Water Resources Research Institute of The University of North Carolina

Report No. 356

METHOD DEVELOPMENT FOR THE OCCURRENCE OF RESIDUAL ANTIBIOTICS IN DRINKING WATER

By
Howard S. Weinberg and Zhengqi Ye
Department of Environmental Sciences and Engineering
School of Public Health
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-7431

and

Michael T. Meyer
U.S. Geological Survey
Lawrence, Kansas 66049-3839

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ABSTRACT

More than 50 million pounds of antibiotics are produced in the United States each year, approximately one-half of which are prescribed for human use and one-half for agriculture. When used with livestock (for example, swine, poultry, and cattle), the majority of these antibiotics are given in subtherapeutic doses as feed additives to enhance growth. Antibiotics are also used in aquaculture and sprayed on fruit trees to inhibit fungal growth. These findings have raised concern, not only that pharmaceutical compounds will find their way into our water systems, but also that they may have detrimental effects on ecological and human health. Because little is known about the occurrence, fate, and transport of antibiotics in the environment, it is important to begin assessing where they may occur and at what levels. Some of these compounds have been detected at low concentrations in some of the lakes, rivers, and ground waters in Europe and across the United States but no surveillance of source waters that supply drinking water treatment plants had been previously undertaken in North Carolina. With evidence that these chemicals survive wastewater treatment and given the geographical location of many of the state's drinking water treatment plants downstream of wastewater treatment plant (WWTP) discharges, this study set out to evaluate whether pharmaceutical compounds persisted in North Carolina drinking water.

This study set out to first develop and validate analytical methods for determining the occurrence of 22 antibiotics in raw (source) and finished drinking waters. The methods that evolved accounted for interferences from natural organic matter (NOM) and residual disinfectants. The methods were applied in waters from the drinking water treatment plants of several members of the North Carolina Urban Water Consortium (NCUWC). Some of the source waters at these plants were likely impacted by upstream WWTP effluents, while others were located in watersheds in which there are large concentrations of animal feeding operations. Quantitation limits down to 10 ng/L for some of the target antibiotics enabled some preliminary evaluation of the effectiveness of conventional treatment on their apparent removal. Overall, fluoroquinolones were the most frequently detected in source waters, followed by sulfonamides, lincomycin, tetracyclines, and macrolides. Although the detection of ciprofloxacin, which is used only in human medication, seems to indicate impact from human wastewater discharges, the possibility of contamination from animal sources should not be excluded, as the veterinary fluoroquinolone enrofloxacin can, under certain conditions, be metabolized to ciprofloxacin. In most of the treatment plants the finished waters either chlorinated or chloraminated, showed either no detectable antibiotic residues or very much reduced levels. However, it is not known whether these compounds were physically removed during treatment, such as by adsorption to filters or precipitation in coagulation basins, or were merely chemically converted during disinfection processes. Any of these pathways may contribute to accumulation of the target compounds in the

environment, and it is essential that their fate and transport mechanisms be better understood if human health is not to be compromised.

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SUMMARY AND CONCLUSIONS

After extensive development, evaluation, and validation of analytical methodologies for the quantitation of antibiotics in environmental waters, samples of raw and finished water from several drinking water plants within the NCUWC were analyzed for 22 antibiotic residues. The results of these analyses provide the first snapshot of antibiotic occurrence in intakes for drinking water plants in North Carolina. The levels mirror those concentrations found in studies carried out by the U.S. Geological Survey (USGS) in other parts of the United States - albeit at lower concentrations in our study but surprising in the consistency with which specific antibiotics were found. Ciprofloxacin occurred and most frequently in retrospect, this might be expected because this drug is now more heavily prescribed as a broad-screen antibiotic. As a potent drug, Ciprofloxacin is expected to survive conventional wastewater treatment and persist to some degree in the environment. The parts-per-trillion levels found in surface waters, while extremely low due to a combination of dilution effects and adsorption onto sediments, are of concern as they indicate prevalence in other parts of the environment. Moreover, the discovery of these residues in source waters that utility managers hitherto expected to find no documented upstream discharges is troubling as it points to the possibility of non-point sources such as those suggested above as non-trivial contributors to the pollutant load. This study also provided the first occurrence data of antibiotic residues in finished drinking waters in North Carolina. The trends for each treatment plant, while indicating some degree of removal during drinking water treatment, indicate that removal is not always consistent and that conventional treatment may not protect the consumer against daily exposure to low levels of these and other so-called "emerging" contaminants. Antibiotics are probably as a good a surrogate indicator of chemical contamination by synthetic organics in drinking water as any other group of compounds; as communities continue to take advantage of the therapeutic and life-supporting benefits of drugs, many of these compounds will be found in the environment. The onus for protecting drinking water from such contamination lies with utilities, which will have to assess the capabilities of their current and alternate treatment processes in removal at the source.

RECOMMENDATIONS

Pharmaceutical compounds are currently not regulated as contaminants in water supplies. However, there is much interest in their presence in surface water and groundwater. Antibiotics are of particular concern because of the development of antibiotic-resistant bacteria and potential immunosuppressant properties. Also, many pharmaceutical compounds have similar chemical functional groups to pesticides and appear to not be efficiently removed by conventional treatment processes. Federal agencies such as the Centers for Disease Control and U.S. Environmental Protection Agency are beginning to conduct studies to assess occurrence of antibiotic residues and bacterial resistance in relation to potential exposure pathways that may impact human or ecosystem health.

The levels of antibiotics found in raw-water intakes at the drinking water plants of NCUWC members suggest that these drugs are in the aquatic environment as a result of inadequate wastewater treatment and/or nonpoint source runoff from agricultural operations such as confined animal feeding operations. Moreover, some of these compounds are also surviving drinking water treatment with the result that small quantities are most certainly being consumed on a daily basis by the population at large. The long-term health implications of these exposures are unknown, but there are some initial, though controversial, suggestions that their occurrence in the aquatic environment could contribute to the growth of antibiotic-resistant bacteria that could threaten human and animal life in the future. As indicators of the presence of other synthetic chemicals, there is probably a variety of other drugs and heavily used synthetic chemicals in source drinking waters that utilities have very few tools to control. Ongoing research is investigating the use of alternative wastewater and drinking water treatments for the removal of the activity of the chemicals. It is recommended that NCUWC members participate in as many of these studies as possible, so that they can prepare themselves to soon make either subtle or significant changes to their treatment processes to address these concerns. The combination of increased population growth; increase in the aging population; residence of a large proportion of the population in rural communities; dependency on agriculture and animal feeding operations; and the connectivity between upstream WWTPs and downstream drinking water plant intakes create unique needs for North Carolina utilities. With the likely future increase in extreme natural events, such as drought, that will require more conservation and reclamation of water, it is particularly essential that utilities consider the possibility of accumulation of potentially harmful chemicals and take steps to attempt to remove them either at the source or at the point of contact with treatment process.

The next steps in this process could be twofold: (1) evaluate pilot-scale operations using modifications to existing treatment to control the fate of the contaminants using surrogate chemicals, and (2) use the preliminary data from this study and a follow-up study as suggested in (1) to permit a tailored collaboration through a larger agency with more resources to support the unique needs of North Carolina utilities.

This research team stands ready to support the NCUWC in taking the next steps to protect the environment and, in particular, drinking water from contaminant accumulation.

1.0 INTRODUCTION

1.1 Cause for Concern

Natural and synthetic antimicrobials are of human health concern because of increased bacterial resistance possibly caused by the presence of antibiotic residues in the environment. Some of these compounds are known to have immunosuppressant properties. Since this group of pharmaceutically active compounds (PhACs) is among the most frequently used in human therapy and animal husbandry, more effort has been expended in developing techniques for detection of PhACs in the environment, both by microbial and immunoassay techniques. As with other PhACs, a large amount of the ingested dosage is excreted in an unmetabolized form and finds its way into the environment via sewage plants, runoff, and groundwater contamination especially, from confined animal feeding operations (CAFOs).

More than 50 million pounds of antibiotics are produced in the United States each year, approximately one-half of which are prescribed for human use and one-half for agriculture (Levy 1998). Approximately 40 percent of the antibiotics produced are used for livestock (for example, swine, poultry, and cattle). The majority of these antibiotics are given in subtherapeutic doses as feed additives to enhance growth (Levy 1998). Antibiotics are also used in aquaculture and sprayed on fruit trees to inhibit fungal growth. When administered in human medicine, the active compounds are often only partially metabolized (Table 1) and are then excreted via urine or feces (Forth et al. 1992). In this way, quantities of antibiotics are likely to reach WWTPs; it has been demonstrated (Ternes 1998) that up to 80% of the incident concentrations pass through the plant and consequently reach receiving streams. In addition to impacting stream ecology comes in contact, if the wastewater discharge is upstream of a drinking water treatment plant, it may produce unacceptable levels of antibiotics in the raw water intake. Furthermore, if the stream flows into a lake or other surface water source, these residues may accumulate in the basin, affecting the use of the water for both recreational and drinking purposes (Kolpin et al. 2004).

The interest in pharmaceutical compounds in the environment is fairly new. For the most part, suitable methods have only recently been developed to concentrate and analyze these compounds at levels at which they probably occur in the environment. These methods have had a limited application in recent reconnaissance studies of stream water and liquid waste and surface waters and groundwater that drain CAFOs and indicate that antibiotics can be transported from potential sources into our nation's water resources (Kolpin et al. 1999; Meyer et al. 1999a-b; Meyer et al. 2000). The extent to which drinking water supplies and drinking water treatment facilities are impacted from upstream sources, such as hospitals, pharmaceutical manufacturers, and CAFOs that have the potential to release pharmaceutical compounds into the environment is, for the most part, not known.

Table 1. Human Prescription Dosages of Antibiotics and Excretion Rates

Substance	Prescribed daily doses 1995 (Mio)	Daily dose (mg)	Prescribed amount (t/a)	Excretion in % Unchanged	Glucuronide	Other metabolites
Amoxicillin	34	750–2250	25.5–127.5	80–90	–	10–20
Ampicillin	0.6	3000–6000	1.8–3.6	30–60	–	20–30
Penicillin V	70	2000	140	~40	–	~60
Oxacillin	–	–	–	~40	–	~60
Penicillin G	0.1	240–720	1.8–3.6	50–70	–	30–50
Dicloxacillin	–	–	–	~65	–	~20
Sulfamethoxazole	41.6	400–1600	16.6–76	~15	–	–
Trimethoprim	41.6	80–360	3.3–15	~60	–	–
Erythromycin	19.8	200–1000	3.9–19.8	>60	–	–
Roxithromycin	20.8	150–300	3.1–6.2	>60	–	–
Clarithromycin	10.5	125–250	1.3–2.6	>60	–	–
Chloramphenicol	20.1	–	–	5–10	70–90	–
Chlortetracyline	1.9	–	–	>70	–	–
Tetracycline	–	–	–	80–90	–	–
Minocycline	8	100–200	0.8–1.6	~60	–	~40
Oxytetracycline	–	–	–	>80	–	–
Doxycycline	80.2	100–200	8–16	>70	–	–

Reproduced from Ternes et al. 1998.
Mio= million
t/a = tonnes per year

Examples of antimicrobials that have been found in some groundwater in Germany at low microgram/Liter levels (Hirsch et al. 1999) include macrolides, sulfonamides, penicillins (which are susceptible to hydrolysis), and tetracyclines (which can precipitate with calcium and other cations). These findings have raised concern, not only that pharmaceutical compounds are present in our water systems, but also that they may have detrimental effects on ecological and human health. For example, it has been shown that tetracycline-resistant bacteria in swine outflow can pass this resistance on to bacteria commonly found in soil (Golet et al. 2003; Halling-Sorensen et al. 2002). Because little is known about the occurrence, fate, and transport of antibiotics in the environment, it is important to begin assessing where they may occur and at what levels. Additionally, the ecological or human health effects from long-term exposure to low levels of antibiotics have not been studied.

There is currently no data on the occurrence of antibiotics in North Carolina drinking water supplies, many of which may be impacted by upstream wastewater discharges. There are pockets across the state where heavy antibiotic use is prevalent. Studies in Europe have shown that such pockets increase the incidence of antibiotic residues in drinking water plants, so this suggests that antibiotic residues are present in some of the raw water sources. Whether these contaminants can survive conventional drinking water treatment and persist into finished water is unknown.

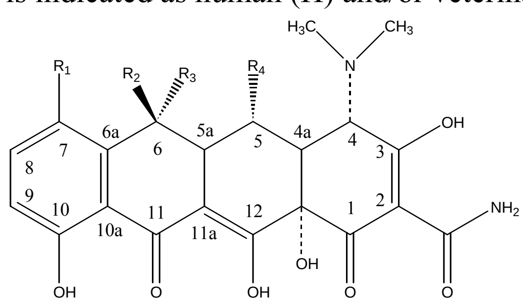
1.2 Background

Antibiotics are widely used in human and veterinary medicine and as animal growth promoters, and can enter the environment via various pathways. They have been detected in the aquatic environment at levels from ng/L to sub µg/L. As microcontaminants, antibiotics in the environment can potentially be transported to drinking water supplies. To enable environmental risk assessment, it is important to know the occurrence level of antibiotics in drinking water sources and also to understand their fate in drinking water treatment.

1.2.1 Major -Use Antibiotics

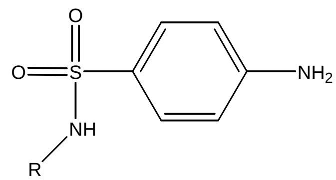
Antibiotics are classified according to their chemical and structural properties. Major antibiotics used in humans and animals include sulfonamides, tetracyclines, β-lactams (penicillins and cephalosporins), fluoroquinolones, macrolides, aminoglycosides, lincosamides, and diaminopyrimidines. Among the top 200 prescribed human use drugs in U.S. in 2002 (www.rxlist.com/top200.htm) are 14 antibiotics belonging to the previously described classes. Antibiotics are also heavily used in animal husbandry (i.e. animal feeding operations and CAFOs) and aquaculture for treating or preventing diseases and promoting growth.

Generic structures and some examples of tetracyclines, sulfonamides, and β-lactams are shown in Figures 1-3. Structures of some fluoroquinolones, aminoglycosides, and macrolides are given in Figures 4-6. Structures of trimethoprim and lincomycin, which represent the groups diaminopyrimidines and lincosamides, are shown in Figure 7. The application of each antibiotic is indicated as human (H) and/or veterinary (V).

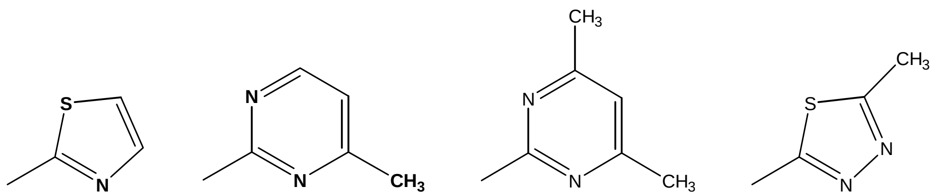


	—R_1	—R_2	—R_3	—R_4	Application
Minocycline	$N(CH_3)_2$	H	H	H	H
Oxytetracycline	H	OH	CH_3	OH	H, V
Tetracycline	H	OH	CH_3	H	H, V
Demeclocycline	Cl	OH	H	H	H
Chlortetracycline	Cl	OH	CH_3	H	H, V
Doxycycline	H	H	CH_3	OH	H

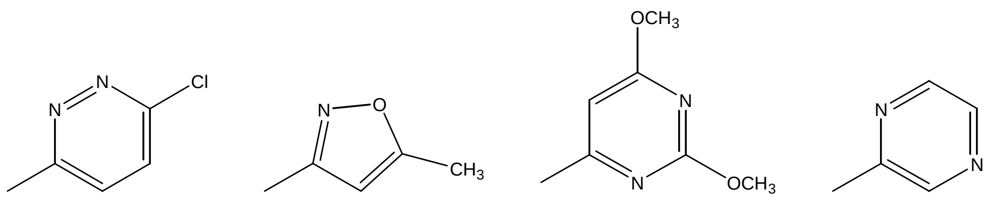
Figure 1. Structures of Tetracyclines



R group



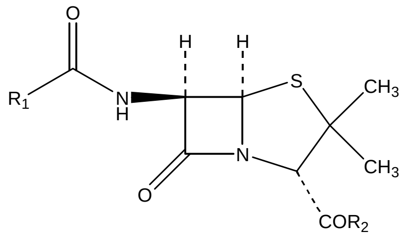
Sulfathiazole (H, V)	Sulfamerazine (V)	Sulfamethazine (V)	Sulfamethizole (H, V)

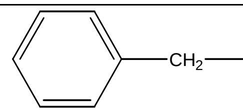
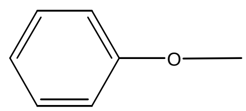
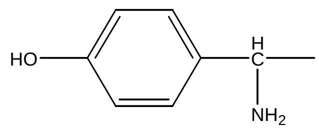
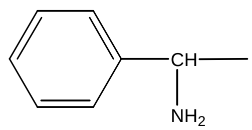


Sulfachlorpyridazine (V)	Sulfamethoxazole (H, V)	Sulfadimethoxine (V)	Sulfadiazine (H, V)

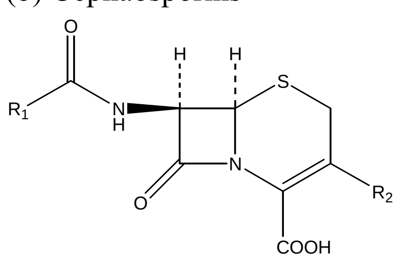
Figure 2. Structures of Sulfonamides

(a) Penicillins



	R_1	R_2	Application
Penicillin G		—OH	H, V
Penicillin V		—OH	H
Amoxicillin		—OH	H, V
Ampicillin		—OH	H, V

(b) Cephaosporins



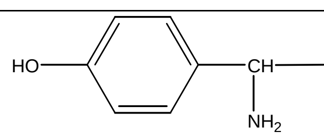
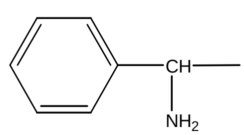
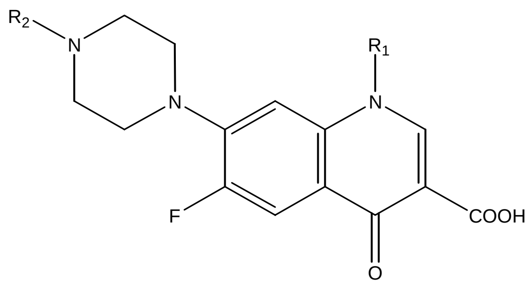
	R_1	R_2	Application
Cefprozil		—CH=CH—CH_3	H
Cephalexin		—CH_3	H

Figure 3. Structures of β-lactams



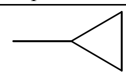
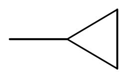
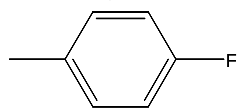
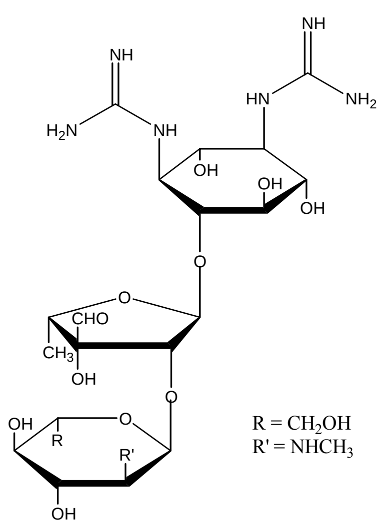
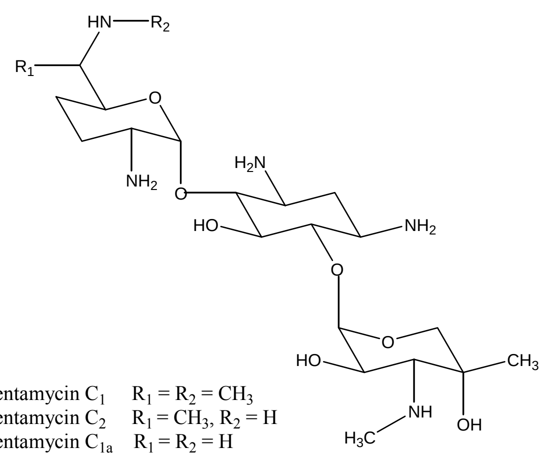
	R_1	R_2	Application
Ciprofloxacin		—H	H
Norfloxacin	—C_2H_5	—H	H
Enrofloxacin		—C_2H_5	V
Sarafloxacin		—H	V

Figure 4. Structures of Some Fluoroquinolones



R = CH_2OH
R' = $NHCH_3$

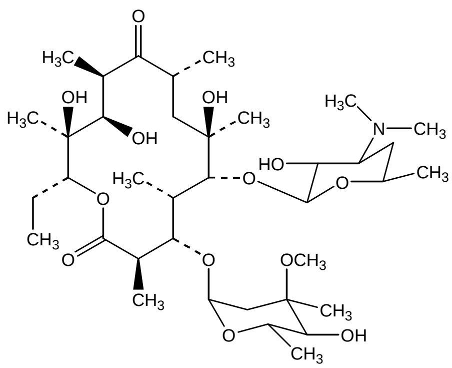


Gentamycin C_1 $R_1 = R_2 = CH_3$
Gentamycin C_2 $R_1 = CH_3$, $R_2 = H$
Gentamycin C_{1a} $R_1 = R_2 = H$

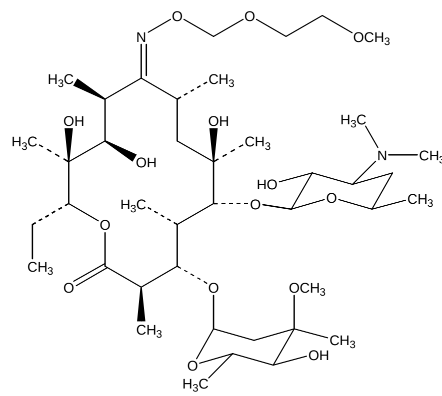
Streptomycin (H, V)

Gentamycin (H, V)

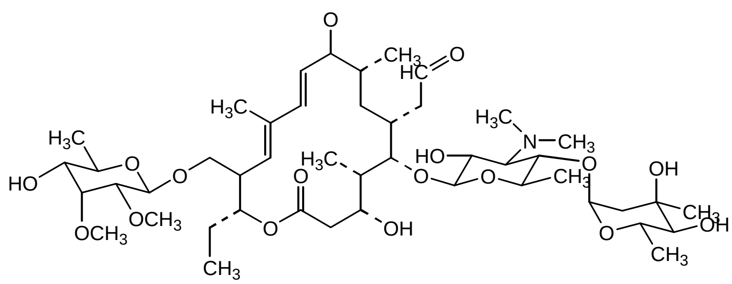
Figure 5. Structures of Some Aminoglycosides



Erythromycin (H, V)

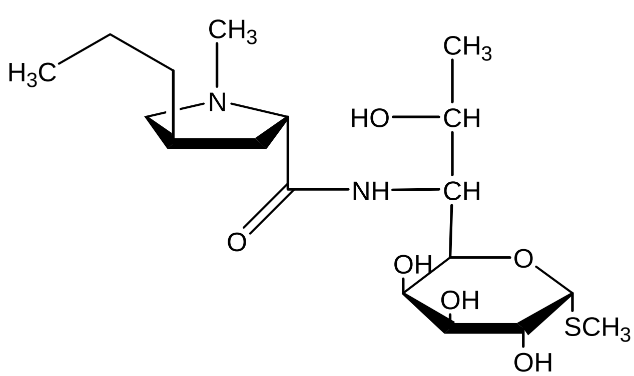


Roxithromycin (H)

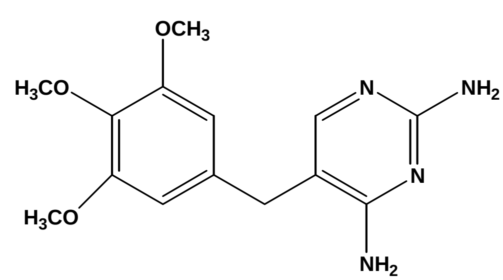


Tylosin (V)

Figure 6. Structures of Some Macrolides



Lincomycin (H, V)



Trimethoprim (H, V)

Figure 7. Structures of Lincomycin and Trimethoprim

According to the American Hospital Formulary Service Drug Information (AHFS) (McEvoy 2003), a benzene ring with a sulfonamido group and a primary amino group para to the sulfur side-chain are essential for antibacterial activity of sulfonamides; the carboxyl and ketone groups are essential for antimicrobial activity of fluoroquinolones; for penicillins, the cleavage at any point in the penicillin nucleus, including the β-lactam ring, results in complete loss of their antibacterial activity. The major cause of inactivation of β-lactams is hydrolysis of the β-lactam ring.

1.2.2 Pathways into the Environment

After human consumption and excretion, the residual antibiotics end up in sewage systems. They can be discharged into the receiving waters via wastewater effluent if not completely removed in treatment or concentrated in biosolids that may, subsequently, be used in land application. Antibiotics have been detected in treated municipal wastewater effluents at sub µg/L or µg/L (Hirsch et al. 1999; Hartig et al. 1999; Golet et al. 2001; Mcardell et al. 2003), indicating their incomplete removal in wastewater treatment.

Residual antibiotics in animal waste, which are stored in lagoons or dispersed on field as fertilizer, may leach into soil and be transported to groundwater. They may also enter surface water via runoff or water exchange. Surface waters and groundwater proximal to some CAFO sites were found to be contaminated with antibiotics at concentrations up to 10 µg/L (Campagnolo et al. 2002).

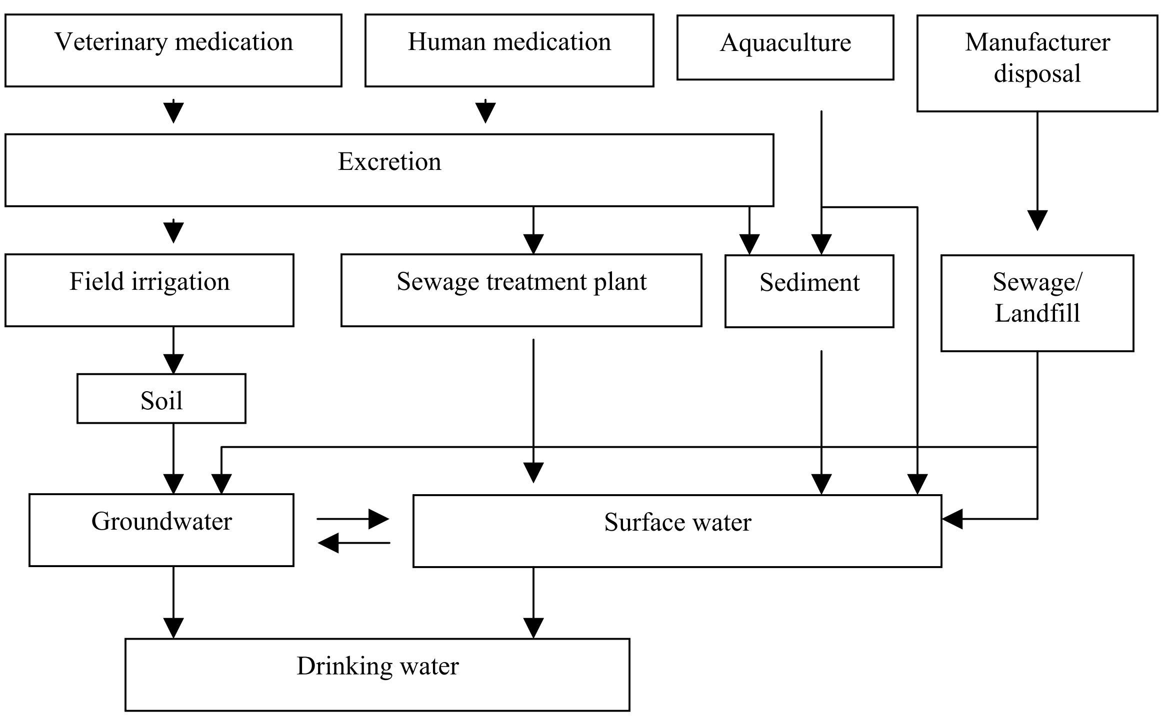


Figure 8. Possible Pathways of Antibiotics into the Aquatic Environment

When applied in aquaculture, the unused antibiotics end up in the sediments, where they are either degraded (Lai et al. 1995) or slowly leach back into the water (Smith and Samuelsen 1996). Oxytetracycline and sulfadimethoxine were detected at 0.17 to 10 μg/L and 0.20 to 1.2 μg/L, respectively, in fish hatcheries (Thurman et al. 2002). Antibiotics could also enter the environment by leaching through landfills (Barnes et al. 2004) or from disposal after antibiotic production (Hirsch et al. 1999). The pathways of antibiotics into the aquatic environment are proposed in Figure 8.

1.2.3 Physiochemical Properties

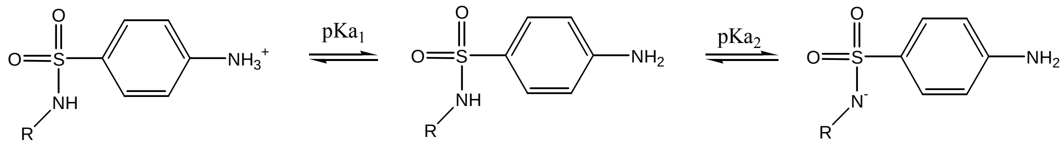
Once released into the environment, antibiotics are subject to various natural attenuation processes, including dilution, sorption, hydrolysis photodegradation, and biodegradation. Their physiochemical properties (Table 2) influence their fate and transport in natural and engineered environments. The water solubility (S_w) measures the tendency of the compound to remain in water. The octanol-water distribution coefficient K_{ow} indicates the tendency of the compound to partition into organic phase. The relatively high water-solubility and low K_{ow} values of these antibiotics, as given in Table 2, indicate their low hydrophobicity. The acidity constant pKa determines the speciation of a compound (ionic versus nonionic) in water as a function of pH. The speciation patterns of some antibiotics are shown in Figure 9. At environmental pH (6-9), sulfonamides are mainly present in anionic and neutral forms; tetracyclines are present in zwitterionic and anionic forms; fluoroquinolones are present in zwitterionic, anionic, and neutral forms; macrolides are present in neutral and/or protonated forms.

Table 2. Physicochemical Properties of Selected Antibiotics

Compound	S_w (mg/L) [(a)] (25°C)	Log K_{ow} [(a)]	pKa			
			pKa_1	pKa_2	pKa_3	Reference
Oxytetracycline	1,000 [(b)]	-1.22	3.27	7.32	9.11	(b)
Tetracycline	231	-1.30	3.3	7.68	9.69	(b)
Chlortetracycline	630	-0.62	4.5	n.a.	9.68	(c)
Penicillin G	5,000-10,000	n.a.	2.7	n.a.	n.a.	Prescott and Baggot 1993
Amoxicillin	4,000[(d)]	0.61[(c)]	2.6	6.9		(c)
Cefprozil	n.a.	0.15 [(c)]	2.7	7.4	9.7	(e)
Cephalexin	1,790	0.65	n.a.			
Ciprofloxacin	11,500	0.28	6.43	8.49		Barbosa et al. 2001
Norfloxacin	280 [(e)]	-1.03	6.34	8.75		(e)
Enrofloxacin	n.a.	1.1 [(b)]	6.27	8.3		(c)
Sarafloxacin	100 [(b)]	n.a.	6.0	8.6		(c)
Sulfathiazole	372	0.05	2.3	7.2		(c)
Sulfamerazine	202	0.14	1.58	6.98		(c)
Sulfamethazine	1,500	0.89	2.65	7.65		(b)
Sulfamethoxazole	610	0.89	1.69	5.57		(e)
Erythromycin	1.44	3.06	8.88			(a)
Tylosin	n.a.	3.5 [(b)]	7.1			(b)
Streptomycin	n.a.	-2.5	7.4			(c)
Trimethoprim	400	0.91	1.32	7.45		Cao and Cross 1995; Cocco et al. 1983
Lincomycin	927	0.56	7.6			(d)

n.a.: not available. Data source: (a) Syracuse Research Corporation 1995, unless otherwise stated; (b) Tolls 2001; (c) Chemical Abstract Registry Database 2004; (d) Merck Index 1996; (e) McEvoy 2003.

(a)



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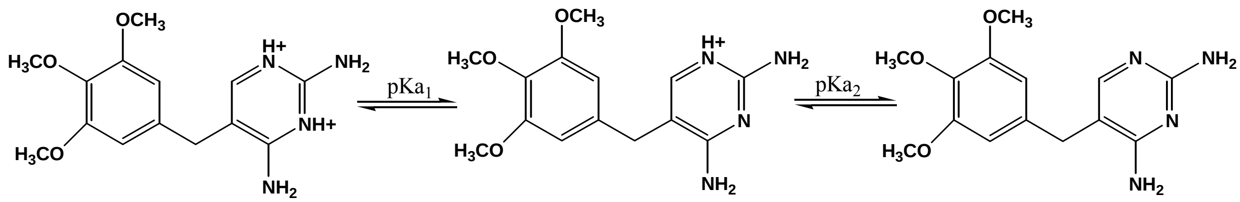

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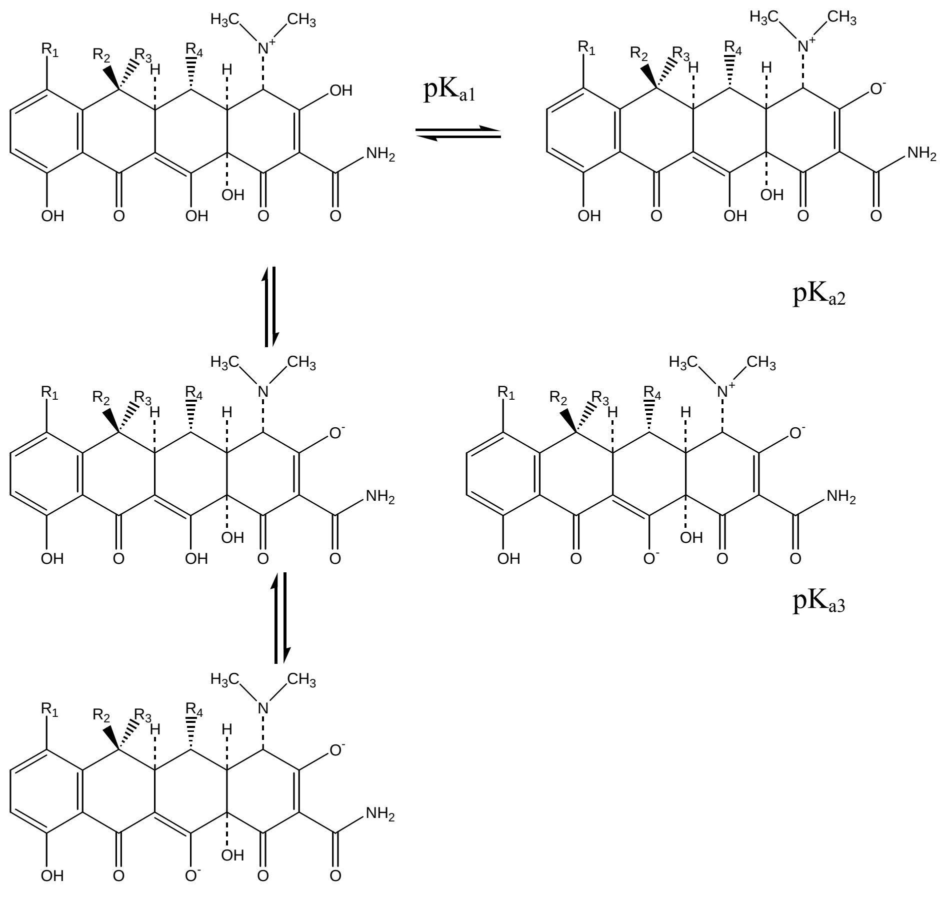


Figure 9. Speciation of Antibiotics: (a) sulfonamides; (b) trimethoprim; (c) tetracyclines; (d) relevant fluoroquinolones (continued)

(d)

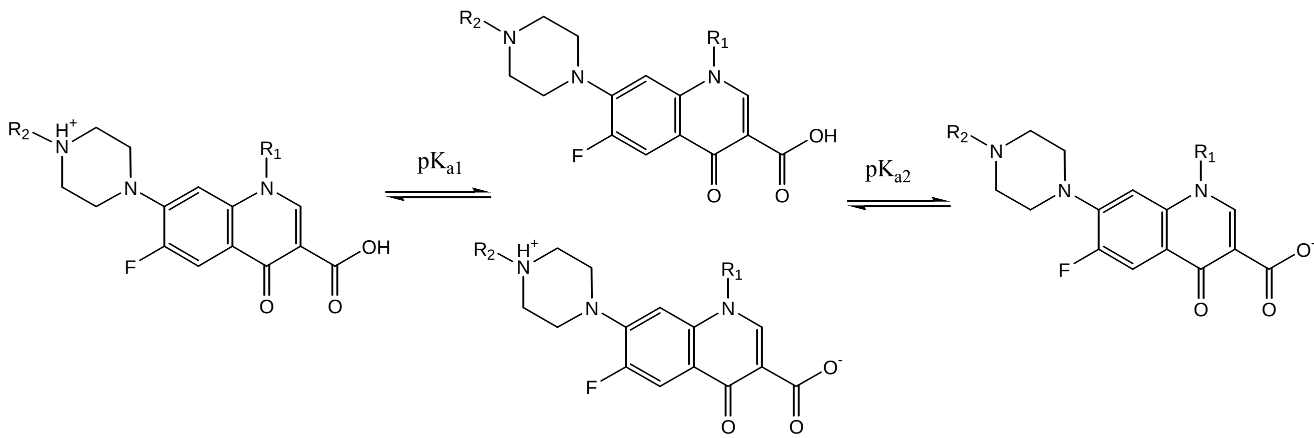


Figure 9. (cont.) Speciation of Antibiotics: (a) sulfonamides; (b) trimethoprim; (c) tetracyclines; (d) relevant fluoroquinolones.

1.2.4 Sorption and Mobility

In general, organic pollutants that have weak sorption to soil or sediments are more likely to leach into groundwater or transport in surface water. In addition, sorption of organic pollutants to dissolved organic matter (DOM) may facilitate their transport in water.

1.2.4.1 Sorption to Soil and Sediments. Sorption of organic molecules to soil and sediments involves various mechanisms, including association with natural organic matter (NOM), interaction with mineral surfaces, and electrostatic attraction to surface sites. In groundwater environments where the organic fraction of the aquifer solids is very small, association of organic pollutants with mineral surfaces may become significant. The solid-water distribution coefficient, K_d, indicates the degree of sorption of the organic compound to soil and sediments. Experimental data of K_d, as given in Table 3, are available for some antibiotics from different groups, except for β-lactams and aminoglycosides.

The multifunctionality and ionic nature of tetracyclines and fluoroquinolones suggest various mechanisms involved in their sorption to soil and sediments, which leads to their strong sorption as evidenced by the experimentally measured high K_d values (Table 3). Hydrophobic interaction with NOM is not likely a major sorption mechanism for these compounds, as their high K_d values are beyond the prediction using a model simply based on hydrophobic interaction (Tolls 2001). At environmental pH values, the positive-charged moieties in tetracyclines and fluoroquinolones are likely to be electrostatically attracted to NOM that is negatively charged. Tetracyclines and fluoroquinolones might also associate with NOM through hydrogen bonding (Lindsey et al. 2001) or cation bridging (Tolls 2001), respectively. These compounds may form complexes with multivalent ions in the mineral surface (Tolls 2001). Considering their pH-dependent speciation and ionic interaction with NOM or mineral surfaces, the sorption of tetracyclines and fluoroquinolones could be affected by several factors in the soil environment, including pH, ionic strength, and NOM concentration. (Figueroa et al. 2004).

Table 3. Sorption Coefficients of Antibiotics in Soils, Sediments, and Slurry (Modified from Thiele-Bruhn 2003)

Antibiotic	Concentration (μg/g)	Sample / pH	K_d (L/Kg)	Reference
Oxytetracycline	2.5-50	loamy sand / 6.1	680	Rabølle and Spliid 2000
	2.5-50	sand / 5.6	670	
Tetracycline	n.a.	SOM / 4.55	1,620	Sithole and Guy 1987a, 1987b
	n.a.	SOM / 6.14	1,140	
Ciprofloxacin	2-200	loamy sand / 5.3	427	Nowara et al. 1997
Enrofloxacin	2-200	clay / 4.9	3,037	Nowara et al. 1997
	2-200	loam / 5.3	5,612	
Tylosin	1.25-25	loamy sand / 6.1	128	Rabølle and Spliid 2000
	1.25-25	sand / 5.6	10.8	
Sulfamethazine	0.2-25	sand / 5.8	1.2	Langhammer and Büning-Pfaue 1989
	0.2-25	loamy sand / 5.2	3.1	
Sulfachlorpyridazine	0.05-20	clay loam / 6.5	1.8	Boxall et al. 2002
	0.05-20	sandy loam / 6.8	0.9	
Sulfadimethoxine	1.0-10	silt loam / 7.0	2.3	Tolls 2001

SOM = soil organic matter

In contrast, experimental data of K_d were relatively low for sulfonamides and macrolides. Hydrophobic interaction with NOM was suggested to be the major mechanism for their sorption (Tolls 2001) because their sorption coefficients fit well with the model prediction based on partitioning into NOM. The higher K_d values of macrolides as compared to those of sulfonamides could be explained by the additional electrostatic attraction of positive-charged macrolides to negatively charged NOM, which is not likely to happen for sulfonamides that predominantly exist in neutral and anionic forms at environmental pH of 6-9.

1.2.4.2 Sorption to Dissolved Organic Matter. Organic pollutants can associate with DOM via the same mechanism as their sorption to NOM in particular phase. $K_{d,\ DOM}$ values of tetracyclines and fluoroquinolones show their strong sorption to DOM at certain pH range (Table 4). The relatively low $K_{d,\ DOM}$ of fluoroquinolones at pH 9.2 could be explained by electrostatic repulsion between negatively charged NOM and anionic fluoroquinolones.

Table 4. Sorption Coefficients of Antibiotics to Dissolved Organic Matter (DOM)

Compound	Sample/pH	$K_{d, DOM}$ (L/Kg)	Reference
Tetracycline	AHA/4.55	2,060	Sithole and Guy 1987a,
	AHA/6.14	1,430	1987b
Sarafloxacin	AHA/3-8	18,700 – 52,700	Holten Lützhøft et al. 2000
Ciprofloxacin	HAS/9.2	250	Schmitt-Kopplin et al. 1999
Enrofloxacin	HAS/9.2	110	

AHA = Aldrich humic acid
HAS = humic acid from a soil

Overall, mobility of sulfonamides and macrolides is expected to be relatively high, and these compunds are likely to leach through soil to groundwater if applied on fields. Tetracyclines and fluoroquinolones are more likely to be associated with soil and sediments. On the other hand, association of tetracyclines and fluoroquinolones with DOM may facilitate their transport in soil solutions and in waters. Sorption of antibiotics to soil and DOM decreases the fraction of free species that may be susceptible to degradation and therefore enhances their persistence in the environment.

1.2.5 Stability in Water

Penicillins hydrolyze fairly quickly under mild acidic and basic conditions (Hou and Poole 1969). The presence of unstable β-lactam ring in the penicillin structures make them susceptible to degradation. Natural penicilllins (penicillin G and penicillin V) are most stable in solution at pH range 6-7.2 and are rapidly inactivated at pH<5 or pH>8, while the semisynthetic amoxicillin and ampicillin are more resistant to acid-catalyzed hydrolysis than the natural penicillins (McEvoy 2003). In solution, most cephalosporins are stable for only short periods of time, unless frozen (McEvoy 2003). Overall, β-lactams are not likely to persist in natural waters at pH 6-9. Erythromycin is dehydrated under acidic conditions to form erythromycin-H_2O (Figure 10), which has been detected in the aquatic environment (Hirsch et al. 1999; Kolpin et al. 2002), though its degradation at neutral pH is very slow (Volmer and Hui 1998).

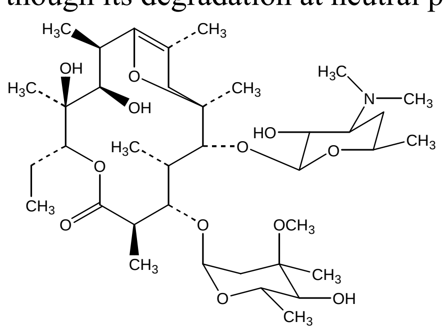


Figure 10. Structure of Erythromycin-H_2O

Tetracyclines can form anhydrotetracyclines at pH< 7.5 or 4-epitetracyclines by epimerization in weak acid (pH 3) (Figure 11). In general, aminoglycosides are stable at pH 2-11 and are most active at alkaline pH (McEvoy 2003).

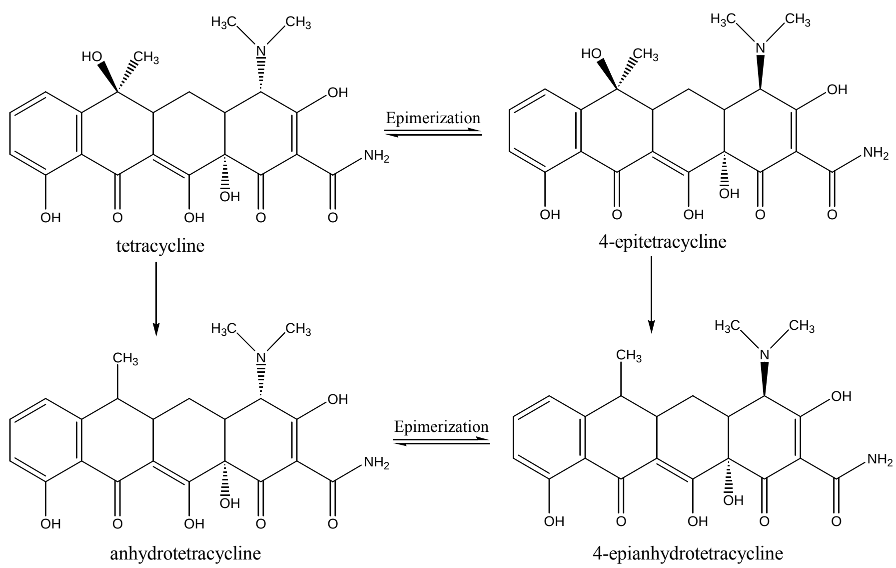


Figure 11. Transformation of Tetracycline in Water

1.2.6 Photostability

Many antibiotics, such as fluoroquinolones, sulfonamides, and tetracyclines, are susceptible to photodegradation. Ciprofloxacin was found to decompose photochemically in aqueous solutions at acidic pH under ultraviolet (UV) irradiation (Torniainen et al. 1997), forming two major products (Figure 12a). Sulfamethoxazole was found to be extremely susceptible to photodegradation in acidic aqueous solution under UV radiation, forming sulfanilic acid; aniline; 3-amino-5-methylisoxazole, a product from photoisomerization of the isoxazole ring; and a hydrated product (Zhou and Moore 1994). Trimethoprim undergoes oxidation at the benzyl position as well as hydrolysis of the amino groups in the pyrimidine ring and of the 4'-methoxy function under UV radiation (Figure 12b). Photodegradation of antibiotics under simulated environmental conditions has been reported. Oxytetracycline was found to experience photodegradation in direct sunlight, while sulfadiazine and sulfadimethoxine examined in the same study were photodegraded to a lesser degree (Sigve et al. 1993). Oka et al. (1989) studied photolysis of tetracyclines under conditions similar to a natural fish-culture pond: irradiation with wavelengths longer than 290 nm in air-saturated aqueous solution at pH 7.0 at 30ºC. Photodecomposition products were identified to be lactones and hydroxyl carboxylic acids.

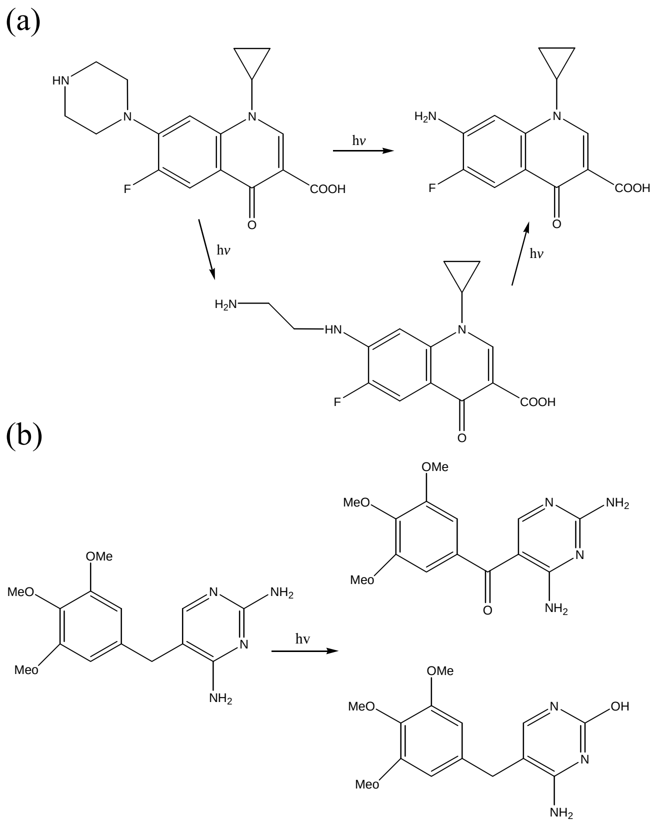


Figure 12. Photodegradation Products of (a) Ciprofloxacin and (b) Trimethoprim

The persistence of tetracyclines, sulfonamides, and fluoroquinolones could be affected by photolysis if they are present in the surface layers of water bodies that receive large amounts of sunlight. Their photodegradation is expected to decline with increasing water depth and turbidity. The association of antibiotics with subsurface sediments would increase their persistence due to inhibition of photodegradation.

1.2.7 Biodegradability

The persistence of antibiotics in soil is mainly affected by biological processes. The soil biomass consists of a large number of bacteria species and may have a great capacity for biodegrading antibiotics. On the other hand, sorption of antibiotics to particles reduces their bioavailability and thus the biodegradability.

Natural penicillins (penicillin V and penicillin G) are susceptible to the action of many β-lactamases in bacteria (McEvoy 2003). Al-Ahmad et al. (1999) assessed the biodegradability of some antibiotics, including ciprofloxacin, penicillin G, and sulfamethoxazole, by municipal wastewater bacteria. Penicillin G was found to be biodegradable to some degree (27%), while sulfamethoxazole and ciprofloxacin were less biodegradable. Gavalchin and Katz (1994) examined the stability of some antibiotics in soil and observed that their persistence was in the order of chlortetracycline > erythromycin > tylosin > penicillin and streptomycin. The removal of antibiotics was attributed to biodegradation. Marengo et al. (1997) reported very slow aerobic biodegradation of sarafloxacin.

Ingerslev and Nyholm (2001) conducted a primary biodegradation study of some veterinary antibiotics in aerobic and anaerobic surface-water simulation systems. Half-lives for aerobic degradation were determined to be 9.5-40 days for tylosin and 42-46 days for oxytetracycline, assuming first-order degradation kinetics. Biodegradation was significantly lower in the absence of oxygen. Addition of sediment or activated sludge from treated wastewater increased the biodegradation potential, which was mostly likely due to the increase of biomass concentrations in the test solution. The overall assessment of the results suggests that tylosin and oxytetracycline might be considered moderately persistent in surface-water systems.

1.2.8 Occurrence in Natural Waters

1.2.8.1 Analytical Methodologies and Occurrence Level. Due to their high water-solubility, antibiotics are compatible with liquid chromatography (LC) separation and can be detected by UV, fluorescence, or mass spectrometry (MS). MS detection is advantageous because of its high selectivity and ability to provide confirmation, as compared to conventional UV or fluorescence detection. Atmospheric pressure ionization (API), including atmospheric chemical ionization and electrospray, is currently the most widely used LC-MS interfacing technique (Niessen 1998). Solid phase extraction (SPE) is commonly used to concentrate analytes from ultra-trace levels in environmental samples to instrumentally detectable levels. LC electrospray tandem mass spectrometry (LC-ESI-MS/MS) provides high sensitivity and selectivity for analyzing compounds at trace levels in complex matrices, and this technique in combination with SPE has been increasingly applied to analysis of antibiotics in environmental samples (Hirsch et al. 1998; Hartig et al. 1999; Hilton and Thomas 2003; Vanderfold et al. 2003).

Occurrence studies of antibiotics were conducted in Europe and the United States in recent years; some data are summarized in Tables 5 and 6. The relatively frequent occurrence of sulfamethoxazole is consistent with the fact that it is the most used sulfonamide antibiotic in human medicine and the relatively high mobility and persistence predicted for sulfonamides in the environment. In contrast, none of the targeted penicillins were detected by Hirsch et al. (1999) and Sacher et al. (2001), which could be due to the predicted instability of penicillins in the environment. The nondetection of tetracyclines targeted in the groundwater survey (Lindsey et al. 2001) seems to correspond to their relative low mobility in the environment.

Table 5. Occurrence of Antibiotics in Surface Waters

Analyte	C_{max} (μg/L)	C_{median} (μg/L)	Reference
Sulfamethoxazole	0.48	0.03	Hirsch et al. 1999
	0.085		Hartig et al. 1999
	1.02		Lindsey et al. 2001
	1.9	0.15	Kolpin et al. 2002
	0.02		Vanderford et al. 2003
Sulfadimethoxine	0.24		Lindsey et al. 2001
Sulfamethazine	0.12		Kolpin et al. 2002
	0.22		Lindsey et al. 2001
Sulfathiazole	0.08		Lindsey et al. 2001
Oxytetracycline	1.34		Lindsey et al. 2001
Tetracycline	0.11	0.11	Kolpin et al. 2002
Chlortetracycline	0.69	0.42	
Ciprofloxacin	0.03	0.02	
Norfloxacin	0.12	0.12	
Erythromycin-H_2O	1.7	0.1	Kolpin et al. 2002
	1.7	0.15	Hirsch et al. 1999
	0.098		Vanderford et al. 2003
Tylosin	0.28	0.04	Kolpin et al. 2002
Roxithromycin	0.18	0.05	Kolpin et al. 2002
	0.56	0.20	Hirsch et al 1999
Trimethoprim	0.71	0.15	Kolpin et al. 2002
	0.20	0.09	Hirsch et al. 1999
	0.025		Vanderford et al. 2003
Lincomycin	0.73	0.06	Kolpin et al. 2002

Table 6. Occurrence of Antibiotics in Groundwater

Analyte	C_{max} (μg/L)	Reference
Sulfamethoxazole	0.41	Sacher et al. 2001
	0.22	Lindsey et al. 2001
Erythromycin-H_2O	0.049	Sacher et al. 2001

Although antibiotics have been detected in the aquatic environment in various occurrence studies, there is little information on whether and to what extent these microcontaminants are transported to our drinking water supplies and, if so, whether they are removed or transformed in drinking water treatment. Therefore, occurrence studies of a broad range of antibiotics in source and finished drinking waters are necessary to elucidate this issue.

1.2.8.2 Existing Analytical Methods. For a broad-range occurrence study, a method for analyzing multiple classes of antibiotics is needed. Subjected to various natural attenuation processes, antibiotics are expected to be present at low ng/L levels in water supplies, and even lower levels in treated water, requiring highly sensitive and selective methods for their detection.

Many methods for analysis of antibiotics in aqueous environmental samples were developed for determination of a single class of antibiotics, such as sulfonamides (Hartig et al. 1999), fluoroquinolones (Golet et al. 2001; Turiel et al. 2003), and macrolides (Mcardell et al. 2003), or for two classes of antibiotics, such as sulfonamides and tetracyclines (Lindsey et al. 2001; Yang and Carlson 2003) or tetracyclines and fluoroquinolones (Reverte et al. 2003).

Hirsch et al. (1998) and Sacher et al. (2001) developed SPE-LC-MS/MS methods for analyzing multiple classes of antibiotics, including sulfonamides, trimethoprim, penicillins, and macrolides, in environmental water samples using a single SPE method. However, the SPE method developed by Hirsch et al. (1999) failed to extract tetracyclines from water. Another major-use antibiotic group, fluoroquinolones, were not included as target analytes in both methods. In the USGS national reconnaissance survey (Kolpin et al. 2002), 21 target antibiotics, including tetracyclines, sulfonamides, fluoroquinolones, and macrolides, were extracted simultaneously using a tandem SPE method and detected by LC-MS with reporting limits varying from 20 to 100 ng/L for different groups. For occurrence studies in drinking water, however, a more sensitive method for detecting multiple classes of antibiotics needs to be developed based on the existing methods; and in order to provide accurate and reliable occurrence data, the current analytical methodologies, whose weaknesses will be described here, need to be improved.

Matrix effect on electrospray ionization (ESI) is commonly observed in LC-MS analysis. Co-eluting matrix components may reduce or enhance the ion intensity of the analytes and affect the reproducibility and accuracy of the assay. In analyses of surface water samples, NOM in water, which is often co-extracted in SPE and co-eluted with analytes in chromatography, is a major contributor to matrix effects on ionization of analytes. Ion enhancement and, more often, ion suppression have been observed in the analysis of antibiotics in water samples (Hirsch et al. 1999; Lindsey et al. 2001; Vanderford et al. 2003; Yang and Carlson 2003; Hilton and Thomas 2003).

Quantitation using solvent-based external standard calibration, as reported in some methods (Vanderford et al. 2003; Hilton and Thomas 2003), can generate biased results if matrix interferences are not accounted for in the method. Hirsch et al. (1998) and Sacher et al. (2001) used external calibration extracted in mountain spring water and tap water, respectively, to quantify the antibiotics, which compensates for the loss of analytes throughout the whole analytical processes and, to some degree, the signal variation in MS analysis. However, this quantitation method could still lead to biased results when applied to raw water with higher NOM content, causing more severe matrix effects. The method of standard addition has been used to compensate for the variation of signal in analysis of tetracyclines in raw water (Lindsey et al. 2001; Yang and Carlson 2003). Although this method could compensate for signal variation, it may not prevent loss of sensitivity due to ion suppression. The strategies to minimize matrix interference include (1) improved chromatographic separation of analytes from interferences and (2) improved sample preparation (Reemtsma 2001). In multiresidue analysis in a complex matrix, however, it may not be possible to separate a variety of analytes with differing physiochemical properties from matrix components. Therefore, it is worth investigating how to minimize matrix co-extraction in the solid-phase extraction process.

1.2.9 Fate and Transformation in Drinking Water Treatment

In order to provide a basis for health-risk assessment associated with antibiotics in source drinking waters, it is not only necessary to monitor the parent compounds and their metabolites, but also to track the fate and transformation of these compounds and their degradates in drinking water treatment. Conventional drinking water treatment processes generally include coagulation/flocculation, activated carbon adsorption, and disinfection using chlorine-based disinfectants. Antibiotics in raw water could partition into solid phase during coagulation or adsorption, or undergo chemical transformation during disinfection.

1.2.9.1 Coagulation. In the coagulation/flocculation process, coagulants destabilize colloids in water and promote the aggregation of small particles to larger ones that can be subsequently removed by sedimentation and filtration. Aluminum and ferric iron salts are commonly used coagulants. DOM is among the colloidal suspensions that can be removed by coagulation (AWWA 1999). Pollutants with strong affinity to natural organic colloids are likely to be removed together.

As discussed previously, tetracyclines and fluoroquinolones are likely to associate with DOM, which provides opportunity for being removed together during coagulation. Antibiotics such as sulfonamides and macrolides, are less likely to associate with DOM and are therefore not expected to be efficiently removed by coagulation. Adams et al. (2002) found that no significant removal of sulfonamides was achieved by coagulation in natural water systems at pH 6.8.

The fate of antibiotics in sludge/sediments produced from coagulation is also of concern, because residual antibiotics accumulated in sludge/sediments, which are often used as field fertilizer, may leach into soil and contaminate the environment.

1.2.9.2 Activated Carbon Sorption. Dissolved organic pollutants can be sorbed onto activated carbon and thus removed from water. The extent of adsorption of a compound is a function of its affinity to the activated carbon as compared to its affinity to water. Hydrophobic interaction is the main mechanism for the removal of most nonpolar organic compounds, while ionic interaction can remove polar ionic compounds (Snyder et al. 2003). Granular activated carbon (GAC) is more efficient than powdered activated carbon (PAC) in removing micropollutants, due to its greater surface area and adsorption capacity. NOM in water can decrease the capacity of GAC and PAC for adsorption of micropollutants by competing for the adsorption sites (Matsui et al. 2002). On the other hand, the association of polar, ionic micropollutants with NOM might provide an opportunity for being removed together with NOM.

Adams et al. (2002) studied the removal of sulfonamides and trimethoprim by PAC in deionized (DI) and natural water systems. The percent removal of each of the antibiotics in DI water ranged from 57% to 97% and 81% to 98% for PAC dosages of 10 and 20 mg/L, respectively. The percent removal of each of the antibiotics in natural water ranged from 49% to 73% and 65% to 100% for a PAC dosage of 10 and 20 mg/L, respectively. The results show that activated carbon adsorption is a promising technology for removal of sulfonamide and trimethoprim.

The adsorption of NOM to activated carbon can remove it from water. Karanfil et al. (2003) investigated the modification of GAC surface by creating specific functionalities and mesoporosity to enhance adsorption of DOM by enhancing ionic interaction. The enhanced basicity or acidity of GAC surface could also lead to stronger ionic interaction of polar, ionic micropollutants with GAC surface, hence enhancing their sorption and subsequent removal. Whether these modified GAC materials can facilitate the removal of antibiotics in raw water has not yet been examined.

1.2.9.3 Chlorine Disinfection. Chlorine disinfectants such as free chlorine and chloramines are widely used for drinking water disinfection among NCUWC member utilities. Free aqueous chlorine is formed by dissolving chlorine gas or hypochlorite into water. Chloramines are formed by reaction of free chlorine with ammonia. Monochloramine (NH_2Cl) predominates at pH>7.5 whereas dichloramine ($NHCl_2$) prevails down to pH 5, except at high ammonium concentrations, where it converts back to NH_2Cl. Trichloramine (NCl_3) occurs in appreciable concentrations only at pH<3.5 (Larson and Weber 1994).

Chlorine disinfectants are also oxidants that can react with many micropollutants. Free chlorine, a strong oxidant, is reactive toward many organic pollutants and produces chlorination byproducts. Chloramines, which are weaker oxidants, are expected to react much more slowly with organics (Rice and Gomez-Taylor 1986). Tetracyclines with phenolic structures are expected to be reactive with free chlorine. The presence of tertiary and/or secondary amino groups within the structure of fluoroquinolones would make this group of antibiotics quite susceptible to oxidative attack via chlorine as well. Similarly, the presence of primary amino groups in sulfonamides would promote their reactivity toward free chlorine.

The use of free chlorine was shown to be effective in "removing" sulfonamides from both distilled and natural water systems (Adams et al. 2002). Fifty micrograms per liter of each antibiotic was chlorinated by 1 mg/L of free chlorine. Ninety percent of sulfonamides were removed from distilled and natural water within 13 and 30 minutes, respectively. Chlorination byproducts were observed in the LC-UV monitoring of the parent compounds but were not structurally identified in this study.

Dodd (2003) studied oxidation of selected antibiotics by free available chlorine (FAC) and chloramines in buffered DI water and environmental water matrices. Second-order rate constants were measured to be 4.80×10^{2} $M^{-1}s^{-1}$, 1.53×10^{3} $M^{-1}s^{-1}$, and 4.74×10^{1} $M^{-1}s^{-1}$ for the oxidation of enrofloxacin, sulfamethoxazole, and trimethoprim by FAC at pH 7, respectively. Reaction pathways, given in Figures 13-15, were proposed based on a number of reaction products identified by LC-MS. The reactions of studied compounds with preformed chloramines were much slower compared to reaction with free chlorine. Chlorination of ciprofloxacin, sulfamethoxazole, and trimethoprim were also conducted in real water systems, and these compounds were substantially degraded by free chlorine under simulated water-treatment conditions. The target compounds were all spiked into aquatic matrices at elevated levels, leaving open the possibility of different reaction fates at environmentally relevant concentrations (ng/L to sub µg/L) under water-treatment conditions. Other major-use antibiotics, such as lincomycin, macrolides, and tetracyclines, have been detected in surface water and could potentially enter drinking water supplies, but the transformation of these compounds via chlorination has not yet been studied. It should also be noted that instead of using preformed chloramines, utilities often accomplish chloramination is often practiced by adding ammonia to chlorinated water. Given the rapid reaction of antibiotics with free chlorine, the antibiotics could be completely or partially transformed by the time they are in contact with chloramines. Hence, the effect of different uses of chloramines on degradation kinetics and resulting product formation remains unknown.

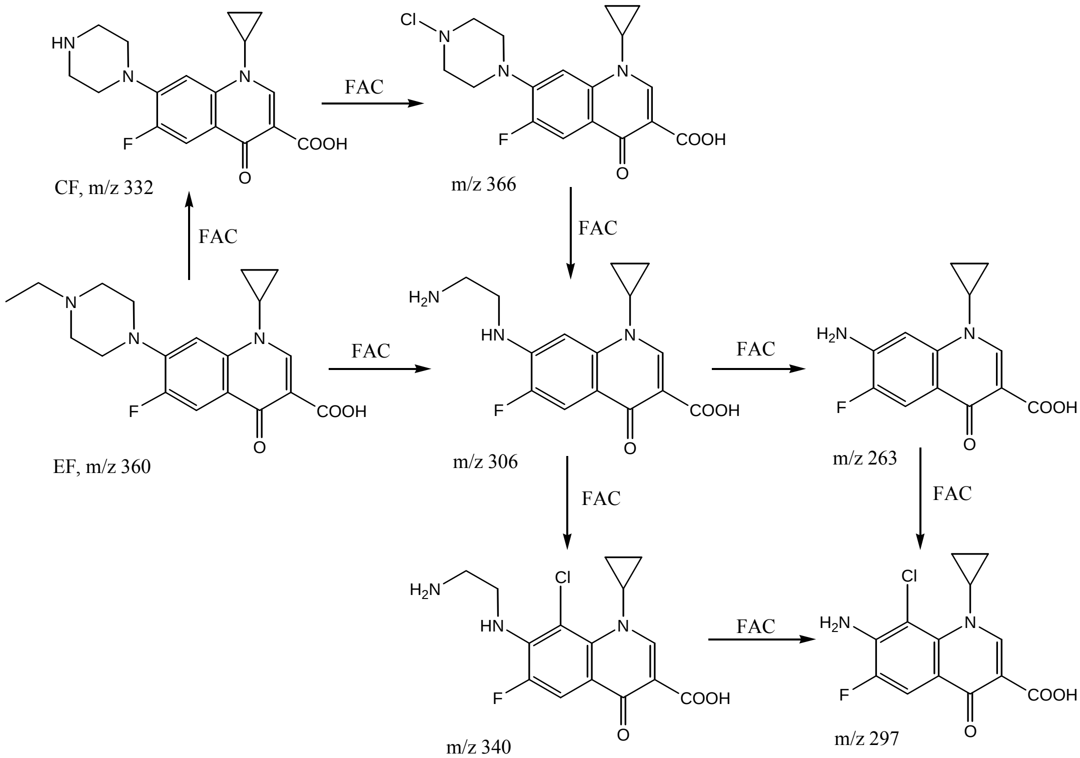


Figure 13. Proposed Reaction Pathways for Reaction of Fluoroquinolones with Free Available Chlorine (FAC) (Dodd 2003)

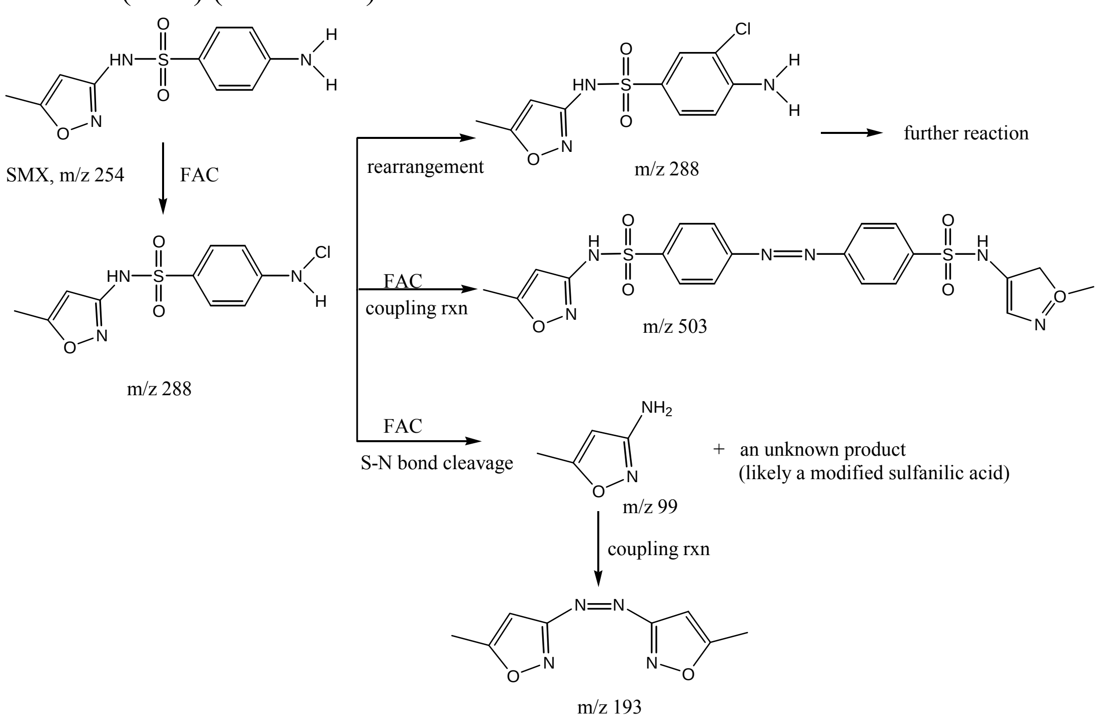


Figure 14. Proposed Reaction Pathways for Reaction of Sulfamethoxazole with Free Available Chlorine (FAC) (Dodd 2003)

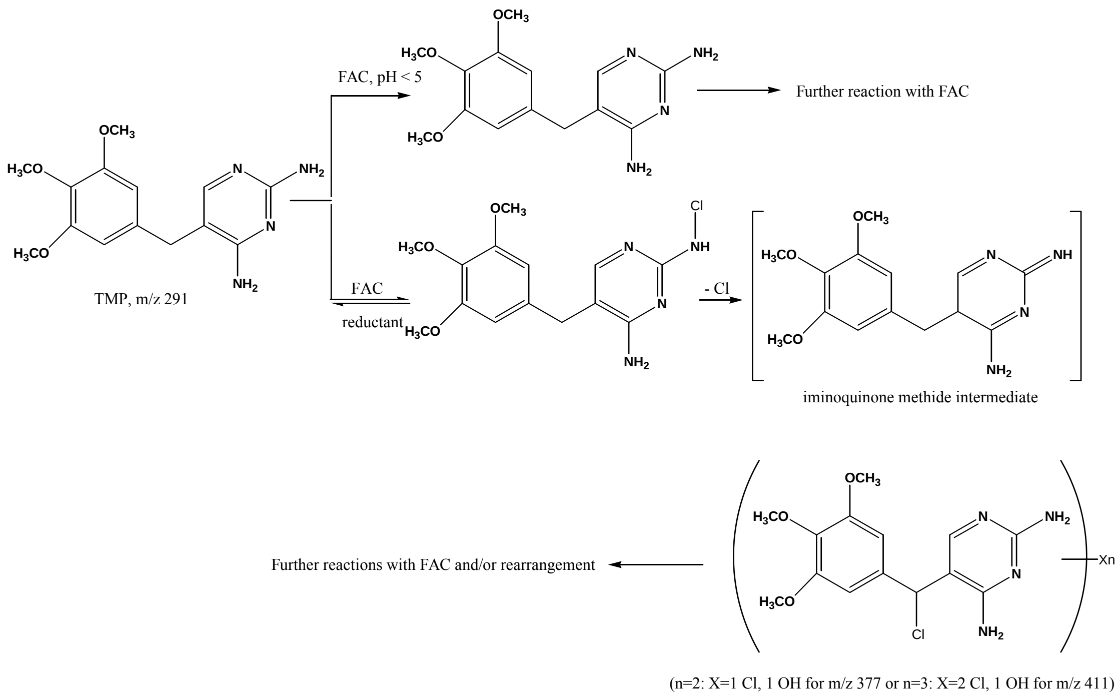


Figure 15. Proposed Reaction Pathways for Reaction of Trimethoprim with Free Available Chlorine (FAC) (Dodd 2003)

1.3. Objectives

The premises upon which this study was based included the following:

1. Many North Carolina drinking water treatment plants are located downstream of WWTP discharges and incomplete removal of antibiotics during wastewater treatment would cause antibiotic residues to be transported downstream.
2. Even if drinking water treatment plants are not downstream from a point source discharge, nonpoint sources may contribute antibiotic residues to surface waters.
3. Conventional drinking water treatment processes do not remove all of the antibiotics present in drinking water supplies that have intakes downstream of potential sources such as pharmaceutical manufacturers, WWTPs, hospitals, and CAFOs.
4. Antibiotic residues may be present in finished drinking water.

In order to examine these issues, the drinking water treatment plants of NCUWC members were surveyed for the presence of 22 antibiotics in raw and finished water. Beforehand, rugged analytical procedures were developed that accounted for interferences from NOM and residual disinfectants. The specific objectives were:

1. To determine the occurrence of antibiotics at the intakes of selected drinking water treatment facilities that are downstream of potential sources of where pharmaceutical compounds.
2. To determine the levels of antibiotics in finished drinking water at these facilities.

2.0 EXPERIMENTAL APPROACHES

This chapter introduces the different experimental approaches that will be subsequently developed. A detailed discussion of each follows in Chapter 3.

2.1 Analytical Method Development

The target analytes were selected from the major-use antibiotic groups, namely sulfonamides, tetracyclines, fluoroquinolones, and macrolides. For the purpose of determining the fate of single compounds in kinetic studies with chlorine or determining suitability of quenching agents, a simpler LC method with photodiode array (PDA) detection was evolved. However, for the analysis of target analytes in surface and finished waters, a more selective method was developed that involved LC with triple quad mass spectrometric detection (LC-MS/MS). For the purpose of simplifying extraction of all 22 target analytes, method development also focused on the use of a single SPE with sensitivity enhancement utilizing concentration of the extract.

2.1.1 LC with UV Detection Using A PDA

Separation and detection of the target antibiotics was performed on a Waters™ 616 liquid chromatography pump equipped with Waters 717plus autosampler and Waters™ 996 PDA. One hundred microliters of sample injection was made to a Supelco (Bellefonte, PA) Discovery C-18 (25 cm, 4.6 mm, 5 µm) LC column. The mobile phase and operating conditions were selected as a result of preliminary tests that were run to explore the optimum resolution of the target species. The mobile phase employed an isocratic 80%A:20%B (phase A was 0.3% formic acid in organic-free water; phase B was acetonitrile) stored in two separate glass-feed bottles and supplied to the column at a flow rate of 1mL/min by a mixing valve. The mobile phases were prepared by filtering the relevant solvents through 0.45 µm membranes before use. All of the analytes were eluted within 25 minutes and their response measured at a detection wavelength of 268 nm with the Waters Millennium software. Peak identification was based on comparing retention time of the analyte in SPE extracts to that of standards prepared directly in methanol, and quantification was based on comparing the peak area of the analytes in SPE extracts with the peak area of standards. The instrument detection limit (IDL) was estimated by analyzing a series of dilutions of the standard mixtures of analytes prepared in mobile phase and selecting the lowest concentration at which a signal response was detected significantly and consistently above background noise. At this stage in the study, we have not yet performed statistical analysis of detector responses.

2.1.2 LC-MS/MS Method

The LC-MS/MS method was developed on a Varian model 1200L triple quadrupole LC mass spectrometer (Varian, Walnut Creek, CA). The system consists of a ProStar 210 solvent delivery module connected to a ProStar 430 autosampler and 1200L triple stage quadruple mass spectrometer with a dual off-axis ESI interface. The target analytes were separated on a Pursuit C-18 column (15 cm, 2 mm, 3 µm) (Varian, Walnut Creek, CA) using gradient mobile phases. The ESI conditions, such as drying gas temperature and capillary voltage, were optimized to achieve efficient ionization of each analyte. Collision-induced dissociation (CID) using argon as collision gas was performed to obtain major product ions of each analyte and the optimal collision voltage for formation of each product ion. The two most intense product ions of each analyte were selected for monitoring in MS/MS. The collision gas pressure was then optimized to achieve maximum response of the fragment ions. The effect of the raw-water matrix on LC-MS analysis of individual analyte was then studied by comparing its response in a clean solvent matrix and in a raw-water extract.

2.1.3 Method Improvement for Sample Preparation

In the SPE, 250 ml of raw water or 500 ml of finished water sample was adjusted to a specific pH, then Na_2EDTA was added and the sample extracted onto a polymeric sorbent. The analytes were then eluted and further concentrated to about 250 µL and analyzed using LC-MS/MS methods. The surrogate standards were spiked into the aqueous sample before SPE to monitor the extraction process. An internal standard was spiked into the concentrated sample just before LC-MS/MS analysis to compensate for the variation in final sample volume.

For each antibiotic group, a surrogate standard was selected that is structurally similar to the analytes but not likely present in the environment. The internal standard was selected so that it eluted between analytes in the chromatography and ionized efficiently in ESI in addition to being absent in the water samples.

For extraction of raw-water samples, different eluting solvents were investigated in order to reduce co-elution with NOM. Samples extracted at acidic and neutral pH values were compared for the effect on reduction of NOM co-extraction.

The residual chlorine disinfectants in finished water were quenched at the time of sample collection. A quenching agent was selected that could efficiently quench chlorine disinfectants without interfering with the analysis of target analytes.

2.2 Occurrence Study

Raw and finished drinking water samples were collected from the conventional surface water treatment plants of NCUWC members. Raw-water samples were collected in the plant intake prior to any treatment, while finished water was collected after the final disinfection stage but before entering the distribution system. Samples of finished water were collected at a time lag after collection of the raw water, representing the approximate hydraulic residence time (HRT) of raw water through the treatment plant. The value of HRT was provided by plant operators.

2.3 Chlorination

A preliminary study was conducted to examine the reactivity of a specific antibiotic toward free chlorine. For each antibiotic, chlorination was conducted in buffered DI water at pH 7 with a molar ratio of free-chlorine-to-antibiotic varying from 1:1 to 10:1 or higher. The selected parent antibiotic is starting concentration was in the high μg/L range to allow ease of monitoring of the kinetics. The reaction mixture was sampled at specific time intervals, quenched by the appropriate quenching agent, and analyzed by LC-UV. The free chlorine concentration was determined at the end of monitoring.

3.0 RESULTS

3.1 Analytical Method Development

LC-UV methods were developed for kinetic experiments and for monitoring the decay of tetracyclines and sulphonamides in synthetic waters where single components in a relatively organic-free matrix were targeted. The occurrence-and-fate study in source and finished drinking water examined 22 antibiotics that represented the most widely used groups in both human and animal therapy and for which standards were readily available. A more sensitive and selective analytical method using LC-MS/MS was developed for the monitoring of these compounds that evaluated SPE using different eluting solvents and extraction pH values in order to reduce the co-extraction of interfering NOM. Additionally, the use of ascorbic acid as a disinfectant quenching agent for finished water-sample analysis was evaluated.

3.1.1 Materials

Tetracycline standards (minocycline, oxytetracycline, tetracycline, demeclocycline, chlortetracycline, doxycycline) in their hydrochloride form; sulfonamides (sulfathiazole, sulfamerazine, sulfamethazine, sulfamethizole, sulfachlorpyridazine, sulfamethoxazole, sulfadimethoxine), macrolides (tylosin tartrate, roxithromycin, erythromycin); trimethoprim; lincomycin hydrochloride; and meclocycline sulfosalicylate salt were purchased from Sigma (St. Louis, MO) and were all reagent grade. Ciprofloxacin hydrochloride was purchased from ICN Biochemicals, Inc. (Irvine, CA). Enrofloxacin hydrochloride (≥ 98%) was purchased from Fluka (Buchs, Switzerland). $^{12}C_6$-sulfamethazine was purchased from Cambridge Isotope Laboratories (Andover, MA). Simatone was purchased from Accustandards (New Haven, CT).

Stock solutions of ciprofloxacin, norfloxacin and enrofloxacin were individually prepared at 400 mg/L in water (HPLC grade): methanol (1:1 v/v, with 0.2% HCl v/v). The stock solution of sarafloxacin was prepared at 100 mg/L in HPLC-grade water. Stock solutions of other antibiotics were individually prepared at 400 mg/L or 1,000 mg/L in methanol.

Water, acetonitrile, and methanol (all HPLC grade) and formic acid (98%) were purchased from EM Science (Gibbstown, NJ). Isopropanol and dichloromethane were purchased from EMD Chemicals (Gibbstown, NJ). Diethyl ether was purchased from Mallinckrodt (Paris, KY). Ascorbic acid (100%) and sodium thiosulfate · $5H_2O$ (100%) were purchased from Fisher Chemicals (Fair Lawn, NJ). Sodium sulfite (99.5%), sodium hypochlorite reagent solution (10-13% active chlorine) and sulfuric acid (trace metal grade) were purchased from Fisher Scientific (Pittsburgh, PA).

3.1.2 LC-UV

Tetracyclines were eluted in the order of minocycline, oxytetracycline, tetracycline, demeclocycline, chlortetracycline, and doxycycline as illustrated in the chromatogram of Figure 16. Sulfonamides were eluted in the order of sulfathiazole, sulfamerazine, sulfamethazine, sulfamethizole, sulfachloro-pyridazine, sulfamethoxazole, and sulfadimethoxine as illustrated in the chromatogram of Figure 17.

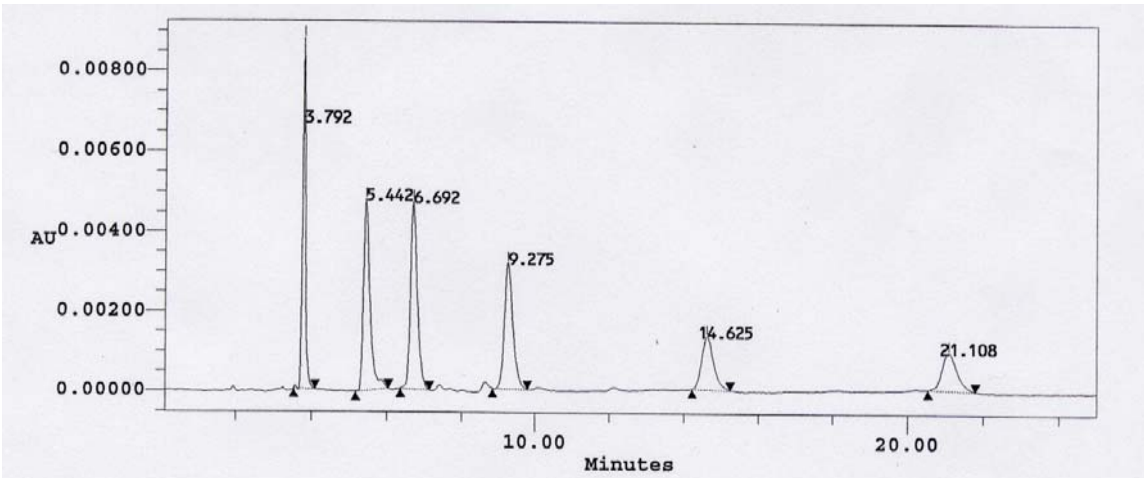


Figure 16. Separation of Tetracyclines (1mg/L each in mobile phase) by HPLC-UV. Order of Elution is Minocycline, Oxytetracycline, Tetracycline, Demeclocycline, Chlortetracycline, and Doxycycline.

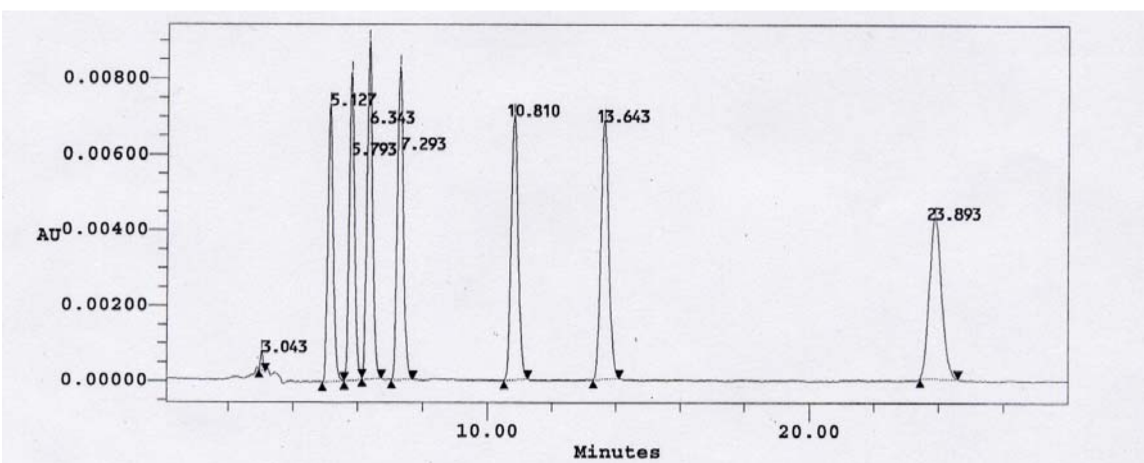


Figure 17. Separation of Sulfonamides (1mg/L each in mobile phase) by HPLC-UV. Order of Elution is Sulfathiazole, Sulfamerazine, Sulfamethazine, Sulfamethizole, Sulfachloro-pyridazine, Sulfamethoxazole, and Sulfadimethoxine.

A series of calibration standard solutions (prepared in mobile phase 80%A:20%B- see Section 2.1.1) were analyzed by HPLC-UV at concentrations of 0, 0.05, 0.1, and 1 mg/L for each tetracycline and 0, 0.01, 0.05, 0.1, and 0.5 mg/L for each sulfonamide. A plot of peak area against concentration yielded calibration curves with excellent regression coefficients. The IDL was estimated as the lowest non-zero calibration point of the calibration curve. For tetracyclines, this value was 50 µg/L (5 ng on column) and for sulfonamides, 10 µg/L (1 ng on column). However, when a 1:1000 concentration factor was used to extract settled water spiked with 10µg/L tetracycline mix, co-extracted NOM produced inferior chromatograms (see Figure 18), even though at pH 2.5, recoveries exceeded 80%.

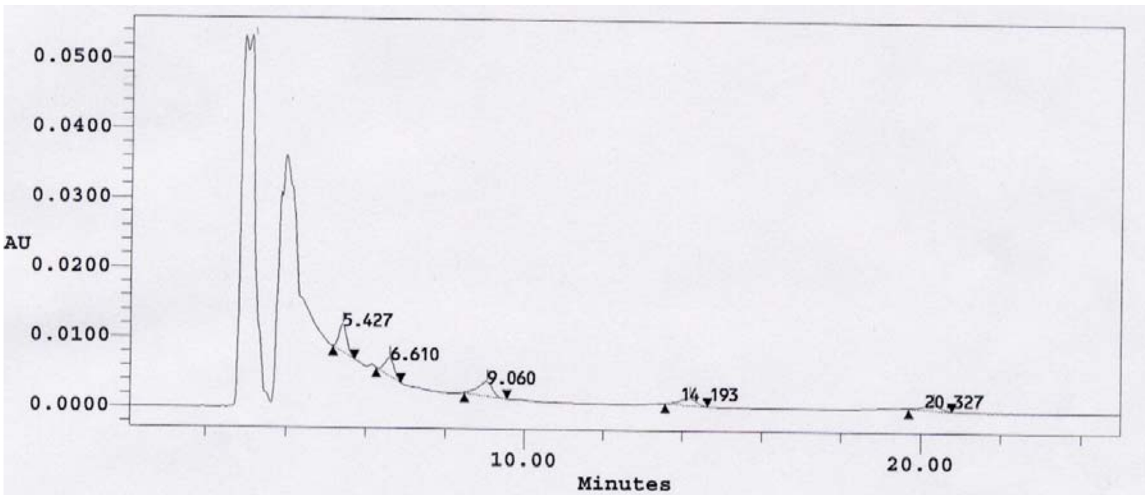


Figure 18. LC-UV Chromatogram of SPE extract of Tetracyclines from Settled Water (pH 2.5). Order of Elution is Oxytetracycline, Tetracycline, Demeclocycline, Chlortetracycline, and Doxycycline.

3.1.3 LC Separation for MS Analysis

The 22 antibiotics were separated on a Pursuit C-18 column (15 cm, 2 mm, 3 µm) (Varian, Walnut Creek, CA) using three chromatographic runs each with different gradient described in (Table 7) at a flow rate of 0.2 mL/min with an injection volume of 20 µL. The LC-MS/MS chromatograms are shown in Figure 19 for the analytes at 10 µg/L, each in a solvent mixture of 90% water (with 0.1% formic acid) and 10% methanol.

3.1.4 Optimization of ESI Response

All the analytes were ionized in positive ionization mode. Nitrogen was used both as a drying and nebulizing gas. The electrospray settings (Table 8), such as nebulizer needle position, drying gas temperature, and capillary voltage, were optimized by infusing a mixture of 22 antibiotics at 1 mg/L into the electrospray interface at a flow rate of 10µL/min using a Model 11 single syringe pump (Harvard, Holliston, MA), accompanied with a background mobile phase of 50% 0.1% formic acid:50% acetonitrile delivered by the LC pump at a flow rate of 0.2 mL/min.

Table 7. Gradient Elution Program for LC Separation of Antibiotics

(a) Tetracyclines

Time (min)	A (%)	B (%)
0	82	18
6	50	50
8	50	50
10	0	100
12	0	100
14	82	18
29	82	18

(b) Fluoroquinolones

Time (min)	A (%)	B (%)
0	90	10
22	0	100
24	0	100
26	90	10
41	90	10

(c) Others

Time (min)	A (%)	B (%)
0	85	15
8	50	50
12	50	50
14	0	100
16	0	100
18	85	15
33	85	15

Mobile phase A: 0.1% formic acid in water; B: acetonitrile

Others: sulfonamides, macrolides, lincomycin, and trimethoprim

3.1.5 Optimization of Product Ion Intensity

CID was conducted for each analyte using argon as collision gas. The optimal collision voltage for formation of a specific product ion was identified on the MS/MS breakdown curves, which show the change of product ion intensity as a function of collision voltage. The two most intense product ions of each analyte were selected for monitoring in MS/MS by comparing the maximum signal intensity of product ions in the MS/MS breakdown curves and by running a standard at a specific concentration (10 or 20 µg/L) with all the major product ions being scanned and comparing peak areas of the ions. The MS/MS methods are shown in Tables 9-11.

Table 8. Optimized ESI Conditions

Parameter	Optimal setting
Drying gas temperature	300˚C
Drying gas pressure	20 psi
Nebulizer gas pressure	50 psi
ESI chamber temperature	50˚C
Shield voltage	600 V
Needle voltage	5,000 V
Capillary voltage	60 V

Table 9. MS/MS Method for Tetracyclines

Analyte	Parent ion $[M+H]^+$	Product ion	Collision energy (V)
Minocycline	458	441	18
		283	41
Oxytetracycline	461	443	11
		426	17
Tetracycline	445	427	12
		410	18
Demeclocycline	465	448	17
		430	26
Chlortetracycline	479	444	19
		154	24
Doxycycline	445	428	17
		154	24
Meclocycline	477	460	18

Table 10. MS/MS Method for Sulfonamides, Macrolides, Trimethoprim, and Lincomycin

Analyte	Parent ion $[M+H]^+$	Product ion	Collision energy (V)
Lincomycin	407	126	23
		359	18
Sulfathiazole	256	156	13
		108	20
Sulfamerazine	265	156	16
		108	22
Sulfamethazine	279	186	16
		92	21
Trimethoprim	291	230	23
		123	21
Sulfamethizole	271	156	12
		108	19
Sulfachlorpyridazine	285	156	15
		92	23
Sulfamethoxazole	254	156	14
		108	20
Sulfadimethoxine	311	156	19
		108	24
Erythromycin-H_2O	716.4	158	22
		558	16
Tylosin	917	174	32
		101	38
Roxithromycin	838	158	26
		159	26
$^{13}C_6$-sulfamethazine	285	186	18

Table 11. MS/MS Method for Fluoroquinolones

Analyte	Parent ion $[M+H]^+$	Product ion	Collision energy (V)
Norfloxacin	320	<u>276</u>	17
		233	23
Ciprofloxacin	332	<u>288</u>	17
		245	23
Enrofloxacin	360	<u>316</u>	18
		245	25
Sarafloxacin	386	<u>342</u>	17
		299	26

The effect of collision gas pressure on the signal intensity of major product ions of each analyte was investigated by infusing an antibiotic mixture at 1 mg/L into the ESI at a flow rate of 10 µL/min while monitoring the intensity of product ions at collision gas pressures between 1.6 and 3.3 mTorr. For each analyte, the intensity of major product ions increases as collision gas pressure increases from 1.6 to 2.0 mTorr. Fluoroquinolones show maximum intensity at 2.7-3.0 mTorr. Macrolides, sulfonamides, and tetracyclines mostly show maximum intensity at 2.7 mTorr, but the intensity decreased as the collision gas pressure further increased to 3.3 mTorr.

The effect of collision gas pressure was further studied in the presence of background mobile phase (Figure 20). A mixture of 22 antibiotics at 1 mg/L was infused into the ESI at 1 mg/L at a flow rate of 10µL/min, accompanied by a background mobile phase of 50% 0.1% formic acid: 50% acetonitrile at a flow rate of 0.2 mL/min. The intensity of the product ions was monitored at argon pressures 2.0, 2.6, and 3.0 mTorr. Overall, 2.6 mTorr appeared to be an optimal collision gas pressure.

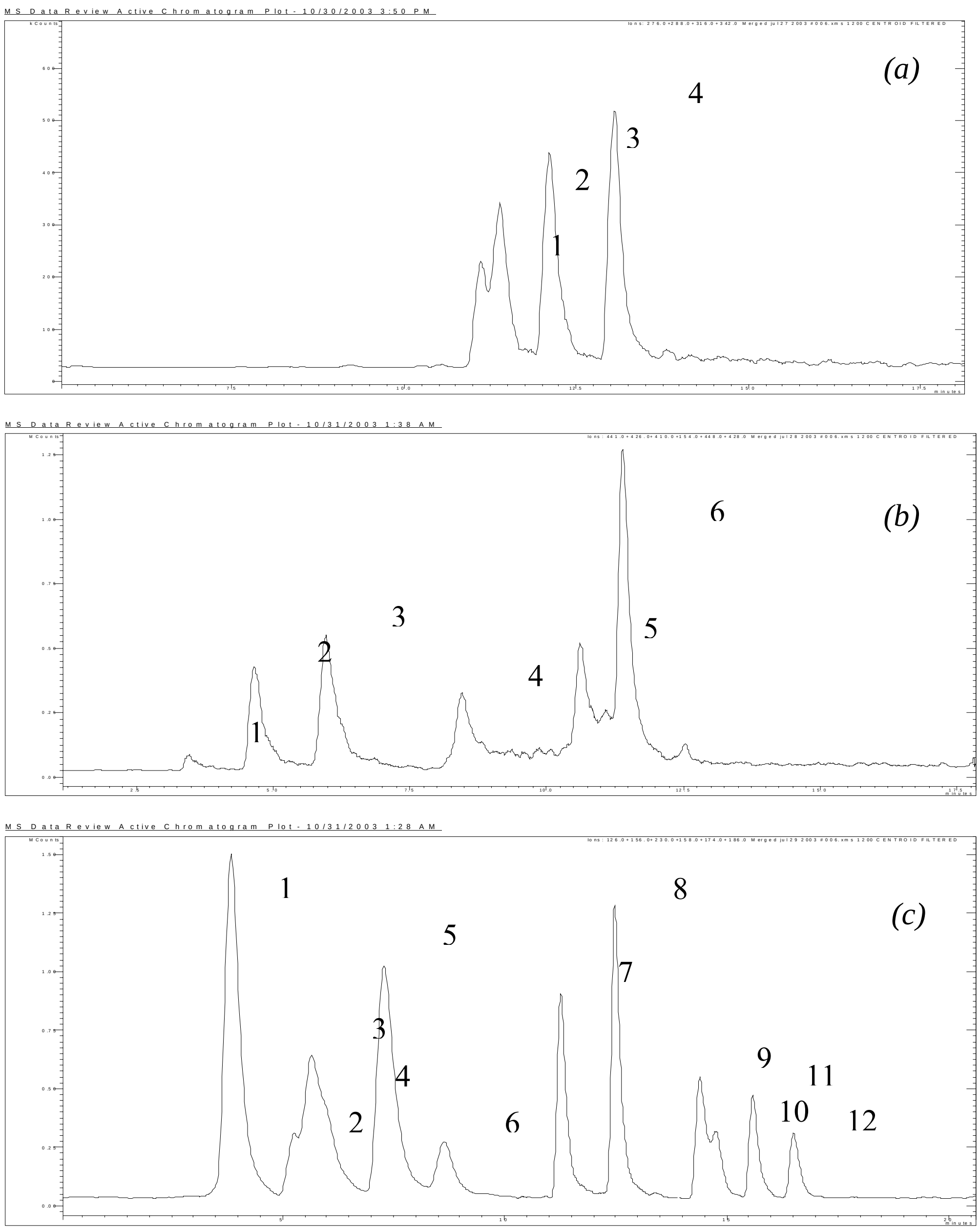


Figure 19. LC-MS/MS Chromatograms of Antibiotics at 10 µg/L in Mixture of Water (0.1% Formic Acid)/Methanol (9:1 v:v)

(a) Tetracyclines: 1. minocycline; 2. oxytetracycline; 3. tetracycline; 4. demeclocycline;
(b) 5. chlortetracycline; 6. doxycycline
(b) Fluoroquinolones: 1. norfloxacin; 2. ciprofloxacin; 3. enrofloxacin; 4. sarafloxacin
(c) Others: 1. lincomycin; 2. sulfathiazole; 3. trimethoprim; 4. sulfamerazine; 5. sulfamethazine; 6. sulfamethizole; 7. sulfachlorpyridazine; 8. sulfamethoxazole; 9. sulfadimethoxine;
(d) 10. erythromycin-H_2O; 11. tylosin; 12. roxithromycin

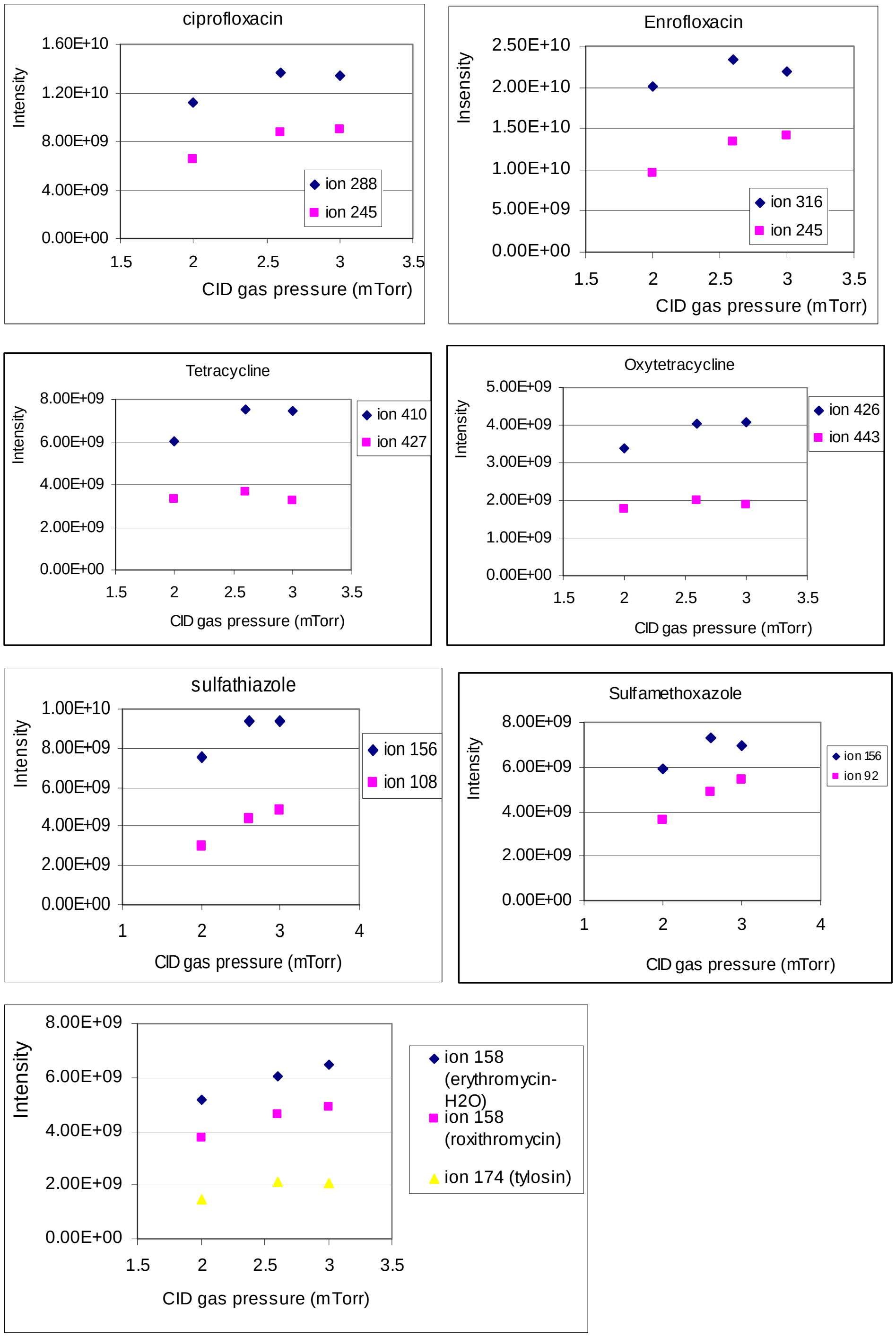


Figure 20. Effect of Argon Pressure on Product Ion Intensity of Selected Analytes in the Presence of Mobile Phase

3.1.6 Calibration Linearity in Solvent

Standard solutions of 22 antibiotics were prepared in a mixture at 0-100 μg/L in a solvent mixture of 90% 0.1% formic acid 10% methanol. The calibration curves were made by plotting peak area of a major product ion of each analyte (the underlined ions in Tables 9-11) against concentration. The correlation coefficients (r^2) of calibration curves of tetracyclines vary from 0.995 (minocycline) to 1.000 (oxytetracycline) in the range of 5-100 μg/L. The correlation coefficients of calibration curves of sulfonamides vary from 0.997 (sulfamethoxazole) to 1.000 (sulfachlorpyridazine) in the range of 0.5-100 μg/L, except for sulfadimethoxine, which has an r^2 of 0.979. The correlation coefficients of calibration curves of macrolide, fluoroquinolone, trimethoprim, and lincomycin are all 0.999.

IDLS, estimated as the lowest point in the linear calibration, were 0.5 μg/L for sulfonamides and macrolides, 1 μg/L for fluoroquinolones, 5 μg/L for tetracyclines, and 0.1 μg/L for trimethoprim and lincomycin.

3.1.7 Matrix Effect

3.1.7.1 ESI-MS/MS Response. In order to assess the applicability of the method to the analysis of raw drinking water, the matrix effect was evaluated. Calibration curves were made using two sets of samples that were prepared by spiking a mixture of 22 antibiotics into a solvent mixture of water/methanol (9:1, v:v) or a surface-water extract at concentrations of 20, 50, and 100 μg/L. The raw-water extract was prepared by extracting a 250 ml raw-water sample at pH 3 through a hydrophilic-lyophilic balance (HLB) SPE column (Waters, Milford, MA), and the methanol eluate was further concentrated to near dryness and reconstituted up to 250 μL with the solvent mixture of water/methanol (9:1). The matrix effect was evaluated by the change of response of each analyte in raw-water extract as compared to that in solvent. The percent change, calculated as follows, is given in Table 12.

Detector Change (%) = {[response (raw extract) – response (solvent)]/response (solvent)} * 100

The negative values of detector response change (%) indicate ion suppression, while the positive values indicate ion enhancement at a specific concentration. Matrix effect on ionization was observed on sulfonamides (except for sulfadimethoxine) and trimethoprim with various degrees of ion suppression throughout the calibration range. The response of analytes in the raw-water extract was much lower than that in the clean solvent, and the calibration linearity in the raw-water extract was poorer. Figure 21 shows the matrix effect on sulfathiazole, as an example. Ion enhancement was observed for sulfadimethoxine throughout the calibration range. Ion enhancement was observed for most of the fluoroquinolones at 20 and 50 μg/L, except for sarafloxacin. At 100 μg/L, the matrix effect on ciprofloxacin and norfloxacin was negligible, while ion suppression was observed for enrofloxacin and sarafloxacin. Ion suppression was also observed for oxytetracycline, chlortetracycline, and doxycycline throughout the calibration range of 20-100 µg/L and for tetracycline at 100 µg/L. For tylosin and roxithromycin, ion enhancement was observed at the 100 µg/L spike level, while ion suppression was observed at 20 and 50 µg/L. The matrix effect on ionization of lincomycin was negligible throughout the calibration range.

Table 12. Matrix Effect on Target Analytes in Raw Water Compared to Solvent

	Detector response change (%)		
	20 µg/L	50 µg/L	100 µg/L
Sulfathiazole	-70	-87	-79
Sulfamerazine	-86	-73	-38
Sulfamethazine	-76	-74	-31
Sulfamethizole	-86	-84	-55
Sulfachlorpyridazine	-83	-86	-92
Sulfamethoxazole	-88	-89	-68
Sulfadimethoxine	198	138	1,057
Trimethoprim	-42	-43	-21
Ciprofloxacin	144	149	0
Norfloxacin	57	33	-6
Enrofloxacin	18	33	-26
Sarafloxacin	-34	-47	-52
Oxytetracycline	-24	-5	-31
Tetracycline	4	6	-40
Demeclocycline	-11	11	-45
Chlortetracycline	-16	-9	23
Doxycycline	-45	-28	-15
Tylosin	-25	-9	214
Roxithromycin	-43	-26	275
Lincomycin	-9	-3	-1

These observations show that the matrix effect on ionization of antibiotics varies between different groups. Whether the matrix causes ion suppression or enhancement and to what degree appear to be concentration-dependent for some analytes in this study. Due to the variety of NOM composition and concentration in natural water, the nature and degree of matrix effect may vary in different raw drinking water. This suggests the need for the method of standard additions in such waters.

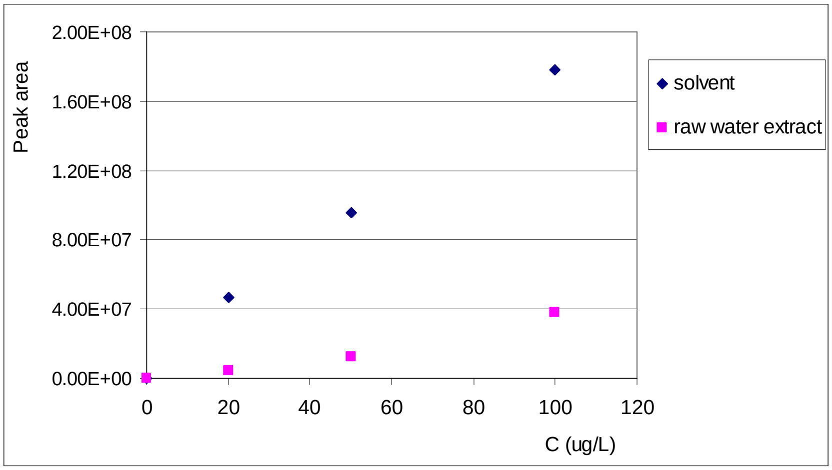


Figure 21. Calibration of Sulfathiazole in Solvent and in Raw-Water Extract

3.1.7.2 Peak Shape. Besides effect on ionization, peak shapes of most of the analytes were broadened in the raw water extract, particularly at a spike level of 100 µg/L as shown in Figures 22-24

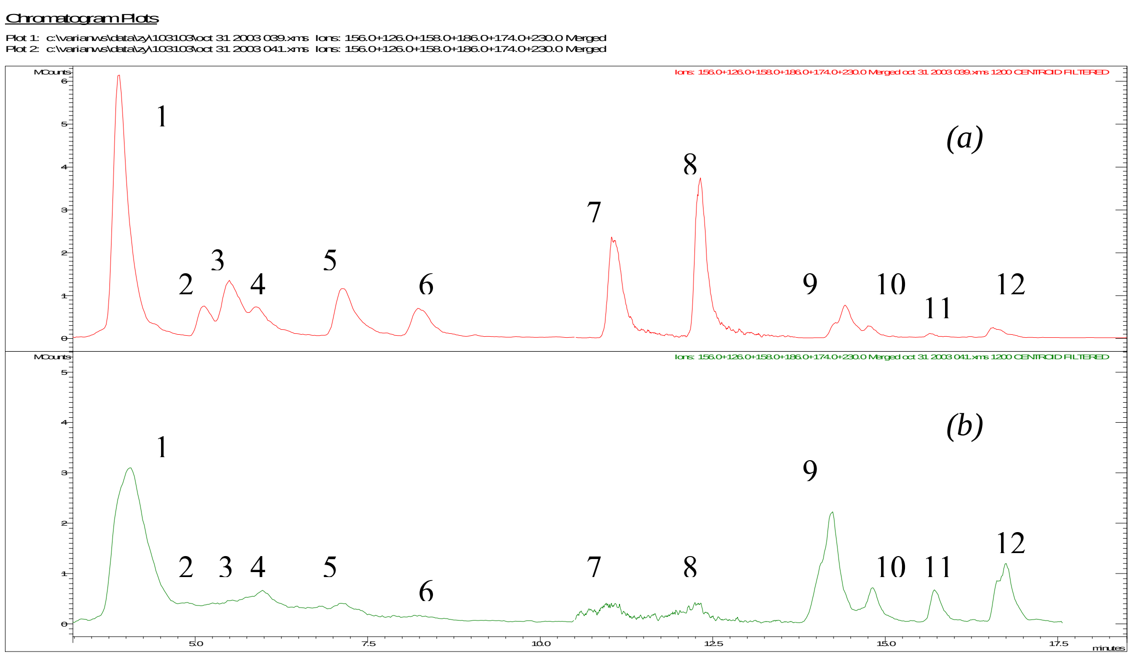


Figure 22. LC-MS/MS Chromatograms of Sulfonamides, Macrolides, Trimethoprim, and Lincomycin at 100 µg/L in (a) Pure Solvent and (b) Raw-Water Extract (on same scale) Analytes: 1. lincomycin; 2. sulfathiazole; 3. trimethoprim; 4. sulfamerazine; 5. sulfamethazine; 6. sulfamethizole; 7. sulfachlorpyridazine; 8. sulfamethoxazole; 9. sulfadimethoxine; 10. erythromycin-H_2O; 11. tylosin; 12. roxithromycin.

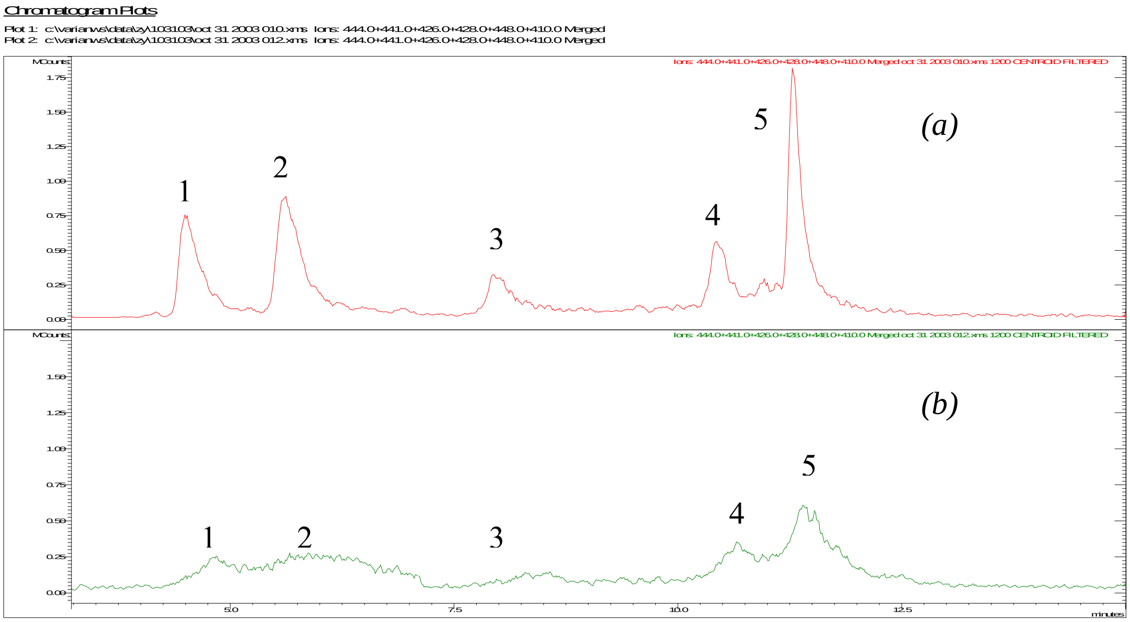


Figure 23. LC-MS/MS Chromatograms of Tetracyclines at 100 µg/L in (a) Pure Solvent and (b) Raw-Water Extract (on same scale): Analytes: 1. oxytetracycline; 2. tetracycline; 3. demeclocycline; 4. chlortetracycline; 5. doxycycline.

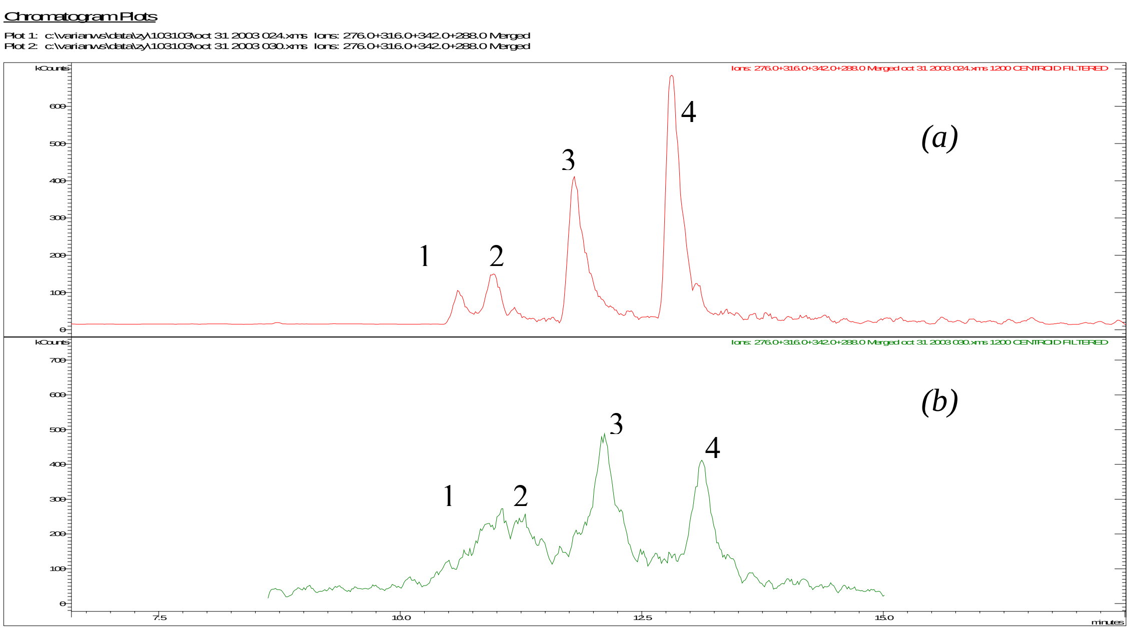


Figure 24. LC-MS/MS Chromatograms of Fluoroquinolones at 100 µg/L in (a) Pure Solvent and (b) Raw-Water Extract (on same scale). Analytes: 1. norfloxacin; 2. ciprofloxacin; 3. enrofloxacin; 4. sarafloxacin.

3.1.8 SPE of Antibiotics in Surface Water — Evaluation of Eluting Solvents in Reducing Matrix Co-elution

In the SPE of organic pollutants in surface water, NOM can be retained on the sorbent and subsequently co-eluted with analytes, resulting in a matrix effect on LC-MS analysis. This study aimed to explore extracting multiple classes of antibiotics in natural water using a single extraction, while attempting to reduce the co-extraction of NOM. Eluting solvents of different polarities were tested. The study also provided an opportunity to look into the interaction of the antibiotics with the SPE sorbent and the NOM matrix.

A raw drinking-water sample was collected from the OWASA drinking water treatment plant (Carrboro, NC). A 250 ml sample of this water was spiked with the 22-component antibiotic mixture at 2 µg/L for each analyte, adjusted to pH 3, with sulfuric acid and Na_2EDTA added at 1 mg/L. The sample was extracted through a 200 mg HLB SPE cartridge, which was preconditioned with methanol, DI water, and the specific solvent used for elution. A 60 mg HLB cartridge was attached to the bottom of some 200 mg HLB columns to determine if breakthrough occurred. After extraction, the column was rinsed with DI water. The columns for elution with solvents containing methylene chloride and/ether were dried under a nitrogen flow before elution. The cartridges were then eluted with 6 ml of a specific solvent. In order to know if there were any residual analytes on the column after the elution using a specific solvent, the column was eluted with 6 ml of methanol after the first elution, and the extract was collected separately. The 60 mg HLB column was eluted with 3 ml of methanol and collected separately. The solvents tested for elution were:

1) methanol
2) acidic methanol (with 0.1% formic acid)
3) isopropanol
4) dichloromethane/ether/isopropanol (5:1:4, v:v:v)
5) dichloromethane/isopropanol (2:8)

After elution, 3 ml of isopropanol were added to those extracts that contained dichloromethane and/or ether. All the extracts were then blown down to about 20 µL under a nitrogen pressure of 10-15 pounds per square inch (psi) and at a temperature of 30-45°C, and reconstituted with 230 µL of a solvent mixture of water/methanol (9:1). The final volume of each extract was measured by a 500 µL syringe. The samples were filtered by 0.45 µm syringe filters (Waters, Milford, MA), transferred to 2 ml amber autosampler vials, and stored in a freezer at -15°C until analysis.

The extracts were analyzed using the LC-MS/MS methods previously described. The concentration was calculated from peak areas of the analytes in sample, using external standard calibration curves prepared in neat solvents. The concentration was corrected for the final volume of the extracts. The overall recovery was calculated by dividing the calculated concentration by the theoretical concentration of 2 mg/L, assuming 100% recovery from sample preparation and no matrix effect during LC-MS analysis.

3.1.8.1 Solvent Reduction Test. A mixture of 22 antibiotics was spiked at 0.5 mg/L into 8 ml of a specific solvent in a glass tube. The samples were blown down at a temperature of 45-50˚C and nitrogen pressure 12-15 psi to a final volume of 20 µL, and reconstituted with 230 µL of 90% water/10% methanol. The samples were vortexed and filtered by syringe filter (0.45 µm, 4 mm), transferred to amber autosampler vials, and analyzed by LC-MS/MS. A three-point external calibration curve was made by analyzing standard solutions prepared at 0, 0.25, and 0.5 mg/L. The recoveries were calculated by comparing the peak area of analyte in the samples to those in the external calibration curves.

To reduce loss of analytes during evaporation of the less polar solvents, another test was done. The antibiotic mixture was spiked into 6 ml of a solvent mixture of 50% dichloromethane:10% ether:40% isopropanol or 20% dichloromethane:80% isopropanol, and 3 ml of isopropanol were added into each solvent. The solvent was evaporated to 20 µL and reconstituted with 90% water/ 10% methanol up to 500 µL. The standard solutions of 0, 250, and 500 µg/L were analyzed along with the samples. The samples were analyzed by the LC-MS/MS method.

3.1.8.2 Predicted Interaction of Antibiotics with SPE Sorbent and NOM. In SPE, the analytes are retained on the sorbent via various mechanisms and eluted with an appropriate solvent that can disrupt the analyte-sorbent interaction. HLB sorbent is a copolymer of [poly(divinylbenzene-*co*-N-vinylpyrrolidone)] (Figure 25). The antibiotics could be retained on the HLB sorbent by hydrophobic interaction, hydrogen bonding, π-π interaction, etc. NOM in surface water may affect the extraction of analytes by competing for the sorption sites on the column, or by associating with analytes, resulting in either co-extraction or prevention of the retention of analytes onto the column.

Tetracyclines are protonated in acidic condition and exist predominantly in zwitterionic form under mildly acidic and neutral pH. The targeted fluoroquinolones are protonated in mildly acidic conditions and exist in zwitterionic form under neutral conditions. Sulfonamides are protonated in acidic pH, exist in neutral form in mildly acidic pH, and carry a negative charge at neutral pH. Macrolides are positively charged under both acidic and neutral conditions.

Tetracyclines, the target fluoroquinolones, and macrolides with a positive charge are likely to be electrostatically attracted to NOM that is negatively charged at environmental pH 6-9. Tetracyclines and fluoroquinolones might also associate with humic substances through hydrogen bonding and cation bridging, respectively. The $K_{d,\,DOM}$ values for sorption to purified humic substances were determined to be 53,000 L/kg for quinolone carboxylic acid and range between 1,500 and 2,000 L/kg for tetracyclines (Tolls 2001).

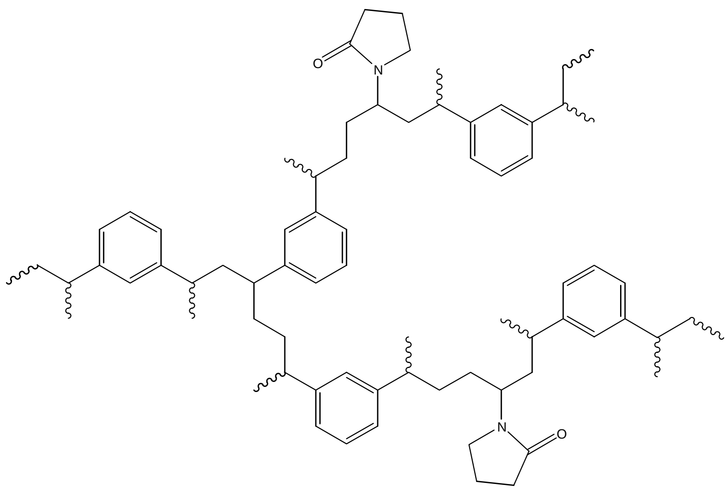


Figure 25. Structure of the HLB SPE Sorbent

Among the analytes, tetracyclines and fluoroquinolones are most likely to be associated with NOM. Macrolides are also expected to associate with NOM, though to a lesser extent.

3.1.8.3 Properties of Eluting Solvents. Methanol was shown to be an effective eluting solvent for sulfonamides and tetracyclines (Lindsey et al. 2001). However, methanol can also dissolve humic substances that are retained on the column and cause co-elution of the matrix. Acidic methanol was tested in order to keep the carboxylic acid moiety of humic substances in nondissociated form, which is more likely to be retained on the column during elution. Solubility of humic substances is lower in isopropanol than in methanol (Lindsey et al. 2001), so they are expected to be less eluted with isopropanol.

The solvents with less polarity than methanol were tested in order to reduce the co-elution of relatively polar NOM, which may co-elute with some polar analytes in chromatography. The polarity of the solvent is reflected in the polarity index, which is as follows: methanol (5.1) > isopropanol (4.0) > dichloromethane (3.0) > ether (2.8). The polarity index (P') of a solvent mixture can be calculated using the polarity index of individual solvent components and their volume fraction (ϕ): $P' = \phi_A P'_A + \phi_B P'_B + \cdots\cdots + \phi_i P'_i$

The resulting values are shown in Table 13.

3.1.8.4 Recovery of Analytes After Solvent Reduction. Most of the analytes could be recovered after the solvent-reduction step (without SPE) in acidic methanol and isopropanol solvents. Sulfonamides and trimethoprim can also be satisfactorily recovered in the solvent mixture of dichloromethane/ether/isopropanol (5:1:4, in volume), while loss of macrolides and fluuoroquinolones occurred as shown in Table 14. Mixture 1 and Mixture 2 refer to the solvent mixture of dichloromethane/ether/isopropanol (5:1:4) and mixture of dichloromethane/isopropanol (1:4), respectively, in Tables 14-23.

Table 13. Polarity Index of the Tested Eluting Solvents

Solvent	Polarity index
Methanol	5.1
Acidic methanol	5.1
Isopropanol	4.0
Dichloromethane/isopropanol (1:4)	3.8
Dichloromethane/ether/isopropanol (5:1:4)	3.4

Table 14. Recovery (%) of Selected Analytes After Solvent Reduction

	Eluting solvent		
	Acidic methanol	Isopropanol	Mixture 1
Sulfathiazole	86	91	81
Sulfamethoxazole	95	101	98
Roxithromycin	88	109	7.1
Tylosin	69	104	37
Lincomycin	105	109	12
Ciprofloxacin	123	85	32
Enrofloxacin	107	82	17
Oxytetracycline	74	64	n.m.
Tetracycline	38	63	n.m.

nm = not measured

The loss of these analytes in the evaporation of solvents containing dichloromethane, ether, and isopropanol was suspected to be caused by partitioning of these polar compounds out of solvents to the glass surface. The addition of 3 ml of isopropanol into each SPE extract before solvent reduction improved the recoveries of tetracyclines, fluoroquinolones, macrolides, and lincomycin after the solvent-reduction step (Table 15), most likely because of an increase in the polarity of the solvent mixture and hence the likely increased retention of the polar analytes in the solvent.

3.1.8.5 Breakthrough in SPE. The experimental data show that 6% of lincomycin was detected in the methanol extract of the 60 mg HLB column both in DI and raw-water extractions, indicating breakthrough of this analyte. Breakthrough did not occur for any of the other analytes either in DI or raw water extractions.

Table 15. Recovery (%) of Selected Analytes After Solvent Reduction
(with additional 3 ml of isopropanol)

	Eluting solvent	
Analyte	Mixture 1	Mixture 2
Sulfamethoxazole	89	102
Sulfadimethoxine	80	112
Roxithromycin	86	106
Tylosin	86	100
Lincomycin	109	130
Trimethoprim	86	92
Norfloxacin	56	73
Ciprofloxacin	57	75
Enrofloxacin	58	74
Sarafloxacin	68	85
Tetracycline	75	87
Oxytetracycline	72	82
Chlortetracycline	60	81
Doxycycline	87	91

3.1.8.6 Overall Recovery of Analytes. With 100% percent of sorption on the column (no breakthrough), the recoveries of analytes extracted from DI water indicate the effectiveness of these solvents in eluting the analytes. However, recovery of analytes in raw water can be affected not only by the effectiveness of elution, but also by the degree of co-elution of NOM which can affect the ionization of analytes.

3.1.9 Sulfonamides, Macrolides, Trimethoprim, and Lincomycin

3.1.9.1 Recovery in DI Water. Recoveries of the analytes in DI water are given in Tables 16 and 17. The elution using acidic methanol produces the highest recoveries for sulfonamides in DI water, while the solvent mixture of dichloromethane/ether/isopropanol (5:1:4) produces the highest recovery for macrolides. There is little difference among these solvents as to elution of lincomycin and trimethoprim in DI water. Although the sum of the recovery in the two extracts is not equal to 100%, the loss could be due to the degradation of analytes, possibly during storage of extracts or loss during solvent reduction.

Table 16. Recovery (%) of Analytes in DI Water in the First Extracts

	Methanol	Acidic methanol	Isopropanol	Mixture 1	Mixture 2
Sulfathiazole	80	81	33	71	73
Sulfamerazine	74	74	69	71	67
Sulfamethazine	93	85	92	90	83
Sulfamethizole	12	65	n.d.	n.d.	40
Sulfachlorpyridazine	43	83	1.4	n.d.	54
Sulfamethoxazole	85	94	48	30	79
Sulfadimethoxine	57	89	27	27	89
Trimethoprim	80	84	75	77	72
Lincomycin	25	25	23	23	22
Tylosin	55	62	64	81	66
Erythromycin-H_2O	67	87	78	92	73
Roxithromycin	36	46	42	51	41

Table 17. Recovery (%) of Analytes in DI Water in the Second Extracts in Methanol

Solvent in first elution	Methanol	Acidic methanol	Isopropanol	Mixture 1	Mixture 2
Sulfathiazole	0.2	0.3	22	0.7	0.7
Sulfamerazine	0.5	0.3	4.8	0.7	0.5
Sulfamethazine	0.4	0.3	3.7	0.7	0.6
Sulfamethizole	0.2	0.4	0.3	0.2	0.8
Sulfachlorpyridazine	0.2	0.4	2.0	0.2	0.7
Sulfamethoxazole	0.1	0.4	7.9	0.5	0.5
Sulfadimethoxine	0.5	2.3	27	1.9	3.2
Trimethoprim	0.6	0.3	5.3	2.7	1.8
Lincomycin	0.1	0.1	0.5	0.3	0.2
Tylosin	0.1	0.2	0.3	0.5	0.2
Erythromycin-H_2O	0.1	0.1	0.2	0.3	0.2
Roxithromycin	n.d.	n.d.	0.2	0.3	0.2

3.1.9.2 Recovery in Raw Water. Recoveries of the analytes in raw water in the first elution using the specific solvent and second elution using methanol are given in Tables 18 and 19, respectively. Sulfonamides and trimethoprim can be mostly eluted during the first elution with methanol, but their recoveries are low due to the ion-suppression effect caused by co-eluted NOM. Elution by acidic methanol slightly increased the recovery, possibly due to co-elution of NOM. Using less-polar solvents results in a much higher increase of recovery, indicating these solvents are more effective in reducing the co-elution of NOM. The solvent mixture of dichloromethane/ether/isopropanol (5:1:4) turns out to be the best solvent for elution of these analytes in raw-water extraction. Although isopropanol could reduce the co-elution of NOM, it is not effective in eluting the analytes and, overall, not a good choice.

Table 18. Recovery (%) of Analytes in Raw Water in the First Extracts

	Methanol	Acidic methanol	Isopropanol	Mixture 1	Mixture 2
Sulfathiazole	7.8	11	9.2	32	25
Sulfamerazine	9.8	14	24	32	26
Sulfamethazine	18	23	35	48	41
Sulfamethizole	7.6	9.4	13	32	26
Sulfachlorpyridazine	7.3	8.8	4.1	22	18
Sulfamethoxazole	5.1	6.4	9.7	17	14
Sulfadimethoxine	27	33	36	94	68
Trimethoprim	58	65	30	98	84
Lincomycin	63	64	80	82	73
Tylosin	72	76	115	108	92
Erythromycin-H_2O	45	47	64	72	60
Roxithromycin	32	38	50	69	55

Table 19. Recovery (%) of Analytes in Raw Water in the Second Extracts in Methanol

Solvent in first elution	Methanol	Acidic methanol	Isopropanol	Mixture 1	Mixture 2
Sulfathiazole	1.0	1.0	12	1.1	1.7
Sulfamerazine	0.4	0.7	4.9	1.2	1.6
Sulfamethazine	0.7	0.7	4.7	1.3	1.7
Sulfamethizole	0.8	1.1	7.1	0.9	1.0
Sulfachlorpyridazine	0.9	0.9	13.4	0.7	0.9
Sulfamethoxazole	0.8	0.9	5.6	0.6	0.8
Sulfadimethoxine	3.3	3.2	59	3.1	2.9
Trimethoprim	0.4	0.3	45	5.0	3.6
Lincomycin	0.3	0.2	9.0	5.6	4.0
Tylosin	0	0.1	1.3	1.3	1.5
Erythromycin-H_2O	0.2	0.1	1.2	1.1	0.9
Roxithromycin	0.1	0.1	1.5	1.2	1.1

The recoveries of macrolides were also increased by elution with less-polar solvents. It is interesting to see higher recovery of lincomycin in raw-water extraction compared to DI water extraction. Perhaps the retention mechanism of lincomycin to the solid phase column is different in the presence of a matrix. Whether the possible association of lincomycin with NOM facilitates its retention onto the SPE column is unknown.

3.1.10 Tetracyclines and Fluoroquinolones

Recoveries of the analytes in DI water in the first elution using the specific solvent and second elution using methanol are given in Tables 20 and 21, respectively. Recoveries of the analytes in raw water in the first elution using the specific solvent and second elution using methanol are given in Tables 22 and 23, respectively.

Table 20. Recovery (%) of Analytes in DI Water in the First Extracts

	Methanol	Acidic methanol	Isopropanol	Mixture 1	Mixture 2
Minocycline	53	71	52	87	56
Oxytetracycline	94	89	87	93	83
Tetracycline	129	118	119	133	119
Chlortetracycline	71	77	63	59	62
Doxycycline	76	85	71	105	78
Meclocycline	85	111	63	82	76
Norfloxacin	30	66	15	28	30
Ciprofloxacin	43	83	23	44	45
Enrofloxacin	47	83	38	43	53
Sarafloxacin	39	66	29	47	41

Table 21. Recovery (%) of Analytes in DI Water in the Second Extracts in Methanol

Solvent in first elution	Methanol	Acidic methanol	Isopropanol	Mixture 1	Mixture 2
Minocycline	n.d.	n.d.	8.9	n.d.	15
Oxytetracycline	4.3	3.3	13	5.6	7.4
Tetracycline	6.7	5.5	20	8.2	12
Chlortetracycline	5.7	6.0	15	9.6	15
Doxycycline	7.2	6.2	14	6.5	15
Meclocycline	n.d.	n.d.	16	11	36
Norfloxacin	n.d.	n.d.	16	11	36
Ciprofloxacin	n.d.	n.d.	n.d.	n.d.	3.0
Enrofloxacin	n.d.	0.59	0.86	0.22	3.5
Sarafloxacin	n.d.	n.d.	1.0	n.d.	2.4

nd = none detected

Table 22. Recovery (%) of Analytes in Raw Water in the First Extracts

	Methanol	Acidic methanol	Isopropanol	Mixture 1	Mixture 2
Minocycline	12	11	8.7	**16**	11
Oxytetracycline	41	39	7.4	**44**	36
Tetracycline	66	60	8.9	**72**	50
Chlortetracycline	19	18	5.5	**26**	17
Doxycycline	15	13	5.9	**17**	13
Meclocycline	14	13	n.d.	**16**	12
Norfloxacin	8.2	7.0	n.d.	5.4	1.3
Ciprofloxacin	8.7	7.5	n.d.	8.3	1.5
Enrofloxacin	10	8.1	0.4	16	5.6
Sarafloxacin	2.9	1.8	n.d.	4.3	1.0

Table 23. Recovery (%) of Analytes in Raw Water in the Second Extracts in Methanol

Solvent in first elution	Methanol	Acidic methanol	Isopropanol	Mixture 1	Mixture 2
Minocycline	n.d.	9.7	26	17	12
Oxytetracycline	5.6	7.8	81	46	32
Tetracycline	7.5	11	137	68	48
Chlortetracycline	n.d.	27	100	57	62
Doxycycline	29	34	100	66	67
Meclocycline	n.d.	10	44	34	28
Norfloxacin	n.d.	0.27	15	12	8.5
Ciprofloxacin	n.d.	n.d.	18	12	10
Enrofloxacin	0.47	1.2	22	5.2	11
Sarafloxacin	n.d.	n.d.	11	3.3	4.7

SPE of antibiotics in natural water is complicated by their potential association with NOM due to their multifunctionality and ionic nature, which also poses difficulty in separating antibiotics from NOM to reduce matrix co-extraction. Tetracyclines and fluoroquinolones are likely to associate with NOM, while sulfonamides and macrolides are less likely to be associated.

Using solvents less polar than methanol, such as a mixture of dichloromethane, ether, and isopropanol, sulfonamides and trimethoprim could be effectively eluted, while the NOM components that cause suppression of these analytes appear to be less eluted, resulting in less matrix effect on these compounds in LC-MS/MS analysis. However, tetracyclines and fluoroquinolones could not be effectively eluted with these solvents in raw-water extraction. Use of isopropanol as a rinsing solvent and acidic methanol as an elution solvent is, therefore, suggested for elution of tetracyclines. Although a unified approach to effectively extract these analytes while reducing NOM co-extraction has not yet been accomplished, these results will be helpful for selective elution of antibiotics classified according to their likely association with NOM.

3.1.11 SPE of Antibiotics from Surface Water — Evaluation of Extraction pH in Reducing Matrix Co-extraction

In most of the published methods for analysis of antibiotics in environmental water samples, samples were acidified before extraction by SPE. Acidification of samples can increase the retention of acidic compounds on the solid phase but also increase the co-extraction of humic and fulvic acids from natural water. Humic substances are less likely to be retained on polymeric columns (Pichon 1996). This study evaluated the likelihood of effective extraction of antibiotics from surface waters without adjusting to an acidic pH, while reducing the co-extraction of humic substances.

A 250 ml water sample was spiked with the 22-component antibiotic mixture at 0.1 µg/L (for DI water) and 0.5 µg/L (for raw water), the pH adjusted to 3 or 6 with sulfuric acid, and Na_2EDTA added at 1 mg/L. The sample was extracted through a preconditioned 200 mg HLB column, rinsed with DI water after extraction, and eluted with 6 ml acidic methanol (0.1% formic acid).

The extracts were blown down to about 20 µL under nitrogen pressure 10-15 psi and temperature 30-45°C, and reconstituted with 230 µL of 90% water/ 10% methanol. The final volume of the extract was measured by a 500 µL syringe. The samples were filtered through 0.45 µm syringe filters and stored in a freezer until analysis. The extracts were analyzed using the LC-MS/MS methods previously described.

3.1.11.1 SPE in DI Water. A comparison of extraction efficiency at pH 3 and 6 was made by comparing the peak area of each of the analytes in the samples extracted at pH 3 and 6 (Figure 26). For sulfonamides, macrolides, tetracyclines, and trimethoprim, there was little difference between the peak responses of analytes in the samples extracted at the two different pH levels. However, the extraction efficiency of fluoroquinolones was greater at pH 3. This could be explained by the fact that under acidic conditions, the quinolone carboxylic acids are in nondissociated form and thus more hydrophobic, favoring the retention on the column by hydrophobic interaction. In contrast, the extraction efficiency of lincomycin was much higher at pH 6, but could not be explained at this stage. Lincomycin has a macroscopic pKa of 7.6 (Table 2), though the ionization state of lincomycin is not clear.

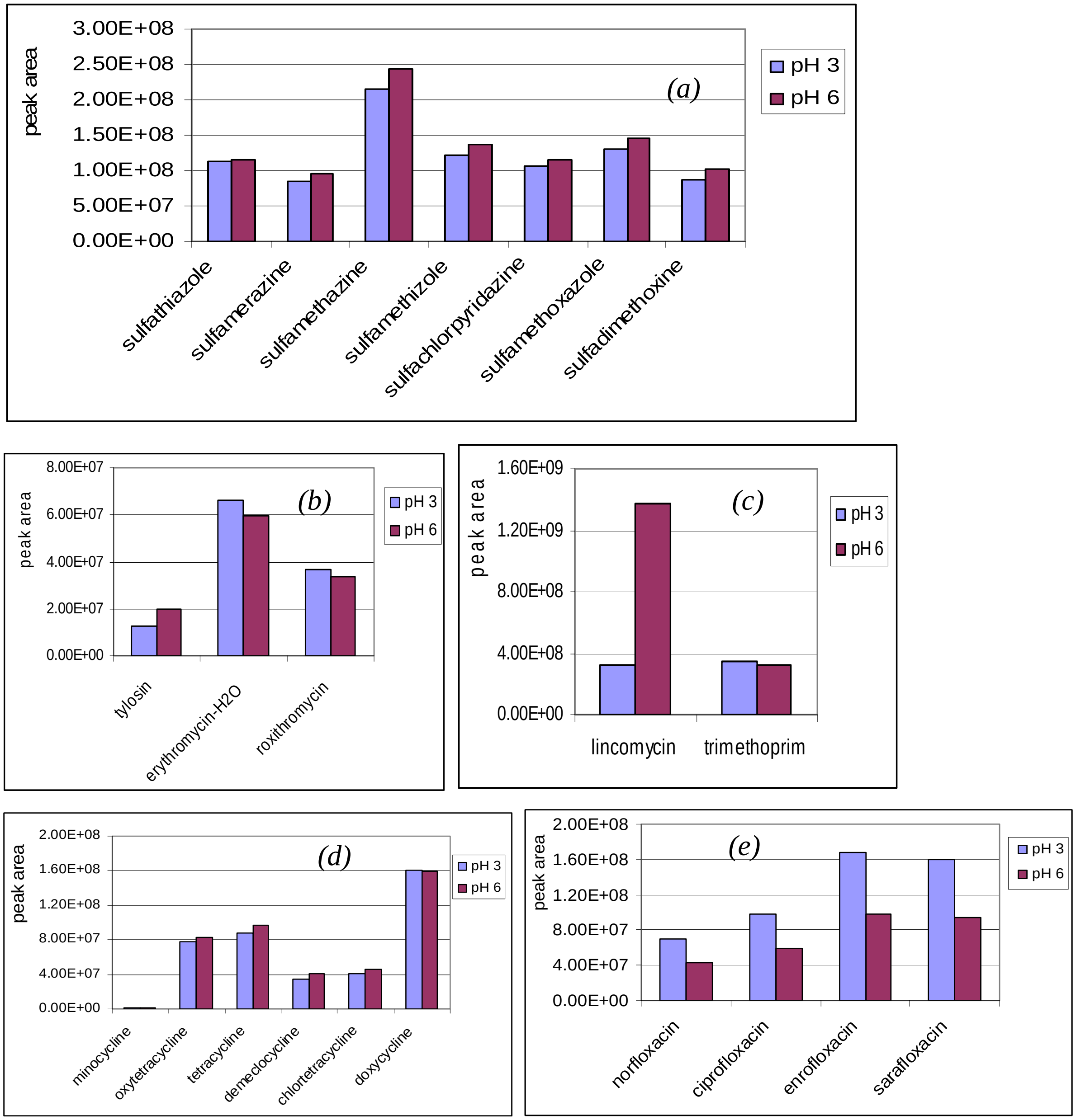


Figure 26. Comparison of Extraction Efficiency at pH 3 and 6 in DI water: (a) sulfonamides; (b) macrolides; (c) trimethoprim and lincomycin; (d) tetracyclines; (e) fluoroquinolones.

3.1.11.2 SPE in Raw Water. For most of the analytes, peak areas in the sample extracted at pH 6 was higher than those at pH 3 (Figure 27), except that minocycline was not detected due to its low sensitivity. This could be explained by less NOM being co-extracted at pH 6 and the ion-suppression effect on ionization being reduced, leading to the increase of signal response. Although fluoroquinolones are more effectively extracted at pH 3 in DI water, the reduction of matrix co-extraction seems to compensate for the loss of analytes during extraction at pH 6 in raw water.

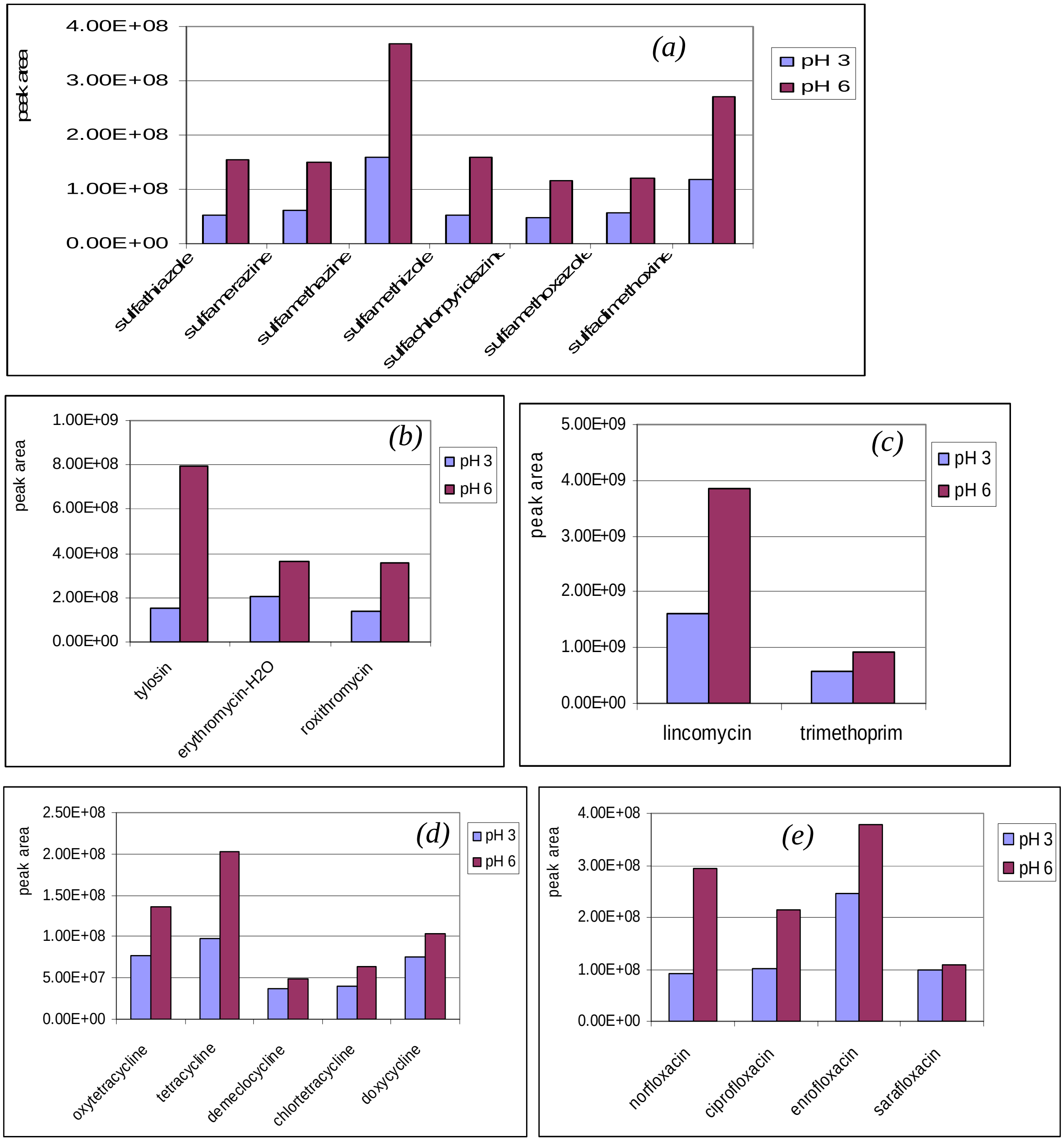


Figure 27. Comparison of Extraction Efficiency at pH 3 and 6 in Raw Water: (a) sulfonamides; (b) macrolides; (c) trimethoprim and lincomycin; (d) tetracyclines; (e) fluoroquinolones.

However, due to the variety of NOM composition and concentration in natural waters, different raw waters need to be tested to explore the applicability of this extraction method.

3.1.12 Evaluation of Ascorbic Acid as a Disinfectant Quenching Agent for Finished Waters

The residual chlorine disinfectants in finished water may react with antibiotics, so they need to be quenched by an appropriate quenching agent. Ascorbic acid (vitamin C) is known to react rapidly with hypochlorous acid (Folkes et al. 1995) to produce inorganic halides and dehydroascorbic acid which exists as several forms in aqueous solution and can itself decompose (Hvoslef and Pedersen 1979). Ascorbic acid can also remove chloramines (Wiseman 1997).

In addition to rapid removal of disinfectants/oxidants, the quenching agent should be chemically inert toward the analytes and have negligible effects on their quantitation. The following experiments were conducted to test whether ascorbic acid is an appropriate choice for quenching residual chlorines in finished water for antibiotic analysis. Other quenching agents, including sodium sulfite, sodium bisulfite, and sodium thiosulfate, were also tested for comparison.

Stock solutions of the quenching agents were individually prepared at 10 g/L in DI water. A free-chlorine working solution was prepared at 1 g/L from dilution of sodium hypochlorite reagent solution, and its concentration was checked right after the experiment using DPD colorimetric method with a Hach colorimeter (Hach Co., Loveland, CO). A tap water sample was collected in the lab, with free and total chlorine concentration measured to be 0.07 and 3.0 mg/L, respectively, using DPD colorimetric method, and pH measured to be 8.5 with a Fisher Accumet Meter.

3.1.12.1 Reduction of Free and Combined Chlorine by Quenching Agents. A specific quenching agent was added to 1 mg/L of free chlorine solution prepared in DI water from dilution of the free chlorine working solution, at molar ratio of ascorbic acid versus free chlorine of 1:1 and 2:1. The quenching agent was also added to the tap water sample with 3 mg/L of chloramines, at concentrations of 6 to 12 mg/L. The residual free and total chlorine of each solution after the addition of quenching agent were measured using DPD colorimeter method.

3.1.12.2 Effect Of Quenching Agents on LC-MS/MS Analysis of Antibiotics. A specific quenching agent was added at 20 mg/L into a 3 mg/L free chlorine solution or the tap water sample to form a mixture that contained quenched chlorine and an excess of ascorbic acid. The solution mixture was then spiked with the 22-component antibiotic mixture at 0.2 mg/L. A similar control sample was prepared without addition of quenching agent.

All the above samples were adjusted to pH 3 with 10% formic acid, and directly analyzed by the LC-MS/MS method.

3.1.12.3 Effect of Ascorbic Acid on SPE of Antibiotics. Two hundred and fifty milliliters of the 22-component antibiotic mixture in DI water was added to ascorbic acid at 20 mg/L, adjusted to pH 3 with 10% formic acid, and extracted through a 200 mg HLB column, and eluted with 6 ml of acidic methanol (0.1% formic acid). The extracts were blown down to about 250 µL, reconstituted up to 2.5 ml with a solvent mixture of water/methanol (9:1), and analyzed by LC-MS. The sample passing through the column was also collected and analyzed directly by LC-MS to determine if there were any analytes not retained on the column. The SPE recovery was calculated by comparing the peak area of the analyte in the SPE extract to that in a standard solution of 5 mg/L.

3.1.12.4 Reduction of Free and Combined Chlorine by Quenching Agents. All the tested quenching agents could stoichiometrically (1:1) remove free chlorine. Three milligrams per liter of chloramines could be completely reduced by sodium bisulfite, sodium sulfite, sodium thiosulfate, and 12 mg/L of ascorbic acid of 7, 9, 11, and 12 mg/L. Because quenching agents are usually added in at least twofold in excess in the drinking water sample, in the following tests each quenching agent was added at 20 mg/L into 3 mg/L of free chlorine solution and tap water sample with 3 mg/L of chloramines.

3.1.11.5 Effect of Ascorbic Acid on LC-MS/MS Analysis of Antibiotics. Results show that MS/MS response of most of the analytes was not affected by the presence of excess ascorbic acid and/or the pre-quenched chlorine disinfectants at pH 3 (Figure 28). However, the response of tetracyclines was reduced in the mixture of pre-quenched free or combined chlorine with excess ascorbic acid (Figure 28). The response of fluoroquinolones was reduced in the mixture of pre-quenched free chlorine with excess ascorbic acid (Figure 28). Response of lincomycin was greatly reduced in the presence of pre-quenched chloramines with excess ascorbic acid (Figure 28).

3.1.12.6 Comparison of Ascorbic Acid with Other Quenching Agents. The responses of analytes in the presence of prequenched free or combined chlorine with excess quenching agents were compared, as shown in Figures 29 and 30. Little difference was observed among the use of ascorbic acid, sodium sulfite, and sodium thiosulfate.

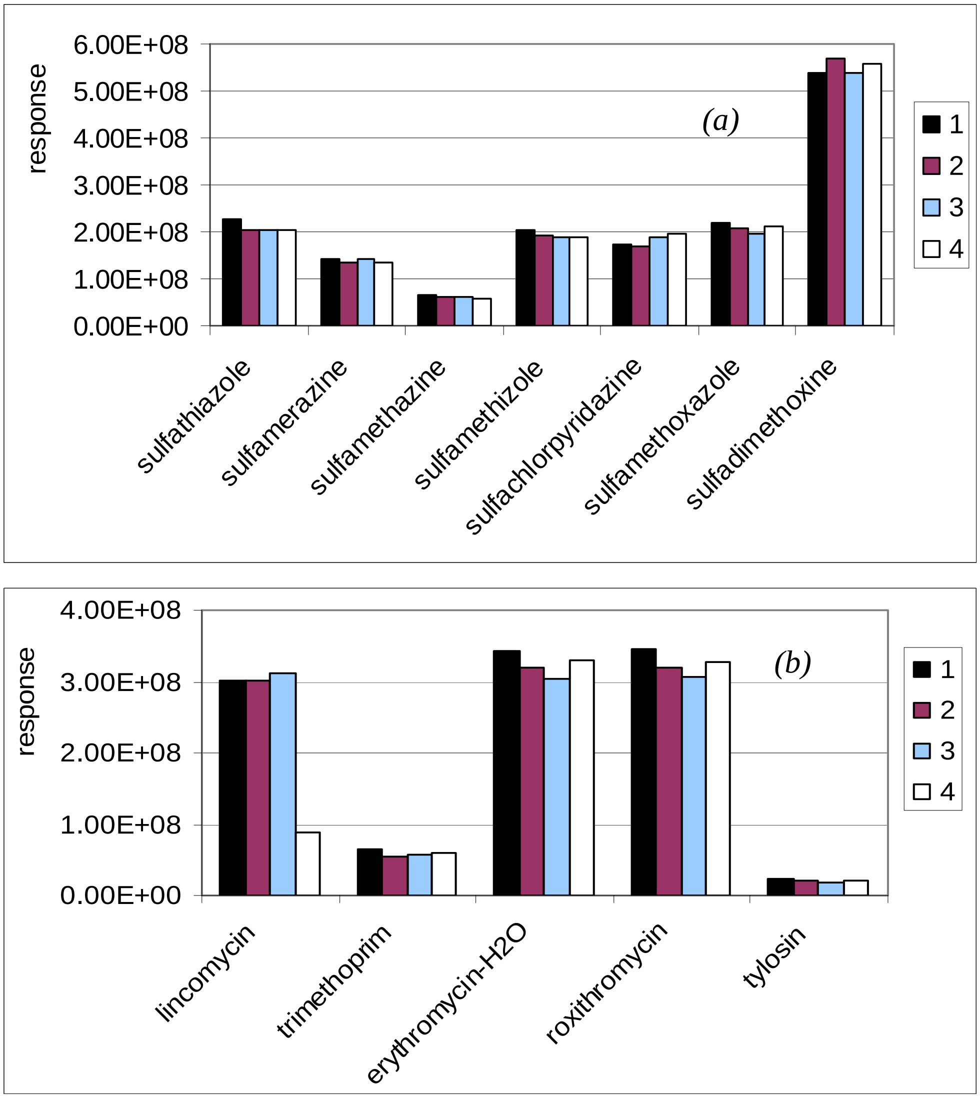


Figure 28. Comparison of Analyte Response With/out Quenching Agent: (a) sulfonamides; (b) macrolides, trimethoprim, and lincomycin; (c) tetracyclines; (d) fluoroquinolones.

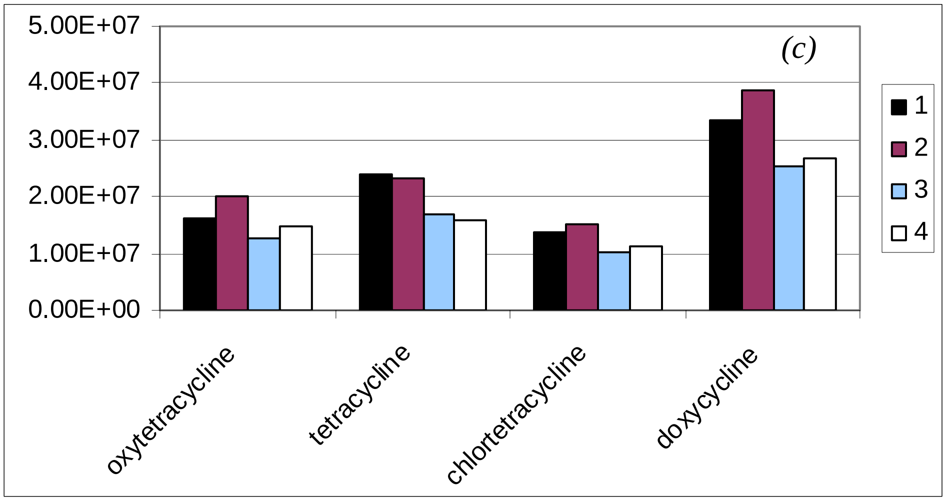
Sample 1: antibiotic mixture at 200 μg/L.

Sample 2: antibiotic mixture at 200 μg/L with the addition of 20 mg/L ascorbic acid.

Sample 3: antibiotic mixture at 200 μg/L in free chlorine solution prequenched with 20 mg/L ascorbic acid.

Sample 4: antibiotic mixture at 200 μg/L in tap water prequenched with 20 mg/L ascorbic acid.

(continued)



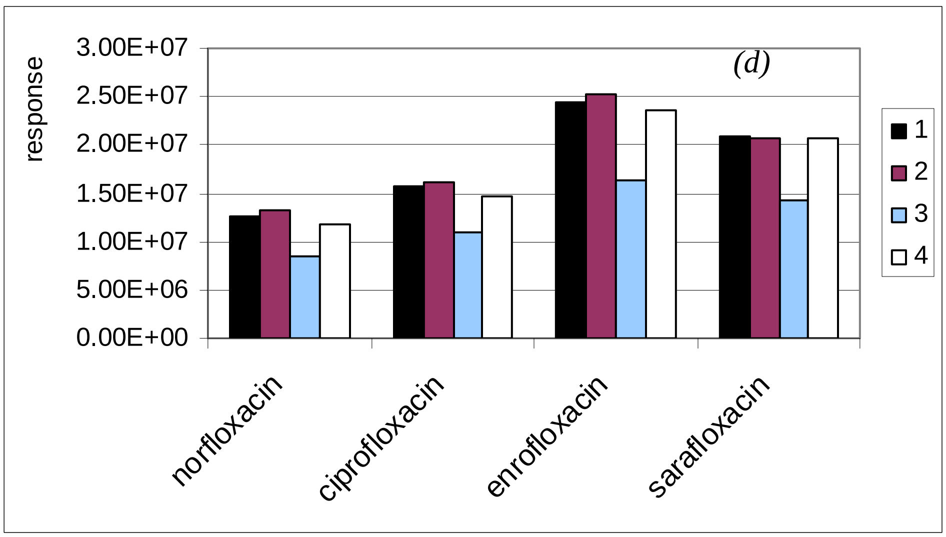


Figure 28 (cont). Comparison of Analyte Response With/out Quenching Agent: (a) sulfonamides; (b) macrolides, trimethoprim, and lincomycin; (c) tetracyclines; (d) fluoroquinolones.

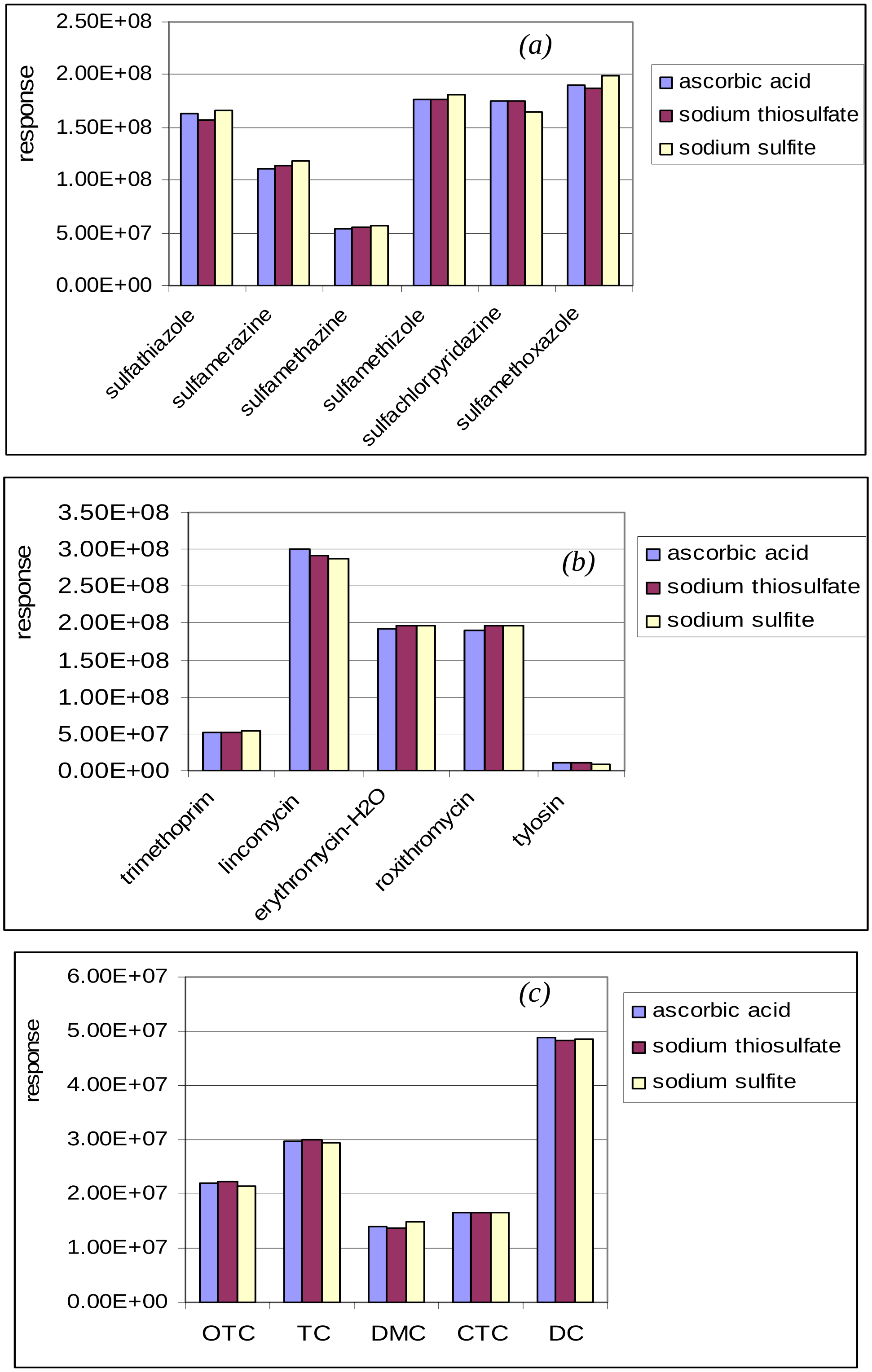


Figure 29. Comparison of Analyte Response in the Presence of Prequenched Free Chlorine with Excess Ascorbic Acid: (a) sulfonamides; (b) macrolides, trimethoprim, and lincomycin; (c) tetracyclines; (d) fluoroquinolones. (continued)

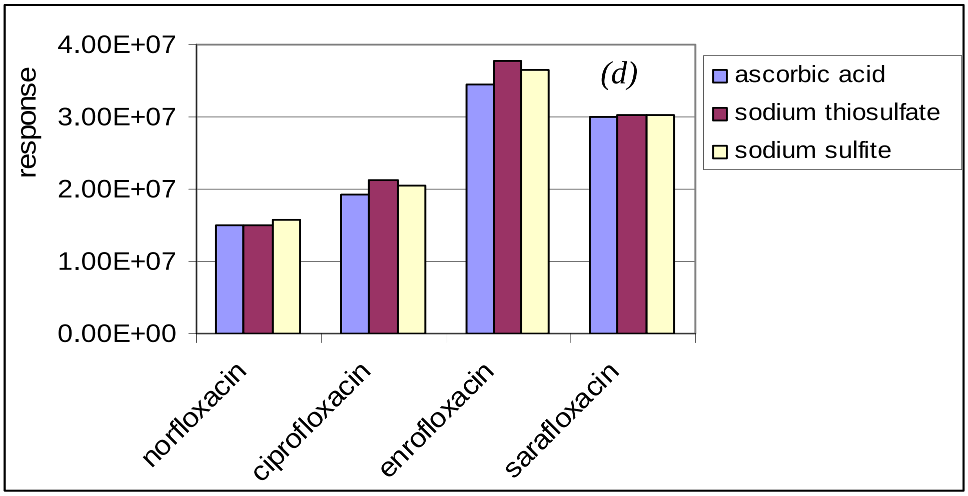


Figure 29 (cont). Comparison of Analyte Response in the Presence of Prequenched Free Chlorine with Excess Ascorbic Acid: (a) sulfonamides; (b) macrolides, trimethoprim, and lincomycin; (c) tetracyclines; (d) fluoroquinolones.

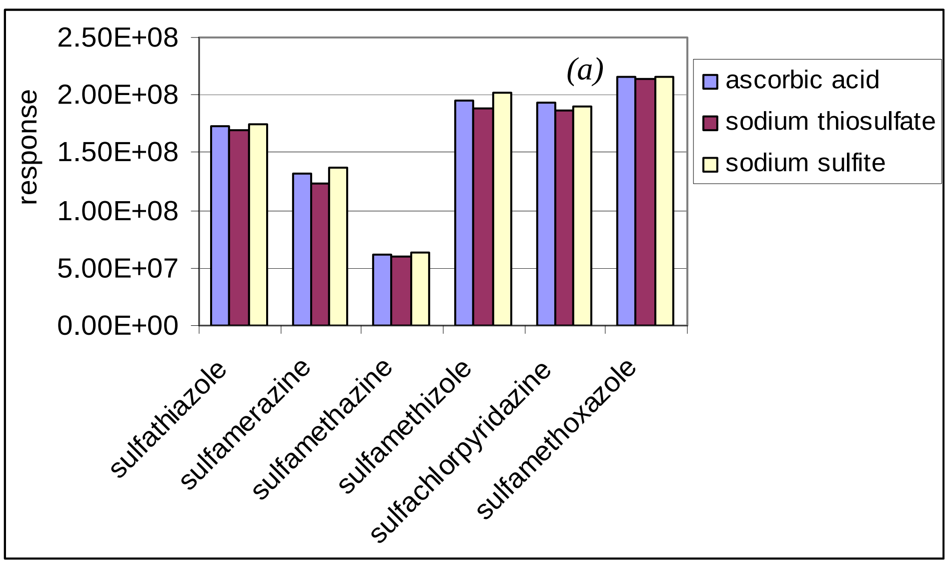
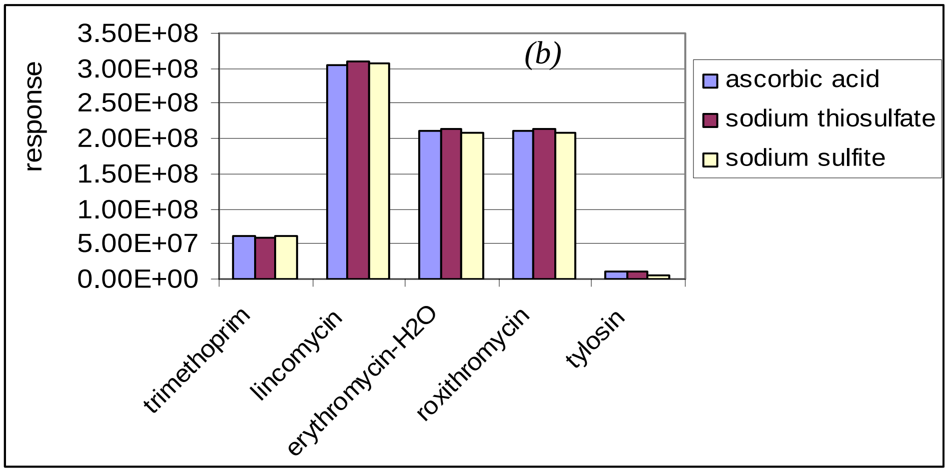
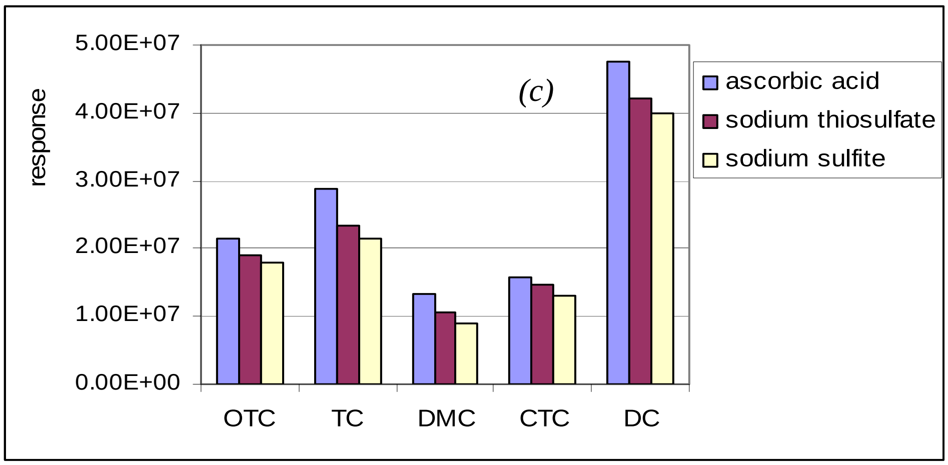


Figure 30. Comparison of Analyte Response in the Presence of Prequenched Chloramines with Excess Ascorbic Acid: (a) sulfonamides; (b) macrolides, trimethoprim, and lincomycin; (c) tetracyclines; (d) fluoroquinolones. (continued)





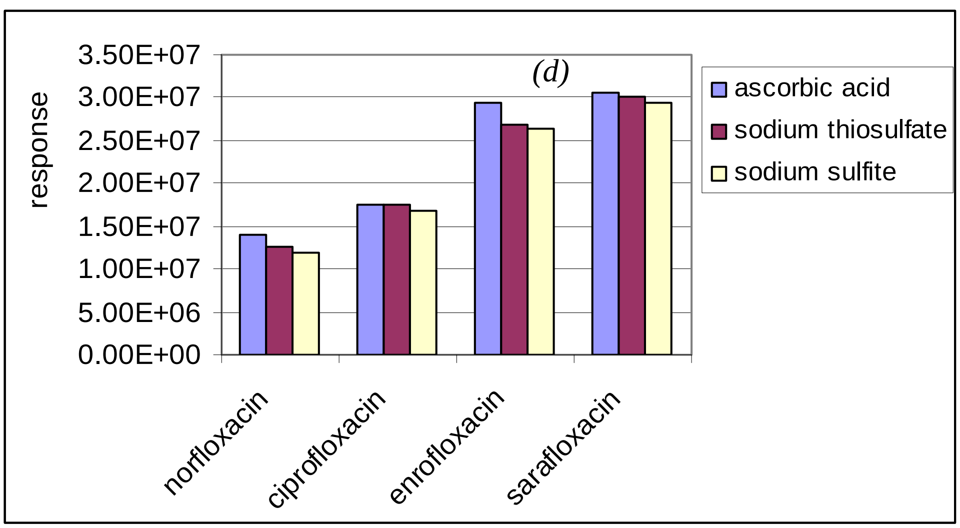


Figure 30 (cont). Comparison of Analyte Response in the Presence of Prequenched Chloramines with Excess Ascorbic Acid: (a) sulfonamides; (b) macrolides, trimethoprim, and lincomycin; (c) tetracyclines; (d) fluoroquinolones.

3.2 Chlorination Study

Sulfamethoxazole (SMX), tetracycline (TC) and doxycycline (DC), whose structures are given in Figure 31, were chosen as model compounds for a preliminary examination of reactivity of sulfonamide and tetracycline antibiotics toward free chlorine. Transformation products of these compounds were observed in LC-UV chromatograms. The molecular ions of transformation products of tetracyclines were identified by LC-MS. This study provides a basis for further examinations of reaction pathways and product formation of chlorination of antibiotics, both in DI and drinking-water matrices.

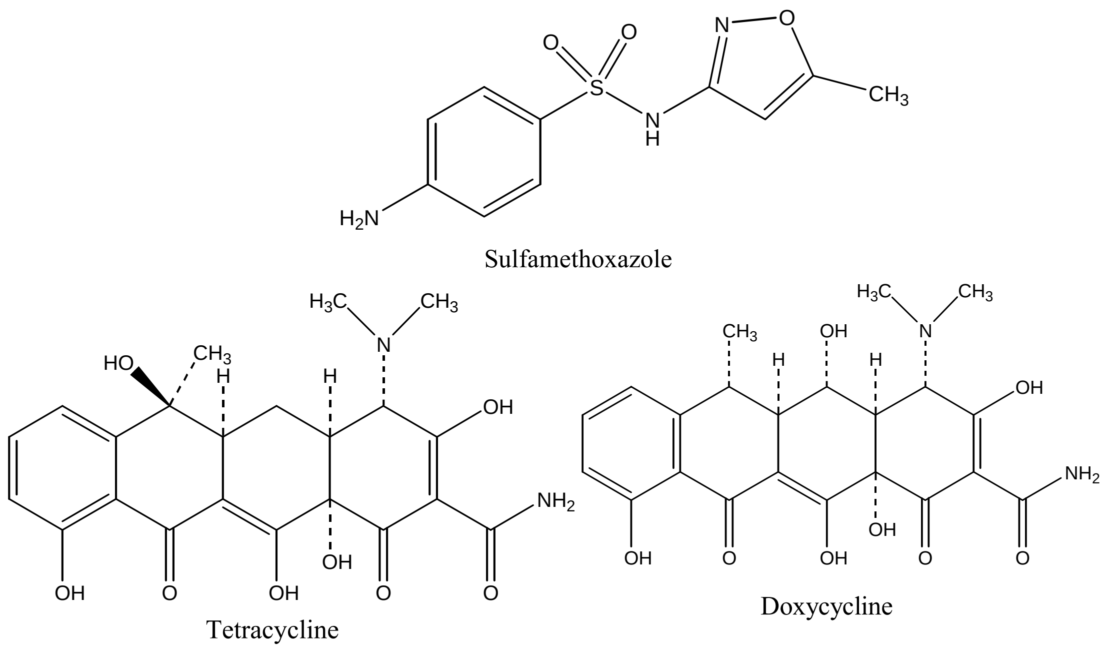


Figure 31. Structures of Model Compounds

3.2.1 Chlorination of Sulfamethoxazole

Chlorination of sulfamethoxazole was conducted with excess free chlorine at pH 7 at an ambient temperature of about 21.5ºC. A 500 ml amber glass bottle with a dispenser system mounted onto the screw-top was used as a reaction vessel. The reaction was initiated by addition of sodium hypochlorite at 1 mg/L into 500 µg/L sulfamethoxazole solution in 10 mM phosphate buffer at pH 7. At scheduled time intervals, aliquots of the reaction mixture were withdrawn and quenched instantly with 15 µL of a fresh 1 g/L sodium sulfite solution. The chlorination samples were immediately analyzed by LC-UV.

The parent compound and reaction products were separated on a Luna C-18 column (15cm, 2.1mm, 3 µm) using an isocratic mobile phase of 63% A: 37% B (A: 0.3% formic acid in water; B: acetonitrile) at a flow rate of 0.2 ml/min. The compounds were detected at a wavelength of

268 nm. The UV spectra were acquired by a PDA detector in the wavelength range of 200 nm to 500 nm.

3.2.1.1 Removal of SMX by Free Chlorine. The SMX initial concentration of 500 µg/L is not environmentally realistic but analytically convenient for monitoring the parent compounds and identifying the transformation products. The reaction pH 7 is within the pH 6-8 range that is likely to be encountered during drinking water treatment. Figure 32 shows the removal of sulfamethoxazole by free chlorine at pH 7 under these conditions.

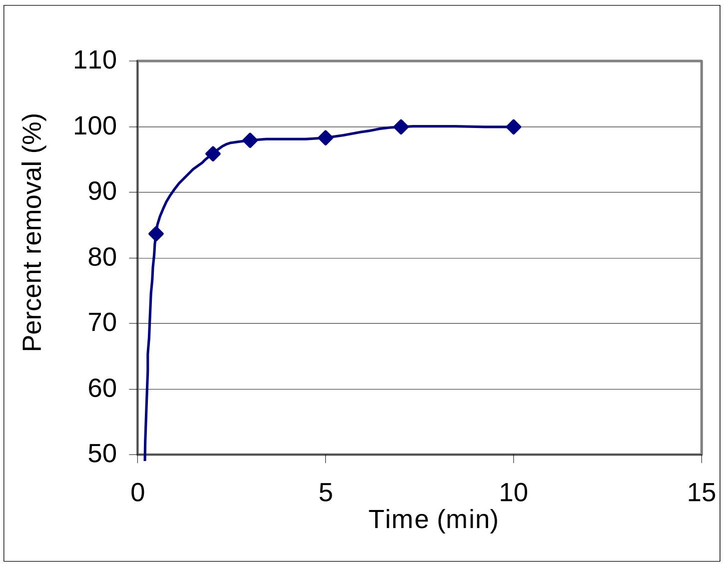


Figure 32. Removal of SMX by Free Chlorine at pH 7

3.2.1.2 Product Formation. Figure 33 shows the LC-UV chromatograms of the degradation mixture quenched at different times during the SMX (λ_{max} = 271 nm; retention time = 5.45 min) chlorination. As is apparent, at least one UV-detectable byproduct was produced during the first stage of SMX chlorination (unknown #1 at retention time 8.5 min). A minor byproduct appeared at a longer reaction time (unknown #2 at retention time 4.9 min) when the unknown #1 was not observed. Unknown #1 could be an intermediate byproduct formed during the chlorination. The UV spectra of the unknown #1 and #2 were found to have a λ_{max} of 285 nm and 261 nm, respectively. In previous work, the structure of the product was proposed based on the protonated molecular ion and its fragmentation ions identified by LC-MS (Dodd 2004). UV spectra of the following three compounds were provided in subsequent discussion: the parent compound sulfamethoxazole (λ_{max} = 275nm), an N-chloramine intermediate (λ_{max} = 254nm), and a major product (λ_{max} = 295nm) that was later identified as N-chloro-p-benzoquinoneeimine. By comparing their UV spectra alone, it is not possible to determine if the byproducts found in the two studies are identical. We propose that the major product (unknown #1) with λ_{max} = 285 nm in our study could possibly be a chlorine-substituted SMX based on the following reasoning:

1) chlorine substitution on benzene can lead to a positive shift in the compound's absorbance. In this case, λ_{max} increases from 271 nm for the parent compound to 285 nm for the byproduct;
2) chlorine substitution on the benzene ring would increase the product's hydrophobicity as demonstrated, for example, in the case of benzene and chlorobenzene where the $\log K_{ow}$ values are 2.13 and 2.84, respectively. An increase in hydrophobicity would most likely increase the compound's retention with the stationary phase on the LC column, which is the case for unknown #1 compared to the parent compound.

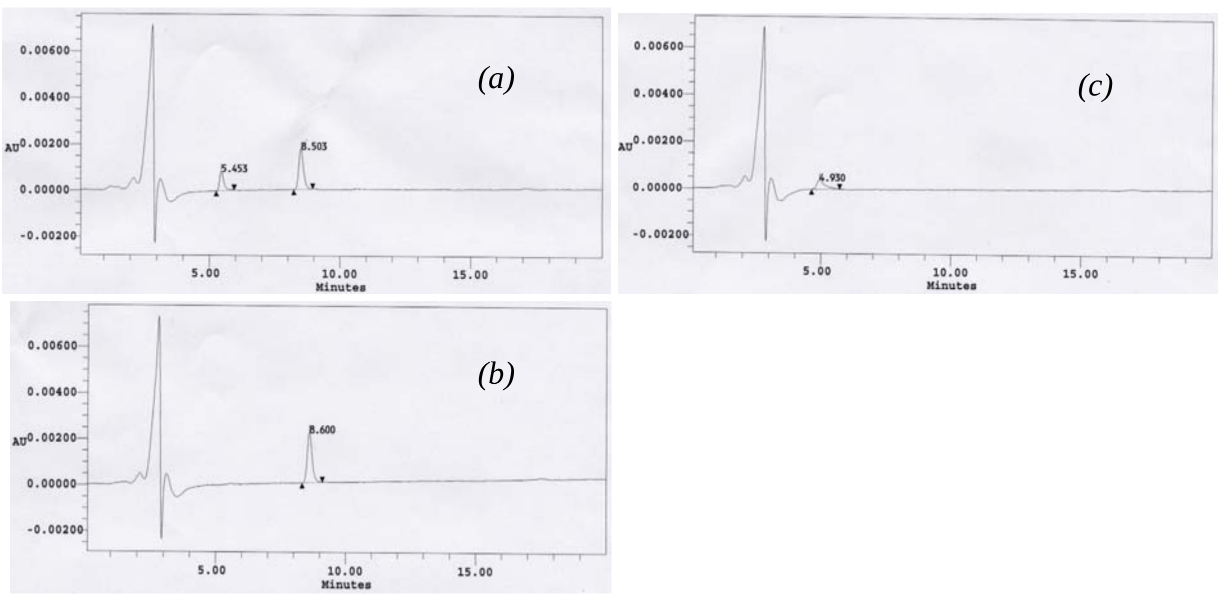


Figure 33. LC-UV Chromatograms (λ = 268 nm) of the SMZ Chlorination Reaction Mixture at Reaction Times of (a) 0.5 min; (b) 7 min; and (c) 40 min

3.2.2 Chlorination of Tetracyclines

Reactions were initiated by addition of sodium hypochlorite into 10 mg/L tetracycline or doxycycline solutions in 10 mM phosphate buffer at pH 7.0 with a tetracycline-to-free-chlorine molar ratio ranging from 2:1 to 1:10 for tetracycline and 1:1 for doxycycline. Reactions were conducted under ambient temperature 25°C for 1 hour. The residual free-chlorine concentration was monitored during chlorination. Ascorbic acid was used to quench residual chlorine. The chlorination samples were immediately analyzed by LC-UV or stored in a refrigerator at 4˚C and analyzed by LC-MS within 6 hours.

The parent compound and reaction products were separated by a Luna C-18 column (15 cm, 2.1 mm, 3 μm) using a binary gradient mobile phase at a flow rate of 0.2 ml/min for both LC-UV and LC-MS analysis. The gradient mobile phase started with 10% B, increased to 100% B within 22 minutes, stayed at 100% B for 2 minutes, and returned to the initial conditions and re-equilibrated for 15 minutes (A: 0.1% formic acid in water; B: acetonitrile). The UV spectra were acquired by PDA in the wavelength range of 200 nm to 500 nm. The reaction mixtures were also analyzed by LC-MS using EPI mode in full scan in the range of m/z 50-350 and m/z 350-500, respectively.

3.2.2.1 Removal of Tetracycline by Free Chlorine. The percent removal of tetracycline, shown in Table 24, was calculated by comparing the LC-UV peak area of the tetracycline-in-chlorination sample to that in 10 mg/L tetracycline solution. One hundred percent removal was achieved at molar ratio of 1:10 with a reaction time of 1 hour. The occurrence level of tetracyclines in raw drinking water is expected to be very low, around, ng/L concentration, while free chlorine is usually dosed at the mg/L level in disinfection. Hence, the molar ratio of free-chlorine-to-tetracycline in drinking water disinfection should be much higher than 10 (the highest ratio used in this study), suggesting removal of tetracyclines in real water treatment. However, due to chlorine demand from other sources in the drinking water matrix, the efficiency of chlorine in removing tetracycline may be very different in real waters from that in DI water.

Table 24. Removal of Tetracycline by Free Chlorine

Molar ratio of tetracycline:free chlorine	Percent removal (%)
2:1	35
1:1	70
1:2	94
1:4	99
1:10	100

3.2.2.2 Transformation of Tetracyclines. A number of transformation products of tetracycline and doxycycline were observed in the LC-UV chromatograms of the chlorination mixtures, and their molecular ions were identified by LC-MS (Figures 34 and 35). A prominent transient peak at m/z 399 was identified as a major intermediate in the chlorination of tetracycline. The structures of these products are, however, not yet elucidated. The absence of peaks in the LC-UV chromatogram of the reaction mixture at a molar ratio of 1:10 suggests the loss of aromaticity in the tetracycline structure. Product ions at m/z 443, m/z 479, and m/z 461 were identified in the chlorination mixture of doxycycline at an initial molar ratio of free-chlorine-to-doxycycline of 1:1. Product m/z 479 (445-1+35) is a chlorine-substitution product, as confirmed by the chlorine isotopic ratio.

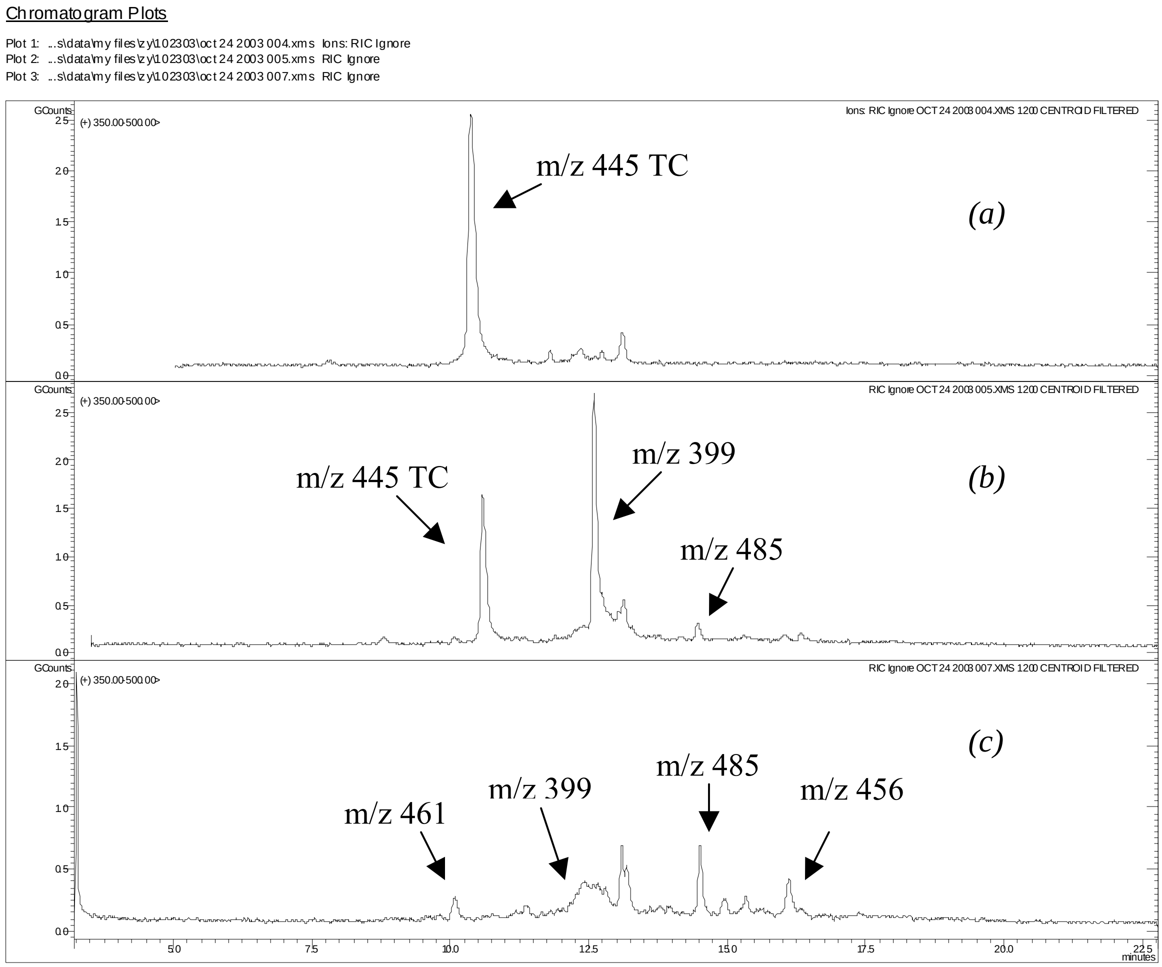


Figure 34. LC-MS Chromatograms (full scan: m/z 350-500) of (a) tetracycline solution at 10 mg/L; (b)-(c) tetracycline/free chlorine reaction mixture at molar ratios of (b) 1:2 and (c) 1:4.

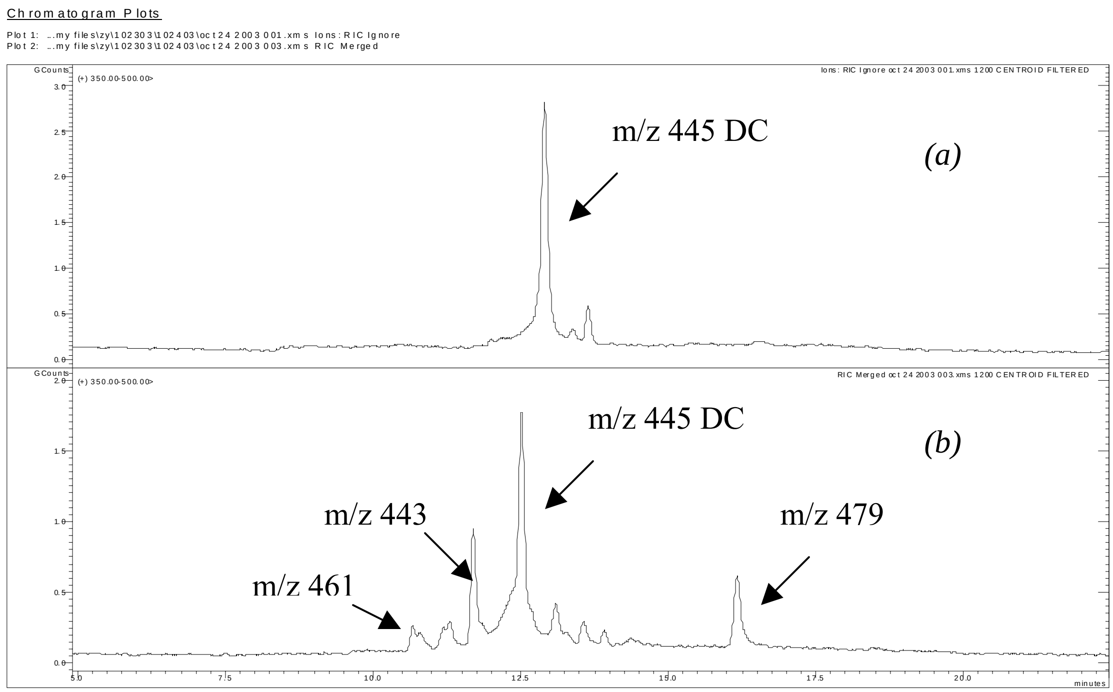


Figure 35. LC-MS Chromatograms (full scan: m/z 350-500) of (a) doxycycline solution at 10 mg/L; (b) doxycycline/free chlorine reaction mixture at molar ratio of 1:1

3.3 Occurrence Study

The method development described in Section 3.1 created the selection of protocols and procedures that would permit accurate and confirmed quantitation of 22 antibiotics in both raw and finished water at drinking water treatment plants.

3.3.1 Sample Collection

Raw-water samples were collected at the plant intakes prior to any water treatment process. Finished water samples were collected at the point after disinfection and before entry into the distribution system. Residual chlorine disinfectants in finished water were quenched by excess ascorbic acid that was pre-placed in the sample bottles. Grab samples were collected in the acid-washed 1 L amber glass bottles, which were packed into coolers with ice packs and transported to the University of North Carolina laboratory by overnight shipping. Samples were stored in a refrigerator at 4˚C and extracted within 3 days.

3.3.2 Methods Applied in Occurrence Study

Raw-water samples were filtered through 0.7 µm glass fiber (Ahlstrom, Windsor Locks, CT) to remove particles. Since the method of standard addition is used for quantitation, a 250 ml sample was spiked with analytes from 0 to 100 ng/L, and surrogate standards ($^{13}C_6$-sulfamethazine and meclocycline) at 100 ng/L. Na_2EDTA was added into samples at 1 mg/L, and sample pH was adjusted to 6 with dilute sulfuric acid (trace-metal grade). Samples were extracted through 200 mg HLB SPE columns at flow rates of approximately 5 mL/min. After extraction, the SPE column was rinsed with 6 ml of DI water and dried under vacuum for 2 min. The analytes were subsequently eluted with 8 ml of acidic methanol containing 0.1% formic acid.

Finished water samples were spiked with analytes and surrogate standards, Na_2EDTA added at 1 mg/L, and the pH adjusted to 3 with 10% formic acid. 500 ml sample aliquots were extracted on 200 mg HLB SPE cartridges at flow rates of approximately 5 ml/min. After extraction, the columns were rinsed with 6 ml DI water and dried under vacuum for 2 min. The analytes were subsequently eluted with 8 ml of acidic methanol containing 0.1% formic acid.

All the extracts were blown down to 50-100 µL under a gentle nitrogen flow at 42-45ºC, reconstituted to about 250 µL with a solvent mixture of water/methanol (9:1), and spiked with the internal standard simatone at 100 µg/L for raw-water extracts and 25 µg/L for finished-water extracts. The samples were votexed, filtered though 0.45 µm syringe filters (Water, Milford, MA), transferred to amber autosampler vials, and stored in a freezer before LC-MS/MS analysis. Figure 36 summarizes schematically the entire analytical procedure.

3.3.3 Initial Survey of Source Waters

During analytical method development, treatment plants from four utilities were surveyed for 22 antibiotics in their source surface waters. The drinking water treatment plants located in Burlington and Greensboro used free chlorine for disinfection, while those in Durham and Fayetteville added ammonia into the chlorinated water to form chloramines. The water-quality data measured by the plant personnel are shown in Tables 25 and 26. The samples of finished water collected from these plants were also used in the development of an approach to quench residual disinfectants, as described in Section 3.1.12.

Table 25. Source Water-Quality Data (06/03/03)

Plant (location)	WTP1 (Burlington)	WTP2 (Fayetteville)	WTP3 (Durham)	WTP4 (Greensboro)
pH	6.7	6.9	6.4	7.4
TOC	6.3	7.3	8.9	4.9

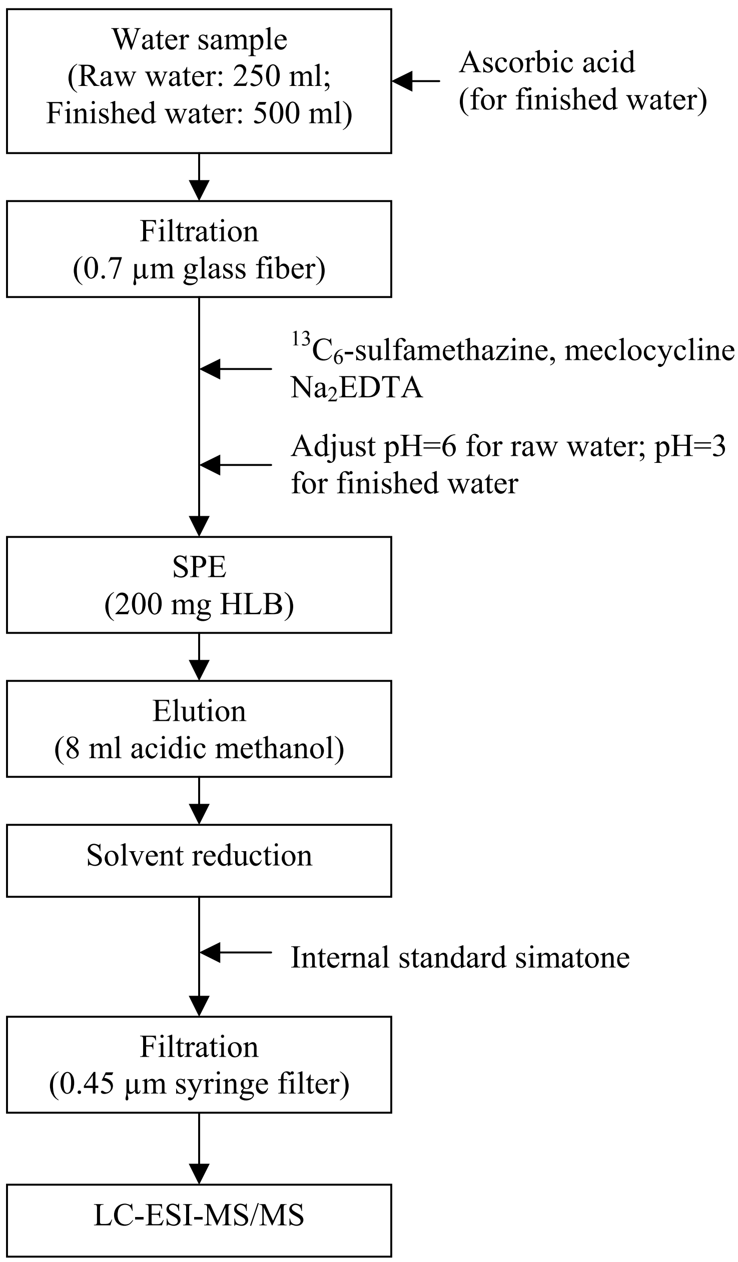


Figure 36. Schematic of the Analytical Procedure for Antibiotics in Raw and Finished Drinking Water Samples

Table 26. Finished Water-Quality Data (06/03/03)

	WTP1	WTP2	WTP3	WTP4
pH	7.0	7.1	8.3	7.6
TOC (mg C/L)	2.2	2.9	2.5	2.5
Residual free chlorine (mg/L)	0.0	0.29	1.4	1.2
Residual total chlorine (mg/L)	3.4	4.0	1.6	1.7

3.3.3.1 Results. Lincomycin at 4.8 ng/L was detected at WTP1 and erythromycin-H_2O was detected at all four plants, viz WTP1:3.1ng/L; WTP2:2.6 ng/L; WTP3:2.1 ng/L; and WTP4:2.2 ng/L. No other analytes were detected in the raw-water samples, and all finished waters detected no analytes.

3.3.4 Second Survey

3.3.4.1 Sampling Sites. Raw and finished drinking-water samples were collected from five drinking water treatment plants Oct. 23, 2003. The plant located in Burlington used chlorine-only as its disinfectant while the other four plants added ammonia into the chlorinated water to form chloramines. The water-quality data measured by plant personnel are shown in Tables 27 and 28.

Table 27. Source Water Quality Data (10/23/03)

Plant (location)	WTP1 (Burlington)	WTP2 (Fayetteville)	WTP3 (Durham)	WTP5 (Greenville)	WTP6 (Raleigh)
pH	6.5	7.3	6.6	7.3	6.6
TOC	6.4	n.a.	5.9	10.2	5.0

Table 28. Finished Water Quality Data (10/23/03)

	WTP1	WTP2	WTP3	WTP5	WTP6
pH	7.1	7.4	8.6	7.9	8.5
TOC (mg C/L)	2.7	n.a.	3.5	3.8	2.0
Residual free chlorine (mg/L)	1.7	0.05	n.a.	n.a.	n.a.
Residual total chlorine (mg/L)	n.a.	3.9	4.0	3.5	4.0

n.a.: not available

3.3.4.2 Results. All the raw-water samples were spiked with the 22-component antibiotic mixture at 20, 50 and 100 ng/L before extraction. Finished-water samples from treatment plants in Fayetteville (WTP2) and Durham (WTP3) were spiked with this mixture at 20, 50 and 75 ng/L, while those from treatment plants in Burlington (WTP1) and Raleigh (WTP6) were spiked at 10, 20 and 50 ng/L. The finished-water sample from Greenville (WTP5) was spiked at only one level (75 ng/L) due to sample loss as a result of bottle breakage during shipment.

Antibiotics were detected in raw-water and finished-water samples (Tables 29 and 30, respectively). The concentrations of analytes that were not detected were reported as less than the lowest detectable spike level. For those analytes in which a signal response was detected and an MS/MS ion positively identified, a linearly extrapolated concentration is presented, shown in parentheses. WTP2 showed the largest number of antibiotics in its source water, which comes from the Cape Fear River. Overall, fluoroquinolones were the most frequently detected antibiotics in source waters, followed by sulfonamides, lincomycin, tetracyclines, and macrolides.

Table 29. Occurrence of Antibiotics in Source Waters During Second Survey

Concentration (ng/L)	WTP1	WTP2	WTP3	WTP5	WTP6
Ciprofloxacin	8.3	70	68	55	17
Norfloxacin	< 20	64	37	38	< 20
Enrofloxacin	< 20	43	24	20	49
Sarafloxacin	< 20	(15)	< 20	< 20	< 20
Lincomycin	< 20	(8.0)	< 20	(14)	(7.0)
Sulfamethoxazole	< 20	62	< 20	< 20	< 20
Sulfadimethoxine	< 20	(15)	< 20	< 20	< 20
Sulfathiazole	< 20	< 20	< 20	< 20	< 20
Sulfamerazine	< 20	< 20	< 20	< 20	< 20
Sulfamethazine	< 20	< 20	< 20	< 20	< 20
Sulfamethizole	< 20	< 20	< 20	< 20	< 20
Sulfachlorpyridazine	< 20	< 20	< 20	< 20	< 20
Trimethoprim	< 20	< 20	< 20	< 20	< 20
Oxytetracycline	< 20	(15)	< 20	< 20	< 20
Tetracycline	< 20	25	(13)	< 20	< 20
Doxycycline	< 20	< 20	22	< 20	< 20
Chlortetracycline	< 20	< 20	< 20	< 20	< 20
Demeclocycline	< 20	< 20	< 20	< 20	< 20
Minocycline	< 100	< 100	< 100	< 100	< 100
Tylosin	< 20	21	(12)	< 20	< 20
Erythromycin-H_2O	< 20	(6.7)	< 20	< 20	< 20
Roxithromycin	< 20	< 20	< 20	< 20	< 20

Note: Values in parentheses indicate positive identification of the species, but at concentrations less than the lowest spiked level.

Table 30. Occurrence of Antibiotics in Finished Waters During Second Survey

Concentration (ng/L)	WTP1	WTP2	WTP3	WTP5	WTP6
Ciprofloxacin	< 10	18	13	7.6	< 10
Norfloxacin	< 20	< 20	(5.5)	< 75	< 10
Enrofloxacin	< 10	< 20	< 20	< 75	< 10
Sarafloxacin	< 10	< 20	< 20	< 75	< 10
Lincomycin	< 10	(9.6)	< 20	< 75	<10
Sulfamethoxazole	< 10	< 20	< 20	< 75	< 20
Sulfadimethoxine	< 10	< 20	< 20	< 75	< 10
Sulfathiazole	< 20	< 20	< 20	< 75	< 20
Sulfamerazine	< 10	< 20	< 20	< 75	< 20
Sulfamethazine	< 10	< 20	< 20	< 75	< 10
Sulfamethizole	< 20	< 20	< 20	< 75	< 20
Sulfachlorpyridazine	< 10	< 20	< 20	< 75	< 10
Trimethoprim	< 10	< 20	< 20	< 75	< 10
Oxytetracycline	< 10	< 20	< 20	< 75	< 10
Tetracycline	< 10	< 20	< 20	< 75	< 10
Doxycycline	< 10	< 20	4.5	< 75	< 10
Chlortetracycline	< 10	< 20	< 20	< 75	< 10
Demeclocycline	< 10	< 20	< 20	< 75	< 10
Minocycline	< 50	< 75	< 75	< 75	< 50
Tylosin	< 10	< 20	12	< 75	< 10
Erythromycin-H_2O	< 10	< 20	< 20	< 75	< 10
Roxithromycin	< 10	< 20	< 20	< 75	< 10

Note: Values in parentheses indicate positive identification of the species, but at concentrations less than the lowest spiked level.

Although the detection of ciprofloxacin, which is used only in human medication, seems to indicate the impact of wastewater discharge, the possibility of contamination from animal sources should not be excluded, as the veterinary fluoroquinolone enrofloxacin can, under certain conditions, be metabolized to ciprofloxacin.

3.3.5 Discussion of Antibiotic Occurrence Survey Results

The occurrence levels of antibiotics obtained in this study are generally lower than the data included in the USGS national reconnaissance (Kolpin et al. 2002), in which the selection of sampling streams was biased toward those that were susceptible to wastewater contamination.

Although the occurrence level of individual antibiotics is low, the presence of a broad range of antibiotics in some source waters suggests that the overall effect of antibiotics as a contaminant group should not be underestimated.

In an attempt to discern fundamental reasons for the different findings during the two sampling events, hydrologic conditions at the time of each sample collection were compared and are presented in Table 31.

Table 31. Hydrologic Conditions at Time of Sample Collection

City	Plant ID	County	Source Water	Daily Mean Flow into Plant (ft^3/second)	
				06/03/03	10/23/03
Burlington	WTP1	Alamance	Haw River	450	160
Fayetteville	WTP2	Cumberland	Cape Fear*	19	12.2
			Little River	880	250
Durham	WTP3	Durham	Little River	56	20
			Eno River	88	105
Greensboro	WTP4	Guilford	Reedy Fork	50	15 (not sampled)
Greenville	WTP5	Pitt	Tar River	not sampled	800
Raleigh	WTP6	Wake	Falls Lake *	252	251
			Neuse River near Falls Lake	3500	180

Source data is from http://nwis.waterdata.usgs.gov/nc/nwis/sw
*for Falls Lake and Cape Fear River, only gauge height (feet) is available

Information supplied from plant records indicates that there was heavy rainfall in the 14 days preceding the June 2003 sampling (e.g., 5.92" at WTP1) compared to the relatively dry 14-day period preceding the October 2003 sampling (e.g., 0.03" at WTP1). This is reflected in the lower stream-flow rates for most of the river sources feeding the plants intakes during the October sampling compared to June. Given that less than 5% of the inflow to most of the plants comes from upstream WWTP effluents or nonpoint sources, the increased flow rates resulting from storm events will be more likely to dilute the levels of contaminants from either of these sources. This is, therefore, the most likely explanation for the much lower levels of antibiotics found during the first (June 2003) survey. In the second (October 2003) survey, the larger number of analytes and their higher concentrations found in the source water of WTP2 are most likely due to swine farm operations identified in the upstream watershed and a higher percentage of point and nonpoint wastewater discharges than the 5% average for all plants.

WTP5 plant personnel also suggested that there are likely several waste plant discharges into the Tar River upstream of the drinking water plant intake that could be responsible for the elevated levels of fluoroquinolones in WTP5's source water. WTP1 lacks any identified wastewater discharges upstream of its plant intake and very few small-scale cattle farms in its protected watershed. This could account for the near total lack of quantifiable antibiotics in that plant's source water.

Most of the antibiotics detected in source water are not detected in finished drinking water or are present at a much lower level if detected, indicating their partial removal during conventional treatment processes. This study did not set out to identify which treatment process would be most effective at antibiotic removal, although it was noted that chlorination, as shown in Section 3.2, does cause some loss of functionality that would render the parent compound nondetectable by the analytical process employed. The incomplete removal of some antibiotics, such as ciprofloxacin, is of concern due to the unknown health effects if these compounds persist in finished drinking water, even at ng/L levels.

4. CONCLUSIONS

4.1 Analytical Method Development

LC-ESI-MS/MS methods were developed for analysis of 22 antibiotics, including sulfonamides, tetracyclines, fluoroquinolones, macrolides, trimethoprim, and lincomycin, with reporting limits ranging from 10 to 20 ng/L depending on the concentration factor used during extraction and the effect of the NOM. The nature and degree of the matrix effect on LC-MS/MS analysis vary for different analytes and appear to be dependent on concentrations for some analytes. SPE using eluting solvents with differing polarities appears to reduce the co-elution of NOM and improve LC-MS/MS analysis for sulfonamides, trimethoprim, and macrolides. Extraction of surface waters of pH 6 appeared to reduce co-extraction of NOM, as compared to extraction at pH 3.

Overall, a single SPE with liquid chromatography/tandem mass spectrometry detection was developed to analyze multiple classes of antibiotics in raw and finished drinking water samples down to low range ng/L (with the exception of minocycline, which is less sensitive).

4.2 Chlorination Study

Sulfonamides and tetracyclines were found to be reactive with free chlorine in aqueous solutions at pH 7. A number of reaction intermediates or products were observed by LC-UV monitoring of reaction mixture, and the molecular ions of some products were identified by LC-MS.

4.3 Occurrence Study

A survey of several of the treatment plants in the NCUWC indicated the presence in their source waters of some of the antibiotics targeted in this study. Overall, fluoroquinolones were the most frequently detected in source waters, followed by sulfonamides, lincomycin, tetracyclines, and macrolides. Although the detection of ciprofloxacin, which is used only in human medication, seems to indicate the impact of wastewater discharges, the possibility of contamination from animal sources should not be excluded, as the veterinary fluoroquinolone enrofloxacin can, under certain conditions, be metabolized to ciprofloxacin. In most of the treatment plants, the finished waters showed either no detectable antibiotic residues or very reduced levels. However, it is not known whether these compounds were physically removed during treatment, such as via adsorption to filters or precipitation in coagulation basins, or were merely chemically converted during disinfection processes. Any of these pathways may contribute to accumulation of the target compounds in the environment. It is essential that fate of these compounds and transport mechanisms be better understood if human health is not to be compromised.

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