

FINAL PROJECT REPORT

for

OCCURRENCE OF ANTIBIOTICS IN WASTEWATER EFFLUENTS AND THEIR MOBILITY IN  
SOILS: A CASE STUDY FOR WISCONSIN

DNR Project # 169

Submitted to

Wisconsin Department of Natural Resources  
Wisconsin Department of Agriculture, Trade, and Consumer Protection

by

PIs: K.G. Karthikeyan and William F. Blear  
Departments of Biological Systems Engineering and Soil Science  
University of Wisconsin-Madison

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## PROJECT SUMMARY

Title: OCCURRENCE OF ANTIBIOTICS IN WASTEWATER EFFLUENTS AND THEIR MOBILITY IN SOILS: A CASE STUDY FOR WISCONSIN

Project I.D.: DNR Project #169

Investigators: **K.G. Karthikeyan**, Assistant Professor, Department of Biological Systems Engineering, University of Wisconsin, Madison  
**William F. Bleam**, Professor, Department of Soil Science, University of Wisconsin, Madison

Research Assistant: **Cheng Gu**, Graduate Research Assistant, Biological Systems Engineering University of Wisconsin, Madison

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### Background/Need:

Recent reports on the detection of pharmaceuticals in water bodies and waste streams in Europe and the United States have raised some environmental concerns. Sulfonamides have been detected in leachate from a Danish landfill, in Berlin drinking water wells for which 80% of the groundwater was bank-filtered surface water, and in groundwater in Germany. Studies in the U.S. have identified antibiotics (sulfonamides and trimethoprim) in groundwater down gradient from a landfill containing hospital waste, in water supply wells in a Nebraska bank filtration site and in groundwater from Washington. There is an increasing interest among scientists, policy makers and industry personnel in the U. S. to survey the nation's water resources for human and veterinary pharmaceuticals, steroidal hormones, etc. The United States Geological Survey (USGS) is leading the efforts by collecting stream samples from several locations across the country to obtain baseline information regarding these emerging organic contaminants. Their efforts need to be augmented at the local level by systematically screening potential sources (e.g., wastewater treatment effluent, on-site wastewater systems, animal waste lagoons) to obtain a better understanding of the transport pathways and environmental fate of pharmaceuticals. Compared to conventional organic contaminants (e.g., pesticides, PAHs, PCBs), little information is available on the environmental fate and transport of antibiotics. The molecular properties of the antibiotics would favor sorption to soil components and interaction with metal ions in soils. However, our ability to predict mobility and fate of antibiotics in surface and subsurface systems is hampered by a lack of understanding of the fundamental processes governing their environmental reactivity.

### Objectives:

- i) Conduct a screening for five different classes of antibiotics, namely, sulfonamides, macrolides, tetracyclines, fluoroquinolones, and beta-lactams, in the effluents from wastewater treatment systems, and in adjacent groundwater monitoring wells, and
- ii) Determine the environmental fate and transformation of selected antibiotics, namely, tetracyclines and fluoroquinolones.

### Methods:

Our sampling activities spanned a range of wastewater treatment methods, community size served by the facility, receiving water body impacted by effluent discharge and geographical locations within the state. Samples were collected from seven different wastewater treatment facilities (Green Bay, Oshkosh, Lake Geneva, Barron-Cameron, Hayward, Spooner, Middle River Health Care), adjacent groundwater monitoring wells at two sites (Lake Geneva, Spooner), and two on-site wastewater treatment systems. In addition, three water supply wells in La Crosse under the influence of surface water were sampled. Filtered water/wastewater samples collected from the study sites were shipped overnight on ice (4 °C) to the USGS, Ocala FL for antibiotic analysis. The samples were extracted following established procedures

and analyzed using a LC/MS fitted with atmospheric pressure ionization (LC/API-MS) interfaces. Dr. Michael T. Meyer (USGS) who has developed a method to extract and analyze 25 antibiotic compounds in water/wastewater/manure extracts, provided analytical and interpretive support for this study.

Batch experiments were used to quantify the sorption of tetracycline and fluoroquinolone to well-known soil minerals (hydrrous oxides of Fe and Al). These experiments involved reacting  $\text{Al}(\text{OH})_3/\text{Fe}(\text{OH})_3$  to the selected antibiotics (tetracycline and ciprofloxacin) in centrifuge tubes. After equilibration at 25 °C at 7 rpm for 24 h, the suspensions were centrifuged and filtered (0.2  $\mu\text{m}$ ). Antibiotic concentrations (HPLC, by UV detection),  $^3\text{H}/^{14}\text{C}$  activities (liquid scintillation counting, LSC), and pH were determined in the supernatant. LSC provided a measure of the total activity contributed by these compounds, while HPLC allowed us to determine whether significant degradation occurred during the batch experiments. The amount of antibiotics sorbed was calculated from the difference between solution-phase  $^{14}\text{C}/^3\text{H}$  activities in equilibrated blanks and suspensions (corrected for any degradation).

### Results and Discussion:

A total of eight antibiotic compounds were detected (1-5 compounds per site), including three sulfonamides (sulfamethazine, sulfamethoxazole, and sulfadimethoxine), two tetracyclines (tetracycline, chlortetracycline), one fluoroquinolone (ciprofloxacin), one macrolide (erythromycin) and trimethoprim. Detected antibiotics fall into the following classes for the frequency of detection: sulfonamides > tetracyclines > fluoroquinolones > trimethoprim > macrolides. The USGS nationwide stream survey lists the above compounds among the 30 most frequently detected organic contaminants and the levels detected by us are in agreement with those found in the literature.

Contrasting sorption behavior of tetracycline and ciprofloxacin to Al and Fe hydroxides was observed. Compared to tetracycline the following trends were observed for ciprofloxacin: (i) strong pH-dependence (ii) minor differences between LSC and HPLC measurements after both 1 day and 1 week, and (iii) higher removal level - maximum sorption of  $\approx 72\%$ , unaffected by reaction time, around neutral pH. Tetracycline sorption exhibits mild pH-dependence and a significant difference existed between LSC- and HPLC-determined removal levels, highlighting the importance of transformation reactions. Our efforts to identify degradation products did not reveal the presence of hydrolyzed forms (most plausible reaction products) or other compounds. However, elevated soluble Al levels observed in the presence of tetracycline indicated that ligand-promoted dissolution of  $\text{Al}(\text{OH})_3$  could be occurring. The presence of Al-tetracycline complexes in solution helps explain the difference between HPLC and LSC quantifications. Our research findings will increase understanding of the environmental occurrence, fate and transformation characteristics of these emerging organic contaminants.

### Conclusions/Implications/Recommendations:

Antibiotics were detected in wastewater influent/effluent, adjacent groundwater monitoring wells and in samples from on-site wastewater treatment systems. However, the soluble levels were extremely low (< 10  $\mu\text{g}/\text{L}$ ), and importantly were unaffected by the size of the wastewater treatment facility. Future monitoring programs can be limited to the eight compounds detected in our study.

### Key Words:

Antibiotics, wastewater treatment, sulfonamide, tetracycline, fluoroquinolone, macrolide, trimethoprim, ciprofloxacin, sorption, Al hydroxide, Fe hydroxide, transformation

### Funding:

Wisconsin Department of Natural Resources

Wisconsin Department of Agriculture, Trade, and Consumer Protection

## INTRODUCTION

Recent reports on the detection of pharmaceuticals in water bodies and waste streams in Europe, albeit at low levels, have raised some environmental concerns (Buser et al., 1998; Halling-Sorensen et al., 1998). There is an increasing interest among scientists, policy makers and industry personnel in the United States to survey the nation's water resources for human and veterinary pharmaceuticals, steroidal hormones, etc. The United States Geological Survey (USGS) is leading the efforts by collecting stream samples from several locations across the country to obtain base-line information regarding these "emerging contaminants" (Kolpin et al., 2000). Their efforts need to be augmented at the local level by systematically screening potential sources, such as effluents from wastewater treatment, animal waste lagoons, in addition to ground and surface waters, to obtain a better understanding of the transport pathways and environmental fate of pharmaceuticals.

Among a wide variety of pharmaceutical compounds, antibiotics assume special significance due to: i) their extensive use (> 50,000,000 lbs produced annually in the US) in human therapy and veterinary medicine (Levy, 1998), ii) contribution from numerous sources (sewage treatment plants (STPs), confined animal feeding operations, (CAFOs)), iii) their ability to alter microbial community structure facilitating the development of antibiotic-resistant human pathogens (Meyer et al., 2000), and iv) the potential to serve as indicators for the presence of resistant pathogens. The presence of numerous antibiotics and analytical constraints will dictate the scope of any investigation. The USGS has developed a liquid chromatography-mass spectrometry (LC/MS) based analytical protocol to quantify the presence of 25 antibiotics (sulfonamides, macrolides, tetracyclines, fluoroquinolones, beta-lactams) at parts-per-trillion [ng/L] levels. A screening study conducted for these different classes of antibiotics in liquid waste from CAFOs reported the following order in terms of frequency of detection: tetracycline>sulfonamides>beta-lactams>macrolides. Tetracyclines (TCs) have been detected at  $\mu\text{g/L}$  concentrations or greater in river water ( $\approx 1 \mu\text{g/L}$ , (Watts et al., 1983)), sediments near a fish farm ( $0.1 - 11 \mu\text{g/g}$  sediment, (Watts et al., 1983)), groundwater samples collected near waste and wastewater lagoons ( $> 1 \mu\text{g/L}$ , (Thurman and Hostetler, 1999)), and liquid hog lagoon samples [5 to 700  $\mu\text{g/L}$ , Meyer et al. (2000)]. Several fluoroquinolone (FQ) antibiotics (e.g., enrofloxacin for cattle; levofloxacin and sparfloxacin for humans) have been approved over the past three years by the Centre for Veterinary Medicine and Food and Drug Administration (CVM, 1998). As a result, significant quantities of FQs are being used as antibacterial agents in large-scale animal operations. Exposure of other bacteria that constitute the natural microbial flora of cattle and poultry, specifically *Campylobacter*, has resulted in development of resistance to FQs (Gaunt and Piddock, 1996). In the case of *C. jejuni*, an important human pathogen, such an exposure can result in a high FQ minimal inhibitory concentration of 4 mg/L. Fluoroquinolone-resistant *C. jejuni* has been already detected in US poultry products (Smith et al., 1999) and extensive use of FQs in cattle is likely to substantially increase the risk imposed by this species (Anderson et al., 2001). Development of resistance to FQs typically occurs within 2 years of its widespread application in veterinary medicine (Endtz et al., 1991).

Unlike pesticides/agrochemicals that are applied sporadically, antibiotics are being continually released to the environment via STPs and discharge of untreated sewage to the rivers (Daughton and Ternes, 1999). In comparison to conventional organic contaminants (COCs), such as pesticides, PAHs, and PCBs, there is very little or no information available on the fate and transformation (bio- and photo-) of antibiotics in soil/water. The antibiotics possess high polarity and low volatility compared to the COCs and, hence, their environmental distribution will be controlled by aqueous transport. However, their lipophilic character increases environmental persistence, which along with their non-volatile nature increases the tendency to bioaccumulate in terrestrial ecosystems (Daughton and Ternes, 1999).

The current scientific literature, drawn from our understanding of the reactivity of COCs in soils and the pharmaceutical and medicinal studies on antibiotics, suggest that the molecular properties of FQs/TCs would favor sorption to soil components and interactions with metal ions in soils. The affinity of FQs for inorganic mineral surfaces is being exploited in certain bio-medical applications. Hydroxyapatite binds the FQs ciprofloxacin/norfloxacin via cation-bridging, a relatively moderate interaction that facilitates slow release of antibiotics to control infection during bone tissue growth (Martin and Goissis, 2000). Similarly, titanium based orthopedic materials deposited with ciprofloxacin have potential for use in prosthetic devices to deliver the desired amount of antibiotics to surrounding tissues (Dunn et al., 1994). In addition, kaolin and Mg-trisilicate are effective FQ sorbents and have been proposed as replacements for activated charcoal to manage ciprofloxacin poisoning (Ofoefule and Okonta, 1999). It is evident that, although the above studies provide useful information regarding sorptive interactions of antibiotics, they are not directly applicable to soil/aquatic environments since the geochemical conditions (e.g., pH, background ionic composition) are quite different. Nowara et al. (1997) investigated the ability of soils (pretreated to remove organic matter) and phyllosilicates to sorb FQs. Both the soils and clay minerals retained a high amount of enrofloxacin. The extent of sorption to soils correlates well with the percent clay content. Similar to the FQs, oxytetracycline interacts with hydroxyapatite through cation-bridging and the extent of sorption is affected by the presence of competing ligands (Ibsen and Urist, 1967).

Groundwater contamination is a serious environmental concern in the US, especially in the rural areas where it is the major source of drinking water (Moody, 1990). It is well known that the transport of an organic contaminant to groundwater depends on the nature of its sorption to soil components (i.e., strength, degree of irreversibility), its resistance to degradation and the presence of preferential flow pathways. Our ability to predict mobility, fate and effects of antibiotics is hampered by a lack of information on fundamental processes governing their behavior in the environment. Sorption processes in particular warrant investigation because association of antibiotics with particles and colloidal organic matter influences their mobilization and transport in surface runoff, determines their potential to leach into groundwater, controls their mobility in aquifer, and affects their propensity to undergo transformation reactions. Sorption affinities will control whether the soil provides a long-term “sink” or merely a temporary reservoir that will release the contaminants when the solution concentration decreases (Gao et al., 1998). Several reports highlighting the importance of sorption on pesticide leaching can be found in the literature (Kladivko et al., 1991; Matthies and Behrendt, 1991; Truman and Leonard, 1991).

Although our major goal was to determine the occurrence of antibiotics in groundwater, due to low environmental concentrations of these compounds we initially targeted potential environmental sources. Therefore, the approach used in this study was to collect samples from municipal wastewater treatment facilities with a special focus on those with land-based effluent discharge systems. Based on the background information presented in this section, specific **objectives** of the proposed work are to:

- (i) Conduct a screening for five different classes of antibiotics, namely, sulfonamides, macrolides, tetracyclines, fluoroquinolones, and beta-lactams, in the effluents from wastewater treatment systems and in adjacent groundwater monitoring wells, and
- (ii) Determine the reactivity of selected antibiotics (e.g., tetracycline, ciprofloxacin) with important soil minerals (e.g., hydrous oxides of Al and Fe).

## PROCEDURES AND METHODS:

### Wastewater/water Sample Collection:

Sites for our Wisconsin antibiotics survey were selected based on differences in wastewater treatment method, size of the community served by the facility, receiving water body impacted by effluent discharge, and geographical location. In coordination with the WI Department of Natural Resources (DNR), contacts were established with regional DNR engineers and treatment plant operators in different regions of the state. We identified several wastewater treatment facilities, listed in Table 1, satisfying the above requirements for our screening protocol.

**Table 1. Sites for wastewater sampling and antibiotics survey in Wisconsin.**

<i>Location</i>	<i>County</i>	<i>Average Daily Flow (MGD)</i>	<i>Community Size</i>	<i>Secondary Treatment Process</i>	<i>Effluent Discharge</i>
City of Oshkosh WWTP <sup>1</sup>	Winnebago	9.6	73,192 <sup>a</sup>	Activated sludge	Fond du Lac River [Lake Winnebago] <sup>b</sup>
Green Bay Metropolitan Sewerage District	Brown	≈30 <sup>c</sup>	≈150,000	Activated sludge	Mouth of Fox River discharging to Green Bay
City of Lake Geneva WWTP <sup>1</sup>	Walworth	1.3	several 1000s	Oxidation ditch	groundwater <sup>d</sup> [Fox River] <sup>b</sup>
Barron-Cameron WWTF <sup>2</sup>	Barron	1.3		Aerated lagoons & seepage cells	groundwater <sup>d</sup>
Hayward City WWTF <sup>2</sup>	Sawyer	0.772		Aerated lagoons & seepage cells	groundwater <sup>d</sup>
Spooner City WWTF <sup>2</sup>	Washburn	0.375		Aerated lagoons & seepage cells	groundwater <sup>d</sup>
Middle River Health Care <sup>3</sup>		0.018			Middle River [Lake Superior] <sup>b</sup>
La Crosse water supply well <sup>4</sup>	La Crosse	[2500 gpm, 100 ft sandstone wells under the influence of surface water]			

<sup>1</sup>wastewater treatment plant

<sup>2</sup>wastewater treatment facility

<sup>3</sup>dedicated wastewater treatment unit for this health care facility

<sup>4</sup>currently under viral & endocrine disruptor sampling study, USGS

<sup>a</sup>plus leachate from 2 landfills

<sup>b</sup>water body ultimately impacted

<sup>c</sup>domestic + 2 paper mills

<sup>d</sup>monitoring wells active

At each site, samples influent to and effluent from the secondary treatment systems (e.g., activated sludge, oxidation ditch, seepage cells) were collected in amber glass bottles, transported on ice and stored at 4°C prior to solid-liquid separation and analysis. Treatment plants in certain locations, such as Green Bay and Oshkosh, are required to follow a 24-hr flow-based composite sampling protocol. In such cases, one sample corresponding to a 24-hr period (e.g., 7 am to 7 am next day) was obtained. For grab



sampling events at other facilities, replicate samples were collected. Groundwater monitoring wells located downstream of effluent discharge points at the Lake Geneva and Spooner wastewater treatment facilities were also sampled. Relevant sample characteristics are provided in Table 2.

**Table 2. Characteristics of wastewater collected from various sampling locations.**

<i>Location</i>	<i>Flow<sup>s</sup></i> (MGD)	<i>BOD</i> (mg/L)	<i>TSS</i> (mg/L)	<i>TDS<sup>@</sup></i> (mg/L)	<i>TS<sup>@</sup></i> (mg/L)	<i>Total P</i> (mg/L)	<i>N</i> (mg/L)	<i>pH</i>	<i>Sampling date</i>
GB MSD <sup>1</sup> – Influent	23.6	137 162	174 137	1068.6	1317	5.14 N/A	21.9 (TKN) N/A	7.25	10/21/01 6/12/02
GB MSD <sup>1</sup> – Effluent		< 2 N/A	3.5 4	940.8	1072	0.46 0.32	1.18 (TKN) 1.15 (TKN)	7.42	10/21/01 6/12/02
COS WWTF <sup>2</sup> Influent	9.6	184 122	230 232	672	877	5.05 3.88	23.1 (NH <sub>3</sub> ) 14.7 (NH <sub>3</sub> )	7.5 7.3	10/21/01 6/12/02
COS WWTF <sup>2</sup> Effluent		6 24	4 6	575.4	765	0.84 0.37	0.15 (NH <sub>3</sub> ) 7 (NH <sub>3</sub> )	6.9 7	10/21/01 6/12/02
COLG WWTP <sup>3</sup> Influent	1.3	234	227	864			34 (TKN)	7.4	12/6/01
COLG WWTP <sup>3</sup> Effluent		6	8	852			1.1 (TKN)	7.6	12/6/01
COLG <sup>3</sup> well #217*		1.03		857 760			<1 (TKN)	7.45	12/6/01 6/11/02
COLG <sup>3</sup> well #220*		1.48		827 780			< 1 (TKN)	7.41	12/6/01 6/11/02

<sup>1</sup>Green Bay Metropolitan Sewerage District; <sup>2</sup>City of Oshkosh wastewater treatment facility;

<sup>3</sup>City of Lake Geneva wastewater treatment plant; <sup>s</sup>Average daily flow (million gallons per day)

\*Groundwater monitoring well [Depth to groundwater: Well # 217 - 3.45 ft; Well # 220 - 2.76 ft]

<sup>@</sup>determined in our laboratory

Water/wastewater samples were initially centrifuged in 250 mL polypropylene co-polymer bottles on a Sorvall Super T 21 high-speed, bench-top centrifuge. Following centrifugation, the supernatant was filtered through a 0.45 µm polypropylene membrane filter and stored at 4°C in 500 mL amber glass bottles. The supernatant samples obtained after centrifugation and filtration were shipped overnight on ice (2-4 °C) to Dr. Michael T. Meyer U.S. Geological Survey, Ocala, FL for antibiotic analysis. The low environmental concentrations of antibiotics require careful sample pretreatment and use of sophisticated instrumentation. Dr. Meyer has developed a LC/MS method to extract and analyze 25 antibiotic compounds (Table 3) in wastewater, ambient water, and sediment and is an expert in developing and adapting methods for the analysis of “*emerging organic contaminants*” in environmental samples. These compounds are in the USGS target list for “National Reconnaissance of Emerging Contaminants in US Streams” (Kolpin et al., 2000). We have obtained analytical and data interpretive support for this project through a joint funding agreement with Dr. Meyer.

External QA/QC consists of field and equipment blanks, field spikes, and blind samples. One field blank, equipment blank, field spike, and blind sample were submitted with each sampling round or every 10 samples. Spiking solutions were prepared by the Ocala Water Quality and Research Laboratory. The samples received were logged in and refrigerated at 2-4 °C until extracted. The samples were extracted within 14 days after they were received and the extracts were frozen until analysis, which were within 30 days after they are extracted. Extraction of samples was performed in sets of 16 that include 11 samples, one duplicate, two standards and two blanks. The extraction conditions are as follows: 50 mL of sample was pumped through a C<sub>18</sub> solid phase extraction [SPE] disk and eluted with a solvent mix

comprising 0.1% trifluoroacetic acid, methanol, acetonitrile, and water. The samples were then analyzed by USGS personnel using a LC/MS equipped with atmospheric pressure ionization interfaces (LC/API-MS). Duplicate seven point standard curves were generated by extracting 500mL ultra pure water samples spiked at 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0  $\mu\text{g/L}$  with a standard mix containing 2.5 ng/ $\mu\text{l}$  of each analyte. The standards of each sample set were checked against the existing standard curve. Blanks were used to check for potential sample carryover. Stock solutions of each analyte were prepared from analytical grade standards at a concentration of 1 mg/mL in methanol. The standard mix was prepared in 100 mL batches from individual stock solutions in ultrapure water with 20 mM ammonia acetate adjusted to pH 6. The standard mix was divided into 20 mL aliquots that were kept frozen until use.

**Table 3. Antibiotics analyzed by LC/MS in water/wastewater extracts and their quantitation limits [Source: Dr. M.T. Meyer].**

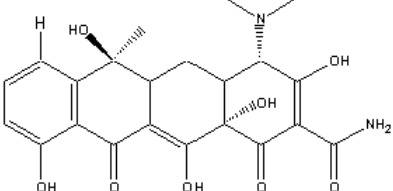
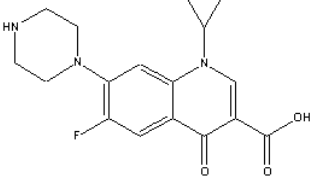
<i>Antibiotic</i>	<i>Quantitation Limit [<math>\mu\text{g/L}</math>]</i>	<i>Antibiotic</i>	<i>Quantitation Limit [<math>\mu\text{g/L}</math>]</i>
<u>Sulfonamides</u>		<u>Fluoroquinolones</u>	
Sufamethizole	0.1	Norfloxacin	0.05
Sulfathiazole	0.1	Enrofloxacin	0.05
Sulfamerazine	0.05	Ciprofloxacin	0.05
Sulfamethazine	0.05	Sarafloxacin	0.05
Sulfachloropyridazine	0.1		
Sulfamethoxazole	0.05		
Sulfadimethoxine	0.05	<u>Others</u>	
<u>Macrolides</u>		Lincomycin	0.05
		Carbadox	0.1
Tylosin	0.05	Trimethoprim	0.05
Roxithromycin	0.05	Virginiamycin	0.1
Erythromycin-H <sub>2</sub> O (dehydrated metabolite of erythromycin)	0.05	Spectinomycin	2.0
		Vancomycin	2.0
		Roxarsone	2.0
<u>Tetracyclines</u>			
Tetracycline	0.05		
Oxytetracycline	0.1		
Chlortetracycline	0.05		
Doxycycline	0.05		

**Batch Sorption Experiments:**

Batch sorption experiments with well-known model sorbents (e.g., minerals or organic matter fractions) provide the ability to isolate reaction pathways and minimize competing fate processes possible in whole soils. Compared to dynamic flow-through column studies, static equilibration studies offer an economical way to quickly evaluate the effect of numerous environmental variables (e.g., pH, ratio of antibiotics to sorbents) as well as reaction time on the fate and transformation of antibiotics. The batch sorption technique has been often used to assess the capacity of minerals and organic materials for various chemical contaminants and its simplicity accounts in part for its popularity. Macroscopic batch experiments were performed to quantify the sorption of tetracycline and ciprofloxacin to poorly crystalline Al and Fe hydroxides. Selected physico-chemical properties of these compounds are provided in Table 4. Experimental stock solutions were prepared by mixing radiolabeled forms of these antibiotics

(7-<sup>3</sup>H-tetracycline obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO and 2-<sup>14</sup>C-ciprofloxacin from Moravek Biochemicals, Brea, CA) with unlabeled compounds (obtained from Sigma Aldrich, St. Louis, MO and ICN Pharmaceuticals, Inc. Costa Mesa, CA). Al hydroxide was synthesized by gradual neutralization of a 0.5 M AlCl<sub>3</sub> solution to pH 7 using 0.5 M NaOH (Huang et al., 1977). This material has been characterized using X-ray diffraction as poorly crystalline gibbsite and it possesses a surface area of 80 m<sup>2</sup>/g as determined by BET-N<sub>2</sub> analysis. Fe hydroxide (6-line ferrihydrite) was synthesized by adding 10 g of Fe(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O to 1 L of distilled water heated to 75 °C. The suspension was maintained at 75 °C for 10 min and then cooled rapidly, dialyzed to remove excess NO<sub>3</sub><sup>-</sup> and then freeze dried (Schwertmann and Cornell, 2000).

**Table 4. Selected physico-chemical properties of tetracycline and ciprofloxacin** (Stephens et al., 1956; Huang et al., 1997; Tolls, 2001).

Property	Tetracycline	Ciprofloxacin
Structure		
Molecular weight	444.43	331.35
Aqueous solubility (mg/L)	1,700	30,000
Octanol water partition coefficient (log K <sub>ow</sub> )	-1.19	0.4
Acid-base equilibrium constants (pK <sub>a</sub> )	3.3, 7.68, 9.69	6.0, 8.8
Melting point (°C)	172.5	258
Stability on exposure to light	photodegradation fast	slightly photosensitive

For each batch system, 30 mg of Al(OH)<sub>3</sub>(s) were added to tared 16 mL round-bottomed culture tubes with PTFE lids (solid-to-solution ratio - 1:500) along with variable proportions of 0.01 M NaCl, NaOH, and HCl to obtain a range of final pH values. Stock solution of tetracycline or ciprofloxacin was added to obtain final concentrations ranging from 0.1 to 50 mg/L. Controls [no Al(OH)<sub>3</sub>] were identically prepared to account for losses as sorption to PTFE tubes, volatilization and transformation. All experiments were conducted in dark in triplicates using MilliQ-grade deionized water. The suspensions were equilibrated at 25°C by end-over-end rotation at 7 rpm for 24 h. At the end of the reaction period, suspensions were centrifuged and then passed through 0.45 μm filters. Supernatant solution pH was determined using an Accumet AR-50 pH/conductivity meter. Tetracycline and ciprofloxacin concentration in the filtrate were measured using HPLC on a Waters<sup>TM</sup> Spherisorb column using UV detection. Solution <sup>14</sup>C and <sup>3</sup>H activities were determined using liquid scintillation counting [LSC]. HPLC measurement is specific for tetracycline and ciprofloxacin. Conversely, since each mol of 7-<sup>3</sup>H-tetracycline and 2-<sup>14</sup>C-ciprofloxacin contains 1 mol of <sup>3</sup>H and <sup>14</sup>C, respectively, LSC provides a measure of the total carbon contribution from these compounds, i.e.,

$$[^{14}\text{C}/^3\text{H}]_{\text{LSC}} = [\text{antibiotics}]_{\text{HPLC}} + [\text{antibiotics}]_{\text{transformed}} \quad (1)$$

Therefore, the difference between concentrations measured by LSC and HPLC will provide an estimate of the amount of ciprofloxacin and tetracycline subjected to transformation reactions.

## RESULTS AND DISCUSSION:

### Antibiotics Analysis:

Table 5 provides the soluble concentration of antibiotics measured in wastewater samples collected from the treatment plants in Green Bay (GB), Oshkosh (COS), Lake Geneva (for COLG - treatment plant plus 2 groundwater monitoring wells), Barron, Hayward, Spooner (including a monitoring well) and the Middle Health Care Facility near Superior. A total of eight antibiotic compounds were detected (1-5 compounds per site), including three sulfonamides (sulfamethazine, sulfamethoxazole, and sulfadimethoxine), two tetracyclines (tetracycline, chlortetracycline), one fluoroquinolone (ciprofloxacin), one macrolide (erythromycin) and trimethoprim. Compounds that are not listed in this table were found at levels below their quantitation limits.

**Table 5. Concentration ( $\mu\text{g/L}$ ) of antibiotics in wastewater influent and effluent samples.**

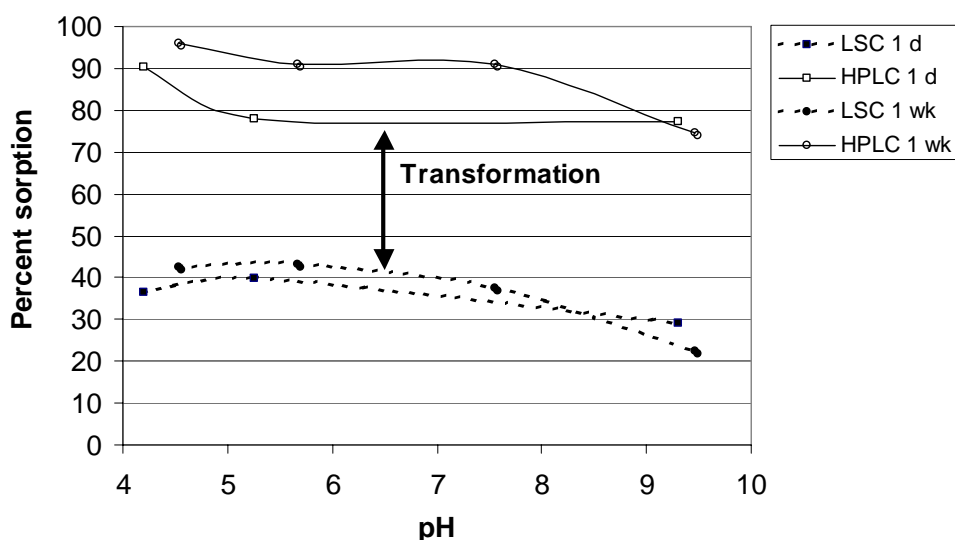
<i>Location</i>	<i>Sampling Date</i>	# 1	#2	#3	#4	#5	#6	#7	#8
GB MSD Influent	10/22/01 6/12/02	0.21	<0.05		1.1 0.27	1.2		0.17	0.49
GB MSD Effluent	10/22/01 6/12/02	0.1	0.5		0.23	< 0.02		0.38	< 0.01
COS WWTF Influent	10/22/01 6/12/02	0.11	0.14		4 1.2	1			0.2
COS WWTF Effluent	10/22/01 6/12/02	< 0.05	0.05		0.57 0.25	< 0.02		0.29	0.55 0.07
COLG WWTP Influent	12/6/01 6/11/02		0.06		0.7		0.05		
COLG WWTP Effluent	12/6/01 6/11/02		<0.05		1.00		0.04 0.08	0.11	0.05
COLG well #217	12/6/01 6/11/02		0.01 0.08						
COLG well #220	12/6/01 6/11/02				0.5				
Barron WWTF Influent	5/9/02		0.05		0.97		0.07		
Barron WWTF Effluent	5/9/02			0.01	0.17			0.33	
Hayward WWTF Influent	5/9/02		0.06		0.28				0.02
Hayward WWTF Effluent	5/9/02		0.04		0.3				
Spooner WWTF influent	5/9/02		0.28		1.2				
Spooner WWTF effluent	5/9/02		0.23		0.46				
Spooner monitoring well	5/9/02				0.34				
Middle River Health Care - Inf	5/9/02						0.08		0.08
Middle River Health Care - Eff	5/9/02		0.11		0.37		0.08		

*Compound #1 - sulfamethazine; #2 - sulfamethoxazole; #3 - sulfadimethoxine; #4 - tetracycline; #5 - chlortetracycline; #6 ciprofloxacin; #7 - erythromycin; #8 - trimethoprim*

A recent USGS stream survey lists the above compounds among the 30 most frequently detected organic contaminants (Kolpin et al., 2002). In addition, the levels reported in Table 5 are in agreement with those found in the literature (Hirsch et al., 1999). Our preliminary results indicate that the secondary treatment processes used in these treatment facilities are capable of reducing the concentration of antibiotics in the soluble phase. However, no correlation was observed between antibiotics removal levels and the reduction in BOD and total suspended solids (TSS). Soluble concentration of antibiotics is relatively low ( $< 4 \mu\text{g/L}$ ) and more importantly are independent of the treatment capacity of a wastewater treatment facility. Samples collected from two on-site wastewater treatment systems contain oxytetracycline and higher levels of tetracycline (between 2.2 and 11  $\mu\text{g/L}$ ) than detected in municipal wastewater influents and effluents.

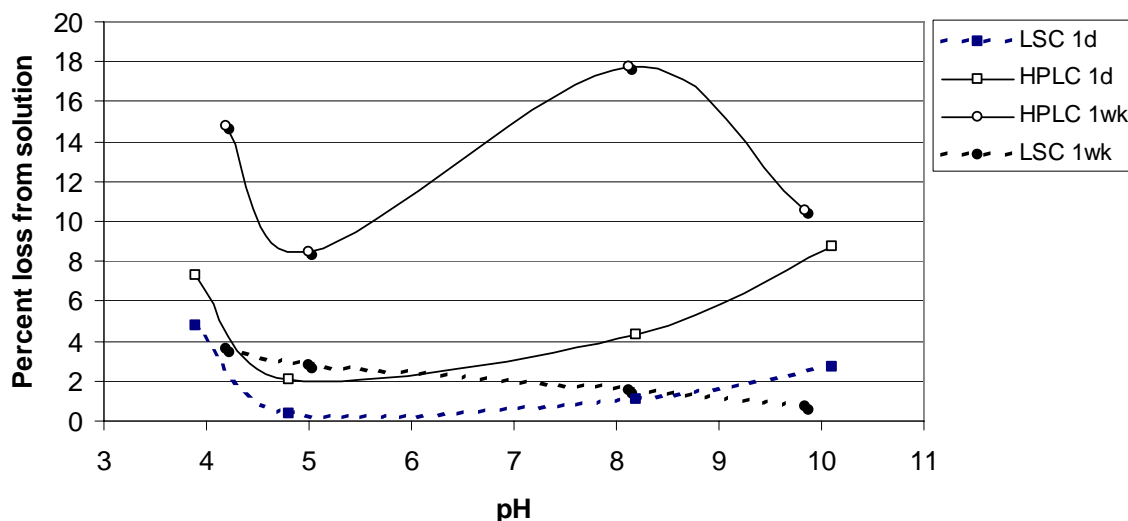
***Batch Sorption Experiments:***

Figure 1 shows the amount of tetracycline sorbed onto  $\text{Al}(\text{OH})_3(\text{s})$  (measured as the loss from solution of  $^3\text{H}$  label (LSC) and tetracycline (HPLC)) as a function of pH and reaction time (1 d and 1 wk). LSC data revealed a slight increase in tetracycline sorption between pH 4 and pH 6.0, above which it decreased with increasing pH values. Longer reaction time did not affect the pH-dependent trend as well as the extent of removal as determined by LSC. Loss of tetracycline from solution as measured by HPLC could be due to abiotic transformation mediated by  $\text{Al}(\text{OH})_3(\text{s})$  (e.g., acid-catalyzed hydrolysis), besides sorptive removal. Based on a comparison of the data obtained from these independent analytical methods, it appears that significant amount of transformation of tetracycline is occurring under our solution conditions. Possible degradation products include anhydrotetracycline and epi-anhydrotetracycline (Halling-Sorensen et al., 2002). Since these products remained in solution (counted by LSC), they probably do not have a strong affinity towards  $\text{Al}(\text{OH})_3(\text{s})$ . Our efforts to identify degradation products did not reveal the presence of hydrolyzed forms (most plausible reaction products) or other compounds. However, elevated soluble Al levels (measured using Atomic Absorption Spectrometry in our laboratory) were observed in the presence of tetracycline indicating that ligand-promoted dissolution of  $\text{Al}(\text{OH})_3$  could be occurring. The presence of Al-tetracycline complexes in solution can help explain the difference between HPLC and LSC quantifications. Longer reaction time appears to promote  $\text{Al}(\text{OH})_3$  dissolution thereby resulting in a greater difference in removal levels determined independently by LSC and HPLC.



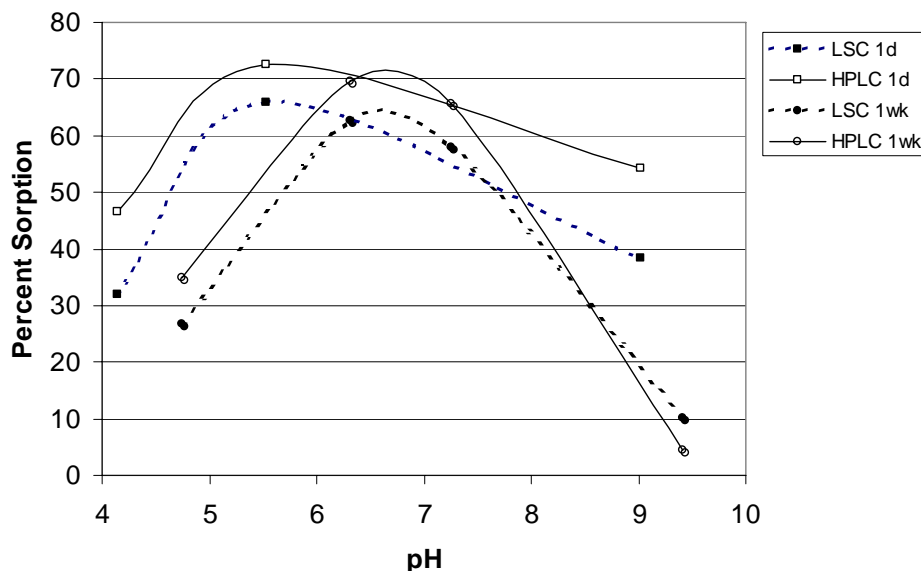
**Figure 1. Sorption of tetracycline [measured as loss from solution of  $^3\text{H}$  label (LSC) and tetracycline (HPLC)] to  $\text{Al}(\text{OH})_3(\text{s})$  as a function of pH.**

Tetracycline levels in solution were monitored by HPLC and LSC in the absence of  $\text{Al}(\text{OH})_3(\text{s})$  to account for losses due to sorption to glassware, volatilization, and hydrolysis. LSC always yielded higher levels in solution and the recovery of  $^3\text{H}$  label was greater than 95.2 and 96.4 % after 1 d and 1 wk, respectively (Figure 2). The difference between HPLC and LSC measurements (2.5 - 6% after 1 day; 5.6 - 11.2% after 1 wk) increased with increasing reaction time.



**Figure 2. Loss of tetracycline from solution in the absence of  $\text{Al}(\text{OH})_3(\text{s})$  as measured using LSC ( $^3\text{H}$ ) and HPLC.**

Figure 3 shows the amount of ciprofloxacin sorbed onto  $\text{Al}(\text{OH})_3(\text{s})$  [measured by LSC and HPLC] as a function of pH and reaction time (1 d and 1 wk). Compared to tetracycline the following trends were observed: (i) strong pH-dependence - sorption level increases sharply between pH 4.5 and 7, reaches a maximum in the circum-neutral pH range, and then decreases with increasing pH, (ii) minor differences between LSC and HPLC measurements after both 1 d and 1 wk, and (iii) higher removal level (determined by LSC) - maximum sorption of  $\approx 72\%$ , unaffected by reaction time, around neutral pH.



**Figure 3. Sorption of ciprofloxacin [measured as loss from solution of  $^{14}\text{C}$  label (LSC) and ciprofloxacin (HPLC)] to  $\text{Al}(\text{OH})_3(\text{s})$  as a function of pH.**

### On-going Research Activities – Determining Antibiotic Levels in Sludge Samples:

Treatment processes used for municipal wastewater can be highly effective in removing antibiotics as evidenced from low effluent soluble levels in this study and by other researchers (Golet et al., 2002). We are currently (in collaboration with Dr. Michael Meyer, USGS, Ocala FL and Dr. Joel Pedersen, Soil Science, UW-Madison) developing new, robust and powerful extraction methods which will provide high recoveries and precision to determine antibiotic levels in a variety of solid environmental sample matrices (e.g., sewage sludge, animal manure, septic tank solids). One of our initial project objectives was to determine antibiotics concentration in manures and in soils subjected to frequent animal waste application. Due to the complexities involved in developing a procedure with widespread applicability, we were unable to make significant progress with this particular objective. However, as a part of our on-going research activities we anticipate analyzing several manure and soil samples to determine the concentration of particulate-bound antibiotics. Extraction and cleanup procedure for antibiotics will involve accelerated solvent extraction (ASE, available with Dr. Pedersen) followed by solid-phase extraction (SPE). Sewage sludge samples have been collected from the treatment plants listed in Table 1 and are being preserved in a freezer. Prior to extraction, the samples will be dried, finely ground and stored in amber bottles. Dried sludge samples will be extracted using ASE in various extraction mixtures for different classes of antibiotics.

### **CONCLUSIONS/IMPLICATIONS/RECOMMENDATIONS:**

Antibiotics were detected in wastewater influent/effluent, adjacent groundwater monitoring wells and in samples from on-site wastewater treatment systems. However, the soluble levels were extremely low ( $< 10 \mu\text{g/L}$ ), and importantly for the wastewater treatment systems were unaffected by the size of the facility. Future monitoring programs can be limited to the eight compounds detected in our study. Contrasting sorption behavior of tetracycline and ciprofloxacin to Al and Fe hydroxides was observed. Compared to tetracycline the following trends were observed for ciprofloxacin: (i) strong pH-dependence (ii) minor differences between LSC and HPLC measurements after both 1 day and 1 week, and (iii) higher removal level - maximum sorption of  $\approx 72\%$ , unaffected by reaction time, around neutral pH. Tetracycline sorption exhibits mild pH-dependence and a significant difference existed between LSC- and HPLC-determined removal levels, highlighting the importance of transformation reactions. Our research findings will increase understanding of the environmental occurrence, fate and transformation characteristics of these emerging organic contaminants.

### **REFERENCES:**

- Anderson, S.A., Yeaton Woo, R.W., and L.M. Crawford. 2001. Risk assessment of the impact on human health of resistant *Campylobacter jejuni* from fluoroquinolone use in beef cattle. Food Control. 12:13-25.
- Buser, H-R., Muller, M.D., and N. Theobald. 1998. Occurrence of the pharmaceutical drug clofibric acid and the herbicide mecoprop in various Swiss lakes and in the North Sea. Environ. Sci. Technol. 32:188-192.
- CVM. 1998. CVM approves fluoroquinolone product for use in cattle. FDA, Center for Veterinary Medicine, Rockville, MD [<http://www.fda.gov/cvm/infores/updates/fluoroquinolone.htm>].
- Daughton, C.G., and T.A. Ternes. 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environ. Health Perspectives. 107:907-938.

- Dunn, D.S., Raghavan, S., and R.G. Volz. 1994. Ciprofloxacin attachment to porous-coated titanium surfaces. *J. Appl. Biomaterials*. 5:325:331.
- Endtz, H.P., Ruijs, G.J., van Kungeren, B., Jansen, W.H., van der Reyden, T., and R.P. Mouton. 1991. Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. *Antimicrobial Agents & Chemo*. 27: 199- 208.
- Gao, J.P., Maguhn, J., Spitzauer, P., and A. Kettrup. 1998. Sorption of pesticides in the sediment of the Teufelsweiher pond (Southern Germany). I: Equilibrium assessments, effect of organic carbon content and pH. *Wat. Res*. 32:1662-1672.
- Gaunt, P.N., and J.V. Piddock. 1996. Ciprofloxacin resistant *Campylobacter* spp. in humans: an epidemiological and laboratory study. *Antimicrobial Agents and Chemotherapy*. 37:747-757.
- Golet, E.M., Strehler, A., Alder, A.C., and W. Giger. 2002. Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. *Anal. Chem*. 74:5455-5462.
- Halling-Sorensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lutzhoft, H.C., and S.E. Jorgensen. 1998. Occurrence, fate and effects of pharmaceutical substances in the Environment – A review. *Chemosphere*. 36:357-393.
- Halling-Sorensen, B., Sengelov, G., and J. Tjornelund. 2002. Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria, including selected tetracycline-resistant bacteria. *Arch. Environ. Contam. Toxicol*. 42:263-271.
- Hirsch, R., Ternes, T., Haberer, K., and K.-L. Kratz. 1999. Occurrence of antibiotics in the aquatic environment. *Sci. Total Environ*. 225:109-118.
- Huang, P.M., Wang, T.S.C., Wang, M.K., Wu, M.H., and N. Hsu. 1977. Retention of phenolic acids by noncrystalline hydroxy-aluminum and -iron compounds and clay minerals of soils. *Soil Sci*. 123:213-219.
- Huang, Z., Huang, H. and C. Ruxiu. 1997. Fluorescence investigation of some fluoroquinolones in various media: ground-state acid-base equilibria. *Anal. Sci*. 13.
- Ibsen, K.H., and M.R. Urist. 1967. Competition between oxytetracycline and citrate for binding sites on hydroxyapatite crystals. *Calc. Tiss. Res*. 1:243-245.
- Kladivko, E.J., Van-Scoyoc, G.E., Monke, E.J., Oates, K.M., and W. Pask. 1991. Pesticide and nutrient movement into subsurface tile drains on a silt loam soil in Indiana. *J. Environ. Qual*. 20:264-270.
- Kolpin, D.W., Furlong, E.T., Meyer, M.T., Barber, L.B., and S.D. Zaugg. 2000. A national reconnaissance for pharmaceuticals and other emerging contaminants in U.S. streams. In *Emerging Issues Conference Abstracts*, Minneapolis, MN [<http://toxics.usgs.gov/regional/emc.html>].



- Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., and H.T. Buxton. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. *Environ. Sci. & Technol.* 36:1202-1211.
- Levy, S.B. 1998. The challenge of antibiotic resistance. *Sci. Am.* 278:46-53.
- Martin, V.C.A., and G. Goisis. 2000. Nonstoichiometric hydroxyapatite-anionic collagen composite as support for the double sustained release of gentamicin and norfloxacin/ciprofloxacin. *Artificial Organs.* 24:224-230.
- Matthies, M., and H. Behrendt. 1991. Pesticide transport modeling in soil for risk assessment of groundwater contamination. *Toxicol. Environ. Chem.* 31/32:357-365.
- Meyer, M.T., Bumgarner, J.E., Varns, J.L., Daughtridge, J.V., Thurman, E.M., and K.A. Hostetler. 2000. Use of radioimmunoassay as a screen for antibiotics in confined animal feeding operations and confirmation by liquid chromatography/mass spectrometry. *Sci. Total Environ.* 248: 181-187.
- Moody, D.W. 1990. Groundwater contamination in the United States. *J. Soil Water Conserv.* 45:170-179.
- Nowara, A., Burhenne, J., and M. Spiteller. 1997. Binding of fluoroquinolone carboxylic acid derivatives to clay minerals. *J. Agric. Food Chem.* 45:1459-1463.
- Ofoefule, S.I., and M. Okonta. 1999. Adsorption studies of ciprofloxacin: evaluation of magnesium trisilicate, kaolin and starch as alternatives for the management of ciprofloxacin poisoning. *Boll. Chim. Farma.* 138:239-242.
- Schwertmann, U. and R.M. Cornell. 2000. Iron oxides in the laboratory: Preparation and Characterization. Wiley, New York, pp 104-112.
- Smith, K.E., Besser, J.M., Hedburg, C.W., Leano, F.T., Bender, J.B., Wicklund, J.H., Johnson, B.P., Moore, K.A., and M.T. Osterholm. 1999. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992-1998. *New England J. Medicine.* 340:1525-1532.
- Stephens, C.R., Murai, K., Brunings, K.J., and Woodward, R.B. 1956. Acidity constants of tetracycline antibiotics. *J. Amer. Chem. Soc.* 78.
- Thurman, E.M., and K.A. Hostetler. 1999. Analysis of tetracycline and sulfamethazine antibiotics in ground water and animal-feedlot wastewater by high performance liquid chromatography/mass spectrometry using positive-ion electrospray. In Conference Proceedings: Effects of Animal Feeding Operations (AFOs) on Water Resources and the Environment. Fort Collins, CO [<http://water.usgs.gov/owq/AFO/proceedings/afo/proceedings.html>].
- Tolls, J. 2001. Sorption of veterinary pharmaceuticals in soils: A review. *Environ. Sci. Technol.* 35:3397-3406.
- Truman, C.C., and R.A. Leonard. 1991. Effects of pesticide, soil, and rainfall characteristics on potential pesticide loss by percolation – A GLEAMS simulation. *Trans. ASAE.* 34:2461-2468.

Watts, C.D., Craythorne, M., Fielding, M., and C.P. Steel. 1983. Identification of non-volatile organics in water using field desorption mass spectrometry and high performance liquid chromatography. pp 120-131. Presented at the 3<sup>rd</sup> European Symposium on Organic Micropollutants in Water (ed.) G. Angeletti and A. Bjorseth. DD. Reidel Publishing Co – Dordrech.