INVESTIGATION OF TETRACYCLINE, SULFONAMIDE, AND FLUOROQUINOLONE ANTIMICROBIAL COMPOUNDS IN MANURE AND AGRICULTURAL SOILS IN NORTH MARMARA REGION

by

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ABSTRACT

Occurrence of antibiotic residuals in the environment is of concern because of the emergence and development of antibiotic-resistance in pathogen bacteria, and the ecotoxicological behaviour of these compounds. Investigation of antibiotic pollution in animal manure has special importance since they constitute the major source for the dissemination of them into the environment. Hence, this study was conducted to determine the level of antimicrobial pollution in eight animal manure and nine agricultural soil samples collected from different sampling points located in the North part of Marmara Region. While tetracyclines (TCs) and sulfonamides (SAs) extraction were carried out by ultrasonic agitation followed by tandem solid phase extraction, fluoroquinolones (FQs) were extracted by ultrasonic agitation followed by 0.45 μm filtration. The antibiotic analysis was performed by high performance liquid chromatography (HPLC).

TCs, SAs, and FQs were determined in manure up to 0.47, 35.5, and 0.057 mg kg^{-1}, respectively. In manure amended agricultural soils, TCs, SAs, and FQs were detected at the maximum concentrations of 0.50, 0.40, and 0.053 mg kg^{-1}, respectively. At least one antimicrobial compound was detected in all the agricultural soil and animal manure samples. The recovery rates of the antimicrobial compounds from soil and manure were assessed, and the relationship between these results and sample characteristics was statistically evaluated.

ÖZET

Çevrede antibiyotik kalıntılarının varlığı, patojen bakterilerde antibiyotik direncinin ortaya çıkması ve gelişmesi ve bu bileşiklerin ekotoksikolojik davranışlarından dolayı endişe yaratmaktadır. Hayvan gübresinde antibiyotik kirliliğinin araştırılması özellikle önemlidir, çünkü hayvan gübresi, antibiyotiklerin çevrede yayılmaları açısından önemli kaynak rolü oynamaktadır. Bu nedenle, Marmara Bölgesi'nin kuzey kesiminde bulunan farklı örnekleme noktalarından toplanan sekiz hayvan gübresi ve dokuz tarım toprağı örneğinde antimikrobiyal kirlilik seviyelerinin belirlenmesi amacıyla bu çalışma gerçekleştirilmiştir. Tetrasiklin ve sülfonamid ekstraksiyonu, ultrasonik titreşim ve tandem katı faz ekstraksiyonu ile yapılırken, fluorokinolon ekstraksiyonu, ultrasonik titreşim ve 0.45 μm-filtreleme ile yapıldı. Antibiyotik analizi, yüksek performanslı sıvı kromatografisi (HPLC) ile gerçekleştirildi.

Tetrasiklin, sülfonamid ve fluorokinolonlar gübrede, sırasıyla azami 0.47, 35.5 ve 0.057 mg kg^{-1}'a kadar tespit edildi. Gübre uygulanan tarım topraklarında tetrasiklin, sülfonamid ve fluorokinolonlar, sırasıyla azami 0.50, 0.40 and 0.053 mg kg^{-1} derişimlerde tespit edildi. Tüm tarım toprağı ve hayvan gübresi örneklerinde, en az bir antimikrobiyal bileşik tespit edildi. Antimikrobiyal bileşiklerin toprak ve gübreden geri kazanım miktarları saptandı ve bu sonuçlar ile örnek karakteristikleri arasındaki ilişki istatistiksel olarak değerlendirildi.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Antibiotics are one of the most predominant pharmaceutical classes prescribed for both humans and animals all over the world. They constitute more than 6 % of all prescriptions in human medicine and more than 70 % of all consumed pharmaceuticals in veterinary medicine (Thiele-Bruhn, 2003). Among the veterinary antibiotics, approximately 1600 tons of growth promoters and 3500 tons of therapeutic antibiotics were estimated to be used in 1997 in European Union and Switzerland to accelerate the growth of animals and to treat and/or prevent diseases, respectively (Christian et al., 2003). In the United States of America, approximately 70 % of the estimated 16 million kg of antibiotic compounds consumed in 2000 has been used for non-therapeutic purposes (Sarmah et al., 2006). In Turkey, antibacterial usage has been reported to be 33 % of the total veterinary pharmaceutical consumption (İlaç Sanayii Özel İhtisas Komisyonu, 2006).

TCs, SAs, and FQs are three important antibiotic groups due to their extensive use in veterinary medicine. These compounds administered to animals are excreted from the body as parent compounds or metabolites after a short time of residence (Thiele-Bruhn, 2003). TCs and SAs can be excreted at a rate up to 90 % (Kemper, 2008) and similarly, FQs are excreted at the rates varying between 60 and 85 % largely unchanged (Morales-Munoz et al., 2004). As a result, the active antimicrobial compounds or metabolites can end up in the manure. TCs, SAs, and FQs have been detected in liquid manure and dung samples up to 46, 91, and 8.3 mg kg^{-1}, respectively (Martinez-Carballo et al., 2007). Fertilization of agricultural soils with animal manure can, in turn, lead to a non-point source contamination of the terrestrial environment with these substances (Accinelli et al., 2006). The strong sorption of particularly TCs and FQs to soil results in the persistence of them in the environment without any changes in their chemical structures. As a result, they may accumulate in agricultural soils after repeated fertilization with animal manure (Kay et al., 2004). Recent studies of the occurrence of various antibiotics in different soils fertilized with animal manure were conducted in Europe. The reported maximum concentrations for TCs, SAs, and FQs screened in these studies were 0.3 mg kg^{-1} (Hamscher et al., 2005),

0.015 mg kg^{-1} (Christian et al., 2003), and 14 mg kg^{-1} (Morales-Munoz et al., 2004), respectively.

Repeated fertilization with manure contaminated with antimicrobial compounds may lead to the development of antibiotic-resistance in agricultural soils (Rooklidge, 2004). Resistance genes can be transferred from soil bacteria to other bacteria living in the aquatic compartments such as surface water and ground water (Kümmerer, 2004). In addition, they may be transferred directly to humans by the consumption of crops which are grown in arable soils containing antibiotics (Batchelder et al., 1982; Kumar et al., 2005; Boxall et al., 2006). Furthermore, some antibiotics may suppress the activity and growth of certain kinds of microorganisms and lead to changes in the population of indigeneous soil microorganisms even at low concentrations (Halling-Sørensen et al., 2002b). In a recent study, sulfapyridine and oxytetracycline (OTC) have been shown to inhibit the soil microbial activity by 10 % at the effective dose ranges of 0.003-1.14 mg kg^{-1} and 5.50-7.35 mg kg^{-1}, respectively (Thiele-Bruhn and Beck, 2005). Moreover, enrofloxacin (ENR) has been reported to induce both toxic effect and hormesis in plants at the concentration range of 0.05-5 mg l^{-1} (Migliore et al., 2003).

Due to the low concentrations of antimicrobial compounds typically encountered in the environment and the complexity of the environmental matrices, sensitive and selective techniques are needed for the extraction and analysis of them in environmental samples. To date, various extraction methods of TCs, SAs, and FQs for different matrices such as food, tissue, and groundwater and surface water samples have been reported (Vinas et al., 2004; Cherlet et al., 2003a; Batt et al., 2006; Turiel et al., 2003) although there are few studies involving the extraction of these three groups of antimicrobial compounds in soil and animal manure matrices (Martinez-Carballo et al., 2007; Jacobsen and Halling-Sorensen, 2006). The strong interactions of TCs and FQs with soil or manure reduce the extraction efficiency of these compounds. The recovery rates and limit of detections (LODs) of the antimicrobial compounds have generally been matrix-dependent. The simultaneous extraction of three different antimicrobial classes (TCs, SAs, and macrolides) from four different matrices (soil, slurry, groundwater, and surface water) using the same extraction method with little modification has been recently reported (Blackwell et al., 2004a,b). Clean-up of soil and manure extracts commonly using solid phase extraction (SPE) is

essential in most instances to reduce the effects of the unwanted matrix components on detection and quantification of the analytes. The use of liquid chromatography-mass spectrometry (LC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS^2) techniques for the environmental analysis of many antimicrobial compounds provides lower LODs compared to HPLC-UV. However, despite the superiority of MS as a detection technique, LC in combination with ultraviolet-diode array detection (UV-DAD) or fluorescence detection (FLD) is still used in many laboratories due to their relatively low cost.

Extraction efficiencies can be strongly influenced by manure and soil composition, since the sorption of TCs, SAs and FQs is highly dependent on several variables such as clay, organic carbon (OC), di- and trivalent metal content, cation exchange capacity (CEC), and pH of the environment (Jones et al., 2005; Thiele-Bruhn et al., 2004; Uslu et al., 2008). Therefore, the efficiency of extraction method should be evaluated together with the physical and chemical characteristics of manure and soil samples.

Although in Europe, some investigations (Haller et al., 2002; Jacobsen et al., 2004) have been conducted on the occurence of veterinary antimicrobial compounds in animal manure and agricultural soils, which are considered as a main source for water pollution, there is still lack of data on this issue in Turkey. Therefore, the aim of this study was to investigate the presence of TCs, SAs, and FQs in fertilized soil and manure samples collected from different agricultural fields located on North – Marmara Region in Turkey. These three antibiotic classes were selected as target substances in the present study due to their extensive use in animal husbandry. This study also aimed to establish a relationship between different physical and chemical characteristics of soil and manure samples and the recovery rates of antibiotics.

2. THEORETICAL BACKGROUND

2.1. Environmental Effects of Veterinary Antibiotics

Antibiotics are defined as naturally occurring, semi-synthetic and synthetic compounds with antimicrobial activity that can be applied parenterally, orally or topically (Kemper, 2008). They are one of the most predominant therapeutic classes prescribed for both humans and animals all over the world. Among all pharmaceuticals, antibiotics constitute more than 6 % of all prescriptions in human medicine, and more than 70 % of all consumed pharmaceuticals in veterinary medicine (Thiele-Bruhn, 2003).

Despite the extensive use of antibiotics for decades, they were recently regarded as a concern by the recognition of antibiotic-resistant bacteria. As a consequence of the concerns about the environmental effects of antibiotics, most studies have focused on the occurrence of human and veterinary antibiotics in the environment since mid to late 1990s. Bacteria may share their resistance traits with one another and so, diseases would not be eliminated by using the conventional antimicrobial compounds (Levy, 1998). Other possible effects of antibiotics on the environment are both acute and chronic effects on different organisms such as aquatic species and soil bacteria (Blackwell et al., 2004a), and transfer of these substances to food chain via uptake by plants (Boxall et al., 2006).

2.1.1. Antibiotic Resistance

The main concern about the occurrence of antibiotics in the environment is the development of resistant bacterial strains. Widespread use and long-term exposure to low doses of antimicrobial compounds have resulted in the emergence of resistant bacteria which are no longer sensitive to these compounds (Rooklidge, 2004). In addition, the inherent property of bacteria to be able to adapt to different environmental conditions facilitates this process. Many of the bacteria have been reported to be able to show multiple resistance patterns (Hirsch et al., 1999).

Bacterial resistance to antimicrobial compounds can occur in several ways. Although many bacteria inherit the resistance genes from their forerunners, genetic mutations are readily occur in bacteria and spontaneously produce a new resistant trait or will strengthen an existing one contributing to defense against an antibiotic via exchange of resistance genes between the species (Levy, 1998). Genetic material is exchanged between bacteria through transformation (the exchange of DNA), transduction (bacteriophage), and conjugation by plasmids (extrachromosomal DNA) (Neu, 1992). In transformation, after a bacterium dies and releases its content into the environment, another bacterium will scavenge the free DNA from a dead cell in their vicinity. In transduction, resistance genes are transferred by viral delivery. Conjugation by plasmids, tiny loops of DNA, is the most favoured pathway of resistance-gene transfer and refers to the transfer of resistance genes via exchange of plasmids between bacteria (Levy, 1998).

The resistance patterns to individual antimicrobial compounds can be found in different environmental compartments. Hospital effluent has been identified as an important source of antibiotics and antibiotic resistance. Gram-negative bacteria with extended spectrum β-lactamase and multi-resistant pneumococcal bacteria have been isolated from hospitals all over the world and this may cause to problems in treatment and prevention of human illnesses (Kemper, 2008). Ciprofloxacin (CF) residues detected in hospital effluent up to 125 $\mu g\ l^{-1}$ was concluded to be the cause of genotoxicity in this matrix (Hartmann et al., 1998). Schwartz et al. (2003) detected vanA resistance genes in bacteria present in hospital effluent.

Municipal sewage and activated sludge of STPs were found to be an important source of antibiotic-resistant bacteria and antibiotic resistance (Kümmerer, 2004). It is the wide acceptance that the main origin of antibiotic-resistant bacteria in municipal sewage is the effluents of hospitals even though some resistance may be contributed from the domestic use of antibiotics. Reinthaler et al. (2003) studied antibiotic resistance of *E. coli* in sewage and sludge, and resistance rates were highest for tetracycline. Vancomycin-resistant *enterococci* carrying the resistance gene "vanA" were detected in Swedish sewage by Iversen et al. (2002). Although the overall contamination with bacteria would be much less than in municipal STP effluents, surface water and ground water may act as a reservoir of antibiotic resistance (Hirsch et al., 1999). Goni-Urriza et al. (2000) investigated the

influence of an urban effluent on antibiotic resistance in rivers, and these authors concluded that antibiotic resistance increased with increasing urban effluent input to rivers.

Fish farms are generally the main reason of the development of antibiotic-resistance in sediments (Halling-Sørensen et al., 1998). Samuelsen et al. (1992) found sediment bacteria resistant to OTC which is extensively fed to fish. Similar to sediments, antibiotic-resistance may occur in agricultural soils after repeated fertilization with manure contaminated with antimicrobial compounds (Rooklidge, 2004). Fründ et al. (2000) have detected TC-resistant microorganisms in soil after the fertilization of agricultural soil with manure containing TC residues. Sengeløv et al. (2003) have studied bacterial resistance levels in agricultural soil as a result of treatment with pig manure, and concluded that elevated amounts of pig manure slurry amendment to farmland may lead to higher TC resistance levels in soil.

MacKie et al. (2006) detected both TC residues and TC-resistant genes in groundwater influenced by swine production facilities. This study clearly demonstrated that agricultural use of antibiotics has an impact on the development of antibiotic-resistance in groundwater. Even in drinking water, antibiotic-resistance patterns have been found likely due to faecal contamination. Schwartz et al. (2003) detected resistance genes to vancomycin and ampicillin in heterotrophic bacteria present in drinking water biofilms.

Antibiotic-resistance may also develop in humans via exposure to residues of veterinary antibiotics in the environment such as soil, water, and sediment. In addition, resistance genes may be transferred directly to humans by the consumption of crops, livestock, fish, and water that have accumulated substances from various sources (Batt et al., 2006). It is also noteworthy that veterinary antibiotics can cause resistance to those used for humans due to the chemical structures similar enough although antibiotics prescribed for humans are generally not the same as those given to animals (Lindsey et al., 2001).

2.1.2. Ecotoxic Effects

Antibiotics are biologically active substances, and may act efficiently at low doses against microorganisms and bacteria found in both humans and animals. This effectiveness at low concentrations is a significant threat for other organisms present in the environment (Sarmah et al., 2006). Some antibiotics may suppress the activity and growth of certain kinds of microorganisms, and lead to changes in the population of indigeneous water and soil microbes even in low concentrations.

Antibiotics may have ecotoxic effects on various organisms living in different environmental compartments. There are a number of studies involving the acute and chronic effects of antibiotics on a range of aquatic species. Wollenberger et al. (2000) investigated the acute and chronic effects of nine veterinary antibiotic compounds used in intensive farming on a freshwater crustacean *Daphnia Magna*, and reported that oxolinic acid and tiamulin exerted acute toxic effects to *D. Magna* with 48-h EC_{50} values of 4.6 and 40 mg l^{-1}, respectively. In addition, reproduction toxicities were observed for OTC, sulfadiazine (SDZ), TC, and tiamulin in the range of 5-50 mg l^{-1}. Ferreira et al. (2007) studied the acute toxicity of OTC and florfenicol to the microalgae *Tetraselmis chuii* and the crustacean *Artemia parthenogenotica*, and found that OTC and florfenicol inhibit the growth of *T. chuii* with 96 h IC_{50} values of 11 and 6 mg l^{-1}, respectively. These authors concluded that these antibiotics were more toxic to *T. chuii* than to *A. parthenogenotica*. Sanderson et al. (2004) focused on the evaluation and categorization of the toxicity of antibiotics, antineoplastics, cardiovascular, and sex hormones on daphnid, fish, and algae. This study depicted that antibiotics pose the highest risk in terms of the human and environmental health. The most vulnerable species to antibiotics was the daphnids, while the algae have been found to be least susceptible to these compounds. All of these studies demonstrate that antibiotics may disturb the communities of aquatic species having severe toxic effects on them.

Even though most studies have concentrated on the toxic effects of antibiotics on aquatic species, some authors have evaluated antibacterial ecotoxicity on soil-dwelling organisms. Thiele-Bruhn and Beck (2005) investigated the toxicity of TCs and SAs compounds on soil microorganisms, and found that the antibiotics significantly reduced the

number of soil microbes. This study showed that environmentally relevant concentrations of antibiotic compounds in soil may have ecotoxicological impacts on soil microorganisms. On the other hand, the effects of antimicrobial compounds on soil-dwelling organisms mainly depend on several factors including antibiotic dose, persistence time, and bioavailability. Since antibiotic doses required to inhibit the microorganisms are generally much smaller than required for the higher organisms (Wollenberger et al., 2000), even environmentally relevant concentrations of many antibiotics may have short- and long-term toxic effects on a range of microbial communities. Persistence of antibiotics in soil may increase their toxic effects on soil-dwelling organisms (Al-Ahmad et al., 1999), since their persistence leads to the accumulation of antibiotics in soil. Bioavailability of antibiotics mainly depends on the soil properties, availability of nutrients, and the presence of root exudates. For instance, sorption and the presence of multivalent cations have been reported to inhibit the antibiotic potential of some antimicrobial compounds (Jjemba, 2002; Froehner et al., 2000).

Besides the parent compounds, the degradation products of antibiotics may be potent to different soil organisms. This potency may be even similar as the parent compounds. For example, several degradation products of tetracycline, chlortetracycline (CTC), and OTC have been found to have the same potency to sludge and TC-sensitive soil bacteria as the parent compounds (Halling-Sørensen et al., 2002). As a consequence, degradation products of antibacterial compounds may pose a risk in terms of the ecotoxicity, and should be monitored in environmental matrices together with the parent compounds.

2.1.3. Uptake of Antibiotics by Plants

Uptake of the antimicrobial compounds is influenced mainly by two factors: type of the plant and antibiotic compound (Jjemba, 2002). For example, Kumar et al. (2005) have found that CTC was taken up by corn to the greatest extent followed by cabbage and green onions, whereas tylosin could not be detected in any plant species. This result was surprising since CTC is known to have higher adsorption coefficient (K_d) for soils compared to that of tylosin, and adsorption to soil organic matter can reduce bioavailability of antimicrobial compounds to plants. As a concequence of the differences of plant species to take up the antibiotic compounds, vulnerability to different antibiotics may also differ

from one plant species to another. For instance, pinto beans have been found to be negatively affected by OTC and CTC, whereas the growth of corn was unaffected by these two antibiotics (Batchelder, 1982). Moreover, the growth of radish and wheat has been observed to be increased in the presence of OTC and CTC.

As a result of the uptake and accumulation of antibiotics in plants, various toxic reactions may take place in plants. ENR has been reported to alter post-germinative development of *Cucumis*, *Lactuca*, *Phaseolus*, and *Raphanus* resulting in the reduction of the lengths and/or numbers of primary roots and leaves (Migliore et al., 2003). This study also demonstrated that the plants may able to convert a significant amount of ENR into its metabolite CF leading to a cross-contamination in the environment. Being parallel to these findings, Boxall et al. (2006) have showed that the growth of lettuce and carrot plants was negatively affected by the exposure to the antibiotic compounds OTC and ENR. Although there are several papers about the uptake and phytotoxicity of antibiotics in plants, there is still the lack of knowledge on the toxicity of antibiotics at realistic environmental concentrations. Furthermore, toxic effects of the degradation products in plant species should also be evaluated in order to be able make a comprehensive risk assessment for human health.

2.2. Consumption of Antibiotics

The main groups of antibiotics used in human and veterinary medicine are represented in Table 2.1. According to European Federation of Animal Health (FEDESA, 2001), approximately 7700 tons of antibiotics were consumed by human, and 5100 tons of antibiotics were prescribed for both livestock and poultry in 1997 only in European Union and Switzerland. Among the veterinary antibiotics, approximately 1600 tons of growth promoters and 3500 tons of therapeutic antibiotics were estimated to be used in 1997 in European Union and Switzerland to accelerate the growth of animals and to treat and/or prevent diseases, respectively (Christian et al., 2003). In human, antimicrobials are used to treat microbial diseases and 26 % of the total consumption of human antibiotics has been reported to be used in hospitals in Germany (Kümmerer, 2001). This information also demonstrates that hospital effluent accounts for a significant portion of human antibiotic compounds discharged into the wastewater.

Table 2.1. The important antibiotic groups used for humans and animals (Kemper, 2008).

Antibiotic Class	Antibiotic Compounds	Primary Usage	Antibiotic Class	Antibiotic Compounds	Primary Usage
Aminoglycosides	Apramycin	Pigs only	**Fluoroquinolones**	Ciprofloxacin	Humans
	Gentamycin	All animals, humans		Enrofloxacin	All animals
	Kanamycin	Dogs, pigs, cattle, horses		Marbofloxacin	All animals
	Neomycin	All animals		Flumequin	Humans
	Sisomycin	Humans only		Ofloxacin	Humans
	Spectinomycin	Pigs, cattle, poultry, sheep	**Lincosamides**	Clindamycin	Dogs, animals
	Streptomycin	No longer in use		Lincomycin	Pigs, cats, dogs, cattle
β-lactams	Amoxicillin	All animals	**Macrolides**	Azithromycin	Humans
	Ampicillin	All animals		Clarithromycin	Humans
	Azlocillin	Humans		Erythromycin	Humans, cattle, chicken
	Benzylpenicillins	All animals		Roxithromycin	Humans
	Cloxacillin	Cattle		Spiramycin	All animals
	Dicloxacillin	Cattle		Tylosin	Animals only
	Flucloxacillin	Humans		Vancomycin	Humans
	Methicillin	Humans			
	Mezlocillin	Humans	**Sulfonamides**	Sulfanilamide	Humans
	Nafcillin	Humans		Sulfadimethoxine	Cattle, pigs, chicken
	Oxacillin	Cattle		Sulfadimidine	Cattle, sheep, chicken
	Piperacillin	Humans		Sulfamethoxazole	Humans
	Phenoxymethylcillin	Humans		Sulfapyridine	Pigs
	Penicillin G	Humans		Sulfathiazole	Humans
Cephalosporins	Cefalexin	Dogs	**Trimethoprim**		Sulfonamides synergist
	Cefalotin	Humans	**Tetracyclines**	Chlortetracycline	Cattle, pigs
	Cefazolin	Humans		Doxycycline	Humans, cats, dogs
	Ceftiofur	Cattle, pigs		Oxytetracycline	Humans, cattle, sheep, pigs
	Cefotaxim	Humans		Tetracycline	Humans, horse, sheep, pigs
	Cefotiam	Humans			

Antibiotics are used in food producing animals to treat and prevent illnesses, and to promote the growth of animals (O'Connor and Aga, 2007). The frequent use of growth promoters in livestock and poultry is of greater concern, since these compounds are long-term used at sub-therapeutic concentrations, and subtherapeutic concentrations of antibiotics frequently administered to farm animals can cause occurrence and development of antibiotic resistance (Kemper, 2008). For this reason, growth promoting antibacterial agents are withdrawn from the market of many European countries (Rooklidge, 2004). The use of avoparcin, ardacin, zinc-bacitracin, tylosin, spiramycin, virginiamycin, carbadox, and olaquindox, the eight major growth promoters, has been banned within the European Community (EC) between 1997 and 1999 (Butaye et al., 2003). This ban has limited the use of antibiotic compounds as growth promoters. For instance, in 1999, the antibiotic consumption as feed additives has been estimated to decrease to its half value probably due to this prohibition (Christian et al., 2003). In Turkey, the four growth promoters monencin sodium, salinomycin sodium, avilamycin, and flavophospholipol were banned in 2006 remaining no more growth promoters in the market (Tuncer, 2007).

The veterinary antibiotic groups of aminoglycosides, β-lactams, macrolides, sulfonamides, tetracyclines, lincosamides, ionophores, and fluoroquinolones are the major classes used in animal husbandry (Sarmah et al., 2006). Approximately 2600 tonnes of TCs have been estimated to be consumed especially by livestock for therapeutic purposes in the European Union (EU) in 1999, and this amount corresponded to 66 % of all veterinary antibiotics (Thiele-Bruhn, 2003). SAs have been reported to have the consumption ratios of 11-23 % for veterinary purposes in many European countries. Total sales of FQs for veterinary purposes in the EU and Switzerland in 1997 have been reported to be 43 tonnes (Pico and Andreu, 2007). In addition to application of TCs and SAs to animals for therapeutic purposes as described in Table 2.1, these compounds are used as growth promoters / feed additives in countries such as Canada and USA. For instance, OTC and CTC may be fed to swine, beef cattle and chicken for growth-promoting purposes in USA (Sarmah et al., 2006). Similarly, the SA compound sulfamethazine has been registered for use as growth promoters/feed efficiency in swine and cattle in Canada. Various SAs are used in combination with trimethoprim, the synergist of SAs, to enhance the spectrum of activity of the compounds.

2.3. Physicochemical Properties of Tetracyclines, Sulfonamides, and Fluoroquinolones, and Behaviour in the Environment

The physicochemical properties of TCs, SAs, and FQs, three antibiotic classes selected in this study, and their resulting behaviour in the environment are explained in this section. For this purpose, chemical structures and some selected physicochemical characteristics that are most environmentally relevant are given in Table 2.2.

2.3.1. Tetracyclines

2.3.1.1. Physicochemical Properties. TCs are formed from a partially conjugated four-ring structure with a carboxyamide functional group (Sarmah et al., 2006). TCs have three pK_a values demonstrating their amphoteric nature. They are not stable in bases forming salts in both acidic and basic media (Halling-Sørensen et al., 2002b). TCs form reversible chelate complexes with di- and tri-valent metal ions such as Mg^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, and Fe^{3+} (Wessels et al., 1998). Moreover, they have been reported to be able to bind to humic acids, proteins, organic matter, and silanol groups (Loke et al., 2002). TCs are highly soluble in alcohols, whereas they all are only sparingly soluble in non-polar solvents such as chloroform (O'Connor and Aga, 2007). Although they are sparingly water soluble, their water solubility may be further increased by the introduction of a HCl molecule into the chemical structure (Thiele-Bruhn, 2003). Light exposure causes photodecomposition of TCs due to the strong absorption of light (Pouliquen et al., 2007).

2.3.1.2. Behaviour in the Environment. Due to the three pK_a values of TCs, they always carry a local charge throughout the pH range, and they are zwitterionic in the pH range of approximately 3-9 (O'Connor and Aga, 2007). Giving the common pH range of 6-8 and 7-9 for soil and manure, respectively, it may be concluded that zwitterionic TCs would be predominant in manure and soil matrices, and they can interact with both anionic and cationic sites in these matrices.

Table 2.2. Chemical structures and some physicochemical properties of studied antibiotic compounds.

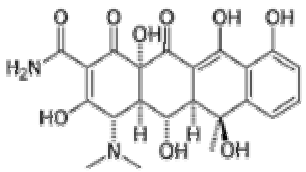
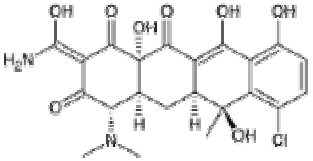
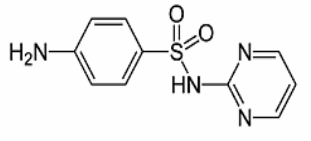
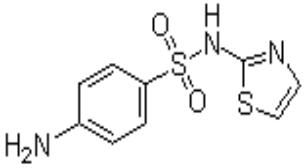
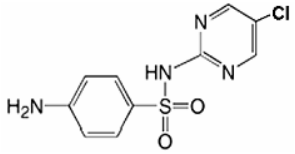
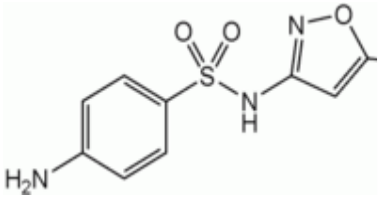
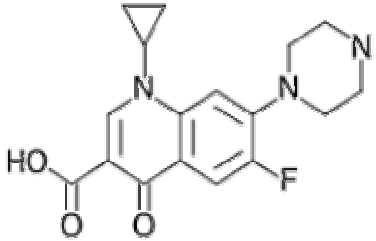
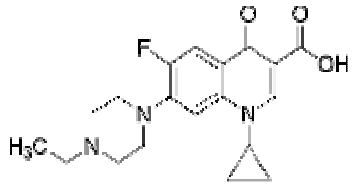
	Structure	M_w (g mol^{-1})	Water Solubility (mg L^{-1})	log K_{ow}	pK_a	K_d (L kg^{-1})
Tetracycline						
Oxytetracycline		460.4	1000 [a]	-1.22 [i]	3.2, 7.5, 8.9 [d]	417-1026 in loamy sand and sand; 83.2/77.6 in pig manure [a]
Chlortetracycline		478.9	600 [a]	-0.41 [i]	3.3, 7.6, 9.3 [d]	1280-2386 in clay loam and sandy loam [a]
Sulfonamide						
Sulfadiazine		250.3	77 [a]	-0.09 [a]	2.0, 6.4 [f]	1.4-2.8 in whole soil, clay, and sand fraction [a]
Sulfathiazole		255.3	600 [b]	0.05 [b]	2.4, 7.1 [f]	0.6 in clay loam [a]
Sulfachloropyridazine		284.7	8200 [c]	-0.52 [d]	1.8, 5.7 [d]	0.9-1.8, in clay loam and sandy loam [a]

Table 2.2. (Continued)

	Structure	M_w (g mol^{-1})	Water Solubility (mg L^{-1})	log K_{ow}	pK_a	K_d (L kg^{-1})
Sulfonamide						
Sulfamethoxazole		253.3	n.a.	0.89 [e]	1.8, 6.0 [g]	260 in secondary sludge [h]
Fluoroquinolones						
Ciprofloxacin		331.4	n.a.	-1.74 [d]	5.9, 8.9 [j]	427 in loamy sand [a]
Enrofloxacin		359.4	130000 [a]	2.53 [a]	6.3, 8.3 [j]	260-5612 in clay, loam, and loamy sand [a]

a: Sarmah et al., 2006 b: Tolls, 2001 c: Kay et al., 2005a d: Batt, 2006
e: Peng et al., 2006 g: Stoob et al., 2006 h: Joss et al., 2005
i: O'Connor and Aga, 2007 j: Pico and Andreu, 2007
n.a.: No information is available.

Acidic and basic conditions of the environment may catalyze the chemical transformation processes such as isomerization and epimerization of TCs. For example, CTC is converted to isochlortetracycline under basic conditions, whereas the formation of epimerization products is favoured in acidic environments in the pH range of 2-6 (Eichhorn and Aga, 2004). Since the epimerization of TCs is facilitated from pH environment around 6, such as that in organically fertilised soil, epi-TCs may be encountered in agricultural soils (Brambilla et al., 2007). On the other hand, the relatively high pH of animal manure may cause complete isomerization of TCs making the presence of TCs isomerization products in this matrix probable.

The formation of chelate complexes between TCs and particularly metal ions may be expected in terrestrial environment, since animal manure, and especially agricultural soil, typically contain high amounts of metal ions. This complexation behaviour of TCs with di- and tri-valent cations can cause the formation of biologically inactive compounds. Furthermore, due to strong binding of TCs to mineral cations, they are almost immobile in terrestrial environment (Loke et al., 2002).

Although the hydrophillic character of TCs, they exhibit strong sorption to soil and manure as indicated by their high K_d values (Table 2.2.). TCs sorption to soil is governed by several factors including organic carbon content, pH, cation exchange capacity (CEC), and texture of soils. The increase in pH leads to a reduction in the sorption of TCs to soil making them more mobile. TCs have a negative charge at higher pH, and so will sorb less to soil which also has a negative charge. For instance, Sassman and Lee (2005) investigated the sorption of three TCs, namely TC, OTC, and CTC, by several soils with a pH range of 3.8-7.5 and a CEC range of 0.8-26.5 cmol kg^{-1}, and found that all three TCs are highly sorbed especially in acidic soils. Manure application to agricultural soils may affect the sorption and mobility of TCs by increasing the soil pH due to the high levels of ammonia present in manure. On the other hand, significant changes in the sorption characteristics are not expected via manure amendment due to small changes in soil pH (Kay et al., 2005b).

Cation exchange is the primary sorption mechanism responsible for sorption of TCs especially under acidic conditions. Moreover, it has been shown that cation exchange

mechanism also remains important in terms of the sorption of TCs even at high pH despite the negative charge of these compounds under basic conditions (Sassman and Lee, 2005). Thus, CEC plays a major role in the sorption of TCs to soil particles. Higher values of CEC have been reported to result in higher sorption of TCs to soils (Jones et al., 2005).

Soil texture and percent organic carbon play a large role on the CEC of the soil, and so it has been demonstrated that these two characteristics also have a significant influence on the sorption of TCs. Humic and fulvic acids are the natural organic compounds present in the composition of soil, whereas manure primarily contains fatty acids, different types of phenols, and proteins. These organic matters have varying ability to interact with TC molecules. For instance, TCs have been found to have the K_d value of 32000 L kg^{-1} in dirty humic acid that had no acidification or cation exchange resin treatments, while no sorption was observed when using cellulose (MacKay and Canterbury, 2005). It has been indicated that increasing aromaticity of the soil components results in higher sorption of TCs (Jones et al., 2005). Furthermore, organic matter with bound metals have been found to cause higher sorption coefficients for OTC than organic matter that could not complex metals indicating that metal bridging plays a significant role in the sorption of metal-complexing compounds such as TCs (MacKay and Canterbury, 2005). This may also explain the strong sorption of TCs onto manure which commonly contains high amounts of organic carbon and bound divalent metal cations such as Ca^{2+} and Mg^{2+} despite their negative log K_{ow}.

Clay content of soil is also an important factor in the assessment of TCs sorption onto soil, since TCs may strongly bind to clay minerals. In the study of Pils and Laird (2007), the sorption of TC and CTC has been evaluated using clays, humic substances, and clay-humic complexes, and it has been shown that TCs are more dominantly sorbed to soil clays than to humic substances. These authors have concluded that humic substances may reduce the sorption of TCs to clay by masking sorption sites on clay surfaces or inhibiting interlayer diffusion of TCs. This is also the case when liquid manure is applied to agricultural soils, since it contains high amounts of dissolved organic matter which may reduce the sorption of TCs to clay, and in turn, increase their mobility in natural environment (Kulshrestha et al., 2004). TCs sorption to clay minerals is suggested to be governed by complexation with divalent cations to the greatest extent. For instance, Figueroa et al. (2004) showed that ionic functional groups of the base compound structure

are responsible for the interaction between TCs and clays. On the other hand, the dominant mechanism in the sorption of zwitterionic TC species and clay minerals is hydrophobic interactions (Kulshrestha et al., 2004).

TCs are rather persistent in manure and soil, and so they have a high potential to accumulate in agricultural soils. Total TCs have been demonstrated to decline slowly in manure amended soil 5 months after fertilization (Aga et al., 2005). Similarly, in a previous study, OTC has been shown to be still detectable in manure (0.82 mg kg^{-1}) after 5 months maturation (De Liguoro et al., 2003). These findings have been confirmed by a recent study in which OTC was found to have DT_{50} and DT_{90} (dissipation time) values of 21.7 and 98.3 days, respectively, in a sandy loam soil (Blackwell et al., 2007). Biodegradation of TCs is rarely encountered in manure and soil probably due to their strong sorption to organic matter and clay fraction, which makes them unavailable for microbial attack. The degradation of TCs may be catalyzed by the exposure to light, however, this type of decomposition is negligible for soil and manure under field conditions. This is because the penetration of light may only occur in the first millimeters of manure heaps and soils (Beausse, 2004).

2.3.2. Sulfonamides

2.3.2.1. Physicochemical Properties. The SAs chemical structure is formed from a benzene ring, an $–NH_2$ group, and a sulfonamide ($-SO_2NH_2$) group. They are amphoteric compounds, and are characterized by two pK_a values. Despite their amphoteric character, they generally function as weak acids at physiologic range. SAs are known to form salts at strongly acidic or basic solutions (Thiele-Bruhn, 2003). The solubility of their sodium salts increases as pH increases. SAs may form complexes with heavy metal cations (Zimmermann, 2006). The antibacterial activity of SAs is characterized by their aniline group.

2.3.2.2. Behaviour in the Environment. SAs may occur in the environment as three different species depending on environmental pH: cationic, neutral, or anionic forms. A zwitterionic form also exists in tautomeric equilibrium with the neutral form, however, this

zwitterionic form has been reported to constitute only a minor portion of all the SAs species present in the environment (Kahle and Stamm, 2007b).

As can be seen from their log K_{ow} and K_d values for different types of solid matrices (Table 2.2.), SAs are not hydrophobic, and have low tendency to sorb to soil. SAs sorption to soil is facilitated by several factors. The first factor is the soil pH. It has been found that SAs are less strongly sorbed to soil particles as pH increases due to the prevalence of anionic SAs species in soil at high pH (Boxall et al., 2002). On the other hand, although the addition of manure results in an increase in soil pH, a significant reduction in the sorption of SAs in manure-amended soil is unlikely due to their pK_a values outside the range normally encountered in agricultural soils (Kay et al., 2005b). For instance, in the study in which the sorption of STZ to clay minerals and ferrihydrite has been investigated for varying pH values and contact times (Kahle and Stamm, 2007b), it has been found that STZ cations were most important for sorption to clay minerals while ferrihydrite, a specific anion sorbent, showed significant sorption only between pH 5.5-7. Similar to inorganic constituents of soil, sorption of SAs to organic matter has been demonstrated to increase as pH decreases (Kahle and Stamm, 2007a).

The second important factor determining the strength of SAs sorption to soil is contact time. Increased contact times result in a substancial increase in K_d values of SAs in soil. For instance, it has been demonstrated that K_d values of SAs observed after 14 days contact time were significantly higher than those observed after one day in both clay minerals, and various organic matters of different origin (Kahle and Stamm, 2007a,b). Particularly for manure, the increase in the sorption of STZ with increasing contact time was most pronounced (Kahle and Stamm, 2007a). The reason of the increase in SAs sorption with increasing contact time may be time-dependent formation of nonextractable SAs residues and transformation products (Stoob et al., 2006; Hamscher et al., 2005).

Clay content of soil is the third factor affecting the strength of SAs sorption to soil. SAs have been found to bind most strongly to clay and fine silt whereas sorption to coarse and medium silt, and sand was lowest (Thiele-Bruhn et al., 2004). This difference in the sorption capacity of sand and clay particles may be caused by higher CEC, amorphous iron oxides which are commonly found in pedogenic clay minerals, and specific surface area of

clays compared to sand fractions. Cation exchange is a very common mechanism especially between cationic SAs and exchangeable metal cations initially saturating negatively charged clay particles, while ligand exchange may also occur between the anionic SAs and a metal cation of iron oxides as well as clay minerals (Zimmermann, 2006). However, it has been reported that clay minerals play a minor role for SAs sorption in many soils compared to organic matter (Kahle and Stamm, 2007b).

The fourth, and the final, factor having a significant impact on SAs sorption to manure and soil is the organic carbon content. Thiele-Bruhn et al. (2004) showed not only the influence of the quantity of organic carbon on SAs sorption, but also the effect of organic matter composition. Phenolic and carboxylic groups, N-heterocyclic compounds, and lignin decomposition products have been demonstrated to be preferred for SAs sorption. Furthermore, the authors investigated the influence of molecular structures of SAs on their sorption behaviour, and found that K_d values increase as aromaticity and electronegativity increase. Stronger sorption of SAs to manure compared to soil may be anticipated due to higher organic carbon content of manure. For example, it has been found that SAs sorption to pig slurry was much stronger than to soil (Thiele-Bruhn and Aust, 2004). On the other hand, in a mixture of soil and slurry, a significant decrease in the sorption of SAs has been observed probably due to competitive adsorption of dissolved organic matter constituents of manure. In this study, influence of dissolved organic matter present in manure on the SAs sorption was obvious since the investigators eliminated pH effect using acidic manure. For the sorption of SAs to soil organic matter, hydrogen bonds and van der Waals interactions have been suggested to be the preferred sorption mechanisms (Thiele-Bruhn et al., 2004).

Due to their low sorption coefficients in soils, SAs are highly mobile in the environment. Many studies have revealed that these compounds are transported rapidly in soils. For instance, Blackwell et al. (2007) measured SCP concentrations of up to 25.9 and 0.78 $\mu g\ l^{-1}$ in surface run-off and soil water samples at 40 cm depth 20 days after treatment, respectively. In a previous study, the same compound has been demonstrated to be rapidly transported to surface waters, concentrations of up to 590 $\mu g\ l^{-1}$ being observed in drainage waters, although no significant leaching to deeper layers of soil was observed (Boxall et al., 2002). On the other hand, in a recent investigation, SMZ, another SAs compound, has

been detected in measurable concentrations in groundwater wells and lysimeters, indicating that this compound has high potential to reach the groundwater via leaching (Ternes et al., 2007). All these findings suggest that even though SAs have low potential to accumulate in soils, they may contaminate ground and surface waters.

SAs may be more persistent in manure and agricultural fields than would be predicted from the results of laboratory studies. For instance, Christian et al. (2003) showed that measurable concentrations of sulfamethazine was still present in fertilized soil even seven months after the application. Being parallel to this research, De Liguoro et al. (2007) revealed that 0.39 mg kg^{-1} of sulfadimethoxine was still detectable in stable manure after three months maturation. On the other hand, in several papers, SAs have been demonstrated to have limited persistence in soil and manure. For instance, Blackwell et al. (2007) showed that SCP dissipated rapidly in a sandy loam soil with DT_{50} and DT_{90} values of 3.5 and 18.9 days, respectively. Similarly, Accinelli et al. (2007) measured the half-lives of 18.6 and 21.3 days for sulfamethazine and SCP, respectively, in silt loam and sandy soil. The authors concluded that the soil type, manure amendment, and SAs concentration have a significant effect on SAs degradation. Silt loam soil resulted in more rapid dissipation of the two SAs compounds compared to sandy soil likely due to higher number of degrading microorganisms in silt loam soil. Alike, the two SAs were less persistent in liquid swine slurry-amended soil which has higher microbial activity, than in unamended soil. Furthermore, it has been stated that small concentrations of SAs compounds had no influence on the degradation rates. Aside from the biodegradation of SAs, photodegradation of SAs on soil surfaces may also play a key role in their transformation (Wolters and Steffens, 2005).

2.3.3. Fluoroquinolones

2.3.3.1. Physicochemical Properties. Most FQs have two pK_a values due to two possible ionisable functional groups. Most of them are chemically stable, being insensitive to hydrolysis and high temperatures (Pico and Andreu, 2007). However, they may be decomposed when exposed to UV light. FQs form stable complexes with several di-and trivalent metal ions such as Mg^{2+} and Al^{3+} (Turiel et al., 2006). They strongly bind to solid

environmental matrices due to their high liphophilicity. They have weak native fluorescence and can form highly fluorescent complexes with lanthanides.

2.3.3.2. Behaviour in the Environment. Photostability of FQs is an important factor determining their persistence in soil. Even though they are sensitive to UV-light, their phototransformation in soils is not complete, and residual FQs may persist in agricultural soils (Golet et al., 2003). Furthermore, it has been reported that the phototransformation rate of FQs decreased in the presence of humic substances (Schmitt-Kopplin et al., 1999). This finding may be the reason of the incomplete elimination of FQs in topsoil through photolysis.

Sorption of FQs mainly to clay minerals and humic substances is another important factor having a significant impact on their persistence in manure and soil. The large clay fractions and the huge amounts of pure clay minerals present in soils have been found to result in high adsorption and low desorption of FQs (Nowara et al., 1997). The type of pure clay minerals may also affect the sorption strength of FQs. For instance, montmorillonite has been shown to adsorb FQs more strongly than kaolinite. Nowara et al. (1997) have also revealed that β-keto acid structure of FQs is the key element responsible for their high adsorption. They have suggested that an electrostatic interaction between the anionic FQs and the exchangeable cations which are bound to the negatively charged clay mineral surfaces accounts for the high sorption of FQs to clays. Similar to the clay content of soils, lower organic carbon content of soils has been demonstrated to lead to weaker binding of FQs to this matrix. For example, Drillia et al. (2005) demonstrated that ofloxacin, an FQs compound, had a K_d value of 3554 in a soil having an organic carbon content of 7.1 %, while its K_d was 1192 in a soil with an organic carbon content of 0.37 %. Cation bridging may also be regarded as the responsible mechanism for the sorption of FQs to organic matter (Tolls, 2001).

Besides the sorption to layered-clay minerals and organic matter, FQs antimicrobials may also be sorbed to hydrous oxide minerals which are commonly found in soils and are capable of forming strong surface complexes with many metals and organic ligands. The interaction between CF and aluminum and iron oxides has been investigated in the study of Gu and Karthikeyan (2005). The authors found that the sorption of CF is highly pH-

dependent in the pH range of 4-10. They also showed that iron hydrous oxides resulted in higher sorption of CF compared to aluminum hydrous oxides. In this study, surface complexation mechanism has been proposed as the responsible mechanism for the binding of FQs to metal-hydrous oxide minerals.

Persistence of FQs compounds in manure and soil is also influenced by their degradation behaviour. Due to their high chemical stability, FQs are rather persistent in agricultural soils. For instance, CF and norfloxacin have been shown to persist over 21 months in sludge-treated soils in the μg kg^{-1} range (Golet et al., 2003). Considering that they are almost immobile in terrestrial environment, accumulation of FQs in the topsoil readily takes place. Besides the phototransformation, biotransformation also plays a significant role in their degradation. For example, CF and ENR may be metabolized by the soil fungus *Mucor ramannianus* (Pico and Andreu, 2007).

2.4. Analytical Strategies to Determine Antibiotics in Manure and Soil

Unlike the aqueous samples, the monitoring of drugs in solid environmental samples require labor-intensive and time consuming techniques due to complexity of these samples (Diaz-Cruz et al., 2003). To date, various extraction methods of TCs, SAs, and FQs from different matrices such as food, tissue, groundwater and surface water samples have been reported although there are not much studies involving the extraction of these three groups of antimicrobial compounds in soil and animal manure matrices. Therefore, this section will focus on the advances in TCs, SAs, and FQs analysis with special emphasis to solid environmental matrices.

Analytical strategies to determine TCs, SAs, and FQs compounds mainly consist of extraction, clean-up/preconcentration, and analysis. However, some additional steps such as derivatization may be included when necessary. Table 2.3. summarizes all these steps used to extract and analyse TCs, SAs, and FQs in solid environmental matrices using recently published literature.

2.4.1. Extraction of Antibiotics

The aim of the extraction step is to separate the antibiotic compounds from other matrix components with maximum efficiency. In literature, a large number of extraction methods have been proposed for the extraction of TCs, SAs, and FQs from environmental samples. The selected extraction solvents and the most widely used extraction tools are summarized in this section.

2.4.1.1. Extraction Solvents. Antibiotics are the substances most of which are comprised of a non-polar core and polar functional groups. Due to the behavioural changes of antimicrobial compounds at different pH values as explained in Section 2.3, extraction techniques should be optimized depending on pH and polarity of the extraction solvent. For instance, very polar and non-polar extractants cannot completely extract the target antibiotic compounds, and this leads to serious analytical problems (Kemper, 2008).

Due to strong binding of especially TCs to metals which are likely to present in sample matrix, and may reduce the extraction efficiency significantly, use of chelating agents as an extractant has been essential for the extraction of these compounds. Previous studies involving the extraction of particularly TCs and SAs from liquid and solid environmental samples have been based on the use of only one chelating agent as an extractant. For instance, Na_2EDTA has been previously used for the extraction of TCs and SAs from groundwater, surface water, and sewage treatment plant influent and effluent (Lindsey et al., 2001; Hirsch et al., 1999; Karthikeyan and Meyer, 2006). Similarly, citric acid has been used for TC extraction in pig manure in a previous study (Kühne et al., 2000). The use of two or more chelating agents combination has also been common in literature to improve the extraction efficiency of TCs and SAs from various matrices. For example, McIlvaine buffer which is composed of citric acid and Na_2HPO_4 may be combined with EDTA. This combination has been widely used for the extraction of TCs and SAs from the matrices including groundwater and surface water (Blackwell et al., 2004a), foods (Nakazawa et al., 1999), lamb muscle (Castellari and García-Regueiro, 2003), and manure (Arikan et al., 2006).

Weakly acidic McIlvaine buffer combined with MeOH as an organic solvent has been used in several studies to extract TCs and SAs from agricultural soils. Martinez-Carballo et al. (2007) used an extraction buffer composed of EDTA-McIlvaine buffer (pH = 6) in combination with MeOH to extract TCs, SAs, and trimethoprim (TMP) from arable soil.

Despite the common choice of acidic buffers for the extraction of antibiotics from solid environmental matrices, the use of neutral or basic solvents has also been reported in literature. For example, Blackwell et al. (2004b) reported that the use of McIlvaine buffer at pH 7 generally increased the recoveries of OTC from soil. Similarly, Haller et al. (2002) performed the extraction of SAs and TMP from manure using pH adjustment to 9 with KOH followed by the addition of ethyl acetate (EtOAc).

2.4.1.2. Extraction Techniques. The following paragraphs cover both the instrumental and non-instrumental techniques which are employed for the extraction of TCs, SAs, and FQs from environmental samples. Further information is given on their relative merits with respect to their extraction efficiency.

Shake-flask extraction has been employed to extract organic pollutants from environmental matrices, and it has the advantage that it requires minimal glassware, less amounts of organic solvents, and is not a time-consuming technique (Dean and Xiong, 2000). However, it is difficult to obtain a quantitative extract by using shake-flask extraction, and it makes the use of shake-flask methods invalid for accredited laboratories that these methods are not approved for use in such laboratories. Therefore, more sophisticated techniques have been required to detect antimicrobial compounds at environmentally-relevant concentrations.

Soxhlet extraction can be used for the extraction of organic analytes from environmental matrices, and it allows to the extraction of a very wide range of molecules from solid matrices being appropriate for the simultaneous extraction of samples studied. This technique is advantageous due to its low equipment cost although solvent consumption of this method is too high (Dean and Xiong, 2000).

Pressurized liquid extraction (PLE) is based on the use of elevated temperature and pressure to increase extraction efficieny. This technique has been efficiently used to extract TCs and SAs from agricultural soils (Jacobsen et al., 2004). It has been reported to provide satisfactory reproducibilities, to require smaller volumes of extraction solvents and less sample preparation time than other methods. On the other hand, co-extracted matrix components due to high temperature conditions of PLE require intensive clean-up steps. Furthermore, PLE followed by SPE cannot be practically used when high sample loading is needed (Carlson, 2005). The use of elevated temperatures in PLE may also cause thermal degradation of analytes (O'Connor, 2007).

Supercritical fluid extraction (SFE) is another extraction method which uses a supercritical fluid such as CO_2 sometimes together with an organic modifier such as MeOH to extract organic compounds from solid matrices. This technique has been used for the extraction of SAs and FQs from solid biological matrices such as edible animal tissues by several authors, and recoveries of up to 98 and 104 % have been achieved for SAs and FQs, respectively (Maxwell and Lightfield, 1998; Shim et al., 2003). Nevertheless, the application of SFE for the extraction of TCs, SAs, and FQs from solid matrices with environmental importance has not been reported. SFE has several advantages over some other extraction techniques including short extraction time and low solvent consumption (Miege et al., 1998). Additionally, it dissolves less sample matrix eliminating the need of a clean-up step (Hawthorne et al., 2000).

Microwave assisted extraction (MAE) involves the exposure of the sample and organic solvent to microwave irradiation. This emerging technique has been reported to give quantitative recoveries for SAs and FQs in soil (Raich-Montiu et al., 2007; Morales-Munoz et al., 2004). Moreover, MAE is a much more rapid extraction technique compared to traditional techniques such as mechanical shaking (Prat et al., 2006). However, a further clean-up step is needed to remove matrix interferences when using this method. In addition, high temperature and prolonged exposure to irradiation may lead to degradation of thermo-labile compounds such as TCs, so particular attention should be given to the optimization of temperature and irradiation time when TCs are extracted using MAE (Diaz-Cruz and Barcelo, 2007).

Ultrasonic extraction is based on the agitation of sample matrix by ultrasonic waves. This technique has been reported to give higher recoveries due to more efficient contact between solid and solvent, and to be a faster method in the extraction of a wide range of organic compounds than other instrumental and non-instrumental techniques such as Soxhlet, MAE, and SFE (Sun et al., 1998; Popp et al., 1997). Ultrasonic extraction has been succesfully used for the extraction of TCs, SAs, and FQs from agricultural soil and animal manure (Blackwell et al., 2004b; Haller et al., 2002; Turiel et al., 2006). It does not require sophisticated and expensive equipment, qualified researcher, and high solvent consumption. Additionally, ultrasonic extraction is a selective extraction technique which dissolves much less matrix components than shake flask and Soxhlet extraction methods resulting in less interfering peaks in chromatograms (Babić et al., 1998). On the other hand, sonication times should be carefully optimized, since prolonged sonication may result in degradation of some analytes.

2.4.2. Clean-up Techniques

After the extraction of antibiotic compounds from solid environmental matrices using the extraction solvents and techniques discussed above, a clean-up step is generally required. The main goal of the clean-up step is to remove the matrix interferences in the sample without any losses in the amount of analytes present in the liquid extract and/or sample. Due to complex nature of solid samples such as manure and soil, clean-up of the samples has been commonly needed. For this reason, this section aims to discuss the clean-up techniques used for the purification of antibiotic-containing samples with special emphasis to solid phase extraction (SPE).

2.4.2.1. Solid Phase Extraction (SPE). SPE is based on the use of different types of adsorbing materials to selectively retain the target antimicrobial compounds and to remove natural organic matter (Hennion, 1999). This technique is by far the most widely employed clean-up and preconcentration technique probably due to its practical use, low organic solvent requirement, low contamination risk, and possibility to be coupled directly to HPLC (Diaz-Cruz et al., 2003). SPE mainly consists of four stages: (1) conditioning of the cartridges to wet them and to remove the majority of interferences present in the cartridges, (2) loading of the samples in which analytes are aimed to be retained on a suitable

adsorbent with minimal matrix interferences, (3) washing of the cartridges to remove the remaining humic material on the sorbent, and (4) elution of analytes in which retained analytes are transferred to the liquid phase using a convenient solvent. For SPE method development, some parameters including the selection of the type and amount of sorbent, the composition and volume of washing (or clean-up) and elution solution are of greater importance. These parameters affecting SPE extraction efficiency of antibiotic compounds are discussed in the following paragraphs in detail.

Selection of the most suitable SPE cartridge at retaining one or several antibiotic compounds simultaneously is generally the first step in SPE method development. Several kinds of SPE sorbents are used to retain the antimicrobial compounds. Hydrophilic-liphophilic balance (HLB) cartridges are most commonly used among the other SPE sorbents to extract antimicrobial residues and metabolites (Diaz-Cruz and Barcelo, 2006). This sorbent is a copolymer of the hydrophilic monomer "N-vinyl pyrrolidone" and the liphophilic monomer "divinyl benzene", and due to combination of these two monomers, HLB allows the simultaneous extraction of both polar and non-polar compounds from a wide range of chemical classes (Peruzzi et al., 2000). The structure of HLB sorbent is shown in Figure 2.1.

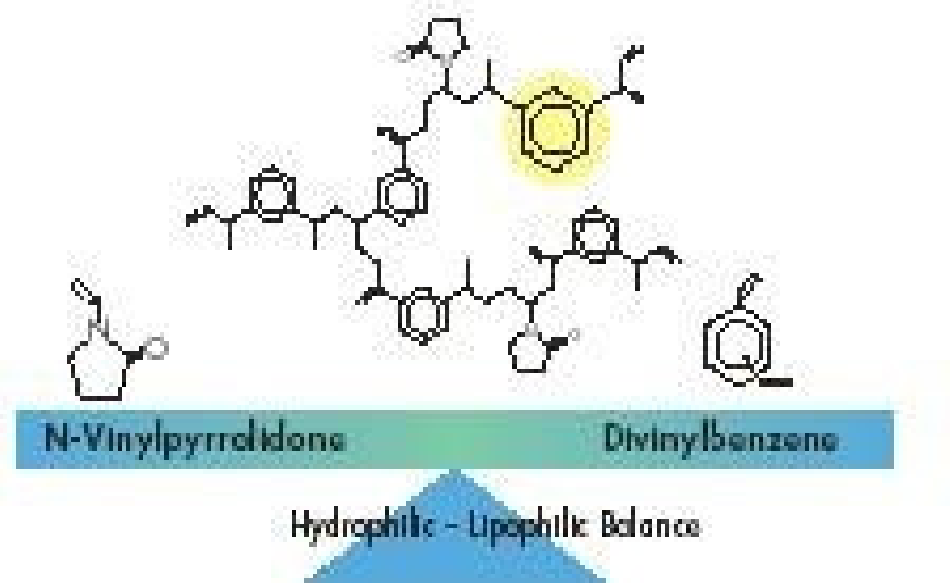


Figure 2.1. Chemical structure of the HLB sorbent.

In literature, HLB cartridges have been efficiently used to retain the target antimicrobial compounds present in various matrices including surface waters (Kolpin et al., 2002), wastewaters (Göbel et al., 2004), groundwaters (Batt et al., 2006), edible animal tissues (Cherlet et al., 2003), feeds (Pecorelli et al., 2003), manure (Eichhorn and Aga, 2004), and soils (Raich-Montiu et al., 2007). This common application of HLB sorbents in literature is primarily due to their more robust extraction efficiency and higher recoveries even in complex matrices (Zhu et al., 2001). For instance, in many studies, HLB cartridges have been reported to give the most reproducible and highest recoveries for both TCs and SAs simultaneously among the other SPE cartridges (Blackwell et al., 2004a; Lindsey et al., 2001). Furthermore, due to the hydrophilic monomer "N-vinyl pyrrolidone" in the composition of HLB cartridges, high and reproducible recoveries have been reported even though HLB cartridges were run dry (Cheng et al., 1997). In addition to robustness and high recoveries, HLB cartridges also have greater capacity than reversed phase chemically bonded cartridges (Hennion, 1999).

Particularly when manure and soil extracts containing high organic carbon are to be purified, a tandem SPE is a good approach in order to improve the efficiency of clean-up process. For this purpose, the combination of strong anion exchanger (SAX) and HLB cartridges has been mostly chosen and efficiently used for the clean-up and preconcentration of TCs and SAs from manure and soil samples (Blackwell et al., 2004b; Aga et al., 2005). The use of SAX cartridges facilitates the elimination of anionic humic substances present in soil extracts. This results in much cleaner samples and less interferences for analysis. However, prior to loading of the samples to SAX-HLB cartridges, their pH should be optimized to maintain the polar analytes such as TCs and SAs in their cationic or neutral form.

Besides the HLB cartridges and HLB-SAX combination, reversed phase SPE sorbents may also be used to purify and concentrate environmental contaminants prior to instrumental analysis. Among the reversed phase SPE sorbents, C_{18} cartridges have been most commonly chosen for the extraction of TCs, SAs, and FQs from various water samples (Turiel et al., 2003; Siemens et al., 2007), animal tissues (Castellari and Garcia-Regueiro, 2003), and manure (Martinez-Carballo et al., 2007). These cartridges are able to retain a large number of compounds effectively and can be easily used. However, silanol

content in C_{18} cartridges may cause irreversible binding of TCs (Schenck and Callery, 1998), and these cartridges generally fail at retaining charged molecules (Blackwell et al., 2004a). Moreover, unlike the HLB sorbents, they have to be remain wetted before sample loading.

The second step of a SPE method development includes the selection of the most suitable conditioning, washing, and elution solutions. While conditioning is not as crucial with HLB cartridges, SAX and C_{18} sorbents should be conditioned prior to sample loading mainly to wet these sorbent materials. The general conditioning procedure is the addition of an organic solvent to remove the organic residues present in the cartridge followed by the addition of deionized water or various buffers to eliminate the remaining impurities such as metal ions in the cartridge (Aga et al., 2003; Blackwell et al., 2004b). Conditioning is commonly performed at acidic pHs due to the successful retention of antimicrobial compounds in the cartridges (Cherlet et al., 2003a; Göbel et al., 2004).

The washing of the cartridges have been reported to result in a significant improvement in the recoveries due to the elimination of substances which may interact with the target antimicrobial compounds and interfere with their analysis (Koesukwiwat et al., 2007). The optimization of the washing solutions is very important since the analyte recoveries should not be negatively affected due to the type and concentration of the washing solution (Blackwell et al., 2004a). For HLB-washing purposes, various buffers usually matched to the concentration of buffer in the samples, dilute solutions of organic salts and/or solvents, and water are generally used (Castellari and Garcia-Regueiro, 2003; Cheng et al., 1997; Turiel et al., 2003; Kay et al., 2005b).

Selection of the most convenient amount and composition of elution solution is very important for the complete extraction of analytes from the solid phase materials. In order to re-extract the antimicrobial compounds retained on SPE cartridges, organic solvents are commonly preferred (Jacobsen et al., 2004; Jacobsen and Halling-Sørensen, 2006). After the elution step using relatively high volumes of usual organic solvents, eluates are generally evaporated to a certain volume under N_2 stream or using rotary evaporator (Martinez-Carballo et al., 2007; Peng et al., 2006). The aim of the evaporation step is to

allow the quantification of smaller amounts of target analytes in HPLC analysis lowering the quantification limit (LOQ).

2.4.2.2. Other Clean-Up Techniques. Although SPE is the leading technology in the clean-up of samples from different environmental compartments, some other techniques may also be used as an alternative to SPE. These alternative techniques and their relative merits are briefly discussed in the following paragraphs.

0.45 µm filtration is simply performed by passing the liquid sample or extract through the disc cartridges with a particle size of 0.45 µm using syringes. This step may also be applied prior to the loading of samples to SPE cartridges, after the elution of analytes from SPE cartridges or reconstitution of evaporated eluate with a suitable solvent. The main aim is to remove suspended matters in the samples which may disrupt SPE performance clogging the pores of cartridges and may interfere with HPLC analysis accumulating on the column. 0.45 µm filtration has been reported to be used in many studies for the clean-up of TCs, SAs, and FQs-containing samples (Koesukwiwat et al., 2007; Haller et al., 2002; Turiel et al., 2003).

Gel permeation chromatography (GPC) is based on the transport of the sample with an organic solvent through the column. This technique is rarely employed for the clean-up of TCs, SAs, and FQs-containing soil extracts. However, several authors have reported the use of GPC as an efficient clean-up technique. For instance, Kreuzig and Höltge (2005) performed the clean-up of SDZ-containing manure-fertilized soil extracts using GPC. Similarly, Boxall et al. (2006) purified extracts from plant samples grown in agricultural fields containing OTC, SDZ, and ENR by using this technique.

Metal chelate affinity chromatography (MCAC) is a clean-up technique based on the chelation of antibiotics with metal ions on the columns. This technique is especially useful for the purification of samples containing TCs and FQs which have the ability to complex with metals. However, the use of MCAC as a clean-up technique of antibiotic-containing samples has been scarcely reported mainly due to the need of a preliminary clean-up step such as SPE in order to achieve acceptable recoveries and detection limits, since the combination of SPE and MCAC is time-consuming and labor-intensive. On the other hand,

Brambilla et al. (2007) used MCAC clean-up for slurry, soil, and corn seeds extracts containing OTC without any preliminary clean-up, and achieved satisfactory recoveries ranging from 83 to 87 %. Besides its combination with SPE, MCAC may be directly coupled to HPLC resulting in increases in throughput (Cooper et al., 1998).

Lyophilization is a universal enrichment and late-stage purification procedure based on freeze-drying of the samples, reducing the surrounding pressure and allowing the frozen water in the sample to sublime directly from solid to gas phase by the addition of heat. Despite its universality, the use of this technique for the clean-up and preconcentration of antibiotics-containing samples has been too scarce. Hirsch et al. (1999) employed lyophilization for the enrichment of wastewater effluents and surface water samples in order to investigate occurrence of TCs and SAs in these matrices. The disadvantages of this technique include high equipment cost, high energy consumption, and long time required for the purification. Furthermore, the addition of too much heat to the sample to sublime the frozen water can cause melting or structural deformations for thermo-labile compounds such as TCs.

2.4.2. Separation and Quantification of Antimicrobial Compounds

Antibiotics separation and quantification is the last step of an antibiotic analysis. The aim of this step is to separate antimicrobial compounds with good peak shapes and resolutions, and to measure their concentrations with optimal detection limits and reproducibilities. Because antibiotics are usually separated using chromatographic techniques, this section primarily focuses on HPLC as the leading separation technique. Other several techniques mainly based on microbiological detection are briefly discussed as well.

2.4.2.1. High Performance Liquid Chromatography (HPLC). HPLC technique is based on the separation of substances in a mixture which are transported with a liquid mobile phase, on a solid stationary phase regarding the differences in migration velocities of the individual substances. This technique is most commonly used among other analytical separation techniques due to its advantages including high sensitivity, ease of applicability for trace quantitative analyses, and suitability for the separation of non-volatile or thermo-

labile species. The main steps of an HPLC analysis include: (1) uptake of the mobile phase from solvent bottles using high pressure pump, (2) transfer of the mobile phase to injection loop, (3) injection of the sample solution followed by transport with mobile phase to column, (4) separation of the components on the column, and (5) signal formation in the detector. For HPLC method development, detector type or detection system used, LC column type, mobile phase composition, and injection volume are of greater importance. These factors affecting the separation and quantification efficiency of HPLC are discussed in detail in the following paragraphs.

Detectors present in, or detection systems coupled to HPLC are the vital components in terms of the selectivity and sensitivity of the analysis. Mass spectrometry (MS) is one of the most popular detection techniques in TCs, SAs, and FQs residue analysis. This detection technique is based on the measurement of the mass-to-charge ratio of ionized molecules. MS offers several advantages over other detection techniques, such as high selectivity, ability to provide structural information by tandem-MS (MS^2) techniques, and lower detection limits (LODs) compared to UV. Therefore, the use of LC-MS or LC-MS^2 techniques for the environmental analysis of many pharmaceuticals increased particularly in the last decades. The rapid development of LC coupled to MS or MS^2 in the environmental field caused for these two systems to become a valuable tool for the quantification of antibiotics in various environmental matrices including water (Hernandez et al., 2007), food (Oka et al., 2000), soil and sediment (Kim and Carlson, 2005). However, these techniques are highly vulnerable to co-extracted matrix components which may lead to ionization suppression or enhancement.

Despite the superiority of MS as a detection technique, HPLC is still used in combination with UV-DAD, or fluoroscence in many laboratories as an alternative technique to LC-MS. Although MS detection has been stated to be most sensitive for most antimicrobial compounds (Niessen, 1998), there are several factors leading to the selection of UV and fluoroscence detectors for detection and quantification purposes. It is the advantage of UV and fluoroscence detectors that these are more accessible for most laboratories than MS or MS^2 detection systems due to their relatively low cost (Croubels et al., 2003b). It makes LC-UV more advantageous over LC-MS further that the intrinsic

variability of an MS detector is higher than a UV detector resulting in less day-to-day variation of LC-UV (Croubels et al., 2003a).

In literature, LC-UV/DAD and –fluoroscence detection (FLD) techniques have been employed in a number of studies to investigate the occurrence and fate of antimicrobial compounds in groundwater, surface water (Blackwell et al., 2004a), animal products (Cooper et al., 1998), honey (Vinas et al., 2004), sewage sludge (Golet et al., 2002), manure and soil (De Liguoro et al., 2003). UV detection is the detection technique employed most for pharmaceutical purposes. The LODs have been reported to vary between 0.01 and 0.2 mg kg^{-1} in solid environmental matrices when UV was employed for detection (Diaz-Cruz et al., 2003). Although fluoroscence detection has also been used successfully, UV detection is the most popular detection technique for TCs (O'Connor and Aga, 2007) due to the presence of UV-chromophores in the chemical structures of these compounds. Similarly, UV detection is also preferred to fluoroscence detection for SAs-residue analysis. Unlike the TCs and SAs, HPLC-FLD technique is traditionally used for routine residue analysis of FQs due to their polarity and native fluoroscence properties (Andreu et al., 2007). Furthermore, fluoroscence detection has been stated to be more sensitive and selective than UV detection achieving LODs around 0.01 mg kg^{-1} in solid matrices (Diaz-Cruz et al., 2003). However, it is the main disadvantage of fluoroscence detection that this technique requires the use of a derivatizing reagent for most compounds making the sample preparation step more time-consuming and labor-intensive (Morales-Munoz et al., 2004).

In addition to the detector type, the quantification efficiency of antibiotics in HPLC is also affected by the injection volume. Higher injection volumes may allow the improvement of the detection limits for the antimicrobial compounds. However, this parameter should be carefully optimized since column performance will simultaneously decrease as sample volume is increased mainly due to the interferences of other species in the mixture with the detection of target compounds, and due to the overloading of the column with these co-eluting interferences (Karger et al., 1974).

The separation efficiency of antimicrobial compounds is influenced primarily by LC column type and mobile phase composition. Reversed-phase (C_{18}) chromatographic

columns are commonly chosen for the separation of TCs (O'Connor and Aga, 2007), SAs, and FQs (Andreu et al., 2007). Despite the frequent use of C_{18} columns, especially TCs and FQs tend to adsorb on the silanol groups present in these columns resulting in the formation of tailing peaks in the chromatograms (Oka et al., 2000; Pico and Andreu, 2007). This situation is overcome by the selection of dilute acids or weakly acidic buffers as the mobile phase components (Thiele-Bruhn, 2003). Volatile organic modifiers such as formic acid are added to the mobile phases to improve ionization efficiencies and control pH (Diaz-Cruz and Barcelo, 2006). In addition, acidification of the mobile phases have been reported to result in better peak shapes (Cheng et al., 1997). The mobile phase, more often, comprise a water/acetonitrile mixture acidified with formic acid for pharmaceuticals (Beausse, 2004).

2.4.2.2. Other Separation/Quantification Techniques. Besides HPLC, there are several techniques which have been employed to separate or detect antimicrobial compounds. These techniques as well as their advantages and disadvantages are briefly discussed in the following paragraphs.

Gas chromatography (GC) is used to separate the components present in a volatile liquid or in a gas mixture. With the exception of mobile phase in the gas phase in GC, its separation principle is very similar to HPLC. Although GC was used to separate the SAs residues in the food samples of animal origin in previous studies (Reeves, 1999), there is a tendency to use HPLC in the studies recently conducted to measure the antimicrobial compounds. The fact that HPLC is preferred to GC for the separation of antibiotics is due to easy decomposition of many antibiotics, such as TCs, by heat (Diaz-Cruz et al., 2003). Moreover, the elevated number of functional groups is present in the chemical structure of many pharmaceuticals such as antibiotics, and this makes the efficient derivatization essential when using GC-MS (Ternes, 2001). Additionally, GC-MS has been showed to fail in the analysis of some polar pharmaceuticals even after derivatization which could be determined using LC-MS^2, and to provide higher RSDs compared to LC-MS^2 (Ternes et al., 1998).

Microbiological assays are based on the detection of substances with antimicrobial activity by a growth inhibition zone surrounding the sample plot, by alteration in the colour

of the growth agar due to changes in the pH caused by the production of bacterial waste, or by photometric means. These techniques have been routinely used for the determination of TCs, SAs, and FQs in food due to its low cost and practical use (Ferrini et al., 2006), and can be coupled with HPLC. In addition to food samples, microbiological assays have been efficiently used for the detection of TCs and their possible biologically active metabolites in manure and soil samples (Sczesny et al., 2003). However, these assays could not detect a test substance specifically sometimes ignoring the antimicrobial activities of the target compounds (Nakazawa et al., 1999).

Enzyme-linked immunosorbent assay (ELISA) is a commonly used analytical technique based on antigen-antibody interactions. In literature, this technique has been effectively used to determine the presence of TCs and SAs in complex soil and manure samples (Christian et al., 2003; Aga et al., 2003). ELISA has several advantages including practical use without time-consuming sample preparation steps, inexpensive instrumentation, and suitability for large sample loadings. However, this technique cannot respond to an individual compound specifically in the presence of structurally related compounds, epimers, or transformation products.

All the steps used to extract and analyse TCs, SAs, and FQs in solid environmental matrices and explained in this section in detail are summarized in Table 2.3 using recently published literature.

Table 2.3. Selected methods to determine TCs, SAs, and FQs in solid environmental samples.

Compound	Matrix	Extraction	Clean-up	Separation	Detection	Recovery (LOD) % (µg/kg)	Reference
Tetracyclines							
OTC	Slurry Soil Seeds	Ultrasonication with citrate buffer (pH 4.7) followed by LLE with ethyl acetate	MCAC	HPLC Purospher RP-C8 (250 x 4 mm, 5 µm), glycine buffer: ACN/water, 1 ml min^{-1}	UV/DAD	83-86 (20) 85-87 (20) 83-85 (20)	Brambilla et al., 2007
OTC	Soil	Ultrasonication with a mixture of McIlvaine buffer (pH 7), EDTA, and MeOH	SPE Isolute SAX + Oasis HLB	HPLC Genesis C18 (150 x 4.6 mm, 4 µm), THF: ACN: TFA, 1 ml min^{-1}	UV 355 nm	65-75 (18)	Blackwell et al., 2007
OTC CTC TC	Dung	Ultrasonication with EDTA-McIlvaine buffer (pH 4)	SPE Isolute C18	HPLC Luna C8 (150 x 2 mm, 5 µm), water: ACN: HCOOH, 0.25 ml min^{-1}	MS-MS ESI-PI	91 (8.5*) 78 (16*) 89 (8.6*)	Martinez-Carballo et al., 2007
	Soil	Ultrasonication with MeOH: EDTA McIlvaine buffer (pH 6)				66 (3.4*) 66 (6.4*) 74 (3.3*)	
Sulfonamides							
SDM	Feces Bedding Manure Soil	ACN	Filtration GF-8 glass fiber	HPLC Supelco LC18 DB (150 x 4.6 mm, 5 µm), KH_2PO_4: ACN, 1 ml min^{-1}	UV 268 nm	64 (ND) 65 (50) 65 (50) 88 (10)	De Liguoro et al., 2007
SDZ SDD SMD SCP SDM SQ	Soil	MAE with ACN	SPE Oasis HLB	HPLC LiChrospher 100 RP-18, (250 x 4 mm, 5 µm), acetate buffer: ACN, 1 ml min^{-1}	FLD after derivatizing with fluorescamine 405/485 nm	73-97 (1.2) 80-98 (1.0) 75-85 (2.4) 60-74 (3.0) 75-79 (2.4) 69-76 (6.0)	Raich-Montiu et al., 2007

Table 2.3. (Continued)

Compound	Matrix	Extraction	Clean-up	Separation	Detection	Recovery (LOD) % (µg/kg)	Reference
SDZ SDD SDX	Manure	PLE with citric acid buffer (pH 4.7) followed by MeOH citric acid (pH 3) combination LLE with heptane	0.6 µm filtration followed by SPE Isolute SAX + Oasis HLB	HPLC Xterra MS-C18 (100 x 2.1 mm, 3.5 µm), MeOH (95 %):MeOH (20 %)	MS-MS ESI-PI	59-73 (8.7) 84-109 (8.2) 82-101 (2.7)	Jacobsen and Halling-Sørensen, 2006
Quinolones							
ENR CF	Soil	Ultrasonication with phosphate buffer (pH 3)-ACN combination	SPE SAX + Oasis HLB	HPLC Luna RP-C18 (2) (150 x 4 mm, 5 µm), H_3PO_4:ACN, 0.8 ml min^{-1}	FLD 280/450 nm	71-100 (ND) 61-89 (ND)	Uslu et al., 2007
ENO NOR CF DAN ENR	Soil	Ultrasonication in small columns with basic $Mg(NO_3)_2$ solution	Whatman No. 1 filter papers	HPLC Atlantis dC18 (150 x 3 mm, 3 µm), HCOOH:ACN	UV 280 nm	90-95 (80) 100-102 (60) 86-104 (50) 88-92 (80) 84-90 (40)	Turiel et al., 2006

* Data are given as method quantification limits.

SDM, sulfadimethoxine; SDD, sulfadimidine; SMD, sulfamethoxidiazine; SQ, sulfaquinoxaline; SDX, sulfadoxine; ENO, enoxacin; NOR, norfloxacin; DAN, danofloxacin; LLE, liquid-liquid extraction; EDTA, ethylene diamine tetraacetic acid; MeOH, methanol; ACN, acetonitrile; RP, reversed phase; THF, tetrahydofuran; TFA, trifluoroacetic acid; ESI-PI, electrospray ionization-positive ion; ND, not determined.

2.5. Exposure Routes and Presence of Both Veterinary and Human Antibiotics in the Environment

Antibiotics can enter the environment through sewage tratment plant (STP) effluents, disposal of unused or expired compounds, landfill disposal of industrial wastes, manifacturing plant wastewater, overland flow runoff, and leaching after the fertilization or during the storage of animal wastes in lagoons. All these entry routes are shown in Figure 2.2. The environmental compartments subjected to the antimicrobial contamination are classified as aquatic environment and solid environmental matrices in this section. These compartments are further divided into sub-parts such as surface water and soil, respectively.

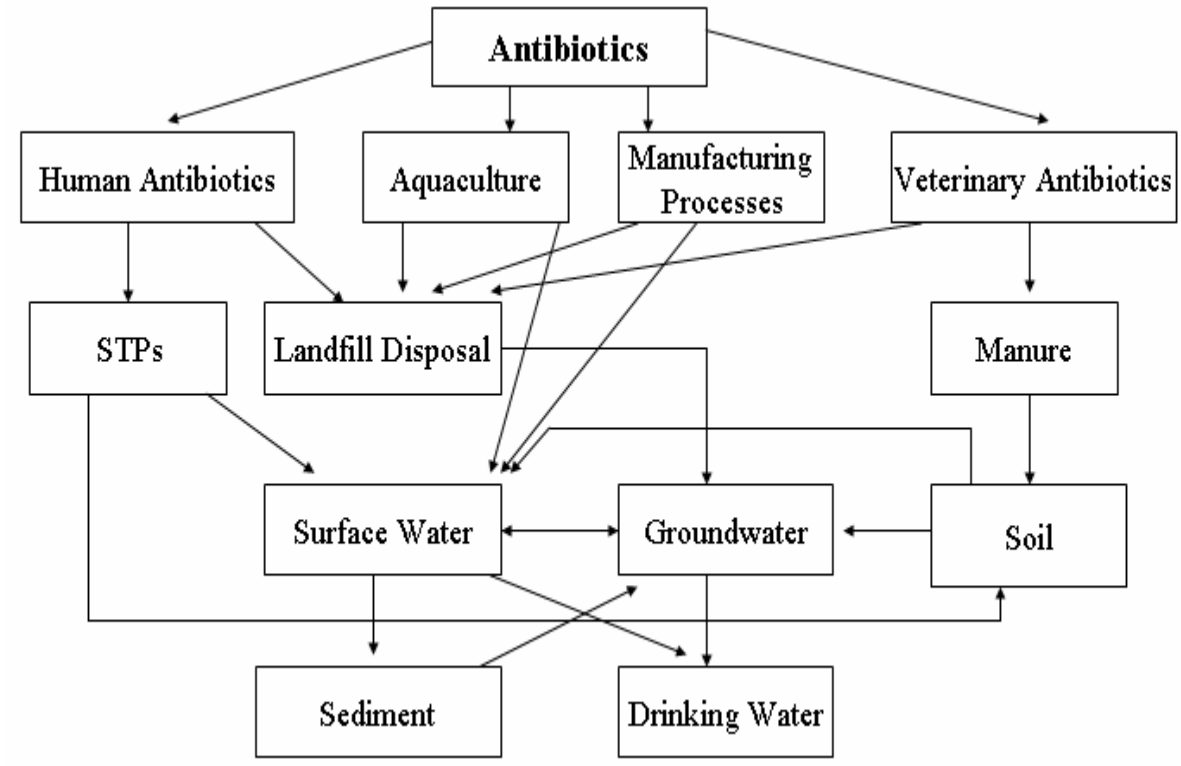


Figure 2.2. Anticipated pathways of human and veterinary antibiotics in the environment.

2.5.1. Occurrence of Tetracyclines, Sulfonamides, and Fluoroquinolones in the Aquatic Environment

2.5.1.1. Occurrence in STP influents and effluents. The major source of antibiotics in wastewater influents is their therapeutic use for humans. After administration to humans, up to 90 % of antibiotic compounds may be excreted into raw sewage via feces and urine. For instance, Hoeverstadt et al. (1986) determined trimethoprim and doxycycline ranging from 3 to 40 mg kg^{-1} in human feces during 6 days of regular application. This means that vast amounts of human antibiotics are continuously loaded into STPs. Soluble concentrations of antibiotics in STP influents have been generally in μg l^{-1} range. For example, Karthikeyan and Meyer (2006) detected two SAs, one TC, and one FQ in different STPs located in Wisconsin, USA, up to 1.25 μg l^{-1}. The authors have concluded that tetracycline (TC) was the most frequently detected antimicrobial compound in STP influents (80 %) while sulfamethazine, an SAs compound, was detected only in 10 % of the overall influents. Comparably, Peng et al. (2006) detected SDZ, SMZ, and ofloxacin in the raw sewages of the two STPs in China in the concentration range of 3.5-8 μg l^{-1}.

Among the wastes of human origin, effluents from hospitals is one of the main sources of STP influents. In fact, some studies have revealed that some antibiotics may be present in hospital sewage at high concentrations. For instance, Turiel et al. (2005) detected CF in hospital sewage water at the concentration of 5.6 μg l^{-1}. Another important source of antibiotics and their degradation products resulting in high loads into STPs is antibiotic production wastewater from manufacturing plants. In a newly published paper, Li et al. (2008) reported the concentration of OTC of 920 mg l^{-1} in the raw wastewater of an OTC production facility in China. It is clear that this concentration is much higher than those normally detected in municipal STP influents.

Besides the human antibiotics, veterinary antibiotics may also be excreted into sewage via wastewater from animal feeding operations. Even though the waste generated by animals also contributes to the wastewater from such facilities, the major contribution is from the water added to the waste from sources such as flushwater to remove manure from alleys and barns, water for cleaning, rainfall runoff from roofs and open lots and direct rainfall on pretreatment facilities (Huang et al., 2001). Antibiotics have been reported to be

detectable in the wastewater from animal husbandry activities. For example, Malintan and Mohd (2006) analysed 300 wastewater samples collected from the swine facilities from three states in Malaysia, and found that more than one-third of the samples contained SAs in the concentration range of 5-95 ng l^{-1}. The most frequently detected SAs in swine wastewater were sulfanilamide, SDZ, sulfamerazine, and sulfamethoxypyridazine, whereas the least frequently detected ones are sulfamethazine and sulfaquinoxaline.

Occurrence of antibiotics in STP effluents is primarily dependent on their removal during wastewater treatment. They can potentially occur in the effluent unchanged or as their metabolites if the removal is incomplete. Municipal wastewater treatment consisting of mechanical treatment, biological treatment, and sand filtration as an advanced treatment has been shown to fail in the complete elimination of antimicrobial compounds such as SAs (Göbel et al., 2004). Furthermore, in the case of the simultaneous presence of SAs metabolites, some of them have been shown to be retransformed to the antibiotic itself throughout the wastewater treatment process, leading to an increase in the antibiotic loads in the final effluents (Göbel et al., 2007). Due to this inefficiency of conventional wastewater treatment techniques in the complete elimination of antibiotics, other techniques, such as ozonation of wastewater (Balcıoğlu and Ötker, 2003) and reverse osmosis/nanofiltration (Košutić et al., 2007), have been proposed to further reduce the antimicrobial load in the wastewater effluents.

Concentrations of antibiotics determined in wastewater effluent are generally in ng l^{-1}-μg l^{-1} range. For example, Yang et al. (2005) detected CTC, doxycycline, and SMZ in the effluents of an STP in USA in which activated sludge treatment and chlorination are performed, with a concentration range of 0.06-0.21 μg l^{-1}. SMZ has been found to have the highest concentration in the effluent wastewater. Comparably, Kim et al. (2007) detected SMZ in 4 wastewater effluent samples among seven total samples from the STPs in South Korea at relatively high concentrations (up to 0.41 μg l^{-1}). The frequent detection of some SAs such as SMZ in STP effluents is mainly due to the polar nature of these compounds, since a large part of elimination is achieved by adsorption on activated sludge partly mediated through hydrophobic interactions which is inefficient in the removal of polar antibiotics.

2.5.1.2. Occurrence in Surface Waters. Due to partial elimination of antibiotic substances in STPs, they may be expected to be found in surface waters. For surface water samples, the maximum concentrations of antibiotics are generally one order of magnitude lower than the maximum concentrations for the STP effluents. For instance, Hirsch et al. (1999) detected SMZ with a maximum concentration of 0.48 μg l^{-1} in surface waters in Germany, while its concentration reached the maximum value of 2.00 μg l^{-1} in STP effluents. Similar trend was observed by Kim et al. (2007). These authors measured SMZ concentrations reaching the maximum value of 0.41 μg l^{-1} in STP effluents, whereas the maximum concentration of this SAs compound in surface waters was 0.04 μg l^{-1}.

In addition to STP effluents, agricultural practices may also lead to antimicrobial contamination of surface waters. For instance, Campagnolo et al. (2002) reported sarafloxacin, sulfadimethoxine, CTC, TC and OTC in field streams and in a river proximal to large-scale poultry feeding operations at concentrations up to 4 μg l^{-1}. In a more recently conducted study, Yang et al. (2004) investigated the influence of the variation in land use settings, i.e. urban or agricultural sites, on the occurrence of TCs and SAs in surface water samples collected from different locations on a river in USA. The authors found that OTC, CTC, SMZ, sulfadimethoxane, and sulfamethazine were present at the highest concentration detected along the river with the greatest influence of agriculture. These studies demonstrate that animal waste applied to agricultural fields as fertilizer may act as a non-point source of antimicrobial residues in surface waters.

A direct route through which antimicrobial agents are released into surface waters, is aquacultural practices. In such practices, a large fraction of antibacterial agents is administered to farmed fish, and in turn, is excreted via feces and urine into the aquatic environment, or remains in the water without ingestion by fish (Pouliquen et al., 2007). Antibiotic concentrations in aquaculture ponds may extend up to several mg l^{-1}. For example, Le and Munekage (2004) detected SMZ and norfloxacin in different shrimp ponds and surrounding canals in Viet Nam at the highest concentrations of 2.4 and 6.1 mg l^{-1}, respectively. Similarly, Hamscher et al. (2006) detected SDZ (0.23 mg l^{-1}) or sulfadimethoxine (0.14-0.88 mg l^{-1}) in four of 15 water samples originating from aquaculture systems.

Detection frequency and maximum concentrations of antibiotic compounds in surface waters may be influenced by some factors such as compound class, sampling season, and size of the surface waters. Compound class mainly affects the stability of antibiotics in solid and aquatic environments. For example, TCs and FQs have been rarely detected as free molecules in surface waters due to their strong sorption to soil, sediment, sludge, and suspended solid particles (Hirsch et al., 1999; Kolpin et al., 2002). On the other hand, SAs have been commonly found in surface waters due to their hydrophillic nature, leading to negligible sorption in soils and sewage sludge (Lindsey et al., 2001).

Variation of antibiotics residues in differing seasons is probable due to the differences in usage amounts of antibiotic compounds, temperature and flow rate of surface waters. Generally, the highest antibiotic concentrations in surface waters have been measured in winter season, since low flow conditions and cold-water temperatures might enhance the persistence of many compounds (Kim and Carlson, 2007). The usage frequency of antibiotic compounds in different seasons also determines the presence and concentrations in surface waters. For instance, SMZ, an SAs compound used for the first-line treatment of bacterial sinusitis, which is prevalent during fall, winter, and spring seasons, have been frequently detected during these seasons in STPs (Karthikeyan and Meyer, 2006). This finding also suggests that SMZ has a high potential to occur in surface waters with higher coincidences during fall, winter, and spring, compared to summer season.

The size of the receiving surface water mostly determines the maximum concentrations of antibiotic compounds in this matrix. Surface waters with a small area are more susceptible to contamination, and thus, generally contain a high percentage of STP effluents and run-off waters from agricultural fields. This trend has been shown in several studies. For example, Christian et al. (2003) analyzed a series of river waters for up to 29 antibiotic compounds, and found that in some small creeks, eleven antibiotics out of 29 could be found due to stronger impact of an input of STP effluent in small creeks than in a larger river.

2.5.1.3. Occurrence in Groundwater. Contamination of groundwater with antimicrobial compounds used in human medicine is primarily caused by the leakage from landfill, direct disposal of sewage sludge to soils, and irrigation of treated or untreated wastewater (Kot-

Wasik et al., 2007; Ternes et al., 2007; Siemens et al., 2007). On the other hand, contamination of groundwaters with veterinary antibiotics is known to be the consequence of the application of animal manure to agricultural fields, and direct excretion by pasture-reared animals (Kemper, 2008). The reported concentrations of antimicrobial compounds detected in groundwater samples have been generally at sub-μg l^{-1} level or even below the LOQ (Hirsch et al., 1999). Detection frequency and concentrations of antibiotics in groundwater are strongly related to their physicochemical properties. For instance, SAs which have low K_d of adsorption to soil, have been the most frequently detected antimicrobial class at the concentration range of 0.04-0.47 μg l^{-1} (Hirsch et al., 1999; Batt et al., 2006; Lindsey et al., 2001). On the other hand, the strong sorption of TCs and FQs to soil renders them fairly immobile in the environment, so they have not been detected in groundwater samples in many studies (Zhu et al., 2001; Hamscher et al., 2005; Batt and Aga, 2005). Despite the strong binding of TCs to soil, however, total concentration of TC and OTC in groundwater samples from a field well proximal to a poultry farm has been reported to be as high as 1 μg l^{-1} (Campagnolo et al., 2002).

2.5.1.4. Occurrence in Drinking Water. There are almost no reports of antimicrobial compounds being detected in drinking water due to possible maximum concentrations in the order of low ng l^{-1} which require very sensitive and reliable analytical methods. Since the source waters of drinking water treatment plants have been previously shown to contain SMZ residues (Stackelberg et al., 2004), antimicrobial compounds might be present in consumers' drinking water if drinking water treatment is unable to fully remove these chemicals. In a preliminary occurrence study conducted in USA (Ye et al., 2007), SMZ has been revealed to be present in drinking waters in the concentration range of 3.0-3.4 ng l^{-1}. However, due to limited occurrence data, drinking water standards have not yet been established for antibiotic compounds. Furthermore, the presence of transformation products in drinking water should also be considered in risk assessment, since antibiotics, e.g. SAs, are known to react with chlorine disinfectants (Huber et al., 2005).

2.5.2. Occurrence of Tetracyclines, Sulfonamides and Fluoroquinolones in Solid Environmental Matrices

Since their identification in aquatic environment, antimicrobial compounds have been targeted as emerging environmental contaminants. However, depending on their physicochemical properties, they also show a tendency towards persistence in solid environmental matrices (Beausse, 2004). Table 2.4 represents recently conducted studies in which TCs, SAs and FQs have been detected in various solid environmental matrices.

2.5.2.1. Occurrence in Sewage Sludge. The parent antimicrobial compounds or their metabolites are more or less persistent in the STP. Therefore, depending on the polarities or other binding properties, a part of the substance will be retained in the sludge (Jorgensen and Halling-Sorensen, 2000). For instance, CF and norfloxacin, two FQs compounds have been determined in sewage sludges from several wastewater treatment plants with concentrations ranging from 1.40 to 2.42 mg kg^{-1} (Golet et al., 2002). This finding also refers to the high affinity of FQs to sewage sludge during municipal wastewater treatment because of their strong sorption properties. On the other hand, prevalence of the occurrence of antimicrobial compounds in sludge could not be explained only considering their physicochemical properties, but also taking their consumption amounts into account. For example, Göbel et al. (2005) reported the presence of two SAs compounds which include a family of antimicrobial agents widely used in human and veterinary medicine, in activated sludges from different STPs in Germany and Switzerland at the concentrations of up to 0.20 mg kg^{-1}. Similarly, in a recent study conducted in Spain (Nieto et al., 2007), four SAs were detected in sewage sludge samples from two domestic STPs, even though concentrations of all of the SAs compounds were below the LOQ (0.02-0.1 mg kg^{-1}).

2.5.2.2. Occurrence in Animal Manure. Antimicrobial compounds administered to animals are excreted from the body after a short time of residence as parent compounds or metabolites (Thiele-Bruhn, 2003). Even though the elimination of antibiotics from the organism is not complete, TCs and SAs may be excreted at a rate up to 90 % (Kemper, 2008). Similarly, FQs are excreted at the rates varying between 60 and 85 % largely unchanged (Morales-Munoz et al., 2004). In addition to the single antibiotic substances, excretion rates may also vary among the treated species and depending on the mode of

Table 2.4. Tetracyclines, Sulfonamides and Fluoroquinolones in Solid Matrices.

Class	Compound	Matrix	Concentration mg kg^{-1}	Reference
Tetracyclines	TC, DC, CTC, OTC	Manure	0.005-6.1	Aga et al., 2003
	TC, CTC	Manure Soil	0.09-4.0 0.004-0.20	Hamscher et al. 2002
	CTC	Soil	0.0006-0.016	Jacobsen et al., 2004
	TC, OTC, CTC	Soil	0.004-0.25	Zilles et al., 2005
Sulfonamides	SDM	Manure Soil	1.0-1.1 0.015	Christian et al., 2003
	SDM, STZ	Manure	0.10-12.4	Haller et al., 2002
Fluoroquinolones	CF, NOR	Sludge Soil	1.40-2.42 0.27-0.40	Golet et al., 2002
	CF, NOR	Soil	9-14	Morales-Munoz et al., 2004
	ENR, CF	Soil	0.013-0.20	Uslu et al., 2007
Multiple classes	SDM, CTC	Manure Soil	Up to 9.99 Up to 0.087	Aust et al., 2008
	TC, CTC, SDM TC, CTC, SDM, SDZ	Soil Manure	0.002-0.30 0.9-41.2	Hamscher et al. 2005
	TC, OTC, CTC, DC, SDZ, SDX	Manure	0.015-15.7	Jacobsen and Halling Sorensen 2006
	SMZ, NOR	Mud	4.8-2616.0	Le and Munekage, 2004
	TC, OTC, CTC, SDM, SDZ, ENR, CF	Manure	0.1-91	Martinez-Carballo et al., 2007

DC : Doxycycline, SDM : Sulfadimidine, NOR : Norfloxacin, SDX: Sulfadoxine.

application. Following their excretion in urine and faeces, the active antimicrobial compounds or metabolites largely end up in the manure. In addition, antibiotic metabolites which may also be bioactive, can be transformed back to the parent compound in manure application. Following their excretion in urine and faeces, the active antimicrobial compounds or metabolites largely end up in the manure. In addition, antibiotic metabolites which may also be bioactive, can be transformed back to the parent compound in manure (Halling-Sorensen et al., 1998). For instance, chloramphenicol glucoronide and N-4-acetylated sulfadimidine present in liquid manure have been shown to be converted to chloramphenicol and sulfadimidine, respectively, and thus to be reactivated (Berger et al., 1986). This means that a significant percentage of the administered antibiotics may be excreted into the environment in active forms.

Manure often has antimicrobial levels that could potentially be harmful to soil biota, and these levels are generally well enough to promote antibiotic resistance. TCs, SAs, and FQs have been detected in liquid manure and dung samples up to 46, 91, and 8.3 mg kg^{-1}, respectively (Martinez-Carballo et al., 2007). In another study, TCs and SAs residual concentrations in swine manure samples from finishing pigs, sows and mixed production sites have been reported to be up to 30 and 2 mg kg^{-1}, respectively (Jacobsen and Halling-Sorensen, 2006). Zilles et al. (2005) and Campagnolo et al. (2002) have reported a range of 0.17-4.26 and 0.025-1.00 mg l^{-1}, respectively, for concentrations of TCs in swine wastes, which are consistent with each other. The latter has also determined the presence of SAs in animal waste samples at the concentrations ranging from 0.0025 to 0.40 mg l^{-1}. These relatively large concentrations of antibiotics being introduced directly into the environment via manure amendment are the major concern in terms of the ecotoxicity and promotion of antibiotic resistance. To prevent excessive antibiotic loadings to agricultural fields via fertilization with animal manure, prolonged maturation times of at least five months have been proposed, since OTC and sulfadimethoxine have been demonstrated to be present in stable manure at the concentrations of 0.82 and 0.39 mg kg^{-1} after about five and three months maturation, respectively (De Liguoro et al., 2003; 2007).

2.5.2.3. Occurrence in Agricultural Soils. Soil is a natural source of antimicrobial compounds, since numerous antibiotics producing microorganisms are present in soil. Therefore, even though no antibiotic input took place, occurrence of antibiotic residues in

soil may be anticipated (Thiele-Bruhn, 2003). However, the major transfer of antimicrobial compounds to agricultural soils occurs via anthropogenic as well as agricultural inputs. The primary entry route of human-use antimicrobial compounds to agricultural fields is via direct disposal of sewage sludge to soils. For instance, norfloxacin and CF were determined in two sludge-treated soil samples near Zurich (Golet et al., 2002). Furthermore, these compounds have been demonstrated to be persistent in sludge-treated soils up to several months after application. In another study conducted by the same group (Golet et al., 2003), FQs have been found to accumulate in the topsoil during the initial phase of sludge application to soils, and at the following weeks, only a limited mobility to the subsoil has been observed due to their high sorption coefficients to soil. This finding indicates the possibility of a continuous increase of FQs concentration in the topsoil with each addition of sludge.

Irrigation of agricultural fields with untreated or treated wastewater can also possibly lead to contamination of these soils with antimicrobial compounds. Particularly in developing countries, municipal wastewater is discharged without prior treatment through channels, and is used for irrigation of agricultural lands. For instance, sulfasalazine has been detected in the channels which are used to transport the municipal wastewater through an agricultural area in Mexico (Siemens et al., 2007). As a consequence of irrigation of agricultural fields with the wastewater containing this substance, sulfasalazine has been detected in soil solution in the concentration range of 0.12-0.16 $\mu g\ l^{-1}$. Since the treated or untreated wastewater may contain appreciable amounts of SAs compounds due to their widespread consumption in human medicine and low affinity to sewage sludge, these compounds may also contaminate the groundwater after the application of wastewater to soil, as shown by Ternes et al. (2007).

Despite the fact that antimicrobial compounds may also enter into the terrestrial environment via anthropogenic sources as described above, veterinary antibiotics contribute more significantly to the occurrence of antimicrobial compounds in agricultural soils due to the application patterns. After animal-origin antibiotics are excreted and stored in storage or manure, the residual can enter into the agricultural field via fertilization with animal manure or even directly through grazing livestock. Transfer of antibiotics such as

OTC from manure to soil is possible due to stronger binding to soil than the binding to manure (Loke et al., 2002).

The environmental concentrations of antimicrobial compounds detected in soil are substantially lower than that detected in manure, mainly due to the dilution of the antibiotics when contaminated manure is tilled into the soil, or the compounds are washed into surface water or leached to groundwater by rainfall or irrigation. Aust et al. (2008) detected sulfamethazine and CTC in permanent grassland, an experimental and commercial feedlot soils in Canada up to 0.087 mg kg^{-1}. The authors have also showed that horizontal and vertical flow within feedlots caused dislocation of the two compounds from soil. In another study, OTC has been investigated as the environmental contaminant in arable soil in Italy where crops are cultivated for animal feeding purposes (Brambilla et al., 2007). Although results of this study indicated that OTC occurred at mg kg^{-1} levels in soil exposed to contaminated pig manure fertilization, these contamination levels have been concluded to be insufficient for the uptake of OTC by main seeds. In the study conducted by Martinez-Carballo et al. (2007), CTC, ENR and CF have been detected approximately in one-fifth of the agricultural soils located in Austria up to a maximum concentration of 0.37 mg kg^{-1}. The authors have emphasized that FQs and CTC should be regarded critically in terms of ecotoxicity since they have been suspected to be accumulated in agriculturally used soils.

2.5.2.4. Occurrence in Sediments. Occurrence of human- and animal-used antibiotics in the sediment matrix has not been studied to a large extent. Antibiotics used widely in aquaculture are generally supposed to be responsible for most of the contamination observed in sediments, since these compounds are directly released into the aquatic environment as feed additives and the excess is deposited at the bottom. On the other hand, human-used antimicrobial compounds present in municipal wastewaters can also result in the contamination of sediments. Ferdig et al. (2005) measured ofloxacin concentration of 0.058 mg kg^{-1} in sediment taken from the river water some 20 m downstream of the STP effluent discharge.

Antimicrobial compounds can be present in sediments at the concentrations which may cause harmful effect on ecosystems. For instance, SMZ and norfloxacin have been

shown to occur in mud from shrimp ponds in mangrove areas in Viet Nam at the concentrations of 820 and 2616 mg kg^{-1}, respectively (Le and Munekage, 2004). TCs and SAs compounds tend to be deposited on the sediment due to their high rate of partitioning to this matrix, and hence their concentrations in sediment have been generally shown to be considerably higher than that of the overlying water matrix (Kim and Carlson, 2007a,b). The concentrations of antimicrobial compounds in sediment can be affected by the flow rates of rivers. In the study conducted by Pei et al. (2006), TCs and SAs concentrations in sediments of a river landscape have been found to be higher during the low-flow sampling event (February) compared to the high-flow sampling event (April).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemical Substances

Standards of veterinary antibiotics used in this study and some information about these compounds are given in Table 3.1.

Table 3.1. Standards of veterinary antibiotics investigated in this study.

Antibiotic Standards	Molecular Formula	Molecular Weight (g mol^{-1})	CAS Number	Supplier
Tetracyclines				
Oxytetracycline hydrochloride	$C_{22}H_{25}ClN_2O_9$	496.90	2058-46-0	Sigma
Chlortetracycline hydrochloride	$C_{22}H_{24}Cl_2N_2O_8$	515.35	64-72-2	Riedel-de Haën
Sulfonamides				
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	250.28	68-35-9	Sigma
Sulfathiazole	$C_9H_9N_3O_2S_2$	255.32	72-14-0	Sigma
Sulfachloropyridazine	$C_{10}H_9ClN_4O_2S$	284.72	80-32-0	Riedel-de Haën
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.28	723-46-6	Sigma
Fluoroquinolones				
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.35	85721-33-1	Fluka
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	359.40	93106-60-6	Fluka

Stock solutions of TCs and SAs were at the concentration level of 1 mg ml^{-1}, and stock solutions of FQs were at the concentration level of 0.1 mg ml^{-1}. All the stock solutions were prepared by dissolving the antibiotics in methanol. Stock solutions were stored at -20ºC up to one month. Because it is known that especially TCs and FQs may interact with UV–light and can undergo photodegradation, volumetric flasks in which

stock solutions were stored, were covered with aluminum foil in order to minimize photochemical degradation.

The chemicals used for antibiotic extraction from manure and soil are given in Table 3.2.

Table 3.2. Chemical substances used for the extraction.

Chemical	Molecular Formula	Step In Which It Is Used	Supplier
TCs and SAs Extraction			
Orthophosphoric acid	H_3PO_4	pH adjustment	Merck
Sodium hydroxide pellets	NaOH	pH adjustment	Riedel-de Haën
Methanol	CH_3OH	Extraction/SPE	Sigma-Aldrich
Citric acid monohydrate	$C_6H_8O_7 \cdot H_2O$	Extraction	Merck
Disodium hydrogenphosphate anhydrous	Na_2HPO_4	Extraction	Merck
Ethylene diaminetetraacetic acid disodium salt dihydrate	$Na_2EDTA \cdot 2H_2O$	Extraction/SPE	Fluka
Sodium acetate trihydrate	$NaOAc \cdot 3H_2O$	SPE	Riedel-de Haën
Milli-Q water	H_2O	SPE	Millipore
FQs Extraction			
Ammonium hydroxide	NH_4OH	pH adjustment	Riedel-de Haën
Magnesium nitrate hexahydrate	$Mg(NO_3)_2 \cdot 6H_2O$	Extraction	Merck

Three buffer solutions, namely McIlvaine buffer, extraction buffer, and conditioning buffer, were used in the extraction of TCs and SAs, and SPE purification of the extracts. McIlvaine buffer was prepared by mixing 0.2 M citric acid and 0.4 M Na_2HPO_4 solutions at a ratio of 90:60 (v/v). Extraction buffer was prepared by mixing McIlvaine buffer, 0.1 M Na_2EDTA solution, and MeOH at a ratio of 25:25:50 (v/v/v), and pH was adjusted to 7.2 by adding 6 N NaOH solution. Conditioning buffer was prepared by diluting 3.75 ml

extraction buffer to a total volume of 100 ml by adding MilliQ water and pH was adjusted to 2.9 by adding H_3PO_4.

The chemicals used for soil and manure characterization studies are given in Table 3.3.

Table 3.3. The chemical substances used in the characterization of manure and soil samples.

Chemical	Molecular Formula	Purpose of Use	Supplier
Potassium dichromate	$K_2Cr_2O_7$	OC	Merck
Glacial acetic acid	CH_3COOH	CEC	Merck
Concentrated sulphuric acid	H_2SO_4	OC, Digestion of the samples	Riedel-de Haën
Hydrogen peroxide	H_2O_2	Digestion of the samples	Park Scientific Limited
Isopropyl alcohol	$(CH_3)_2CHOH$	CEC	Sigma-Aldrich
Ferrous ammonium sulfate	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	OC	Merck
Potassium chloride	KCl	pH	Merck
TKN indicator		TKN	Hach
Mineral stabilizer		TKN	Hach
Polyvinyl alcohol	$(CH_2CHOH)_n$	TKN	Hach
Nessler reagent		TKN	Hach
Phenolphthalein	$C_{20}H_{14}O_4$	TP	Hach
PhosVer pillows		TP	Hach

Chemical substances described in Table 3.4 was used in the analysis of antimicrobial compounds.

Table 3.4. Chemical substances used in the analysis of antimicrobial compounds.

Chemical	Molecular Formula	Purpose of Use	Supplier
Methanol	CH_3OH	Mobile phase	Sigma-Aldrich
Formic acid	HCOOH	Mobile phase	Sigma-Aldrich
Acetonitrile	CH_3CN	Mobile phase	Sigma-Aldrich
Milli-Q water	H_2O	Mobile phase	Millipore

3.1.2. Manure and Soil Samples

In this investigation, total nine manure-amended agricultural soil and eight animal manure samples were used as environmental samples. Sampling was performed in December 2006 and January 2007 and from four zones. Soil samples were collected from agricultural areas amended with poultry or calf manure. Cattle, poultry, and mixed (cattle + poultry) manure samples were taken from manure heaps stockpiled adjacent to the fields in which soil samples were obtained. Only the manure samples K-3 and K-4 were taken from the coordinates unconnected with those of the agricultural fields K-1 and K-2. The samples were not only used for the determination of antimicrobial compounds, but also for the recovery experiments. The sampling zones located in the North part of Marmara Region are represented on a basic map in Figure 3.1. Sampling locations, sampling dates and types of crops cultivated in the manure-amended fields are represented in Table 3.5. Geographic coordinates of the different sampling locations were determined by using a Geographical Positioning Systems (GPS) device (Garmin eTrex).

Table 3.5. Description of the agricultural fields and manure samples.

Soil Samples					
Sample ID	**Crops Grown**	**Manure Type for Fertilization**	**Fertilization Date/ Sampling Date**	**Sampling Location**	**Coordinates**
D-1	Wheat	Poultry	Jun. 2006/Dec. 2006	Babaeski	N 41º 37’ 22.5” E 27º 17’ 4.7”
D-2	Wheat	Cattle	Jun. 2006/Dec. 2006	Babaeski	N 41º 37’ 17.2” E 27º 17’ 0.3”
M-1	Wheat, sunflower	Cattle	Sept. 2005/Dec. 2006	Malkara	N 40º 55’ 10.1” E 26º 58’ 17.4”
M-2	Fruit	Poultry	Jun. 2006/Dec. 2006	Malkara	
B-1	Vetch	Poultry	Jun. 2006/Jan. 2007	Balcik	N 40º 56’ 2.1” E 29º 27’ 58.2”
B-2	Vegetable (lettuce, garden orache)	Poultry	Jun. 2006/Jan. 2007	Balcik	N 40º 56’ 4.2” E 29º 27’ 58.5”
B-3	Vegetable (lettuce)	Poultry	Jun. 2006/Jan. 2007	Balcik	N 40º 56’ 4.2” E 29º 28’ 55.6”
K-1	Ploughed field	Cattle	Jun. 2006/Jan. 2007	Kandira	N 41º 01’ 32.4” E 29º 54’ 33.4”
K-2	Ploughed field	Cattle	Jun. 2006/Jan. 2007	Kandira	N 41º 01’ 32.3“ E 29º 54’ 33.6“

Table 3.5. (Continued)

Sample ID	Manure Type	Manure Samples Sampling Date	Sampling Location	Coordinates
D-3	Poultry	Dec. 2006	Babaeski	
D-4	Cattle	Dec. 2006	Babaeski	
M-3	Mixed	Dec. 2006	Malkara	
B-4	Poultry	Jan. 2007	Balcik	
B-5	Poultry	Jan. 2007	Balcik	
B-6	Poultry	Jan. 2007	Balcik	
K-3	Poultry	Jan. 2007	Kandira	N 41º 01’ 20,7" E 024º 54’ 39,1"
K-4	Poultry	Jan. 2007	Kandira	N 40º 54’ 08" E 029º 58’ 47,8"

Figure 3.1. Representation of the study zones on a basic map.

3.2. Methods

3.2.1. Sampling, Storage and Sample Preparation

Agricultural soil and animal manure samples were collected at a depth of 10 cm below the surface layer of soils and manure heaps. Sampling was achieved by using a gardening trowel, and 1 to 10 discrete subsamples were collected depending on the size of the agricultural fields and manure heaps. Each subsample to be used for the preparation of the composite samples was put into separate, clean, sealed plastic bags, and immediately transported to the laboratory after collection.

The samples were left to be air-dried for minimum one day in order to prevent any errors which may be caused by variable moisture content. After air-drying, composite samples were prepared by weighing the equal amount of discrete subsamples and mixing them in one clean pochette. Composite samples were sieved from a sieve with a mesh size

of 2 mm in order to remove any large constituents and stored at + 4ºC until analysis to minimize the microorganisms growth.

3.2.2. Extraction of the Antimicrobial Compounds

3.2.2.1. Extraction of Tetracyclines and Sulfonamides. Simultaneous extraction of TCs and SAs from soil and manure, and SPE clean-up of the extracts were carried out using an optimized version of the method developed by Blackwell et al. (2004b). In the antibiotic analyses, three replicate samples were used. Extraction method was applied for the extraction of OTC, CTC, SDZ, STZ, SCP, and SMZ from agricultural soil and animal manure samples with different characteristics. The whole extraction procedure is schematically represented in Figure 3.2.

In this extraction procedure, 4 g of soil or 1 g of manure was weighed on an analytical balance (Scaltec SBA 31) and transferred carefully into a centrifuge tube. 5 ml extraction buffer was added into each tube and the centrifuge tubes were vortexed (Nüve, NM 110) for 30 s. The centrifuge tubes were put into an ultrasonic bath (Bandelin Sonorex Super RK 510) for 10 min and the samples were then centrifuged (PSelecta centrifuge) at 3300 rpm for 15 min. The supernatant was decanted into a 500 ml glass bottle and extraction was repeated two times more without ultrasonication step. At the end of extraction with totally 3 cycles, approximately 15 ml extract (supernatant) was present in 500 ml glass bottle. Supernatants were then diluted to approximately 400 ml by adding MilliQ water in order to reduce the methanol content to < 2% to prevent that the antibiotics were eluted from HLB cartridges during SPE, and pH was adjusted to 2.9 by adding H_3PO_4 (WTW, pH 330).

Strata strong anion exchanger (SAX) cartridges (6 ml/500 mg, Phenomenex Inc., Torrance, CA) which were used to remove interfering natural organic matter (NOM) of soil and manure, and Oasis hydrophilic-liphophilic balance (HLB) cartridges (6 ml/200 mg, Waters, Watford, UK) which were used to retain tetracyclines and sulfonamides in this study, were set up in tandem and connected to SPE apparatus (Phenomenex) supplied with a vacuum pump (KIF LAB Laboport) with a platinum-cured silicone vacuum hose (Masterflex). The cartridges were conditioned with 2 ml of MeOH and 2 ml of

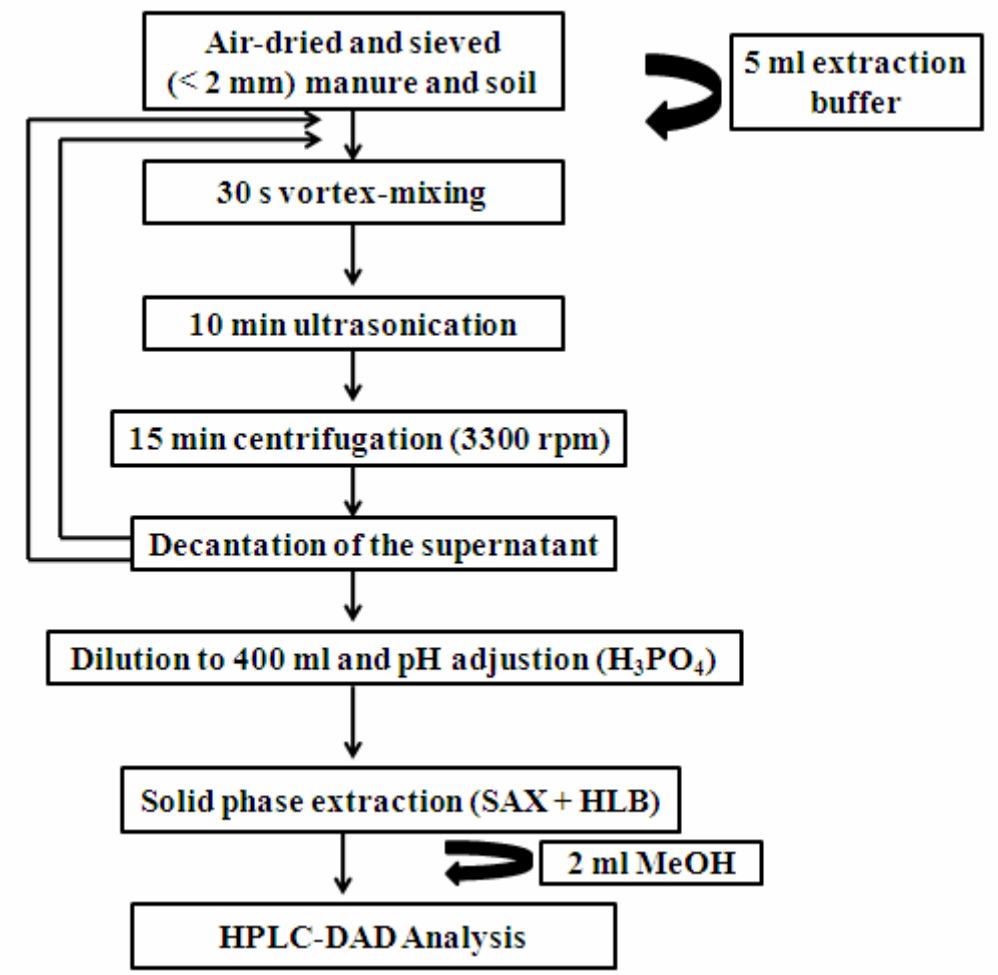


Figure 3.2. Schematic representation of the extraction and SPE.

conditioning buffer in order to activate the cartridges and to remove any impurities in the cartridges. The supernatant diluted to approximately 400 ml before it was passed through the cartridges at a loading rate of about 5 ml min^{-1}. After the entire sample was loaded, SAX cartridges were removed and HLB cartridges were then washed with 5 ml conditioning buffer, 5 ml 0.1 M NaOAc solution, 5 ml Milli-Q water, and 2 ml 20% MeOH sequentially in order to remove humic substances which may interfere the analysis. HLB cartridges were then air dried for 10 min to minimize moisture in the cartridges which may be eluted with MeOH. Finally, elution was performed 2 times as 1 ml of MeOH. Before HPLC analysis, the extracts were vortexed and transferred into amber HPLC vials. The extracts were stored at - 20ºC until the day of analysis.

3.2.2.2. Extraction of Fluoroquinolones. Extraction of FQs from manure and soil was based on the method developed by Turiel et al. (2006). In the FQs analyses, three replicate samples were used. The extraction method was applied for the extraction of ENR and its metabolite CF from five agricultural soil and poultry manure samples with different characteristics. Extraction and analysis steps of the soil and manure samples are presented in Figure 3.3.

1 g of soil or manure in a centrifuge tube was mixed with 8 ml of an aqueous solution of $Mg(NO_3)_2$ (29 % (w/v); pH: 8.1 ± 0.1) in the vortex for 30 s and subsequently in the ultrasonic bath for 30 min and the samples were then centrifuged at 7000 rpm for 10 min. The resulting supernatants were passed through 0.45 μm filters to produce the extract for analysis.

3.2.3. Recovery Studies of the Antimicrobial Compounds

In order to assess performance of the method used to extract the antimicrobial compounds from soil and manure simultaneously and to establish a relationship between the recovery ratios and soil-manure characteristics, recovery studies were performed. For the determination of the recovery levels of antibacterial compounds in soil and manure, 25 μl of mastermixes prepared from the stock solutions were added to 1 g of soil and manure sample to achieve the antibiotic concentrations of 0.2 and 1 mg kg^{-1}. Spiked soil and manure samples were mixed thoroughly by using a vortex and kept in dark conditions overnight at room temperature in order to allow the formation of an equilibrium state between antimicrobial compounds and soil and manure matrices. Three replicates for both spiking level were analysed for most of the soil and manure samples. Antibiotic concentrations quantified in some unfortified samples were subtracted from the concentrations measured in spiked samples. The recovery ratios were calculated by using the following equation:

$$\text{Recovery (\%)} = \frac{\text{Concentration of antibiotics quantified by HPLC in mg kg}^{-1}}{\text{Spiking level (0.2 or 1.0 mg kg}^{-1}\text{)}} \times 100 \quad (3.1)$$

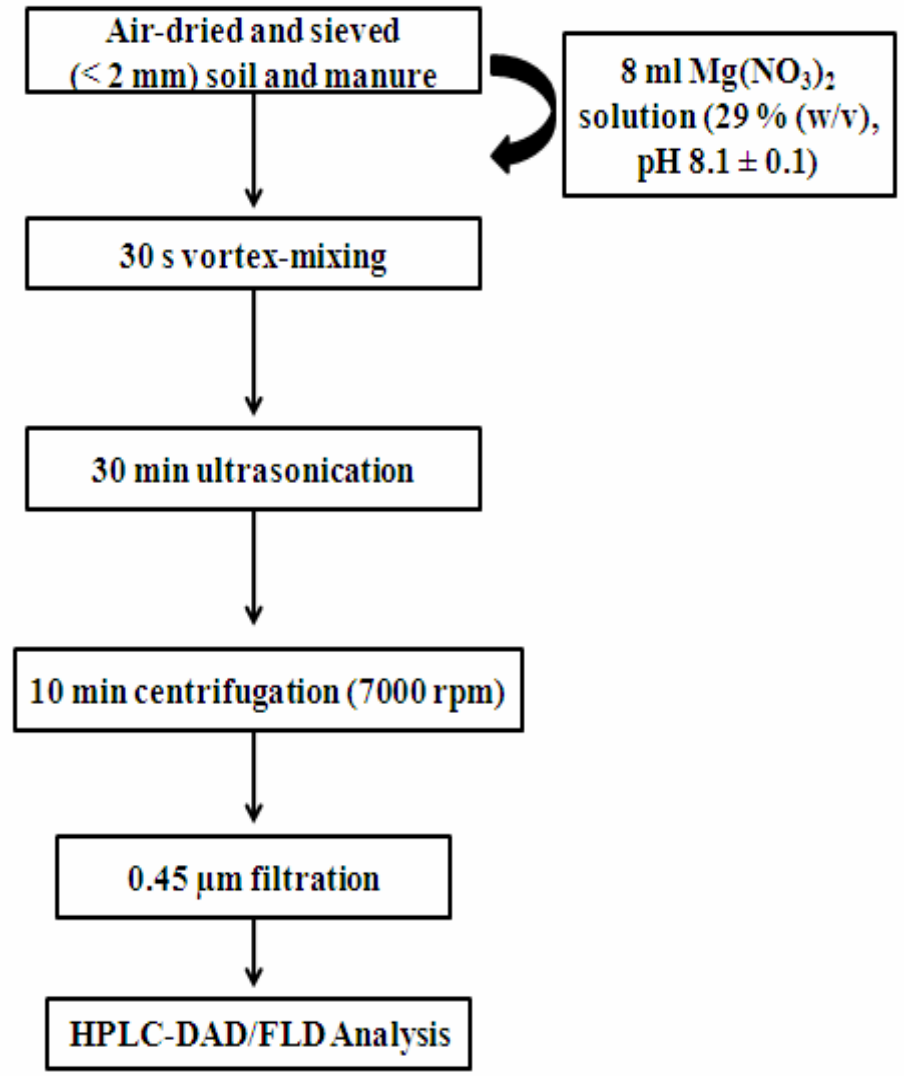


Figure 3.3. Overall flow scheme of FQs extraction from soil and manure.

The relative presicion of the measurements were expressed as the relative standard deviation (RSD, %). Microsoft Excel 2002 was used for the mathematical calculations.

3.2.4. HPLC Analysis of the Antimicrobial Compounds

Detection and quantification of TCs, SAs, and FQs were carried out using an Agilent 1100 series HPLC system equipped with a binary pump, diode array detector (DAD), fluoroscence detector (FLD), and an autosampler, and controlled by ChemStations software. Separations were performed on a 50 x 4.0 mm, 3 µm, YMC-Pack ODS-AQ column at 26ºC with an injection volume of 20 µl. Gradient elution was carried out with

0.1 % formic acid in ACN (solvent A) and 0.1 % formic acid in water (solvent B). The separation of all the analytes was carried out in 10 min, and after each run, the solvents were passed through the column without sample injection for 2 min. The gradient solvent program used is shown in Table 3.6.

Table 3.6. The gradient program used.

Time (min)	Flow Rate (ml min^{-1})	Solvent A (%)	Solvent B (%)
0	0.7	5	95
7	0.7	30	70
8.5	0.7	30	70
10	0.7	5	95

Simultaneous detection was carried out at 360 nm for TCs, 270 nm for SAs, and 280 nm for FQs. FQs were also analyzed using FLD at the excitation and emission wavelengths of 280 and 450 nm, respectively. Standard solutions at six concentration level (0.05, 0.1, 0.5, 1, 2, 5 μg ml^{-1} for TCs and SAs; 0.01, 0.05, 0.1, 0.2, 0.5, 1 μg ml^{-1} for FQs) were prepared freshly from stock solutions just before HPLC analysis, and external calibration curves were constructed using these standard solutions. Concentrations of the analytes in sample matrices were determined by using peak areas which correspond to the unknown concentrations in the calibration curve. The calibration curves obtained for each antimicrobial compound are presented in Appendix A.

3.2.5. Characterization of Manure and Soil Samples

Total 17 manure and soil samples were characterized by using different parameters to obtain a comprehensive data about the samples and to establish a relationship between the recoveries and sample characteristics. These characteristics include pH, organic carbon (OC), total Kjeldahl nitrogen (TKN), total phosphorus (T-P), Cr, Cu, Pb, Ni, Zn, Fe, Cd, K, Na, Ca, Mg contents, cation exchange capacity (CEC), and texture.

3.2.5.1. pH. pH values of 5 g of the air-dried agricultural soil and animal manure samples were assessed in 12.5 ml of 1 M KCl solution according to the method described by Forster (1995).

3.2.5.2. Organic Carbon Content. OC content of the soil and manure determined by the oxidation reaction with dichromate in H_2SO_4 as decribed in method no. TS 8336 of Turkish Standards Institute (TSE) which has been based on Walkley-Black Method.

0.1 g of soil and 0.02 g of manure samples were digested with 3 ml 1 N $K_2Cr_2O_7$ and 3 ml concentrated H_2SO_4 in closed COD tubes at 150ºC for 2 h. To determine the amount of dichromate reacted with soil and manure, excess dichromate was back titrated with 0.5 N standard ferrous ammonium sulfate (FAS) solution by the addition of ferroin indicator. The OC content of soil and manure was calculated using the following formula.

$$OC\ (\%) = \frac{[(B-S) \times N_k \times 0.389]}{T} \qquad (3.2)$$

B = ml of FAS used for the blank sample
S = ml of FAS used for the sample
N_k = Normality of FAS solution (N)
T = g of soil or manure sample used

3.2.5.3. Digesdahl Digestion Method for the Determination of Total Kjeldahl Nitrogen, Total Phosphorus and Metal Content. Prior to the measurement of nitrogen, phosphorus and metal concentrations in the soil and manure samples, a digestion procedure was performed using sulphuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) (Hach Company, 1996, 1997). 0.5 g of the soil and manure samples were digested with 6 ml concentrated H_2SO_4 until the temperature increased up to 440ºC (Digesdahl, Hach). After a further 4 min at 440ºC, the sample was treated with 15 ml H_2O_2 and digested for 1 min at 440 ºC. Digested sample was diluted to a volume of 100 ml with deionized water.

3.2.5.4. Total Kjeldahl Nitrogen Content. TKN content of the digested soil and manure samples was determined according to Nessler method (Hach DR/2010 Spectrophotometer

Handbook, 1996, 1997). The method includes the treatment of digested samples with one drop TKN indicator, 8 N KOH dropwise until the permanent blue color, deionized water to 20 ml, 3 drops mineral stabilizer, and 3 drops polyvinyl alcohol (PVA), sequentially. The sample was then diluted to 25 ml with deionized water, and finally, 1 ml Nessler reagent was added into the mixture. For the calibration of spectrophotometer, blank sample prepared using the same procedure was read in the unit of mg l^{-1} TKN in the spectrophotometer, and the value was then adjusted to zero. The samples were put into spectrophotometer cuvettes with a volume of 25 ml, and the TKN contents were measured at a wavelength of 460 nm in the unit of mg l^{-1} TKN. For the calculation of TKN content, the following formula was used.

$$\text{TKN (mg kg}^{-1}) = \frac{75 \times A}{B \times C} \qquad (3.3)$$

A = mg l^{-1} read from display
B = g of sample taken for digestion
C = ml volume of digested sample

3.2.5.5. Total Phosphorus Content. T-P content of the digested soil and manure samples was spectrophotometrically determined according to Ascorbic acid method (Hach Com., 1996, 1997). The method includes the addition of one drop phenol phythalein, 8 N KOH dropwise until the first permanent dark pink color, and H_2SO_4 dropwise until the disappearance of the color. The samples were then diluted 1:10 to 1:1000 with deionized water, and 10 ml of the samples was put into spectrophotometer cuvettes with a volume of 10 ml. In the final step, PhosVer pillow containing ascorbic acid was added into the mixture. Reading was performed at the wavelength of 890 nm as mg l^{-1} P, P_2O_5 and PO_4^{3-}, and the phosphorus content was calculated using the following formula.

$$\text{P (mg kg}^{-1}) = \frac{A \times DF}{B} \times 100 \qquad (3.4)$$

A = mg l^{-1} read from display
B = g of sample taken for digestion
DF = Dilution factor

3.2.5.6. Metal Content. Cr, Cu, Pb, Ni, Zn, Fe, Cd, K, Na, Ca, and Mg concentrations in the digested soil and manure samples were measured using atomic absorption spectrometry (AAS, AAnalyst 300, Pelkin Elmer). Formula 3.4 was also used for the calculation of metal contents of each sample.

3.2.5.7. Cation Exchange Capacity. CEC of the soil samples was determined according to Method 9081 of Environmental Protection Agency (EPA). pH values of 1 N NaOAc and 1 N ammonium acetate (NH_4OAc) reagents used in the method was adjusted to 8.2 and 7.0, respectively.

Briefly, the method includes mixing of 4 g soil sample with 33 ml 1 N NaOAc, 99 % isopropyl alcohol and 1 N NH_4OAc, sequentially, on a mechanical shaker (nüve, SL 350) followed by centrifugation (Universal 16 A). The combined extract obtained at the final step was diluted to 100 ml with NH_4OAc solution and Na concentration was determined using AAS. CEC was calculated using the following formula.

$$\text{CEC (mEq 100 g}^{-1}) = \frac{[Na^+] \text{ x DF}}{23m} \text{ x } 10 \qquad (3.5)$$

$[Na^+]$ = mg l^{-1} read from display
DF = Dilution factor
m = g of weighed sample

3.2.5.8. Texture. For the assessment of texture of the soil samples, hydrometer method was used (Bouyoucos, 1962). This method is based on Stokes' law which gives the relationship among the velocity of fall of spheres in a fluid, the diameter of the sphere, the specific weights of the sphere and of the fluid, and the fluid viscosity.

According to the hydrometer method, 50 g of air-dried soil whose hygroscopic moisture content has been previously determined, was transferred into the containers, and approximately ½ of these containers was filled with tap water. 10 ml of Calgon was added

into the mixture to facilitate the dispersion of soil constituents. This mixture was allowed to stand overnight.

In the next day, soil sample was mechanically and horizontally mixed for 2 h, and sequentially transferred into the sedimentation cylinders without any loss of material. The cylinders were filled with water to 1000 ml, and mixed with a metallic mixing bar up and down. The cylinders were set down, and after 20 s, the hydrometer (ASTM No. 152 H) was inserted carefully into the suspension for the first reading at an elapsed time of 288 s. The temperature was also recorded for the first reading. The second reading was performed after 2 h, and the temperature was recorded. To correct the hydrometer values in the first and second readings, 0.36 was added to or subtracted from the hydrometer values for each degree above or below 19.4ºC, respectively. Sand, silt and clay fractions (%) of the soils were calculated using the following formula.

$$\text{Sand (\%)} = 100-(100D/B) \quad (3.6)$$

$$\text{Silt (\%)} = 100(D-D')/B \quad (3.7)$$

$$\text{Clay (\%)} = 100D'/B \quad (3.8)$$

B = g of absolute dry soil (for 50 g: 50-(moisture (%)/2)
D = Corrected value for the first reading
D′ = Corrected value for the second reading

The soil type was determined marking the sand %, silt %, and clay % values calculated as outlined above on an international soil texture triangle.

3.2.5.9. Moisture Content. Determination of the moisture level of soil and manure is based on weight difference between moist samples and dried samples kept at 105ºC in an oven overnight. Moisture content of the samples was calculated using the following formula.

$$\text{Moisture (\%)} = [(\text{Moist weight}-\text{Dry weight})/\text{Moist weight}] \times 100 \quad (3.9)$$

3.2.6. Statistical Analysis

Relationship between each data obtained from the experimental work was investigated using the statistical analysis program SPSS (Statistical Package for the Social Science) 11.5 for Windows. The influence of sample characteristics on the recovery levels of antimicrobial compounds and relationship of these characteristics with each other were evaluated using Bivariate Correlations Test. Significance of the correlations were determined to predict the most important parameters affecting the behaviour of TCs, SAs, and FQs in soil and manure. For this evaluation, probability (p) values, a statistical measure of the values between compared groups, were used. $p < 0.05$ and $p < 0.01$ mean that there is a 5 and 1 % chance respectively, that the correlation was randomly obtained. To illustrate the effects of parameters used to determine the soil and manure characteristics on the recovery levels of antimicrobial compounds, diagrams were constructed using scatter-plot technique.

For the determination of which means differ from each other, one-way analysis of variance (one-way ANOVA) test in SPSS was used when equal variances were present for both groups compared. However, when the test variable was ordinal, median test which is a nonparametric test was used. $p < 0.05$ obtained from one-way ANOVA and median tests means that both groups are significantly different from each other.

4. RESULTS

4.1. Agricultural Soil and Manure Characteristics

4.1.1. Agricultural Soil Characteristics

Some physical and chemical characteristics of the soil samples collected from agricultural fields were investigated in order to obtain extensive characterization data about the studied regions, and to establish a relationship between the recoveries and sample characteristics. Because pH, organic carbon content, metal content, texture, and cation exchange capacity are known to affect adsorption of tetracycline, sulfonamide, and fluoroquinolone group antibiotics to soil and manure matrices (Jones et al., 2005; Tolls, 2001; Loke et al., 2002), special attention has been paid to analysis of these parameters.

Soil characteristics investigated include pH, organic carbon content, TKN, total P, metal content, moisture content, cation exchange capacity, and texture. pH, moisture content, cation exchange capacity, and texture are represented together in Table 4.1.

Table 4.1. pH, moisture content, CEC, and texture of soil samples.

Sample ID	pH	Moisture (%)	CEC (mEq·100 g^{-1})	Sand (%)	Clay (%)	Silt (%)	Soil Type
D–1	6.93	9.60	15.89	77.98	10.85	11.17	Sandy loam
D-2	6.91	11.62	23.35	76.15	10.19	13.66	Sandy loam
M–1	6.90	23.37	37.17	48.98	1.80	49.22	Silty loam
M–2	6.94	21.11	34.90	50.93	9.51	39.56	Loam
B–1	6.99	13.73	28.15	73.12	15.83	11.05	Sandy clay loam
B–2	6.72	14.25	43.10	56.63	22.05	21.32	Clay loam
B–3	6.54	16.25	29.24	32.14	3.71	64.15	Silty loam
K–1	5.99	24.97	34.57	51.10	8.02	40.88	Loam
K–2	6.47	35.29	65.87	41.71	23.34	34.95	Clay loam

pH values of soil samples was found to be in the range of 6-7, indicating that all the agricultural soils analyzed in the present study were almost neutral. This pH range is typical of mineral soils in humid regions which have the pH values ranging from about 5 to approximately 7. At the pH values measured in the current study the mobility of some heavy metals such as Fe, Zn and Cu can be expected to be somewhat reduced, leading to a partial subsupply of plants (Manz et al., 1999).

The moisture contents of soil samples varied from 10 to 35 %. Relatively higher moisture content of the agricultural soil samples K–1 and K–2 compared to the other soils analyzed were due to collection of these two samples during a rain episode.

The CEC of soils is known to increase with increasing clay and OC content (Manz et al., 1999; Peinemann et al., 2000). Correspondingly, CECs of soil samples analyzed in the current study were found to be highest for clay loam soils (43 and 66 mEq·100 g^{-1} soil), and lowest for sandy loam soils (16 and 23 mEq·100 g^{-1} soil).

OC and total N and P content of soil samples are shown in Table 4.2. P contents were presented as total P, PO_4^{3-}-P, and P_2O_5-P. The average concentrations of duplicate samples and standard deviations of each parameter are given in the table.

Table 4.2. Organic carbon, nitrogen and phosphorus content of soil samples.

Sample ID	**OC (%)**	**T-P (g kg^{-1})**	**P_2O_5–P (g kg^{-1})**	**PO_4^{3-}-P (g kg^{-1})**	**TKN (g kg^{-1})**
D–1	2.42 ± 0.35	2.75 ± 0.35	6.60 ± 0.85	8.80 ± 1.13	2.74 ± 1.96
D–2	4.40 ± 0.21	1.18 ± 0.03	2.71 ± 0.07	3.62 ± 0.11	3.38 ± 0.11
M–1	3.71 ± 0.49	1.40 ± 0.08	3.22 ± 0.20	4.31 ± 0.27	3.68 ± 0.32
M–2	3.90 ± 0.21	4.50 ± 0.42	10.20 ± 1.13	13.60 ± 1.41	4.05 ± 0.00
B–1	3.20 ± 0.06	4.20 ± 0.28	9.50 ± 0.71	12.70 ± 0.99	3.00 ± 0.00
B–2	3.41 ± 0.35	3.50 ± 0.42	8.00 ± 0.85	10.60 ± 1.13	3.75 ± 0.42
B–3	1.66 ± 0.00	1.40 ± 0.00	3.10 ± 0.14	4.20 ± 0.28	2.85 ± 0.00
K–1	2.12 ± 0.18	0.80 ± 0.00	1.80 ± 0.28	2.40 ± 0.28	2.55 ± 0.21
K-2	8.14*	5.80 ± 2.26	13.20 ± 5.37	17.60 ± 7.07	7.20 ± 1.91

* N = 1.

OC contents of the soil samples varied from 2 up to 8 %. CEC of the agricultural soils were found to increase with increasing OC content ($p < 0.05$). This was expected, since in agricultural soils maintained at pH of 6-8, nearly 40-50 % of CEC may be associated with soil OC, and the addition of organic matter to soil further increases the pH dependent fraction of CEC (Loveland and Webb, 2003). Correspondingly, relatively high CEC and OC content of the soil sample K-2 was attributed to its high manure content.

Total N and P contents of the soil samples were in the range of 3-7 and 1–6 g kg^{-1}, respectively. Soil total N content was found to increase with increasing OC ($p < 0.01$) and T-P content ($p < 0.05$), while the positive correlation between soil OC and T-P content was not significant at the 0.05 level. These results are in agreement with those found in earlier studies (Hountin et al., 1997) and may indicate that accumulation of soil organic matter is affected by the amount of plant residues returned and animal wastes dispersed to the soil. Agricultural soils fertilized with poultry manure were generally found to contain highest phosphorus concentrations, indicating that poultry manure can be an efficient P source for plants grown in the agricultural fields.

Since metals bound to organic matter and clay have been previously shown to influence the sorption coefficients for TCs (MacKay and Canterbury, 2005; Figueroa et al., 2004), extraction efficiencies for metal-complexing compounds such as TCs, SAs and FQs can be affected by the metal content of soils. In addition to the influence on extraction efficiencies of antimicrobial compounds, excessive and repeated fertilization with animal manure can lead to heavy metal pollution in agricultural soils (De Temmerman et al., 2003) and this, in turn, can have toxic effects on humans, animals and plants. For all these reasons, metal contents of the samples were determined in the present study. The results of metal contents of the soil samples are shown in Tables 4.3 as the average concentration of duplicate samples and standard deviations.

K, Na, Ca, and Mg concentrations of the agricultural soil samples were determined to be in the range of 2-9, 0-1, 0-17, and 1-10 g kg^{-1}, respectively. Na content of the agricultural fields located in Tekirdağ and Kırklareli was found to be significantly higher ($p = 0.05$) compared to those in Kocaeli. This significant difference is considered to be unrelated to the differences in agricultural practices between these regions, since there was

Table 4.3. Metal contents of the soil samples.

Metal	D-1	D-2	M-1	M-2	B-1	B-2	B-3	K-1	K-2
K (g kg^{-1})	1.95 ± 0.32	1.77 ± 0.11	3.64 ± 0.02	6.42 ± 1.00	4.38 ± 0.04	6.56 ± 0.36	9.24 ± 0.24	5.04 ± 0.09	8.15 ± 0.78
Na (g kg^{-1})	0.97 ± 1.01	0.80 ± 0.30	0.36 ± 0.04	0.70 ± 0.01	0.25 ± 0.01	0.43 ± 0.02	0.49 ± 0.00	0.48 ± 0.06	0.43 ± 0.01
Ca (g kg^{-1})	3.93 ± 0.35	1.45 ± 0.05	12.53 ± 0.34	17.06 ± 1.16	1.50 ± 0.19	1.38 ± 0.06	0.52 ± 0.18	0.38 ± 0.05	2.65 ± 0.79
Mg (g kg^{-1})	1.16 ± 0.18	1.74 ± 0.06	7.17 ± 0.07	10.14 ± 0.01	1.88 ± 0.06	2.36 ± 0.01	2.90 ± 0.07	2.66 ± 0.05	3.74 ± 0.36
Cr (mg kg^{-1})	10.80 ± 15.27	10.20 ± 1.41	216.70 ± 18.24	177.20 ± 4.53	29.50 ± 5.23	38.70 ± 6.08	71.80 ± 0.85	127.50 ± 0.71	39.00 ± 2.55
Cu (mg kg^{-1})	14.70 ± 0.42	13.10 ± 1.56	30.50 ± 0.14	62.60 ± 3.68	24.80 ± 4.80	17.00 ± 4.81	28.10 ± 2.40	28.00 ± 0.57	36.30 ± 4.38
Pb (mg kg^{-1})	ND	ND	10.80 ± 15.27	ND	2.60 ± 3.68	ND	ND	ND	ND
Ni (mg kg^{-1})	1.30 ± 1.84	5.60 ± 7.92	108.60 ± 7.35	93.60 ± 4.24	3.40 ± 0.28	6.00 ± 6.22	10.40 ± 1.98	40.10 ± 2.69	9.20 ± 2.55
Zn (mg kg^{-1})	65.60 ± 3.68	32.80 ± 5.09	67.50 ± 1.27	157.80 ± 4.81	93.80 ± 5.94	88.40 ± 3.96	80.70 ± 2.69	43.90 ± 4.10	187.80 ± 65.90
Fe (g kg^{-1})	6.35 ± 1.96	7.03 ± 0.36	25.27 ± 0.13	26.46 ± 1.00	14.26 ± 0.33	13.72 ± 0.73	23.67 ± 2.99	16.99 ± 0.16	19.16 ± 2.38
Cd (mg kg^{-1})	0.50 ± 0.71	1.60 ± 2.26	1.30 ± 1.84	5.20 ± 0.57	ND	ND	ND	ND	0.20 ± 0.28

ND: Not detected.

not any significant difference between Na content of the agricultural soils fertilized with cattle and poultry manure. Heavy metal concentrations of the agricultural soil samples were found to be in the range of 10-217 mg kg^{-1} for Cr, 13-63 mg kg^{-1} for Cu, 1-109 mg kg^{-1} for Ni, 33-188 mg kg^{-1} for Zn, and 6-27 g kg^{-1} for Fe. Pb was quantified only in the samples M-1 and B-1 at the amounts of 10.8 and 2.6 mg kg^{-1}, and Cd was measured in five soil samples (D-1, D-2, M-1, M-2 and K-2) at the range of 0-5 mg kg^{-1}. In the study conducted by De Temmerman et al. (2003), Zn, Cu, and Pb have been shown to occur in arable soils in Northern Belgium in the concentration range of 12.7-264, 1.48-88 and 3.2-191 mg kg^{-1}, respectively, and the contents of these heavy metals measured in the present study were within the mentioned ranges.

Upper limit values of Pb, Cd, Cr, Cu, Ni, Zn, and Hg in soils in Turkey are defined in Soil Pollution Control Regulation (http://www.iso.org.tr/tr/Documents/Cevre/MEVZUAT LISTESI/3 TOPRAK KIRLILIGININ KONTROLU YON.doc). These limit values and the samples exceeding the limits are given in Table 4.4. In the current study, agricultural soil samples M-1, M-2 and K-1 were found to contain Cr and Ni above the limit values set for these metals. Cr and Ni pollution observed in the samples M-1 and M-2 can be linked to the use of animal manure, since the manure sample taken from the same location (M-3) contained the highest Cr and Ni concentrations among all the manure samples analyzed. In addition to Cr and Ni, agricultural soil sample M-2 was also found to have higher Cd content than the limit value defined in Soil Pollution Control Regulation. The source of

Table 4.4. The upper limit values of heavy metals permitted in Soil Pollution Control Regulation, and number of samples exceeded these values.

Heavy Metal (Total)	**Upper Limit Concentration**		**Samples Exceeding The Limit**
	pH 5-6 mg kg^{-1} dry weight	**pH > 6 mg kg^{-1} dry weight**	
Cr	100	100	M-1, M-2, K-1
Cu	50	140	-
Pb	50	300	-
Ni	30	75	M-1, M-2, K-1
Zn	150	300	-
Cd	1	3	M-2

this metal may be manure application to the agricultural field, because the manure sample M-3 also contained this heavy metal. Correspondingly, cattle and poultry solid manure applications to agricultural soils have been shown to be an important source of Cd pollution in agricultural fields (Nicholson et al., 1999). Cr, Ni and Cd content of the agricultural soil samples M-1, M-2 and K-1 can be regarded as critical in terms of human and environmental health, since these metals, particularly Cd, have the potential to be bioaccumulated in plants and, in turn, in humans causing toxic effects (De Temmerman et al., 2003).

4.1.2. Manure Characteristics

pH and moisture contents of the manure samples are represented in Table 4.5. The pH values of manure samples were alkaline from 7.3 to 8.2. The moisture contents of the manure samples were determined to vary from 22 % up to 57 %, and the fresh poultry manure samples K-3 and K-4 were observed to contain significantly less moisture than the stored ones ($p < 0.05$). The loss of dry matter during manure storage can extend to considerable values (Petersen et al., 1998) and the correlation obtained in the current study may refer to this situation. In this study, animal manure samples were found to contain significantly higher amounts of moisture compared to the agricultural soils ($p < 0.01$).

Table 4.5. pH and moisture contents of the manure samples.

Sample	pH	Moisture (%)
D–3	8.24	45.06
D–4	8.07	48.24
M–3	7.37	32.47
B–4	7.77	52.78
B–5	8.15	52.24
B–6	7.89	57.43
K–3	7.30	39.82
K-4	7.97	22.26

The OC, P (total P, PO_4^{3-}-P, and P_2O_5–P), and total N contents of the manure samples are represented in Table 4.6. The results given in the table are represented as the average concentration of duplicate samples and standard deviations.

Table 4.6. Organic carbon, nitrogen, and phosphorus contents of manure.

Sample ID	OC (%)	T-P (g kg^{-1})	P_2O_5–P (g kg^{-1})	PO_4^{3-}-P (g kg^{-1})	TKN (g kg^{-1})
D–3	26.92 ± 0.35	31.00 ± 1.41	70.00 ± 2.83	94.00 ± 2.83	22.72 ± 1.38
D–4	10.38 ± 1.39	4.60 ± 0.28	10.40 ± 0.85	13.90 ± 0.99	8.25 ± 0.21
M–3	23.46 ± 1.75	6.90 ± 0.99	16.00 ± 2.26	21.40 ± 3.11	17.18 ± 2.44
B–4	25.12 ± 3.23	23.00 ± 1.41	54.00 ± 2.83	72.00 ± 2.83	22.20 ± 0.21
B–5	26.78 ± 2.06	23.25 ± 0.35	53.50 ± 1.41	71.75 ± 1.77	19.35 ± 0.42
B–6	31.98 ± 1.76	25.75 ± 0.35	59.00 ± 0.71	79.00 ± 0.71	22.88 ± 0.53
K–3	45.68 ± 1.76	12.75 ± 0.35	29.00 ± 0.71	38.75 ± 1.06	43.50 ± 0.85
K-4	34.88 ± 2.35	17.25 ± 0.35	39.50 ± 0.71	53.00 ± 0.71	23.85 ± 1.48

OC contents of the manure samples were determined to be between 10 and 46 %. Organic C contents of the samples increased in the following order: Cattle manure < Mixed (Cattle + Poultry) manure < Poultry manure. Total OC content of cow and chicken manure has been reported to be in the range of 13-39 % and 28-39 %, respectively (Moral et al., 2005) and manure OC contents determined in the present study were comparable with those measured in the mentioned investigation. Total N and P contents of the manure samples were in the range of 8-44 g kg^{-1} and 5-31 g kg^{-1}, respectively and these values were also highest for all the poultry manures.

Metal contents of the animal manure samples are shown in Table 4.7, and are represented as the average concentration of duplicate samples and standard deviations. The macroelements (K, Na, Ca and Mg) were found in lower concentrations in the cattle manure sample D-4 compared to all the poultry manure samples. Zn, Cu, Pb, Ni, and Cr concentrations measured in the present study were in similarity with those reported in literature for cattle and poultry manure (Nicholson et al.,1999, 2003; Westing et al., 1985; Jackson et al., 2003). Cu and Zn content of the poultry manure samples was observed to be higher than those of the cattle manure D-4, while maximum Cr, Ni, and Fe concentrations

Table 4.7. Metal contents of the manure samples.

Metal	D-3	D-4	M-3	B-4	B-5	B-6	K-3	K-4
K (g kg^{-1})	30.48 ± 4.24	6.83 ± 0.36	14.49 ± 2.51	27.99 ± 0.33	24.01 ± 0.81	28.32 ± 0.35	26.19 ± 0.25	23.07 ± 0.25
Na (g kg^{-1})	8.08 ± 3.82	1.55 ± 0.03	1.31 ± 0.19	4.01 ± 0.18	4.26 ± 0.22	4.32 ± 0.14	2.39 ± 0.17	5.34 ± 0.29
Ca (g kg^{-1})	25.63 ± 2.01	4.05 ± 0.28	6.67 ± 1.14	19.60 ± 0.99	12.86 ± 0.93	14.40 ± 0.63	4.62 ± 0.53	7.93 ± 0.43
Mg (g kg^{-1})	8.67 ± 0.33	3.76 ± 0.08	6.42 ± 0.09	7.64 ± 0.43	8.86 ± 0.57	7.45 ± 0.08	5.44 ± 0.00	6.91 ± 0.12
Cr (mg kg^{-1})	19.70 ± 2.40	21.20 ± 12.16	159.20 ± 8.20	11.40 ± 3.39	6.20 ± 1.98	11.30 ± 5.52	4.50 ± 6.36	10.60 ± 5.66
Cu (mg kg^{-1})	73.90 ± 0.71	21.20 ± 0.28	28.50 ± 15.98	53.20 ± 3.68	40.30 ± 6.08	48.30 ± 5.80	42.90 ± 4.38	46.30 ± 3.25
Pb (mg kg^{-1})	ND	0.60 ± 0.85	3.60 ± 5.09	ND	1.10 ± 1.56	ND	2.40 ± 1.98	ND
Ni (mg kg^{-1})	8.30 ± 3.54	29.80 ± 12.45	54.60 ± 15.84	5.80 ± 8.20	8.30 ± 2.40	11.30 ± 4.38	11.40 ± 1.70	8.50 ± 0.42
Zn (mg kg^{-1})	757.10 ± 81.32	68.10 ± 0.14	176.90 ± 41.44	650.70 ± 251.02	420.70 ± 24.75	485.70 ± 10.61	418.20 ± 28.28	535.70 ± 152.03
Fe (g kg^{-1})	1.78 ± 0.16	7.94 ± 0.71	14.47 ± 1.78	1.72 ± 0.11	1.51 ± 0.08	1.59 ± 0.01	0.97 ± 0.08	1.35 ± 0.05
Cd (mg kg^{-1})	2.90 ± 1.56	ND	2.70 ± 0.14	ND	ND	ND	ND	ND

ND: Not detected.

were measured in D-4. These differences may be caused by the dissimilarities between the diets of cattle and poultry.

In Turkey, heavy metal concentrations in animal manure are governed by The Regulation on the Production, Importation, Exportation, Introduction to the Market and Control of Organic, Organomineral, Soil Conditioners and Microbial Fertilizers Used in Agriculture(http://www.tarim.gov.tr/mevzuat/yonetmelik_son/tarimda_kullanilan_organik.doc). The maximum concentration values of heavy metals permitted in this regulation are shown in Table 4.8. Cd concentrations in the manure samples D-3 and M-3 were close to the maximum concentration level of 3 mg kg^{-1}. Therefore, immediate care must be taken for the presence of Cd in animal manure since manure amendment to soil can cause accumulation of this toxic metal in agricultural soils leading to long-term contamination.

Table 4.8. The upper limit values of heavy metals in organic fertilizers permitted by the Turkish regulation.

Heavy Metal	**Maximum Permittible Concentration (mg kg^{-1} dry matter)**
Cr	270
Cu	450
Pb	150
Ni	120
Zn	1100
Cd	3

The fresh poultry manure samples K-3 and K-4 were found to contain significantly higher amounts of OC compared to the stored manure samples ($p < 0.05$). Correspondingly, organic matter losses have been previously shown during storage of cattle and pig manure due to the decomposition of manure organic material during storage (Petersen et al., 1998). Unlike organic matter, nutrients such as P and K has been reported to be enriched during maturation of animal manure (Tiquia et al., 1998). Comparably, the stored poultry manure samples D-3, B-4, B-5, and B-6 were observed to have significantly higher P and Mg concentrations than the fresh ones ($p < 0.05$). pH, OC, total N and P, Ca, Mg, Zn, K, and Na contents of the animal manure samples were all found to be

significantly higher than those of the agricultural soils (for Ca and Mg: $p < 0.05$; for the others: $p < 0.01$). On the other hand, Fe concentration of the agricultural soils was significantly higher compared to that of the manure samples ($p < 0.01$). All these findings suggest that fate and behaviour of TCs, SAs, and FQs would considerably differ depending on the matrix investigated due to significant differences in physicochemical characteristics.

4.2. Extraction and Analysis of Tetracyclines, Sulfonamides, and Fluoroquinolones from Soil and Manure Matrices

4.2.1. Simultaneous Extraction and Analysis of Tetracyclines and Sulfonamides

In the present study, the extraction method developed by Blackwell et al. (2004b) was employed without any significant changes in the original procedure for the TCs and SAs. As a first step, soil and manure matrices were treated with an extraction solvent, a mixture of McIlvaine buffer, EDTA, and MeOH, to separate the TCs and SAs compounds from solid to liquid phase. Due to the ability of TCs to form chelate complexes with metal ions present in agricultural soil and animal manure which can affect the binding strength of TCs to these matrices (Thiele-Bruhn, 2003), the chelating agents EDTA and McIlvaine buffer were used in the extraction buffer to prevent the formation of such complexes.

McIlvaine buffer prepared in this study consisted of 0.2 M citric acid and 0.4 M Na_2HPO_4 instead of 0.1 M citric acid and 0.2 M Na_2HPO_4 which was the composition of McIlvaine buffer used by Blackwell et al. (2004b). The McIlvaine concentration used in the current study has also been employed for the extraction of OTC and SCP from soil (Kay et al., 2005b). Increasing TCs extraction from sediment has been shown with increasing citric acid concentration in McIlvaine buffer (Kim and Carlson, 2007b) and the use of higher concentration of McIlvaine buffer in the present study was due to the improvement in the extraction efficiencies of TCs. Being a polar solvent, MeOH was also added to the extraction buffer in order to improve the extraction efficiency of TCs and SAs which include several polar functional groups in their chemical structure.

The stabilities of the extraction buffers at pH 5 and 7 were evaluated, and in pH 5 extraction buffer, a precipitate formation was observed a short time after the preparation of

the buffer. Therefore, final pH of the extraction buffer was set as 7.2 at which no precipitation occurred. The selection of an extraction buffer with a relatively high pH (pH: 7.2) is also based on the fact that the sorption of TCs decreases with increasing pH (O'Connor and Aga, 2007). This pH was not further increased, since TCs are not stable in alkaline conditions (Sarmah et al., 2006).

Extraction was performed in three cycles in order to improve the extraction efficiency of particularly OTC and CTC which have the ability of strong binding to soil and manure matrices (Tolls, 2001). Vortex-mixing was used in order to provide a large contact surface area between the solid matrix and the extraction buffer used, and to re-suspend the soil and manure samples after the centrifugation steps. However, the vortexing time of 30 s was exceeded sometimes especially when the blank soil analyses were carried out with a soil weighing of 4 g which is somewhat hard to re-suspend. The present extraction method mainly depends on ultrasonic agitation of the samples. Ultrasonic extraction was selected as the extraction technique due to its advantages such as high recoveries for most compounds, short extraction time (Popp et al., 1997), low instrument cost and solvent consumption, its simplicity of use (Sun et al., 1998), and selectivity (Babić et al., 1998).

However, the use of MeOH resulted in the formation of highly coloured extracts (dark yellow for soil samples, and dark brown for manure samples) probably caused by dissolution of natural organic materials such as humic and fulvic substances as also reported by Blackwell et al. (2004b). Therefore, a further SPE clean-up step was needed to eliminate the humic substances. Since particularly poultry manure samples analyzed in this study contained high OC, SAX cartridges were employed in tandem with HLB cartridges to prevent the contamination and overload of the solid phase of the HLB cartridges. TCs and SAs have been documented to be more efficiently extracted during SAX-HLB SPE when the sample pH was below 3 due to the prevalence of positively charged or neutral species which can be easily separated from co-eluting matrix components (Diaz-Cruz and Barcelo, 2006). Therefore, the diluted extracts prior to SPE were acidified to a pH of 2.90 ± 0.04 by the addition of H_3PO_4 before loading onto the cartridges. During SPE clean-up of the manure extracts, capacity of the SAX cartridges were generally exceeded, and this resulted in clogging of the cartridges. In these cases, a second SAX cartridge was used to remove the remaining humic substances in the extract.

In the chromatographic separation and quantification of antimicrobial compounds, peak tailings and high standard deviations between the recovery measurements are the main problems arising when conventional octadecyl silica (ODS) columns are used (Zhu et al., 2001). In the present study, YMC Pack ODS-AQ column was selected as the stationary phase in the present study due to its ability to reduce the activity of acidic unreacted silanol groups which can cause the mentioned problems. To improve retention of neutral and cationic TCs and SAs, and thus separation of these analytes in the HPLC column, formic acid was added into the mobile phases ACN and water.

Prior to HPLC analysis of the analytes, the column was conditioned with 100 % ACN, and 0.1 % formic acid in Milli-Q water and ACN for 20 min by using a gradient elution. This step was useful in terms of the elimination of metal ions, some traces of analytes, and other impurities which are likely to sorb onto the column. After the column was conditioned, the mobile phases were passed through the column at a constant flow rate of 0.7 ml min^{-1} for approximately 30 min in order to achieve a flat baseline. Before starting the analysis, pure MeOH was injected into the column to ensure the absence of TCs and SAs on the column in each case, and no peaks were detected indicating that there were not any carry-over from the LC column, and the washing steps were efficient in the elimination of all the impurities.

OTC which is more polar than CTC and the more polar SAs such as SDZ and STZ possessed very rapid elution times. Therefore, providing the high initial aqueous percentage in the mobile phase, an initial mobile phase composition of 0.1 % formic acid in Milli-Q water (95 %): 0.1 % formic acid in ACN (5 %) resulted in good chromatographic peak resolution. The mobile phase composition, the gradient elution, LC column type, and the flow rate allowed for all of TCs and SAs to be analysed in a short time as 10 min and to be efficiently separated. A sample chromatogram representing all the TCs and SAs in a mixed standard solution which contains all the antibiotics investigated at a concentration of 5 μg ml^{-1} is given in Figure 4.1.

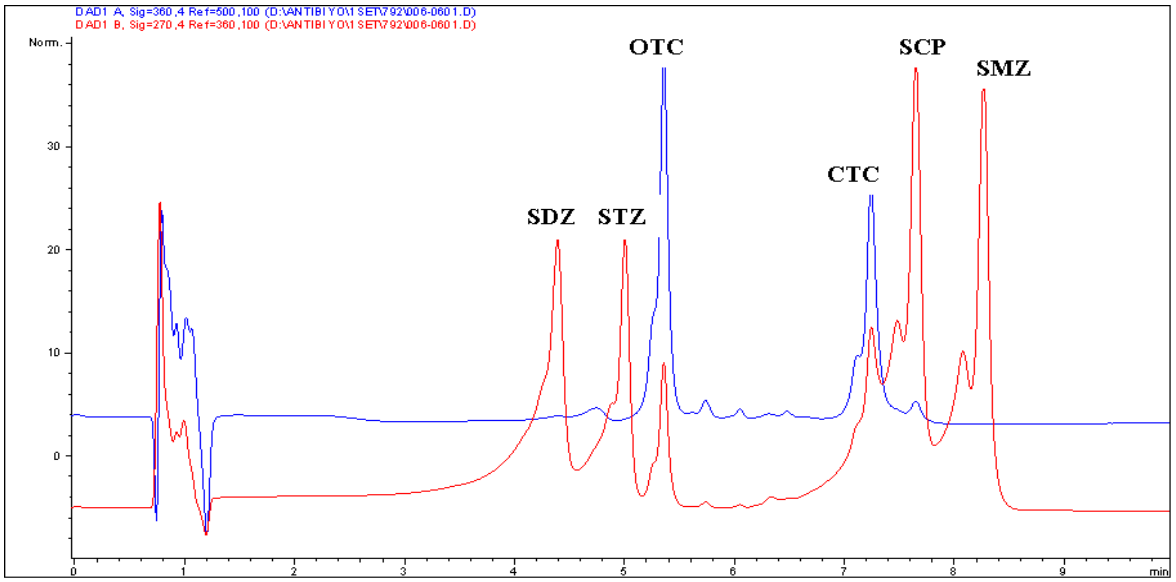


Figure 4.1. The chromatograms of TCs and SAs in a standard mixture at the concentration of 5 µg ml^{-1}. Blue and red peaks refer to the groups of TCs and SAs, respectively.

No significant numbers of co-eluting peaks originating from the soil and manure matrices occurred at 360 nm at which the TCs compounds were analyzed. However, for the SAs analysis at 270 nm, a number of interfering peaks were observable because most dissolved natural organic compounds in the composition of soil and manure extracts which have been retained on HLB cartridges and eluted with MeOH strongly absorbed UV-light at this wavelength and this situation sometimes resulted in “drifts” in the chromatograms.

Integration for quantification of the analyte concentrations was performed manually drawing peak baselines and then integrating. Calibration curves were linear within the concentration range 0.05-5 µg ml^{-1} and good correlations above 0.998 were achieved for all of TCs and SAs investigated in the present study. RSDs for retention times, slopes of the resulting calibration curves, and peak areas in the standard mixtures at the concentration of 5 mg l^{-1} were determined after replicate measurements made on the same or different days, and satisfactory RSDs below 15 % were achieved. Some calibration parameters, and between- and within-day RSDs of several parameters involving calibration are shown in Table 4.9. Between- and within-day RSDs of the retention times, slopes, and peak areas at 5 mg l^{-1} given in the table are represented as the mean values of three replicate

measurements. Within-day RSDs of the retention times are presented as the mean values of six replicate measurements.

Table 4.9. Calibration parameters, and between- and within-day RSDs of some calibration parameters for the TCs and SAs investigated in the present study.

	Compound					
	OTC	**CTC**	**SDZ**	**STZ**	**SCP**	**SMZ**
$\lambda_{detection}$ (nm)	360	360	270	270	270	270
Retention Time (min)	5.40	7.30	4.42	5.04	7.70	8.33
Slope	23.48	19.01	35.84	17.28	32.41	37.82
R^2	0.9994	0.9993	0.9983	0.9999	0.9997	0.9996
Peak Area (5 mg l^{-1})	116.72	93.22	179.39	86.44	161.71	185.22
Between-Day RSDs (%) of the Retention Times	1.05	1.04	0.76	0.80	1.04	1.25
Within-Day RSDs (%) of the Retention Times	0.16	0.14	0.20	0.18	0.13	0.15
Between-Day RSDs (%) of the Slopes	6.02	5.13	4.87	6.19	14.63	5.22
Within-Day RSDs (%) of the Peak Areas (5 mg l^{-1})	6.69	4.85	4.41	6.57	14.22	4.12

To determine the LODs for both soil and manure, the calculation method based on the S/N ratio > 3 was used in this study (Hamscher et al., 2002; Jacobsen and Halling-Sørensen, 2006). In this method, calibration curves based on heights of the analyte peaks instead of the peak areas were reconstructed, and noises on blank soil and manure chromatograms were scanned in a time range close to emergence time of the analyte peaks. Signals corresponding to 3 times the noise were determined, and minimum concentrations of the analytes than can be measured were calculated from the Height-Concentration calibration curve. Sandy loam soil D-2, clay loam soil B-2, cattle manure D-4, and poultry manure D-3 was chosen as the representatives of the samples analyzed, and used for the LOD determination. The LOD values of the TCs and SAs compounds analyzed are given in Table 4.10 as the mean of triplicates and standard deviations. The LODs obtained in the present study clearly demonstrated that clay loam soils and poultry manures had higher

LODs than the sandy loam soils and cattle manure. This finding may be attributed to that increasing clay and OC content resulted in higher background noise in the chromatograms, and as a result of this, minimum detectable amounts of the analytes increased. LOD values obtained in the current study for TCs and SAs in agricultural soil and animal manure were in the range in which TCs and SAs may be expected to occur in the environmental samples of soil and manure (Hamscher et al., 2005; Haller et al., 2002). The sufficiently low LODs in complex solid matrices such as soil and manure with varying characteristics refer to the suitability of the method for the application to environmental samples.

Table 4.10. The LODs for soil and manure samples having different clay and OC contents.

	OTC (mg kg^{-1})	**CTC (mg kg^{-1})**	**SDZ (mg kg^{-1})**	**STZ (mg kg^{-1})**	**SCP (mg kg^{-1})**	**SMZ (mg kg^{-1})**
Soil Type						
Sandy loam	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
Clay loam	0.04 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.05 ± 0.00	0.05 ± 0.00
Manure Type						
Cattle	0.13 ± 0.01	0.16 ± 0.02	0.10 ± 0.00	0.10 ± 0.00	0.09 ± 0.03	0.09 ± 0.03
Poultry	0.57 ± 0.09	3.67 ± 0.34	0.65 ± 0.04	0.70 ± 0.04	0.97 ± 0.04	0.89 ± 0.04

4.2.2. Extraction and Analysis of Fluoroquinolones

The extraction method developed by Turiel et al. (2006) and used in the current study with some modifications to extract ENR and its metabolite CF from soil and manure is based on the formation of FQs-Mg^{2+} complexes. Mg^{2+} ions were selected as the complexing agent due to their higher efficiency to desorb FQs from soil than Ca^{2+} and Al^{3+} ions. The extraction with Mg^{2+} ions made the desorption and quantitative extraction of FQs in a single step possible which is impossible using a conventional organic solvent (Andreu et al., 2007). FQs extraction was performed under alkaline conditions (pH 8) due to their completely negative charge which provides minimum interaction with, thus maximum desorption from negatively charged soil sites and due to their improved water solubilities (Golet et al., 2002). However, pH of the $Mg(NO_3)_2$ was not further increased due to the precipitation of Mg^{2+} ions in the form of $Mg(OH)_2$ at higher pH values than used in the present study. Extraction was carried out using the pure water solutions of $Mg(NO_3)_2$

instead of a combination of an organic solvent such as MeOH and water due to the dissolution of more unwanted matrix components when using MeOH. The extraction volume chosen was 8 ml, since the lower volume of the extraction solution (4 ml) was observed to result in the lower CF and ENR recoveries from soil. The positive influence of the increasing volume of $Mg(NO_3)_2$ solution on the extraction efficiencies of quinolones and FQs from soil has also been shown by Turiel et al. (2006).

Vortex-mixing for 30 s was added to the extraction procedure to provide a larger contact surface area between the extraction solution and solid matrices of soil and manure. Centrifugation at 7000 rpm for 10 min was also added to the extraction procedure to settle down the suspended solids and to provide a clear supernatant liquid for the HPLC analysis. However, the resulting extracts, especially those of manure samples, still contained some suspended solids which may further accumulate in HPLC column, and in turn cause interferences in the analysis. For this reason, extracts were finally passed through 0.45 μm filters to remove the remaining suspended particles.

Extraction efficiencies from soil spiked with CF and ENR at the concentration of 1.0 mg kg^{-1} were also tested using rotary evaporator at a maximum speed of 150 rpm and at a maximum temperature of 50ºC. Because 8 ml of extraction solution contained more $Mg(NO_3)_2$ than 4 ml solution, $Mg(NO_3)_2$ crystallized during evaporation due to over-saturation of the former one. Hence, approximately 1 ml of the sample obtained after the evaporation of 4 ml extract was injected into the HPLC column, and the recovery was compared to that of the sample obtained after the extraction of 4 ml sample without evaporation. It was found that recovery of CF and ENR from soil achieved without the evaporation step was higher than that of the sample evaporated to approximately 1 ml, while more peaks belonging to soil matrix components were visible in the chromatogram of evaporated sample due to sample concentration. The lower extraction efficiency obtained for the evaporated sample was likely due to the losses of FQs which might have been caused by the possible sorption to glass during sample evaporation (Golet et al., 2002) and dissociation of Mg^{2+} - FQ complexes at the high temperature in the rotary evaporator.

The sample volume to be injected into the HPLC column was set at 20 µl since higher injection volumes did not allow the quantification for FLD detector. The same chromatographic conditions as applied for the TCs and SAs investigated provided an excellent baseline separation, peak shapes and resolutions for the two FQs. A sample FLD chromatogram of a standard mixture involving CF and ENR at the concentration of 1.0 µg ml^{-1} is represented in Figure 4.2.

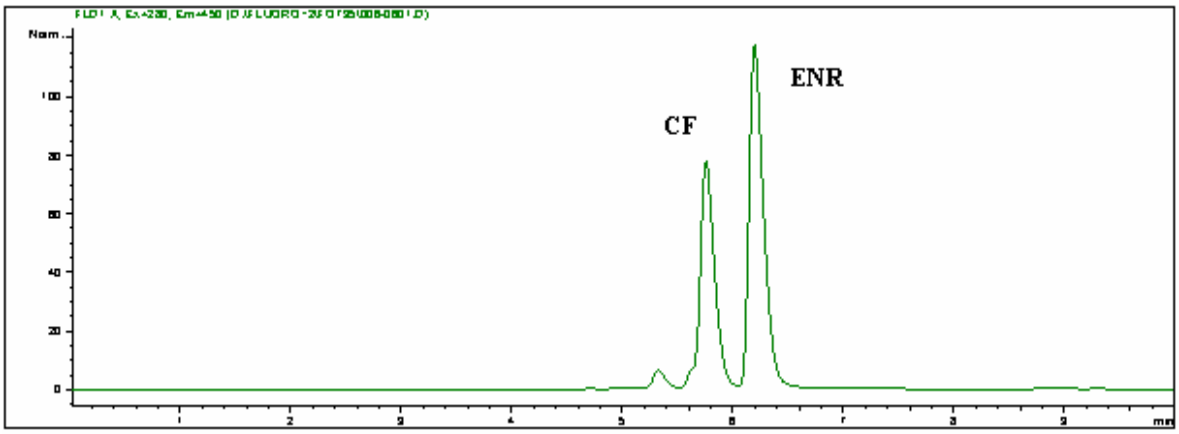


Figure 4.2. The FLD chromatogram of a standard mixture containing CF and ENR at the concentration level of 1.0 µg ml^{-1}.

A cattle manure sample which does not contain any antimicrobial residuals was extracted and analyzed using the same procedure to ensure the absence of any co-eluting matrix components at the same retention time as CF and ENR. Although there were not any peaks at the same retention time as the ENR peak, a small peak was observed at the excitation wavelength of 280 nm close to the retention time of CF when HPLC analysis was performed with FLD. This peak was present in all of the soil and manure chromatograms throughout the chromatographic run. In addition, some unknown peaks around the retention time of ENR were visible for some of the poultry manure samples. Therefore, to increase the selectivity of the analysis, resulting chromatograms were quantitatively evaluated using DAD at the wavelength of 280 nm, while fluoroscence detector was employed to confirm the positive results.

Standard solutions were prepared in the extraction solution and at the pH value of approximately 8, since a different solvent matrix and pH could significantly affect the

signal intensities of the Mg^{2+}-FQs complexes and retention times of the analytes. Calibration curves constructed for CF and ENR were linear over the concentration range of 0.01-1 μg ml^{-1}. RSDs between the retention times obtained from six replicate measurements performed within the same day, were found to be less than 0.25 % for both DAD and FLD detection. Some calibration parameters are shown in Table 4.12 for both DAD and FLD detection.

Table 4.11. Calibration parameters for ciprofloxacin and enrofloxacin when using diode array and fluoroscence detection.

			DAD		
Compound	**$\lambda_{detection}$ (nm)**	**RT (min)**	**Slope**	**R^2**	**Peak Area at 1 mg l^{-1} (mAU·s)**
CF	280	5.74	221.18	0.99999	221.11
ENR	280	6.17	198.94	0.99995	198.62
			FLD		
	$\lambda_{excitation}/\lambda_{emission}$ (nm)	**RT (min)**	**Slope**	**R^2**	**Peak Area at 1 mg l^{-1} (LU·s)**
CF	280/450	5.78	608.47	0.99959	606.41
ENR	280/450	6.22	1020.83	0.99931	1017.60

RT: Retention time, mAU: Milliabsorbance units, LU: Luminescence units.

LODs for CF and ENR in soil and manure samples were calculated using S/N method which has also been used for LOD determination of the TCs and SAs analyzed. LODs for CF and ENR in soil were estimated to be 13 and 5.44 μg kg^{-1}, respectively, while LODs in manure was found to be 0.21 and 0.31 mg kg^{-1} for CF and ENR, respectively. Despite the high clay content of soil samples, LODs obtained in the present study were lower than the values reported by Turiel et al. (2006; 50 and 40 μg kg^{-1} for CF and ENR, respectively), Morales-Muñoz et al. (2004; 150 μg kg^{-1} for CF), and Golet et al. (2002; 50 μg kg^{-1} for CF) for soil. The sufficiently low LODs obtained for both soil and manure in the current study can refer to high sensitivity of the method and suitability for the scanning of ENR and its metabolite CF in complex matrices such as agricultural soil and animal manure.

4.3. Occurrence of Tetracyclines, Sulfonamides, and Fluoroquinolones in Manure and Agricultural Soil

4.3.1. Occurrence of Tetracyclines, Sulfonamides, and Fluoroquinolones in Manure

The manure samples collected from the heaps adjacent to agricultural fields or taken from individual locations were extracted and analysed in an effort to detect and quantify the TCs, SAs, and FQs antimicrobial compounds in manure matrices. The information of antimicrobial use for individual farms could not be collected as part of this study. The results of positive and negative samples are represented in Table 4.12 for the TCs, SAs, and FQs as the mean of triplicate samples, and RSDs (%) in the parantheses.

Table 4.12. Identification and quantification results of TCs, SAs, and FQs in animal manure.

	Antimicrobial Compound					
Sample ID	**OTC (mg kg^{-1})**	**CTC (mg kg^{-1})**	**SDZ (mg kg^{-1})**	**STZ (mg kg^{-1})**	**SCP (mg kg^{-1})**	**SMZ (mg kg^{-1})**
D-3	< LOD	0.32 (2.35)	< LOD	< LOD	NQ	0.10 (12.69)
D-4	0.06 (3.22)	< LOD	< LOD	< LOD	< LOD	< LOD
M-3	0.20 (1.03)	< LOD	< LOD	< LOD	< LOD	< LOD
B-4	0.33 (0.62)	0.39 (2.05)	< LOD	< LOD	< LOD	2.08 (2.92)
B-5	0.27 (4.62)	0.24 (1.23)	< LOD	< LOD	0.22 (0.49)	2.63 (2.13)
B-6	0.23 (2.61)	0.25 (1.34)	< LOD	< LOD	< LOD	< LOD
K-3	< LOD	< LOD	1.43 (2.71)	3.71 (0.23)	35.5 (1.21)	< LOD
K-4	0.47 (9.13)	< LOD	< LOD	5.51 (2.86)	10.3 (1.87)	3.76 (16.55)

Sample ID	**CF (mg kg^{-1})**	**ENR (mg kg^{-1})**
B-4	< LOD	< LOD
B-5	< LOD	< LOD
B-6	NQ	< LOD
K-3	< LOD	0.06 (3.06)
K-4	< LOD	NQ

NQ: Detected but not quantified due to the smaller value of the peak area than the calibration curve intercept.

The most frequently detected antimicrobial compound in animal manure was OTC, while the SDZ and CF were the least frequently detected ones. The lowest antimicrobial concentration was detected in the cattle manure D-4 and this manure sample was found to contain only OTC residues. The UV chromatogram of the sample D-4 is shown in Figure 4.3. The mixed manure sample M-3 was also observed to contain only OTC, but at the higher concentration than D-4. On the other hand, the highest antimicrobial concentrations were in general detected in the fresh poultry manure samples K-3 and K-4. The most frequent occurrence of the antimicrobial compounds was also observed in K-4. This sample was found to contain at least a member of the three antimicrobial classes.

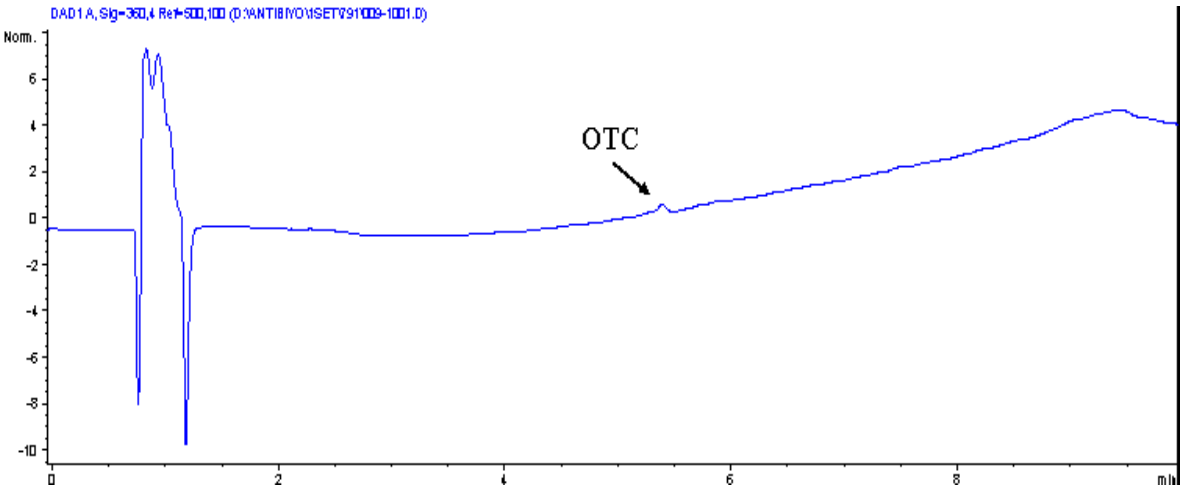


Figure 4.3. The chromatogram of the cattle manure sample D-4 containing OTC at the concentration of 0.06 mg kg^{-1}.

TCs class of antimicrobial compounds was commonly detected in the animal manure samples analyzed, indicating that these compounds have been fed to the animals. Although TCs can also be excreted in faeces and urine in the form of their 4-epimers (Brambilla et al., 2007), isomerized at basic pH which is the condition generally observed in animal manure, and photodegraded by UV-light (Eichhorn and Aga, 2004), these transformation products were not be analyzed in the present study. However, influence of the latter on the degradation of TCs can be neglected for this study, since the light can penetrate through only a few millimeters of the manure heaps.

In the present study, higher OTC concentrations were detected in the manure samples with higher OC, total N and Na content ($p < 0.05$) and CTC was detected only in the poultry manure samples. The less frequent detection of CTC compared to OTC may be due to the possibility that CTC could have been less frequently fed to animals and degraded completely or to levels below LOD into other compounds such as isochlortetracycline that were not targeted in the present study. The concentrations of the TCs compounds in animal manure samples measured in the present study were similar to those reported in literature (Martinez-Carballo et al., 2007; Jacobsen and Halling- Sørensen, 2006). On the other hand, even higher concentrations up to 136 mg kg^{-1} have also been reported for OTC in pig manure (Winckler et al., 2003).

SAs were detected in the animal manure samples at significantly higher concentrations compared to TCs ($p < 0.05$). SCP concentration was the highest one measured for all of the eight antimicrobial compounds and was also detected in the freshly taken poultry manure sample K-3. Similarly, the maximum SMZ concentration was detected in the fresh manure sample K-4 from breeder chickens. On the other hand, relatively small amounts of these two SAs compounds in the poultry manure sample D-3 suggest that a significant portion of these compounds could have been degraded or eliminated during storage in manure heaps. Although it is known that SMZ is mainly available for human use in most countries (Christian et al., 2003), it is also applied to farm animals in some countries such as Austria with its synergist trimethoprim (Martinez-Carballo et al., 2007), and for the same reason, it was detected in the poultry manure samples D-3, B-4, B-5 and K-4 in the present study. SCP concentrations were observed to decrease with increasing Fe content ($p < 0.01$) and pH ($p < 0.05$) of the poultry manure samples, while SMZ concentrations measured in the samples were found to decrease with increasing P and Ca content ($p < 0.05$).

The other two SAs compounds, namely SDZ and STZ, were less frequently detected than SCP and SMZ, and were only measured in the fresh poultry manure samples K-3 and K-4 in the concentration range of 1.43-5.51 mg kg^{-1}. These concentration values were comparable with those reported in literature (Haller et al., 2002; Hamscher et al., 2005; Jacobsen and Halling-Sørensen, 2006).

The FQs compounds ENR and CF were not detectable or quantifiable in most of the poultry manure samples. Only the fresh poultry manure sample K-3 was found to contain ENR residues at the concentration of 0.06 mg kg^{-1}. The FLD chromatogram of the poultry manure sample K-3 is given in Figure 4.4. This FQs compound has been shown to be present in chicken manure at the maximum concentration of 2.8 mg kg^{-1} (Martinez-Carballo et al., 2007), which was much higher than reported in the current study. In this study, CF could be detected just in the poultry manure sample B-6, but could not be quantified. Occurrence of CF traces in the sample B-6 was attributed to transformation of ENR into this compound during storage, since ENR is known to be transformed to CF by deethylation during the storage of animal manure (Andreu et al., 2007). On the other hand, freshly taken poultry manure samples K-3 and K-4 contained only the parent compound ENR, indicating that no significant degradation of this compound occurred without long-term storage.

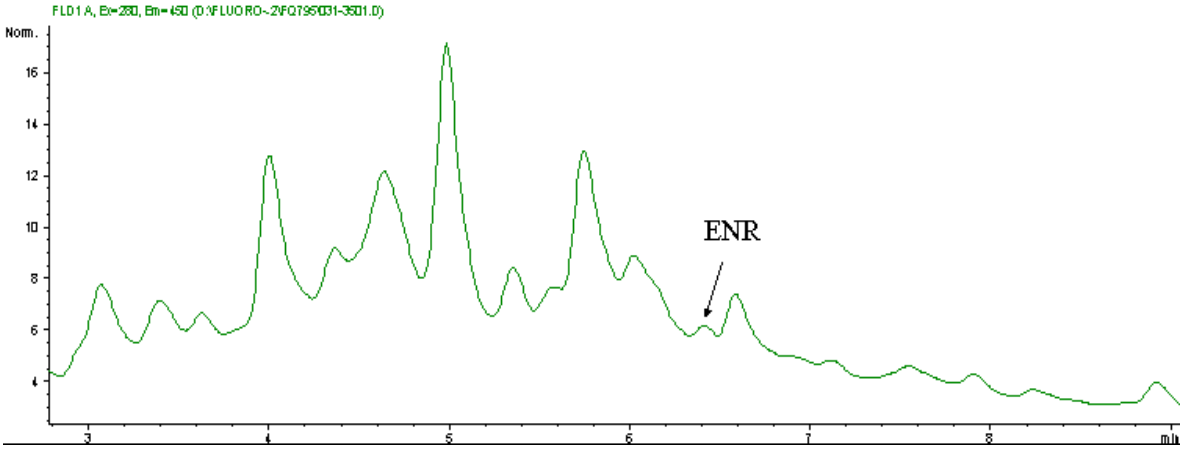


Figure 4.4. FLD chromatogram of the poultry manure sample K-3 containing 0.06 mg kg^{-1} ENR.

It can also be concluded from this study that more frequent detection of TCs and SAs in the samples from Kocaeli compared to Tekirdağ and Kırklareli refers to more common administration of antibiotics in this region. Frequent occurrence of veterinary antibiotics in the manure samples particularly taken from Kocaeli may pose a significant risk in terms of ecotoxicity and public health. In one study, minimum inhibition concentrations of SAs (e.g., for *E. Coli*) has been showed to be typically below 1 mg kg^{-1} (Neuman, 1981), and it is visible that SAs concentrations measured in some of the manure samples investigated

(e.g., K-3 and K-4) may inhibit certain kinds of microorganisms. Furthermore, it is known that even low concentrations may present optimal conditions for the development of antibiotic resistance (Kümmerer, 2003).

4.3.2. Occurrence of Tetracyclines, Sulfonamides, and Fluoroquinolones in Agricultural Soils

The results of positive and negative agricultural soil samples in terms of the presence of TCs, SAs, and FQs residues are shown in Table 4.13. The antimicrobial concentrations given in the table show the mean of triplicate samples, and RSDs (%) were represented in the parantheses.

Table 4.13. Identification and quantification results of TCs, SAs, and FQs in agricultural soils.

	Antimicrobial Compound					
Sample ID	**OTC ($mg\ kg^{-1}$)**	**CTC ($mg\ kg^{-1}$)**	**SDZ ($mg\ kg^{-1}$)**	**STZ ($mg\ kg^{-1}$)**	**SCP ($mg\ kg^{-1}$)**	**SMZ ($mg\ kg^{-1}$)**
D-1	0.50 (15.93)	< LOD	< LOD	< LOD	< LOD	NQ
D-2	0.01 (3.40)	< LOD	< LOD	0.04 (0.04)	< LOD	NQ
M-1	0.03 (8.05)	< LOD	< LOD	0.05 (4.70)	< LOD	NQ
M-2	0.02 (6.56)	< LOD	< LOD	< LOD	< LOD	< LOD
B-1	0.23 (42.97)	< LOD	< LOD	< LOD	0.11 (4.55)	0.04 (20.75)
B-2	0.14 (11.28)	0.06 (0.55)	< LOD	< LOD	0.04 (6.11)	0.10 (4.43)
B-3	0.13 (11.12)	0.06 (0.60)	< LOD	< LOD	0.04 (6.51)	0.07 (3.77)
K-1	0.11 (1.11)	< LOD	< LOD	0.40 (1.29)	< LOD	< LOD
K-2	0.14 (2.03)	0.10 (10.60)	< LOD	< LOD	< LOD	0.11 (21.62)

Sample ID	**CF ($mg\ kg^{-1}$)**	**ENR ($mg\ kg^{-1}$)**
B-1	< LOD	< LOD
B-2	< LOD	0.05 (15.77)
B-3	< LOD	< LOD
K-1	< LOD	< LOD
K-2	< LOD	0.02 (27.93)

OTC was detected in all of the agricultural soil samples, while SDZ and CF could not be detected in any agricultural soil samples investigated. The lowest measured antimicrobial concentration was recorded for the field D-2 fertilized with the cattle manure D-4 which has been demonstrated to contain the minimum OTC concentration among all the animal manure samples analyzed. On the other hand, no significant difference was observed between the agricultural soils fertilized with cattle and poultry manure. The most frequent detection of the antimicrobial compounds was observed in the agricultural soil sample B-2 fertilized with poultry manure. This sample was found to contain at least one member of the three antimicrobial classes investigated.

Similar to the results obtained for the animal manure samples studied, TCs compounds were frequently detected in the agricultural soils, indicating that their transfer from manure to soil was responsible for their occurrence in these soils. The UV-chromatogram of the agricultural soil sample D-1 which was found to contain maximum OTC concentration obtained in the present study, is shown in Figure 4.5. OTC concentrations in the range of 0.13-0.22 mg kg^{-1} in arable land which are comparable with the current OTC concentrations detected in the agricultural soils, have been recently demonstrated to be insufficient for the contamination of main seeds (Brambilla et al., 2007). However, the relatively high concentrations of OTC in agricultural soils in the present study may pose a significant risk for the environment. Furthermore, insignificant difference between the OTC concentrations in manure and soil can indicate the accumulation potential of this compound in fields due to its negligible mobility in soils (Rooklidge, 2004).

CTC concentrations measured in the agricultural soils were found to increase with increasing moisture content observed in the fields ($p < 0.01$). Despite their structural similarities, CTC was less frequently detected in agricultural soils compared to OTC in parallel with their detection frequencies in animal manure samples. Although, to the current knowledge, no previous studies have been conducted to compare the degradation rates of OTC and CTC in manure and soil, the difference in their occurrence and concentrations in the studied agricultural soils is supposed to be caused by the difference in their stability in manure and soil. This hypothesis is supported by a research in which CTC could not be detected in soil from a field amended with swine waste treatment byproducts

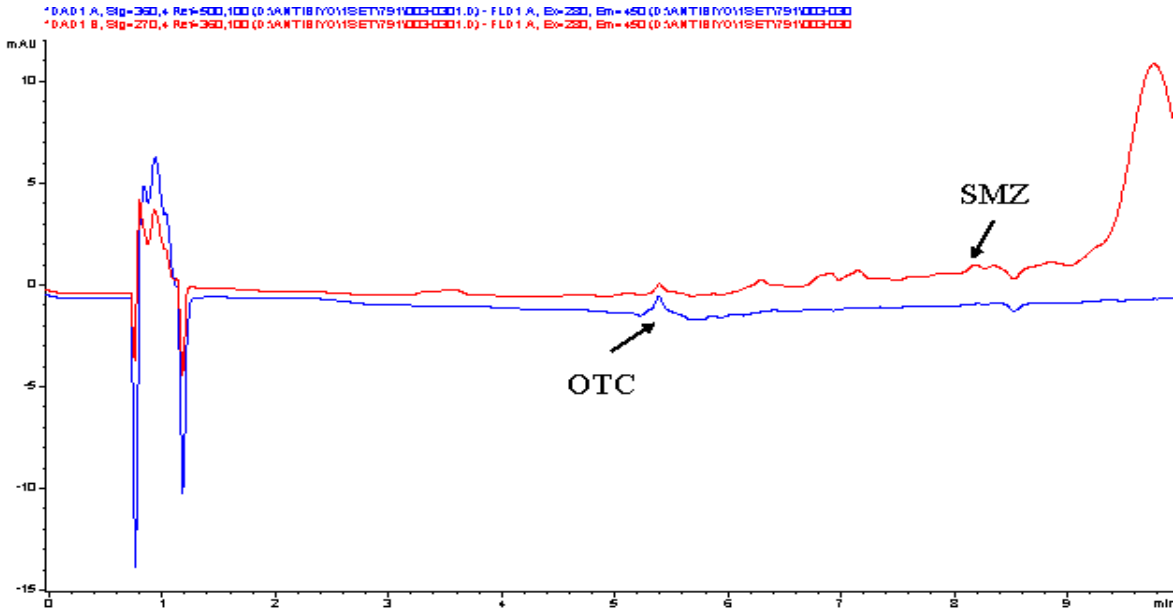


Figure 4.5. The chromatogram of the agricultural soil sample D-1.

despite its routine use, while OTC was detected in soil samples at the concentration of 0.25 mg kg^{-1} (Zilles et al., 2005).

Although SAs are known to be highly mobile in terrestrial environment and to have a high tendency to be transported into surface waters via run-off and leach through the soil column into groundwater, findings obtained in the present study demonstrate that this antimicrobial class of compounds can occur in agricultural soils amended with animal manure at high concentrations up to 0.40 mg kg^{-1}. However, insignificant difference between the concentrations of TCs and SAs in these agricultural soils suggests that the mobility of SAs result in a decrease in their concentrations in soil, since SAs have been found to be present at significantly higher concentrations than TCs in the animal manure samples investigated in the current study. The detection frequencies of SDZ and SMZ in soil can be explained by the detection frequencies of these SAs compounds in the animal manure samples investigated in this study. The detection of SCP only in the agricultural fields fertilized with poultry manure may indicate that poultry manure can be responsible for the most of SCP pollution in agricultural soils. Correspondingly, this compound has been only found in poultry manure samples in this study. STZ concentrations measured in the agricultural soils were found to decrease with increasing soil pH ($p < 0.01$).

The FQs compound ENR was found to be present in the agricultural soil samples B-2 and K-2 at the concentrations of 0.05 and 0.02 mg kg^{-1}, respectively. The FLD chromatogram of B-2 is given in Figure 4.6. The concentration values of ENR measured in the agricultural soils B-2 and K-2 indicate the high stability of this compound in soil especially when it is considered that these two agricultural soils were fertilized seven months before the sampling.

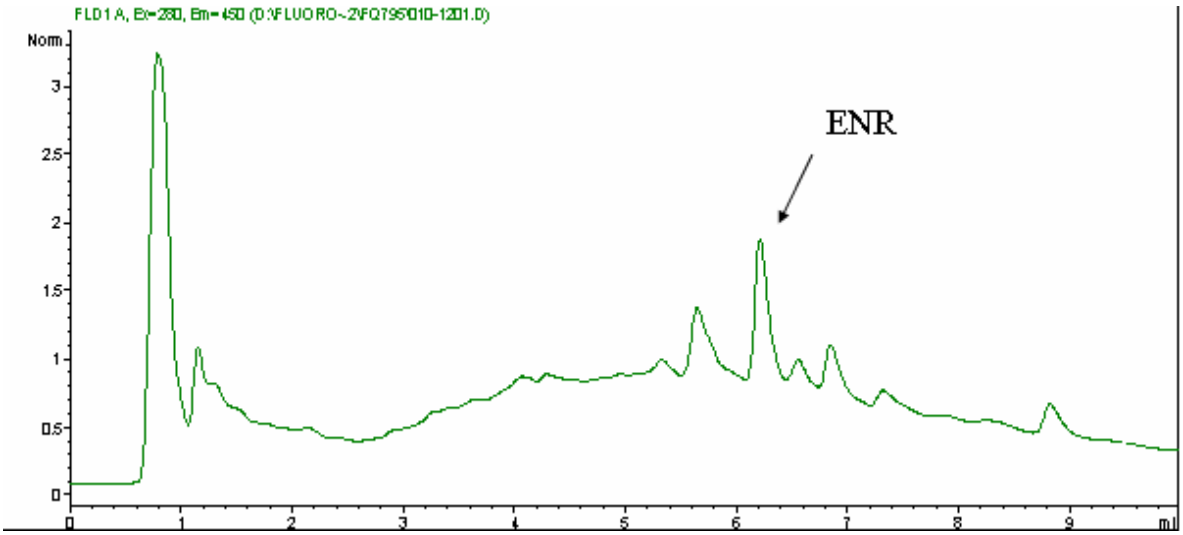


Figure 4.6. FLD chromatogram of the clay loam agricultural soil sample B-2 containing 0.05 mg kg^{-1} of ENR.

In June 2001, the Steering Comittee of the Veterinary International Comittee on Harmonization (VICH) set the trigger value of 0.10 mg kg^{-1} for antibiotic concentrations in soil based on their ecotoxic effects on a range of organisms. Antibiotic concentrations measured in the present study were evaluated with respect to this value and the results are shown in Table 4.14. Since antimicrobial concentrations measured in this study were found to exceed the trigger value of 0.10 mg kg^{-1} in the agricultural soil samples D-1, B-1, B-2, B-3, K-1 and K-2, some toxic effects on soil communities can be expected. Particularly, immediate attention should be paid for the occurrence of OTC in soil, since this compound was found to be present in all of the six mentioned soils above the concentrations of 0.10 mg kg^{-1}. Furthermore, it should not be ruled out that antibiotic resistance can occur even if the measured concentrations of antimicrobial compounds in soil fall below the trigger value of 0.10 mg kg^{-1} for risk assessment (Hamscher et al., 2002). On the other hand, the

soil subsamples used for the preparation of the composite samples were generally collected from the points which had higher manure intake relative to the other points in agricultural fields. The soil lying under dried liquid manure aggregates has been previously shown to contain high antibiotic concentrations due to persistence of these aggregates undamaged after incorporation into the soil (Hamscher et al., 2002). Therefore, antibiotic concentrations measured in the present study may describe a worst case scenario for the agricultural soils.

Table 4.14. The agricultural soil samples exceeding the trigger value of 0.10 mg kg^{-1} soil set by VICH.

Antimicrobial Compound	Sample
OTC	D-1, B-1, B-2, B-3, K-1, K-2
CTC	-
SDZ	-
STZ	K-1
SCP	B-1
SMZ	K-2
CF	-
ENR	-

4.4. Recoveries of tetracyclines, sulfonamides and fluoroquinolones from soil and manure

For recovery studies, fortification levels of 0.2 and 1.0 mg kg^{-1} were used both for soil and manure samples collected from different fields, since in most field studies, TCs, SAs, and FQs compounds have been detected in soil and manure at this concentration range (Hamscher et al., 2002; Sengeløv et al., 2003; Golet et al., 2002). The recovery rates obtained for the three groups of antimicrobial compounds are separately discussed in the following sections in detail.

4.4.1. Tetracyclines recovery from soil and manure matrices

The recovery rates of TCs in soil and manure collected from different agricultural areas are shown in Table 4.15 as the mean of triplicate samples for both spiking level together with the RSDs (%).

Table 4.15. The recovery rates of OTC and CTC in soil and manure.

	Agricultural Soil							
	OTC				**CTC**			
	Spiking Level							
	0.20 mg kg^{-1}		**1.00 mg kg^{-1}**		**0.20 mg kg^{-1}**		**1.00 mg kg^{-1}**	
Sample ID	**R (%)**	**RSD (%)**	**R (%)**	**RSD (%)**	**R (%)**	**RSD (%)**	**R (%)**	**RSD (%)**
D-1	89.56	25.18	82.64	4.48	82.04	24.19	92.91	6.13
D-2	88.12	1.19	84.36	3.51	41.73	7.26	79.76	16.92
M-1	107.44	5.34	95.94	5.16	70.25	27.81	86.59	6.09
M-2	92.69	16.29	90.35	8.82	100.85	8.87	97.00	18.08
B-1	93.33	6.57	78.01	7.83	34.94	5.79	68.73	25.02
B-2	93.27	1.46	71.99	3.45	51.80	6.76	69.20	4.36
B-3	89.76	12.17	65.07	8.04	51.62	18.30	58.61	18.18
K-1	67.89	25.77	64.95	11.23	34.22	6.30	66.52	13.74
K-2	54.71	9.78	86.46	4.00	68.31	3.28	66.28	18.87
	Animal Manure							
D-3	93.66	7.95	97.51	1.30	30.61	12.65	88.28	7.77
D-4	107.43	7.38	87.29	5.86	87.43	18.46	73.41	10.94
M-3	90.64	7.76	59.49	21.30	63.31	24.92	10.81	26.12
B-4	81.88	8.69	73.97	14.74	43.27	44.04	40.34	26.30
B-5	33.62	3.23	70.77	0.20	31.15	11.10	86.19	0.09
B-6	54.30	1.90	66.51	0.35	68.96	24.53	80.67	7.95
K-3	75.59	0.36	95.55	1.29	86.07	5.73	72.20	6.13
K-4	41.43	0.45	66.94	8.65	83.72	10.19	77.86	7.42

R: Recovery.

The TCs recoveries achieved in the present study for agricultural soil and animal manure matrices were not significantly different from each other. Slightly higher values than 100 % observed in the agricultural soil samples M-1 and M-2 and the cattle manure D-4 were probably due to the co-eluting matrix components of soil and manure. Furthermore, the formation of TCs-metal complexes has been reported to lead to the recovery rates above 100 % (Scheborg et al., 2004). Hence, such an interaction between TCs and metals may also be the reason of the recoveries slightly higher than 100 %. The recovery of CTC from soil and manure was generally lower than the recovery of OTC from the same matrices. It can be seen that in general, the recovery of OTC was higher at lower concentration (0.2 mg kg^{-1}). However, for CTC, recovery increased generally with increasing concentration, especially in soil samples. The UV-chromatograms of TCs in the unspiked and spiked agricultural soil sample D-1 and cattle manure sample D-4 at the spiking levels of 0.2 and 1.0 mg kg^{-1} are given in Figure 4.7.

The OTC recoveries from soil achieved in the present study were significantly higher than those obtained by Blackwell et al. (2004b). Since the main difference in the two extraction method was the concentrations of citric acid and Na_2HPO_4 in the extraction buffer which were two times higher in the current study, the chelating agent concentration in the extraction of TCs from soil can be regarded as critical in terms of extraction efficiency. The recoveries of OTC from soil and manure achieved in the present study were comparable with those obtained in a recent study (Brambilla et al., 2007) in which a recovery range of 83-86 % in slurry and 85-87 % in soil was achieved for OTC. On the other hand, the higher variability between the recovery rates observed in the current study compared to the mentioned investigation was due to the evaluation of extraction efficiencies using the manure and soil samples with different physical and chemical characteristics in this study. CTC recoveries from soil obtained in the present study were similar to those achieved by Hamscher et al. (2002) who reported CTC recoveries from soil in the range of 57-76 %. However, CTC recoveries from manure reported by the same authors were substantially higher (94-127 %) than obtained in the current study likely due to the stronger sorption of CTC to solid manure particles than to the aqueous phase, as has also been documented by Loke et al. (2002).

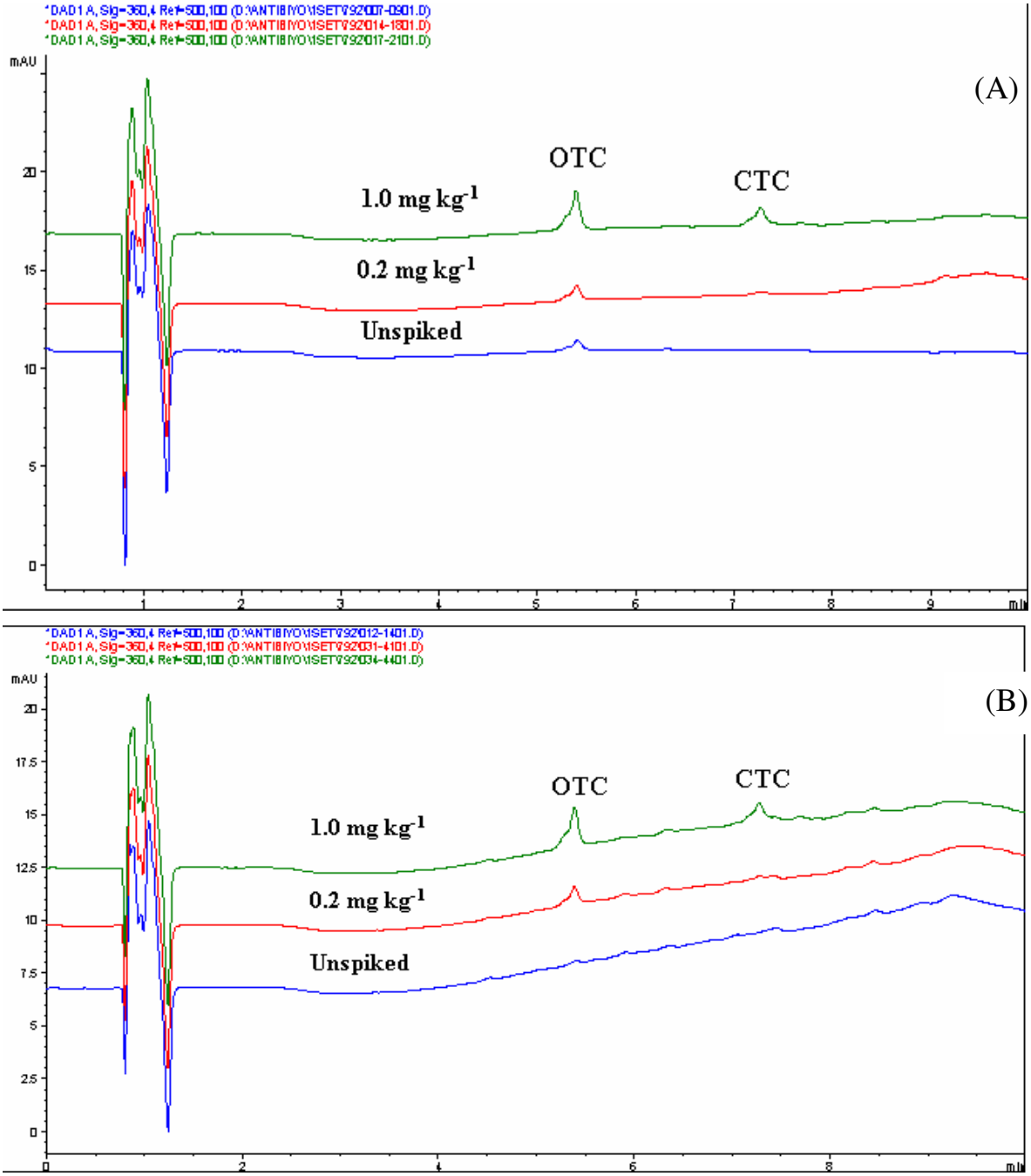


Figure 4.7. The UV-chromatograms of TCs in the unspiked and spiked (A) agricultural soil sample D-1 and (B) cattle manure sample D-4 at two spiking levels.

4.4.2. Sulfonamides recovery from soil and manure matrices

The recoveries of the SAs compounds SDZ, STZ, SCP, and SMZ from soil and manure matrices are shown in Table 4.16 as the mean of triplicate samples for both spiking level together with the RSDs (%). The UV-chromatograms of SAs in the unspiked and spiked agricultural soil sample D-1 and cattle manure sample D-4 at the spiking levels of 0.2 and 1.0 mg kg^{-1} are given in Figure 4.8.

RSDs of the recoveries were calculated to be generally smaller than 34 % for all the SAs compounds investigated indicating that there were no significant mismatches for complicated matrices such as manure and soil. However, RSDs of SAs recoveries from manure samples were generally found to be higher than observed in the soil samples. The difference between the RSDs observed in soil and manure samples were most pronounced for SCP for which RSDs of recoveries from manure were found to be significantly higher than those from soil ($p = 0.05$). This finding may indicate that especially co-eluting matrix components of the manure samples can have a significant influence on the reliability of HPLC-UV analysis of SAs compounds.

SAs recoveries were generally higher than those of TCs. This was in agreement with the fact that adsorption coefficients of the SAs are smaller than those of TCs (Thiele-Bruhn et al., 2004). SAs antimicrobial compounds have been previously shown to have higher sorption tendencies to pig manure than to a Chernozem soil likely due to the higher particulate organic matter content of manure (Thiele-Bruhn and Aust, 2004). In the present study, SAs recoveries from manure were generally lower in agreement with the results obtained by the mentioned authors. Recoveries of SAs obtained in the present study were generally concentration-dependant, i.e. recovery values increased with increasing spiking concentration in most cases with the exception of a few manure samples.

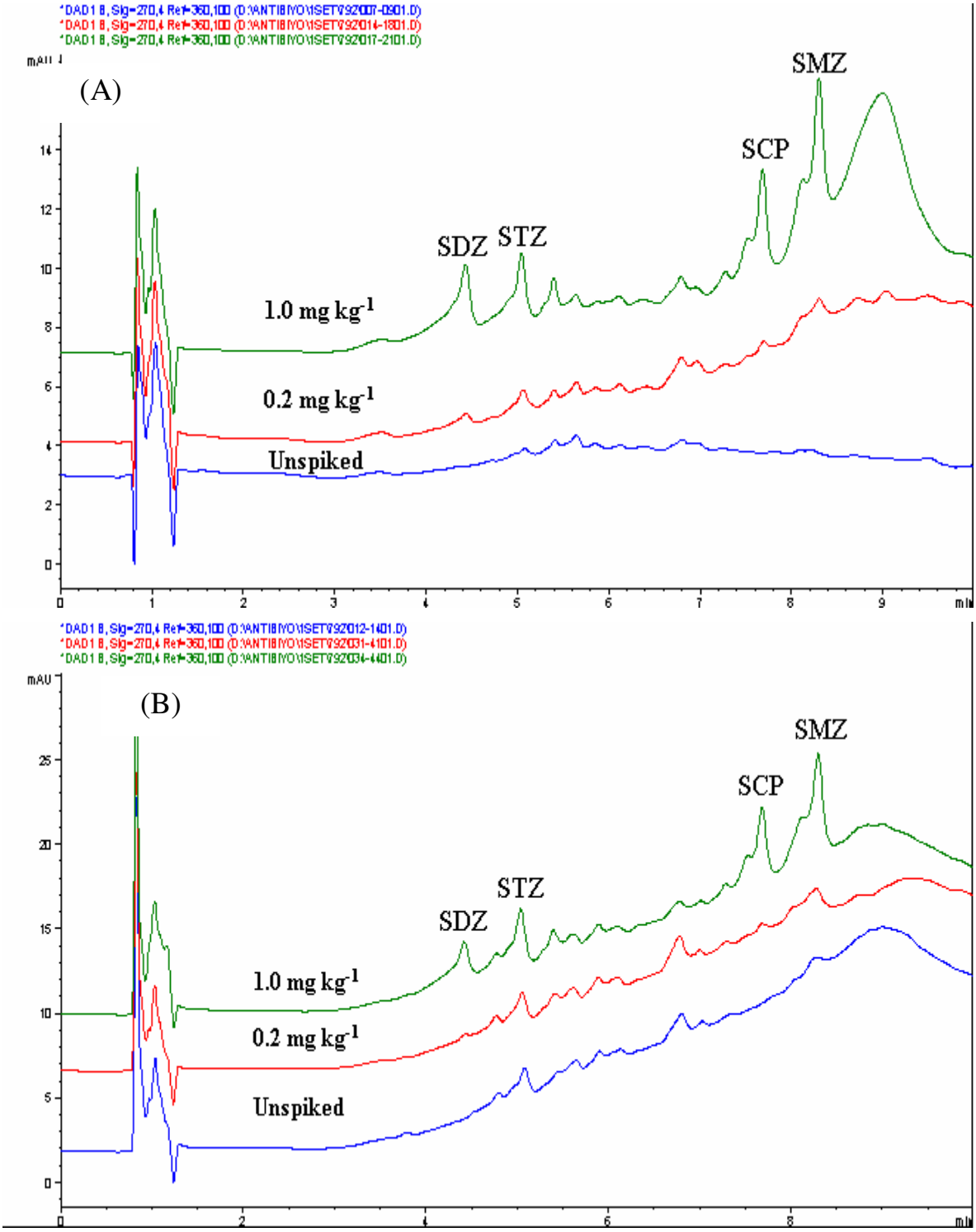


Figure 4.8. The UV-chromatograms of SAs in the unspiked and spiked (A) agricultural soil sample D-1 and (B) cattle manure sample D-4 at two spiking levels.

Table 4.16. The recovery rates of SDZ, STZ, SCP and SMZ in soil and manure.

	Agricultural Soil															
	SDZ				STZ				SCP				SMZ			
	Spiking Level															
	0.20 mg kg^{-1}		1.00 mg kg^{-1}		0.20 mg kg^{-1}		1.00 mg kg^{-1}		0.20 mg kg^{-1}		1.00 mg kg^{-1}		0.20 mg kg^{-1}		1.00 mg kg^{-1}	
Sample ID	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
D-1	83.57	4.97	95.81	4.54	72.30	21.11	88.87	5.89	61.79	14.56	91.28	0.86	79.51	7.23	104.11	2.08
D-2	82.52	7.23	99.24	1.30	245.80	16.42	93.90	0.91	40.06	21.58	95.00	11.23	85.08	6.60	101.06	0.90
M-1	87.51	5.42	119.11	7.49	114.89	17.04	103.04	7.05	78.11	5.12	95.43	7.86	110.84	13.03	120.70	5.72
M-2	56.32	5.73	95.05	17.37	72.23	11.91	93.99	6.11	70.37	1.52	91.50	11.02	93.09	4.13	98.44	11.60
B-1	61.84	2.37	87.09	15.38	59.55	24.44	110.99	9.46	82.59	25.84	80.90	33.00	43.47	42.72	60.35	17.75
B-2	65.06	0.54	82.27	3.32	96.07	1.38	77.34	3.94	87.14	17.50	96.89	1.43	76.06	0.77	83.25	4.07
B-3	64.23	7.55	98.68	1.42	101.05	3.54	96.48	2.42	76.30	2.72	88.94	1.39	53.63	27.30	75.77	0.66
K-1	69.59	0.54	97.89	1.57	48.42	10.17	94.94	0.30	71.96	1.49	94.68	0.46	26.13	131.70	98.64	1.34
K-2	88.22	1.21	96.39	0.46	101.39	7.25	92.83	3.15	70.48	3.63	79.01	1.43	52.59	3.47	92.97	1.38
	Animal Manure															
D-3	ND	ND	61.63	4.05	64.83	22.46	99.05	2.75	45.26	23.17	96.76	4.56	86.58	10.47	97.16	3.48
D-4	21.76	3.55	89.44	3.77	62.95	88.25	118.26	10.24	72.18	15.84	102.53	4.35	90.21	3.59	97.69	1.38
M-3	ND	ND	60.77	18.79	124.10	40.71	ND*	ND*	86.29	8.73	49.38	8.50	69.77	10.36	75.76	1.97
B-4	ND	ND	18.38	24.27	81.78	1.63	93.74	0.27	36.99	33.34	22.63	5.93	91.65	27.30	89.95	28.23
B-5	ND	ND	ND	ND	81.52	2.27	98.64	0.40	49.16	20.26	11.90	23.64	95.30	4.17	68.55	17.18
B-6	ND	ND	ND	ND	84.17	0.57	93.55	0.22	42.11	3.89	9.58	11.63	49.27	10.43	60.13	0.82
K-3	ND	ND	ND	ND	88.31	10.15	11.77	51.39	91.79	28.85	84.56	10.77	44.79	14.84	37.65	4.58
K-4	94.08	1.08	70.21	20.29	69.55	13.78	35.69	0.70	53.84	26.13	75.81	24.41	81.13	9.05	74.40	1.27

ND: The compound could not be recovered at this concentration, ND*: Recovery could not be calculated due to intensive matrix interferences.

4.4.3. Recoveries of fluoroquinolones from soil and manure

The recoveries of the FQs compound ENR and its metabolite CF from the agricultural soil and animal manure samples are represented in Table 4.17 as the mean of triplicate samples for both spiking level together with the RSDs (%). The recovery ratios obtained in the present study were significantly less than the ratios obtained by Turiel et al. (2006), although the same procedure was followed to a certain extent. However, this difference was stemmed from the fact that an extraction solvent containing less Mg^{2+} ions was used in this study instead of 50 % $Mg(NO_3)_2$ solution optimized by Turiel et al. (2006). On the other hand, the extraction method developed by Turiel et al. (2006) for an alkaline (pH: 7.69) clay loam soil with the OC content of 0.97 % was firstly applied to the agricultural soil samples with varying characteristics (sandy clay loam, clay loam, silty loam and loam; pH: 5.99-6.99; OC content: 1.66-8.14) and poultry manure samples (pH: 7.30-8.15; OC content: 25.12-45.68) with some modifications in the current study.

The recovery ratios of CF and ENR from soil and poultry manure obtained in the present study were not concentration dependent. This independence of recovery ratio from the concentration level was also found by other authors (Turiel et al., 2006; Uslu et al., 2007). There were not a significant difference between the recovery ratios determined for soil and manure samples. However, for the poultry manure samples K-3 and K-4, CF could not be detected at the spiking level of 0.2 mg kg^{-1}. Furthermore, ENR could also not be detected in the manure sample B-3 at the same spiking level. This situation is probably caused by the relatively high OC contents of these manure samples (32-46 %) and may indicate the strong binding of CF and ENR to the faecal material (Sunderland et al., 2004). The recovery ratios obtained for both CF and ENR were also not significantly different from each other and represents that the extraction method recovers these two compounds with similar efficiencies regardless of stronger and specific adsorption of CF on soil compared to that of ENR. The RSDs were lower than 18 % in all cases and this proved the suitability of the present extraction method for the analysis of CF and ENR in complex matrices including poultry manure and agricultural soil samples. The FLD chromatograms of FQs in the unspiked and spiked agricultural soil sample B-3 and poultry manure sample B-4 at the spiking levels of 0.2 and 1.0 mg kg^{-1} are given in Figure 4.9.

Table 4.17. The recovery rates of CF and ENR in soil and manure.

	Agricultural Soil							
	CF				ENR			
	Spiking Level							
	0.20 mg kg^{-1}		1.00 mg kg^{-1}		0.20 mg kg^{-1}		1.00 mg kg^{-1}	
Sample ID	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
B-1	54.24	3.53	49.77	3.23	89.26	4.64	58.19	4.01
B-2	54.28	3.66	55.82	17.52	57.22	6.67	51.69	3.76
B-3	50.48	8.72	49.65	1.03	47.27	7.68	50.08	2.18
K-1	22.36	1.78	35.16	0.63	35.54	8.01	38.35	0.38
K-2	46.16	2.07	41.33	6.10	46.75	2.25	35.42	6.79
	Animal Manure							
B-4	8.31	17.66	40.35	3.45	29.79	2.01	48.57	2.87
B-5	53.69	4.88	48.08	4.74	31.17	3.89	44.63	10.25
B-6	56.23	8.58	47.80	2.18	ND	ND	28.86	4.43
K-3	ND	ND	34.06	3.54	50.72	3.75	44.38	10.55
K-4	ND	ND	18.06	2.50	31.60	14.03	44.59	8.96

ND: The compound could not be recovered at this concentration.

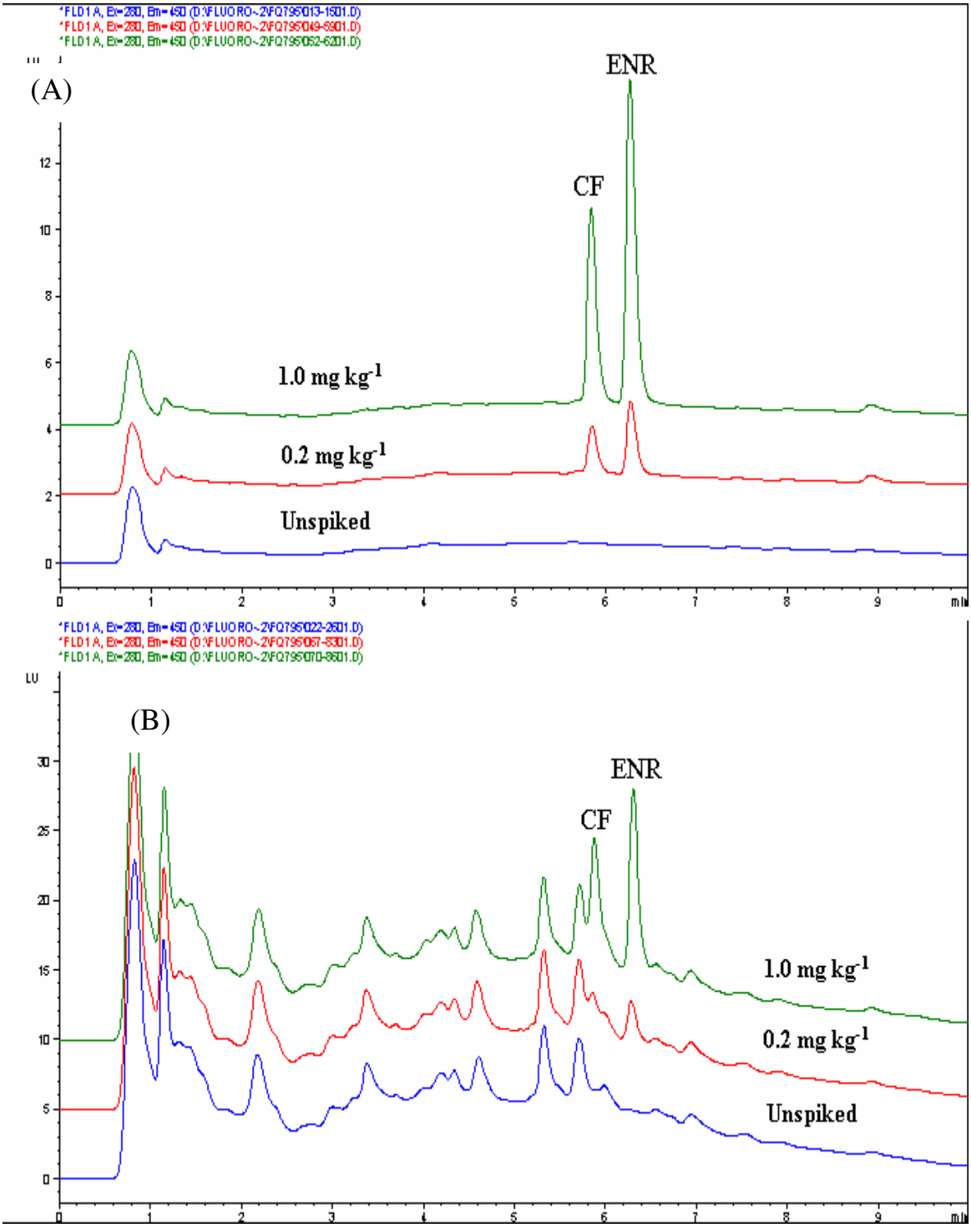


Figure 4.9. The FLD-chromatograms of FQs in the unspiked and spiked (A) agricultural soil sample B-3, and (B) poultry manure sample B-4 at two spiking levels.

4.6. Relationships between Sample Characteristics and the Recoveries

To evaluate the relationship between sample characteristics and the recoveries, and determine the significance levels of the significant correlations, statistical Bivariate Correlation Test was used, and significant correlations are given in Table 4.18 together with their significance levels.

Table 4.18. Significance (2-tailed) of the bivariate correlations.

Correlation	Pearson Correlation (r)	Significance (p)	Significance Level
Soil Samples			
OTC (0.2) – pH	0.705	0.034	*
OTC (1.0) – Ca	0.737	0.024	*
CTC (0.2) – Ca	0.798	0.010	*
CTC (0.2) – Mg	0.686	0.041	*
CTC (1.0) – Ca	0.774	0.014	*
SDZ (1.0) – Clay	- 0.721	0.028	*
SCP (0.2) – Na	- 0.738	0.023	*
SCP (1.0) – T-P	- 0.685	0.042	*
SMZ (0.2) – pH	0.702	0.035	*
SMZ (0.2) – Ca	0.706	0.033	*
CF (0.2) – pH	0.925	0.025	*
CF (0.2) – Cr	- 0.914	0.030	*
CF (0.2) – Ni	- 0.986	0.002	**
ENR (0.2) – pH	0.896	0.039	*
ENR (0.2) - Na	- 0.950	0.013	*
Manure Samples			
CTC (0.2) – Ca	- 0.818	0.013	*
CTC (0.2) – Mg	- 0.849	0.008	**
CTC (1.0) – Cr	- 0.822	0.012	*
CTC (1.0) – Ni	- 0.719	0.044	*
CTC (1.0) – Fe	- 0.743	0.035	*
STZ (0.2) – pH	- 0.765	0.027	*
STZ (0.2) – Cr	- 0.865	0.012	*
STZ (1.0) – OC	- 0.877	0.010	**
STZ (1.0) – TKN	- 0.853	0.015	*
SCP (0.2) – pH	- 0.707	0.050	*
SCP (0.2) – T-P	- 0.816	0.013	*
SCP (0.2) – Ca	- 0.777	0.023	*
SMZ (1.0) – OC	- 0.824	0.012	*
SMZ (1.0) – TKN	- 0.780	0.022	*
ENR (0.2) - TKN	0.984	0.016	*

* and ** Correlation is significant at the 0.05 and 0.01 level (2-tailed), respectively.

Statistical results obtained in the present study demonstrated that the primary factor having an effect on the recovery rates is the soil pH. pH of the agricultural soil samples was found to be determinant of the sorption behaviour of three groups of antimicrobial compounds. An increase of 1 unit in the soil pH caused substantial increases in the recoveries of OTC and SMZ (Figure 4.10A). Since no statistically significant correlation was obtained between pH and the other sample characteristics investigated, the decrease in the recoveries may be attributed to the decrease in soil pH. The decrease in the sorption of TCs and SAs with increasing soil pH has been already shown in parallel to our findings (Sassman and Lee, 2005; Boxall et al., 2002). Lower OTC recoveries obtained in the soils with lower pH suggest that cation exchange with anionic clay surfaces is a significant sorption mechanism at pH values where it exists as a zwitterion (pH 3.27-9.11). The recoveries of ENR from soil were also pH-dependant on the range of 5.99-6.99. At this pH range, ENR are predominantly present in the zwitterionic form due to their pKa_1 values of 5.90 and 6.27, respectively (Pico and Andreu, 2007). Similar to OTC, an increase in the recoveries of ENR with increasing soil pH was observed (Figure 4.10B). This finding suggests that the repulsion between the negatively charged soil sites and FQs with an increasing negative charge results in weaker sorption of these compounds to soil.

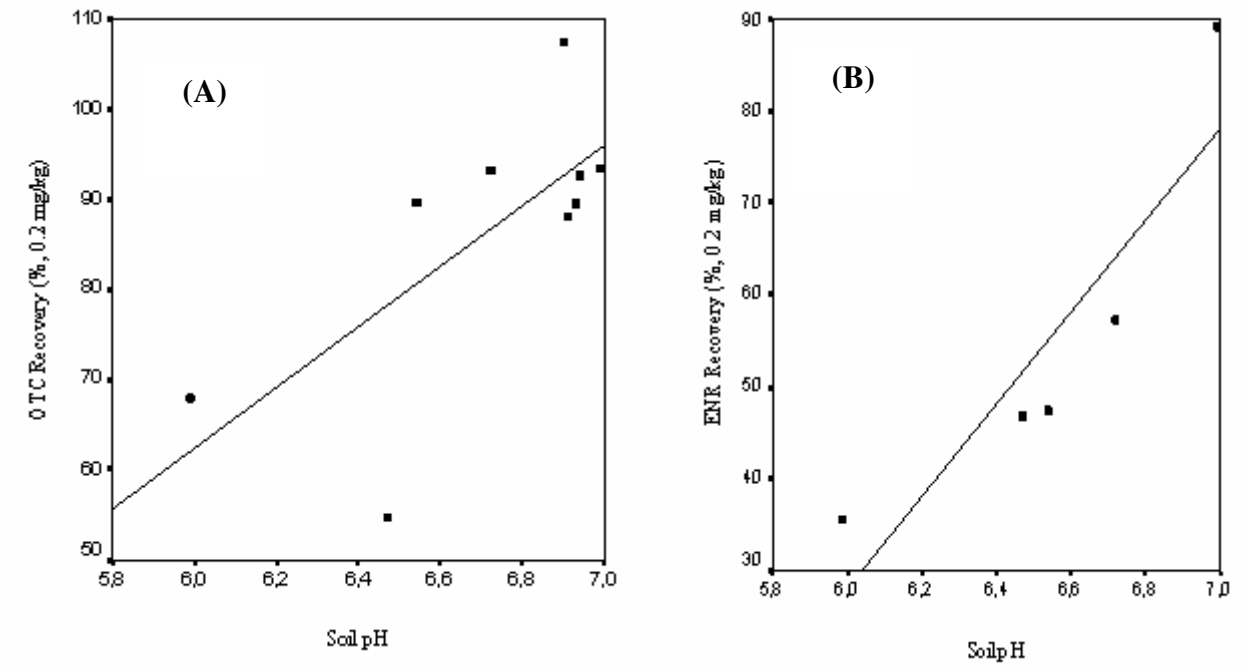


Figure 4.10. Correlation between (A) OTC and (B) ENR recoveries (0.2 mg kg^{-1}), and soil pH.

Correspondingly, zwitterionic FQs have been shown to be better isolated from soil by use of basic solutions probably due to the same reason (Turiel et al., 2006). A cation exchange mechanism should be responsible for the interaction of FQs with soils. All these results reveal that one of the important factors affecting the sorption behaviour of OTC and ENR is the environmental pH.

STZ and SCP recoveries were found to decrease with increasing manure pH. The increasing SAs sorption to manure at pH range of 7.30-8.24 suggests that a sorption mechanism involving metal bridging is dominant rather than cation exchange in manure. This mechanism can be proposed to account for the lower recoveries of STZ and SCP which have a net negative charge in the pH range of analyzed manure samples, from the samples with higher Cr and Ca content, respectively, and pH (Figure 4.11). Since STZ and SCP are present in their anionic form in manure due to their pK_a values of 7.1 and 5.7, respectively (Haller et al., 2002; Batt, 2006), these compounds can strongly bind to metal ions complexed with organic matter which is abundantly present in manure. On the other hand, the sorption of sulfamethazine to manure has been previously shown to increase with decreasing pH value at the range of 4.5-9 (Zimmermann, 2006). Therefore, this demonstrates that the sorption behaviour of SAs to manure cannot be explained by a limited pH range alone. Furthermore, it should not be ruled out that each member of SAs class of compounds may have a different sorption characteristic.

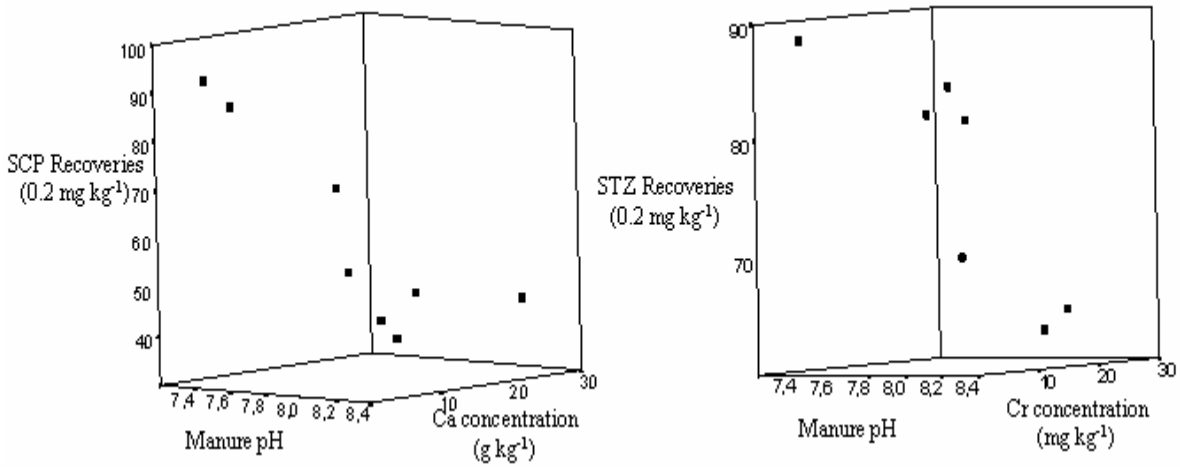


Figure 4.11. Correlation between STZ and SCP recoveries (0.2 mg kg^{-1}), manure pH, and Ca and Cr content.

Increasing Ca content of the agricultural soils resulted in a significant increase in the recoveries of OTC, CTC and SMZ. Furthermore, CTC recoveries were also found to increase as Mg content of the soils increases. The reduction in the sorption strength of competing TCs with increasing amount of the bound Ca^{2+} and Mg^{2+} to the negatively charged soil sites supports the suggestion that the cation exchange is the controlling process in the sorption of TCs to soil. The suppression of the magnitude of TCs sorption to soil with increasing Ca^{2+} cation concentration has been previously shown in correspondence with the present findings (Sassman and Lee, 2005). Moreover, because the solubility of OTC in the presence of Ca^{2+} shows a substantial increase at pH 6.8-8 (Tongaree et al., 1999), an alternative reduction in the magnitude of OTC sorption to soil may be expected. The increasing SMZ recoveries from soil with increasing Ca concentration and pH may also be due to higher stability of Ca^{2+}-SMZ complexes than that of the complexes formed between Ca^{2+} and the negative sites of soil at higher pH values, although such a comparison has not been previously reported (Figure 4.12).

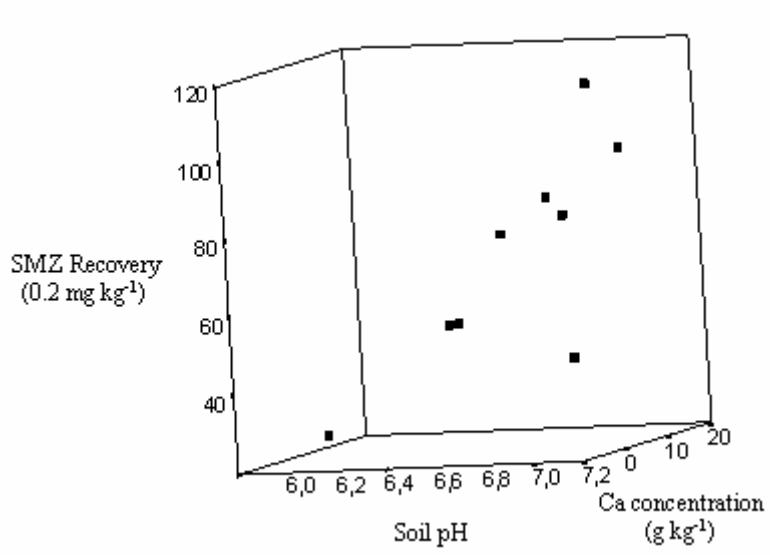


Figure 4.12. Correlation between SMZ recoveries, pH, and Ca content of soil.

In contrary to Ca and Mg, Na concentration of the agricultural soils were found to negatively influence the extraction efficiencies of SCP and ENR, while CF recoveries showed a decreasing trend with increasing Cr and Ni contents of soils. Decreased SCP recoveries can be explained by increased sorption caused by decreased electrostatic repulsion of negatively charged sorbate molecules due to increased Na^{+} concentrations

near the negatively charged soil surfaces. The similar phenomenon has been observed by ter Laak et al. (2006) in sorption studies with SCP. The lower ENR recoveries obtained in the soils with higher Na concentration suggest that a cation exchange mechanism exists between the weakly adsorbed Na^+ ions and positively charged/zwitterionic ENR species. The presence of NH_4^+ ion has been previously shown to enhance the adsorption of ENR on zeolite, an ion-exchange aluminosilicate, in accordance with the present findings (Ötker and Balcıoğlu, 2005). On the other hand, decreasing CF recoveries with increasing pH, Cr, and Ni content of soil was likely due to binding of the zwitterionic CF to divalent and trivalent cations via cation bridging (Figure 4.13). This type of sorption mechanism has been already demonstrated to play a key role in the sorption of FQs to clay minerals (Nowara et al., 1997) and mineral oxides-rich soils (Gu and Karthikeyan, 2005b). All the findings obtained in the present study suggest that the metal concentrations would be contribute to mobility of the antimicrobial compounds in terrestrial environment or their sorption to soil.

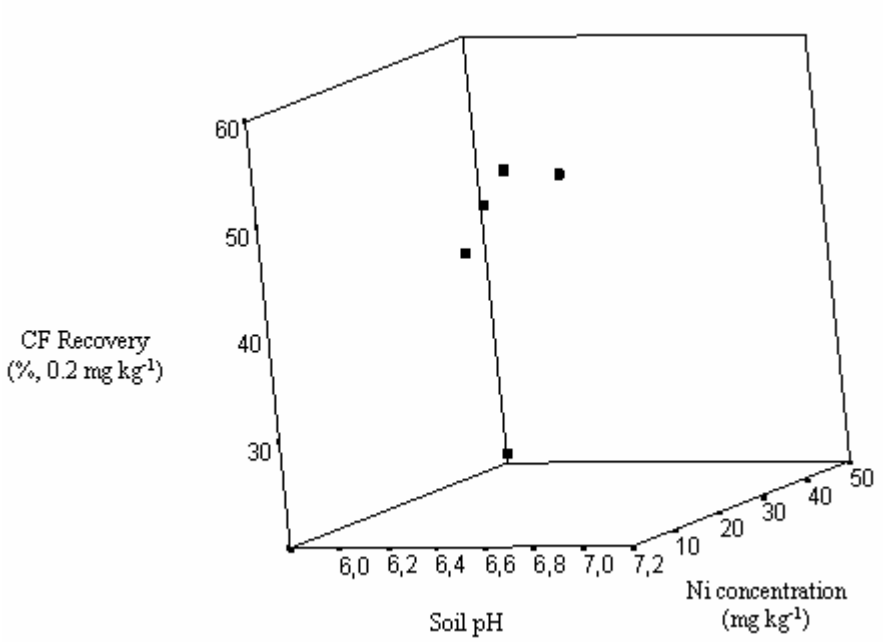


Figure 4.13. Correlation between CF recoveries, pH, and Ni content of soil.

In manure samples, CTC recoveries were observed to decrease with increasing Ca, Mg, Cr, Ni and Fe concentrations in contrary to the increasing trend of the recoveries in soil samples with increasing Ca and Mg contents. This reveals that a surface complexation mechanism with di- and trivalent cations is preferred rather than cation exchange in

manure when anionic CTC (CTC^-) species are predominant. The sorption of OTC to manure has been previously reported to be influenced by ionic binding to divalent metal ions such as Ca^{2+} and Mg^{2+} as well as other charged compounds in the matrix (Loke et al., 2002). The similar mechanism has also been observed in the studies conducted using Al- and Fe-amended humic acid (MacKay and Canterbury, 2005), Fe and Al oxides (Figueroa and MacKay, 2005; Gu and Karthikeyan, 2005a), and clays (Figueroa et al., 2004). All these studies demonstrated that the increasing metal concentration contributed to TCs sorption when these compounds are predominantly anionic, or increasing pH led to an increase in the sorption of TCs when cation bridging is the controlling mechanism.

In addition to pH and metal content, SAs recoveries were also found to be influenced by the changes in the amount of clay in soils. The negative significant correlation ($p < 0.05$) between SDZ recoveries and clay content of the soil samples revealed that this compound is sorbed more strongly by clay loam soils relative to sandy loam soils. In accordance with the results obtained in the present study, adsorption of SAs has been shown to be larger for the clay fractions of soils than for the sand fractions due to highest abundance of pedogenic oxides with positively charged surfaces which may interact with anionic SAs, in the clay fraction (Thiele-Bruhn et al., 2004). This finding indicates that cation bridging is an important mechanism for the sorption of SAs to clay minerals as reviewed by Tolls (2001). Cation- and water bridging has also been emphasized to be the possible sorption mechanisms of neutral sulfamethazine to clay minerals (Gao and Pedersen, 2005).

Besides the pH and metal content of manure samples, SAs recoveries were negatively influenced by the increases in OC and TKN content of the samples. Similarly, K_{oc} values of adsorption of SMZ has been shown 8.5 times greater in a soil with an OC content of 7.1 % than in another one with the OC content of 0.37 % (Drillia et al., 2005). Soil sorption has been concluded to be governed by nonbonding van der Waals forces and hydrogen bonding (Thiele-Bruhn et al., 2004). The similar mechanism has been proposed for the sorption of both neutral and anionic STZ species to organic materials of different origin (Kahle and Stamm, 2007a), and may also be the case responsible for the sorption of SAs to manure OC. Substantially higher OC content of manure compared to soil may account for much stronger sorption of SAs compounds to manure (Thiele-Bruhn and Aust, 2004).

Decreasing STZ and SMZ recoveries with increasing TKN content of manure obtained in the present study may be due to positive significant correlation between OC and TKN content of manure ($p < 0.01$). On the other hand, ENR recoveries were found to increase with increasing TKN content of manure. Manure typically contains high amounts of ammonia, and the higher recoveries in the presence of elevated concentrations of TKN indicate that an ammonia-facilitated desorption of ENR takes place. Correspondingly, elevated concentrations of sulfamethazine, sulfadimethoxine and ammonia have been detected simultaneously in groundwater samples nearby a confined animal feeding operation (Batt et al., 2006).

Increasing T-P contents of both soil and manure samples were found to result in decreasing SCP recoveries. Phosphorus may be enriched especially in clay soils (Solis and Torrent, 1989), and may be present in manure in the structure of various organic and inorganic compounds. Phosphorus compounds have the ability to form complexes with metal ions, so they have been shown to be sorbed by soil minerals (Penn et al., 2005). Correspondingly, significant positive correlations between soil and manure T-P and various metal contents (Zn, Na, K, Ca, Mg and Cu; $p < 0.01$) were obtained in the present study. Decreasing trend in the recoveries of SCP which is predominantly present as anionic species in manure samples analyzed in this study, with increasing pH and T-P content is likely due to enhanced sorption to metals bound to the phosphorus-containing sites of manures (Figure 4.14). However, there is lack of knowledge about the influence of phosphorus compounds on the sorption of antimicrobial compounds to soil and manure, and further research is needed to clarify this relationship.

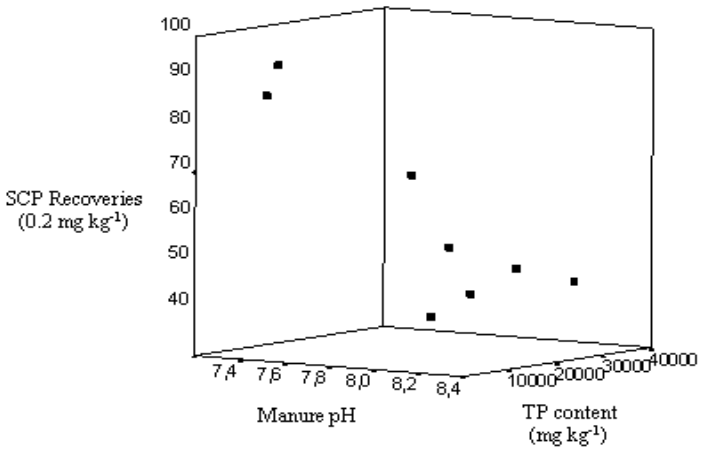


Figure 4.14. Correlation between SCP recoveries, pH, and TP content of manure.

5. CONCLUSIONS

Veterinary antibiotics are largely consumed in animal feeding operations. After administration, antibiotics are excreted by urine and feces mainly without undergoing any change in their structure due to their poor metabolization in the body of the animal. Hence significant amounts of the antibiotic residuals can enter into the agricultural fields via fertilization with animal manure and can cause adverse effects in the environment. Although there are some studies about the antibiotic pollution in animal manure and soil throughout the world in Turkey there is not any knowledge on the occurrence of veterinary antibiotics in the animal manure. Such information could be necessary to regulate the pollution from animal feeding operations.

This study consisted of three experimental parts. In the first part, physical and chemical characteristics, namely pH, CEC, texture, moisture, OC, T-P, TKN and metal content of the samples, were determined. In the second part of the study, occurrence of three commonly used veterinary antibiotic classes, namely TCs, SAs, and FQs in eight different animal manure and nine different agricultural soil samples was investigated. Two different methods were used to extract (1) TCs and SAs simultaneously, and (2) FQs. In the third part of the study, a recovery assay was conducted for all of the samples with different physicochemical characteristics. Following conclusions were drawn from this study:

- TCs, SAs and FQs were determined in manure up to 0.47, 35.5, and 0.057 mg kg^{-1}, respectively. Soil concentrations of antibiotics were in general significantly lower than the residual amounts measured in manure. In manure amended agricultural soils sampled from nine different coordinates in the north of Marmara Region, TCs, SAs, and FQs were detected at the maximum concentrations of 0.50, 0.40 and 0.053 mg kg^{-1}, respectively. At least one antimicrobial compound was detected in all of the total 17 agricultural soil and animal manure samples.
- OTC, STZ, SCP, and SMZ concentrations were found to exceed the trigger value of 0.10 mg kg^{-1} set by Steering Comittee of the Veterinary International Comittee on Harmonization, which has been based on the ecotoxic effects of antimicrobial compounds on a range of organisms, in six soil samples out of nine. Particularly,

immediate attention should be paid for the occurrence of OTC in soil, since this compound was found to be present in all of the six soils above the concentrations of 0.10 mg kg^{-1}. Although the measured concentrations of CTC and ENR in agricultural soils were below the trigger value, antibiotic resistance can occur at these subtherapeutic concentrations.

- The detection of antimicrobial compounds in soil 6-15 months after fertilization with animal manure demonstrated that these substances could be persistent and accumulate in these agricultural soils. Since OTC was detected in all of the agricultural soils and OTC concentrations measured in soil and manure samples were not significantly different from each other, special attention should be paid to persistence and accumulation of this antimicrobial compound in agricultural soils.
- Comparable antimicrobial concentrations measured in agricultural soils amended with cattle and poultry manure can be an evidence of that cattle and poultry manure are of equal importance in terms of the antimicrobial contamination of agricultural soils. On the other hand, poultry manure can be an important source of some veterinary antimicrobial compounds such as SCP, as shown by the presence of this compound only in poultry manure samples and in agricultural soils fertilized with poultry manure. However, higher sample numbers may be required to more accurately evaluate the influence of cattle, poultry and mixed manure on the antimicrobial contamination levels in agricultural fields.
- The method used for the simultaneous extraction of two TCs (OTC and CTC) and four SAs compounds (SDZ, STZ, SCP and SMZ) gave recovery rates in the range of 34-107 % and 26-121 % for TCs and SAs, respectively, in soil samples. In case of animal manure samples, recoveries of TCs and SAs compounds were in the range of 11-107 % and 10-124 %, respectively. Only for SDZ, recoveries were not detected in six manure samples in which OC content was greater than 26 %. In most of the cases, RSDs in spiked soil and manure samples were smaller than 34 % for both TCs and SAs compounds.
- The method used for the extraction of two FQs compounds (CF and ENR) from soil and manure gave recoveries ranging from 22 to 89 % and from 8 to 56 %, respectively. Similar to the results obtained with SDZ antibiotic, CF and ENR recoveries were not detected in manure samples with OC content above 30 %. The RSDs in spiked soil and manure samples were lower than 18 % in all cases.

- Six out of the nine agricultural soil samples analyzed in the present study contained heavy metals below the limit values permitted by Soil Pollution Control Regulation. While Cr and Ni contents exceeded the limit values set for these metals in three agricultural fields, Cd concentration was found high only in one soil sample. The occurrence of Cr, Ni, and Cd in these agricultural soils can be important due to the potential transfer of these metals to the animals and humans via plants grown in these contaminated lands.
- Increasing TCs recoveries from soil with increasing pH, Ca, and Mg content can be attributed to that CEC and dissolution promoted by the formation of metal-TCs complexes can be the dominant processes in the binding of TCs to soil and in their desorption from soil, respectively, within the environmental pH range.
- SAs recoveries from manure were found to be affected by the changes in Ca, Cr, OC, TKN, T-P content, and pH. A mechanism involving metal bridging and sorption to organic matter via nonbonding van der Waals forces and hydrogen bonding could be dominant rather than cation exchange in manure. Clay, Na, Ca, T-P content, and pH of the soil samples were shown to influence the recoveries of SAs compounds. These findings suggest that although no significant sorption of SAs compounds to soil is anticipated at pH values mostly encountered in soil due to the prevalence of neutral/anionic species, these compounds can be more strongly sorbed to soil by the introduction of some nutrients such as Na and phosphorus into the environment. Therefore, soil amendment with animal manure, resulting in high contents of Na and phosphorus, will increase the immobilization of SAs in soil and consequently increase the risk of accumulation in agricultural soils.
- The only factor influencing the recoveries of FQs from manure was the TKN content which has been found to be closely correlated with OC content. Na, Cr, Ni content, and pH were the leading factors determining their fate and behaviour in agricultural soils. These findings suggest that metal pollution in agricultural soils can lead to stronger sorption of FQs compounds to soil, resulting in their accumulation in this environmental compartment.

The findings obtained in the present study demonstrated that veterinary antimicrobial compounds can lead to the contamination of agricultural soils via fertilization with animal manure. Since cattle or poultry manure have been used to supply the nutrition demand of

plants grown in the target agricultural fields, environmentally harmful components of manure such as veterinary drugs and metals have the potential to be transferred into the environment via animal manure. The occurrence data of TCs, SAs, and FQs antibacterial compounds in manure and agricultural soil samples collected from four separate districts located in the north of Marmara Region are anticipated to be an important reference to other studies to be conducted in this issue. These data are especially significant when considering the lack of information on the presence of antimicrobial residues in agricultural fields in Turkey.

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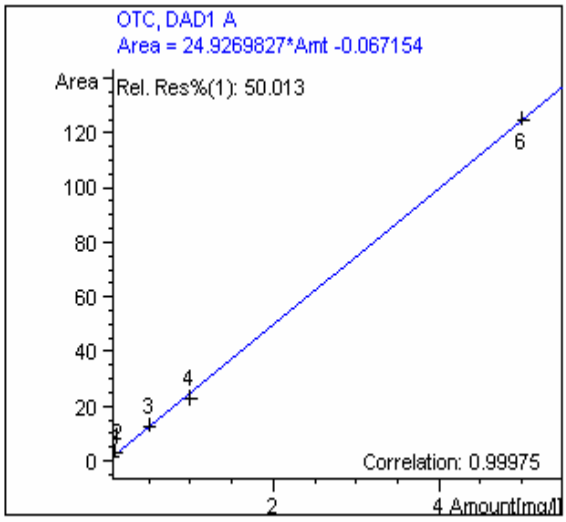
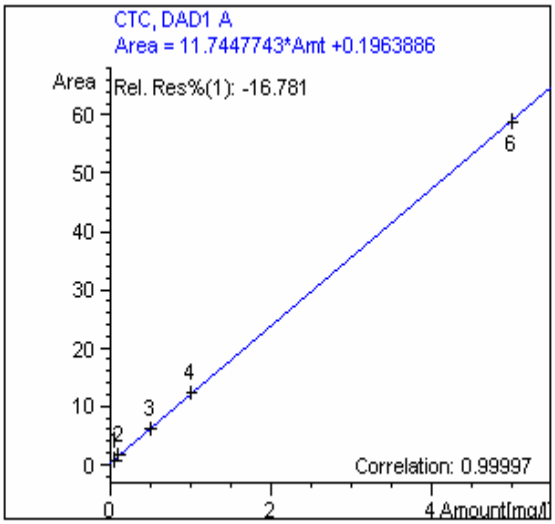
APPENDIX A

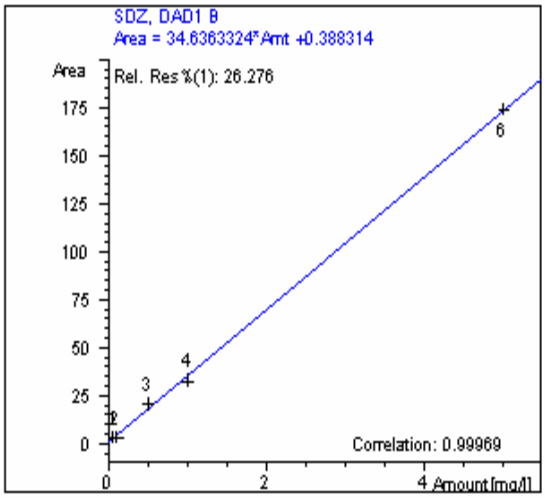
CALIBRATION GRAPHS OF THE ANTIMICROBIAL COMPOUNDS



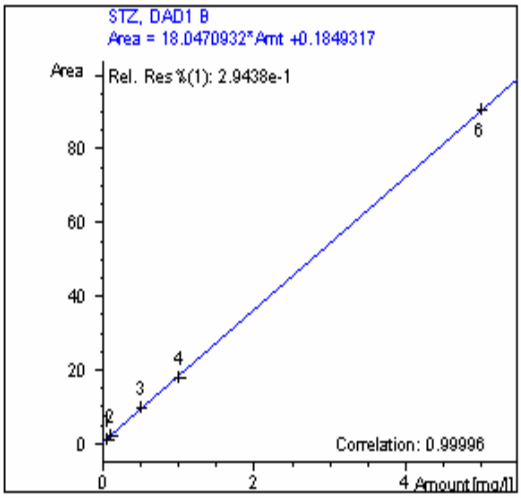
Calibration Curve of OTC



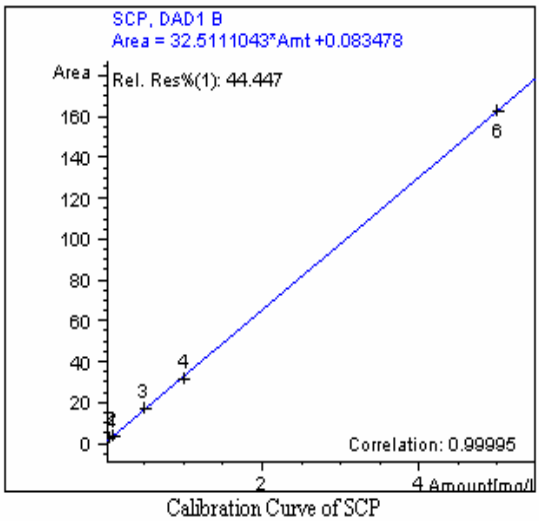
Calibration Curve of CTC



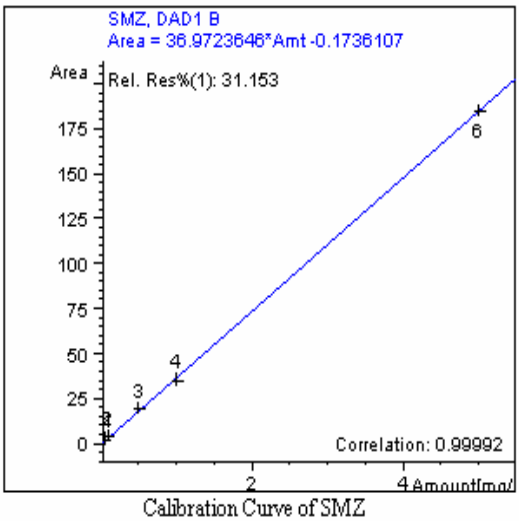
Calibration Curve of SDZ



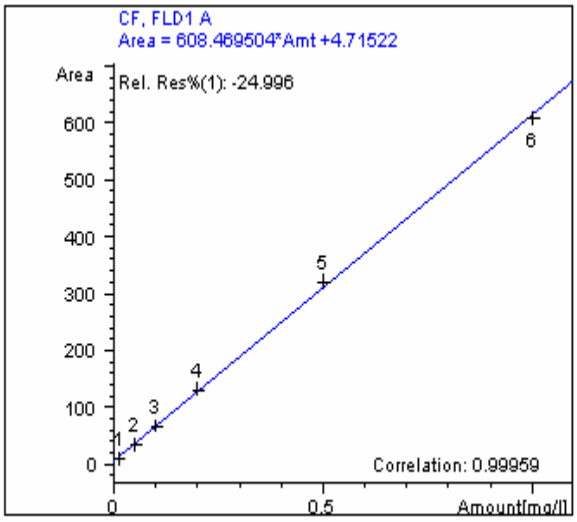
Calibration Curve of STZ



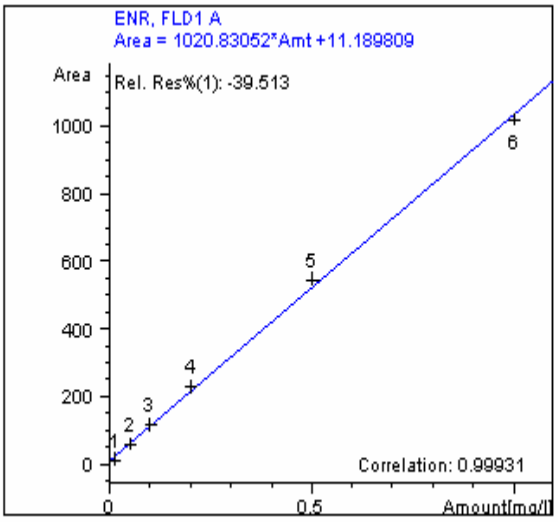
Calibration Curve of SCP



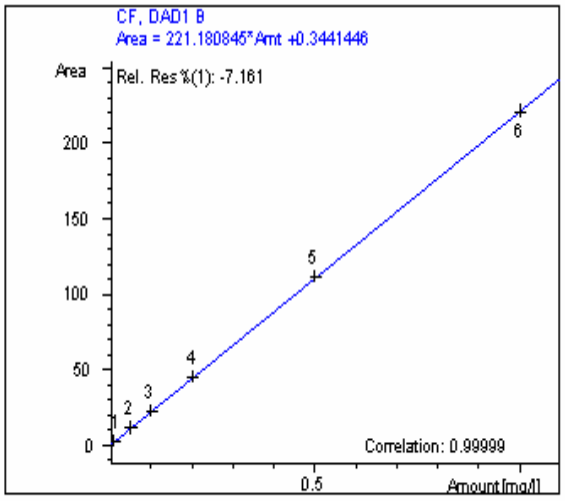
Calibration Curve of SMZ



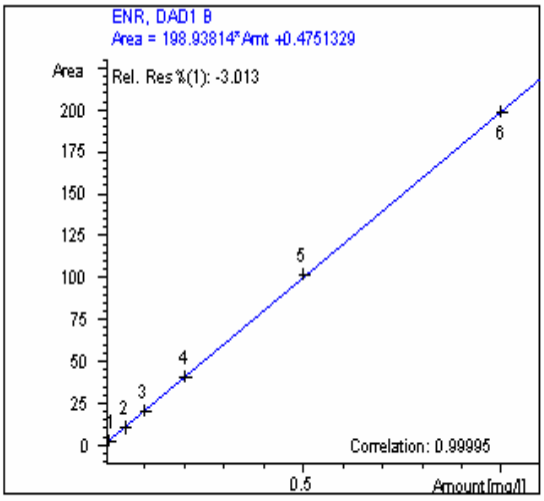
FLD Calibration Curve of CF



FLD Calibration Curve of ENR



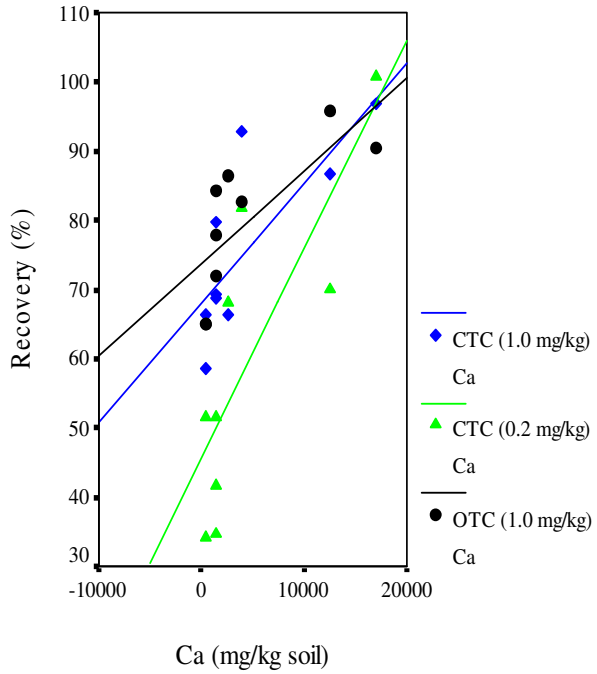
DAD Calibration Curve of CF

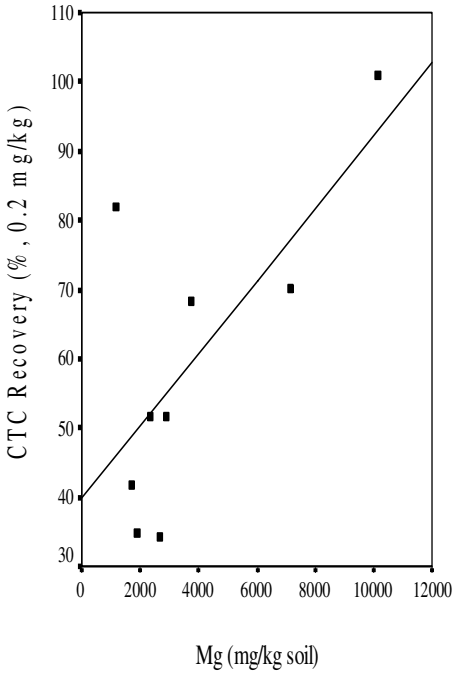


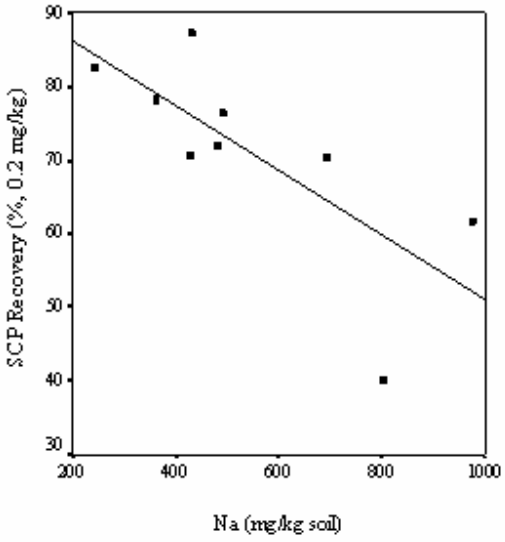
DAD Calibration Curve of ENR

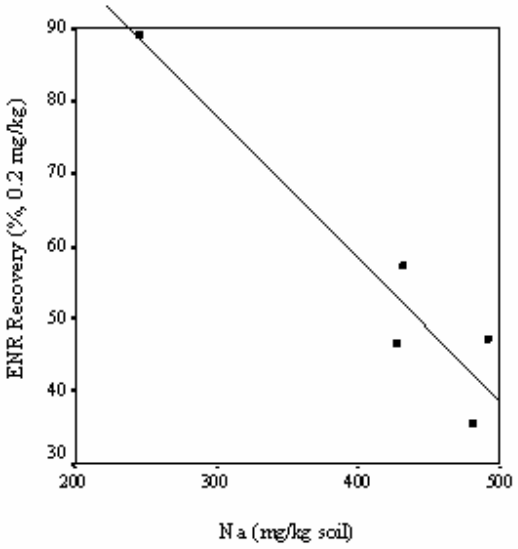
APPENDIX B

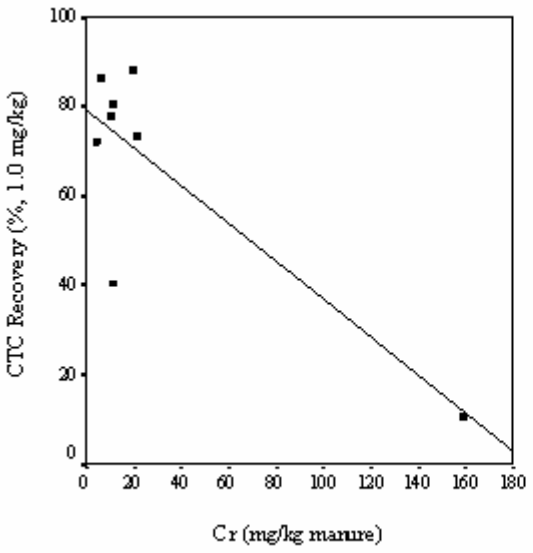
SCATTER GRAPHS OF THE SIGNIFICANT CORRELATIONS

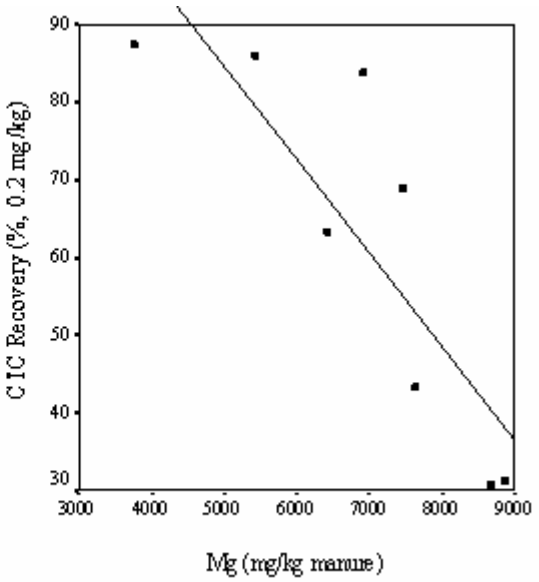

Recovery (%)
110
100
90
80
70
60
50
40
30
-10000
0
10000
20000
Ca (mg/kg soil)
CTC (1.0 mg/kg) Ca
CTC (0.2 mg/kg) Ca
OTC (1.0 mg/kg) Ca

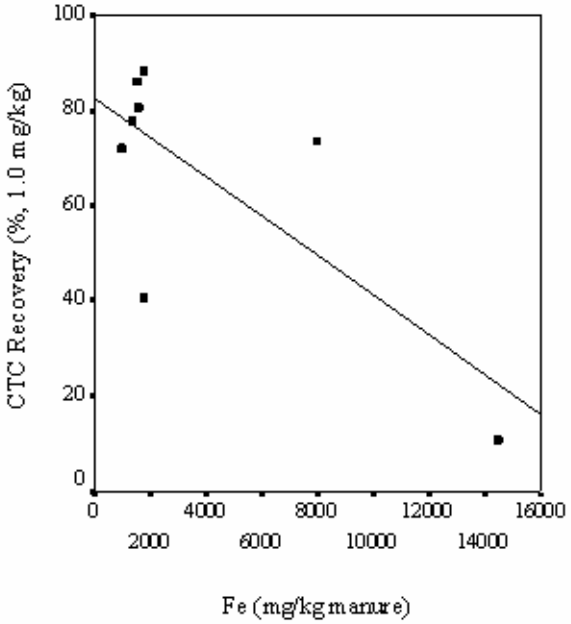

CTC Recovery (%, 0.2 mg/kg)
110
100
90
80
70
60
50
40
30
0
2000
4000
6000
8000
10000
12000
Mg (mg/kg soil)

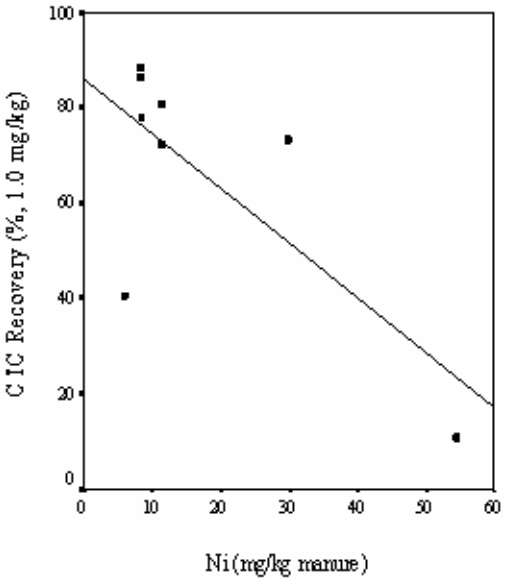

SCP Recovery (%, 0.2 mg/kg)
90
80
70
60
50
40
30
200
400
600
800
1000
Na (mg/kg soil)

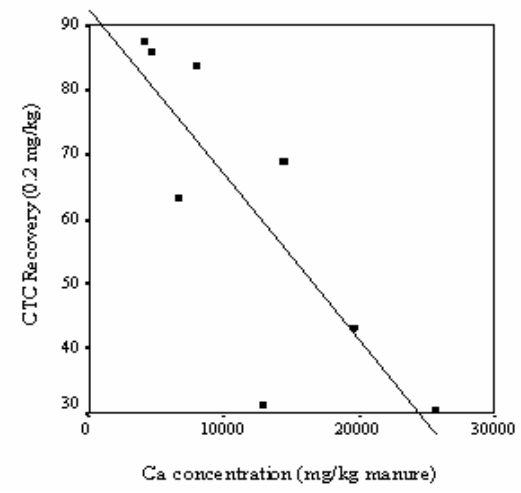

ENR Recovery (%, 0.2 mg/kg)
90
80
70
60
50
40
30
200
300
400
500
Na (mg/kg soil)

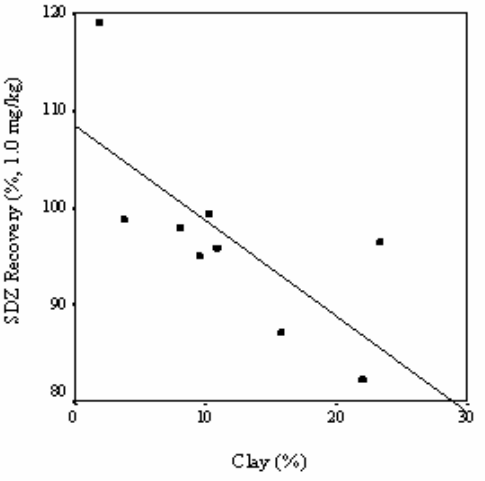

CTC Recovery (%, 1.0 mg/kg)
Cr (mg/kg manure)

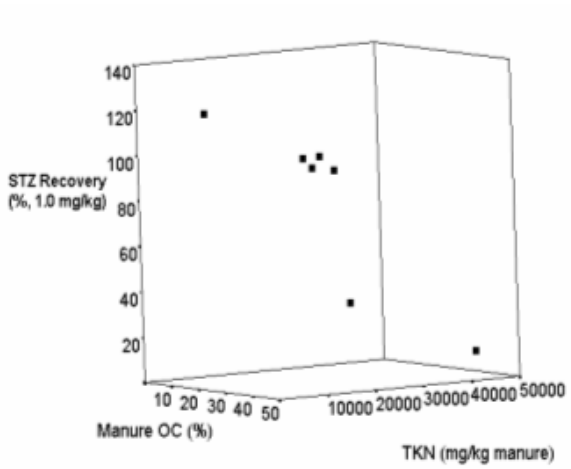

C IC Recovery (%, 0.2 mg/kg)
Mg (mg/kg manure)

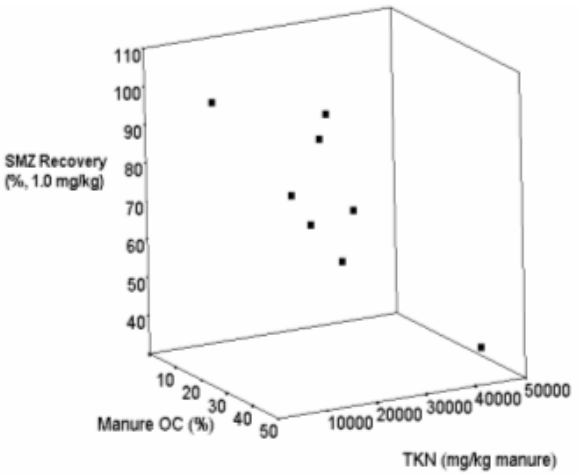

CTC Recovery (%, 1.0 mg/kg)
Fe (mg/kg manure)

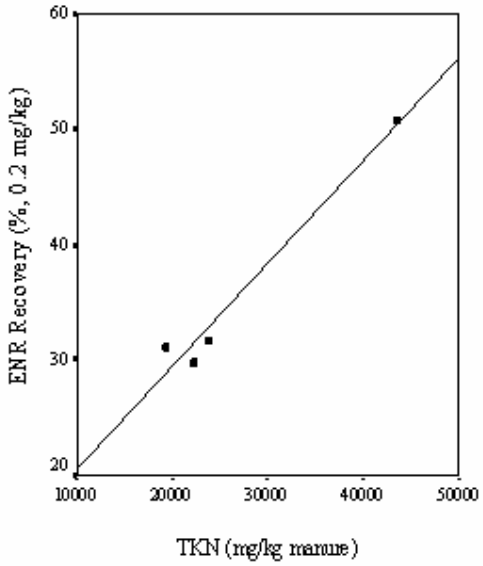

C IC Recovery (%, 1.0 mg/kg)
Ni (mg/kg manure)

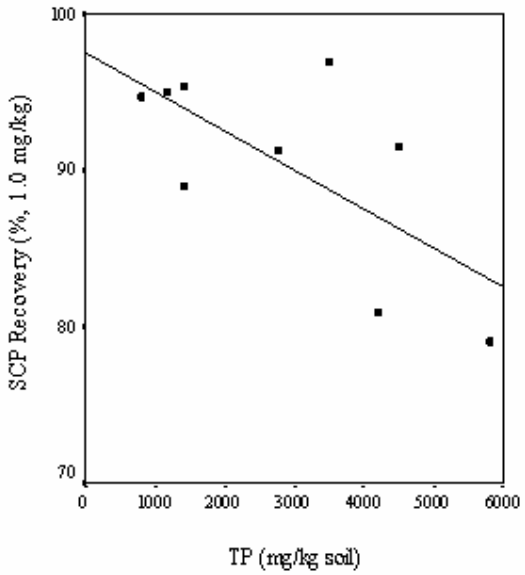

CTC Recovery (0.2 mg/kg)
Ca concentration (mg/kg manure)

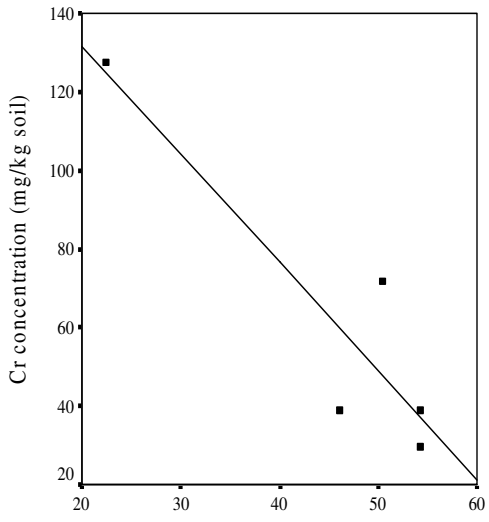

SDZ Recovery (%, 1.0 mg/kg)
Clay (%)


STZ Recovery
(%, 1.0 mg/kg)
140
120
100
80
60
40
20
10 20 30 40 50
Manure OC (%)
10000 20000 30000 40000 50000
TKN (mg/kg manure)


SMZ Recovery
(%, 1.0 mg/kg)
110
100
90
80
70
60
50
40
10 20 30 40 50
Manure OC (%)
10000 20000 30000 40000 50000
TKN (mg/kg manure)


ENR Recovery (%, 0.2 mg/kg)
60
50
40
30
20
10000 20000 30000 40000 50000
TKN (mg/kg manure)


SCP Recovery (%, 1.0 mg/kg)
100
90
80
70
0 1000 2000 3000 4000 5000 6000
TP (mg/kg soil)


Cr concentration (mg/kg soil)
140
120
100
80
60
40
20
20 30 40 50 60
CF Recovery (%, 0.2 mg/kg)