0.63**	1.00										
CTCexpos	-0.16	-0.16	0.10	0.02	0.03	-0.17	-0.23	-0.08	0.20	0.40	1.00									
pH	-0.11	0.23	0.06	0.56	0.08	-0.54	-0.24	0.22	0.33	-0.21	0.00	1.00								
SCOD	-0.03	0.58	-0.38	0.62	-0.04	-0.36	-0.45	0.13	0.39	-0.13	-0.34	0.62	1.00							
TNH3	0.58	0.41	**-0.88**	0.35	-0.04	-0.30	-0.42	-0.30	-0.04	-0.28	-0.19	0.11	0.43	1.00						
TVFAs	**0.88**	0.22	**-0.64**	-0.24	-0.54	0.35	0.37	-0.55	**-0.71**	-0.52	-0.30	-0.09	0.09	0.52	1.00					
Acetic	**0.83**	0.16	**-0.65**	-0.36	**-0.66**	0.48	0.35	**-0.65**	**-0.71**	-0.49	-0.21	-0.23	0.05	0.48	**0.96**	1.00				
Propionic	**0.69**	0.01	-0.25	-0.43	-0.52	0.41	**0.63**	-0.52	**-0.89**	**-0.70**	-0.32	-0.05	-0.25	0.17	**0.80**	**0.71**	1.00			
Buytiric	0.03	0.10	0.23	-0.61	-0.29	**0.65**	**0.72**	-0.10	-0.47	-0.20	-0.40	-0.35	-0.22	-0.50	0.32	0.32	0.45	1.00		
CH4	-0.02	-0.13	0.12	-0.15	-0.10	0.38	0.22	0.09	-0.18	-0.04	**-0.79**	-0.37	0.14	-0.14	0.10	0.14	0.04	0.51	1.00	
TCODremv	0.50	0.38	-0.40	0.36	0.01	-0.07	0.05	0.12	-0.15	0.01	**-0.59**	0.07	0.51	0.36	0.58	0.47	0.30	0.29	0.47	1.00

M-: methano