ENVIRONMENTAL SURVEILLANCE OF PHARMACEUTICALS AND SORPTION TO CLAY MINERALS

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ABSTRACT

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Pharmaceuticals are commonly used to animals and humans for disease control and treatment. Large fractions of administered pharmaceuticals are released into the environment via land application of animal manures and biosolids from sewage sludge of wastewater treatment plants (WWTPs). In this study, we investigated the occurrence and fate of amprolium, carbadox, monensin and tylosin in an agricultural farm. Amprolium and monensin were frequently detected in surface runoff with concentrations at nanograms per liter of water, whereas tylosin or carbadox was rarely found. Soil is a reservoir for these veterinary pharmaceuticals. Accompanied with this study, we developed analytical methods to determine pharmaceuticals in water and biosolids. Among commonly used fifteen pharmaceuticals, fourteen pharmaceuticals were frequently detected in the collected biosolids from WWTPs. This result suggests that pharmaceuticals survive wastewater treatment process and are disseminated into ecosystems via land application. Soil is a major media for retention of pharmaceuticals in the environment, and soil mineral is a major component for sorption of pharmaceuticals. Tetracycline sorption mechanism to clays from aqueous solution was investigated. Cation exchange reaction and metal-tetracycline complexation were identified to be the major processes responsible for tetracycline sorption on minerals. Overall, this study provides useful information to evaluate occurrence and fate of pharmaceuticals in the environment.

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CHAPTER I

SELECTED VETERINARY PHARMACEUTICALS IN AGRICULTURAL WATER AND SOIL FROM LAND APPLICATION OF ANIMAL MANURE

Abstract

Veterinary pharmaceuticals are commonly administered to animals for disease control, and added into feeds at subtherapeutic levels to improve feeding efficiency. As a result of these practices, a certain fraction of the pharmaceuticals are excreted into animal manures. Land application of these manures contaminates soils with the veterinary pharmaceuticals which can subsequently lead to contamination of surface and ground waters. Information on the occurrence and fate of pharmaceuticals in soil and water is needed to assess the potential for exposure of at-risk populations and the impacts on agricultural ecosystems. In this study, we investigated the occurrence and fate of four commonly used veterinary pharmaceuticals (amprolium, carbadox, monensin and tylosin) in a farm in Michigan. Amprolium and monensin were frequently detected in nearby surface water with concentrations ranging from several to hundreds of ngL^{-1}, whereas tylosin or carbadox was rarely found. These pharmaceuticals were more frequently detected in surface runoff during non-growing season (October to April) than during growing season (May to September). Pharmaceuticals resulting from post-harvest manure application appeared to be more persistent than those from spring application. High concentrations of pharmaceuticals in soils were generally observed at the sites where the respective concentrations in surface water were also high. For monensin, the ratios of soil-sorbed to aqueous concentrations obtained from field samples were within the order of the distribution coefficients obtained from laboratory studies. These results suggest that soil is a reservoir for veterinary pharmaceuticals that can be disseminated to nearby surface water via desorption from soil, surface runoff, and soil erosion.

Introduction

Veterinary pharmaceuticals are commonly administered to animals for disease control, and added into feeds at subtherapeutic levels to improve livestock growth via enhanced feeding efficiency. Such pharmaceutical use facilitates the success of large-scale concentrated animal feeding operations (CAFOs). It was estimated that in the United States, approximately 11.2 million kilograms of antibiotics were used as nontherapeutic feed additives, corresponding to about 70% of the annual antibiotic production (Mellon et al., 2001). Large fractions (e.g., up to 95%) of animal feeding pharmaceuticals are excreted into manure either as parent compounds or as bioactive metabolites (Aga et al., 2005; Jacobsen et al., 2004; Kay et al., 2004). After a period of storage, the manure is usually land-applied for its fertilizer value. This practice introduces manure-borne pharmaceuticals into agricultural ecosystems where their fate is largely unknown (Halling-Sorensen et al., 2005; Hamscher et al., 2005; Kolpin et al., 2002; Snow et al., 2008). There is growing concern regarding the environmental fate and potential impacts of these veterinary pharmaceuticals on human and ecosystem health.

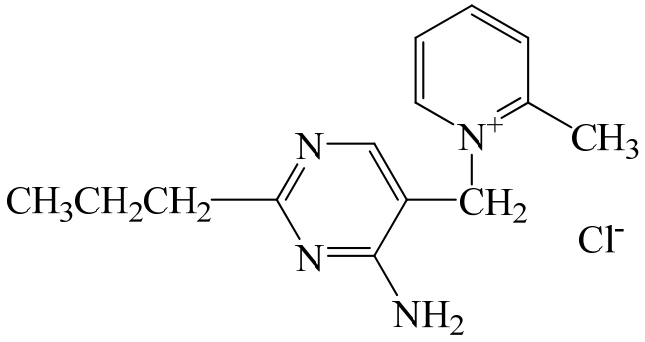
Compared to point sources such as wastewater treatment plants, non-point sources of contaminant release such as those related to agricultural practice, e.g., surface runoff from manure-treated fields, has received much less attention. Land application of animal manures can lead to veterinary pharmaceutical contamination of surface water and groundwater via surface runoff and leaching (Dolliver and Gupta, 2008; Kay et al., 2004; Kay et al., 2005; Kuchta et al., 2009; Larsbo et al., 2009). Reliable and robust analytical methods have been developed to measure the levels and frequencies of pharmaceuticals present in water and soil. This information is needed to assess adverse effects of pharmaceuticals on environmental quality and public health, and for the development of regulations to limit the release of these unregulated

bioactive compounds. Solid-phase extraction (SPE) coupled with liquid chromatography/tandem mass spectrometry (LC-MS/MS) is currently the most effective approach to identify and quantify trace levels of pharmaceuticals in environmental samples. Using this capability, several classes of veterinary pharmaceuticals, including tetracyclines, sulfonamides, macrolides, lincosamides, and ionophores, have been detected in surface water adjacent to concentrated animal feeding facilities, with the concentration ranging from low to hundreds of nanograms per liter (Brown et al., 2006; Hamscher et al., 2005; Hao et al., 2006; Kolpin et al., 2002; Song et al., 2007). The pharmaceuticals manifesting relatively weak sorptive interactions with soils, such as some of sulfonamides, have been shown to leach through soil profile and into groundwater. For example, sulfonamides have been detected in groundwater at concentrations up to 215 ng L^{-1} (Batt et al., 2006).

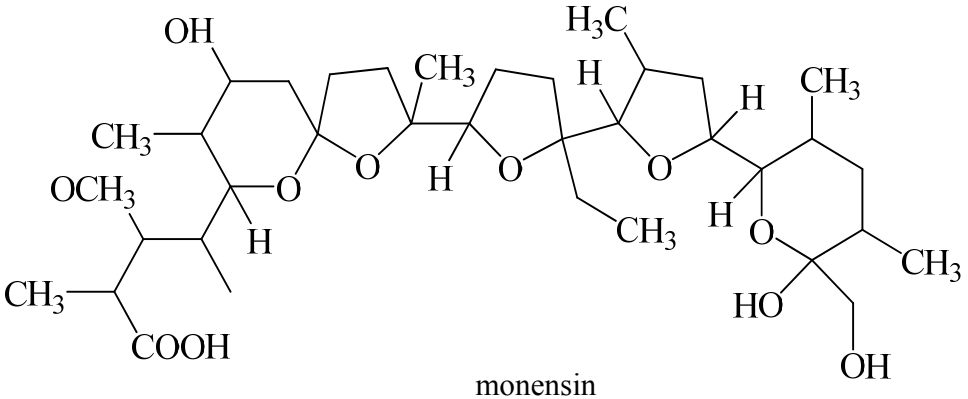
Soil is the primary reservoir for many veterinary pharmaceuticals released from land-applied livestock manures (Jacobsen et al., 2004; Kim and Carlson, 2007; Stoob et al., 2007). Pharmaceuticals commonly contain several polar and/or ionic functional groups that may engage in multiple interactions with soil components, such as by ion exchange, metal-organic complexation, and hydrogen bonding. Partitioning into soil organic matter (SOM) is, by itself, insufficient to account for pharmaceutical retention by soils, as evidenced by the wide range (up to 4 orders of magnitude) of SOM-normalized sorption data (e.g., K_{om} values) for individual pharmaceutical among soils (Tolls, 2001). In comparison, other nonionic compounds with similar lipophilic properties but lacking polar/ionic functionalities display a much narrower range of K_{om} values (Tolls, 2001). The relatively strong affinity of certain pharmaceuticals with soil reduces their availability for biotic or abiotic transformations, leading to enhanced

persistence in the environment. For example, Hamscher et al. (2002) reported up to 200 μg kg^{-1} of tetracycline in the soils fertilized with livestock liquid manures. In a two-year field study, they found that the rate of tetracycline accumulation in soils exceeded the rate of degradation suggesting that antibiotics might persist in soils over years with continuous applications of animal manures (Hamscher et al., 2002). As a result, precipitation and irrigation can disseminate manure-borne veterinary pharmaceuticals and their metabolites from soil to surface and ground waters via surface runoff or leaching.

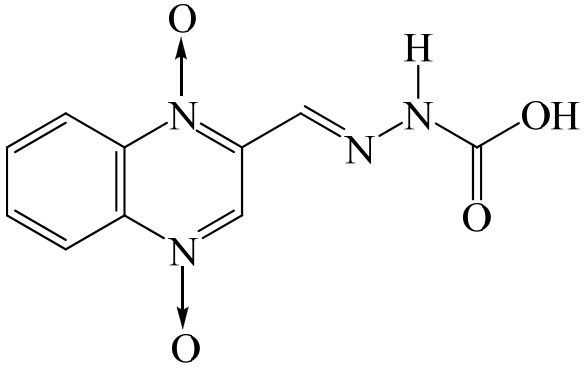
The objective of this study was to document the concentrations of selected veterinary pharmaceuticals in agricultural soil and surface runoff in an agricultural farm near Lansing, Michigan. Four veterinary pharmaceuticals, amprolium, carbadox, monensin, and tylosin were selected as target compounds, and their levels in soil and water were quantified at various time points for a period of one year. The selected pharmaceuticals had been used as livestock feed additives at the study site (farm), and encompass a range of distinct structures and physicochemical characteristics (Figure I-1). Amprolium is a cationic compound added to livestock feeds to inhibit the growth of protozoan coccidian. Carbadox is commonly used to treat dysenteries for swine and promote their growth. Monensin is a polyether ionophore widely used in dairy cows and beef cattle to protect against certain types of gram-positive bacteria. The macrolide tylosin is a broad-spectrum antibiotic commonly used to treat livestock infection and promote livestock growth. The measured concentrations of these pharmaceuticals in soil and water over the period of one year were used to evaluate their environmental fate in an agricultural setting.



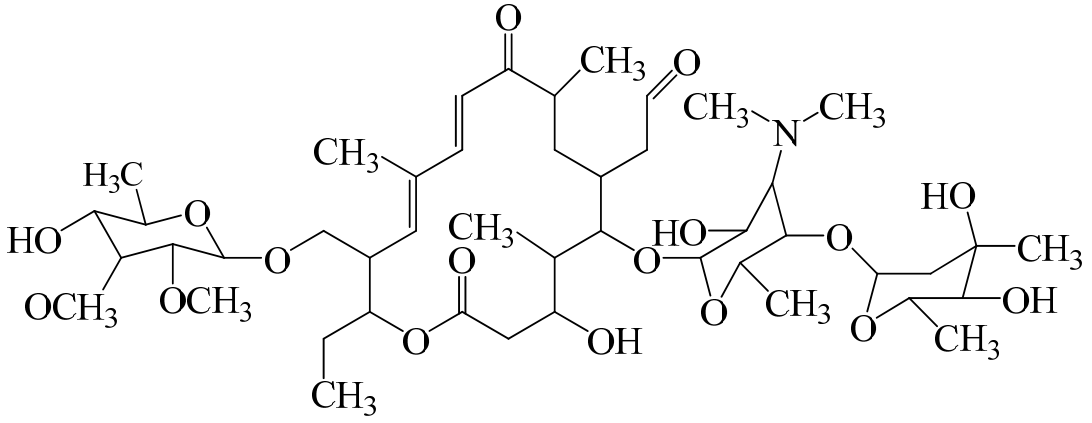
amprolium
molecular weight = 278 g/mol
log Kow = -2.5



monensin
molecular weight = 693 g/mol
pKa = 4.26
log Kow = -2.5



carbadox
molecular weight = 262 g/mol
pKa = 1.8, 10.5; log Kow = -1.37



tylosin
molecular weight = 916 g/mol
pKa = 7.39, 13.06; log Kow = 3.5

Figure I-1. Molecular structures and selected properties of amprolium, carbadox, monensin, and tylosin

Materials and Methods

Chemicals. Amprolium (5-[(2-methylpyridin-1-ium-1-yl)methyl]-2-propyl-pyrimidin-4-amine chloride), carbadox (methyl(2E)-2-[(1,4-dioxidoquinoxalin-2-yl)methylene]hydrazinecarboxylate), monensin (4-[2-[5-ethyl-5-[5-[6-hydroxy-6-(hydroxymethyl)-3,5-dimethyl-oxan-2-yl]-3-methyl-oxolan-2-yl]oxolan-2-yl]-9-hydroxy-2,8-dimethyl-1,6-dioxaspiro[4.5]dec-7-yl]-3-methoxy-2-methyl-pentanoic acid), and tylosin ([(2R,3R,4E,6E,9R,11R,12S,13S,14R)-12-{[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy}-2-ethyl-14-hydroxy-5,9,13-trimethyl-8,16-dioxo-11-(2-oxoethyl)oxacyclohexadeca-4,6-dien-3-yl]methyl 6-deoxy-2,3-di-O-methyl-β-D-allopyranoside) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Formic acid, ammonium acetate, and heptafluorobutyric acid (HFBA) were also obtained from Sigma-Aldrich Chemical Company. Methanol was of HPLC-purity purchased from J. T. Baker Chemical Company (Phillipsburg, NJ). Water was obtained from a Millipore Mill-Q (Billerica, MA) water system then filtered through a 0.22 μm membrane.

Site Description. This study was conducted in a CAFO located near Lansing, Michigan. The facility consists of swine, beef cattle, horse, sheep, dairy, and poultry farms. The farms produce approximately 1.2×10^7 L of liquid and 9.5×10^6 kg of solid manures each year. The animal manures produced are stored in the farm for a few months or composted, and then spread over 650-acre agricultural lands adjoining these farms. Surface runoff from the agricultural farm is released into the surrounding surface water. The animal manures are usually applied to the lands twice (spring and fall seasons) per year. During our study period, animal manures were applied in April, May, August, October and November 2006, and again in April and May 2007. The more intensive application of manure to the fields occurred in spring season in which 4.2 x

10^6 L of liquid and 7.5 x 10^5 kg of solid manures were applied in April and May 2006, and 4.1 x 10^6 L of liquid and 1.4 x 10^6 kg of solid manures were applied in April and May 2007. In August 2006 a relatively small amount of liquid animal manure (1.6 x 10^5 L) was applied to the cropland. The application during post-harvest season (October and November 2006) was 1.9 x 10^6 L of liquid manure.

Water Sampling and Preparation. Surface water samples were collected from the agricultural farm once per month from September 2006 to September 2007. The samples were taken at six locations from drainage tile channels exiting from the farm, and five additional locations in ditches surrounding the cropland. These samples were collected within two days after a moderate to heavy precipitation event (i.e., > 10 mm)**.** Water samples were placed in 1-L amber glass bottles, transferred to laboratory, passed through 0.4-μm glass fiber filter (Millipore Corp., Billerica, MA) to remove suspended solid particles, and stored in a refrigerator at 4 ºC. A PrepSep 12-port vacuum manifold (Fisher Scientific, Pittsburgh, PA) connected to a filtration pump was used for solid-phase extraction. A hydrophilic-lipophilic balance (HLB) cartridge (Waters Oasis, Milford, MA) (30μm, 6cc/200mg) was used for sample extraction, cleanup, and preconcentration. The cartridge was conditioned with 6 mL of methanol and 6 mL of water prior to use. Water sample (50 mL) was mixed with 0.2 mL of 1.0 M ammonium acetate, and passed through the preconditioned cartridge at a rate of 1.5 mL min^{-1}. The cartridge was washed with 5 mL of water, vacuum-dried for 3 min, and eluted with 5.0 mL of methanol containing 0.5% formic acid at a rate of ~1 mL min^{-1}. Pharmaceuticals in the eluate were analyzed using a LC-MS/MS (described below). For samples containing low levels of the target compounds, the

volume of eluate (5.0 mL) was reduced to 1.0 mL or less using a gentle N_2 flow to concentrate the samples. The quantification limits of this method were 5, 15, 0.6, and 20 ng L^{-1} for amprolium, carbadox, monensin, and tylosin, respectively. The average extraction recoveries (%) were 116 ± 20 for amprolium, 74 ± 2 for carbadox, 87 ± 12 for monensin, and 71 ± 7 for tylosin. A detailed description of the method development is presented in the supporting information.

Soil Sampling and Preparation. Soil samples were collected from the top 10 cm of soil profile in the farm, air dried in the dark, and manually homogenized using stainless steel spatulas. Five to ten grams of soil samples were weighed into 25-mL Corex glass centrifuge tubes (Thermo Scientific, Waltham, MA), mixed with 3 g of Ottawa sand (20-30 mesh) (Fisher Scientific, Pittsburgh, PA), and extracted with 15 mL of 4/1 (v/v) methanol/0.3 M ammonium acetate solution by vigorously shaking for 2 h at 200 rpm on a platform shaker. The tubes were then centrifuged at 4300 g for 20 min, and the supernatants were transferred to 15-mL Pyrex graduated glass vials. The volume of extractant was reduced to 3 mL using a nitrogen-stream evaporator apparatus. Approximately 12 mL of deionized water was added to the graduated vial to reach 15 mL, and the entire solution was then extracted via the SPE procedure described above. The average overall recoveries (%) were 53 ± 3 for amprolium, 67 ± 2 for carbadox, 116 ± 10 for monensin, and 80 ± 3 for tylosin. The quantification limits for the soil extraction were 0.035, 0.06, 0.004, and 0.09 μg kg^{-1} for amprolium, carbadox, monensin, and tylosin, respectively.

LC-MS/MS Analysis. The LC-MS/MS system used in this study was a Shimadzu HPLC (Columbia, MD) consisting of two LC-20AD pumps and an autosampler (SIL-20A) coupled with an Applied Biosystems (Foster City, CA) Sciex 3200 triple quadrupole mass spectrometer.

High-purity gases required for mass spectrometer were supplied by a gas generator (NM20ZA, PEAK Scientific, Billerica, MA). The Turbo IonSpray source of mass spectrometer was operated with electrospray ionization source in positive mode. The target pharmaceuticals were quantified using multiple reaction monitoring mode. Three pairs of precursor/product ion transitions were simultaneously monitored in order to unambiguously identify the target analytes in the environmental samples (Song et al., 2007). In this study the precursor/product ion pairs (m/z) selected were: 243/150, 243/122, 243/94 for amprolium, 263/231, 263/145, 263/130 for carbadox, 693/675, 693/479, 693/461 for monensin, and 916/174, 916/772, 916/145 for tylosin. The first pair of transition in each set was used to quantify the analyte.

A binary gradient mobile phase for the liquid chromatography system was programmed with a flow rate of 300 μL min^{-1}. Phase A (95% water and 5% methanol) and phase B (95% methanol and 5% water) both contained 2 mM ammonium acetate and 20 mM HFBA. Separation of the four analytes was achieved using a Thermo Hypersil Gold column (Thermo Scientific, Waltham, MA, 50 x 2.1 mm, particle size, 5 μm) with the following mobile phase gradient program: 0 to 2.0 min, A:B = 95:5; 2.0 to 7.0 min, phase B linearly increased to 70%; 7.0 to 7.5 min, phase B ramped to 100% and held until 10.5 min; 10.5 to 11.0 min, phase B reduced to 5% and equilibrated in the flow system until 12.0 min. The injection volume was 10 μL. After each sample injection, the first five-minute elution volume was diverted to waste through a built-in valve, then switched to the inlet of the mass spectrometer.

Sorption Isotherm Measurement. Sorption isotherms of monensin and amprolium were measured using a batch equilibration method for four soils collected from the sites nearby the farm which contained no detectable target pharmaceuticals. Selected soil properties are summarized in Table I-1. A series of initial monensin (50 to 1000 μg L^{-1}) and amprolium (100 to

2000 μg L^{-1}) solutions were prepared, then mixed with a known weight of soil placed in glass centrifuge tubes. The mixtures were equilibrated in an end-over-end shaker at 40 rpm for 24 h at room temperature (~23 ºC). Preliminary studies indicated that sorption approached equilibrium within 24 h. The tubes were then centrifuged at 4000 g for 30 min. An aliquot of supernatant was sampled and analyzed using LC-MS/MS. Experimental controls consisted of each pharmaceutical initial solution without soil. No changes of pharmaceutical concentrations were detected in the controls. Therefore, sorbed concentrations were calculated from the difference between the initial and equilibrium concentrations of pharmaceuticals in the aqueous phase.

Table I-1. Selected properties of the soils used in measurement of sorption isotherms

Soil	pH	Sand (%)	Silt (%)	Clay (%)	SOM (%)	CEC (cmol(+)/kg)
Capac A	7.3	55	24	21	5.5	15.8
Capac B	7.6	60	20	20	1.1	10.0
Colwood	6.9	64	21	15	6.3	15.3
Oshtemo	4.7	70	12	18	1.4	6.6

Results and Discussion

Water samples were collected monthly at six sites from drainage tile channels (drainage water) exiting the farm, and at five sites from the ditches (stagnant water) in close proximity to the farmland site during a one-year period (September 2006 to September 2007). Due to the snow in January and February 2007, we could not collect surface water samples. In September and December 2006 drainage water samples were not collected because of insufficient flow in the drainage channels. The total number of samples collected was 109 including 55 stagnant water samples and 54 drainage water samples (Table I-2). Among the four target

pharmaceuticals, amprolium and monensin were most frequently detected; the concentrations (without correction by extraction recoveries) are reported in Table I-2, along with the days since last manure application and precipitation during sampling intervals. The concentration ranges and the detection frequencies are presented in Figure I-2. For the stagnant water samples collected 25 out of 55 samples contained amprolium above the detectable level, with concentrations ranging from 12 to 288 ng L^{-1}, and 30 samples contained monensin at concentrations ranging from 1 to 189 ng L^{-1}. For the 54 drainage water samples, amprolium was detected in 27 samples with concentrations between 6 and 53 ng L^{-1}, and monensin was detected in 15 samples at concentrations ranging from 2 to 75 ng L^{-1}. A similar concentration range for monensin (e.g., 20-220 ng L^{-1}) was found in agricultural runoff in the Grand River watershed, Ontario, Canada (Lissemore et al., 2006).

Table I-2. Sampling time, time since last manure application, precipitation during sampling intervals, and amprolium and monensin concentrations (ng L^{-1}) in surface water

Sampling time	Days since last manure application	Precipitation (mm)	Analytes	Drainage water[a]					
				Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
September 15, 2006	15	48.0	Amprolium	--[c]	--	--	--	--	--
			Monensin	--	--	--	--	--	--
October 14, 2006	44	61.2	Amprolium	24	21	13	10	39	10
			Monensin	nd	2	nd	nd	nd	3
November 12, 2006	11	60.2	Amprolium	22	16	53	nd	25	11
			Monensin	nd	12	nd	nd	nd	nd
December 15, 2006	44	85.8	Amprolium	--	--	--	--	--	--
			Monensin	--	--	--	--	--	--
March 24, 2007	143	111.0	Amprolium	nd	nd	nd	nd	6	nd
			Monensin	nd	8	nd	nd	nd	nd
April 27, 2007	3	79.0	Amprolium	53	37	17	34	21	33
			Monensin	15	11	6	4	5	8
May 17, 2007	9	67.1	Amprolium	nd	nd	nd	nd	nd	nd
			Monensin	nd	5	nd	nd	nd	nd
June 20, 2007	37	122.9	Amprolium	nd	nd	nd	nd	nd	nd
			Monensin	nd	4	nd	nd	nd	nd
July 24, 2007	63	9.9	Amprolium	34	18	nd	nd	nd	27
			Monensin	nd	75	nd	nd	nd	nd
August 26, 2007	96	107.7	Amprolium	36	28	21	nd	nd	12
			Monensin	nd	nd	18	nd	nd	nd
September 27, 2007	128	87.9	Amprolium	41	nd	38	nd	nd	nd
			Monensin	nd	17	nd	nd	nd	nd

[a] Drainage water samples were collected from the drainage channels exiting the farm; [b] Stagnant surface water samples were collected from the ditches surrounding the farmlands; [c] not sampled due to insufficient flow in drainage channels; [d] not detected

Table I-2 (cont'd)

Sampling time	Days since last manure application	Precipitation (mm)	Analytes	Stagnant water[b]				
				Site 7	Site 8	Site 9	Site 10	Site 11
September 15, 2006	15	48.0	Amprolium	nd[d]	96	288	26	nd
			Monensin	11	10	29	nd	nd
October 14, 2006	44	61.2	Amprolium	41	82	184	95	nd
			Monensin	2	4	9	3	5
November 12, 2006	11	60.2	Amprolium	17	30	19	nd	18
			Monensin	1	nd	15	nd	2
December 15, 2006	44	85.8	Amprolium	19	34	25	93	23
			Monensin	2	3	33	1	2
March 24, 2007	143	111.0	Amprolium	nd	nd	nd	nd	nd
			Monensin	nd	nd	94	1	nd
April 27, 2007	3	79.0	Amprolium	64	57	260	33	17
			Monensin	29	18	189	10	5
May 17, 2007	9	67.1	Amprolium	nd	nd	nd	nd	nd
			Monensin	nd	nd	20	nd	nd
June 20, 2007	37	122.9	Amprolium	nd	nd	nd	nd	nd
			Monensin	nd	nd	13	nd	nd
July 24, 2007	63	9.9	Amprolium	49	12	282	71	nd
			Monensin	nd	nd	18	nd	nd
August 26, 2007	96	107.7	Amprolium	nd	nd	nd	nd	nd
			Monensin	nd	nd	13	nd	nd
September 27, 2007	128	87.9	Amprolium	nd	nd	nd	nd	nd
			Monensin	nd	5	8	8	nd

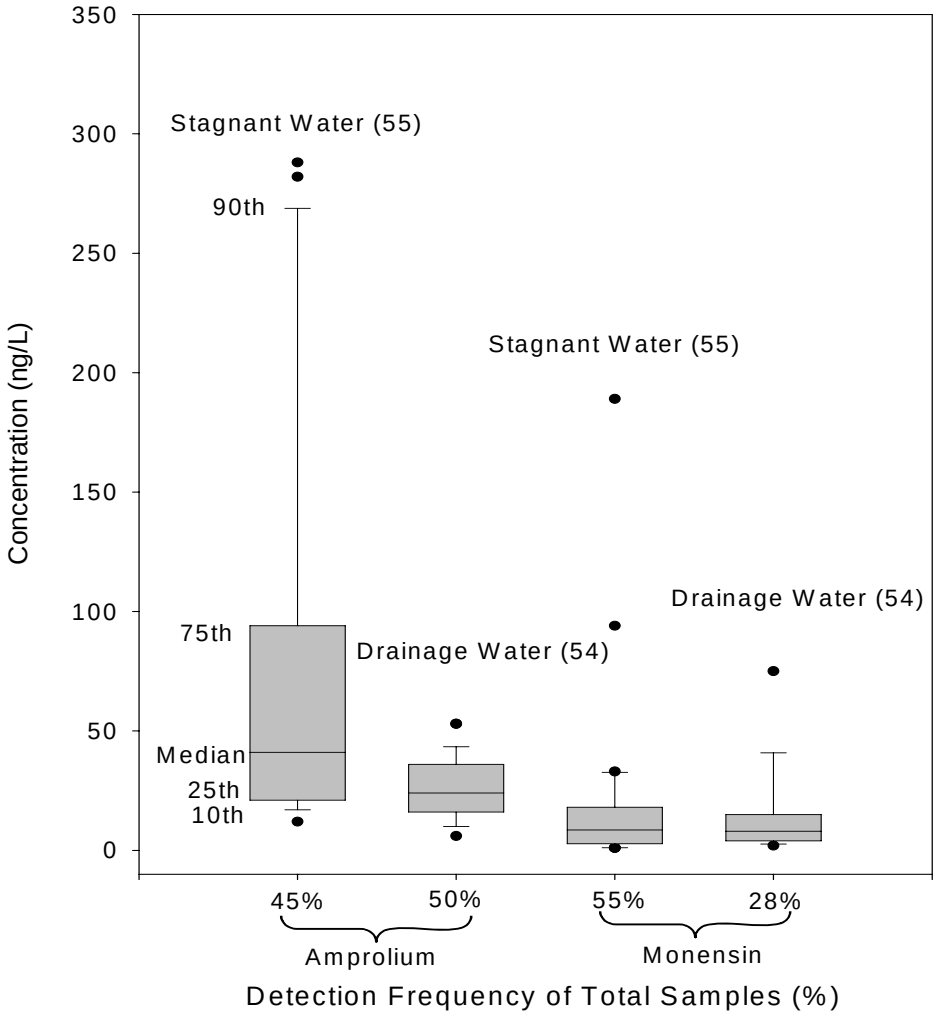


Figure I-2. Distribution of measured concentration and detection frequencies of amprolium and monensin in stagnant surface water and drainage water sampled from September 2006 to September 2007. The box-and-whisker plot represents the range, and the 10^{th}, 25^{th}, median, 75^{th}, and 90^{th} percentile of data distribution

Concentrations of both monensin and amprolium in surface water fell within a relatively narrow range for drainage water samples as compared to those in stagnant water (Figure I-2). This could plausibly be attributed to mixing during the process of surface runoff. Surface runoff from different locations of the farm converged and flowed into drainage channels. It is proposed that this mixing process reduced the variation of pharmaceutical concentrations in drainage water. The veterinary pharmaceuticals present in the stagnant water could result from surface runoff that bypassed the drainage channels, overflow from the nearby livestock waste storage lagoons, and/or desorption from soils contaminated by the pharmaceuticals.

Carbadox and tylosin were not found above detection limits in any of the drainage water samples, but were occasionally detected in stagnant water. Carbadox was detected in three samples of stagnant water at concentrations of 20, 30 and 250 ng L^{-1}, and tylosin was detected in two samples at 100 and 190 ng L^{-1}, respectively. The relatively low occurrence of tylosin and carbadox in this study is consistent with previous laboratory and field studies (Christian et al., 2003; Kay et al., 2004; Kolpin et al., 2002). Kay et al. (2004) reported that in a farm where animal manures were applied, no detectable tylosin was found in tile drainage water whereas sulfachloropyridazine and oxytetracycline were frequently detected. The relatively low occurrence of tylosin in surface water at that farm is possibly due to relatively quick abiotic and/or biotic transformations in soils. Hu and Coats (2007) reported that the dissipation half-life of tylosin in soils was approximately 7 days.

The level and frequency of pharmaceuticals present in surface runoff at the farm are influenced by the timing of animal manure application, plant growth in manure-applied soils, and weather conditions. During our sampling period, a relatively small amount of liquid animal

manure (1.6 x 10^5 L) was applied to the field in August 2006. The application during post-harvest season (October and November 2006) was 1.9 × 10^6 L of liquid manure. The more intensive application of manure to the fields occurred in spring in which 4.1 × 10^6 L of liquid and 1.4 x 10^6 kg of solid manures were applied in April and May 2007. This application schedule resulted in the highest detection frequency, and the highest concentrations of pharmaceuticals, in water samples collected in April 2007 (3 days after manure application); all these samples were found to contain monensin and amprolium. For the stagnant water samples, concentrations ranged from 17 to 260 ng L^{-1} for amprolium, and 5 to 189 ng L^{-1} for monensin. In comparison, concentrations of these pharmaceuticals in drainage water samples were lower. Presumably, convergence of surface runoff into the drainage tile system diluted the pharmaceutical concentrations in water thereby narrowing the concentrations to between 17 and 53 ng L^{-1} for amprolium, and 4 and 15 ng L^{-1} for monensin (Table I-2). The high number of detections of veterinary pharmaceuticals in the April samples is consistent with the study by Dolliver and Gupta (2008) in which the highest detection frequency of antibiotics (i.e., monensin and tylosin) in leachates from a farm in Wisconsin was also in April. A large amount of animal manure was applied in April 2007. The relatively cold weather in Michigan at this time of year likely inhibited pharmaceutical transformations by reducing microbial activity and abiotic reaction rates. Snowmelt commencing during April 2007 in Michigan increased the soil-water contact time thereby enhancing the release of soil-sorbed pharmaceuticals into water. These results suggest that manure application after harvest (i.e., in the late fall and early spring seasons)

in cold regions could lead to the dissemination of pharmaceuticals to the surrounding water during spring snowmelt.

Relatively cold temperatures could be responsible for the enhanced detection frequency of pharmaceuticals in the samples collected in October, November, and December 2006 (Table I-2). Amprolium and monensin were frequently detected in surface runoff samples taken during October to December 2007, even though a comparatively small amount of manure was applied to the field during this period. Although a much larger volume of manure was applied in April and May 2007, pharmaceuticals were only occasionally detected in surface water during the growing season (i.e. from May to September 2007). This is likely due to the enhanced biotic and abiotic pharmaceutical transformations at comparatively high temperatures during the growing season, as well as more biological activities in the plant root zones. It was noted that high concentration and frequency of amprolium were found in the water samples collected in July 2007 due possibly to the small precipitation (9.9 mm) compared to the results in other months during that period. Dolliver and Gupta (2008) reported a significant number of detections of monensin and tylosin in farm runoff and leachate in the non-growing season vs. the growing season. Taken together, these results indicate that land application of manure shortly before the growing season (e.g., early May) could result in less dissemination of veterinary pharmaceuticals to farm runoff water as compared with the application during the non-growing season.

Among all the sampling sites, water samples collected from site 9 had the highest frequency of detection, and contained relatively high levels of amprolium and monensin (Table I-2). This site had a small ditch connecting it to a manure pool. The overflow from the livestock manure pool concurrent with each rainfall event most assuredly resulted in localized high concentrations of pharmaceuticals in the stagnant surface water. In this instance, direct discharge

of animal waste to surface runoff served as a significant point source responsible for contamination of agricultural environment with veterinary pharmaceuticals.

To examine soil contamination by veterinary pharmaceuticals, 13 surface soil grab samples were collected from the farm in May and October 2006. Again, similar to the water samples, amprolium and monensin were the two pharmaceuticals most frequently detected in soils. Amprolium and monensin were found above quantification limits in 21 and 24 of the 26 soil samples, respectively. The concentration ranges were determined between 0.03 to 0.26 μg kg^{-1} for amprolium, and 0.004 to 0.50 μg kg^{-1} for monensin (Figure I-3). The average concentration of samples collected in October 2006 was 0.01 μg kg^{-1} for monensin, and 0.08 μg kg^{-1} for amprolium, which were less than the average concentrations in soils sampled in May 2006 (0.095 μg kg^{-1} for monensin and 0.11 μg kg^{-1} for amprolium). The frequent detection of veterinary pharmaceuticals in soil suggests that soil could be a large reservoir for these bioactive compounds. These contaminants could subsequently be released into water through desorption from soil into water and/or movement of soil particles containing sorbed pharmaceuticals with surface runoff. These processes lead to the dissemination of manure-borne pharmaceuticals in agricultural environment.

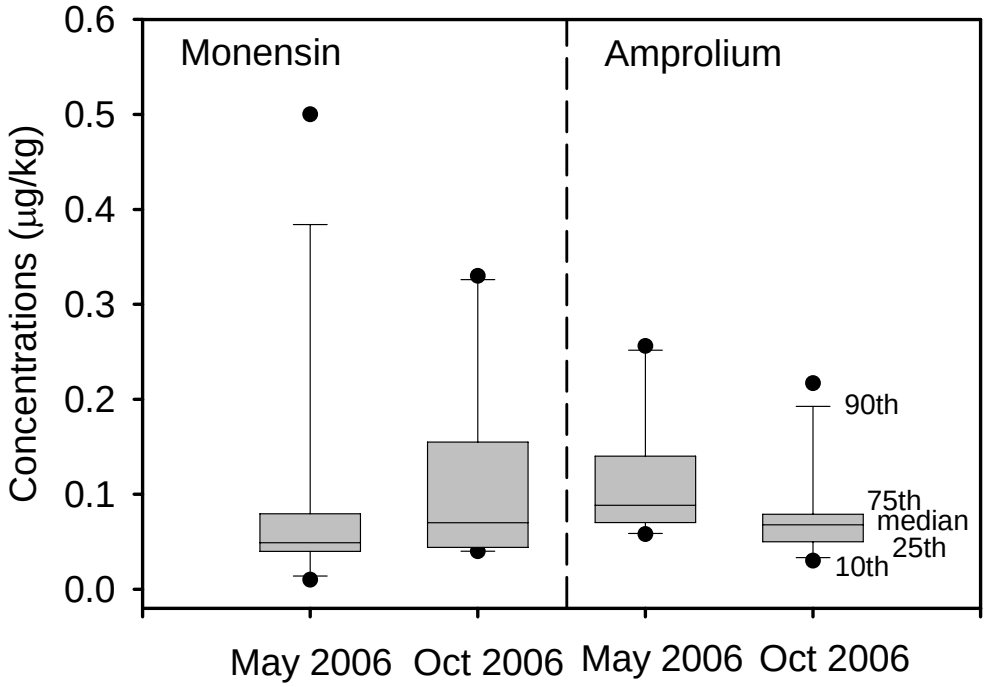


Figure I-3. Distribution of concentration of amprolium and monensin in soils sampled in May and October 2006. The box-and-whisker plot represents the range, and the 10^{th}, 25^{th}, median, 75^{th}, and 90^{th} percentile of concentration distribution

During sampling in October 2006 five soil samples were collected beneath stagnant surface water at site 7 to 11 (Table I-2). At site 11, amprolium was not detected in surface water or in the soil beneath. Monensin, however, was present in both phases. In all other samples amprolium and monensin were detected in both soil and water. The concentrations of amprolium and monensin determined in soils were plotted against their corresponding aqueous concentrations (Figures I-4A and I-4B), and it was observed that the concentrations in soil increased with increasing aqueous concentrations. The distribution coefficients, defined here as

the soil-sorbed concentration divided by the corresponding concentration in water, were ca. 1.4, 2.0, 2.8, 3.2, and 11.0 L kg^{-1} for monensin, and 0.4, 0.7, 0.8, and 2.3 L kg^{-1} for amprolium, at sites 7-11, respectively.

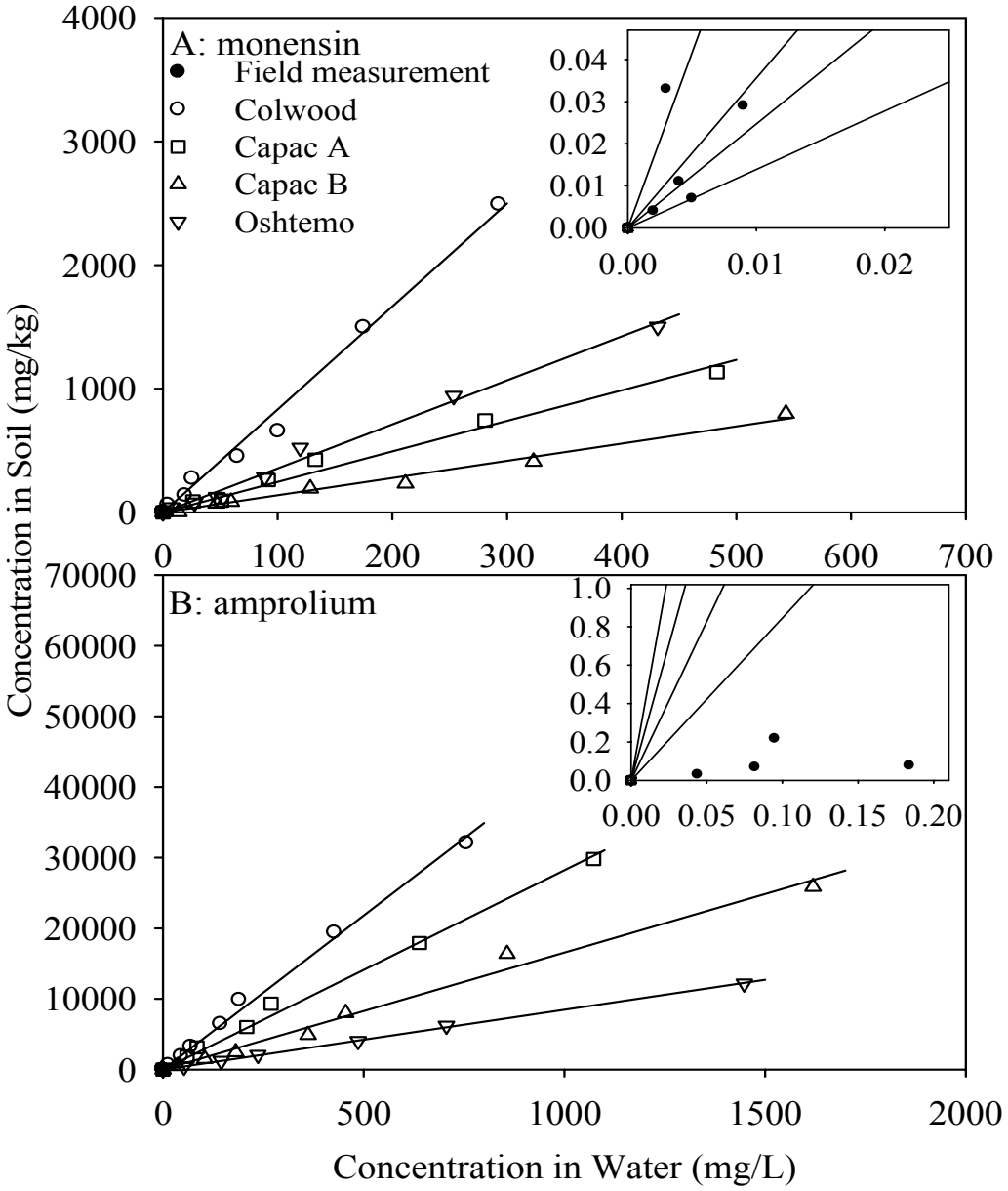


Figure I-4. Sorption isotherms of (A) monensin and (B) amprolium obtained from laboratory studies using soils adjacent to the study site which were devoid of target pharmaceuticals (open symbols). The filled symbols in the inserts represent soil-sorbed concentrations/aqueous concentrations measured for field samples. These data allow comparison of soil-water distribution of monensin and amprolium in actual field samples to that expected based on sorption measurement using soils without manure amendment

Sorption isotherms of monensin and amprolium were measured for four soils obtained from sites nearby the farm which contained no detectable levels of the target pharmaceuticals. Sorption isotherms were nearly linear for the relatively low aqueous concentrations measured, i.e., up to 600 μg L^{-1} for monensin, and 1600 μg L^{-1} for amprolium (Figure I-4). The distribution coefficients (taken from the slopes of the linear isotherms) were from 1.4 to 8.3 L kg^{-1} for monensin, and 8.4 to 43.6 L kg^{-1} for amprolium. Soil organic matter contents of the four soils ranged from 1.1 to 6.3 %. Normalization of the distribution coefficients to SOM (K_{om}) manifests greater variation in the sorption coefficients, implying that soil components other than SOM, e.g., soil minerals, also contribute to sorption. Sassman and Lee (2007) measured the sorption isotherms of monensin by several soils; the isotherms were linear with the distribution coefficients varying from 0.9 to 33.7 L kg^{-1}. Assuming the sorption isotherms remain linear down to ng L^{-1} levels, the estimated distribution coefficients for monensin from field sites are within the same order of magnitude as those obtained from the laboratory measurements (insert in Figure I-4A). This consistency in soil-water distribution of monensin among different sites and studies suggests that monensin sorbed by soils is predictably released into the surface water during rainfall events. In contrast to monensin, the distribution coefficients estimated from field data for amprolium are 4 to 100 times less than the sorption measured in laboratory (insert in Figure I-4B). This distinction can be partially attributed to the relatively low extraction recovery of amprolium from soil (i.e., 53%). However, the recovery-corrected distribution coefficients only increase by approximately two times, which is insufficient to account for the discrepancy in sorption magnitude obtained from laboratory studies vs field samples. Unlike monensin (an

organic acid with $pK_a = 4.2$), amprolium has a permanently positive charge that can engage in strong electrostatic interactions with the negatively charged sites associated with DOM. Several recent studies (Gu et al., 2007; MacKay and Canterbury, 2005; Sibley and Pedersen, 2008) have demonstrated that cationic/zwitterionic antibiotics, such as tetracyclines and clarithromycin, can be strongly associated with dissolved humic acid in aqueous solution. Soil amended with animal manure can result in a significant amount of DOM present in surface runoff (Clark et al., 1998). Hence, it is plausible to suggest that amprolium forms associations with DOM thereby reducing its sorption by soil.

Conclusions

This study indicates that land application of animal manures could lead to the accumulation of veterinary pharmaceuticals in agricultural lands. The pharmaceuticals are more frequently detected in surface runoff from the farm during non-growing season (October to April) than during growing season (May to September). Pharmaceuticals resulting from post-harvest manure application appear to be more persistent than those from spring application. Soil is a reservoir for the veterinary pharmaceuticals that can be disseminated to nearby surface water by rainfall events. Several factors, such as manure application timing and rates, weather conditions, soil types, landscape, and crop growth, are confluent to affect the fate and transport of veterinary pharmaceuticals in the agricultural environment. A better understanding of the major factor(s) controlling veterinary pharmaceuticals in surface runoff is useful for guiding the land application of livestock manures while minimizing the release of veterinary pharmaceuticals to the environment from agricultural practice. More systemic evaluations of the input/output and dissemination of veterinary pharmaceuticals, along with detailed information of agricultural production, are also needed to assess the potential risks posing to human and ecosystem health.

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CHPATER II

DETERMINATION OF PHARMACEUTICALS IN BIOSOLIDS USING ACCERLATED SOLVEN EXTRACTION AND LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

Abstract

An analytical method was developed to quantitatively determine pharmaceuticals in biosolid (treated sewage sludge) from wastewater treatment plants (WWTPs). The collected biosolid samples were initially freeze dried, and grounded to obtain relatively homogenized powders. Pharmaceuticals were extracted using accelerated solvent extraction (ASE) under the optimized conditions. The optimal operation parameters, including extraction solvent, temperature, pressure, extraction time and cycles, were identified to be acetonitrile/water mixture (v/v 7:3) as extraction solvent with 3 extraction cycles (15 minutes for each cycle) at 100 °C and 100 bars. The extracts were cleaned up using solid-phase extraction followed by determination by liquid chromatography coupled with tandem mass spectrometry. For the fifteen target pharmaceuticals commonly found in the environment, the overall method recoveries ranged from 49% to 68% for tetracyclines, 64% to 95% for sulfonamides, and 77% to 88% for other pharmaceuticals (i.e. acetaminophen, caffeine, carbamazepine, erythromycin, lincomycin and tylosin). The developed method was successfully validated and applied to the biosolid samples collected from WWTPs located in six cities in Michigan. Among the fifteen target pharmaceuticals, fourteen pharmaceuticals were detected in the collected biosolid samples. The average concentrations ranged from 2.6 μg/kg for lincomycin to 743.6 μg/kg for oxytetracycline. These results indicated that pharmaceuticals could survive wastewater treatment processes, and accumulate in sewage sludge and biosolids. Subsequent land application of the contaminated biosolids could lead to the dissemination of pharmaceuticals in soil and water environment, which poses potential threats to at-risk populations in the receiving ecosystems.

Introduction

Administration of pharmaceuticals is the most common practice for human disease control and treatments. Large fractions of administrated dosage are, in fact, not assimilated or metabolized in human body, but released into influents to municipal wastewater treatment plants (WWTPs) as either parent compounds or bioactive metabolites (Halling-Sorensen et al., 1998; Leclercq et al., 2009; Plosz et al., 2010). Unfortunately, currently operating WWTPs are not designed for effective removal of pharmaceuticals; as a result, a portion of pharmaceuticals could survive wastewater treatment processes, and disseminate to the environments via effluents from WWTPs in a dissolved form (Daughton and Ternes, 1999; Ternes, 1998) or sorbed pharmaceuticals by sewage sludge (Carballa et al., 2004). Most sewage sludge is treated to produce biosolid and is subsequently applied to agricultural lands for its fertilizer value. This practice undoubtedly introduces sludge-born pharmaceuticals into soil and water environment where their fate and risks remain largely unknown (Kolpin et al., 2002; Lapen et al., 2008; Lindberg et al., 2005; Lozano et al., 2010; Song et al., 2007; Ternes, 2001). There is growing concern regarding the potential impacts of these pharmaceuticals on at-risk populations in the ecosystems.

The information on pharmaceutical compositions and concentrations in land-applied biosolids is critical to assessing the exposure and potential risks. Analytical methods have been developed to analyze pharmaceuticals in water (Lin et al., 2007; Lindberg et al., 2004; Lindberg et al., 2005; Song et al., 2007), animal manure (Blackwell et al., 2004; Haller et al., 2002; Jacobsen and Halling-Sorensen, 2006; Prado et al., 2009) and soil (Blackwell et al., 2004; Morales-Munoz et al., 2004; Turiel et al., 2006; Vazquez-Roig et al., 2010). In several studies analytical methods have been also reported to identify and quantify pharmaceuticals in sewage

sludge from WWTPs (Aparicio et al., 2007; Fernandez-Sanjuan et al., 2009; Garcia-Valcarcel and Tadeo, 2009; Golet et al., 2002; Lillenberg et al., 2009; Lindberg et al., 2005). These methods are worthy of further examination for their applicability to multiple classes of pharmaceuticals in sewage sludge or biosolids (Diaz-Cruz et al., 2009). The procedure of the analytical methodology usually consists of extraction of pharmaceuticals from sludge/biosolid, cleanup of extracts, and analysis using liquid or gas chromatography. Liquid-solid partitioning is the most common approach utilized in combination of ultrasonic-assisted extraction (Blackwell et al., 2004), microwave-assisted extraction (Turiel et al., 2006) and accelerated solvent extraction (ASE). The extraction efficiency is dependent of sample matrices and characteristics of target analytes (Blasco et al., 2009; Navarro et al., 2009; Sanchez-Prado et al., 2010). Among these three extraction techniques, ASE becomes more preferred because it provides apparent advantages such as less solvent assumption, automatic procedure for simultaneous extraction of multiple samples, short sample preparation time, and higher extraction recoveries. Solid-phase extraction (SPE) is usually selected as the cleanup tool for the sludge/biosolid extracts, and the corresponding cartridges commonly used included Waters Oasis hydrophilic-lipophilic balance (HLB), strong-anion exchange (SAX), strong cation-exchange (SCX) or their combinations (Lillenberg et al., 2009; Nie et al., 2009; Vazquez-Roig et al., 2010). The pharmaceuticals are then analyzed using liquid chromatography with fluorescence or ultraviolet detectors (Golet et al., 2002; Raich-Montiu et al., 2007; Rebiere et al., 2007) or gas chromatography coupled to mass spectrometer (Van Hoeck et al., 2009). Recently, liquid chromatography/tandem mass spectrometry (LC-MS/MS) became the most efficient tool for identifying and quantifying trace levels of pharmaceuticals in environmental samples (Diaz-Cruz et al., 2006; Hermo et al., 2005; Nakata et al., 2005; Song et al., 2007; Ternes et al., 2005).

The objective of this study was to develop a robust, sensitive and practical analytical method to simultaneously identify and quantify multiple classes of pharmaceuticals in biosolids. The ASE experimental parameters (i.e., extraction solvent, pressure, temperature, extraction time and cycles) were optimized to achieve the maximum extraction efficiencies for pharmaceuticals. SPE was used to pre-concentrate and clean up the ASE extracts. The amounts of pharmaceuticals were quantitated by a LC-MS/MS equipped with electrospray ionization (ESI) source. The method was validated, and applied to determine multiple classes of pharmaceuticals in biosolid samples collected from several WWTPs of different locations in Michigan, USA.

Experimental

Chemicals and materials

Tetracycline, demeclocycline, chlortetracycline, oxytetracycline, meclocycline, sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole, carbamazepine, acetaminophen, caffeine, tylosin and lincomycin were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Doxycycline was obtained from Fisher Bioreagents (Pittsburgh, PA, USA). $^{13}C_6$-sulfamethazine was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Simeton was purchased from AccuStandard (New Haven, CT, USA). Methanol, sodium ethylenediaminetetraacetate (EDTA), formic acid, and sulfuric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile was purchased from EMD Chemicals (Gibbstown, NJ, USA). Waters Oasis hydrophilic-lipophilic balance (HLB) cartridge was purchased from Waters Corporation (Milford, MA, USA).

Biosolid samples

During the winter season of 2009, six grab biosolid samples were collected from WWTPs of Lansing, East Lansing, St. Clair, Plainwell, Traverse City, and Imlay City, Michigan, USA. The biosolids collected from WWTPs of Traverse City, Plainwell, St. Clair, Imlay City, and Lansing were classified as class B biosolids which meet the criteria for land application. The biosolids collected from East Lansing WWTP was sent off to landfills. At each sampling site, one liter of biosolid was collected, and stored in a polypropylene narrow-necked bottle. The bottles were capped, placed in an iced cooler, and transported immediately to laboratory. The samples were then freeze-dried to obtain dry solids, ground using a mortar and pestle, passed through a 0.5 mm sieve, and stored in a refrigerator (-20 ºC) prior to use.

Sample extraction and cleanup

The dried biosolid sample was weighed (500.0 mg) into cellulose extraction thimble, and mixed with diatomaceous earth (5.0 g) to prevent aggregation during extraction process. $^{13}C_6$-sulfamethazine and meclocycline were used as surrogates for sulfonamide and tetracycline antibiotics, respectively. The collected biosolid samples had been checked not to contain meclocycline. 100 μL of $^{13}C_6$-sulfamethazine (1.0 mg/L) and 200 μL of meclocycline (1.0 mg/L) were spiked into the biosolid samples, which were then placed on a rotator and mixed overnight. The thimbles were then placed into 22 mL stainless steel extraction cells, and extracted using a Dionex ASE 200 accelerated solvent extractor (Sunnyvale, CA, USA). The extraction solvent and operating conditions had been optimized in which acetonitrile and water mixture (v/v = 7:3) was used. The samples were preheated for 5 min, and then extracted at 100 °C under 100 bars. The static extraction period was set 15 min with three extraction cycles. The flushing solvent was 100% of cell volume, and extraction cells were purged with N_2 for 120 s. The extracts were collected and transferred to volumetric flasks, and diluted to 100 mL with deionized water. The pH value of the solution was adjusted to ~3.0 using H_2SO_4. An aliquot of 10.0 mL of the diluted solution passed through a preconditioned HLB solid-phase cartridge (Waters Corporation, Millford, MA, USA) at a rate of 1 mL/min. The analyte-loaded HLB cartridge was rinsed with 3 mL of deionized water, and eluted by 5 mL of methanol-water mixture (v/v = 1:1) containing 150 mg/L EDTA. The elute volume was reduced to 1.0 mL using a gentle N_2 flow to concentrate the samples, which took ~45 min. The prepared samples were transferred to amber LC

autosampler vials, and 10 ng of simeton (internal standard) was added prior to LC-MS/MS analysis.

Liquid chromatography and tandem mass spectrometry

The prepared samples were analyzed using a LC-MS/MS system consisting of a Shimadzu high-performance liquid chromatography (HPLC, Columbia, MD, USA) fully integrated with an Applied Biosystems Sciex 3200 triple quadrupole mass spectrometer (Foster City, CA, USA). A PEAK Scientific gas generator (NM20ZA, Billerica, MA) was used to supply gases required for the mass spectrometer. Turbo IonSpray source of the mass spectrometer was operated with electrospray ionization source in positive mode, ionspray voltage at 5500 V and temperature at 600 °C. Curtain gas pressure was 10 psi, collision gas pressure was 6 psi, and ion source gas pressure was 10 psi. The compound-dependent mass spectrometer parameters were optimized for each analyte (Table II-1). Pharmaceutical concentrations were quantified using multiple-reaction monitoring mode. Two pairs of precursor/product ion transitions were simultaneously monitored during analysis in order to unambiguously identify the target pharmaceuticals in environmental samples (Ghanem et al., 2007; Song et al., 2007). The pair of precursor and product 1 ions were selected to quantify the analytes because the product ion 1 manifested a relatively greater response abundance than product ion 2.

Table II-1. Precursor, product ions and mass spectrometer parameters used to identify and quantify pharmaceuticals

Pharmaceuticals	Precursor ion (m/z)	Product ion 1 (m/z)	Product ion 2 (m/z)	DP[a] (Volts)	EP[b] (Volts)	CEP[c] (Volts)	CE[d] (Volts)	CXP[e] (Volts)
Sulfadiazine	250.9	108.2	156.1	37	9	30	27	36
Sulfamerazine	264.9	155.7	172.4	45	4	20	22	10
Sulfamethazine	279.0	186.1	162.0	37	5	20	23	18
Sulfamethoxazole	254.1	156.2	107.8	38	8	15	22	14
Chlortetracycline	479.2	444.2	462.2	40	4	27	27	40
Demeclocycline	465.0	448.1	430.3	36	9	30	27	36
Doxycycline	445.4	428.2	339.3	33	9	50	31	45
Oxytetracycline	461.3	426.4	444.2	28	7	22	21	41
Tetracycline	445.4	428.2	339.3	33	9	50	31	45
Acetaminophen	152.0	110.0	93.0	40	10	150	23	31
Caffeine	195.0	138.0	110.0	49	5	20	29	16
Carbamazepine	237.1	194.3	165.4	50	3	14	23	18
Erythromycin	734.6	576.4	558.4	17	11	30	32	48
Lincomycin	407.3	126.3	359.2	56	5	22	33	11
Tylosin	916.5	174.0	771.9	63	9	36	47	14

[a] declustering potential. [b] entrance potential. [c] cell entrance potential. [d] collision energy. [e] collision cell exit potential

The Shimadzu HPLC system consisted of two LC-20AD pumps, a SIL-20A autosampler and a DGU-20A degasser operated by a CBM-20A controller. The analytes were separated using a Phenomenex Luna C_{18} column (150 mm × 4.6 mm, particle size: 3 μm, Torrance, CA, USA). A binary gradient mobile phase was applied with a flow rate of 350 μL/min in which phase A (water) and phase B (acetonitrile) both contained 0.1 % (v/v) formic acid. The mobile phase gradient was programmed as: at 0 min 100% phase A; 0 to 2.0 min phase B linearly increased to 12%; 2 to 20 min phase A and phase B held at the ratio of 88:12; 20 to 22 min phase B linearly increased to 25%; 22 to 35 min phase B increased to 40%; 35 to 40 min phase B increased to 80%, and held at this ratio until 42 min. From 42 to 43 min phase B reduced to 0%, and equilibrated in the flow system for 5 min before next injection. The injection volume was 10 μL. After each sample injection, the first three-minute elution was diverted to waste through a built-in valve, and then switched to the inlet of tandem mass spectrometer.

Method validation

External standard solutions were prepared in methanol-water mixture (v/v = 1/1) containing 150 mg/L EDTA. The same background solution was also used to elute analytes from the HLB cartridge during the cleanup step of sample extracts. Standard curves were established with the concentrations spanning 2 orders of magnitude. The method sensitivity was determined by analyzing pharmaceuticals at ~20 μg/L for 10 times using the ASE extracts fortified with target compounds, and standard deviation of these measurements was calculated. Limit of detection (LOD) was estimated as three times the standard deviation, and limit of quantification (LOQ) was defined as ten times the standard deviation. To evaluate extraction efficiency and repeatability of the overall method, the selected target pharmaceuticals and two surrogates (i.e.,

$^{13}C_6$-sulfamethazine and meclocycline) were spiked into biosolid samples, and mixed together overnight. Eight replicates of spiked biosolid samples were prepared followed by accelerating solvent extraction, SPE cleanup and LC-MS/MS analysis (described above). Biosolid control samples without spiked pharmaceuticals were also prepared; the amounts of pharmaceuticals measured in the control were subtracted from the amounts measured in the spiked samples. The sample matrix effects on the responses of tandem mass spectrometer was evaluated via spiking known amounts of target pharmaceuticals into the extract of biosolid which was obtained by ASE followed by the SPE cleanup. The measured pharmaceutical concentrations in the spiked samples were subtracted by the background pharmaceutical amounts originally present in the biosolids, and compared with the amounts of the spiked analytes.

Results and discussion

Selection of extraction solvent

The biosolid sample collected from Lansing WWTP was fortified with carbamazepine, sulfamethazine, sulfamethoxazole, tetracycline, chlortetracycline and oxytetracycline with concentrations ranging from 780 to 1862 μg/kg on dry matter basis, and subjected to ASE using water and organic solvent mixtures. The extracted concentrations of pharmaceuticals were subtracted by the measured concentrations of the corresponding compounds in the unfortified biosolid sample, and reported in Table II-2. Extraction solvents used here were aqueous methanol or acetonitrile mixtures with volume ratios of organic solvent to water 7:3, 5:5 and 3:7. Several previous studies indicated that polar organic solvents (e.g. acetonitrile, methanol) and water mixtures manifested the superior capability to extract pharmaceuticals from sewage sludge and soil (Hermo et al., 2005; Jacobsen and Halling-Sorensen, 2006; Lillenberg et al., 2009). Among the extraction solvents tested, it appeared that higher extraction efficiencies were achieved for the mixtures containing greater contents of organic solvent (Table II-2). Acetonitrile/water mixture (v/v = 7:3) was more effective to remove pharmaceuticals from biosolids than methanol/water mixture. Therefore, acetonitrile/water mixture (v/v = 7:3) was chosen as the extraction solvent in this study. To evaluate the influence of pH on extraction efficiency, pH of acetonitrile/water mixture (v/v = 7:3) was adjusted to ~2.0 using hydrochloric acid, and then extracted the selected pharmaceuticals from the biosolid. The use of acidified extraction solvent, in fact, lowered extraction efficiency by 36% for tetracycline, 42% for chlortetracycline, and 75% for oxytetracycline compared to unacidified acetonitrile/water mixture; no apparent discrepancy was observed for extraction of carbamazepine and sulfonamides. Several previous studies indicated that acidified water-organic solvent mixture

(e.g., pH < 2.5) enhanced extraction recoveries of pharmaceuticals, but the target pharmaceuticals were not the same as those in this study (Golet et al., 2002; Lillenberg et al., 2009). The acidification of extraction solvent protonates the acidic functional groups (e.g., carboxylic acids and phenols) in the organic fractions of sewage sludge/biosolids, and thereby reduces electrostatic interactions between sewage sludge/biosolid and cationic moiety of tetracyclines. In these previous studies, sand was used to mix with sewage sludge or soil samples prior to extraction (Crescenzi et al., 2000; Golet et al., 2002; Lillenberg et al., 2009). Diatomaceous earth, used as the dispersant of biosolid in our study, could release a certain amount of inorganic cations (e.g., Na^{+}, Mg^{2+} etc.) which could effectively suppress the interactions of biosolid with cationic speciation of tetracyclines hence resulting in higher extraction efficiency.

Table II-2. Extracted concentrations of pharmaceuticals vs. spiked concentrations from biosolid by water-organic solvent mixtures using accelerating solvent extraction[a]

Pharmaceuticals	Spiked conc. (g/kg)	Extraction solvents						
		acetonitrile /water (7:3)	acetonitrile/water (7:3), pH 2.0	acetonitrile /water (5:5)	acetonitrile /water (3:7)	methanol / water (7:3)	methanol / water (5:5)	methanol/ water (3:7)
Sulfamethazine	982	**947 ± 21**[b]	920 ± 31	551 ± 25	393 ± 11	758 ± 24	475 ± 23	368 ± 28
Sulfamethoxazole	780	**564 ± 29**	710 ± 13	494 ± 17	266 ± 28	541 ± 27	459 ± 20	339 ± 17
Chlortetracycline	1512	**786 ± 18**	456 ± 5	540 ± 17	306 ± 23	708 ± 20	525 ± 24	373 ± 22
Oxytetracycline	1862	**1008 ± 25**	261 ± 21	627 ± 24	308 ± 12	776 ± 14	655 ± 10	360 ± 10
Tetracycline	1518	**1065 ± 28**	681 ± 76	538 ± 4	282 ± 39	628 ± 36	359 ± 20	302 ± 35
Carbamazepine	840	**700 ± 32**	705 ± 53	480 ± 34	272 ± 19	437 ± 27	260 ± 18	214 ± 15

[a] Operation conditions: 100 bars, 100 °C, 3 × 15 min. [b] measured concentration: mean ± standard deviation (n = 3)

Optimization of ASE operating parameters

High pressure is applied during ASE operation in order to keep extraction solvent in liquid state while temperature is held at or above the boiling point. The application of high pressure also facilitates the contact of extraction solvent with analytes via penetrating into the domains in biosolids where the extraction solvent could not enter under normal atmospheric pressure. In this study four levels of pressure (55, 80, 100, and 130 bars) were applied to the extraction, and the corresponding extracted concentrations are reported in Table II-3. The pharmaceutical concentrations at different extraction pressures were compared using a linear-mixed model (SAS 9.1 software, SAS Institute Inc., Cary, NC, USA). Significant level was set at $p < 0.05$ with 95% confidence. The results indicate that the extraction efficiencies at pressure of 100 bars were significantly higher than those conducted at other pressures ($p = 0.0049$). This extraction pressure falls within the similar range reported in several previous studies (Golet et al., 2002; Lillenberg et al., 2009).

Table II-3. Extraction efficiency of pharmaceuticals under varying accelerating solvent extraction parameters

Pharmaceuticals	Spiked Conc. (μg/kg)	Pressurized liquid extraction conditions							
		Pressure (bar)				Extraction time (min)			
		55	80	**100**	130	2 × 15	**3 × 15**	2 × 25	3 × 25
Sulfadiazine	466	270 ± 6	246 ± 6	**293 ± 13**	243 ± 16	192 ± 12	**264 ± 28**	193 ± 18	250 ± 27
Sulfamerazine	414	249 ± 10	222 ± 17	**289 ± 7**	242 ± 15	200 ± 28	**269 ± 22**	188 ± 23	240 ± 15
Sulfamethazine	491	437 ± 12	385 ± 11	**456 ± 11**	396 ± 26	439 ± 24	**453 ± 25**	420 ± 10	444 ± 35
Sulfamethoxazole	390	274 ± 17	256 ± 33	**279 ± 11**	264 ± 26	203 ± 34	**285 ± 27**	174 ± 12	312 ± 22
Chlortetracycline	756	413 ± 22	488 ± 14	**434 ± 29**	365 ± 20	393 ± 44	**397 ± 19**	401 ± 52	408 ± 60
Demeclocycline	507	316 ± 10	151 ± 8	**304 ± 11**	300 ± 12	193 ± 29	**311 ± 43**	193 ± 23	272 ± 51
Doxycycline	509	335 ± 7	266 ± 5	**355 ± 12**	328 ± 6	335 ± 26	**342 ± 36**	367 ± 26	298 ± 42
Oxytetracycline	931	496 ± 43	483 ± 54	**523 ± 60**	580 ± 20	457 ± 41	**491 ± 52**	438 ± 30	505 ± 21
Tetracycline	759	398 ± 52	315 ± 37	**475 ± 50**	340 ± 49	377 ± 30	**394 ± 24**	466 ± 45	432 ± 38
Acetaminophen	788	617 ± 21	579 ± 12	**761 ± 32**	724 ± 10	432 ± 30	**592 ± 35**	464 ± 34	583 ± 69
Caffeine	391	406 ± 8	397 ± 12	**309 ± 10**	308 ± 10	259 ± 24	**315 ± 12**	248 ± 24	318 ± 11
Carbamazepine	420	377 ± 29	365 ± 38	**389 ± 45**	347 ± 28	281 ± 25	**365 ± 26**	321 ± 14	349 ± 28
Erythromycin	636	231 ± 22	266 ± 28	**408 ± 28**	358 ± 26	432 ± 28	**541 ± 35**	450 ± 20	509 ± 26
Lincomycin	538	424 ± 11	480 ± 21	**449 ± 22**	385 ± 9	356 ± 15	**382 ± 10**	347 ± 15	385 ± 23
Tylosin	484	363 ± 14	359 ± 26	**365 ± 25**	277 ± 14	366 ± 22	**407 ± 54**	371 ± 33	403 ± 19

Table II-3 (cont'd)

Pharmaceuticals	Spiked Conc. (μg/kg)	Pressurized liquid extraction conditions		
		Temperature (ºC)		
		50	75	**100**
Sulfadiazine	466	300 ± 23	342 ± 18	**338 ± 24**
Sulfamerazine	414	277 ± 14	326 ± 33	**308 ± 29**
Sulfamethazine	491	380 ± 19	422 ± 26	**446 ± 32**
Sulfamethoxazole	390	243 ± 11	248 ± 15	**266 ± 24**
Chlortetracycline	756	579 ± 42	556 ± 20	**572 ± 48**
Demeclocycline	507	323 ± 19	313 ± 40	**404 ± 32**
Doxycycline	509	266 ± 24	328 ± 32	**348 ± 45**
Oxytetracycline	931	503 ± 52	614 ± 51	**604 ± 85**
Tetracycline	759	431 ± 42	456 ± 46	**481 ± 35**
Acetaminophen	788	648 ± 43	571 ± 26	**653 ± 39**
Caffeine	391	289 ± 31	299 ± 17	**333 ± 26**
Carbamazepine	420	371 ± 36	384 ± 14	**378 ± 13**
Erythromycin	636	394 ± 38	474 ± 48	**498 ± 53**
Lincomycin	538	377 ± 38	388 ± 35	**406 ± 39**
Tylosin	484	380 ± 36	396 ± 27	**428 ± 21**

Static extraction with 2 or 3 consecutive cycles was conducted at 100 °C and under 100 bars. Two periods of the static extraction time (15 vs. 25 min) were tested for extraction efficiency (Table II-3). In general, prolonging extraction time and more extraction cycles achieve greater extraction efficiency (Bjorklund et al., 1999; Popp et al., 1997). For the static time of 15 min vs. 25 min with the same numbers of extraction cycles, the analysis of the linear-fixed model results indicated no significant difference ($p = 0.897$) of extraction efficiencies. This suggests that 15 min of static period is sufficient for the analytes to approach partition equilibrium between biosolid and solvent. However, increase of consecutive extraction cycles from 2 to 3 significantly enhanced the extraction efficiencies for all pharmaceuticals tested ($p = 0.0002$). In each extraction cycle the induction of fresh solvent into the sample cell reestablishes new partition equilibrium for the analytes between the added solvent and the biosolid residues. This partitioning process drives the release of biosolid-sorbed pharmaceuticals to extraction solvent phase. Taken together, the observation of enhanced extraction efficiencies of 3 consecutive cycles (versus 2 cycles), and no apparent difference with prolonged static time (25 vs. 15 min), warrant that the optimal operative condition consists of 3 extraction cycles with 15 min static time in each cycle.

Increasing temperature in ASE operation resulted in enhanced extraction of the pharmaceuticals from biosolid (Table II-3). The extraction temperature was tested at 50, 75, and 100 °C with the pressure of 100 bars, 3 extraction cycles and static period of 15 min in each cycle. The extraction efficiencies were observed to significantly increase with increasing temperature ($p < 0.0001$). Therefore, higher temperature is preferred in the ASE operation. However, considering thermal stability of analytes, the extraction was not conducted at temperature >100 °C. In this study, the temperature of 100 °C was selected as optimal operating

condition. The increase of temperature enhances dissolution of pharmaceuticals in extraction solvent and hence increasing extraction efficiencies. At the same time, the viscosity of extraction solvent and surface tension on biosolid surfaces decrease as temperature increases, which also facilitates the solvent to access the domains where the analytes are present. In addition, the enhanced extraction efficiency could be due to accelerating mass transfer rate from sample matrices to solvent at increased temperature.

Method validation

The linear ranges of standard curves and correlation coefficients, along with method LOD and LOQ values, are reported in Table II-4. The standard curves were prepared with two order of magnitude, and demonstrated an excellent linearity with the correlation coefficients (r^2) >0.999. The described method was tested for simultaneous extraction and determination of 15 pharmaceuticals in biosolid, which manifested varying levels of LOD and LOQ. For the class of sulfonamides the LOD ranged from 0.6 μg/kg (for sulfamethazine) to 15.0 μg/kg (for sulfadiazine) on dry weight basis of biosolid. This method was less sensitive for tetracycline antibiotics as shown with the LOD ranging from 4.6 μg/kg (for doxycycline) to 146 μg/kg (for tetracycline). The overall method recoveries reported were the average of eight replicates of spiked samples ranging from 49.3% to 94.6%. The standard deviations of the recoveries (n = 8) were < 10% indicating the reported method achieved satisfactory repeatability. It was noted that tetracycline antibiotics manifested the relatively lower recoveries ranging from 49% to 68% (Table II-4). This range of recoveries fell within the low end of the recovery range (i.e., 47.2% to 125 %) reported by Jacobsen et al. (2006) in which tetracyclines were analyzed in swine manure using the similar analytical approach. However, the extraction recovery of tetracycline (53.8%)

measured in this study is significantly greater that (~27%) reported by Lillenberg et al. (2009). The overall recoveries for sulfonamides (from 63.6 % to 94.6%) are comparable with the results in several previous studies (Diaz-Cruz et al., 2006; Jacobsen and Halling-Sorensen, 2006; Lillenberg et al., 2009). For instance, sulfamethazine manifested the highest recovery (94.6%) in this study; the similar results were also reported for extraction of sulfamethazine from swine manure (84.1% to 97.8%) (Jacobsen and Halling-Sorensen, 2006), and from sewage sludge (104%) (Diaz-Cruz et al., 2006).

Table II-4. Linear range and correlation coefficient (r^2) of standard curves, method limit of detection (LOD), limit of quantification (LOQ), and recovery

Pharmaceuticals	Linear range (μg/L)	r^2	LOD (μg/kg)	LOQ (μg/kg)	Recovery (%) (n = 8)
Sulfadiazine	5.8 - 580	0.9992	15.0	50.6	63.6 ± 5.2
Sulfamerazine	5.2 - 518	0.9998	5.5	18.2	71.4 ± 5.3
Sulfamethazine	6.1 - 614	0.9999	0.6	1.9	94.6 ± 2.7
Sulfamethoxazole	4.9 - 488	0.9997	1.0	3.3	77.6 ± 8.0
Chlortetracycline	9.4 - 945	0.9995	37.2	124	49.3 ± 5.4
Demeclocycline	6.3 - 634	0.9996	25.9	86.3	55.0 ± 5.0
Doxycycline	6.4 - 636	0.9993	4.6	15.5	68.3 ± 1.1
Oxytetracycline	11.6 - 1160	0.9997	13.7	45.8	52.4 ± 5.3
Tetracycline	9.5 - 1960	0.9997	146	488	53.8 ± 8.7
Acetaminophen	9.8 - 986	0.9994	30.7	102	84.7 ± 10
Caffeine	4.9 - 488	0.9999	8.4	28.3	79.8 ± 3.8
Carbamazepine	5.2 - 525	0.9997	2.9	9.7	88.1 ± 3.1
Erythromycin	8.0 - 795	0.9993	6.6	21.8	79.0 ± 9.8
Lincomycin	6.7 - 673	0.9999	0.8	2.7	83.6 ± 9.9
Tylosin	6.0 - 605	0.9992	5.4	18.1	77.3 ± 5.4

The manifestation of relatively low recoveries (e.g. ~50%) of the overall method for a certain class of pharmaceuticals such as tetracyclines could be attributed to inefficient extraction of ASE operation, analyte loss during SPE cleanup, and/or matrix effects on mass spectrometer responses from extracts. The loss of target analytes during SPE cleanup step was < 11 % (describe below). To examine sample matrix effects on mass spectrometer responses, standard addition method was used in which the analytes were spiked into biosolid extracts after ASE treatment and SPE cleanup. The spiked pharmaceutical concentrations were tested at two levels: 10-23 μg/L and 100-232 μg/L. The ratios of measured concentrations to the corresponding spiked concentrations ranged from 0.90 to 1.15, indicating matrix effects caused a minimal impact to the overall method recovery. Therefore, the low recoveries measured in this study result primarily from inefficient extraction of pharmaceuticals from ASE procedure, particularly for the class of tetracycline antibiotics which usually demonstrate a great affinity with sorbents.

SPE cleanup

In order to evaluate the extent of pharmaceutical loss during SPE cleanup step, the target compounds were spiked into biosolid extracts after ASE treatment. The solution passed through the preconditioned HLB cartridge, followed by an elution with methanol-water mixture (v/v = 1:1) containing 150 mg/L EDTA and analyzed by LC-MS/MS. Two concentration levels (10-23 μg/L and 98-232 μg/L) of pharmaceuticals were spiked into the biosolid extracts (i.e., after ASE treatment). The measured pharmaceutical concentrations were subtracted by the amount pharmaceuticals originally present in the samples, and compared to the spiked concentrations (Table II-5). For most pharmaceuticals, the recoveries of spiked amount ranged from 86.8 to 120%, indicating that the loss of analytes during SPE cleanup step was minimal. Demeclocycline and lincomycin manifested relatively higher recovery (i.e., 155 % for demeclocycline at 12.7

μg/L, and 176% for lincomycin at 134.6 μg/L), which was attributed to the experimental variations since reasonable recoveries were obtained for these two compounds at another concentration. Therefore, the relatively low recoveries of the overall method for some analytes (e.g., tetracyclines) could be attributed to the inefficient extraction from biosolids during the ASE step.

Table II-5. Recoveries of solid-phase extraction for pharmaceuticals spiked into biosolid extracts after accelerating solvent extraction

Pharmaceuticals	Spiked concentration (µg/L)	Recovery (%) (n = 3)	Spiked concentration (µg/L)	Recovery (%) (n = 3)
Sulfadiazine	11.6	110.9 ± 9.8	116.4	102.6 ± 8.0
Sulfamerazine	10.4	86.8 ± 6.5	103.5	102.5 ± 8.3
Sulfamethazine	12.3	91.0 ± 8.5	122.8	100.9 ± 6.4
Sulfamethoxazole	9.8	115.2 ± 8.6	97.5	97.7 ± 7.1
Chlortetracycline	18.9	95.6 ± 7.5	189.0	89.1 ± 9.4
Demeclocycline	12.7	155.5 ± 16.5	126.8	118.1 ± 14.2
Doxycycline	18.9	116.9 ± 9.1	189.8	111.4 ± 11.3
Oxytetracycline	23.3	98.4 ± 6.1	232.7	121.1 ± 10.9
Tetracycline	12.7	108.5 ± 12.8	127.2	119.4 ± 9.0
Acetaminophen	19.7	102.1 ± 8.2	197.1	113.5 ± 8.9
Caffeine	9.8	103.7 ± 4.6	97.7	110.0 ± 5.2
Carbamazepine	10.5	100.5 ± 8.5	105.0	101.4 ± 8.0
Erythromycin	15.9	112.4 ± 11.1	159.0	107.3 ± 8.1
Lincomycin	13.5	129.2 ± 13.4	134.6	176.5 ± 18.9
Tylosin	12.1	98.2 ± 7.5	120.9	96.8 ± 8.8

Application to biosolid samples

Biosolid samples were collected at WWTPs of six cities (Lansing, East Lansing, St Clair, Plainwell, Traverse City, and Imlay City) in Michigan, USA. These samples were extracted using the optimized method described above. The samples were conducted in triplicates. The measured concentrations and the corresponding standard deviations (n = 3) are presented in Table II-6. Among these samples, 14 out of the 15 target pharmaceuticals were found except tylosin. The average concentrations of the pharmaceuticals manifested a relatively wide range from 2.6 to 743.6 μg/kg (Table II-6). The calculated standard deviations for all the measurements were < 17%, indicating a reasonable repeatability of the analysis. Tetracycline antibiotics were frequently detected with the concentrations ranging from 36.6 to 743.6 μg/kg. Doxycycline was detected in all 6 biosolid samples with concentrations ranging from 159.9 to 292.4 μg/kg. Relatively higher concentrations of tetracycline (281.9 μg/kg) and chlortetracycline (346.6 μg/kg) were found in the sample of Lansing WWTP compared to the samples collected from other five WWTPs. Oxytetracycline was detected in four biosolid samples from 51.9 to 743.6 μg/kg. Trace amounts of demeclocycline were found in three biosolid samples (i.e., 36.6, 98.2 and 131.2 μg/kg) from WWTPs in Plainwell, East Lansing, and St. Clair. Sulfonamides were also frequently detected, and the corresponding concentrations ranged from 4.8 (sulfamethoxazole) to 668.9 μg/kg (sulfamerazine). For other six pharmaceuticals (acetaminophen, carbamazepine, caffeine, erythromycin, lincomycin and tylosin), the measured concentrations spanned from 2.6 to 370.4 μg/kg. Caffeine and carbamazepine, both of which are solely used by humans, were detected in the biosolids from five cities except Imlay City. Interestingly, Imlay City has the least populations among the six cities; this might be the reason responsible for the least occurrence of human medicine in the biosolid of Imlay City WWPT (Peeler et al., 2006). Tylosin is commonly

used as veterinary pharmaceutical to control livestock infection and promote livestock growth. It is reasonable that no samples were found to contain tylosin in municipal WWTP biosolids. Acetaminophen was detected in three biosolid samples from Lansing, East Lansing and St. Clair with concentrations of 88.6 to 370.4 μg/kg. In fact, these three cities have more populations than Plainwell, Traverse City, and Imlay City.

Table II-6. Measured pharmaceutical concentrations and standard deviations (μg/kg dry matter) in the collected biosolid samples

Pharmaceuticals	Sampled wastewater treatment plants					
	Lansing	East Lansing	St. Clair	Plainwell	Traverse City	Imlay City
Sulfadiazine	nd[a]	nd	nd	nd	562.2 ± 61.4	nd
Sulfamerazine	112.0 ± 6.3	668.9 ± 45.5	nd	nd	nd	nd
Sulfamethazine	nd	127.8 ± 15.8	124.8 ± 11.8	nd	nd	131.8 ± 13.0
Sulfamethoxazole	4.8 ± 0.5	26.1 ± 3.0	8.8 ± 1.2	35.9 ± 4.4	nd	nd
Chlortetracycline	346.6 ± 22.1	nd	nd	nd	90.2 ± 10.0[b]	69.6 ± 7.6[b]
Demeclocycline	nd	98.2 ± 6.2	131.2 ± 11.3	36.6 ± 4.0[b]	nd	nd
Doxycycline	291.2 ± 22.0	224.3 ± 30.8	149.6 ± 11.0	292.4 ± 48.5	234.0 ± 26.8	159.9 ± 19.1
Oxytetracycline	nd	51.9 ± 4.4	nd	743.6 ± 21.7	201.4 ± 17.8	174.2 ± 4.6
Tetracycline	281.9 ± 42.3[b]	nd	nd	nd	nd	nd
Acetaminophen	370.4 ± 12.9	101.1 ± 4.7	88.6 ± 7.6[b]	nd	nd	nd
Caffeine	47.9 ± 5.2	47.1 ± 6.1	46.9 ± 3.7	75.5 ± 4.1	33.9 ± 3.7	nd
Carbamazepine	6.2 ± 1.2[b]	4.6 ± 0.5[b]	5.1 ± 0.3[b]	16.4 ± 0.9	22.3 ± 5.9	nd
Erythromycin	62.8 ± 5.8	nd	10.4 ± 0.8	16.8 ± 1.3	nd	nd
Lincomycin	nd	6.2 ± 1.1	8.7 ± 1.4	nd	6.5 ± 2.0	2.6 ± 0.2
Tylosin	nd	nd	nd	nd	nd	nd

[a] Not detected. [b] Detected but less than limit of quantification.

Conclusions

This study describes an efficient and reproducible analytical approach for simultaneous determination of multiple classes of pharmaceuticals in biosolids. The experimental steps consisted of accelerated solvent extraction of freeze-dry biosolid sample, and cleanup by solid-phase extraction followed by simultaneous analysis by liquid chromatography/tandem mass spectrometry. The extraction parameters of ASE including extraction solvent, temperature, pressure, static period and extraction cycle were optimized to achieve satisfactory extraction efficiency. Overall, this method offers less laborious work, automatic extraction procedure, less consumption of organic solvent and relatively high extraction efficiency. The method has been tested successfully for simultaneously determining multiple classes of pharmaceuticals in WWTP biosolids collected from different locations. The results also suggest that many pharmaceuticals could survive wastewater treatment processes and accumulate in solid phase (e.g., biosolids). Land application of the contaminated biosolids could result in the dissemination of pharmaceuticals in soil and water environment. Chronic exposure to low levels of antibiotics might exert selection pressure on development of antibiotic resistant bacterial strains in the environment.

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CHAPTER III

TETRACYCLINE SORPTION BY K- AND Ca-SMECTITE CLAYS FROM WATER

Abstract

Tetracyclines are a class of antimicrobials that have been extensively used to treat diseases and improve health for humans and animals. A large portion of tetracyclines used on this purpose are released into the environment, raising concerns on the potential risks to ecosystems and environmental quality. Sorption by soils is a determinant controlling transport, fate and hence exposure in the environment. Soil mineral fractions are a dominant sorptive domain to retain tetracyclines. In this study, sorption of tetracycline by K^+- and Ca^{2+}-saturated smectite clays was measured using a batch equilibration method. Sorption manifested nonlinear isotherms, and was strongly suppressed by high levels of inorganic cations (e.g., K^+ and Ca^{2+}) in solution. Sorption of tetracycline by K-, and Ca-smectite was concomitant with the release of K^+ and Ca^{2+} from the clays, indicating that cation exchange process is the primary driving force for sorption of tetracycline. In addition, tetracycline sorption to smectite concurred with consumption of protons from solution. X-ray diffraction patterns revealed that the interlayer distances of smectite clays increased as tetracycline sorption increased, and gradually approached a constant basal spacing of 17.5 to 18 Å. These results indicate that the clay-sorbed tetracyclines are intercalated in interlayer regions.

Introduction

Tetracyclines are a class of antibiotics that have been widely administered to human and veterinary treatments to maintain health and prevent diseases since they were first discovered from 1940s. In 2005, it was estimated approximately 3.8 million kilograms of tetracyclines were used to animal treatments and livestock production in the United States; the amount increased about 30% compared to that used in 2004 (Phillips, 2006). Halling-Sorensen et al. (1998) reported that 60 to 90% of tetracyclines administered to animal feeding operations could pass through the digestion system, and excreted into liquid and solid manures. Tetracyclines released from human treatments could survive residential wastewater treatment processes, and disseminate in the environment (Karthikeyan and Meyer, 2006; McArdell et al., 2003; Miao et al., 2004). The short-term lagoon storage (e.g., ~3 months) could remove certain fractions of tetracyclines in animal manures; however, the subsequent land application of animal manures for their fertilizer values results in the contamination of agricultural land and water with the residual tetracyclines (Eichhorn and Aga, 2004; Smith et al., 2004). Kolpin et al. (2002) reported that tetracycline, chlortetracycline and oxytetracycline were found in stream waters collected from 30 states in the U.S. with the detection frequency of 1.2, 2.4 and 1.2%, respectively, and the corresponding maximum measured concentrations were 0.11, 0.69 and 0.34 μg/L. More than fifty-year utilization of tetracyclines and the resultant widespread distributions in environmental media have exerted chronic and selective pressures on the development of antibiotic resistance (Davies, 1994; Witte, 1998; Zhang et al., 2009). Sorption and fate of tetracyclines in the environment is a crucial factor in assessing their exposure and risks in the ecosystems.

Tetracycline contains four fused six-carbon cyclic rings to which amide, tricarbonylmethane, diketone and dimethylammonium functional groups are attached (Figure III-

1). These functional groups are ionized in aqueous solution resulting in cation (+ 0 0), zwitterion (+ - 0), and anion (+ - - , 0 - -) species with the fraction distributions depending on solution pH values (Figure III-1). These species and formed complexes with commonly water-soluble cations (e.g., Ca^{2+} and Mg^{2+}) demonstrated varying steric configuration in aqueous solution (Othersen et al., 2006; Wessels et al., 1998). For instance, in acidic and neutral aqueous solutions dimethylamino functional group is protonated locating above the BCD ring system to reduce the steric crowding with the hydroxyl group OH associated with C12a. In alkaline aqueous phase or nonaqueous solution the dimethylamino functional group is located below the BCD ring planar structure (Gulbis and Everett, 1976; Mitscher et al., 1968; Mitscher et al., 1972). The structure of tetracycline and dissociation to form multiple species could lead to multiple interaction mechanisms with soil or soil components such as replacement with cations affiliating with surfaces, complexation with multivalent cations on surfaces, and possible partitioning into soil organic matter.

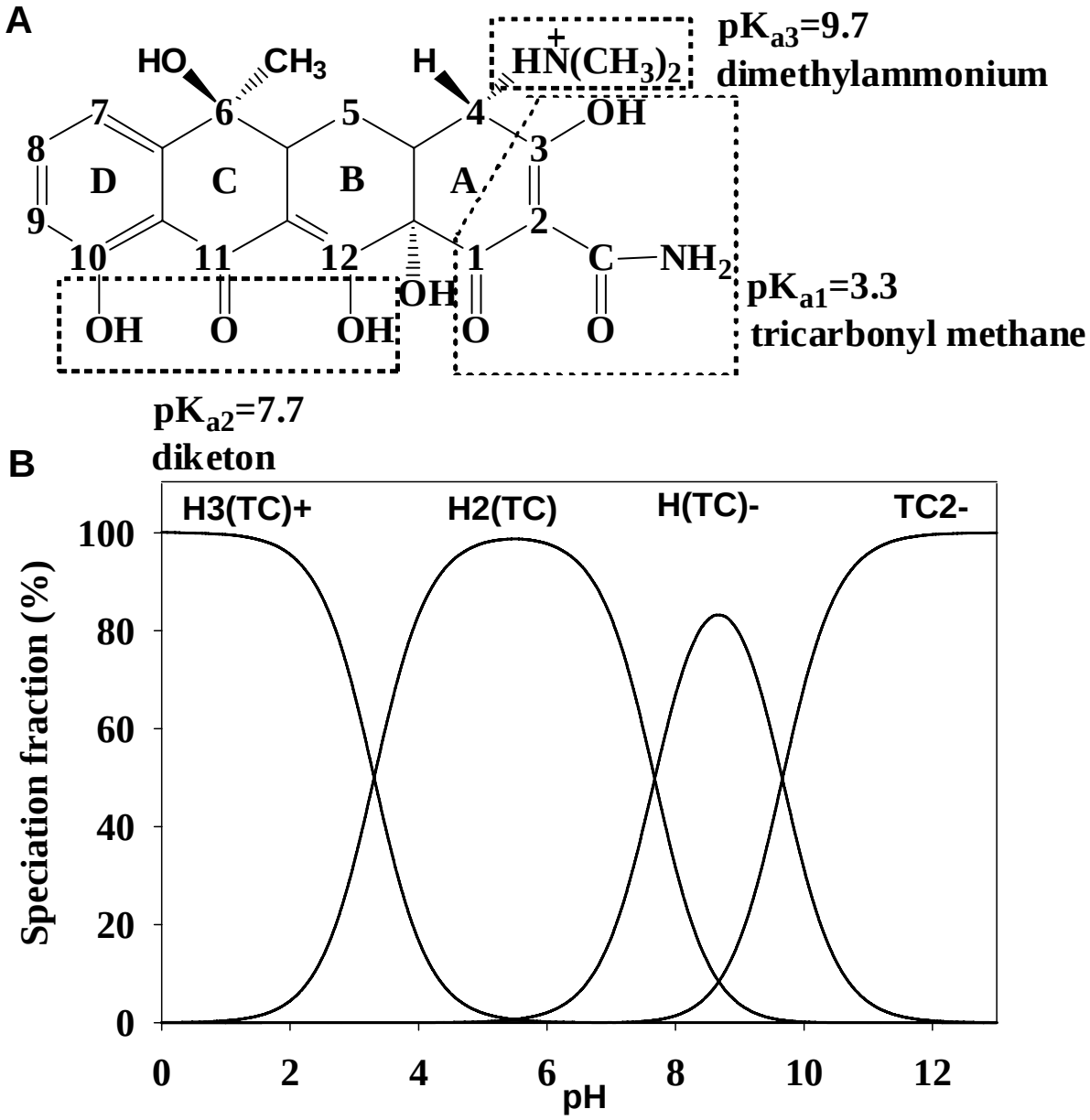

Figure III-1. (A) Tetracycline structure, and (B) speciation distribution as a function of solution pH. $H_3(TC)^+$, $H_2(TC)$, $H(TC)^-$, and TC^{2-} represent the cation, zwitterion, anion, and divalent anion of tetracycline species.

Sorption by soils/sediments is a major process influencing transport, fate and bioavailability of tetracycline in the environment. In soils both soil organic matter and clay mineral fractions are the active domains for sorption of tetracyclines from water solution (Figueroa et al., 2004; Gu et al., 2007; Kay et al., 2004; Kulshrestha et al., 2004; MacKay and Canterbury, 2005; ter Laak et al., 2006). Tetracyclines are adsorbed by clay minerals via exchanging the inorganic cations on clay surfaces and/or complexing with the exchanged multivalent cations associated with clays (Loke et al., 2002; Sithole and Guy, 1987a; Sithole and Guy, 1987b; Wessels et al., 1998). Tetracycline manifest a greater sorption to Na-montmorillonite in solution at pH 1.5 and 5.0 than that in alkaline solutions, and no apparent difference was observed for sorption at pH 1.5 and 5.0 (Kulshrestha et al., 2004). Hence, cation exchange reaction is believed to be the dominant mechanism for tetracycline sorption by soils and minerals (Figueroa et al., 2004; Kulshrestha et al., 2004; Sassman and Lee, 2005). When pH > 7, tetracycline anion is the predominant species in solution, which could complex with metal ions on clay surfaces (Aristilde et al., 2010; Figueroa and Mackay, 2005; Pils and Laird, 2007). Depending on types of ligands and ratio of metal to ligand, stoichiometric 1:1, 1:2 and 2:1 metal-tetracycline complexes could be formed in solution phase (Berthon et al., 1983; Lambs et al., 1988; Martin, 1979; Newman and Frank, 1976; Schmitt and Schneider, 2000; Wessels et al., 1998). Tetracycline can chelate with metal ions at several sites. For example, Ca^{2+} chelate with tetracycline via O12-O1 and O10-O11, whereas Mg^{2+} chelate with tetracycline via O11-O12 and O3-N4 to form Mg-tetracycline complexes (Wessels et al., 1998). Soil organic matter and aqueous dissolved organic matter are other major domains for interacting with tetracycline in the environment, and the corresponding mechanisms involve cation exchange, hydrogen bonding and metal-tetracycline complexation (Gu et al., 2007; MacKay and Canterbury, 2005; McCarthy

and Zachara, 1989; Pils and Laird, 2007; Sithole and Guy, 1987a).

The objective of this study was to improve the understandings of tetracycline sorption mechanism by smectites from aqueous phase. Cation exchange process (i.e. replacement of inorganic cations associated with clay minerals by tetracycline) is hypothesized to be the primary driving force for sorption of tetracycline by clay minerals. To validate this hypothesis, tetracycline sorption from water was measured for smectite with the negatively charged sites compensated by K^{+} and Ca^{2+}. Meanwhile, the release of exchanged inorganic cation was also determined to demonstrate the cation exchange process. In addition, molecular dynamic simulation and X-ray diffractions of tetracycline-sorbed clay films were obtained to provide further insight into the tetracycline-clay interaction mechanisms.

Experiment Section

Smectite Clays and Chemicals. A reference smectite clay (SWy-2) was obtained from the Source Clays Repository of the Clay Minerals Society. The clay has a cation exchange capacity of 800 mmol/kg, and the theoretical surface area of 800 m^2/g. The < 2 μm-sized clay fractions were collected using wet sedimentation method. The cation exchange sites on smectite surfaces were saturated with K^+ and Ca^{2+} separately by washing with 0.5 M KCl or $CaCl_2$ solution three times. The clay suspensions were subsequently washed using Milli-Q water five times to remove the excess KCl and $CaCl_2$ salts. The removal of Cl^- was determined by the negative test result of $AgNO_3$ solution. The clay suspensions were then quickly frozen, and freeze dried to receive clay powders.

Tetracycline hydrochloride (purity > 95%) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO), and was used as received. Sodium acetate, sodium hydroxide, ammonium acetate, heptafluorobutyric acid, KCl, $CaCl_2$, methanol, sodium ethylenediaminetetraacetate (EDTA), formic acid, and sulfuric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA).

Sorption Isotherm Measurements. Tetracycline sorption to K- and Ca-smectites from aqueous solution was measured using a batch equilibration method. A series of tetracycline solutions were prepared with concentrations ranging from 0 to 0.5 μmol/mL in KCl and $CaCl_2$ solutions. The background KCl and $CaCl_2$ solutions were prepared at the concentrations of 0.0, 0.01, 0.10 and 0.80 M. A certain amount of clay was weighed in glass centrifuge tubes, and mixed with a known volume of tetracycline solutions. The tubes were placed on a rotatory shaker at 40 rpm for 18 h at room temperature (~23 ºC). The preliminary study indicated that sorption

reached equilibration within 12 h. The tubes were subsequently centrifuged at 3000 g for 20 min at 23 ºC. The supernatant pH was measured using an Accumet AB15 pH meter (Fisher Scientific, Pittsburgh, PA, USA) equipped with a combination electrode. Experimental controls consisted of the initial tetracycline solutions in the absence of clay. Within the sorption equilibration period (18 h), the loss of tetracycline in these controls was < 5%. Therefore, the sorbed concentration was calculated from the difference between the concentration of initial tetracycline control solution (without clay) after 18 h and the amount remained in aqueous solution after sorption by clay at equilibration. All experimental samples were prepared in duplicate.

An aliquot of supernatant was collected and analyzed using a Perkin-Elmer high-performance liquid chromatography (HPLC, Waltham, MA, USA) for tetracycline concentrations ranging from 0.004 to 0.35 μmol/mL or a Shimadzu HPLC (Columbia, MD, USA) fully integrated with an Applied Biosystems Sciex 3200 triple quadrupole mass spectrometer (MS/MS, Foster City, CA, USA) for tetracycline concentrations from 2×10^{-4} to 0.004 μmol/mL. The Perkin-Elmer HPLC system consisted of a Series 200 pump, a Series 200 UV/vis detector, a Series 200 autosampler, and a Supelco ABZ^{+} column (15cm × 4.6mm, 5 μm). The wavelength of the UV/vis detector was 370 nm. Isocratic binary mobile phase (A:B = 40:60) was used with a flow rate of 1.0 mL/min. Mobile phase A was methanol, and phase B was a aqueous solution (pH = 6.5) containing 50mM sodium acetate, 25 mM calcium chloride, 12.5 mM EDTA and 2 M sodium hydroxide. The samples of lower tetracycline concentrations were analyzed by LC-MS/MS. A binary mobile phase was used, and consisted of phase A water and methanol (v/v = 95/5) and phase B methanol and water (v/v = 95/5) both of which contained 2 mM ammonium acetate and 20 mM heptafluorobutyric acid. A Thermo Hypersil Gold column (50 × 2.1 mm, particle size 5 μm) was used with the flow rate of 0.15 mL/min. Tetracycline concentration was

quantified using multiple reaction monitoring mode with the precursor/product transition of 445.0/410.0.

Release of Exchanged Cation. A certain amount of exchanged cation (i.e., K^+ and Ca^{2+}) associated with smectite surfaces was observed to release into solution during tetracycline sorption. The released cation concentration in aqueous solution was measured only for the experimental setting of tetracycline sorption by smectite without adding the electrolyte in the background solution. K^+ and Ca^{2+} concentrations ware measured using a Perkin-Elmer AAnalyst 400 Atomic Absorption Spectrophotometer (AAS, Branford, CT, USA). To do so, a portion of supernatant (~10 mL) was collected after centrifugation and measured for K^+ and Ca^{2+}. The measured concentrations was subtracted by the amounts of K^+ or Ca^{2+} released from K- or Ca-clays devoid of tetracycline, and normalized on the basis of clay weight. This normalized concentration is referred to as the released exchanged concentration (μmol/g) from clay.

X-ray Diffraction Measurement. After collecting the supernatant for the measurement of tetracycline concentration and the amount of released cation, the remaining supernatant was removed until ~1 to 2 mL of clay residue remained in the tubes. The remaining clay slurry was re-suspended using a vortex mixer, dropped on glass slide, and air dried to obtain oriented clay film. The prepared clay films were analyzed using a Philips APD 3720 automated X-ray diffractiometer (Philips Electronic Instrument Co., Mahwah, NJ) equipped with Cu-*Kα* radiation, an APD 3521 goniometer and a diffracted-beam monochromator. The scanning angle (2θ) ranged from 2 to 14º at step of 0.02º, and the scanning time was 2s per step.

Molecular Dynamic Simulations. The interaction of tetracycline with smectite in

interlayer was conducted using molecular dynamic simulations. A model was created for Ca-smectite (SWy-2) with the composition of $Ca_4(Al_{42}Mg_6)(Si_{95}Al_1)O_{240}(OH)_{48}Cl$ and the corresponding CEC of 790 mmol/kg. Tetracycline was loaded between clay interlayers with the amount equivalent to the concentration of 104 μmol tetracycline per gram of clay. The charge distribution of the added tetracycline was calculated using CHELPG method (Breneman and Wiberg, 1990), and structural configuration was optimized using HF/6-31G method (M. J. Frisch, 2003). Water molecules were randomly added into clay interlayers to maintain the clay basal spacing at 14.9 Å that was experimentally measured. The overall system energies were computed using force filed developed specifically for clay minerals (Teppen et al., 1997) combined with the pcff force field for tetracycline and water molecules (Maple et al., 1994). These molecular dynamics simulations were run in NPT ensemble for 0.1 ns, which is sufficient for the system to reach volume and energy equilibration.

Results and Discussion

Cation Exchange. Figure III-2 shows tetracycline sorption to K- and Ca-smectites from the solution without adding KCl or $CaCl_2$ background electrolytes, and the corresponding release of exchanged K^+ and Ca^{2+} from the minerals. It is showed that the amount of the exchanged cations released into solution from clay increased with increasing tetracycline sorption (Figure III-2). This clearly indicates that tetracycline replaces the inorganic cation associated with mineral surfaces, that is, cation exchange process: tetracycline + M^{n+}-clay ↔ tetracyline-clay + M^{n+} in which $M^{n+} = Ca^{2+}$ or K^+. For K-smectite clay, the amount of tetracycline sorption is equivalent to that of K^+ released from clay (Figure III-2A).

The similar trend of tetracycline sorption vs. the release of exchanged cation was also observed for tetracycline sorption on Ca-clay, but a relatively less amount of exchanged Ca^{2+} was released compared to that magnitude tetracycline sorption (Figure III-2B). It is unlikely to occur that sorption of two tetracycline molecules results in release of one Ca^{2+} due to the significant distinction in size for organic vs. inorganic cations. At the lower tetracycline sorbed concentrations (<350 μmol/g), the apparent distinction between the amount of tetracycline sorption vs. the release of Ca^{2+} was observed (Figure III-2B). In addition, Ca-smectite demonstrated a relatively stronger affinity with tetracycline than K-clay with the aqueous concentration <0.10 μmol/mL. For instance, Ca-smectite approached the sorption of about 200 μmol/g at aqueous tetracycline concentration of 0.01 μmol/mL, whereas K-smectite reached the same level of sorption at aqueous tetracycline concentration of 0.04 μmol/mL. Taken together, in addition to cation exchange reaction, the enhanced sorption by Ca-smectite is plausibly attributed

to formation of cation-organic complexes between tetracycline and Ca^{2+} associated with mineral surfaces (described below).

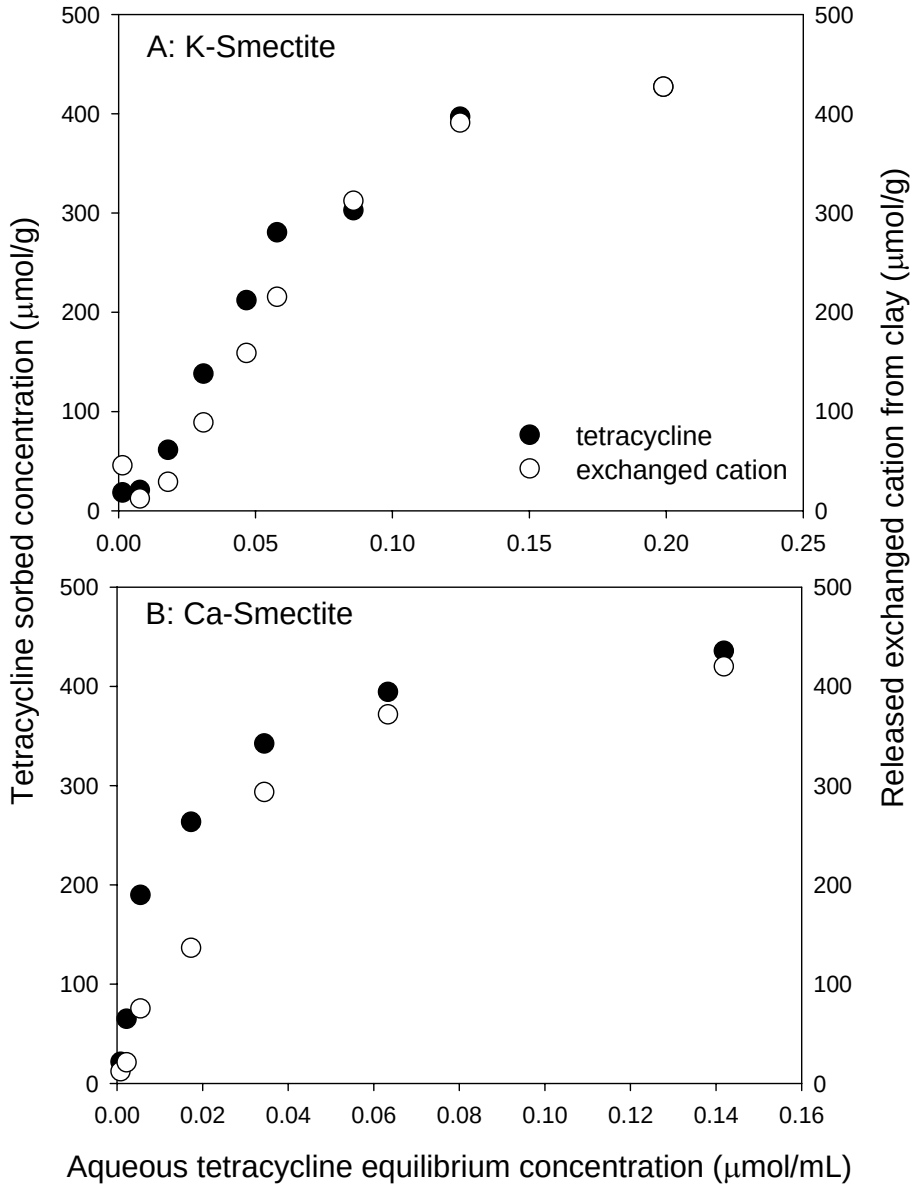

Figure III-2. Relationship between tetracycline sorption and exchanged cation released from clays.

Effects of Competitive Inorganic Cations. Figure III-3 shows tetracycline sorption isotherms to K-smectite from the aqueous KCl solution and to Ca-smectite from $CaCl_2$ solution with concentrations of 0.0, 0.01, 0.10 and 0.80 M, along with sorption from water without added electrolyte for comparison. The sorption isotherms were essentially nonliner for both K- and Ca-smectite. The presence of inorganic cations suppressed sorption of tetracycline by K- or Ca-smectite clays, and the greater suppression was observed with increasing inorganic cation concentrations in solution. For the instance of K-smectite, at aqueous tetracycline concentration of 0.20 μmol/mL tetracycline sorption reduced from 432 μmol/g in solution without the added KCl down to 263, 153 and 36 μmol/g in 0.01 M, 0.10 M and 0.80 M KCl solution, respectively. For Ca-clay similar suppressive effects was also observed; at aqueous tetracycline concentration of 0.15 μmol/mL sorption decreased from 440 μmol/g in the solution without the added $CaCl_2$ to 390, 250 and 79 μmol/g in 0.01, 0.10 and 0.80 M $CaCl_2$ solution. In the experiments conducted, the molar concentrations of added inorganic cations (i.e. K^+ and Ca^{2+}) were 50 to 80000 times the tetracycline molar concentrations in aqueous solution (Figure III-3). Such vast amount of inorganic cation present in aqueous phase effectively competed with tetracycline for the negatively charged sites on mineral surfaces, resulting in a shift of cation exchange equilibration toward less sorption of tetracycline. It is noted that Ca-smectite also manifested a greater sorption for tetracycline compared to K-smectite in the presence of the same level of $CaCl_2$ and KCl. The view of cation exchange reaction would predict that, a priori, more reduced sorption by Ca-smectite (vs. K-smectite) since divalent Ca^{2+} is less favorable for release from mineral surfaces, and is more competitive for cation exchange sites compared to monovalent K^+.

However, the fact that greater tetracycline sorption was observed for Ca-smectite relative to K-smectite suggests that the formation of Ca-tetracycline complexes on mineral surfaces enhances tetracycline sorption by Ca-smectite.

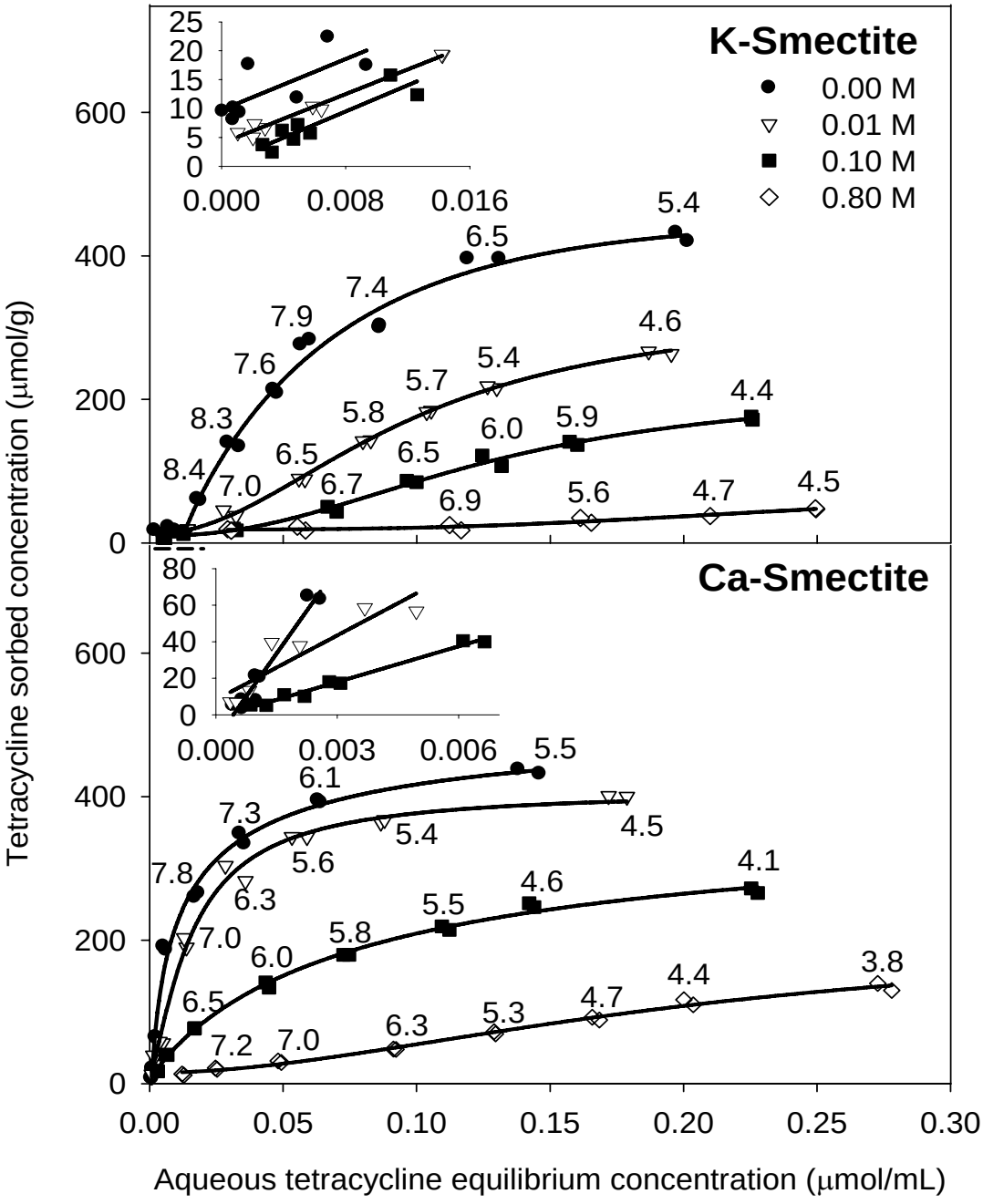

Figure III-3. Tetracycline sorption isotherms for (A) K-smectite from KCl solution and (B) Ca-smectite from $CaCl_2$ solution with the background electrolyte concentrations of 0.0, 0.01, 0.10

and 0.80 M. The pH values measured after sorption equilibrium are marked close to the corresponding sorption data.

pH Changes during Tetracycline Sorption. In this study, no buffer was added into the solution to control pH in order to exclude the possible disturbances from buffer species on tetracycline sorption. The pH values after tetracycline sorption equilibration (labeled in Figure III-3) ranged from 4.5 to 8.4 for K-smectite in which tetracycline primarily manifested zwitterion and anions. For Ca-smectite the pH values ranged from 3.8 to 7.8, and the associated species in aqueous solution were cation, zwitterion, anions and Ca-tetracycline complexes. For the initial tetracycline solution, the pH values ranged from 3.5 to 8.5 corresponding to tetracycline concentrations from 0.34 to 0.01 μmol/mL. As tetracycline was sorbed by the smectite clay, pH values increased from minimal at very lower sorption (< 20 μmol/g) to ~1.5 pH unit at higher sorption (> 250 μmol/g). We estimated the reduction of aqueous tetracycline concentration due to sorption, which is plotted against the corresponding consumption of proton concentration (Figure III-4). It is noted that increased tetracycline sorption (i.e. greater decrease of aqueous tetracycline concentration) concurred with increased consumption of protons, which is consistent with the fact of increase of pH values after tetracycline sorption.

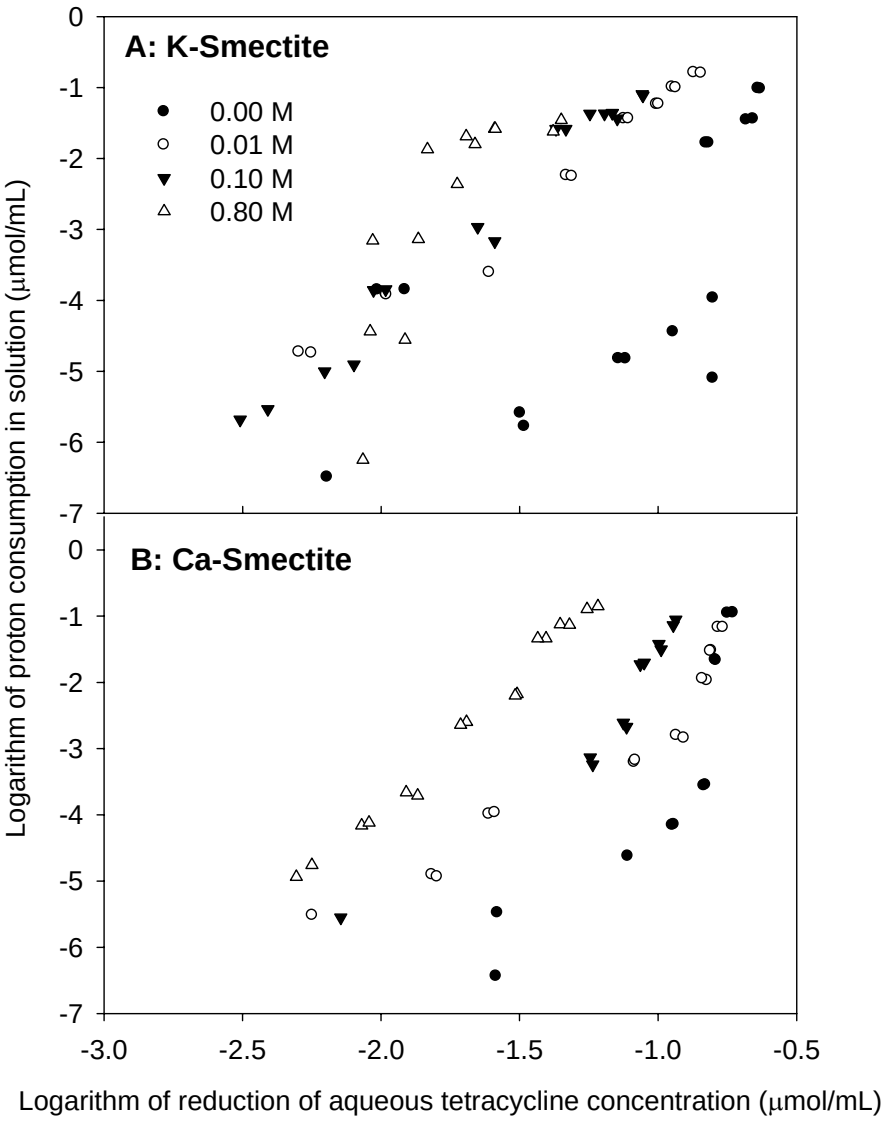

Figure III-4. Relationship between reduction of tetracycline concentration in aqueous phase due to sorption by smectite and the consumption of proton in solution. (A) Sorption by K-smectite in the presence of 0.0, 0.01, 0.10, and 0.80 M KCl, and (B) sorption by Ca-smectite in the presence of 0.0, 0.01, 0.10, and 0.80 M $CaCl_2$.

At lower pH (e.g. < 4.7), tetracycline primarily manifested cationic and zwitterionic species in solution. Cationic tetracycline sorbed onto clay surfaces in concomitant with replacement of exchangeable cations on mineral surfaces. To maintain tetracycline speciation equilibrations in solution, other species such as zwitterion and anions are protonated and shift to cationic species in solution, which consumes proton resulting in increasing solution pH. In addition, zwitterion could be also sorbed by clay through cation exchange reaction, but needs to accompany with proton to balance the negatively charged moiety in tetracycline. Sorption of both tetracycline species consume the protons in aqueous solution leading to an increasing pH. As pH continued to increase, the major species of tetracycline shifted to zwitterions and anions. For $CaCl_2$ solution, tetracycline zwitterion and anions as well as the formed Ca^{2+}-tetracycline complexes were present in the solution with the distributions of each fraction dependent of pH, tetracycline concentration and the amount of Ca^{2+}. Recall that sorption of tetracycline zwitterion via cation exchange reaction consumes the proton from solution to compensate the negatively charged functional moiety of tetracycline molecule as indicated by an increased pH (Figure III-4). Ca-tetracycline complexes usually form at relatively higher pH values in the presence of sufficient amount of $CaCl_2$. For example, at pH 7 approximately 43% of tetracycline formed complexes with Ca^{2+}, 46% and 10% of tetracycline were zwitterions and anion, respectively, in 0.01 M $CaCl_2$. As $CaCl_2$ concentration increased, Ca-tetracycline fractions increased to 95% in 0.10 M $CaCl_2$ solution, and to 99% in 0.80 M $CaCl_2$. Meanwhile, tetracycline sorption was observed to significantly decrease (Figure III-3). The formed Ca-tetracycline complexes manifested a lower affinity for mineral surfaces compared to tetracycline zwitterion due

plausibly to the larger sized metal-tetracycline complexes inhibited the intercalation in smectite interlayers.

It is also noted from Figure III-4 that the presence of KCl and $CaCl_2$ consumed more protons for tetracycline to approach similar sorption levels (i.e., similar reduction of aqueous tetracycline concentrations) compared to sorption from water. The consumed amount of proton increased with increasing electrolyte concentration. In aqueous solution, inorganic cations approximate the negatively charged functional groups of tetracycline. Protons must compete with inorganic cations (K^+ or Ca^{2+} in this study) for these negative charges to convert the species facilitated for sorption. Therefore, more protons are needed to be consumed for achieving the similar sorption levels in the solutions containing a higher electrolyte concentration (Figure III-4).

Complexation of Tetracycline with Ca^{2+} on Mineral Surfaces. Many previous studies have indicated that anionic tetracycline can complex with Ca^{2+} in aqueous phase (Schmitt and Schneider, 2000; Tongaree et al., 1999; Wessels et al., 1998), and minimally complex with monovalent cation such as K^+ (Coibion and Laszlo, 1979). To illustrate the interactions of tetracycline with Ca-smectite, molecular dynamic simulation was used with tetracycline sorption at 104 μmol per gram of Ca-clay which is within the range of our measured sorption. After the computing system reached the static state, tetracycline lay between smectite interlayer with BCD ring system parallel with siloxane surfaces (Figure III-5A). Positively charged dimethylammonium group is positioned towards tetracycline planar structure in the extended conformation (Othersen et al., 2006). Comparing with the twisted conformation in which dimethylammonium group is located above the BCD ring system, tetracycline adopts the extended conformation that could be more ready for intercalation into smectite interlayers. The

enlarged view of tetracycline and Ca^{2+} interaction is shown in Figure III-5B. The simulation results indicated that O11 and O12 of tetracycline approached to Ca^{2+} with a distance of 2.2 Å, suggesting tetracycline forms inner-sphere complexes with Ca^{2+} in smectite interlayers.

To experimentally verify the possible formation of tetracycline-metal cation complexes on mineral surfaces, we measured sorption by Cu- and Fe-smectite clays, and compared the sorption with those by K- and Ca-smectite. The results revealed that sorption by Cu- and Fe-smectite clays was much greater than that by Ca and K-clay. It is known that Cu^{2+} and Fe^{3+} could form a much stronger complexation with tetracycline than Ca^{2+} (Gu and Karthikeyan, 2005; Pei et al., 2010). The magnitude of sorption order implies that tetracycline forms complexes with Ca^{2+}, but the interaction is weaker than those with Cu^{2+} and Fe^{3+} in interlayers.

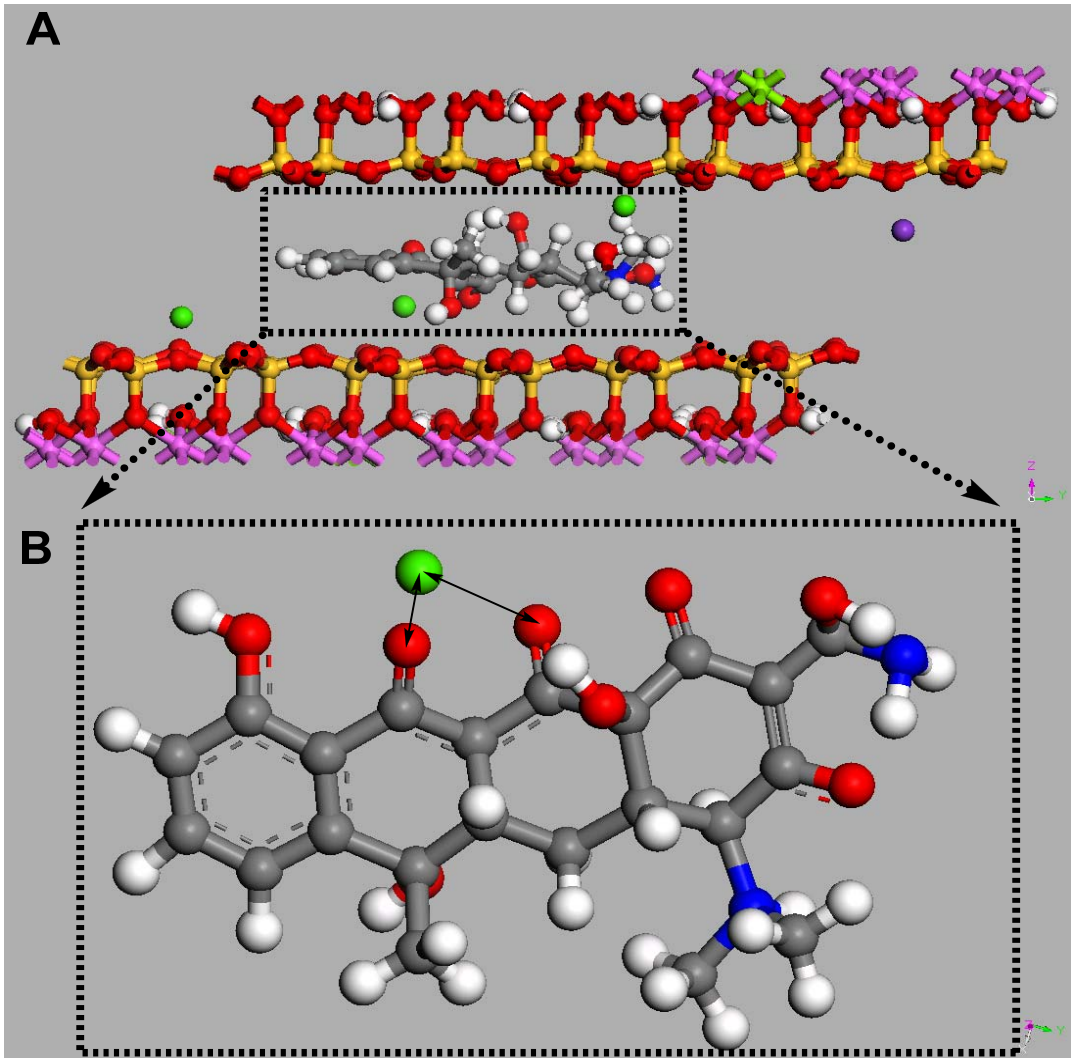

Figure III-5. Molecular dynamic simulations of tetracycline sorption by Ca-smectite. Elements are labeled with color: C (grey), H (white), N (blue), O (red), Ca (green), K (purple), Al (pink), Si (yellow). (A) Tetracycline molecule sits between smectite interlayer with BCD ring system parallel with siloxane sheet. (B) Enlarged view of tetracycline interaction with exchanged Ca^{2+} on mineral surfaces. The distance of 2.2 Å (solid arrows) between Ca and O11, O12 on tetracycline structure indicates tetracycline forms inner-sphere complex with Ca^{2+}. (For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.)

Intercalation of Tetracycline in Smectite Interlayers. X-ray diffraction pattern for K-smectite at several tetracycline sorption levels is presented in Figure III-6A. A diffraction peak was observed at ~7.9º (2θ) for K-smectites without or with low tetracycline sorption (< 56 μmol/g), indicating that smectite platelets maintain relatively parallel structures with the basal spacing of 11.2 Å. As the tetracycline loading increased, the diffraction peak at 7.9º (2θ) gradually became broader with reduced intensity indicating less ordering of K-clay layers. As the sorption reached > 218.4 μmol/g, the diffraction peak at 7.9º became very weak, whereas an additional peak at ~5.0º appeared corresponding to a basal spacing of 17 Å. The similar changes of XRD patterns were also observed for Ca-smectite with varying tetracycline loadings; the diffraction peak shifted from original 6º to 5.2º corresponding to the basal spacing expanding from 14.7 to 17.1 Å (Figure III-6B). For Cu- and Fe-clays, the basal spacings expanded to ~18 Å with tetracycline sorption > 250 μmol/g. These results clearly establish that tetracycline molecules enter interlayers when sorbed by smectite.

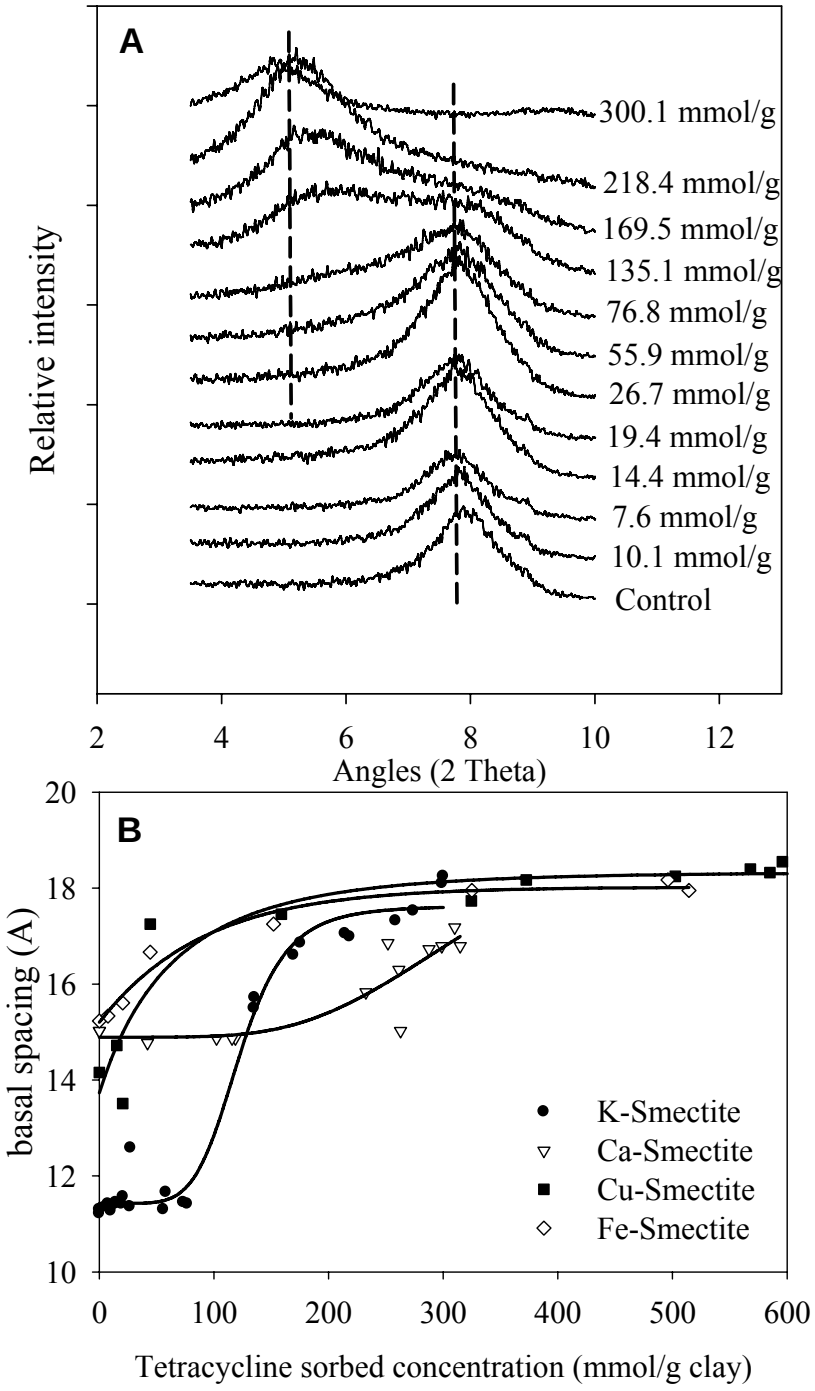

Figure III-6. (A) X-ray diffraction patterns of K-smectite with increasing loading of tetracycline, (B) increase of basal spacing as a function of tetracycline sorbed concentration

Considering that the thickness of smectite is ~9.6 Å, and the basal spacing of smectites were 14.7 Å with tetracycline loadings < 135 μmol/g, and 17.1 Å with loadings > 300 μmol/g, the distance between two adjacent clay sheets was 5.1 Å and 7.5 Å respectively. Recall that the molecular dynamics simulation results indicate that at low loading rate (< 135 μmol/g), tetracycline lays parallel to clay surfaces with ABCD ring systems on the same planar and dimethylammonium group locates beside ring system with extended formation (Figure III-5A). The thickness of the extended structure is around 5.9 Å, which is close to XRD observed result of 5.1 Å. When sorption approaches to 420 μmol/g, which is the sorption plateau in Figure III-2, the clay sheets expands to 7.5 Å. At this distance tetracycline adapts a tilted position in clay interlayers. Molecular dynamic simulation results for sorption at 420 μmol/g indicate that tetracycline molecules adapt vertically tilted position. The disappearance of peak at 7.9º and formed new peak at 5º with increasing tetracycline sorption from 135 to 218 μmol/g (Figure III-6A) indicates that as tetracycline molecules enter clay interlayers, and adapt the position from parallel to vertically tilted positions.

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CHAPTER IV

TETRACYCLINE SORPTION TO DIFFERENT CLAY MINERALS FROM WATER

Abstract

Tetracyclines are a class of antibiotics commonly administered to control diseases and improve health for humans and animals, especially used in the practice of livestock feeding supplement at subtherapeutic levels. Tetracyclines enter the environment via land application of biosolids derived primarily from animal manures and sewage sludge of wastewater treatment plants. Understanding environmental fate and mobilization of tetracyclines is critical to assessing their exposure and potential threats to at-risk populations. Tetracycline usually manifested a great affinity to soil component, such as clay minerals and humic substances. In this study, we investigated sorption of tetracycline onto 2:1 phyllosilicate smectite minerals (including montmorillonite: SW-2, SAz-1; Beidellite: SBCa-1, Panther Creek) and non-expandable 1:1 kaolinite clay. Overall significantly greater sorption of tetracycline was observed for smectite compared to kaolinite. No significant difference in sorption was observed for tetracycline sorption on K- and Ca-smectite in acidic solution (e.g, pH 6.3 and 5.3). However, Ca-smectite manifested much stronger sorption in alkaline solutions (e.g., pH 8.5). The presence of K^{+} or Ca^{2+} in aqueous solution suppressed tetracycline sorption because tetracycline was primarily sorbed to clays via cation exchange reactions. In alkaline solution, sorption on Ca-smectite clays was greater than that by K-smectite, which was attributed to formation of complexes of the negatively charged functional groups of tetracycline with Ca^{2+} on clay siloxane surfaces. K-Panther Creek and K-SWy-2 clay has reached sorption plateau around 450 and 420 μmol/g clay after aqueous equilibrium concentration reaches 0.15 μmol/mL. This sorption amount occupied only ~50% of cation exchange sites on clay surface, but almost all surface area (> 100%) on clay

minerals were occupied, which suggested that limit factor for tetracycline sorption onto smectite was surface area not cation exchange capacity.

Introduction

Tetracyclines are a class of antibiotics widely administered to treat diseases and improve health for humans and animals, especially used routinely as supplementary components in livestock feeds (Chopra and Roberts, 2001). Large fractions of tetracycline used in animal treatment and feeding operations are excreted into livestock liquid and solid manures. After a simple treatment, these manures are land applied to agriculture fields for plant fertilizer values. This process is the primary pathway to disseminate tetracyclines into the environment. Kolpin et al. (2002) found that tetracyclines such as chlortetracycline, oxytetracycline and tetracycline were frequently detected in stream water with concentrations ranging from 0.11 to 0.42 μg/L. Although tetracycline concentrations in water samples were not sufficient high to cause immediate impacts to ecosystems, considering their high affinity to soils/sediments, substantial amount of tetracyclines are still present in sediment/soil and water systems, potentially causing chronic exposure and impacts to at-risk populations. Therefore, understanding the fate and transport of tetracycline in soils is an important factor to assess the risks of tetracycline exposure in the ecosystems.

Sorption to soil/sediment is an essential process affecting the fate, transport and bioavailability of pollutants. It has been viewed that many nonpolar organic contaminants primarily partition to soil organic matter. For tetracyclines and many other pharmaceuticals, those compounds containing multiple types of polar and/or ionic functional groups commonly leading to several speciation existing in aqueous environment; these species could develop several specific interactions with soil organic matter and/or mineral fractions (Gu and Karthikeyan, 2005; MacKay and Canterbury, 2005; Pils and Laird, 2007; ter Laak et al., 2006; Wang et al., 2009). Among soil minerals the 2:1 aluminosilicate smectites are especially

important clay fraction interacting with many classes of organic contaminants including pharmaceuticals (Aggarwal et al., 2006; Boyd et al., 2001; Chappell et al., 2005; Charles et al., 2008; Laird, 1999; Li et al., 2004; Sheng et al., 2002; Wang et al., 2009). Sorption by smectite clay is influenced by many intrinsic properties such as interlayer swelling, high cation exchange capacity (CEC), and large siloxane surface area.

Tetracycline structure and speciation chemistry in aqueous phase indicate that within environmentally-relevant pH range of 5 to 8, tetracycline commonly exists in three species of zwitterion, anion and a minor fraction of cation (Figure IV-1). These species determine sorption mechanisms and driving forces for tetracycline sorption to clay minerals (Aristilde et al., 2010; Figueroa et al., 2004; Sithole and Guy, 1987). Porubcan et al. (1978) studied sorption of tetracycline by montmorillonite, and concluded that at low pH range (1.5 to 5.0) tetracycline sorption to montmorillonite was driven primarily by cation exchange reaction with the major species of cationic (at pH 1.5) and zwitterionic (at pH 5.0) tetracycline to montmorillonite. At pH 5.0, the major species of tetracycline is zwitterion; however, sorption was believed to cation exchange reaction assuming that zwitterion simultaneously accompanied with proton to be sorbed by mineral. Kulshrestha et al. (2004) also observed that tetracycline sorption decreased with increasing pH from 1.5 to 11.0, which was attributed to decreasing cationic fraction of tetracycline in aqueous phase hence reducing the contributions of cation exchange reaction. In addition to cation exchange-driven sorption, formation of complexes between tetracycline and metal ions is another mechanism responsible for tetracycline sorption, particularly at alkaline condition in which minimal amount of cationic tetracycline is present in aqueous phase (Aristilde et al., 2010; MacKay and Canterbury, 2005; Pils and Laird, 2007; Porubcan et al., 1978). Ca^{2+}-saturated soils or clays usually manifested a greater sorption for tetracycline than K^{+}-saturated

sorbents (Pils and Laird, 2007), which was attributed to the formation of complexes between negatively charged functional groups in tetracycline and Ca^{2+} (MacKay and Canterbury, 2005). Using spectroscopic approaches, Aristilde et al. (2010) observed the formation of complexes between deprotonated phenol groups of oxytetracycline and exchanged Na^{+} on clay surfaces, though it is more common to accept that the complexes are more readily formed between tetracycline with multivalent metal cations, which have been reported to occur in aqueous solution (Benet and Goyan, 1965; Berthon et al., 1983; Brion et al., 1981; Lambs et al., 1988; Ohyama and Cowan, 1995; Wessels et al., 1998).

The objective of this study was to compare tetracycline sorption to phyllosilicate smectites and kaolinite under varying solution pH and electrolyte background. Factors such as clay characteristics, type of exchangeable cation, cation exchange capacity, surface area and locations of deficit negative charges were evaluated for their effects on tetracycline sorption in aqueous systems.

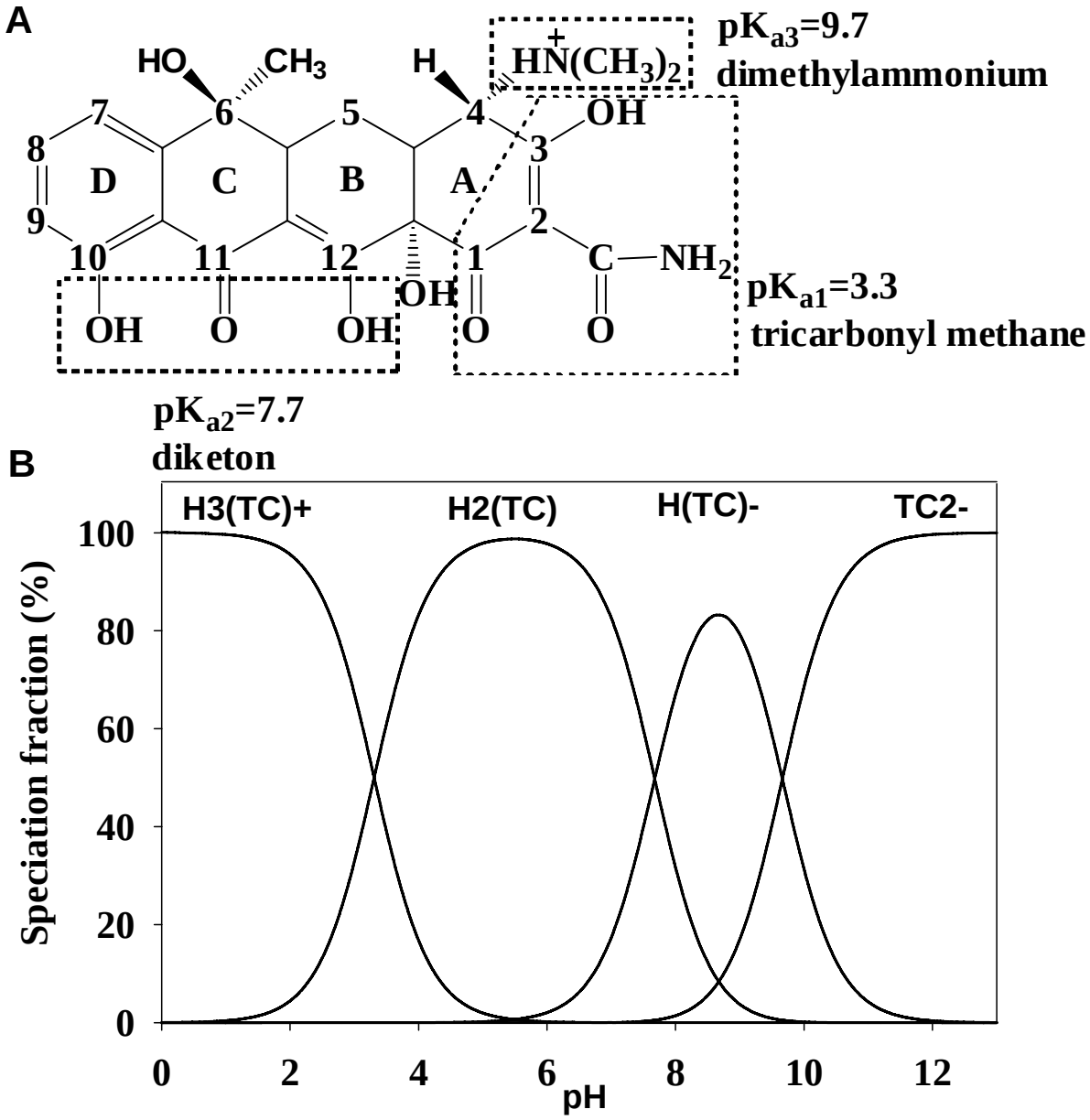

Figure IV-1. (A) Tetracycline structure, and (B) speciation distribution as a function of solution pH. $H_3(TC)^+$, $H_2(TC)$, $H(TC)^-$, and TC^{2-} represent the cation, zwitterion, anion, and divalent anion of tetracycline species.

Materials and Methods

Clay Minerals and Chemicals

Reference clay minerals were purchased from the Source Clays Repository of the Clay Minerals Society. Selected clay mineral characteristics are listed in Table IV-1. Sedimentation method was used to collect < 2 μm-sized clay fractions, which were subsequently K^+ or Ca^{2+} saturated by washing the clay fractions with 0.5 M KCl and $CaCl_2$ solution three times. Excessive KCl and $CaCl_2$ were removed by washing clay with Mili-Q water five times. The removal of Cl^- was examined by negative test result of $AgNO_3$ solution. Suspensions were quickly frozen and freeze dried to receive clay powders.

Tetracycline hydrochloride (≥ 95%) and heptafluorobutyric acid were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO), and was used as received. Methanol, $CaCl_2$, KCl, and ammonium acetate were purchased from J.T. Baker (Phillipsburg, NJ, USA). All aqueous solutions were prepared using water obtained from a Millipore Milli-Q Water purification system followed by 0.2 μm membrane filtration.

Table IV-1. Selected properties of clay mineral used in this study

Minerals	Clay type	Tetrahedral charge (%)	Cation Exchange Capacity (cmol/kg)	Surface area(m^2/g)	Surface charge density ($\mu mol/m^2$)
SWy-2	montmorillonite, dioctahedral	0	78	766	1.02
SHCa-1	hectorite, trioctahedral	0	44	743	0.59
Panther Creek	beidellite, dioctahedral	52	88	619	1.42
SAz-1	montmorillonite, dioctahedral	12	130	768	1.69
SBCa-1	beidellite, dioctahedral	98	66	750	0.88
KGa-1	kaolinite,		2	20	1.00

Soprtion Isotherm Measurement.

Batch equilibration method was used to measure sorption of tetracycline by clay minerals from aqueous solution. Tetracycline initial solutions were prepared with concentrations ranging from 0 to 340 μmol/L. A certain amount of clay was mixed with a known volume of tetracycline initial solution in glass centrifuge tubes on a rotator shaker at 40 rpm for 18 h at room temperature (~23 ^{o}C). The tubes were subsequently centrifuged at 6000 g for 30 min at 23 ^{o}C. The supernatant pH was measured using a pH meter equipped with a combination electrode. All samples were prepared in duplicate. An aliquot of supernatant was collected and analyzed for tetracycline concentrations using a liquid chromatography coupled to a tandem mass spectrometer (LC-MS/MS) system consisting of a Shimadzu high-performance liquid chromatography (HPLC, Columbia, MD, USA) fully integrated with an Applied Biosystems Sciex 3200 triple quadrupole mass spectrometer (Foster City, CA, USA). A PEAK Scientific gas generator (NM20ZA, Billerica, MA, USA) was used to supply gases required for the mass

spectrometer. A binary mobile phase was used consisting of solutions with water and methanol (v/v = 95/5), and methanol and water (v/v = 95/5), both containing 2 mM ammonium acetate and 20 mM heptafluorobutyric acid. A Thermo Hypersil Gold column (50 × 2.1 mm, particle size 5 μm) was used with a flow rate of 0.15 mL min^{-1}. Tetracycline concentration was quantified using multiple reaction monitoring mode with the precursor/product transition of 445.0/410.0. Sorbed concentrations were calculated from the difference between the initial and equilibrium concentrations in aqueous solution.

Results and Discussion

Tetracycline contains three ionic functional groups which can be ionized in water to produce four species (Figure IV-1). This speciation chemistry of tetracycline governs sorption mechanism to clay minerals. In addition, anionic species can form metal-tetracycline complexes in aqueous solution. The distributions of the four species in water as a function of solution pH were calculated based on the relationship between solution acidity and tetracycline pK_a values (Figure IV-1). In this study, equilibrium solution pH values ranged from 3.3 to 9.0 depending on the type of clays and the amount of tetracycline present. Within this pH range, tetracycline exists in the forms of cation, zwitterion and anion containing one negative charge. At pH > 7.7, anionic form is the most abundant species, whereas at pH between 3.3 and 7.7, zwitterion of tetracycline is the dominant species.

Tetracycline sorption on Wyoming montmorillonite (SWy-2), Arizona montmorillonite (SAz-1), Panther Creek beidellite, California beidellite (SBCa-1) and kaolinite (KGa-1) from water was measured with pH value ranging from 3.8 to 8.4 (Figure IV-2). At lower aqueous equilibrium concentrations (< 0.007 μmol/mL), tetracycline sorption to K-Panther Creek mineral

demonstrated a liner relationship. As aqueous tetracycline concentration increased, sorption isotherm became nonliner, and approached to sorption plateau at ~460 μmol/g. Wyoming montmorillonite and California beidellite manifested similar nonlinear sorption isotherms. Arizona montmorillonite demonstrated a S-shape sorption isotherm. Arizona montmorillnite has the highest CEC (130 cmol/kg) and comparable surface area (768 m^2/g), but did not manifest the greatest sorption for tetracycline. This could be attributed to that negatively charged sites on smectite surface is not the limited factor for sorption of relatively large sized pharmaceuticals such as lincomycin (Wang et al., 2009).

Sorption of tetracycline to K^+-saturated Panther Creek clay from aqueous solution containing 0.0, 0.001, 0.01 and 0.10 M KCl (Figure IV-3). Increasing sorption suppressive effect was observed with increasing KCl concentration. Given pH ranges from 3.9 to 6.6, the major species of tetracycline were cation and zwitterion, and the sum of these two species was > 94% in the solutions. This speciation of tetracycline resulted in sorption on smectite mineral to be primarily driven via cation exchange reaction. The protonated dimethylamino functional moiety in tetracycline plausibly replaces the exchangeable potassium and interact with negatively charged sites on mineral surfaces. The results are consistent with previous studies that cation exchange mechanism was predominant mechanism controlling tetracycline sorption to soil minerals and soils (Figueroa et al., 2004; Sassman and Lee, 2005).

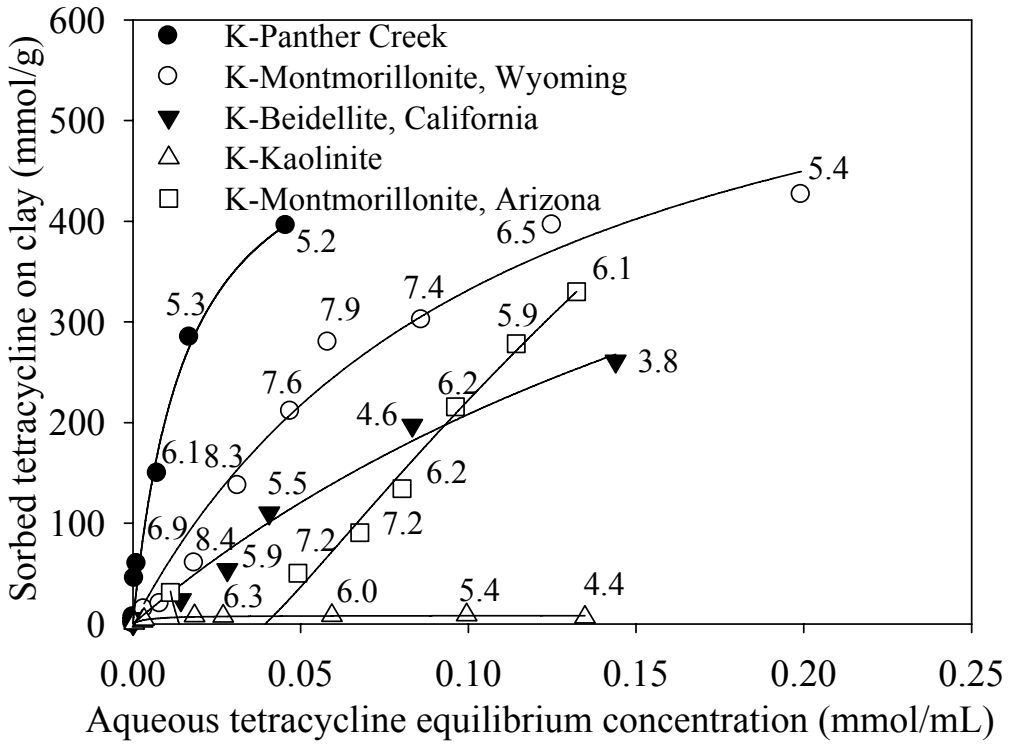

Figure IV-2. Tetracycline sorption to K^+-saturated clay minerals. The solid curves represent the fitting results of Langmuir equation. The equilibrium solution pH values are marked close to sorption data

Tetracycline sorption to clay minerals was fit to the Langmuir equation assuming only one type of surface sites is available sorption (Figueroa et al., 2004). The sorption sites are assumed to be cation exchange sites on mineral surfaces and to demonstrate the same affinity for tetracycline.

$$C_s = \frac{kQ_0C_w}{1+kC_w} \quad (1)$$

where C_s (μmol/g) and C_w (μmol/mL) are tetracycline concentration sorbed by clay and in aqueous phase, respectively. Q_0 (μmol/g) is the maximal sorption capacity, and k (mL/μmol) is Langmuir sorption coefficient. The fitting results are shown as curves in Figures IV-2 and IV-3, and the corresponding parameters are listed in Table IV-2. For different clay minerals, SWy-2 and SBCa-1 clay minerals were estimated to manifest higher sorption (Q_0 = 700 and 768 μmol/g, respectively) than that by Panther Creek mineral (Q_0 = 533 μmol/g). But the fitted *k* values indicate that tetracycline is more strongly sorbed to Panther Creek clay than that by SWy-2 and SBCa-1. The presence of increasing KCl in solution did not result in decreased Q_0 values, but significantly reduced k values, suggesting that K^+ ions present in aqueous solution effectively competed with tetracycline for cation exchangeable sites on clay surfaces.

Table IV-2. Langmuir equation fittings for tetracycline sorption onto clay minerals

Clays and solution conditions	Q_0 (μmol/g)	k (mL/μmol)	r^2
K-Panther Creek			
0.0 M KCl	533 ± 41[a]	63 ± 12	0.99
0.001 M KCl	637 ± 37	35 ± 4	0.99
0.01 M KCl	976 ± 167	10 ± 2	0.99
0.10 M KCl	882 ± 378	3 ± 2	0.98
K-SWy-2			
0.0 M KCl	700 ± 94	9 ± 2	0.97
K-SBCa-1			
0.0 M KCl	768 ± 219	4 ± 1	0.98
K-KGa-1			
0.0 M	8.5 ± 1	248 ± 88	0.90

[a] ± standard error

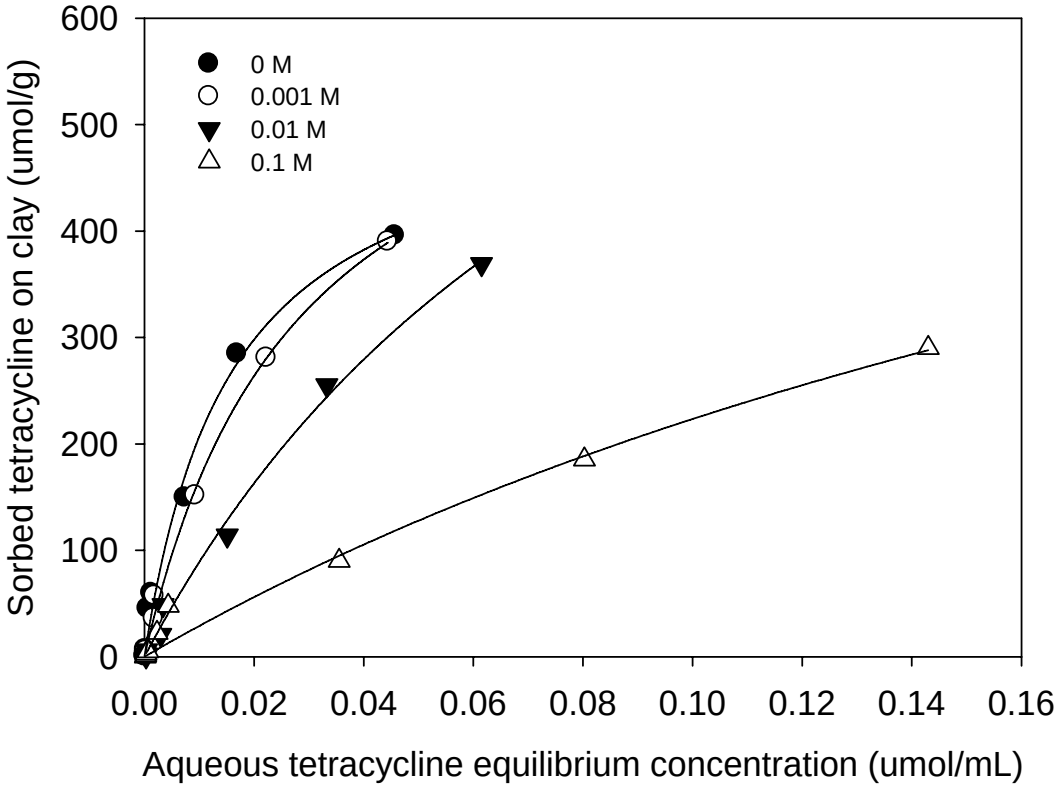

Figure IV-3. Tetracycline sorption to K-Panther Creek from aqueous solution containing 0.0, 0.001, 0.01 and 0.10 M KCl.

For K^+-saturated clay minerals, solution pH change only affects the distribution of tetracycline speciation in aqueous phase hence the extent of cation exchange equilibration. However, for Ca^{2+}-saturated clays as solution pH increases to alkaline condition, formation of Ca-tetracycline complexes on mineral surfaces is believed to enhance sorption. For instance of hectorite, the aqueous solution pH values after tetracycline sorption equilibrium were 8.4 to 9.2 for K- and Ca-SHCa-1 clay. Within this pH range, most tetracycline exists as anion (HTC^-) in aqueous solution. For K-SHCa-1 sorption reached to a plateau at 205 μmol/g when aqueous tetracycline concentration was 0.9 μmol/mL. At the similar pH and aqueous tetracycline concentration, Ca-SHCa-1 sorption of tetracycline reached to 570 μmol/g (Figure IV-4A). The dominant HTC^- species could interact with exchanged Ca^{2+} on mineral surfaces to form complexes, hence demonstrate an enhanced sorption vs. sorption by K-SHCa-1. As for the clay-water system at relatively lower pH such as K- and Ca-Panther Creek clay with pH ranging from 5.2 to 7.3 (Figure IV-4B), there was only a little distinction for tetracycline sorption by K- vs. Ca-Panther Creek clays. Within this pH range, the primary tetracycline species is zwitterion; cation exchange is invoked as the dominant sorption mechanism for tetracycline sorption.

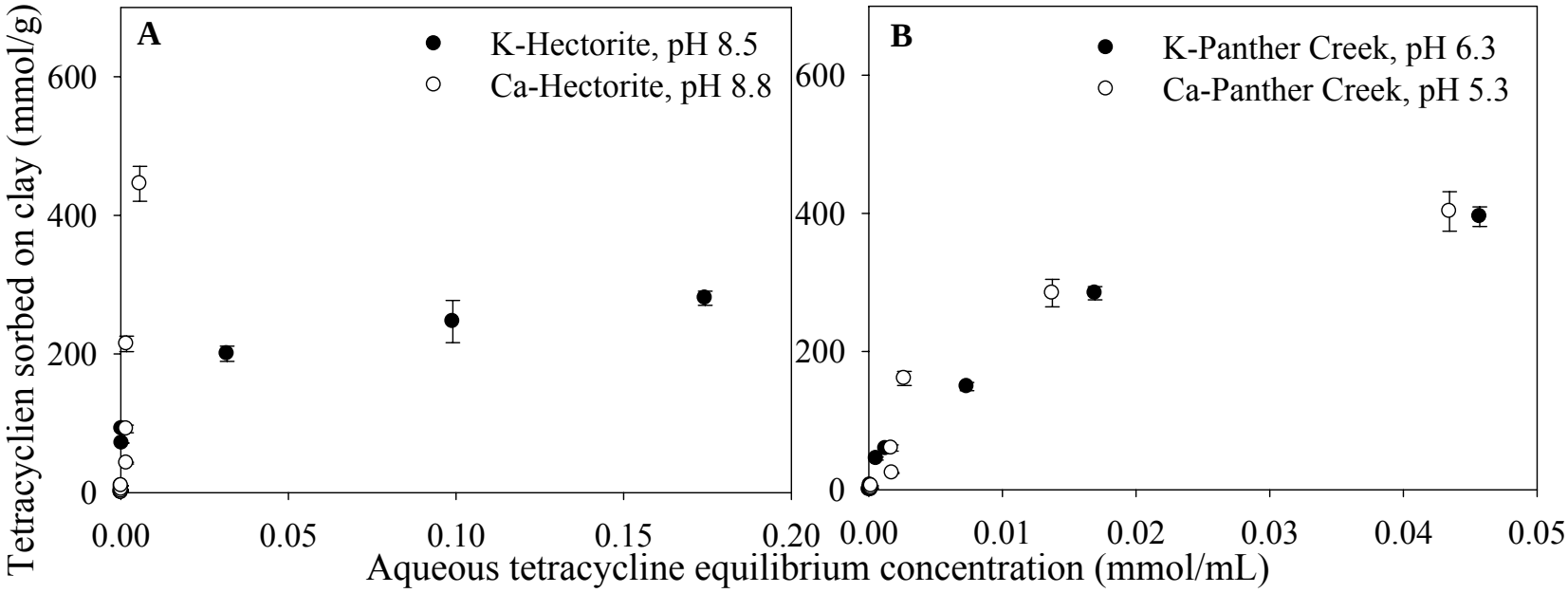

Figure IV-4. Tetracycline sorption to (A) K- and Ca-SHCa-1 and (B) K- and Ca-Panther Creek clays. The pH values for hectorite/water systems were averaged at ~8.6, and the pH values for Panther Creek clays ranged from 5.2 to 7.3.

It has been reported that in aqueous solution tetracycline anionic form (HTC^-) forms complex with Ca^{2+} at metal-to-ligand ratio of 1:1, the corresponding formation constant (log K) was measured at 12.73 (Martin, 1979). Other complexes with metal-to-ligand ratios of 2:1 or 1:2 also formed with the formation constants of 8.67 and 17.62, respectively (Brion et al., 1981; Lambs et al., 1988). These results indicate that strong complexation occurs between tetracycline and Ca^{2+} in water. The formed complexes with varying metal-to-ligand ratios are determined by metal cation and tetracycline concentration, and pH in water. For the complexation occurring on mineral surfaces, Ca^{2+} present on mineral surfaces usually manifests a more condensed scenario compared to that in solution phase. In addition, sorbed tetracycline on clay surfaces enhances the contact probability with exchanged cation due to the condensed sorbent phase. Furthermore, the subaqueous clay interlayer environment plausibly enhances the interaction strength between the negatively charged moiety in tetracycline and Ca^{2+} by formation of inner-sphere complexes.

Tetracycline complexation with exchanged cations on mineral surfaces is expected to be enhanced for transition metal cations such as Cu^{2+} and Fe^{3+} compared to Ca^{2+}. In aqueous solution the complex formation constant between tetracycline and Cu^{2+} is six order of magnitudes greater than that for Ca^{2+} and Mg^{2+} of 1:1 metal-to-ligand complex (MacKay and Canterbury, 2005; NovakPekli et al., 1996). Therefore, it is expected that Cu-clay would manifest a strong affinity for tetracycline than Ca-clay. Our measured results are consistent with this expectation that tetracycline sorption onto Cu- and Fe-SWy-2 was higher than Ca-SWy-2 (Data not shown here).

In addition to the factors of solution acidity/alkalinity and exchangeable cations compensating negative charges on mineral surfaces, clay type also influences tetracycline sorption. In general, swelling clay minerals (i.e. smectites) displayed much greater sorption for tetracycline than non-expandable kaolinite mineral (Figure IV-2). Kaolinite is a 1:1 tetrahedral : octahedral silicate with a small amount of cation exchange sites due to dissociation of –OH groups on broken edges. Strong hydrogen bonding between the adjacent clay layers prevents kaolinite from expanding when hydrated (Schroth and Sposito, 1997). Therefore, kaolinite has the least sorption sites for tetracycline due to the insufficient amount of cation exchange sites and small surface area available for sorption. On the other hand, smectites are 2:1 phyllosilicates containing high cation exchange capacity (i.e. negatively charged sites) and large surface area available for tetracycline sorption when expanded. As expected, tetracycline displayed a much stronger sorption for smectite clays compared to sorption by kaolinite (Figure IV-2).

For tetracycline sorption on smectites, sorption approached close to plateaus at 420, 450 and 280 μmol/g for K-SWy-2, K-Panther Creek and K-SBCa-1, respectively (Figure IV-2). The molecular surface area of tetracycline was estimated at ~178 $Å^2$. Assuming cation exchange sites and surface area are fully accessible for tetracycline sorption, and the sorbed tetracycline are positioned with ABCD ring systems flat on mineral surfaces, the occupation of cation exchange sites and surface area was calculated for tetracycline sorption by K-SWy-2, K-Panther Creek and K-SBCa-1 as reaching to plateaus (Table IV-3). Approximately 42 to 54% of cation exchange sites are occupied by tetracycline assuming that tetracycline is sorbed singly by cation exchange reaction. And ~40 to 78 % of smectite surface area was estimated to be occupied by tetracycline molecules. The sorbed tetracyclines plausibly adapt a flat position with the molecular plane parallel to clay siloxane sheets at lower sorption rate. As sorption increases, the

accessible mineral surface areas become a limiting factor for tetracycline to adapt that parallel position, though sufficient amount of cation exchange sites are still available. To compromise this limiting factor, tetracycline molecules may form a double-layer or vertically tilted configurations in clay interlayers, resulting in further expansion of smectite basal spacing. For many large-sized cationic pharmaceuticals sorption by smectites is not limited by the amount of negatively charged sites (i.e. cation exchange sites) on mineral surfaces but by accessible surface area, despite smectite has very large surface area of ~800 m^2/g.

Table IV-3. Cation exchange site and surface area of clay mineral occupied by tetracycline

Minerals	Clay Type	CEC Occupied (%)	Surface area occupied (%)
SWy-2	montmorillonite, dioctahedral	54	58
Panther Creek	beidellite, dioctahedral	51	78
SBCa-1	beidellite, dioctahedral	42	40

Conclusions

Tetracycline structure and solution chemistry determine the formation of cationic, zwiterrionic and anionic species in solution, which pose significant impacts to sorption by soil/sediment. Results from this study revealed that tetracycline sorption to clay minerals was highly dependent on solution pH. Cation exchange reaction and metal-ligand complexation are

the two major mechanisms responsible for tetracycline sorption on clay surfaces. For acidic and neutral solution, replacement of metal cation associated with clay with tetracycline is the dominant mechanism responsible for the overall sorption process. In alkaline solution complex formation between tetracycline anion and multivalent metal cation on mineral surface also contributes to the overall sorption. Theoretically, tetracycline could be sorbed on smectites up to reaching cation exchange capacity when cation exchange reaction dominates the overall sorption; however, the large molecular size of tetracycline results in that the accessible mineral surface area becomes the limiting factor in spite of the large surface areas of smectite.

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