보건학석사 학위논문

Removal of Antibiotics from Livestock Wastewater using Constructed Wetlands

인공습지로 처리한 축산폐수 내

항생제 제거효율 및 영향 인자 연구

2015 년 2 월

서울대학교 보건대학원

환경보건학과 환경보건학 전공

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지도교수 조 경 덕

이 논문을 보건학 석사학위논문으로 제출함

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서울대학교 보건대학원

환경보건학과 환경보건학 전공

최 영 주

최영주의 석사학위논문을 인준함

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Abstract

The occurrence and removal of antibiotics (sulfamethoxazole (SMZ), sulfathiazole (SFI), sulfamethazine (SMA), trimethoprim (TMP), tetracycline (TC), oxytetracycline (OTC), chlorotetracycline (CTC), and enrofloxacin (EFX)) using constructed wetlands (CWs) system for treating livestock wastewater were investigated by measuring the concentrations of the influents and the effluents in the CWs flowing into the Geum River Basin, Korea. The concentration of antibiotics was measured using solid phase extraction (SPE) followed by LC-MS/MS analysis.

The results showed that the levels of antibiotics in the effluents of the CWs were in the order of CTC, SFI, SMA, SMZ, TMP, OTC and EFX, ranging from 47 to 6,834.66 μg/L, respectively. There was an inverse correlation ($p < 0.0493$) between the removal of sulfonamide-group (SMZ, SFI and SMA) and tetracycline-group (TC, OTC and CTC) in the concentrations of effluents of the CWs, indicating that sulfonamide-group-antibiotics were more effectively removed in the CWs. The sulfonamide-antibiotics have usually higher pKa values, resulting in more effective adsorption with the negative charged soils by the electrostatic interaction.

The microcosm antibiotics adsorption experiment by wetlands soil with two sets (biotic system without sterilization and abiotic system with sterilization) showed that average removals of 20, 50 and 100ng/L of antibiotics in biotic system effectively removed (e.g. SMZ (73%), SFI (64%), SMA (76%), TMP (91%) and EFX (87%)) within 48 hours. However, the removals using abiotic system (e.g. SMZ (66%), SFI (36%), SMA (63%), TMP (63%) and EFX (78%)) were relatively low. Sunlight photo-degradation experiments showed that EFX was removed effectively (70%), compared to the removals of sulfonamide-antibiotics with SMZ, SFI and SMA with 23%, 44% and 28%, respectively.

The microcosm experiments of antibiotics adsorption by wetlands plants (*Phragmites australis*) were also performed with two sets (biotic and abiotic systems). The results showed that the biotic system effectively removed sulfonamide-antibiotics such as SMZ (71%), SFI (54%) and SMA (62%) within 3 hours. The effective removals of sulfonamide-group might be attributed to microbial biodegradation compared to lower removals (SMZ (3%), SFI (25%) and SMA (50%)) by abiotic system. Overall, these results imply that the removals of antibiotics are mediated from biodegradation by microbial activity and adsorption into soil and plants which are the main removal mechanisms in the CWs.

Keywords: Antibiotics, Constructed Wetlands, pKa, Photo-degradation, Plants, Soil

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Ⅰ. Introduction

1. Background

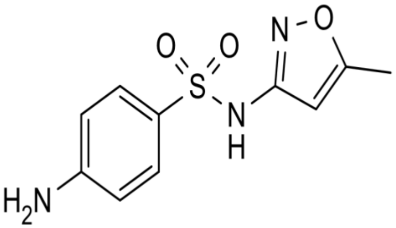
1.1. Information on the study compounds

A number of pharmaceutical compounds are recently considered as emerging contaminants compared to legacy contaminants, and became the great threat because of their widespread use, continuous release, persistence and the increasing evidence for their eco-toxicological (if not human health) effects (Buser et al. 1999). Among the pharmaceuticals, the worldwide use of antibiotics is reported as 100,000 - 200,000 tons in 2003 (Kümmerer et al. 2003). The antibiotic usage for animal feeds varies from 3.0 to 220.0 g/kg, depending on sizes and types of animals, and medicines in the world (Zhao et al. 2010).

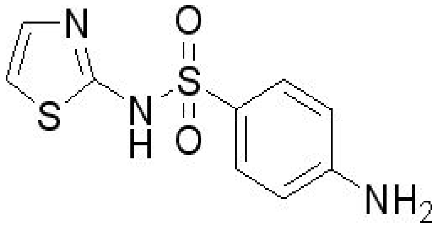
In Korea, antibiotics have been widely used in livestock farms as prophylaxis (therapy) and growth promoters, and the usage was reported as amounted to 1,460 tons in 2003 (Jung, 2003). According to health insurance claim data in 2003, Korea had the sixth highest antibiotics usage in OECD countries (Kim et al. 2006; Kim et al. 2011). Among the antibiotics, the most frequently used antibiotics in livestock farms are sulfonamide-antibiotics (e.g. sulfamethoxazole, sulfathiazole and sulfamethazine), tetracycline-antibiotics (e.g. tetracycline, oxytetracycline and chlortetracycline) and fluoroquinolones (e.g. enrofloxacin) which are the most commonly found in the swine wastewater and livestock farm, respectively (Zhao et al. 2010; Chen et al. 2012). Although trimethoprim is not actually a sulfonamides, it often used in combination with sulfamethoxazole as co-trimoxazole (Dan et al. 2013). Figure 1 and Table 1 show the details for molecular structures and physical and chemical properties of the eight selected antibiotics.

The reported concentrations range in the swine wastewater is from 23.80 to 685 μg/L, and concentrations of some antibiotics vary from trace amounts to those as high as the ppm levels in manure slurry and wastewater (Kumar et al. 2005; Wei et al. 2011; Zhang et al. 2011; Chen et al. 2012). In Korea, National Institute of Environmental Research (N.I.E.R.) reported concentration of sulfathiazole and chlortetracycline in livestock wastewater effluents is 659.74 and 523.60 μg/L, respectively. Table 2 and Table 3 show details for occurrence and fate of eight selected antibiotics in water environment.

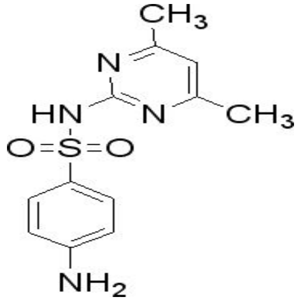
These high concentration of antibiotics found in the swine wastewater is caused by the poor antibiotics absorption rate of animals since 30 ~ 90% of the antibiotics in their diet excreted as an unchanged form or as metabolites (Wei et al. 2011). The antibiotics contamination by in sewage, agricultural wastewater, surface water and both influent and treated water of drinking water treatment plants (Choi et al. 2007; Adams et al. 2003) occur a threat to management of water quality owing to their potential adverse effects on the nature environment and humans (Yang et al. 2003; Qiang et al. 2004). They may cause microbial resistance among pathogen organisms or death of microorganisms, which are effective in wastewater treatment (Aksu et al. 2004).When untreated antibiotics reach to river or lakes, the potential toxicity of antibiotics to humans by through drinking water treatment plants is a concern (Yang et al. 2003). Therefore, it is important to investigate the occurrence and removal of antibiotics in the environment, especially near the livestock farm.



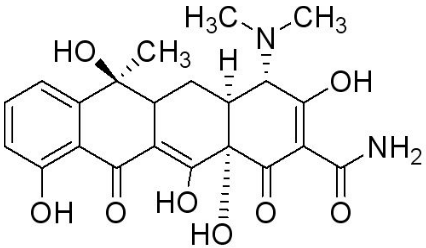
Sulfamethoxazole (SMZ)



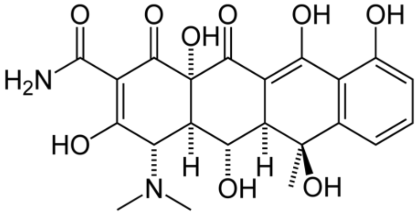
Sulfathiazole (SFI)



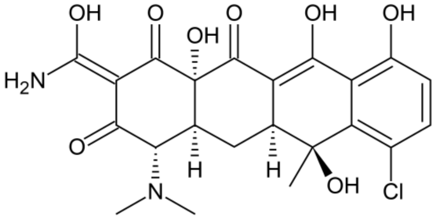
Sulfamethazine (SMA)



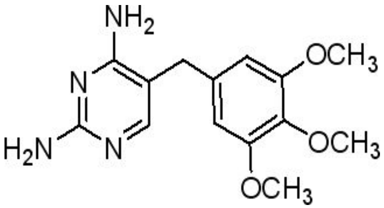
Tetracycline (TC)



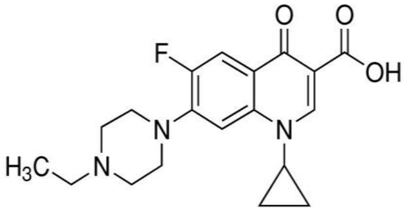
Oxytetracycline (OTC)



Chlortetracycline (CTC)



Trimethoprim (TMP)



Enrofloxacin (EFX)

Figure 1. Molecular structures of the eight selected antibiotics

Table 1.   Physical and chemical properties of eight selected antibiotics

Type	Compound	Cas Number	Molecular Weight	Molecular Formula	log K_{ow}	pKa
Sulfonamide	Sulfamethoxazole (SMZ)	723-46-6	253.28	$C_{10}H_{11}N_3O_3S$	0.48 ~ 0.88	5.7 ~ 5.9
	Sulfathiazole (SFI)	72-14-0	255.32	$C_9H_9N_3O_2S_2$	0.72	7.1 ~ 7.2
	Sulfamethazine (SMA)	57-68-1	278.33	$C_{12}H_{14}N_4O_2S$	0.28 ~ 0.76	7.3 ~ 7.7
-	Trimethoprim (TMP)	738-70-5	290.32	$C_{14}H_{18}N_4O_3$	0.73	6.6 ~ 6.8
Tetracycline	Tetracycline (TC)	64-54-8	444.44	$C_{22}H_{24}N_2O_8$	-1.33	3.3 ~ 4.5
	Oxytetracycline (OTC)	79-57-2	460.34	$C_{22}H_{24}N_2O_9$	-4.04 ~ -2.87	3.2 ~ 4.5
	Chlortetracycline (CTC)	57-62-5	478.88	$C_{22}H_{23}ClN_2O_8$	-3.60	3.3 ~ 4.5
Fluoroquinolone	Enrofloxacin (EFX)	93106-60-6	359.40	$C_{19}H_{22}FN_3O_3$	0.70	2.7 ~ 3.9

Table 2. Occurrence and fate of sulfonamide-group (SMZ, SFI and SMA) and trimethoprim (TMP) in water environment

Type	Compound	Source	Concentration (μg/L)	Country	References
Sulfonamide	Sulfamethoxazole (SMZ)	Livestock wastewater influent	1,314.30	Korea	N.I.E.R. (2008)
		Livestock wastewater effluent	840	Italy	Aukidy et al. (2012)
		Surface water	1.02	USA	Michele et al. (2001)
		Secondary wastewater effluent	0.28	Switzerland	ANKE et al. (2005)
		Hospital wastewater effluent	22.30	Korea	Ministry of Environment (2010)
		Livestock wastewater effluent	5.61	Korea	Sim et al. (2011)
	Sulfathiazole (SFI)	Swine farm wastewater effluent	4.66	China	Wei et al. (2011)
		Surface water	0.08	USA	Michele et al. (2001)
		Livestock wastewater effluent	659.74	Korea	N.I.E.R. (2007)
		Livestock wastewater influent	2,293.90	Korea	N.I.E.R. (2006)
		Livestock wastewater effluent	72.20	Korea	Sim et al. (2011)
	Sulfamethazine (SMA)	Water reclamation and clarifier effluent	ND / 0.64	USA	Shin et al. (2003)
		Swine wastewater effluent	13.20	China	Lin et al. (2013)
		Livestock wastewater influent	658.50	Korea	N.I.E.R. (2006)
		Lagoon wastewater	0.60	China	Li et al. (2013)
		Livestock wastewater effluent	69.69	Korea	N.I.E.R. (2008)
-	Trimethoprim (TMP)	Pharmaceutical factory effluent	77.20	Korea	Ministry of Environment (2010)
		Livestock wastewater influent	547.80	Korea	N.I.E.R. (2008)
		Lagoon wastewater	0.60	China	Li et al. (2013)
		Swage and agricultural wastewater effluent	0.23	China	Gulkowska et al. (2008)
		Livestock wastewater effluent	23.60	Korea	Sim et al. (2011)

* N.I.E.R. : National Institute of Environmental Research

Table 3. Occurrence and fate of tetracycline-group (TC, OTC and CTC) and enrofloxacin (EFX) in water environment

Type	Compound	Source	Concentration (μg/L)	Country	References
Tetracycline	Tetracycline (TC)	Swage and agricultural wastewater effluent	1.41	Korea	Choi et al. (2007)
		Lagoon wastewater	1.19	China	Li et al. (2013)
		Lake water and wastewater effluent	11.50 / 0.90	China	Lei et al. (2009)
	Oxytetracycline (OTC)	Lagoon wastewater	1.41	China	Li et al. (2013)
		Swage and agricultural wastewater effluent	6.53	Korea	Choi et al. (2007)
		Livestock wastewater effluent	35	Korea	N.I.E.R. (2007)
		Lake water and wastewater effluent	8.20 / 11	China	Lei et al. (2009)
		Livestock wastewater influent	741	Korea	N.I.E.R. (2007)
		Swine farm wastewater effluent	13.60	China	Wei et al. (2011)
		Swine wastewater effluent	4.34	China	Lin et al. (2013)
	Chlortetracycline (CTC)	Livestock wastewater effluent	99.90	Korea	Ministry of Environment (2010)
		Swage and agricultural wastewater	90.90	Korea	Choi et al. (2007)
		Livestock wastewater effluent	523.60	Korea	N.I.E.R. (2007)
		Livestock wastewater effluent	2.93	China	Li et al. (2013)
		Livestock wastewater influent	2,960	Korea	N.I.E.R. (2007)
Fluoroquinolone	Enrofloxacin (EFX)	Lake water and wastewater effluent	10.60 / 0.80	China	Lei et al. (2009)
		Livestock wastewater effluent	0.18	Korea	Ministry of Environment (2010)
		Livestock wastewater effluent	0.59	Korea	Woo et al. (2011)

* N.I.E.R. : National Institute of Environmental Researc

1.2. Information on the study site

Wetlands are used around the globe to treat domestic and industrial sewage wastewater, which have been used to treat wastewater ranging from raw sewage to tertiary-treated waste streams (White et al. 2006). Among the wetlands system, constructed wetlands (CWs) have been used in the swine wastewater treatment process as a post treatment due to providing ease of operation, low input requirements, and low operational cost compared to the conventional technical solutions for water treatment (Kadlec et al. 1995; Knight et al. 2000; Poach et al. 2003).

A number of studies have been examined using CWs used for the treatment of wastewater effluents in order to control organics, nutrients, and heavy metals, as well as many other components, and their findings indicate that the CWs have commonly showed 60 ~ 99% of high removal for organics, such as BOD and COD, and moderate (or low) removal efficiencies for nutrients, such as ammonia, nitrate, and total phosphate, etc. (Brix et al. 2005; Vymazal, 2005; Maine et al. 2006; Song et al. 2006).

The occurrence and fate of antibiotics in the livestock wastewaters is the major concern recently, and the detection of antibiotics has been reported in a sustained manner. Recently, the suitability of CWs for the removal of some PPCPs has been assessed (Matamoros et al. 2005). Park et al. (2009) analyzed the removal of sulfapyridine and sulfamethoxazole in CWs and found sulfamethoxazole removal of 30 ~ 50% in a full scale surface CWs. However, relatively only a few studies examine the removals of pharmaceuticals such as antibiotics using CWs (Matamoros et al. 2006; Conkle et al. 2008; Park et al. 2009). Therefore, it is in great needs to examine the effectiveness for removal of the antibiotics using the CWs.

The elimination of antibiotics in the CWs can be achieved by physicochemical decomposition, photo-degradation, adsorption by wetland soil and plants, and biodegradation (microbial activity). Andreozzi et al. (2003) and Matamoros et al. (2009) have suggested that some PPCPs can be removed by photo-degradation. And Conkle et al. (2010) observed that sorption was an important removal pathway for fluoroquinolone antibiotics in wetlands soil. Also, the plants growing in CWs typically exhibits several functional characteristics (e.g. transform or contain contaminants, oxygenate the systems, provide surface for periphyton attachment). It has been shown that the plants can be accumulated antibiotics via water transport and passive absorption, and excessive levels of antibiotics in water or soil can exhibit significant toxic influences on plants growth and biochemical activities (Liu et al. 2009; Boonsaner et al. 2010; Hillis et al. 2011; Li et al. 2011; Luo et al. 2011). Among the plants in CWs, the commonly used plant is *Phragmites australis* (common reed) (Vymazal, 2011) that has abilities to adsorb pollutants directly into their own tissues, and functions as catalyst for purification (Hadad et al. 2006).

2. The objectives of this study

In order to improve the antibiotics removal effectiveness in the CWs for livestock wastewater treatment, occurrence and the removal mechanisms of antibiotics in the CWs need to be examined.

The overall purpose of this work is to investigate the residual concentration of antibiotics in the livestock wastewater, and to examine the mechanisms of antibiotics removal in the CWs for treating livestock wastewater flowing into the Geum River Basin in Korea.

First, the effect of physicochemical properties (e.g. molecular weight, pKa value, ionic bond and functional groups) on removal of antibiotics in a wetlands system was examined. And, lab-scale microcosm experiments were performed to examine which mechanisms such as sunlight photo-degradation (photolysis), microbial degradation, and accumulation or adsorption by wetlands soil and plants are important when treating antibiotics by the CWs.

Ⅱ. Materials and Methods

1. Chemicals and materials

Eight antibiotics such as sulfamethoxazole (SMZ; Irritant), sulfathiazole (SFI; Irritant), sulfamethazine (SMA; VETRANAL™, analytical standard), trimethoprim (TMP; ≥ 99% (HPLC)), tetracycline (TC; ≥ 98% (NT)), oxytetracycline-HCl (OTC; ≥ 95% (HPLC)), chlorotetracycline-HCl (CTC; VETRANAL™, analytical standard), enrofloxacin (EFX; ≥ 98% (HPLC)) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Simetone (PESTANAL®, analytical standard) from Sigma (St. Louis, MO, USA) and $^{13}C_6$-sulfamethoxazole from Cambridge Isotope Labs. (Andover, MA, USA) were used as the internal standards at 10 and 100 ng/L to compensate for matrix effects. Each standard solution of the antibiotics at a concentration of 0.1 g/100 mL was dissolved in methanol (JT Baker, Phillipsburg, NJ. USA). All standard solutions were stored at 4°C in the dark. Formic acid (98%, Kanto Ltd), ethylendiamin etetraacetic acid di-sodium salt dihydrate (Na_2EDTA, Sigma-Aldrich, St. Louis, MO, USA) and hydrochloric acid (HCl, 37%) were purchased from Fisher Scientific Corporated.

All solvents and the samples were filtered through 1.2 μm glass microfiber filters GF/C (47 mm circles, Whatman, UK) and 0.45 μm Mixed Cellulose Esters MCE (47 mm circles, Advantec MFS Inc., USA). Oasis hydrophilic-lipophilic balance (HLB) (3 cc, 60 mg) was purchased from Waters Oasis (Milford, Mass., USA). Deionized water was purified using a Milli-Q system at 18.3 MΩ/cm throughout all experiments.

2. Constructed Wetlands (CWs)

2.1. Site description and Sampling

Constructed wetlands (CWs) were built to reduce the concentrations of the secondary piggery wastewater and stormwater runoff in Nonsan City, Chungnamdo Province, Korea in 2007, and its operation started in 2008 (Figure 2).

The CWs consisted of six cells from the influents wastewater entering into the Cell 1 to finally discharging into the Geum River. The influents flowing through the CWs contain organic matters (BOD, COD etc.), nutrients (TN, TP etc.) and other pollutants from livestock wastewater and stormwater runoff (Lee, 2012).

Samplings were conducted from May to December in 2012. The wastewater samples were taken at each unit process (inlet and outlet point) including the influents (after primary treatment) and the effluents (final outlet after Cell 6).

2.2. Constructed wetlands (CWs) design

Table 4 presents the design characteristics of the constructed wetlands (CWs). The CWs has 4,492 m^2 of a total surface area and 4,006 m^3 of a total storage volume and 48.4 hours of hydraulic retention time (HRT) from the inlet to outlet during dry days. The CWs consisted of six cells which were Cell 1 of sedimentation of particulates, Cell 2 of aeration of coarse-bubble diffuser system for enhanced biological treatment and Cells 3 to 6 of enhanced sedimentation of organics in the subsequent regions.

Two typical types of wetlands plants such as *Phragmites australis* (PA) and *Miscanthus sacchariflorus* (MS) were planted surrounding the water zone of the CWs which played role in sediment accumulation, uptake for nutrient and contaminants removal. The plants were selected according to their capability to grow fast, capacity to remove pollutants and tolerance to highly toxic livestock wastewater (Lee et al. 2014).

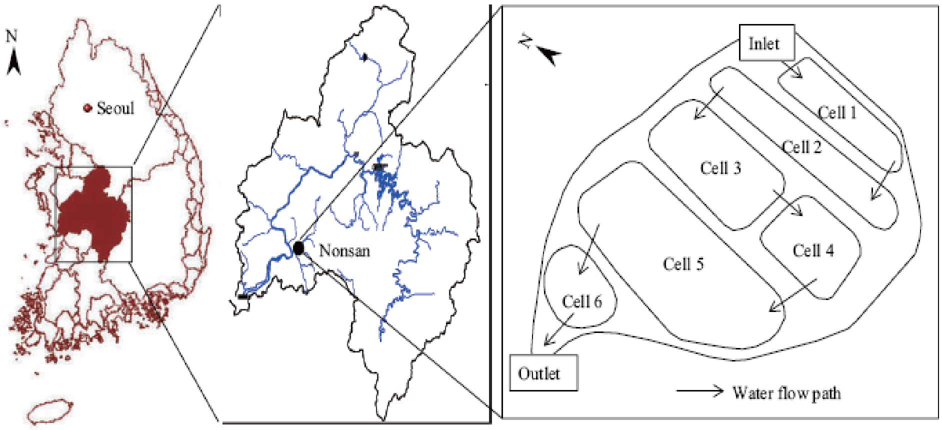


Figure 2. Location of the constructed wetlands (CWs) in Korea (Lee, 2014)

Table 4. Characteristics of constructed wetlands (CWs) in this study

	Settling basin 1 (Cell 1)	Aeration Pond (Cell 2)	Deep Marsh 1 (Cell 3)	Shallow Marsh (Cell 4)	Deep marsh 2 (Cell 5)	Settling Basin 2 (Cell 6)	Total
Surface area (m^2)	560	776	805	527	1,474	350	4,492
Storage volume (m^3)	453	565	810	280	1,626	272	4,006
Water depth (cm)	80.9	72.8	100.6	53.1	110.3	77.7	-
Length (m)	32.7	55.2	26.4	15.6	45.9	10.1	-
HRT for design flow (hours)	5.5	6.8	9.8	3.4	19.6	3.3	48.4
HRT for peak flow (hours)	1.6	2.0	2.9	1.0	5.8	1.0	14.3
Dominant plants	PA	PA	PA	MS	PA		

HRT : hydraulic retention time; PA: *Phragmites australis*; MS: *Miscanthus sacchariflorus*

2.3. Sunlight photo-degradation experiment

The sunlight photo-degradation experiment was performed in a circulating sunlight rector system consisted of a stirred reservoir composed of a 2 L glass bottle and a peristaltic pump (Cole-Parmer Instrument, Vernon Hills, USA) for circulating. The reaction chamber was connected with Teflon tube that was used for connecting six quartz columns (650 mm width and 550 mm length). The initial concentration of antibiotics was a 100 ng/L by adding stock solution in deionized water. The solution in the glass bottle was continuously re-circulated at a 1 L/min flow by a rotary pump (Figure 3).

The sunlight photo-degradation experiments were carried out under sunlight to expose glass bottle from 10 am to 5 pm (7 hours) at the Seoul National University building rooftop in Seoul, Korea in October, 2014. The solar intensity (mW/cm^2), UV-A (365 nm) and UV-B (312 nm) were measured on the surface of the solar reactor by solar irradiator. The samples were taken from the aqueous solutions, and then analyzed for residual antibiotics.

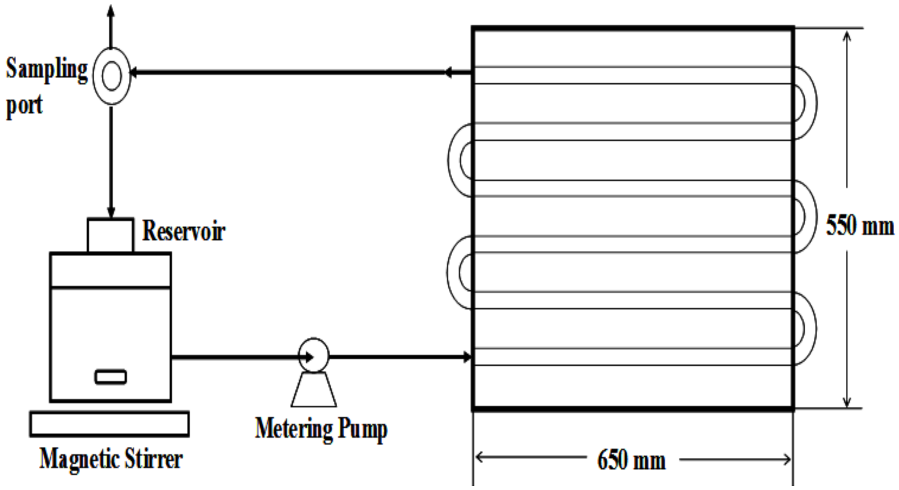


Figure 3. Schematic diagram of the photo-reactor

2.4. The experiments of adsorption by wetlands soil and plants

The soil sources (natural wetlands) were collected from a field located on Jeongneungcheon Stream in Seoul, Korea. After the collection, microcosm experiments of antibiotics adsorption by soil were performed to 20, 50 and 100 ng/L of the antibiotics with two sets which were biotic system without sterilization and abiotic system performed autoclaving at 121°C during 15 ~ 20 min for sterilization. Then, the 2.5 cm of soil was injected into the 1 L deionized water, and agitated at 90 rpm for 48 hours in the sample jar and set at 20°C and 50% humidity in the dark to remove possibility of photolysis of antibiotics.

The wetland plants sources (*Phragmites australis*; common reed) were collected from a field located on near Hangang River and Cheonggyecheon Stream in Seoul, Korea. For microcosm experiments of the plants adsorption, the obtained plants were trimmed and sieved less than 2 mm, and also performed to 5, 10 and 20 g of the plants with two sets (biotic and abiotic systems). The initial concentration of antibiotics was set to the 100 ng/L by adding stock solution in deionized water. Then, the plants was injected into the 1 L deionized water, and agitated at 70 rpm for 3 hours in the sample jar and set at 20°C and 50% humidity. During the reactions of soil and plants adsorption, the supernatant of samples were taken, then filtered by using a 0.45 μm GF/C filter (Whatman, UK) and then, concentrated by solid phase extraction (SPE) for pretreatment, and analyzed the samples by LC-MS/MS.

3. Solid phase extraction (SPE)

All of the samples were immediately stored in a refrigerator below 4°C in the dark before solid phase extraction (SPE). The HLB cartridge was pre-conditioned before loading of the sample with 6 mL of methanol and deionized water to activate the cartridge and remove the impurities in the cartridge. Prior to extraction, 200 mL of livestock wastewater samples were filtered through 1.2 μm GF/C and 0.45 μm MCE and then were conducted to add 5% (v/v) Na_2EDTA into the each filtered samples for extraction preparation. The addition of Na_2EDTA was used to chelate residual metals in the water samples and improve recoveries.

The pH of the samples were adjusted to less than 3.0 (pKa = 3.3) only for tetracycline-group (TC, OTC, and CTC) by adding hydrochloric acid (HCl) and then immediately spiked with two internal standards of $^{13}C_6$-sulfamethoxazole and simeton at 100 and 10 ng/L. Then, the samples were passed through the HLB cartridges at a 3 mL/min flow. After the sample loading, washing with 10 mL of deionized water was performed, and then the cartridges were dried under a gentle stream of air. The dried cartridges were eluted with 6 mL of methanol into a falcon tube (eluted below pH 3 for tetracycline-group). The extracted solutions were rotary evaporated using a gentle nitrogen stream in 40°C (CVE-3100; EYELA, Tokyo, Japan), and then was re-constituted to vortex mixed in 1 ml of deionized water and methanol in 3 to 1 ratio (v/v) in a vial for subsequent LC-MS/MS analysis.

4. LC-MS/MS analysis

High-performance liquid chromatography (HPLC) system (Nexera; Shimadzu, Kyoto, Japan) connected to triple quadrupole mass spectrometer (MS) (ABI-4000; Applied Biosystems, Foster City, CA, USA) with an electron ion spray source, which could operate in both positive and negative modes was used to measure the antibiotics. The antibiotics were separated with a reverse phase C-18 column (Kinetex, 2.6 μm, 50 × 2.1 mm, Phenomenex, Torrance, CA, USA). All aqueous solutions were filtered through a 0.2 μm PTFE, disposable membrane filter (Toyo Roshi Kaisha, Ltd, Japan).

A binary gradient was consisted that 0.1% formic acid in water for mobile phase A and 0.1% formic acid in methanol for mobile phase B in electrospray ionization (ESI) positive complete scan mode for MS detection. The gradient with 2% of mobile phase B was held for 0.1 min, linearly stepped to 74% at 6 min, and decreased to 2% for 7 min then held at 2% for 8 min and stopped in 8.50 min at a flow rate of 0.3 mL/min. The injection volume of 10 μl was used, and the column oven temperature was 40°C. All of the antibiotics were analyzed simultaneously within 8.50 min using a multi-residue method by protonated and detected in the form $[M+H]^+$ in positive ionization. For quality assurance/quality control (QA/QC), the recoveries of the extracted samples were calculated by comparing the spiked wastewater samples with standard samples. Data acquisition was conducted by the multiple reactions monitoring (MRM) which is LC-MS/MS conditions for the analysis of antibiotics by

MRM in positive ion mode in Table 5.

The calibration curve was prepared by spiking the concentration from 1, 2, 5, 10, 20, 50, and 100 ng/L and two surrogates, and they had good linearity with the correlation coefficients (r^2) value of the calibration curves that were exceeded 0.997 for all analysis. It obtained the limit of detection (LOD) calculated using a signal to noise ratio of 3 and a limit of quantification (LOQ) were determined based on the signal to noise ratio greater than 10 for all compounds. LOD and LOQ tests were repeated six times to confirm the accuracy regarding the detected antibiotics of 5, 20, and 100 ng/L. LOD and LOQ of the samples were in the range of 0.49 ~ 4.60 ng/L and 1.50 ~ 13.90 ng/L. The recovery of the internal standards (ISs) and relative standard deviation (RSD) were in the ranged from 72 to 113.83%, and 10.57% for $^{13}C_6$-sulfamethoxazole (SMZ, SFI, SMA and TMP), respectively, and 86.85 ~ 100% and 8.50 ~ 13.91% for simeton (TC, OTC, CTC and EFX), respectively (Table 6).

5. Statistics

The measured results were statistically evaluated using the SAS program (version 9.3, SAS Institute Inc., USA). The correlation coefficients between removal efficiencies of antibiotics, concentration of general parameters and concentration of antibiotics in livestock wastewater were obtained using t-tests.

Table 5. LC-MS/MS conditions for the analysis of antibiotics by MRM in positive ion mode

Compound	Retention time (min)	Exact mass (g)	Precursor ion (m/z)	Product ion (m/z)
Sulfamethoxazole (SMZ)	3.07	253.05 (+)	254.20	108.10, 92.10
Sulfathiazole (SFI)	2.12	255.01 (+)	256.20	156, 92.10
Sulfamethazine (SMA)	2.74	278.08 (+)	279.20	186, 124.10
Trimethoprim (TMP)	2.50	290.00 (+)	291.30	230.10, 123
Tetracycline (TC)	2.73	444.15 (+)	445.10	410.20, 427.10
Oxytetracycline (OTC)	2.80	460.15 (+)	461.20	426.20, 443
Chlortetracycline (CTC)	3.06	478.88 (+)	479.20	444.20, 462.20
Enrofloxacin (EFX)	3.07	359.40 (+)	360.30	342, 316

Table 6. Summary of the detection limits and recoveries for eight selected antibiotics in the CWs

Compound	Retention time (min)	Detection limit (n = 6)		RSD (< 20%)			Recovery (%)		
		LOD (ng/L)	LOQ (ng/L)	Mean ± SD	Max.	Min.	Mean ± SD	Max.	Min.
				IS - $^{13}C_6$-Sulfamethoxazole					
SMZ	3.07	3.34	10.12	10.57	10.57	10.57	81.45 ±16.30	113.83	72.00
SFI	2.12	2.45	7.44						
SMA	2.74	4.60	13.90						
TMP	2.50	1.55	4.70						
				IS - Simeton					
TC	2.73	4.39	13.31	11.46 ± 2.3	13.91	8.50	95.12 ±5.00	100.00	86.85
OTC	2.80	0.49	1.50						
CTC	3.06	2.84	8.63						
EFX	3.07	1.24	3.77						

Ⅲ. Results and Discussion

1. Occurrences of the antibiotics in livestock wastewater

The average concentrations of selected eight antibiotics were measured in influents and effluents of livestock wastewater through constructed wetlands (CWs) (n = 5). Each of the average concentrations of SMZ, SFI, SMA, and TMP in the influents ranged from 10.03 to 11,583.33 μg/L (mean; 2,744.32 ± 4,987.04 μg/L), 1,263.33 to 57,833.33 μg/L (mean; 22,492.66 ± 26,217.42 μg/L), 1,055 to 30,033.33 μg/L (mean; 7,921.99 ± 12,477.81 μg/L), and 1.76 to 673.33 μg/L (mean; 236.75 ± 297.60 μg/L), respectively while in the effluents the respective concentrations were from 0 to 4,778.33 μg/L (mean; 1,387.73 ± 1,748.40 μg/L), 200.67 to 12,833.33 μg/L (mean; 4,080.33 ± 5,659.40 μg/L), 271.16 to 1,546.66 μg/L (mean; 1,187.90 ± 1,398.46 μg/L), and 14.33 to 588.33 μg/L (mean; 231.25 ± 265.05 μg/L), respectively.

The average concentration of TC, OTC, CTC and EFX in the influents ranged from 8.41 to 69.5 μg/L (mean; 36.35 ± 25.62 μg/L), 12.33 to 48.83 μg/L (mean; 31.83 ± 17.58 μg/L), 4,300 to 16,100 μg/L (mean; 9,689.99 ± 4,205.58 μg/L), and 34.26 to 262.16 μg/L (mean; 114.82 ± 91.92 μg/L), respectively while in the effluents the respective concentrations were from 7.27 to 114 μg/L (mean; 47.98 ±

44.52 μg/L), 11.78 to 828.33 μg/L (mean; 188.01 ± 358.29 μg/L), 1,033.33 to 11,600 μg/L (mean; 6,834.66 ± 4,128.61 μg/L), and 24.55 to 225.50 μg/L (mean; 83.52 ± 85.86 μg/L), respectively (Figure 4).

The highest of concentrations of antibiotics in the livestock wastewater were CTC and SFI in this study. It might be attributed by the low efficiency of the CWs for antibiotics treatment and about 2,000 pigs of farms near a primary livestock wastewater treatment plant. The concentrations of the CTC and SFI in the livestock wastewater influents were also reported as high amount 2,960 and 2,293.90 μg/L, respectively in Korea. And SMZ in livestock wastewater effluents was also reported as amount 840 μg/L in Italy (Aukidy et al. 2012) (Table 2 and Table 3).

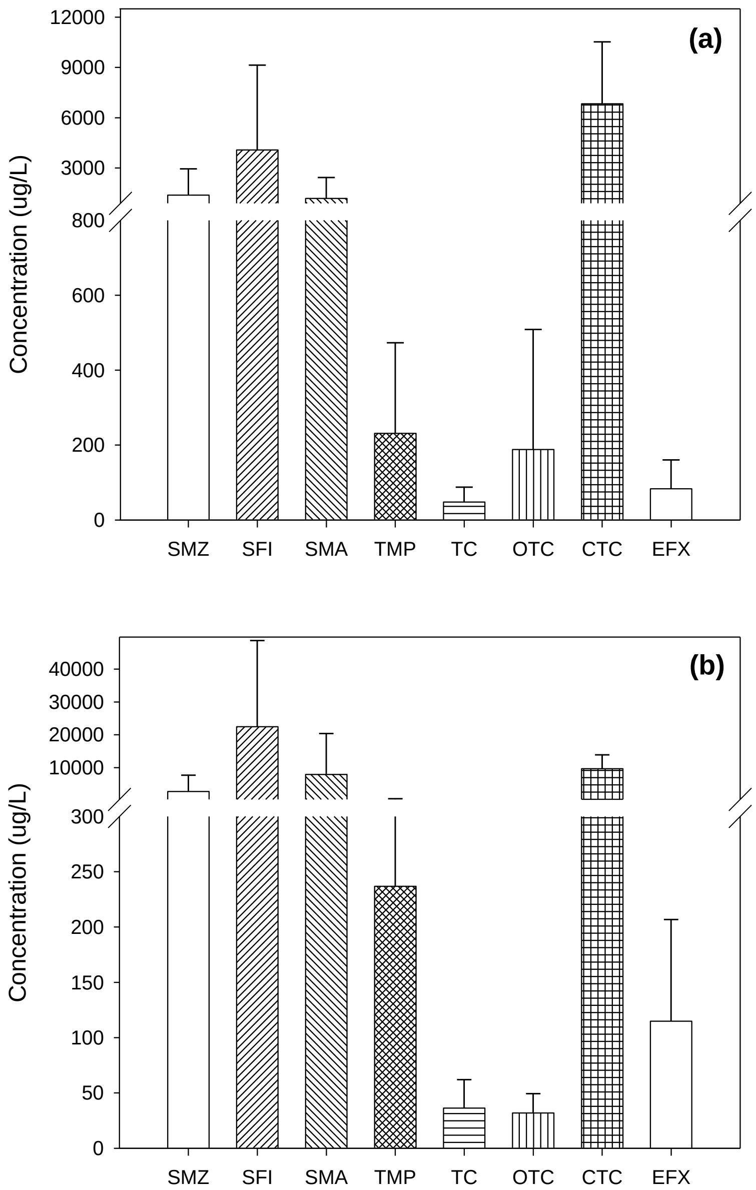


Figure 4. Average concentrations of selected eight antibiotics in livestock wastewater influents (a), and effluents (b) using the CWs (n = 5)

2. Removal efficiency of the antibiotics by CWs

Removal efficiencies of eight selected antibiotics showed various removals in the CWs (0 ~ 85%). Removals of the selected antibiotics at average concentrations were 85% (SMA), 81.86% (SFI), 49.43% (SMZ), 29.47% (CTC), 27.26% (EFX), 2.32% (TMP) and un-removed (TC and OTC) (Figure 5).

Sulfonamide-group-antibiotics (SMZ, SFI and SMA) were relatively effectively removed by the CWs (49.43 ~ 85%), especially SFI and SMA. By comparing removal efficiencies, the antibiotic compounds studied were classified into (i) high removed compounds with the removal efficiency higher than 80% (SFI and SMA); (ii) moderately removed compounds between 40% and 80% (SMZ); (iii) low removed compounds between 10% and 40% (CTC and EFX); (iv) un-removed compounds lower than 10% (TMP, TC and OTC).

While removals of sulfonamide-group-antibiotics (SMZ, SFI and SMA) were higher, the removals of tetracycline-group-antibiotics (TC, OTC and CTC) were lower. According to the SAS t-tests, there was an inverse correlation ($p < 0.0493$, $r^2 = 0.7735$) between the sulfonamide-group (SMZ, SFI and SMA) removals and tetracycline-group (TC, OTC and CTC) removals, indicating that sulfonamide-group-antibiotics were removed effectively in the CWs. Interestingly, the removal of SFI was correlated with the removal of SMA ($p < 0.0143$, $r^2 = 0.8980$), and inverse correlated ($p < 0.0499$, $r^2 = 0.7717$) with the removal of CTC. It might be explained by their different physicochemical properties such as pKa value and ionic state in the water. However, no seasonal differences ($p < 0.05$) were observed for the removal efficiencies of the eight antibiotics during the sampling periods.

The concentrations of TC and OTC in the livestock wastewater effluents were higher than influents (un-removed). The higher effluents concentrations of some antibiotics compared with influents were reported in STPs in Sweden (Lindberg et al. 2005). Possible explanations for these explanations can be as follows; (i) deconjugation of conjugated metabolites during the treatment process (Miao et al. 2002); (ii) an underestimation of the actual amount due to particulate matter with adsorbed antibiotics being filtered out during sample preparation (Gulkowska et al. 2008); and (iii) a change in the adsorption behavior of the analyses to particles during treatment process, influencing the ratio between influents/effluents water (Lindberg et al. 2005).

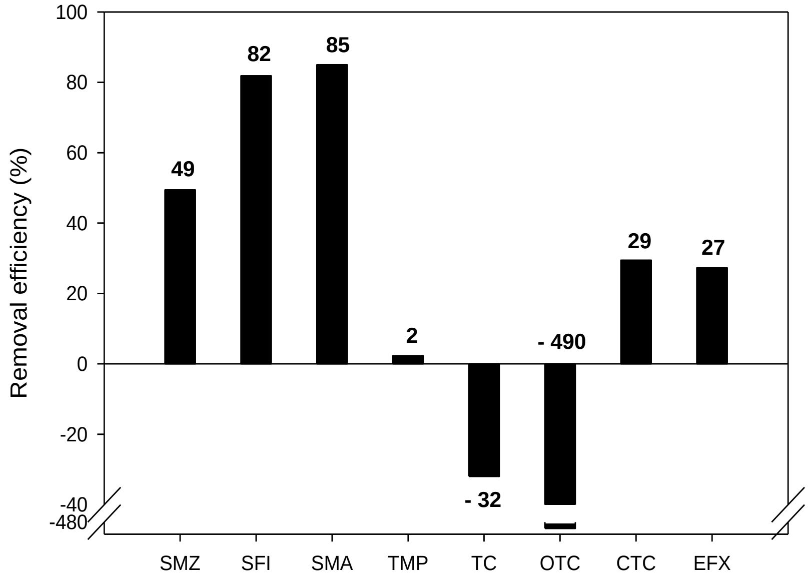


Figure 5. Removal efficiency of eight selected antibiotics in the influents and effluents of livestock wastewater using the CWs (n = 5)

3. General parameters in livestock wastewater

During the experimental periods, the general parameters such as DO, temperature, conductivity, turbidity, TSS, COD_{cr}, COD_{mn}, DOC, BOD, TN, TKN, NH_4-N, NO_3-N, TP, and PO_4-P of the average concentrations in influents and effluents of the CWs were also measured, and the results is shown in Table 7.

Table 7 shows significantly high removal of turbidity, TSS and TKN (> 60%), moderate removal of NO_3-N (30 ~ 60%), and low removal of COD_{cr}, COD_{mn}, BOD and TP (10 ~ 30%), bad removed of TN and NH_4-N (> 10%), and un-removed of DOC and PO_4-P in the CWs system.

According to the t-tests results, there were the correlations between average concentrations of these general parameters and antibiotics in livestock wastewater in the CWs. SMZ had inverse correlation ($p < 0.0559$, $p < 0.0429$) with temperature and BOD, respectively, SFI had correlation ($p < 0.0263$, $p < 0.0656$) with turbidity and COD_{cr}, respectively, and TMP had inverse correlation ($p < 0.0161$, $p < 0.0211$) with BOD and TKN, respectively. TC had inverse correlation ($p < 0.0165$) with temperature, and OTC had correlation ($p < 0.0620$, $p < 0.0769$) with COD_{mn} and TP, respectively, and CTC had correlation ($p < 0.0598$) with TN.

Overall, most of the antibiotics concentrations in the livestock wastewater were in inverse correlation with BOD, temperature, and in correlation with COD, TN and TP in the CWs system. Manoli et al. (2008) examined the seasonal variations of PAHs removal percentages which were observed highest in cold periods and the lowest in warm periods.

Table 7. Average concentrations of general parameters in the influents and effluents of the livestock wastewater using the CWs (n = 5)

	Influents	Effluents	Removal (%)	Removal efficiency
DO (mg/L)	1.50 ± 1.80	1.64 ± 2.30	.	.
Temperature (℃)	18.10 ± 8.70	15.72 ± 11.30	.	.
Conductivity (μS/cm)	3135.67 ± 2542.30	3199.33 ± 1231.10	.	.
Turbidity (NTU)	137.10 ± 81.30	39.44 ± 32.10	71.23	High[a]
TSS (mg/L)	123.80 ± 70.40	49.08 ± 38.80	60.37	High[a]
COD_{cr} (mg/L)	469.30 ± 147.80	334.46 ± 204.10	28.73	Low[c]
COD_{mn} (mg/L)	221.20 ± 78.10	176 ± 99.50	20.45	Low[c]
DOC (mg/L)	79 ± 41.50	83.35 ± 37.90	.	Un-removed[e]
BOD (mg/L)	46.20 ± 2.60	41.20 ± 11.70	10.73	Low[c]
TN (mg/L)	126.90 ± 88.60	114.37 ± 82.70	9.89	Bad[d]
TKN (mg/L)	20.9 ± 0.00	8.10 ± 0.00	61.24	High[a]
NH_4-N (mg/L)	18.90 ±14.10	17.77 ± 14.90	6.19	Bad[d]
NO_3-N (mg/L)	3.30 ± 4.40	2.22 ± 2.40	32.32	Moderate[b]
TP (mg/L)	6.70 ± 1.90	5.51 ± 3.50	18.22	Low[c]
PO_4-P (mg/L)	0.70 ± 0.50	1.67 ± 0.70	.	Un-removed[e]

a High removal efficiency (> 60%)

b Moderate removal efficiency (30 ~ 60%)

c Low removal efficiency (10 ~ 30%)

d Bad removal efficiency (> 10%)

e Un-removed (< 0%)

4. Removal mechanisms of antibiotics in the CWs

4.1. Physicochemical properties

Removal of antibiotics in livestock wastewater can be achieved by a variety of degradation mechanisms such as physicochemical degradation, photo-degradation (photolysis), biodegradation (microbial activity), adsorption in wetlands soil and plants (available organic surfaces), etc. Among of them, these removals generally depended on physicochemical properties of antibiotics (e.g. molecular weight, pKa value, ionic bond and functional groups).

Based on the results, the most relevant mechanism leading to these differences in removal efficiencies can caused by the adsorbed compounds onto the soil by physicochemical properties such as molecular weight and ion state according to pKa value of antibiotics (ionic bond). According to Mitra et al. (2012), the removal efficiency increased with molecular weights of the antibiotics, this result indicates that molecular weight of compounds can be important factor in antibiotics removal.

Moreover, an earlier study had shown that pH value of soil has a possible effect on sorption of the compounds because the relative availability of cations in soil can increase by higher pH (Hussain et al. 2011a). Kurwadkar et al. (2007) reported that certain pharmaceuticals have strong pH dependency for sorption onto sand and loam soil. These studies imply that two parameters values such as molecular weight and pKa of antibiotics with the removal percentages can be closely related in the wetlands system.

Among the removals of the several type of antibiotics (0 ~ 85%), higher removal efficiencies of SFI and SMA (> 80%) can be explained by their low molecular weight and higher pKa value. Since the sulfonamide-group (SMZ, SFI and SMA) has higher pKa values, resulting in more effective adsorption with the negative charged soil by the electrostatic interaction rather than dissolving into the water.

On the other hand, amide and urea functional groups are reported to be biologically transformed via mediated hydrolysis reactions (Chisaka et al. 1970; Englehardt et al. 1973), and poly-halogenated compounds are known to be biodegraded via a microbially mediated reduction (dehalogenation) (Mohn et al. 1992; Scharzenbach et al. 2003).

Although the halogenated compounds (TC, OTC, CTC and EFX) can be biodegraded via microbial mediated reduction indirectly, the un-removals of TC and OTC can be due to their higher molecular weight and pKa values lower than 7 (nature ionic state). CTC and EFX showed the low removal efficiencies by being able to go through biodegraded reduction (poly-halogenated), low pKa value, and high and moderate molecular weight of CTC and EFX, respectively.

Meanwhile, un-removed in TMP is assumed by its extremely symmetric structure (its stable structure state), resulting in persistence in microbial degradation. In fact, Koetzle et al. (1976) observed that TMP molecule has the torsion angles (τ_1 = -89.4°, τ_2 = 153.3°) around the two bonds C(5)-C(7) and C(l)-C(7), respectively. In order better to define the conformational energy surface for rotations τ_1 and τ_2, a plot of potential energy against the two torsion angles was prepared to show that the energy of binding the drug to its receptor is sufficient to stabilize an improbable conformation for the free molecule (Koetzle et al. 1976) (Table 8).

Table 8. Removal efficiency of antibiotics using the CWs based on physicochemical properties (n = 5)

Compound	Molecular weight	Molecular formula	Functional groups	pKa	Charge state	Removal (%)	Removal efficiency
SMZ	253.28	$C_{10}H_{11}N_3O_3S$	- SO_2- (sulfonyl)	5.7 ~ 5.9	(-)	49.43	Moderate[b]
SFI	255.32	$C_9H_9N_3O_2S_2$	- SO_2- (sulfonyl)	7.1 ~ 7.2	(+)	81.86	High[a]
SMA	278.33	$C_{12}H_{14}N_4O_2S$	- SO_2- (sulfonyl)	7.3 ~ 7.7	(+)	85.00	High[a]
TMP	290.32	$C_{14}H_{18}N_4O_3$	Extremely symmetric structure	6.6 ~ 6.8	(-)	2.32	Un-removed[d]
TC	444.44	$C_{22}H_{24}N_2O_8$	-CONR2- (amide) , NH_2 (amine)	3.3 ~ 4.5	(-)	-	Un-removed[d]
OTC	496.65	$C_{22}H_{24}N_2O_9 \cdot HCl$	-CONR2 (amide), NH_2 (amine), -Cl (halide)	3.2 ~ 4.5	(-)	-	Un-removed[d]
CTC	515.38	$C_{22}H_{23}ClN_2O_8 \cdot HCl$	-CONR2 (amide), NH_2 (amine), -Cl (halide)	3.3 ~ 4.5	(-)	29.47	Low[c]
EFX	359.40	$C_{19}H_{22}FN_3O_3$	-COOH (carboxyl), NH_2 (amine), -F (fluoride)	2.7 ~ 3.9	(-)	27.26	Low[c]

a : High removal efficiency(> 80%)

b : Moderate removal efficiency(40 ~ 80%)

c : Low removal efficiency(10 ~ 40%)

d : Un-removed (< 10%)

4.2. Soil adsorption

Degradation of antibiotics in the wetlands soil can occur by biodegradation (microbial activity) and directly adsorbed or accumulated into the soil. Hussain et al. (2011a) showed the higher sorption potential for the sandy clay loam wetlands soil, which was due to the comparatively higher clay and organic matters in loamy soil, so higher organic matter may support a larger and more diverse microbial at community, which in turn could have contributed to biodegradation of the antibiotics.

In this study, based on the antibiotics removal results, higher removal efficiencies of sulfonamide-group-antibiotics (SMZ, SFI and SMA) can be explained by higher their pKa values, resulting in more effective adsorption with the negative charged soil by the electrostatic interaction. However, the other antibiotics were poorly removed.

Therefore, in order to examine the removal mechanisms in details, microcosm adsorption experiments by the soil (natural wetlands) were performed to 20, 50 and 100 ng/L of the antibiotics into 2.5 cm of soil in deionized water with two sets (biotic system without sterilization and abiotic system with sterilization). The results showed that the biotic system effectively removed antibiotics (e.g. SMZ (72.93%), SFI (63.60%), SMA (76.20%), TMP (90.50%) and EFX (86.53%)) within 48 hours; however, the removals of abiotic system (e.g. SMZ (67.00%), SFI (36.60%), SMA

(63.64%), TMP (63.50%) and EFX (78.20%)) were lower than the removals of biotic system (Figure 6 and Table 9).

Higher concentration of antibiotics in the soil had markedly changed the removal of antibiotics, resulting in higher removals of antibiotics in biotic system rather than abiotic system. According to the t-tests results, all of the 100 ng/L of antibiotics (except EFX) in the soil showed statistical differences between biotic and abiotic systems for SMZ ($p < 0.025$), SFI ($p < 0.030$), SMA ($p < 0.012$) and TMP ($p < 0.013$), respectively. Sulfonamide-group (SMZ, SFI, and SMA) showed significant statistical difference in two sets with the 100 ng/L of antibiotics ($p < 0.0005$). Also, 50ng/L of SFI and SMA showed statistical differences in two sets ($p < 0.029$, $p < 0.009$), respectively.

Berns et al. (2008) compared methods of soil sterilization including autoclaving and reported physicochemical modification of soil organic matter. After soil autoclaving, such changes organic matter or physical structure in the soil aggregates can undermine the adsorption results. These results of soil adsorption experiments imply that the important factors of antibiotics removal in wetlands may be adsorbed or accumulated into the soil and biodegradation by microbial activity rather than pKa value of antibiotics. The antibiotics could be also efficiently removed in wetlands soil by higher concentration, especially sulfonamide-antibiotics.

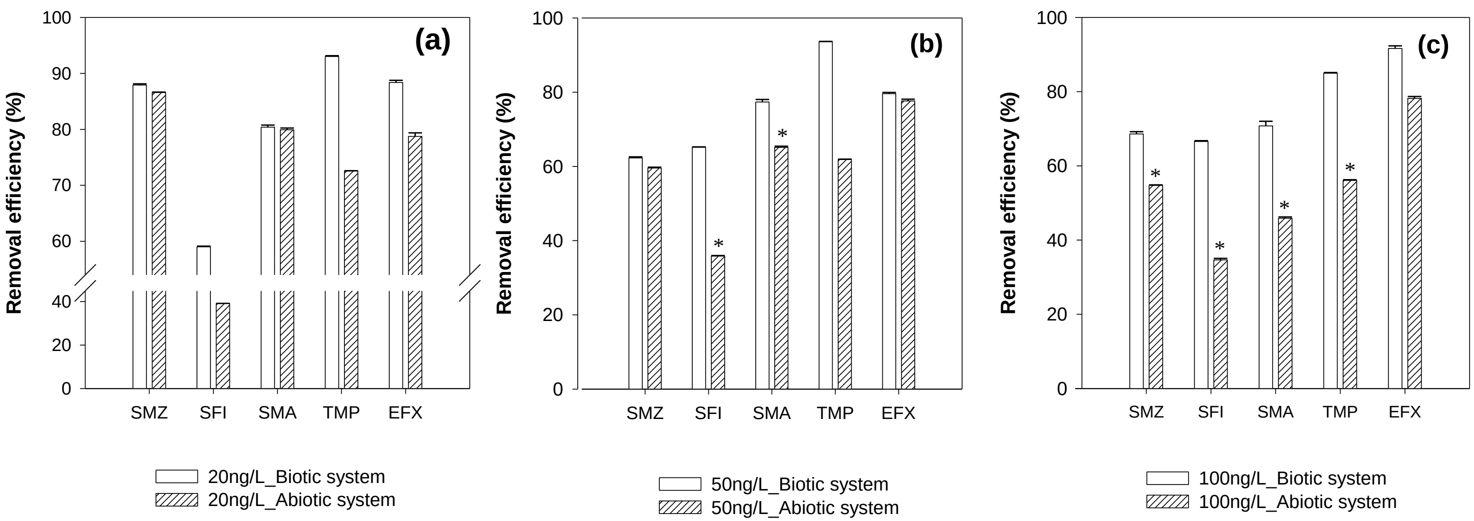


Figure 6. Removal efficiency of each of 20 (a), 50 (b) and 100 ng/L of antibiotics (c) by soil adsorption with biotic and abiotic systems, *: $p < 0.05$ (experimental condition; [antibiotics]$_{initial}$ = 20, 50 and 100 ng/L, [soil] = 2.5 cm/L, [time] = 48 hours, [n] = 2)

Table 9. Removal efficiency of antibiotics by wetlands soil adsorption with biotic and abiotic systems (* : $p < 0.05$)

Concentration of antibiotics (ng/L)	Removal efficiency (%)			
	Biotic system		Abiotic system	
20	SMZ	87.91	SMZ	86.58
	SFI	59.01	SFI	39.15
	SMA	80.38	SMA	79.92
	TMP	93.01	TMP	72.55
	EFX	88.36	EFX	78.75
50	SMZ	62.29	SMZ	59.58
	SFI*	65.15	SFI	35.83
	SMA*	77.34	SMA	65.12
	TMP	93.58	TMP	61.85
	EFX	79.60	EFX	77.61
100	SMZ*	68.60	SMZ	54.74
	SFI*	66.58	SFI	34.67
	SMA*	70.76	SMA	45.87
	TMP*	84.95	TMP	56.06
	EFX	91.65	EFX	78.19
	Average removal efficiency (%)			
20, 50, and 100	SMZ	72.93	SMZ	67.00
	SFI	63.60	SFI	36.60
	SMA	76.20	SMA	63.64
	TMP	90.50	TMP	63.50
	EFX	86.53	EFX	78.20

4.3. Sunlight photo-degradation

Several studies have documented photo-degradation of pharmaceuticals in surface waters including diclofenac, naproxen, and ketoprofen (Buser et al. 1999; Tixier et al. 2003). Ketoprofen was removed from surface and sea waters in a batch reactor process by photolysis (Pereira et al. 2007; Lin et al. 2005). And, Andreozzi et al. (2003) and Matamoros et al. (2009) have also suggested that some pharmaceuticals and personal care products (PPCPs) can be removed by photo-degradation.

In this study, to assess the portion of removal of antibiotics in the CWs, photo-degradation experiment of antibiotics by sunlight was performed. Sunlight intensity during the experimental days from 10 am to 5 pm (7 hours) was measured by solar radiometer (total solar intensity (mW/cm^2), UV-A (365 nm) and UV-B (312 nm), respectively). The average of total sunlight intensity in fall season was ranged from the 8.80 to 94.98 mW/cm^2 (Figure 7 (b)).

Sunlight photo-degradation results showed that EFX was effectively (70%) removed, compared to the removal of sulfonamide-antibiotics with SMZ (23%), SFI (44%) and SMA (28%) in 7 hours, respectively. TMP photo-degradation rate was the lowest (8%) (Figure 7 (a)). In fact, Michela et al. (2012) conducted the photolysis experiment using untreated river water with natural sunlight, and reported a substantial removal of EFX within 60 min.

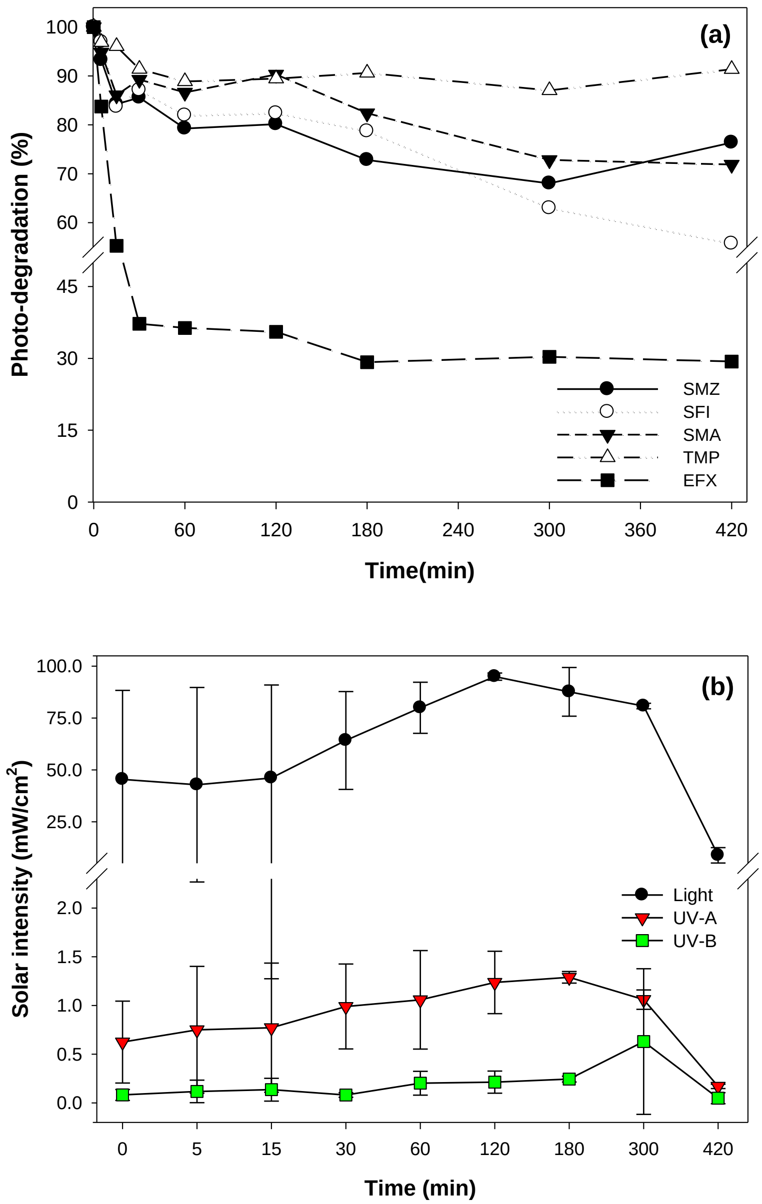


Figure 7. Sunlight photo-degradation rate of antibiotics (a), and solar intensity for sampling duration (b) (experimental condition; [antibiotics]$_{initial}$ = 100 ng/L, [time] = from 10 am to 5 pm (7 hours), [n] = 2)

4.4. Plants adsorption

Degradation of antibiotics in CWs can occur both from the activity of microbes and through physicochemical decomposition process such as biodegradation and hydrolysis in plants. Also, the pharmaceutical compounds could be taken up and transported by living plants via mass flow (in transpiration stream) and through active uptake (Dettenmaier et al. 2008; Kumar et al. 2005; Grote et al. 2007).

The plants can not only uptake pollutants, but also function as a carbon supplier for microbe metabolism, and offer attachment sites for microbes on their extended root system and transfer oxygen through their roots (Stefanakis et al. 2012). The plant roots are most important parts to absorb and secrete exudates, which promote the growth of phosphobacteria, thereby indirectly improve the purification rate in water (Wang et al. 2014). The aboveground biomass such as leaf areas and stomatal conductance appear to be very important plant-specific parameters, including the plants capacities to release oxygen into their rhizosphere (Stottmeister et al. 2003). And, the possible microbial role in the removal of compounds has already been reported (Hussain et al. 2011b).

Therefore, the antibiotics in the wetlands system can be directly adsorbed into the plants (*Phragmites australis*) through its root and leaf via biological uptake (microorganism).

In this study, wetlands plants (*Phragmites australis*) were performed by trimmed and sieved for the microcosm adsorption experiments. To examine the effect of biodegradation, 5, 10 and 20 g of the plants with two sets (biotic system without sterilization and abiotic system with sterilization) were used. The initial concentration of antibiotics was set to the 100 ng/L by adding stock solution in deionized water.

As a result, all of the antibiotics removals by the 5, 10 and 20 g of the wetlands plants showed differences in two sets, resulting in higher removals efficiency of antibiotics in biotic system rather than abiotic system. The biotic system almost effectively removed in sulfonamide-antibiotics (e.g. SMZ (71.40%), SFI (54.02%) and SMA (61.80%)) within 3 hours, however, the lower removals of SMZ (3.30%), SFI (25.01%) and SMA (50.10%) were observed in abiotic system in 3 hours. Meanwhile, there were no significant difference in removals of TMP and EFX in biotic and abiotic systems, which showed 20.30% and 26.43% reduction of TMP and EFX in biotic system, respectively, while the abiotic system yielded 19.94% and 23.83% reduction in TMP and EFX, respectively (Figure 8 and Table 10).

According to the t-tests results, there was a statistical difference between biotic and abiotic removal efficiencies of sulfonamide-antibiotics. SMZ showed statistical differences with 5 g ($p < 0.028$), 10 g ($p < 0.058$) and 20 g of plants ($p < 0.033$) in two sets, respectively. SFI and SMA also showed statistical differences with 20 g of plants ($p < 0.021$, $p < 0.046$) in two sets, respectively. Overall, sulfonamide-group (SMZ, SFI, and SMA) showed significant statistical difference in two sets with 5 g ($p < 0.056$), 10 g ($p < 0.057$) and 20 g of plants ($p < 0.005$) respectively.

Therefore, the effective removals of sulfonamide-antibiotics might be attributed to microbial biodegradation. All the plants adsorption results indicate that plants (*Phragmites australis*) in the CWs can be important factor to sulfonamide-antibiotics (SMZ, SFI and SMA) removal by microbial activity.

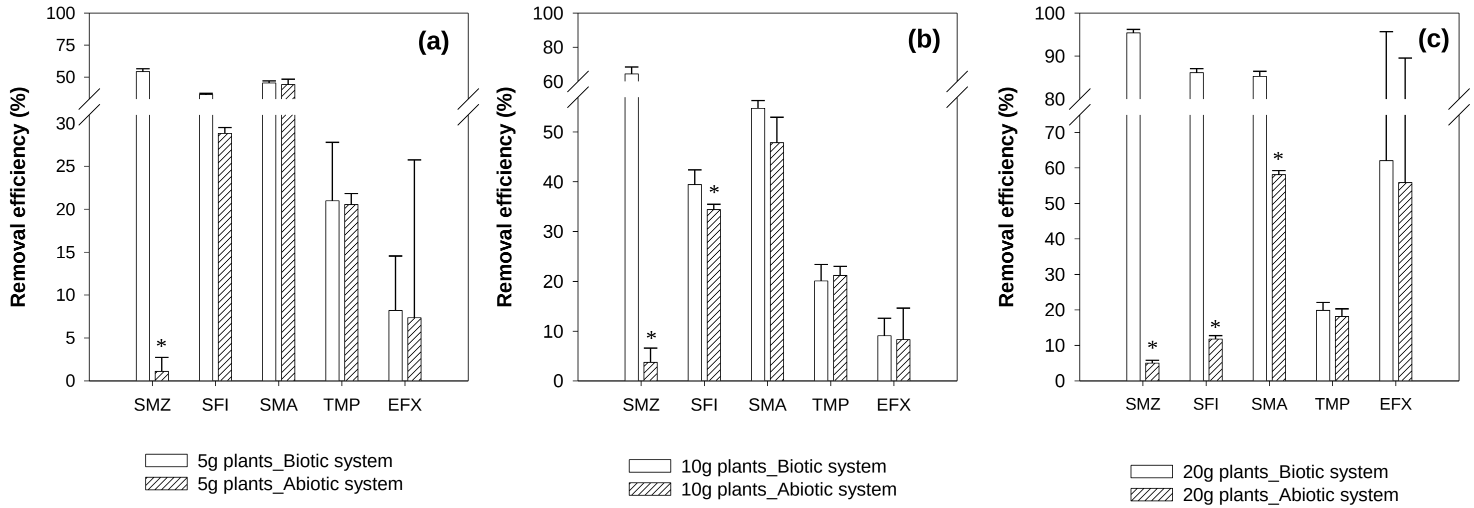


Figure 8. Removal efficiency of antibiotics by 5 g (a), 10 g (b) and 20 g of wetlands plants (c) adsorption with biotic and abiotic systems, * : $p < 0.05$ (experimental condition; [antibiotics]$_{initial}$ = 100 ng/L, [plants mass] = 5, 10, and 20 g/L, [time] = 48 hours, [n] = 2)

Table 10. Removal efficiency of antibiotics by wetlands plants adsorption with biotic and abiotic systems (* : $p < 0.05$)

Wetlands Plants mass (g)	Removal efficiency (%)			
	Biotic system		Abiotic system	
5	SMZ*	54.37	SMZ	1.10
	SFI	36.52	SFI	28.84
	SMA	45.44	SMA	44.34
	TMP	20.97	TMP	20.53
	EFX	8.18	EFX	7.35
10	SMZ*	64.31	SMZ	3.71
	SFI*	39.41	SFI	34.39
	SMA	54.77	SMA	47.85
	TMP	20.06	TMP	21.18
	EFX	9.06	EFX	8.26
20	SMZ*	95.39	SMZ	5.04
	SFI*	86.12	SFI	11.79
	SMA*	85.26	SMA	58.08
	TMP	19.89	TMP	18.10
	EFX	62.04	EFX	55.89
	Average removal efficiency (%)			
5, 10, and 20	SMZ	71.40	SMZ	3.30
	SFI	54.02	SFI	25.01
	SMA	61.80	SMA	50.10
	TMP	20.30	TMP	19.94
	EFX	26.43	EFX	23.83

Ⅳ. Conclusions

This paper examined the occurrence, and the removal of antibiotics in the influents and effluents of livestock wastewater through the constructed wetlands (CWs) flowing into the Geum River Basin in Nonsan, Korea. The results obtained were as follows;

(1) The levels of eight antibiotics were measured in livestock wastewater using constructed wetlands (CWs) in Nonsan, Korea. The concentrations of antibiotics in the effluents were CTC, SFI, SMA, SMZ, TMP, OTC and EFX in order, and ranged from 47 to 6,834 μg/L, respectively.

(2) The removal efficiencies of the selected antibiotics at average concentrations were 85% (SMA), 81.86% (SFI), 49.43% (SMZ), 29.47% (CTC), 27.26% (EFX), and TMP, TC, and OTC were hardly removed. While removals of sulfonamide-group (SMZ, SFI and SMA) were higher, the removals of tetracycline-group (TC, OTC and CTC) were lower. There were inverse correlation ($p < 0.0493$) between sulfonamide-group removals and tetracycline-group removals.

(3) There were correlations between concentration of general parameters (e.g. temperature, BOD, COD, TN, TP, etc.) and the antibiotics ($n = 10$). Most of the antibiotics had inverse correlation with BOD, temperature, while they had correlation with COD, TN and TP ($p < 0.05$).

(4) The physicochemical properties (e.g. molecular weight, pKa value, ionic bond and functional groups) are the possible control factors in the CWs. Based on the physicochemical properties, higher removal of sulfonamide-antibiotics are due to the low molecular weight and higher pKa values, resulting in more effective adsorption with negative charged soils in the CWs by electrostatic interaction.

(5) In the soil adsorption experimental results, most of the higher concentration of antibiotics (except EFX) showed the statistical differences removals in two sets, resulting in higher removal efficiency of antibiotics in biotic system rather than abiotic system ($p < 0.05$). Therefore, the important factors of removal antibiotics in wetlands soil might be absorbed into the soil directly and biodegradation by microbial activity rather than pKa value, especially sulfonamide-antibiotics.

(6) The sunlight photo-degradation results showed that EFX was removed effectively (70%) within 7 hours, however removals of sulfonamide-antibiotics (SMZ (23%), SFI (44%) and SMA (28%)) were low and the TMP photo-degradation rate was the lowest (8%).

(7) In the plants adsorption experiment, the biotic system in the presence of wetland plants (*Phragmites australis*) effectively removed sulfonamide-group such as SMZ (71.40%), SFI (54.02%) and SMA (62.80%), compared to lower removals (SMZ (3.30%), SFI (25.01%) and SMA(50.10%)) by abiotic system, indicating that microbial degradation is effective for sulfonamide- antibiotics in wetlands system ($p < 0.05$). However, there was no significant difference in the removals of TMP and EFX in biotic and abiotic systems. Therefore, the plants (*Phragmites australis*) in the CWs can be important factor to sulfonamide-antibiotics removal by microbial activity.

In this study, the occurrences of antibiotics in livestock wastewater, and removals of antibiotics by the CWs for physicochemical properties were investigated. Also, lab scale experiments as sunlight photo-degradation (photolysis), biodegradation (microbial activity), and the adsorption by wetlands soil and plants were conducted to examine the removal mechanisms in the CWs.

The results showed that biodegradation (uptake into wetlands soil and plants by microorganism) and direct adsorption into soil and plants were the major removal mechanisms for sulfonamide-antibiotics in the wetlands system. TMP removal was low by the wetlands soil in higher concentration, but it seems to be difficult to remove in the CWs due to stable its functional groups. EFX removal in the CWs might be attributed by sunlight photo-degradation and slightly adsorbed into the wetlands soil and plants. Also, the physicochemical properties (e.g. molecular weight, pKa value, ionic bond and functional groups), and each general parameters (e.g. temperature, BOD, COD, TN, TP, etc.) affected the relative contribution among the possible removal mechanisms in the CWs.

This study indicates that the CWs system can be an important option for the removal antibiotics for treating the secondary livestock wastewater treatment. The results would provide the helpful information in comprehending the occurrence and removal of antibiotics in the livestock wastewater treating by the CWs.

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국문초록

인공습지로 처리한 축산폐수 내 항생제 제거효율 및 영향 인자 연구

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축산폐수는 가축분뇨에 포함된 유기물과 질소와 인 등으로 인하여 하천 호수, 하수의 오염, 축산폐수를 처리하는 과정에서 발생하는 악취 등으로 환경에 부정적인 영향을 미친다 (임성근, 2011). 이에 따라 오염부하를 줄이기 위해 추가적인 노력들이 이루어지고 있는데 이 중 하나인 인공습지는 효과적으로 오염물질을 감소시킬 수 있어 유용하다. 그러나 최근 축산폐수 처리수 내 존재하는 미량오염물질이 이슈가 되고 있으며, 이들의 검출이 지속적으로 보고되고 있어 의약물질의 잔류 수준 파악이 시급하다 (Sedlak et al. 2000).

따라서 본 연구는 금강수계로 유입되는 축산폐수 유입수 및 유출수 내 존재 가능한 항생제 농도를 정량 분석하고, 2차 처리인 인공습지에 의한 항생제의 제거효율을 평가하고자 하였다. 또한 인공습지로 인한 항생제 제거 요인으로 물리화학적 특성(분자량, pKa, 이온결합, 기능기 등)연구와 축산폐수 내 general parameters (BOD, COD, TN, TP, etc) 제거효율과의 연관성 연구, lab 실험으로 항생제의 태양광 분해, 미생물에 의한 분해, 습지 내 토양 및 식물(갈대; *Phragmites australis*)의 흡착에 의한 제거 영향을 연구하여 인공습지 내 항생제 제거 메커니즘을 파악하였다.

본 연구에서 선정한 항생제는 Sulfamethoxazole (SMZ), Sulfathiazole (SFI), Sulfamethazine (SMA), Trimethoprim (TMP), Tetracycline (TC), Oxytetracycline-HCl (OTC), Chlorotetracycline-HCl (CTC), Enrofloxacin (EFX)으로 총 8종을 Sigma-aldrich사에서 구입했다. 또한 바탕시료의 내부간섭을 보정하기 위해 내부표준물질로 $^{13}C_6$-Sulfamethoxazole과 Simaton을 이용하였다. LC-MS/MS 분석 시 사용한 컬럼은 phenomenex사의 Kinetex C-18(50×2.1mm, 2.6μm)이며, 이동상은 메탄올과 증류수를 활용하여 측정조건을 구성하였다. 시료는 분석 전 4°C에서 냉장보관하고 Membrane Filter를 이용하여 부유물질을 제거한 후 시료에 내부표준물질(ISs: internal standards)을 넣은 뒤, Oasis HLB 카트리지를 이용하여 항생제 물질을 고체상 추출법(SPE)으로 농축 후 추출하였다. 항생제를 검출하기 위해 사용된 분석기기는 LC-MS/MS로 농도를 정량 분석하였다.

인공습지 처리 후 축산폐수 유출수 내 항생제 농도는 CTC, SFI, SMA, SMZ, TMP, OTC, EFX 순으로 47 에서 6,834 μg/L 까지 범위였으며, 인공습지로 처리한 축산폐수 내 항생제 제거효율은 0 ~ 85%로 물질 별 다양한 차이를 나타내었다. 항생제 중 작은 분자량이면서 높은 pKa(> 7)로 양이온의 성질을 띄는 SFI 와 SMA 는 토양의 이온결합으로 높은 제거효율을 보였지만, TC, OTC, CTC, EFX 은 큰 분자량, 낮은 pKa(< 7) 등으로 잘 제거되지 않았다. 항생제 제거효율의 SAS 상관분석 결과 설파계 (SMZ, SFI, SMA)와 테트라사이클린계 (TC, OTC, CTC)의 제거효율은 서로 역상관 관계로 둘의 제거효율이 상이하게 나타났다 ($p < 0.0493$, $r^2 = 0.7735$).

인공습지로 인한 항생제 제거 요인을 알아보기 위한 microcosm 실험 중 토양 흡착 실험을 항생제 농도 20, 50, 100 ng/L 조건에 멸균 유/무로 나누어 시행하였다. 그 결과, EFX을 제외한 나머지 항생제들은 100ng/L 농도에서 멸균한 토양보다 멸균하지 않은 토양에서 제거효율이 높은 것으로 관찰되었다 ($p < 0.05$). 따라서, 토양에 의한 항생제 제거는 항생제의 pKa에 의한 흡착보다는, 토양 미생물에 의한 분해와 토양 입자로의 흡착이 주로 작용한 것으로 판단되며, 토양에 의한 제거효율은 항생제의 농도가 높을수록 더욱 영향을 미쳤을 것으로 보인다.

또한 항생제의 태양광 분해 실험 결과, 평균적으로 EFX가 70%, SMZ 23%, SFI 44%, SMA 28%의 제거효율을 보여, 태양광에 의한 항생제 분해는 항생제 중 EFX가 주로 영향을 받은 것으로 사료된다.

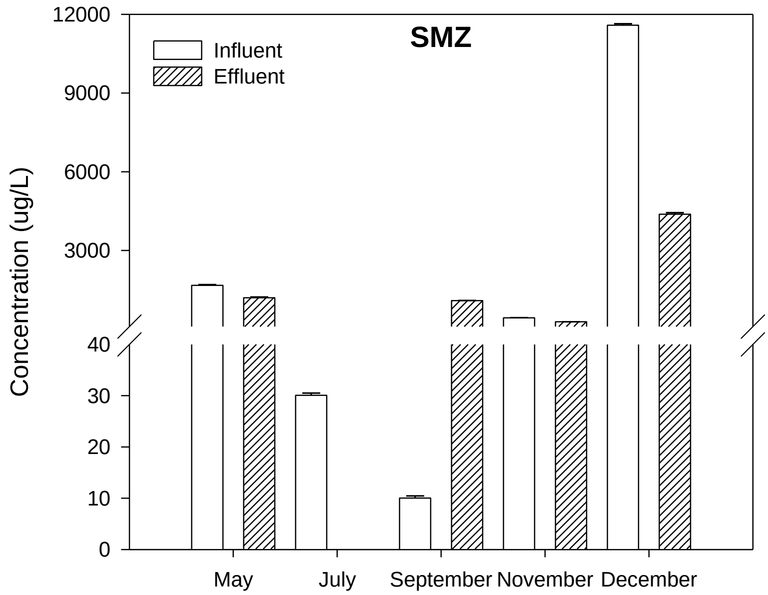
마지막으로, 인공습지 내 식물 흡착 영향을 알아보기 위해 갈대의 항생제 microcosm 흡착 실험을 한 결과, 평균적으로 SMZ 71.40%, SFI 54.02%, SMA 62.80%의 제거효율로 인공습지 내 갈대에 의한 흡착반응이 설파계열의 제거에 주로 작용한 것으로 간주된다. 미생물에 의한 영향을 알아보기 위해 멸균한 갈대로 실험한 결과, 평균 SMZ 3.30%, SFI 25.01%, SMA 50.10%의 제거로 설파계열의 인공습지 내 항생제 제거는 식물 내 미생물 분해 활동이 주된 요소로 작용한 것으로 사료된다 ($p < 0.05$).

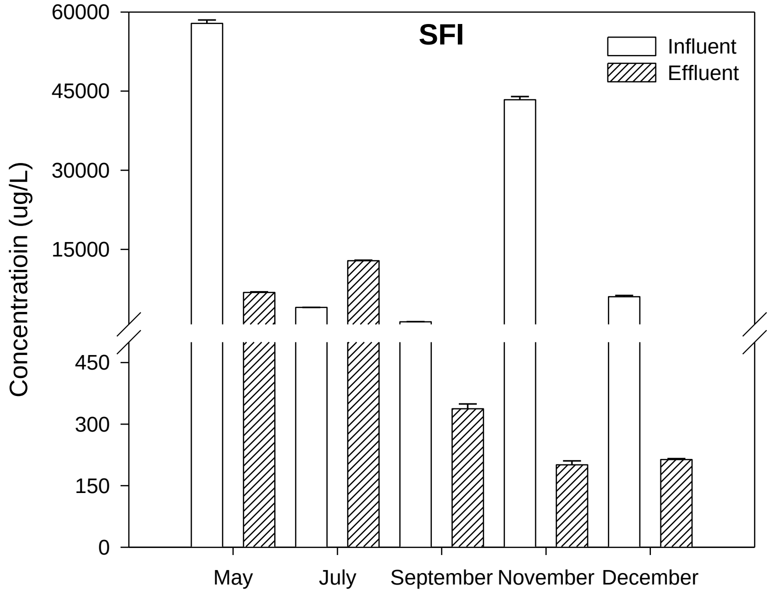
본 결과는 인공습지로 처리한 축산폐수 내 잔류한 항생제 농도를 모니터링 하여, 축산폐수 내 항생제의 제거요인으로 물리화학적 특성 연구와 lab 실험으로 태양광 분해 연구, microcosm 토양 및 식물 흡착 연구로 인공습지의 항생제 제거 메커니즘 및 영향 인자 별 제거 효율성을 제시하여 추후 항생제 제거를 위한 인공습지 설계 및 관련 미량오염물질 관리 방안 연구에 기여할 수 있을 것으로 기대된다.

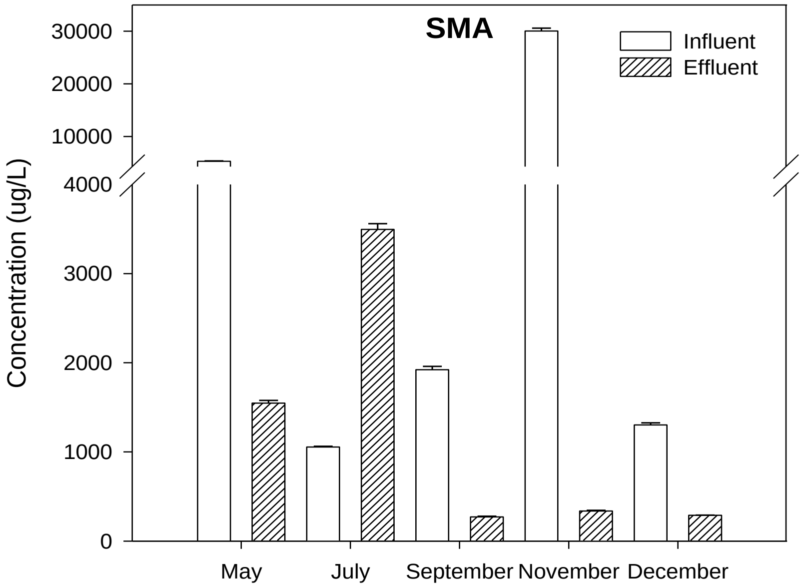
주요어 : 항생제, 인공습지, pKa, 태양광 분해, 토양, 식물

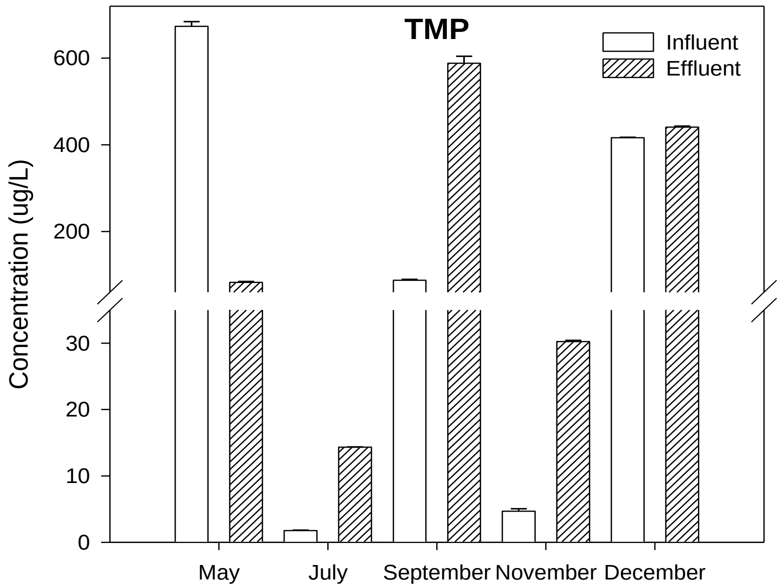
학번 : 2012-21850

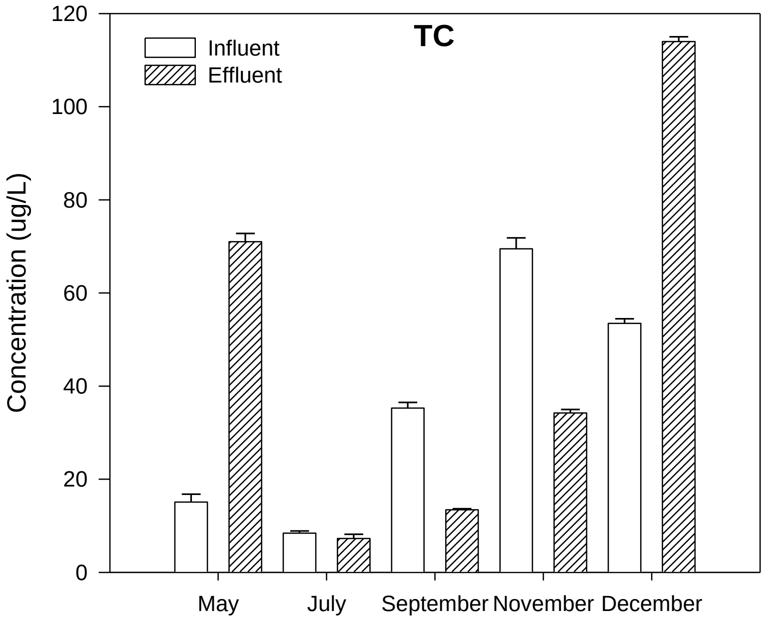
Appendix

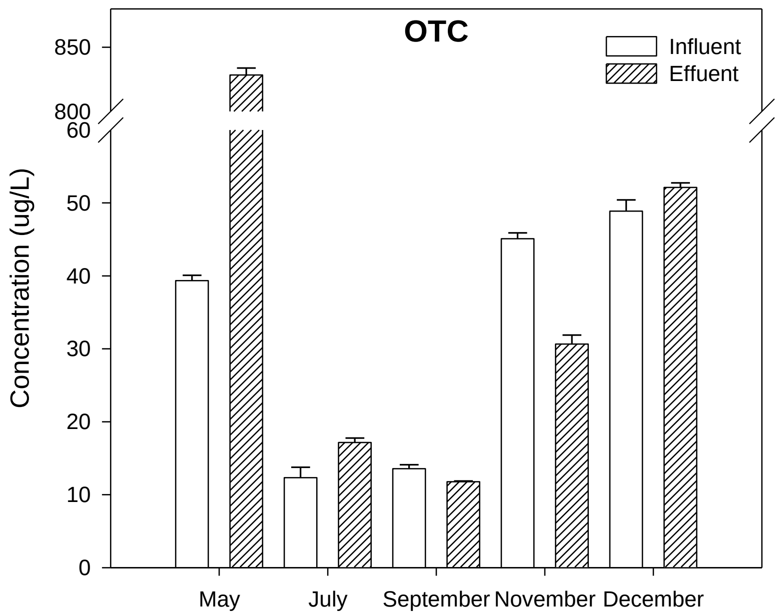

SMZ
Influent
Effluent
Concentration (ug/L)
12000
9000
6000
3000
40
30
20
10
0
May
July
September
November
December

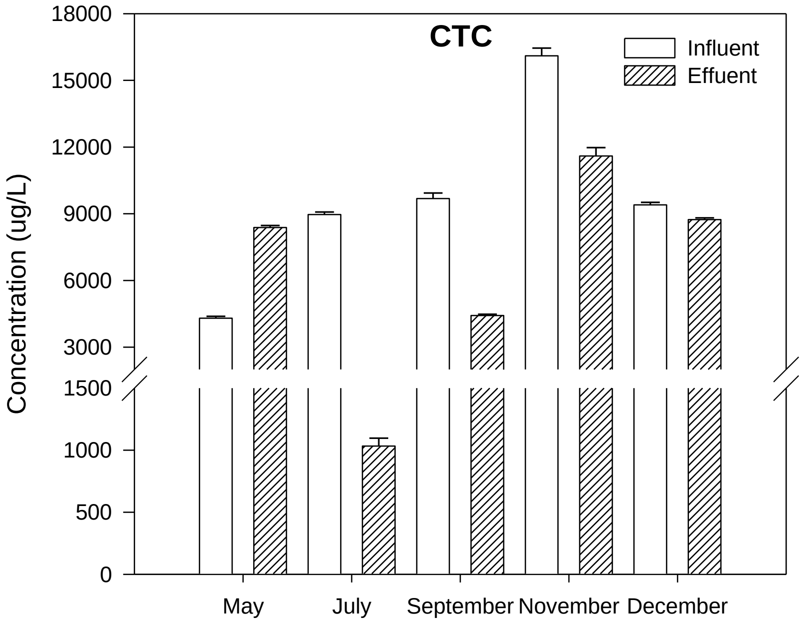

SFI
Influent
Effluent
Concentratioin (ug/L)
60000
45000
30000
15000
450
300
150
0
May
July
September
November
December

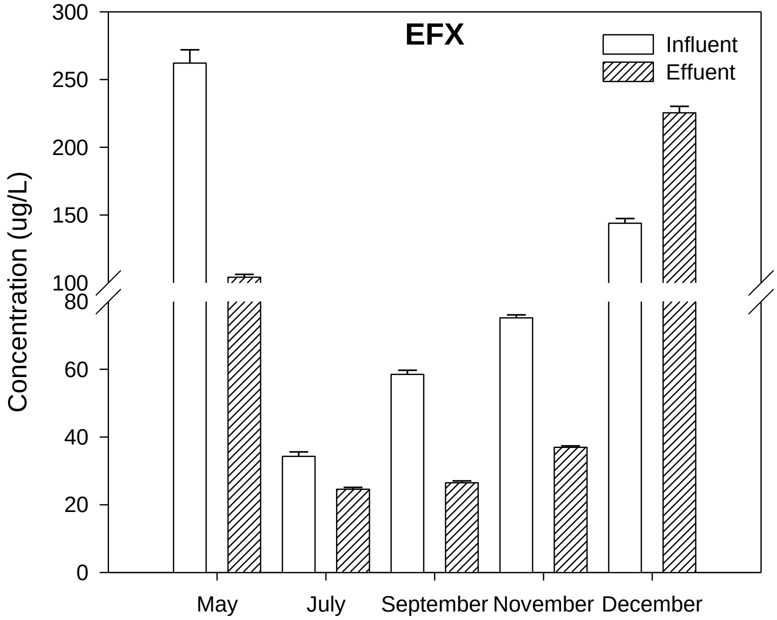

SMA
Influent
Effluent
Concentration (ug/L)
30000
20000
10000
4000
3000
2000
1000
0
May
July
September
November
December

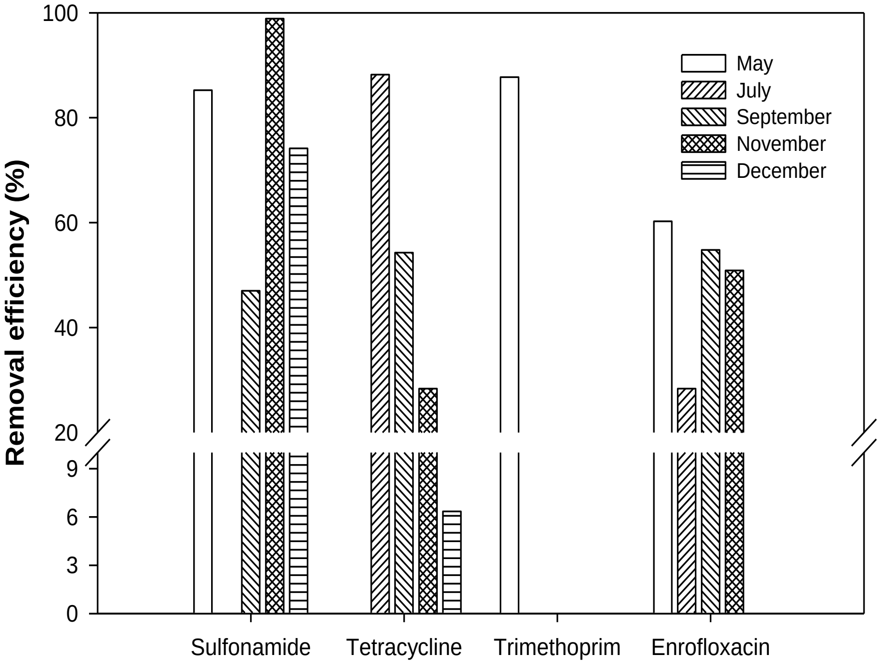

TMP
Influent
Effluent
Concentration (ug/L)
600
400
200
30
20
10
0
May
July
September
November
December


TC
Influent
Effluent
Concentration (ug/L)
120
100
80
60
40
20
0
May
July
September
November
December


OTC
Influent
Effuent
Concentration (ug/L)
850
800
60
50
40
30
20
10
0
May
July
September
November
December


CTC
Influent
Effluent
Concentration (ug/L)
18000
15000
12000
9000
6000
3000
1500
1000
500
0
May
July
September
November
December


EFX
Influent
Effluent
Concentration (ug/L)
300
250
200
150
100
80
60
40
20
0
May
July
September
November
December


Removal efficiency (%)
100
80
60
40
20
9
6
3
0
May
July
September
November
December
Sulfonamide
Tetracycline
Trimethoprim
Enrofloxacin