

**REMOVAL OF ANTIBIOTICS FROM ANIMAL WASTE BY CHEMICAL
OXIDATION PROCESSES**

by

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ABSTRACT

Concern about antibiotic contamination in the environment is growing worldwide because it promotes development of antibiotic resistance in bacterial communities. The residuals of antibiotics in manure have been shown to persist over a long time period and therefore could be considered as an important reservoir for antibiotics and antibiotic resistant bacteria. In the first part of the study, considering the high persistence of tetracycline and fluoroquinolone group antibiotics in solid matrices, their analysis in different field samples were conducted and trace concentrations of oxytetracycline (OTC), enrofloxacin (ENR) and ciprofloxacin (CIP) were detected in sediment and soil samples. Secondly, the study focused on the complete elimination of antibiotics in animal wastes within short time periods to protect their spread on agricultural fields through fertilization. For this purpose, ozone, Fenton's reagent and persulfate oxidants were applied to cow manure contaminated with OTC and sulfamethazine (SMZ) either directly or after the pretreatment with magnesium salt which was applied to desorb OTC from the manure prior to oxidation.

The high desorption of SMZ from the manure was achieved by deionized water, whereas magnesium salt was required for the desorption of OTC. Almost the complete removal of antibacterials in manure was attained by all oxidation processes with the preceding magnesium salt pretreatment. Among the investigated oxidation processes, heat activated persulfate oxidation was the only process which was able to remove antibiotics completely from the manure under the applied experimental conditions. Pretreatment with magnesium salt was not essential for the efficient removal of OTC by subsequent Fenton and persulfate oxidation processes as it was for ozonation process. The use of bedding materials did not significantly affect the efficiencies of magnesium salt desorption and oxidation processes. Ozone treatment increased the humic acid carbon content of the manure improving its quality as soil organic amendment. Furthermore, it also increased the mineral nitrogen content of the manure, which could be utilized by plants as a nutrient source. Ozone and persulfate oxidation were effective in the removal of antibiotics from animal feeding operation wastewater with the production of almost non-toxic and biodegradable by-products.

ÖZET

Doğal ortamların antibiyotik kalıntıları ile kirlenmesi bakterilerde direnç gelişimine sebep olduğundan dünya çapında önem taşımaktadır. Hayvan gübrelerinde düşük seviyedeki antibiyotik kalıntılarının uzun süreler kalabildiği saptanmıştır. Bu yüzden hayvan atıkları, antibiyotik kalıntıları ve antibiyotiğe dirençli bakteriler için önemli bir rezervuar olarak gösterilebilir. Bu çalışmanın ilk kısmında, florokinolon ve tetrasiklin grubu antibiyotiklerin katı matrislerde dirençli olmaları göz önünde bulundurularak değişik saha örneklerinde analizleri yapılmış ve oksitetrasiklin (OTC), enrofloksasin (ENR) ve siprofloksasin (CIP) antibiyotiklerinin düşük konsantrasyonları sediment ve toprak örneklerinde tespit edilmiştir. Çalışmanın ikinci kısmında ise tarımsal alanların kirlenmesini engellemek amacıyla antibiyotik kalıntılarının hayvan atıklarından kısa sürede bertaraf edilmeleri amaçlanmıştır. Bu sebeple, ozon, Fenton reaktifi ve persülfat oksidan maddeleri OTC ve sülfametazin (SMZ) ile kirlenmiş hayvan gübresine doğrudan veya magnezyum tuzu ile ön arıtımından sonra uygulanmıştır.

Sülfametazinin gübreden desorpsiyonu sadece deiyonize su ile sağlanırken, oksitetrasiklinin desorpsiyonu için magnezyum tuzunun gerekliliği saptanmıştır. Magnezyum tuzu ile ön arıtımından sonra uygulanan bütün oksidasyon prosesleri hayvan gübresindeki antibiyotiklerin neredeyse tamamının giderimini sağlamıştır. Uygulanan deneysel şartlar altında, sadece ısı ile aktive edilmiş persülfat oksidasyonu antibiyotiklerin gübreden tamamen giderilmesini sağlamıştır. Gübreye ön arıtım uygulanması ozonlama prosesinin verimliliğini önemli ölçüde artırırken, Fenton ve persülfat prosesleri ile antibiyotik gideriminde çok önemli bir rol oynamadığı görülmüştür. Gübrenin altlık malzemesi ile karıştırılması, antibiyotiklerin gübreden desorpsiyonunu ve oksidasyonunu önemli bir şekilde etkilememiştir. Ozonlama prosesi gübrenin hümitik asit karbonu miktarını artırarak, toprak iyileştirici olarak kullanım kalitesini yükseltmiştir. Buna ek olarak, bitkiler tarafından besin kaynağı olarak yararlanılan mineral azot miktarı da ozonlama prosesi ile artırılmıştır. Ozon ve persülfat oksidasyon proseslerinin hayvan yetiştiriciliğinden kaynaklanan atıksudan antibiyotik gideriminde de etkili olduğu ve biyolojik parçalanabilirliği yüksek ve hemen hemen toksik olmayan yan ürünlerin olduğu saptanmıştır.

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1. INTRODUCTION

Residues of a large number of drugs such as antibiotics, antiparasitic agents, and hormones that are extensively used in both human and veterinary medicine (Kümmerer, 2001), have been found in various environmental compartments such as soil, sediment, and water resources (Sarmah et al., 2006). Among the various groups of pharmaceuticals, antibiotics deserve special attention since they create potential health risk for living organisms through development of antibiotic resistance. Animal feeding operations are an importance source of antibiotic consumption due to their therapeutic and non-therapeutic use.

Tetracyclines, sulfonamides, and fluoroquinolones are important classes of antibiotics commonly used in veterinary medicine. After administration to animals, up to 90 % of the antibiotic is ultimately excreted via urine and feces mostly as a parent compound (Kemper, 2008). Consequently, the residuals of these antibiotics are frequently found in feces and manure at high concentrations (Martinez-Carballo et al., 2007) as well as in lagoon samples from animal feeding operations (Bradford et al., 2008).

The manure derived from animal feeding operations are either stored or subjected to biological treatment (e.g. composting, anaerobic digestion) for maturation and applied to agricultural fields as a fertilizer to reduce soil compaction and to increase soil fertility and productivity via organic matter and nutrient addition. The fate of antibiotics in various manure samples has been investigated in numerous studies and the obtained results exhibited wide variation with the type of animal, the treatment practices of manure, and environmental conditions. Reported half lives of fluoroquinolones and tetracyclines were as high as 90 and 105 days (Winckler and Grafe, 2001; Wetzstein et al., 2002), respectively due to their strong sorption characteristics which could make them unavailable for microbial attack (Thiele-Bruhn, 2003). Although shorter half lives have also been reported for tetracyclines in manure during storage (8.1 days) or composting processes (3.2 days) (Wang and Yates, 2008; Arıkan et al. 2007), their presence in manure samples even after 5 months of maturation (De Liguoro et al., 2003) is an evidence for their persistence. The persistence of a sulfonamide antibiotics in manure have also been denoted by another

study in which relatively high concentrations of sulfonamide antibiotic was detected after 3 months of maturation (De Liguoro et al., 2007).

Once antibiotics are released to soil from contaminated manure, their behavior in the environment differ depending on their physicochemical properties. The strong sorption of particularly tetracyclines and fluoroquinolones to solid matrices leads them to persist in soil, while more mobile sulfonamides tend to threat surface and ground water through surface run-off or leaching (Lindsey et al., 2001).

The main concern regarding the presence of antibiotic residuals in aquatic and terrestrial environment is the development of resistant bacteria strains representing a health risk to humans and animals. Moreover, antibiotic resistant bacteria may further be released to the environment from the waste of applied animal. A wide range of antibiotic resistance has been detected in various environmental compartments such as soil (Sengelov et al., 2003; Schmitt et al., 2006) and natural waters (MacKie et al., 2006; Oliveira et al., 2008) as well as in animal wastes (Bae et al., 2005; Chander et al., 2008). The adverse effect of antibiotics in the environment is not only restricted to resistance development. The toxic effects of several antibiotics to aquatic (Halling-Sørensen, 2000; Wollenberger et al., 2000) and soil organisms (Boleas et al., 2005; Thiele-Bruhn and Beck, 2005) have been demonstrated to occur at mg/l ranges. Inhibition on methane production in anaerobic manure treatment reactors (Masse et al., 2000; Loftin et al., 2005; Arıkan et al., 2006) at mg/l ranges could also be regarded as the adverse effects of antibiotics.

Regarding to these adverse effects, in order to protect terrestrial and aquatic environment from the possible antibiotic contamination originating from animal feeding operations it is necessary to take precautions before the application of manure to agricultural land. Several types of chemical oxidants (Fenton's reagent, ozone, and persulfate) have been efficiently used for the destruction of recalcitrant compounds in different solid (soil, sediment, or sludge) and liquid matrices (water and wastewater). Although the ability of chemical oxidation processes to remove antibiotic substances in water and wastewaters has been verified by numerous studies (Balcıoğlu and Ötger, 2003; Ikehata et al., 2006) their effectiveness on the removal of antibiotics in solid matrices has not been studied. Within these perspectives, the current research mainly aimed to

investigate the removal of two antibiotics, namely oxytetracycline and sulfamethazine from the bovine animal waste by using chemical oxidants, Fenton's reagent, ozone, and persulfate.

The experimental part of this dissertation was divided into five main chapters and the scope of each chapter can be explained as follows;

- In Chapter 6 and 7, analysis methods were developed for fluoroquinolone and tetracycline antibiotics in solid matrices and fertilized soil and sediment field samples were analyzed for these antibiotics.
- In order to prevent the antibiotic pollution in the environment, the treatment of animal waste, which was one of the major sources of this pollution, was investigated in the following chapters. In Chapter 8, ozonation was applied to cow manure from an ecological farm and synthetic animal feeding wastewater for the removal of a tetracycline group antibiotic, oxytetracycline (OTC). Effect of ozone treatment on the manure and wastewater characteristics, and on the toxicity of wastewater were evaluated.
- In Chapter 9 to enhance the removal of antibiotic from the cow manure, a pretreatment, which was the extraction with magnesium salt, was utilized before ozone and Fenton oxidation processes. Efficiencies of the integrated processes were assessed in terms of variations in OTC concentration and treated matrix characteristics.
- Since animal manure may contain multiple antibiotics belonging to different classes, the simultaneous removal of OTC and sulfamethazine (SMZ) from the cow manure was investigated in Chapter 10. Effect of two different bedding materials on antibiotic removal was also assessed in this chapter, since the use of these materials is common in animal feeding operations for the improvement of animal welfare. For this purpose, ozone, Fenton and persulfate oxidations were applied on pretreated cow manure and manure bedding mixtures. Persulfate oxidation was also applied on the animal feeding operation wastewater contaminated with oxytetracycline and sulfamethazine. Effect of persulfate oxidation on the toxicity and characteristics of wastewater was examined.

2. CHEMICAL OXIDATION BY FENTON'S REAGENT, OZONE, AND PERSULFATE

2.1. Theories of Fenton, Ozonation, and Persulfate Oxidation Processes

Chemical oxidation processes, such as Fenton, ozonation, and persulfate oxidation have emerged as important technologies for the treatment of industrial wastewater and for the remediation of groundwater and soil that are contaminated with hazardous/toxic substances. The brief explanations of these chemical oxidation processes are given below.

2.1.1. Fenton Process

Although hydrogen peroxide is a strong oxidant ($E^\circ = 1.8 \text{ V}$), the rate of direct oxidation of it with most organic substances is very slow. The combination of H_2O_2 with Fe^{2+} which is known as the classical Fenton reagent (Walling, 1975) produces highly reactive hydroxyl radicals ($E^\circ = 2.7 \text{ V}$) at acidic pH (~ 3).



When the high concentration of hydrogen peroxide is used which is called as modified Fenton's reagent, the hydroxyl radicals further react with excess hydrogen peroxide and a series of propagation reactions begin (Watts et al., 2005).



Modified Fenton process is different from the classical Fenton process because it includes propagation reactions generating nonhydroxyl radical reactive species (Watts et al., 2005).

When ferric iron is used in Fenton's reagent, the process is called as Fenton like and it produces hydroperoxyl and hydroxyl radical by the following steps;



Introduction of near UV/VIS light ($\lambda > 300 \text{ nm}$) to Fenton process is known to accelerate oxidation of organic compounds (Pignatello, 1992) and this process is called as photo Fenton. The increased efficiency of the photo Fenton reaction is attributable to the photoreduction of the ferric ions formed during Fenton reaction.



2.1.2 Ozonation Process

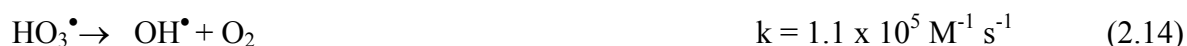
Ozone ($E^\circ = 2.07 \text{ V}$) decomposes at alkaline pH through hydroxyl ions or in the presence of hydrogen peroxide and/or UV light to generate more reactive hydroxyl radicals ($E^\circ = 2.7 \text{ V}$) (Glaze et al., 1987).

2.1.2.1. O_3/OH^- . At alkaline pH values, ozone decomposition could be initiated by hydroxyl ions and reaction kinetics follows the first order (Staehelin and Hoigne, 1982). The initiation and propagation steps for the decomposition of ozone by hydroxyl ions could be as follows (Glaze et al., 1987);

Initiation steps



Propagation steps



The overall reaction for the decomposition of ozone by hydroxyl ions is as follows;



These reactions can be followed by the interaction of hydroxyl radicals with ozone as in the following reaction;



2.1.2.2. $\text{O}_3/\text{H}_2\text{O}_2$ The conjugate base of hydrogen peroxide (HO_2^-) can also initiate the decomposition of ozone to form hydroxyl radicals (Glaze et al., 1987). Hydrogen peroxide is a weak acid and it partially dissociates into hydroperoxide ion in water. The reaction between H_2O_2 and O_3 is very slow whereas the hydroperoxide anion is highly reactive towards ozone. The decomposition rate of ozone by hydrogen peroxide increases with increasing pH (Langlais, 1991). The initiation and propagation reactions for the hydroperoxide anion initiated the decomposition of ozone are:

Initiation steps



Propagation steps





The overall reaction for the hydrogen peroxide initiated decomposition of ozone is represented by the following equation;

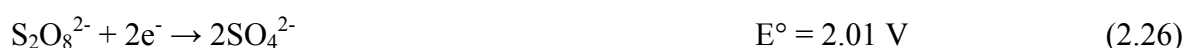


However, at very high concentrations, hydrogen peroxide can act as a hydroxyl radical scavenger (Glaze et al., 1987).



2.1.3. Persulfate Oxidation Process

Persulfate (peroxydisulfate, $\text{S}_2\text{O}_8^{2-}$), is another strong inorganic oxidant known in aqueous solution. Persulfate ion has an oxidation potential of 2.01 V slightly lower than that of ozone ($E^\circ = 2.07 \text{ V}$) (Latimer, 1952).



The oxidation reaction with persulfate anion is kinetically slow. However, it can be thermally or chemically activated to form strong sulfate radicals ($E^\circ = 2.6 \text{ V}$) with as high oxidation potential as hydroxyl radicals (Liang et al., 2008a).



$$k = 1.0 \times 10^{-7} \text{ s}^{-1} (25^\circ \text{C}) \text{ or } 5.7 \times 10^{-5} \text{ s}^{-1} (70^\circ \text{C}), \text{ pH} = 1.3$$



However, in the presence of excess iron concentration in solution, it may act as a sulfate radical scavenger through the following reaction (Liang et al., 2008a)



$$k = 4.6 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \text{ (22}^\circ \text{C, pH = 3-5)}$$

At neutral and basic pH values, $\text{SO}_4^{\bullet-}$ oxidizes OH^- with a moderate rate constant and is thus converted into OH^\bullet at high pH (Neta et al., 1988).



In spite of their similar oxidation potentials, sulfate radicals react with many organic compounds more efficiently than hydroxyl radicals because of their selectivity (Neta et al., 1988). The reactions of sulfate radical with organic compounds mainly involves an electron transfer mechanism (Minisci et al., 1983) while hydrogen abstraction and addition reactions of sulfate radicals occurs at slower rate than that of hydroxyl radicals (Neta et al., 1988).

2.2. Applications of Fenton, Ozonation, and Persulfate Oxidation Processes to Water Matrices

The widespread contamination of aquatic environment, surface and ground water as well as industrial wastewaters with toxic and non-biodegradable organic compounds brings an obligation for the development of treatment technologies for such compounds. The Fenton and ozonation processes have been widely applied to the wastewaters from different industries (e.g. Vogelpohl and Kim, 2004) mainly to improve their biodegradability for subsequent conventional biological processes. Large number of studies have been conducted to evaluate the effectiveness of these processes on the removal of specific organic pollutants such as surfactants, pesticides and pharmaceuticals (Ikehata and El-Din, 2004; Klavarioti, 2009). Moreover persulfate oxidation which is an emerging treatment technology, Fenton and ozonation processes have been reported as an efficient way for in situ remediation of groundwater (Watts and Teel, 2006).

2.3. Applications of Fenton, Ozonation, and Persulfate Oxidation Processes to Solid Matrices

Chemical oxidation is also considered as an efficient process to degrade recalcitrant compounds in solid matrices. Most commonly used chemical oxidation processes for soil, sewage sludge or sediment are iron catalyzed hydrogen peroxide processes.

The common problem encountered in the application of these processes on solid matrices is related with the sorption of contaminants, because oxidants are generally incapable of oxidizing the organic compounds that do not exist in liquid phase. In order to overcome this problem, several modifications have been made in Fenton's systems. The desorption of polycyclic aromatic hydrocarbons (PAHs) from soil which was facilitated by ethanol pretreatment greatly enhanced the efficiency of traditional Fenton oxidation (Lundstedt et al., 2006). The addition of high hydrogen peroxide concentration has also been shown to enhance the oxidative treatment of sorbed contaminants. Watts et al., (1999a) found that more than 300 mM of H_2O_2 is needed to oxidize sorbed contaminants on soil. Moreover, at high hydrogen peroxide concentration, iron oxides in solid matrices could involve in oxidation process making the addition of soluble iron unnecessary (Watts et al., 1999b; Lin and Gurol, 1998).

The addition of soluble iron is not an efficient way to catalyze hydrogen peroxide due to its fast oxidation at environmentally relevant pH values. Furthermore, the acidification of treated matrix can dissolve nontarget compounds that exert oxidant demand. Flotron et al., (2005) observed no significant effect of soluble ferrous iron addition on the oxidation efficiency of PAHs in sludge and sediments with the total indigenous iron content of 94 and 30 mg/g, respectively. Moreover, mineral (hematite $\alpha\text{-Fe}_2\text{O}_3$, goethite $\alpha\text{-FeOOH}$, magnetite Fe_3O_4 and ferrihydrite) catalyzed Fenton like reactions at neutral pH were found to promote more benzo[a]pyrene mineralization in soil with 33.5 mg/g iron oxide than that obtained by the same reactions conducted at acidic pH (Watts et al., 2002). Another study which compares the soluble iron (II) and mineral catalyzed Fenton reaction found that despite of slower reaction, mineral catalyzed one was more efficient in terms of stoichiometry (Watts et al., 1993). However, for the systems where indigenous iron minerals are not efficient to catalyze hydrogen peroxide, the addition of an chelating agent

(HEIDA, N-(2-hydroxyethyl) iminodiacetic acid) to increase the stability of iron in soil slurry systems enhanced the toluene removal from 51 (2 mM Fe^{3+}) to 95 % (2 mM Fe^{3+} and 2 mM HEIDA) in the presence of 300 mM hydrogen peroxide (Kang and Hua, 2005).

Iron is also used to activate the persulfate anion to produce sulfate radicals and due to its unavailability; it is generally added to the solid systems in a complex form with a suitable chelating agent as in case of Fenton oxidation (Ferrerase et al., 2008). On the other hand, the heterogeneous activation of persulfate anion with iron minerals of sediments (14 g/kg) resulted in a 37 % of 2-CB degradation which was only 17 % lower than the case with the addition of soluble iron as a catalyst (Rastogi et al., 2009). Heat activation of persulfate in soil slurry systems have also been reported in the literature. Liang et al. (2003) showed that by using persulfate/contaminant ratio of 10 at 40° C, half life of trichloroethylene (TCE) was 12.91 h in soil slurry systems. Increment of temperature to 50° C decreased the half life of TCE in soil slurries to 4.63 h due to the higher sulfate radical formation at this temperature.

Ozonation is another process to treat contaminants in soil, sludge, or sediments. It is known that ozone readily decomposes via catalytic reaction with metal oxides in solid matrices to form OH radicals (Choi et al., 2001). However, similar to other chemical oxidation processes, the elevated organic carbon content of soil can increase the oxidant demand. It was shown that PAH oxidation requires 0.3 mg ozone per PAH degraded in sand compared to 2.6 mg ozone per PAH degraded in peat (Kulik et al., 2006).

Kulik et al. (2006) also investigated the effect of the water content of solid matrices on the effectiveness of ozone treatment for the organic pollutant degradation. A remarkable decrease in PAH removal efficiency (from 61 % to 41 % in sand) with an increase in ozone consumption was observed when water was added to the system. Similar inhibitory effect of water content on PAH degradation in soil by ozonation was also reported by O'Mahony et al. (2006). They obtained the highest phenanthrene removal in air dried soils. In slurry systems, gas liquid transfer coefficient is another parameter that affects the ozonation efficiency for contaminant removal. Carrere et al. (2006) reported more than 2 times enhancement in PAH removal from sewage sludge by increasing oxygen mass transfer

coefficient through the addition of surfactants to sludge that was assumed to increase interfacial area.

3. ANTIBIOTIC POLLUTION IN THE ENVIRONMENT

3.1. Consumption of Antibiotics

Antibacterial agents are widely used for therapeutic purposes both in human and veterinary medicine and as a growth promoter in animal husbandry. The main groups of these substances are represented in Table 3.1. According to a study by the European Federation of Animal Health in 1999 animals consumed 4,700 tonnes (35 %) of all antibiotics administered in European Union. Of the antibiotics that were given to animals, 3,900 tonnes (29 % of total usage) were administered to help sick animals recover from disease, while 790 tonnes (6 %) were given to farm animals as growth promoters (FEDESA, 2001). The Union of Concerned Scientists has estimated antibiotic consumption in animal feeding operations in USA as 16,000 tonnes and 70 % of antibiotics were used for non-therapeutic purposes (UCS, 2001). Karabay and Hosoglu, (2008) have been reported that total antibiotic utilization based on defined daily dose (DDD) in Turkey was 31.36 DDD/1000 inhabitant-days in the year 2006; however, no distinction was made between veterinary and human consumption.

A result of the use of antibiotics, the high levels of antibiotic resistance is of great concern. For this purpose, use of human related antibiotics as a growth promoter in animals has been banned by the European Union (EU Directive 70/524/EEC) to prevent the occurrence of cross resistance. However, according to Union of Concerned Scientists, the agricultural use of these banned antibiotics in USA was enormous (UCS, 2001). Use of antibiotics as a growth promoter phased out in 2006 by EU leading to a decline in antibiotic consumption in animal feeding operations. Similar to EU, four growth promoters monencin sodium, salinomycin sodium, avilamycin, and flavophospholipol were banned in 2006 remaining no more growth promoters in market in Turkey (Tuncer, 2007).

Table 3.1. Important antibiotics in human and animal medicine, (Kemper et al., 2008).

Class	Compounds	Primary Usage
Aminoglycosides	Apramycin	Pigs
	Gentamycin	All animals, humans
	Kanamycin	Dogs, pigs, cattle, horses
	Neomycin	All animals
	Sisomycin	Humans
	Spectinomycin	Pigs, cattle, poultry
	Streptomycin	Obsolete
β -lactams	Amoxicillin	All animals
	Ampicillin	All animals
	Azlocillin	Humans
	Benzylpenicillin	All animals
	Cloxacilin	Cattle
	Dicloxacilin	Cattle
	Flucloxacillin	Humans
	Methicillin	Humans
	Mezlocillin	Humans
	Nafcillin	Humans
	Oxacillin	Cattle
	Piperacillin	Humans
	Phenoxymethylpeniclin	Humans
	Penicilin G	Humans
Cephalosporines	Cefalexin	Dogs
	Cefalotin	Humans
	Cefazolin	Humans
	Ceftiofur	Cattle, pigs
	Cefotaxim	Humans
	Cefotiam	Humans
	Cefquinom	Cattle, pigs
Fenicoles	Chloramphenicol	Cats, dogs
Fluoroquinolones	Ciprofloxacin	Humans
	Enrofloxacin	All animals
	Marbofloxacin	All animals
	Flumequin	Humans
	Ofloxacin	Humans
Lincosamides	Clindamycin	Dogs, humans
	Lincomycin	Pigs, cats, dogs, cattle
Macrolides	Azithromycin	Humans
	Clarithromycin	Humans
	Erythromycin	Humans, cattle, chicken
	Roxithromycin	Humans
	Spiramycin	All animals
	Tylosin	Animals
	Vancomycin	Humans

Table 3.1 (Continued)

Class	Compounds	Primary Usage
Sulphonamides	Sulphanilamide	Humans
	Sulphadimethoxine	Cattle, pigs, chicken
	Sulphadimidine	Cattle, sheep, chicken
	Sulphamethoxazole	Humans
	Sulphapyridine	Pigs
	Sulphathiazole	Humans
Trimethoprim		In combination with sulphonamides
Tetracyclines	Chlortetracycline	Cattle, pigs
	Doxycycline	Humans, cats, dogs
	Oxytetracycline	Humans, cattle, sheep, pigs
	Tetracycline	Humans, horse, sheep, pigs

3.2. Pathways and Occurrence of Veterinary and Human Antibiotics in the Environment

Excretion is the major means by which human and veterinary medical compounds are introduced into the sewage treatment plants (STPs) and manure, respectively. Antibiotics can enter the environment through the field application of manure and sewage, effluents of sewage treatment plants, manufacturing plants, process effluents, and disposal of unused or expired compounds (Figure 3.1).

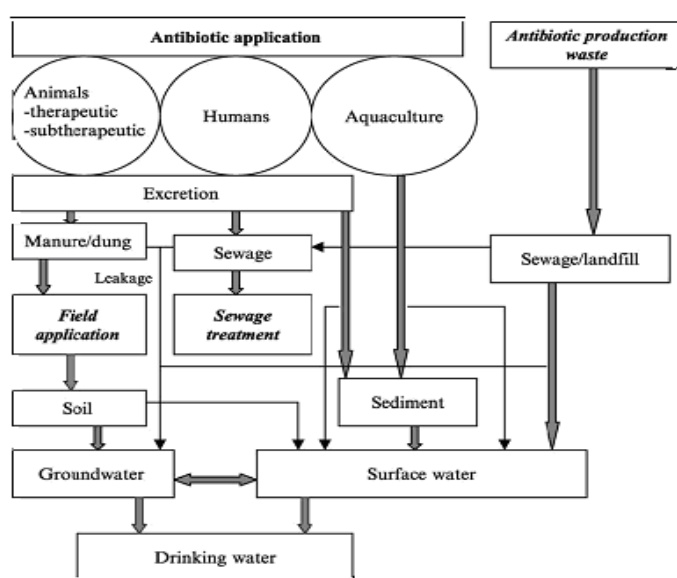


Figure 3.1. Anticipated pathways of human and veterinary antibiotics in the environment (Kumar et al., 2005a).

3.2.1. Antibiotics in Soil

Major pathway for the contamination of soils with human and veterinary antibacterial agents is the field application of sewage sludge and manure for fertilizing purposes. As the antibiotics administered by humans or animals, a large proportion (up to 90 %) of them are excreted via urine and feces as a parent compound passing into sewage treatment plants and manure, respectively. A number of studies, which are summarized in Table 3.2, have been conducted for the occurrence of antibiotics in sewage sludge and manure samples.

During the conventional treatment processes in sewage treatment plants (STPs), a part of antibiotic substances could be retained on sewage sludge depending on their persistence and sorption tendency (Jorgensen and Halling Sørensen, 2000). Antibiotic substances with the strong binding abilities such as fluoroquinolones and tetracyclines have been detected in sewage sludge samples. In spite of considerably lower sorption tendency, sulfonamides have also been determined in sludge samples presumably due to their extensive use in human medicine. Relatively higher concentrations of the same group of antibiotics have also been reported in manure samples (Table 3.2).

In addition to solid wastes like manure and sewage sludge, wastewater from animal feeding operations and municipal sewage treatment plants also contain substantial amount of antibiotics which in turn may end up in soil through the irrigation of agricultural fields (Bradford et al., 2008; Kinney et al., 2006). The environmental concentrations of antibacterial agents detected in soil are given at Table 3.3.

Table 3.2. Antibiotic substances in sewage sludge and manure samples.

Group	Antibiotic ^a	Concentration (mg/kg manure or mg/l slurry)	Reference
<i>Sewage Sludge</i>			
Fluoroquinolones	OFL, NOR	0.04-0.88	Xu et al., 2007
	OFL, NOR, CIP	0.1-4.8	Lindberg et al., 2005
	CIP, NOR	1.4-2.42	Golet et al., 2003
Tetracyclines	DC	1.3-1.5	Lindberg et al., 2005
Sulfonamides	SDM, SMX	0.02-0.03	Xu et al., 2007
	SPY, SMX	0.018-0.19 / up to 0.0012 ^b	Göbel et al., 2005
Macrolides	ROX, TYL	1.3-4	Nieto et al., 2007
	ROX, ERY	0.032-0.195	Xu et al., 2007
	ROX, AZI, CLA	up to 0.158 / up to 0.0023 ^a	Göbel et al., 2005
<i>Manure</i>			
Fluoroquinolones	ENR, CIP	0.13-8.3	Martinez-Carballo et al., 2007
	CIP	0.3-3	Hu et al., 2008
	ENR, CIP	up to 0.06	Karci, 2008
Tetracyclines	TC, DC, CTC, OTC	0.005-6.1	Aga et al., 2003
	TC, CTC	0.09-4	Hamscher et al., 2002
	TC, CTC	0.9-41.2	Hamscher et al., 2005
	CTC, DC	0.6-59.8	Hu et al., 2008
	OTC, TC, CTC	0.1-46	Martinez-Carballo et al., 2007
	CTC	0.4	Aust et al., 2008
	CTC	up to 5.2	Kumar et al., 2004
	TC	0.6-66	Winckler and Grafe, 2001
	OTC, CTC	up to 0.47	Karci, 2008
	OTC, TC, CTC and their epi forms, DC 4-epi-OTC	up to 15.7 1-29	Jacobsen and Halling Sørensen, 2006 Brambilla et al., 2007
Sulfonamides	SMZ, SDZ	3.5-11.3	Hamscher et al., 2005
	SDM	1.0-1.1	Christian et al., 2003
	SMZ, STZ	0.1-12.4	Haller et al., 2002
	SDM, SMX, SCP, SDZ	0.6-24.8	Hu et al., 2008
	SDM, SDZ	up to 91	Martinez-Carballo et al., 2007
	SMZ	up to 9.99	Aust et al., 2008
	SDZ, STZ, SCP, SMZ SDZ, SMZ, SOX	up to 35.5 up to 2.1	Karci, 2008 Jacobsen and Halling Sørensen, 2006
Macrolides	TYL	up to 3.78	Kumar et al., 2004

^aOFL: ofloxacin; NOR: norfloxacin; CIP: ciprofloxacin; ENR: enrofloxacin; DC: doxycycline; OTC: oxytetracycline; CTC: chlorotetracycline; TC: tetracycline; SDM: sulfadimidine; SDZ: sulfadiazine; SMX: sulfamethoxazole; SPY: sulfapyridine; SMZ: sulfamethazine; SCP: sulfachloropyridazine; SMX: sulfamethoxazole; SOX: sulfadoxine; ROX: roxythromycin; TYL: tylosin; ERY: erythromycin; AZI: azithromycin; CLA: clarithromycin;

^b activated sludge / digested sludge

Table 3.3. Environmental concentrations of antibiotics in soil samples^a.

Group	Antibiotic	Concentration (µg/kg)	Reference
Fluoroquinolones	CIP, NOR	270-400 ^b	Golet et al., 2002
	CIP, NOR	9300-14300	Morales-Munoz et al., 2004
	CIP, ENR	13-200	Uslu et al., 2008
	CIP, ENR	up to 50	Karci, 2008
Tetracyclines	TC, CTC	3.7-198	Hamscher et al., 2002
	CTC	0.6-15.5	Jacobsen et al., 2004
	CTC	87	Aust et al., 2008
	TC, CTC	4-295	Hamscher et al., 2005
	TC, CTC, OTC ^c	4-254	Zilles et al., 2005
	4-epi OTC	127 - 216	Brambilla et al., 2007
	OTC, CTC	up to 500	Karci, 2008
	OTC, CTC	up to 144	Cengiz, 2008
Sulfonamides	SDM	15	Christian et al., 2003
	SMZ	10.4	Aust et al., 2008
	SMZ	up to 2	Hamscher et al., 2005
	SDZ, STZ, SCP, SMZ	up to 400	Karci, 2008
Macrolides	TYL	1.8 - 57.4	Jacobsen et al., 2004
	LIN ^{c, d}	9	Zilles et al., 2005
	ERY	15 ^e	Kinney et al., 2006

^a manure amended soil, otherwise indicated^b sludge amended soil^c amended with swine waste treatment sample^d lincomycin^e irrigated with reclaimed water from wastewater treatment facilities

As can be seen from Table 3.3, among different sources, manure amendment has a major contribution on the soil contamination with antibiotics. Direct excretion of veterinary antibiotics through the feces and urine of grazing animals may also be responsible for the occurrence of these compounds in terrestrial environment. Another remarkable conclusion that can be deduced from table is the existence of fluoroquinolones and tetracyclines in soils at higher concentrations compared to sulfonamides and macrolides. The high binding capacities of tetracyclines, fluoroquinolones, and macrolides to a lesser extent induce their accumulation in soils, while sulfonamides can be expected to leach ground and surface water due to their high mobility in the environment.

3.2.2. Antibiotics in Water

Due to the continuous load of human antibiotics into sewage treatment plants through the human excreta they have been detected in STP influents at $\mu\text{g/L}$ level. In addition to human excretion, the effluents from hospitals and antibiotic manufacturing plants are other sources of antibiotic substances in STP influents. In comparison to STP influents, the higher concentrations of antibiotic substances have been detected in these wastewaters. Wastewater originated from animal feeding operations, such as flush water to remove manure from alleys and barns, rainfall runoff from roofs and open lots and direct rainfall on pretreatment facilities could also contribute the contamination of sewage with antibiotic substances. Conventional treatment processes in STPs generally failed in the complete removal of antimicrobial agents as it will be discussed in Section 4.1.1. Therefore, these substances have also been detected in STP effluents. The concentration ranges of several antibiotic compounds in the influents and effluents of the municipal wastewater treatment plants, hospital wastewater, and animal production wastewater are presented at Table 3.4.

Table 3.4. Antibiotic substances in different types of wastewaters.

Group	Antibiotic ^a	Source	Concentration (µg/l)	Reference
Tetracyclines	TC	WWTP ^b Effluent	0.62	Batt and Aga, 2005
	DOX, TC	Hospital wastewater	0.04- 0.2	Watkinson et al., 2009
	DOX, TC, CTC, OTC	WWTP Influent	0.1-0.65	Watkinson et al., 2009
	DOX, TC, CTC, OTC	WWTP Effluent	0.02-0.25	Watkinson et al., 2009
	DOX, TC	WWTP Effluent	0.046-0.977	Miao et al., 2004
	OTC	Swine wastewater	up to 160	Tagiri-Endo et al., 2009
	TC, CTC, DOX, DMC	WWTP Influent	0.05-0.27	Yang et al., 2005
	CTC, DOX	WWTP Effluent	0.06-0.07	Yang et al., 2005
	OTC, MIN, MEC, DMC, DOX, CTC, TC	WWTP Influent	0.11-0.97	Choi et al., 2007a
	DOX, MEC, CTC, DMC	WWTP Effluent	0.03-0.18	Choi et al., 2007a
	OTC, MIN, MEC, DMC, CTC, TC	WWTP Influent ^c	0.98-171	Choi et al., 2007a
	OTC, MIN, DMC, CTC, TC	WWTP Effluent ^c	0.37-90.9	Choi et al., 2007a
	OTC	Antibiotic production wastewater	920000	Li, D et al., 2008
	DOX	Hospital wastewater	0.6-6.7	Lindberg et al., 2004
	CTC, OTC, TC	Hospital wastewater	0.011-0.455	Lin and Tsai, 2009
	CTC, OTC, TC	Antibiotic production wastewater	7.44-13.1	Lin and Tsai, 2009
Sulfonamides	SMX	WWTP Effluent	1.3	Batt and Aga, 2005
	SMZ, SMX, SPY	WWTP Effluent	0.018-0.641	Göbel et al., 2004
	SMX	WWTP Effluent	0.098-2.2	Batt et al., 2008
	SMX	Hospital wastewater	0.3	Watkinson et al., 2009
	SAS, SMX, STZ	WWTP Influent	0.1-3	Watkinson et al., 2009
	SAS, SMX, STZ	WWTP Effluent	0.15-0.6	Watkinson et al., 2009
	SDZ, SMZ, SMX, SPY, SCT, SSZ	WWTP Effluent	0.019-0.151	Miao et al., 2004
	SMZ, SMX, SDX	WWTP Influent	0.07-1.09	Yang et al., 2005
	SMX	WWTP Effluent	0.21	Yang et al., 2005
	SMX, SMMX, SMR, SMZ, STZ, SCP, SDX	WWTP Influent	0.45-10.57	Choi et al., 2007a
	STZ, SCP,	WWTP Effluent	0.06-0.18	Choi et al., 2007a
	SMMX, SMR, SMZ, STZ, SCP, SDX	WWTP Influent ^c	3.45-1158	Choi et al., 2007a
	STZ	WWTP Effluent ^c	4.27	Choi et al., 2007a
	SMX	Hospital wastewater	0.4-12.8	Lindberg et al., 2004
	SDX, SMZ, SMX	Hospital wastewater	0.0028-7.35	Lin and Tsai, 2009

Table 3.4. (Continued)

Group	Antibiotic ^a	Source	Concentration (µg/l)	Reference
Sulfonamides	SDX, SMZ, SMX, SMMX	Antibiotic production wastewater	3.4-1,340	Lin and Tsai, 2009
	SAD, SDZ, SMR, SMZ, SMPD, SDX, SQX	Swine wastewater	0.005-0.094	Malintan and Mohd, 2006
Quinolones	NAL, NOR, ENR, CIP	Hospital wastewater	0.1- 15	Watkinson et al., 2009
	NAL, NOR, ENR, CIP	WWTP Influent	0.04-1.1	Watkinson et al., 2009
	NAL, NOR, ENR	WWTP Effluent	0.05-0.45	Watkinson et al., 2009
	CIP, NOR, OFL	WWTP Effluent	0.112-0.506	Miao et al., 2004
	CIP	Hospital wastewater	32-99	Martins et al., 2008
	CIP, ENR	WWTP Effluent	0.1-0.36	Batt and Aga, 2005
	OFL	WWTP Effluent	0.1	Nakata et al., 2005
	CIP, NOR	Hospital wastewater	0.9-44	Duong et al., 2008
	CIP, OFL	Hospital wastewater	0.2-101	Lindberg et al., 2004
Macrolides	AZI, ERY-H ₂ O, ROX, CLA	WWTP Effluent	0.01-0.374	Göbel et al., 2004
	ROX, OLE	Hospital wastewater	0.04-0.4	Watkinson et al., 2009
	ROX, OLE, TYL	WWTP Influent	0.005-0.5	Watkinson et al., 2009
	ROX, OLE, TYL	WWTP Effluent	0.15-3.40	Watkinson et al., 2009
	CLA, ERY-H ₂ O, ROX	WWTP Effluent	0.008-0.087	Miao et al., 2004
	TYL	Swine wastewater	up to 1.4	Tagiri-Endo et al., 2009
	ROX, ERY, CLA	WWTP Influent	0.054-1.2	Schlüsener and Bester, 2005
	ROX, ERY, CLA	WWTP Effluent	0.038-0.32	Schlüsener and Bester, 2005
	ERY-H ₂ O	Hospital wastewater	6.11	Lin and Tsai, 2009
	ERY-H ₂ O, TYL	Antibiotic production wastewater	7.8-63.9	Lin and Tsai, 2009
Lincosamides	CLI, LIN	Hospital wastewater	0.09- 1.7	Watkinson et al., 2009
	CLI, LIN	WWTP Influent	0.06-0.5	Watkinson et al., 2009
	CLI, LIN	WWTP Effluent	0.07-0.3	Watkinson et al., 2009
	LIN	Swine wastewater	0.12-400	Tagiri-Endo et al., 2009
Pleuromutilin	VLM	Swine wastewater	up to 0.12	Tagiri-Endo et al., 2009
Polyether ionophores	SAL	WWTP Influent	0.3	Watkinson et al., 2009
	MON	WWTP Effluent	0.02	Watkinson et al., 2009

Table 3.4. (Continued)

Group	Antibiotic ^a	Source	Concentration (µg/l)	Reference
β-Lactams	AMO, PEN V, CEP	Hospital wastewater	0.01 -10	Watkinson et al., 2009
	AMO, PEN V, CEP, CEF, PEN G, CLO	WWTP Influent	0.01-64	Watkinson et al., 2009
	AMO, PEN V, PEN G, CEP, CEF, CLO	WWTP Effluent	0.05-2	Watkinson et al., 2009
	AMO, AMP, OXA, CEH, CLOX	WWTP Influent	0.015-0.017	Cha et al., 2006
	AMP	Hospital wastewater	5.08	Lin and Tsai, 2009
Imidazoles	MET	Hospital wastewater	0.1-90.2	Lindberg et al., 2004
	TRI	Hospital wastewater	0.6-7.6	Lindberg et al., 2004

^aMIN: Minocycline; MEC: Meclocycline; DMC: Demeclocycline; SDX: Sulfadimethoxine; SCT: Sulfacetamide; SSZ: Sulfisoxazole; SMPD: Sulfamethoxypyridazine; SQX: Sulfaquinoxaline; SAD: Sulfonilamide; SMMX: Sulfamonomethoxine; SMR: Sulfamerazine; NAL: Nalidixic acid; ERY-H₂O: Dehydroerythromycin; AZY: Azythromycin; CLI: Clindamycin; LIN: Lincomycin; VLM: Valnemulin; SAL: Salinomycin; MON: Monensin; AMO: Amoxycillin; PEN: Penicilin; CEP: Cephalexm; CEF: Cefaclor; CEH: Cephapirin; CLO: Cloxacillin; CLOX: Cloxacillin; OXA: Oxacillin; TRI: Trimethoprim

^bWWTP: wastewater treatment plant; ^c agricultural wastewater treatment plant

Improper discharge of STP effluents, surface runoff, and leaching in agricultural areas, aquaculture practices, and irrigation with treated or untreated wastewater are responsible for the contamination of surface and ground waters by antibacterial agents. The amounts of antibiotic substances reported in surface and ground waters from the literature are presented in Table 3.5. As expected from their mobility in the terrestrial environment, which will be discussed in the following section, sulfonamides are the most frequently detected antibiotic class in surface and ground water. On the other hand, in spite of their strong affinity to bind solid matrices, tetracyclines, and fluoroquinolones have also been detected even at ground water samples (Table 3.5).

Table 3.5. Antibiotic substances in surface and ground water samples.

Group	Antibiotic ^a	Source	Concentration (µg/l)	Reference
Tetracyclines	DOX, TC, CTC, OTC	Surface water	0.08-0.6	Watkinson et al., 2009
	OTC	Surface water	up to 0.68	Feitosa-Felizzola and Chiron, 2009
	TC + OTC	Ground water	1	Campagnolo et al., 2002
	OTC, CTC, TC	Surface water	0.07-1.34	Lindsey et al., 2001
	OTC, CTC, TC	Surface water	0.11- 0.69	Kolpin et al., 2002

Table 3.5. (Continued)

Group	Antibiotic ^a	Source	Concentration (µg/l)	Reference
Sulfonamides	SMX	Surface water	0.14	Batt et al., 2008
	SAS, SMX, STZ	Surface water	0.03 – 2	Watkinson et al., 2009
	SMX, SMZ	Surface water	0.015-0.328	Managaki et al., 2007
	SMX	Drinking water source	0.11	Benotti et al., 2009
	SMX	Drinking water	0.003	Benotti et al., 2009
	SMX	Lake water	0.0044-0.0095	Loos et al., 2007
	SPY, SMX	Surface water	0.005-0.06	Kasprzyk-Hordern et al., 2007
	SMX	Surface water	up to 0.07	Wiegel et al., 2004
	SMZ, SDX	Ground water	0.046-0.22	Batt et al., 2006
	SMX	Ground water	0.041-0.11	Ternes et al., 2007
	SMX	Surface water	0.48	Hirsch et al., 1999
	SMX	Surface water	0.072-0.544	Tamtam et al., 2008
	SMX	Surface water	up to 0.082	Choi et al., 2008a
	SDX	Surface water	0.05	Campagnolo et al., 2002
	SMX	Ground water	0.22	Lindsey et al., 2001
	SDX, SMZ, SMX, STZ	Surface water	0.06-15	Lindsey et al., 2001
	SDX, SMZ, SMX,	Surface water	0.06-1.9	Kolpin et al., 2002
Quinolones	NAL, NOR, ENR, CIP	Surface water	0.30- 1.30	Watkinson et al., 2009
	CIP, NOR	Surface water	0.005 – 0.018	Golet et al., 2002
	ENR, CIP	River water	0.067-0.119	Pena et al., 2007
	CIP	Surface water	up to 9.66	Feitosa-Felizzola and Chiron, 2009
	NOR, SAR, ENR, DAN, OFL, ENO, FLU	Surface water	up to 0.163	Tamtam et al., 2008
	SAR	Surface water	4	Campagnolo et al., 2002
	CIP, NOR	Surface water	0.03-0.12	Kolpin et al., 2002
Macrolides	ROX, OLE, TYL	Surface water	0.02- 0.35	Watkinson et al., 2009
	ERY-H ₂ O	Surface water	0.009-0.041	Managaki et al., 2007
	ERY, AZY	Surface water	up to 0.03	Gros et al., 2006
	CLA	Surface water	0.6-2.33	Feitosa-Felizzola and Chiron, 2009
	ROX, ERY-H ₂ O	Surface water	0.56-1.70	Hirsch et al., 1999
	LIN	Surface water	0.5	Campagnolo et al., 2002
	LIN	Ground water	1.4	Campagnolo et al., 2002

Table 3.5. (Continued)

Group	Antibiotic ^a	Source	Concentration (µg/l)	Reference
Lincosamides	ERY-H ₂ O, ROX, TYL	Surface water	0.18-1.7	Kolpin et al., 2002
	CLI, LIN	Surface water	0.01- 0.05	Watkinson et al., 2009
Polyether ionophores	SAL, MON	Surface water	0.15	Watkinson et al., 2009
β-Lactams	AMO, PEN V, PEN G, CEF, CEP	Surface water	0.01- 0.25	Watkinson et al., 2009
	AMO, AMP, OXA, CEH, CLOX	Surface water	0.009-0.011	Cha et al., 2006
	AMO	Surface water	0.039-0.245	Kasprzyk-Hordern et al., 2007
	TRI	Surface water	0.15	Batt et al., 2008
	TRI	Surface water	up to 0.02	Gros et al., 2006
	TRI	Surface water	0.007-0.044	Managaki et al., 2007
	TRI	Surface water	0.2	Hirsch et al., 1999
	TRI	Surface water	up to 0.04	Wiegel et al., 2004
	TRI	Surface water	0.008-0.027	Kasprzyk-Hordern et al., 2007
	TRI	Drinking water source	0.011	Benotti et al., 2009

^aENO: Enoxacin; OXO: Oxolinic acid; FLU: Flumequine; OFL: Ofloxacin; DAN: Danfloxacin; SAR: Sarafloxacin; OLE: Oleandomycin; AMP: Ampicillin

3.3. Fate of Antibiotics in the Environment

3.3.1. Sorption of Antibiotics

Organic carbon normalized sorption coefficients ($\log K_{oc}$) of hydrophobic compounds in soil are generally predicted from their octanol water partition coefficients ($\log K_{ow}$). However, this prediction could not be used for ionizable compounds since a variety of sorption processes additional to hydrophobic partitioning can occur simultaneously (Tolls, 2001). Sorption of ionizable antibacterial agents, strongly influenced by their physicochemical properties as well as the pH, organic matter content, cation exchange capacity, and texture of solid matrices. Solid distribution coefficients of the antibiotics of different structural classes are given at Table 3.6.

Table 3.6. Sorption of antibiotics on different types of solid matrices.

Antibiotic	Matrix	K _d (l/kg)	Notes	Reference
Tetracycline	Humic acid		Metal complexation, ion exchange, and hydrogen bonding with acidic groups of humic acid were the proposed mechanisms for the sorption of tetracycline on humic acid.	Sithole and Guy, 1987a
	pH = 4.55	2,060		
	pH = 6.14	1,430		
Oxytetracycline	Sandy loam, loamy sand, sand soil	417-1,026	A relation between sorption coefficients and soil properties like pH, OC, CEC was reported to be unimportant.	Rabølle and Spliid, 2000
Oxytetracycline	Pig manure		K _{oc} value estimated from K _d was significantly higher than that estimated from K _{ow} values indicating ionic binding of OTC to divalent metal ions as well as hydrophobic partitioning.	Loke et al., 2002
	6h equilibration	83.2		
	24 h equilibration	77.6		
Oxytetracycline	Montmorillonite	59,800 ^a	K _d values decreased with increasing ionic strength for cationic and neutral OTC. When Ca ²⁺ was present on clay surface instead of Na ⁺ , Ca ²⁺ ions compete with OTC for the sorption of clay surface by cation exchange decreasing K _d values at low pH. At high pH where OTC is in anionic form, sorption to clay occurs through Ca bridging.	Figueroa et al., 2004
	Na- Montmorillonite	5,600-70,800		
	Na-kaolinite	1,390-8,700		
Oxytetracycline	Clay sorbents pH (1.5-11.5)	295-676 ^b	K _f values decreased with increasing pH. Sorption mechanisms were cation exchange at low pH and hydrophobic partitioning at pH 5 where OTC is zwitterionic. The introduction of dissolved organic matter at high concentration decreased the antibiotic sorption, which in turn increases its mobility.	Kulshrestha et al., 2004

Table 3.6. (Continued)

Antibiotic	Matrix	K _d (l/kg)	Notes	Reference
Oxytetracycline	Several types of soil pH = 5.5	486-12,047	A positive correlation was found between sorption coefficients and CEC, soil clay content and iron oxide content of soil having OC content up to 4 %. OC effect on sorption was observed in soil with OC of 9 %.	Jones et al., 2005
Oxytetracycline	Humic acid (HA) pH = 5.5	5,500-250,000	K _d values increased with the order clean HA < Al amended < Fe (III) amended HA indicating that the sorption of oxytetracycline to HA achieved by cation bridging.	MacKay and Canterbury, 2005
Oxytetracycline Tetracycline Chlorotetracycline	Several soil types	1,229-269,097 3,102-312,447 5,706-164,973	Positive correlation was found between sorption coefficients, acidity, and clay content of soils. Sorption decreased upon increasing pH except for one type of soil in which OC was high and CEC value dependent on pH. Almost three fold decreases in CEC of this soil was observed as the pH reduced from 7.98 to 5.6. CEC normalized K _d value also decrease with increasing pH for this soil.	Sassman and Lee, 2005
Tetracycline, Chlorotetracycline	Clay, humic substance and clay humic complexes extracted from different soils	10,000-65,000 10,000-∞ ^c	Sorption was higher by clay with Ca ²⁺ ions than with K ⁺ ions at pH 7 indicating the contribution of cation bridging. Sorption also increased with decreasing pH indicating importance of cation exchange mechanism in overall sorption process. TCs primarily sorbed in the interlayers of clays, and humic substances in clay humic complexes inhibited the interlayer diffusion of TCs.	Pils and Laird, 2007

Table 3.6. (Continued)

Antibiotic	Matrix	K _d (l/kg)	Notes	Reference
Tylosin	Sandy loam, loamy sand, sand soil	8.3-128	Stronger sorption was reported in two sandy loam soils with higher clay contents compared to sand soil.	Rabølle and Spliid, 2000
Tylosin	Pig manure 6 h equilibration 24 h equilibration	45.7 240	Instability of tylosin resulting a decline in its concentration under unsterilized condition was shown to be a reason for an increase in K _d values upon increasing reaction time. K _{oc} values estimated from K _{ow} values were similar to K _{oc} values determined by normalization of K _d to organic carbon content in manure. Therefore, a sorption was attributed to hydrophobic partitioning to organic matter rather than ionic binding.	Loke et al., 2002
Tylosin	Silt clay, clay, sand	5.4-6,690	A positive correlation was found between OC, CEC, and clay content of soil and K _d values. Cation exchange, H-bonding, and hydrophobic partitioning were proposed as sorption mechanisms.	Sassman et al., 2003
Tylosin	Swine manure from anaerobic covered lagoon manure solids(< 2 mm), colloidal material(<1.2 µm)	91.2 38.6	Based on the organic carbon normalized distribution coefficients, importance of organic carbon content of manure on tylosin sorption was declared. Different sorption behavior of tylosin with solid and colloidal material was attributed to differences in organic carbon composition.	Kolz et al., 2005a
Sulfachloropyridazine	Sandy loam Clay loam	0.9 1.8	Sorption coefficients in soils increased as pH decreased. The addition of manure decreased the sorption of antibiotic due to increased pH.	Boxall et al., 2002

Table 3.6. (Continued)

Antibiotic	Matrix	K _d (l/kg)	Notes	Reference
Sulfanilamide	Whole soil and particle	1.7	K _d values in fertilized soil were higher than unfertilized one due to higher quantity and different quality of organic matter. Adsorption to soil fractions increased in the order of coarse silt<whole soil< medium silt <sand<clay<fine silt. A positive correlation was found between K _d values, aromaticity, and electronegativity of functional groups of sulfonamides. H-bonding and van der Waals interaction was proposed for sulfonamide-organic matter complexation.	Thiele Bruhn et al., 2004
Sulfadiazine	size fractions	2.0		
Sulfadimethoxine		2.3		
Sulfadimidine		2.4		
Sulfapyridine		3.5		
Sulfamethazine	Na-Montmorillonite		Increasing ionic strength decreased K _d SMZ ⁺ , but increased K _d SMZ ⁻ due to double layer compression and reduced anion repulsion. The sorption of neutral SMZ (pH 5-7) to Ca amended clay exceeded Na amended one suggesting water bridging and cation bridging as sorption mechanisms.	Gao and Pedersen, 2005
	K _d SMZ ⁺	750-2,620 ^d		
	K _d SMZ ^{neutral}	10.3-12		
	K _d SMZ ⁻	0-8.6		
Sulfathiazole	Compost	12,640-75,015 ^e	Strong dependency to sorption time (1d-14 d) and pH (3.5-9) was detected. For K _{oc} ⁺ sorption mechanism was cation exchange. For K _{oc} ^{neutral} , sorption mechanism was H-bonding and van der Waals forces to aromatic parts of organic sorbents.	Kahle and Stamm, 2007a
	Manure	1,543-10,700		
	Humic acid	77,414-364,661		
Sulfathiazole	Loamy sand pH(2.3-5.8)	5.1-48	Ionization was an important factor in sorption and K _d values increased in the order of cationic>neutral>anionic form. K _d values were highest in sandy loam that has higher CEC and OC content relative to other soils.	Kurwadkar et al., 2007
	Sandy loam pH(4.4-8.0)	11-65		
	Loam pH(3.3-7.5)	6.1-33		

Table 3.6. (Continued)

Antibiotic	Matrix	K _d (l/kg)	Notes	Reference
Sulfadiazine	Different types of soil		Manure amendment increased the sorption of sulfadiazine as well as sorption desorption-hysteresis. The desorbable part of sulfadiazine was attributed to weak van der Waals forces and H-bonding, while chemical reactions between aniline group of antibiotic and soil organic matter was reported to be responsible for non-desorbable part of sulfadiazine.	Sukul et al., 2008
	In the absence of manure	0.1-24.3		
	In the presence of manure	6.9-40.2		
Enrofloxacin	Different types of soil	496-3,037	The type and fraction of clay in soil strongly influenced ENR sorption as well as OC content. Montmorillonite has higher affinity for ENR compared to other clays (illite, kaolinite) due to its ability to sorb ENR between the mineral layers while others sorb only on basal surfaces and edges. Ionized COO ⁻ group was reported to be responsible for ENR sorption.	Nowara et al., 1997
	Different types of clay minerals	3,548-6,310		
Flumequine	Humic acid	2,754-24,547 ^f	Hydrophobic partitioning and electrostatic interactions between carboxylic function of 4-quinolones and positively charged amine groups of humic acid have been proposed as sorption mechanisms.	Holten-Lutzhof et al., 2000
Oxolinic acid	pH (3-8)	7,413-31,622		
Sarafloxacin		33,113-154,881		
Ofloxacin	Soil with OC of 7.1%	3,554	In contrast to K _d values, K _{oc} value was higher in low OC soil. Furthermore, hysteresis was more pronounced for low OC soil. Irreversible sorption of ofloxacin in low OC soil was attributed to other matrix properties than organic carbon and dissociation degree of ofloxacin.	Drillia et al., 2005a
	Soil with OC of			
	0.37%	1,192		

Table 3.6. (Continued)

Antibiotic	Matrix	K _d (l/kg)	Notes	Reference
Enrofloxacin	Zeolite		Sorption increased with decreasing pH and increasing temperature. At acidic pH ionic interactions occur between cationic ENR and negative zeolite surface. Ionized COO ⁻ group interact with cations to sorb negative zeolite surface through cation bridging.	Ötöker and Balçioğlu, 2005
	pH = 7	3.87 ^g		
	pH = 10	2.31		
Enrofloxacin	Sandy, loamy sand,	270-970	Sorption increased as the clay content and organic carbon content of soil increased. Hydrophobic interactions with organic carbon seem to be more important than ionic interactions with clay fraction of soil.	Uslu et al., 2008
Ciprofloxacin	sandy loam	410-1,290		
Efrotomycin	Different soil types	8.3-290	Sorption did not correlate with any of soil properties, e.g. pH, organic matter, texture, or CEC.	Yeager and Halley, 1990
Metronidazole	Sandy loam, loamy sand, sand soil	0.54-0.67	Sorption was higher at low antibiotic concentration.	Rabølle and Spliid, 2000
Olaquinox	Sandy loam, loamy sand, sand soil	0.69-1.67	Sorption was higher at low antibiotic concentration.	Rabølle and Spliid, 2000

^a K_d OTC⁺ (L/equiv); ^b K_f; ^c K_d for chlorotetracycline could not be determined since its solution concentration was below detection limit; ^d K_d values change depending upon ionic strength; ^e 1 day and 14 days K_{oc} STZ⁺ (kg/kg) = K_d/OC where OC (kg/kg); ^f D_{DOC} (distribution coefficient for humic acid); ^g K_f values (mg/g)(l/mg)ⁿ.

As can be deduced from Table 3.6, the sorption affinity of antibiotics to different types of solid matrices varies significantly with the structure antibiotics and the properties of sorbents. Tetracyclines and fluoroquinolones could be classified as strongly adsorbed antibiotics among others. The strong tendency of these antibiotic classes for the complexation with divalent and trivalent cations significantly contributes their sorption to soil and manure.

The sorption affinity of antibiotic compounds significantly affects their mobility in the environment. Rabølle and Spliid (2000) reported the high mobility of metranidazole, an imidazoel group antibiotic, and olaquinox, a quinoxaline derivative, as they completely leached through soil columns. However, no transport of tylosin and oxytetracycline was observed in accordance with their K_d values (Table 3.6). Sulfonamides with relatively low distribution coefficients in soils are also classified as highly mobile substances in the environment. Sulfachloropyridazine has been detected at high concentrations (25.9 $\mu\text{g/l}$) while the concentration of oxytetracycline was only 0.9 $\mu\text{g/l}$ in overland flow water 6 days after application to sandy soil (Blackwell et al., 2007). Authors concluded that oxytetracycline prefers to persist in soil and therefore, the possibility for the contamination of aquatic environment with oxytetracycline is substantially low as compared with that of sulfachloropyridazine. In a similar manner, the decrease in the concentration of fluoroquinolone group antibiotics (enrofloxacin, ciprofloxacin or norfloxacin) as the soil depth increase was attributed to their limited mobility to the subsoil (Golet et al., 2003; Uslu et al., 2008).

3.3.2. Biotic Degradation of Antibiotics

Due to their mode of action, antibacterial agents are generally known to resistant to biotic degradation. The biodegradation of tylosin, metranidazole, and oxytetracycline were examined in aerobic and anaerobic surface water simulation systems (Ingerslev et al., 2001). Half lives ($t_{1/2}$) of antibiotics were ranged for tylosin from 9.5 to 40 days, for metranidazole from 14 to 104 days, and for oxytetracycline from 42 to 46 days depending on the bacterial culture used for the inoculation in simulated systems. The biodegradability of different antibiotics was assessed by performing 28 days closed bottle test with the inoculums of sewage treatment plant of hospitals (Al Ahmad et al., 1999; Kümmerer et al.,

2000). None of the compounds was readily biodegradable at the end of the test. No degradation of sulfamethoxazole, ciprofloxacin, and ofloxacin was observed even the test period was prolonged to 40 days while those of metranidazole, cefotiam and penicillin G were 5 %, 10 % and 36 %, respectively (Al Ahmad et al., 1999; Kümmerer et al., 2000). Although the antibiotic substances were not biodegradable, some of the compounds were completely eliminated due to other physical or chemical processes after 28 days of test period. In another study, Richardson and Bowron (1985) addressed the biodegradation of some antibiotics in tests with a high bacterial density and classified the compounds from non-biodegradable (e.g. Erythromycin) to 48% degradable (Ampicillin).

In manure and soils, microorganisms are mainly responsible for the degradation of antibacterial agents. However, biodegradation of some antibiotics 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 transformation of the half of enrofloxacin in cattle manure was achieved in 90 days and 15 % of enrofloxacin was remained even after 210 days (Wetzstein et al., 2002). In a similar manner, Winckler and Grafe (2001) reported very long periods (55-105 days) for the half life of tetracycline antibiotics in pig slurry. In another study, although the half of oxytetracycline initial concentration degraded in manure bedding mixture within 30 days, it has been shown to be still detectable in bedding pig manure mixture at a concentration of 0.82 mg/kg after 5 months maturation (De Liguoro et al., 2003). On the other hand, a shorter half life ($t_{1/2} = 8.1$ days) was also reported for the oxytetracycline in manure with 80 % moisture content by Wang and Yates (2008). However, the availability of the compound for degradation decreased exponentially with time, which makes their residuals stable in manure. Although the increment of moisture and temperature increased the availability and subsequently, the degradation rate of oxytetracycline, in a water saturated manure, which represents fresh manure, it was very persistent (Wang and Yates, 2008). The persistence of oxytetracycline in calf manure was also reported during 64 days of anaerobic digestion at 35° C with the formation of several oxytetracycline metabolites (Arıkan et al., 2006). Similarly, slower degradation of tetracycline under anaerobic conditions compared to aerobic conditions was found in pig manure (Kühne et al., 2000). Another manure treatment process, composting resulted in more than 99.8 % removal of extractable oxytetracycline after 37 days of treatment while levels of extractable antibiotic in incubated manure decreased only 12 % at

room temperature (Arikan et al., 2007). However, they could not differentiate the reason of degradation during composting whether it is caused by biological degradation, binding to the compost matrix or elevated temperature. During the composting of turkey litter, almost complete removal (> 99%) of chlorotetracycline has also been demonstrated within 22-35 days (Dolliver et al., 2008).

Significantly, different results were reported for the fate of sulfonamide group antibiotics in manure as in case of tetracyclines. The biodegradation was found to be responsible for the degradation of sulfonamide group antibiotic, sulfadimethoxine, in manure since the degradation rate constant in nonsterile manure ($t_{1/2} = 2.56$ days) was significantly higher than that in sterile manure ($t_{1/2} = 10.2$ days) under aerobic conditions (Wang et al., 2006). Similar to oxytetracycline degradation, increasing moisture or temperature were found to increase sulfadimethoxine degradation in manure. On the other hand, with an increase in the initial concentration of antibiotic, the bioactivity of the degrading microorganisms inhibited and then the degradation rate constant of the antibiotic was reduced. In spite of very short half lives of sulfadimethoxine reported by Wang et al. (2006), De Liguoro et al. (2007) detected 0.39 mg/kg sulfadimethoxine in manure after three months of maturation. Moreover, no degradation of another sulfonamide antibiotic, sulfamethazine was observed in manure during the storage or composting (Dolliver et al., 2008).

Tylosin A which is a macrolide group antibiotic was reported as an easily degradable compound in several manure samples. The reported half life of tylosin A in methanogenic manure containing system was less than 2 days (Loke et al., 2000). However, it was not clear whether the biological reactions or sorption is responsible for the dissipation of tylosin, since authors measured only the aqueous phase antibiotic concentration. Oliveira et al. (2002) observed that the degradation of tylosin in liquid manure was rapid during the first 10 days of a laboratory microcosm study and slowed to a steady rate through the formation and accumulation of metabolites in subsequent sampling events. The half life of tylosin was found as 2.4 days which was similar to that (3.6 days) reported earlier by De Liguoro et al. (2003). Similarly, a recent study by Kolz et al. (2005b) found that 90 % of tylosin degraded in anaerobically incubated manure lagoon slurries within less than 5 days.

Aeration of the slurry reduced the degradation period to less than 26 h. However, they detected residual amounts of tylosin in manure even after eight months of incubation.

In spite of rapid degradation rates, the complete elimination of antibiotics could not be always achieved as indicated above and the residuals of them might remain in manure even after very long time periods. Since antibiotics are released to agricultural areas through the application of contaminated manure, their persistence in different soil types has also been studied extensively. For instance, no degradation of oxytetracycline was observed in manure amended soil for 180 days (van Gool, 1993). In another study, total amount of tetracyclines have been demonstrated to decline slowly in manure amended soil within five months after fertilization (Aga et al., 2005). These findings have been confirmed by a recent study in which oxytetracycline was found to have 50 % and 90 % dissipation within 21.7 days and 98.3 days, respectively (Blackwell et al., 2007). For chlorotetracycline half lives in sandy loam and sandy soils have been reported as 25 days and 34 days, respectively (Halling-Sørensen et al., 2005).

The persistence of fluoroquinolone antibiotics due to their strong sorption have also been demonstrated in soil. Ciprofloxacin and norfloxacin have been shown to persist over 21 months in sludge treated soil in the $\mu\text{g/kg}$ range (Golet et al., 2003). Similarly, in different soil types (loam, silt loam and sandy loam), only about 0.6 % of CO_2 was produced from sarafloxacin, another common fluoroquinolone veterinary antibiotic, within 80 days (Marengo et al., 1997).

The residuals of sulfonamide antibiotics have also been found to persist in the terrestrial environment despite of their relatively low half dissipation times. In manure amended sandy loam soil, 50 % and 90 % dissipation times of sulfachloropyridazine were 3.5 and 18.9 days, respectively (Blackwell et al., 2007). However, the antibiotic was still detectable within the top 20 cm of soil even after 127 days. In a similar manner, in spite of initial rapid degradation of several sulfonamides in manure amended soil, more than 15 % of the initial concentration was found to be persistent in three months after manure application (Stoob et al., 2007). In another study, Acinelli et al. (2007) compared the dissipation of sulfonamide antibiotics (sulfamethazine and sulfachloropyridine) in non-amended and manure amended soils. Authors found that the application of 5 % liquid

manure to soil decreased the persistence of antibiotics due to higher microbial activity and the lower sorption of sulfonamides, which makes them more available.

The degradation of macrolide antibiotics, e.g. tylosin and erythromycin, in soil was quite fast and their half lives ranged between 4-20 days (Gavalchin and Katz, 1994; Ingerslev and Halling-Sørensen, 2001; Schlüsener and Bester, 2006). In a recent study in which the dissipation of various antibiotics in manure amended sandy loam soil was evaluated (Blackwell et al., 2007) no observable tylosin residues was detected in soil and leachate samples during 127 days treatment indicating the low persistence of tylosin in soil. On the contrary, longer half lives (up to 67 days) were reported for tylosin A in a study where in situ degradation of it was assessed in Danish sandy soils (Halling Sørensen et al., 2005). Hu and Coats (2007) found that the half of tylosin A concentration was transformed in soil within 8 days either in sterilized or unsterilized conditions. Therefore, the loss of tylosin concentration was attributed to abiotic degradation or sorption rather than biodegradation.

Besides manure and soil, the degradation of some antibiotics has also been evaluated in aquatic sediments and sewage sludges. In aquatic sediments, the insignificant degradation of sulfonamides, tetracyclines, and fluoroquinolones was determined over a period of 180 days (Samuelsen et al., 1994; Hektoen et al., 1995). As opposed to these results, very rapid degradation of several sulfonamides have been reported ($t_{1/2}$ = 0.2-3 days) in sewage sludge with bacterial culture adapted to sulfonamide (Ingerslev and Halling-Sørensen, 2000). On the other hand, non-adapted sludge was able to degrade sulfonamides within a few days only after lag phases of 7 to 10 days at 20°C (Ingerslev and Halling-Sørensen, 2000).

3.3.3. Abiotic Degradation of Antibiotics

Aside from the biodegradation, abiotic processes such as photodegradation can play an important role in the determination of the fate of antibiotics in the environment.

In spite of their stability in solid matrices, tetracyclines and fluoroquinolones are highly susceptible to photodegradation in water. Burhenne et al. (1997) reported that the

photodecomposition of various fluoroquinolones (enrofloxacin, danofloxacin, ciprofloxacin, and norfloxacin) occurred with a half life range of 20.6 - 105.9 min in aqueous solution. In another study, both oxytetracycline and quinolone antibiotics, flumequine and oxolinic acid, degraded by losing their antibacterial activity in seawater after exposure to sunlight for 21 days at sea level (Lunestad et al., 1995). In a recent study, 70 % of oxytetracycline was photodecomposed in sea water after 14 days of light exposure while those of flumequine and oxolinic acid were only 10 % under sterilized conditions (Pouliquen et al., 2007). Turiel et al. (2005) compared the photodegradation of oxolinic acid and ciprofloxacin antibiotics in pure and river water. The rapid degradation of ciprofloxacin compared to that of oxolinic acid was observed confirming the previous studies and this observation was attributed to their structural differences in piperazinyl ring. The authors also observed the lower degradation of both antibiotics in river water samples and explained it by the presence of humic substances, which is known to decrease the photodegradation rate of fluoroquinolones.

Depending on the season and latitude, a half dissipation time of amoxicillin, a β -lactam group antibiotic, was 2-9 days range in pure water and the photodegradation rate increased with increasing pH (Andreozzi et al., 2004a). Another β -lactam antibiotic, ceftiofur sodium, was relatively resistant to photodegradation in water with the half life of about 30 days (Gilbertson et al., 1990). Sulfonamide group antibiotics were found to be highly resistant to photodecomposition and showed no degradation during 21 days of light exposure, either in surface or bottom layer in sea water (Lunestad et al., 1995). Similarly, the photodegradation of tylosin in pure and pond water was very slow with the average half life of 200 days.

Hydrolysis is another common process, which is responsible for the degradation of antibiotics in water. β -lactams generally undergo hydrolysis very quickly under acidic and basic conditions (Hou and Poole, 1969). Hydrolysis also contributes to the limited stability of tetracyclines in natural waters as well as photodegradation. In a study of Pouliquen et al. (2007), the average degradation rate of oxytetracycline was reported as 20 % in deionized water, freshwater, and seawater after 14 days period and it was found to be increased upon increasing pH. On the other hand, flumequine and oxolinic acid, were considerably resistant to hydrolysis with no significant degradation within this time period. In a recent

study (Loftin et al., 2008), a similar sensitivity of tetracycline group antibiotics to pH mediated reactions, such as hydrolysis and epimerization in water has been reported. The hydrolysis rates of tetracyclines were affected by temperature and pH significantly but not by ionic strength. Authors also observed the degradation of tylosin through hydrolysis at low (pH = 2) and high (pH = 11) pH values. On the contrary, the degradation of several sulfonamide antibiotics, trimethoprim and lincomycin were not observed under varying pH, temperature, and ionic strength conditions.

3.4. Adverse Effects of Antibiotics in the Environment

3.4.1. Toxic Effects to Aquatic and Soil Organisms

Specific design of antibiotics to treat infections can make them potentially detrimental to some bacteria and to some other microorganisms in the environment. The presence of the antibiotics in the aquatic and terrestrial environment triggered the interest on studies about the toxic effect of these substances on various organisms.

Lanzky and Halling-Sørensen (1997) investigated that metranidazole exhibited a toxic effect on aquatic organisms (*Chlorella sp.* and *Selenastrum capricornutum*) with 72 h EC₁₀ values of 2.03 and 19.9 mg/l. The study of Halling-Sørensen (2000) about the toxic effect of veterinary antibacterial agents towards algal species clarified that the veterinary antibiotics, benzylpenicillin, tetracycline, chlorotetracycline, olaquinox, tylosin, streptomycin, tiamulin, and spiramycin are toxic with the effective concentrations of (EC₅₀) 0.005 - 5.1 mg/l range to algae cyanobacteria and 0.133-40 mg/l range to green algae (except penicillin which shows no effect up to 100 mg/l). Acute toxicity of oxytetracycline and florfenicol to another algae type *Tetraselmis chuii* has been reported with 96 h IC₅₀ values of 11 and 6 mg/l.

Aside with algae, *Daphnia magna* was also used as a test organism for the evaluation of the toxicity of antibiotics in the environment. Wollenberger et al., (2000) worked with several veterinary antibiotics and found that, oxolinic acid and tiamulin had an acute toxic effect on *Daphnia magna* while oxytetracycline, sulphadiazine, and tetracycline led to reproductive toxicity in the mg/L range. A significant acute toxicity of furazolidone on

Daphnia magna and *Artemia salina* has been investigated by Macri et al., (1988). Migliore et al., (1997) demonstrated the toxicity of several agricultural antibiotics including aminosidine, bacitracin, erythromycin, flumequine and lincomycin, to *Artemia salina*.

In addition to aquatic organisms, the effects of the veterinary antibiotics, oxytetracycline and tylosin on the reproduction of soil organisms and animals have also been studied. For instance, the significant effects of oxytetracycline on soil microbial enzymatic activities (phosphates, dehydrogenase) were observed even at low concentrations (1-10 mg/kg) (Boleas et al., 2005). In another study, which is conducted by Thiele-Bruhn and Beck (2005), the effective doses of (ED₁₀) sulfapyridine and oxytetracycline influencing soil microbial activity as determined by Fe (III) reduction test were ranged between 0.003-1.14 mg/kg and 5.5-7.35 mg/kg, respectively.

The effects of antibiotics on soil organisms are essentially influenced by their bioavailability that depends on the soil properties and the availability of nutrients. The reduction in the antibiotic potency due to sorption is possible (Jjemba, 2002). Chander et al. (2005) found that the antimicrobial activity of oxytetracycline and tylosin adsorbed on soil was higher in loamy sand soil than that in clay loam due to the higher affinity of antibiotics for clay loam.

In addition to parent compounds, the degradation products of antibacterial agents may also exhibit significant potency to different organisms. For example, various metabolites of tetracyclines showed significant bacterial toxicity in sewage sludge and soil (Halling-Sørensen et al., 2002). On the other hand, lower potencies of the degradation products of tylosin, sulfadiazine, ciprofloxacin, olaquinox, and streptomycin than those of parent compounds have been reported (Halling-Sørensen et al., 2003).

Besides their effects on aquatic and terrestrial organisms, the inhibition of several antibiotics on sewage sludge bacteria (Halling-Sørensen, 2001; Halling-Sørensen et al., 2003) and methane production in anaerobic manure treatment reactors (Masse et al., 2000; Loftin et al., 2005; Arıkan et al., 2006) at mg/l ranges have been demonstrated.

3.4.2. Plant Uptake

The uptake of antibiotic by plants is mainly controlled by the structure of compound and the type of plant. For example, among three types of plants, the maximum amount of chlortetracycline has been detected in corn, followed by cabbage and green onions (Kumar et al., 2005b). On the other hand, tylosin which is expected to have higher bioavailability to plants due to its relatively low sorption affinity could not be detected in crops. Authors explained this observation by considerably large molecular structure of tylosin that could not be taken by plants easily. Another example to variable responses of different plants to antibiotics has been given in a study of Batchelder et al. (1982). They have found that pinto beans were negatively affected by chlortetracycline and oxytetracycline, while corn was remained unaffected. Furthermore, the nutrient uptake of radish and wheat was higher in the presence of antibiotics. Sulfonamides were found to affect rice growth in soil significantly, whereas, no inhibitory effects of tetracyclines were observed for this plant (Liu et al., 2009). The higher bioavailability of sulfonamides due their low sorption to soil compared to tetracyclines was proposed for their higher inhibition in soil.

3.4.3. Development of Antibiotic Resistance

The main concern regarding the presence of antibiotic residuals in aquatic and terrestrial environment is the development of resistant bacteria strains representing a health risk to humans and animals. Antibiotic resistance is a threat for humans as they are exposed to antibiotic residuals in different environmental compartments. Moreover, via the consumption of contaminated crops, livestock, fish, and water antibiotic resistance genes could be directly transferred to humans (Batt et al., 2006). The presence of antibiotics at sub therapeutic concentrations and over a long period of time cause the development of resistance and they also facilitate the transfer of resistance genes (Kümmerer, 2004). Furthermore, the ability of bacteria to adapt different environmental conditions may also favor resistance.

The antibiotic resistance could emerge in hospital effluents, municipal sewage, activated sludge of sewage treatment plants, aquaculture, wastes from animal feeding operations, and manure as they are directly exposed to antibacterial residuals. The

contamination of terrestrial and aquatic environment with antibiotics through these sources makes them also an important reservoir for resistant bacteria. Furthermore, the resistance against antibiotics that occur naturally plays an important role in the population dynamics in soils (Kümmerer, 2004). Some examples for different environmental compartments in which antibiotic resistance was detected are listed in Table 3.7.

Table 3.7. Antibiotic resistance in the environment.

Source/Bacteria	Antibiotic	Notes	Reference
Soil	Tetracycline	Antibiotic resistance in soil bacteria was increased with elevated amount of pig slurry application, however, reduced during sampling period.	Sengelov et al., 2003
Turkey manure	Multiple antibiotics	75.4 % and 41.4 % resistances were detected in E.coli and Pasteurella sp, respectively in farms where antibiotics have been used, whereas resistances in bacteria were reduced to 50.8% (E.coli) and 19.6 % (Pasteurella sp) in non antibiotic user farm.	Chander et al., 2008
Sewage sludge	Tetracycline	The production and growth rate of resistant bacteria significantly increased upon increasing influent tetracycline concentration in wastewater.	Kim et al., 2007
Dairy farm manure, water receiving farm runoff, dairy farm soil, garden soil, lake by hospital, public park water canal, residential lake	Tetracycline, Ampicillin, Penicillin G, Vancomycin, Streptomycin	Proportion of bacteria that is resistant at least one of the mentioned antibiotics are ranged between 1.5-95%. Among all antibiotics, the highest resistance was against tetracycline ranging from 47 to 89% of total bacteria.	Esiobu et al., 2002
<i>Enterococcus spp</i> in water and sand of marine beaches	Streptomycin, Erytromycin, Tetracycline, Gentamicin, Ampicillin, Vancomycin	Highest resistance was reported against streptomycin (38.5%) in water, and against erythromycin (38.1%) in sand.	Oliveira et al., 2008.

Table 3.7. (Continued)

Source/Bacteria	Antibiotic	Notes	Reference
<i>Campylobacter spp</i> in cattle farms	Ciprofloxacin Nalidixic acid Erythromycin Doxycycline	Among several antibiotics, most frequent resistance has been found to doxycycline and detected in 42.3 % of 350 isolates.	Bae et al., 2005.
Hospital effluents	Multi antibiotic resistance	Frequency of resistant bacteria could be increased through the extreme use of antibiotics in hospitals.	Chitnis et al., 2000
Hospital wastewater biofilms	Vancomycin	Vancomycin and β -lactam (ceftazidime, cefazolin, penicillin G)	Schwartz et al., 2003
Municipal sewage biofilms	β -lactams	resistant heterotrophic bacteria have been detected in all biofilms.	
River water biofilms			
Drinking water biofilms			
<i>E. coli</i> in sewage and sludge	β -lactams, Quinolones, Tetracyclines, Sulfonamides, Aminoglycosides	The highest resistance rates in β -lactams were found for ampicillin (up to 18%) and cefalothin (up to 35%) and in the group of quinolones for nalidixic acid (up to 15%); and for trimethoprim/sulfamethoxazole (up to 13%) and for tetracycline (57%). STP receiving sewage from hospital has been reported to have higher <i>E.coli</i> resistance than that receives only municipal sewage.	Reinthal et al., 2003
Groundwater near	Tetracycline	Both tetracycline residues and tetracycline resistant genes have been detected in groundwater, which is impacted by swine confinement facilities that use lagoons for manure storage and treatment.	MacKie et al., 2006

Table 3.7. (Continued)

Source/Bacteria	Antibiotic	Notes	Reference
Soil	Tetracyclines	The field study in Swiss showed high diversity of tetracycline and sulfonamide resistance genes even in the absence of slurry amendment. Authors conclude that soil is an important reservoir for resistant genes.	Schmitt et al., 2006
	Sulfonamides		
Fish farm sediment	Oxytetracycline	At the end of 10 d medication of fish with oxytetracycline, resistant bacteria in sediments have been found more than 100 % with subsequent decrease to 20 % after 72 d and remained constant at levels of between 10 and 50%.	Samuelsen et al., 1992.
<i>Salmonella</i> in pig slurry	Tetracycline	Resistance to tetracycline (100%), sulfonamides (84.6%), furazolidone (38.5%), nalidixic acid (15.4%) and streptomycin (15.4%) has been demonstrated.	Watabe et al., 2003
	Sulfonamides		
	Furazolidone		
	Nalidixic acid		
	Streptomycin		

4. TREATMENT OF ANTIBIOTICS IN WATER AND WASTEWATER

Considering the adverse effects of the presence of antibiotic residues in different environmental compartments, their removal from water and wastewater has gained importance. For this purpose, the effectiveness of several treatment technologies including conventional (e.g. biological treatment, chlorination, ozonation, and adsorption) and non-conventional (e.g. reverse osmosis, advanced oxidation processes) has been investigated.

4.1. Conventional Treatment Methods

4.1.1. Biological Treatment

Although most of antibiotics have been reported to be not readily biodegradable (Kümmerer et al., 2000; Al-Ahmad et al., 1999; Ingerslev et al., 2001), the efficiencies of biological treatment methods for the removal of antibacterial agents varied considerably depending on type of antibiotics and treatment processes. While for macrolides the efficiency of upflow anaerobic reactor was as high as 95 % (Chelliapan et al., 2006), it was ranged between 26 and 48 % in different STPs with activated sludge, oxidation ditch and chemical enhanced treatment (Xu et al., 2007).

The complete removal of two different sulfonamide antibiotics, sulfamerazine and sulfamethoxazole in aerobic and anaerobic reactors was reported (Sponza and Demirden, 2007; Drillia et al., 2005b). On the contrary, sulfamethoxazole was detected both in influent and effluent of a sewage treatment plant at high ng/l ranges (Alexy et al., 2006). Furthermore, some of sulfonamide metabolites were reported to be transformed to the parent compound during conventional wastewater treatment process resulting in an increase in the antibiotics of treated wastewater (Göbel et al., 2007).

On the other hand, fluoroquinolones and tetracyclines have been eliminated from sewage due to their sorption to the sludge during biological treatment processes in sewage treatment plants (Golet et al., 2003; Lindberg et al., 2005; Gulkowska et al., 2008).

Similarly, main mechanism for the removal of oxytetracycline in activated sludge was found to be sorption but not biodegradation (Kim et al., 2005).

4.1.2. Adsorption

Several types of adsorbents have been used for the removal of antibiotics from water. Powdered activated carbon (PAC) was found to be highly effective on the removal of sulfonamide group antibiotics from natural water (Adams et al., 2002; Choi et al., 2008b). While the removal efficiencies of sulfonamides by PAC depended on their hydrophobicities and ranged between 22-100 %, that of tetracyclines were more than 98 % regardless of their structures (Choi et al., 2008b). On the other hand, only 60 % of penicillin G could be eliminated from water by using PAC (Aksu and Tunc, 2005). Granulated activated carbon (GAC) easily removed more than 90 % of some types of tetracyclines (oxytetracycline, tetracycline, democlocycline) from river water, whereas efficiency was reduced (< 70 %) for other types of tetracyclines (minocycline, meclocycline) (Choi et al., 2008c).

Treatment of antibiotic containing waters via different kinds of resins and polymers has also been reported in the literature. Anionic MIEX[®] resin was found to be an efficient ion exchange material for the removal of anionic sulfonamides and zwitterionic tetracyclines at neutral pH (Choi et al., 2007b). MIEX treatment seemed to prefer hydrophilic and electronegative antibiotics. Robberson et al. (2006) evaluated the adsorption of quinolone group antibiotic, nalidixic acid onto polymers. The neutral type of polymers exhibited high affinity for the neutral form of nalidixic acid whereas the adsorption of anionic form of nalidixic acid occurred on the anion-exchange polymers. Additionally, the results of adsorption experiments suggested that the aromatic ring interaction between nalidixic acid and the surface of polymers were important in the adsorption process.

4.1.3. Chlorination and Chlorine Dioxide Oxidation

Since the possibility of the complete degradation of most antibiotics via biological treatment is quite low and adsorption process does not offer their degradation, chemical

oxidation processes such as chlorination and chlorine dioxide (ClO_2) oxidation have been applied to antibiotic containing water samples in order to provide their complete degradation. Adams et al. (2002) reported a rapid disappearance of trimethoprim in deionized ($t_{1/2} = 2.8$ min) and river water ($t_{1/2} = 4.3$ min) dosed with 1.0 mg/l of free available chlorine. In accordance to these results, the half-life of trimethoprim both in chlorinated water with free available chlorine dose of 2 mg/l has been reported as 7.3 min (Dodd and Huang, 2007).

For the simultaneous and complete removal of five sulfonamide antibiotics in water, 25 mg/l free chlorine dose was required while that dose was increased to 700 mg/l in untreated swine lagoon wastewater due to the consumption of chlorine by the natural organic compounds of wastewater (Qiang et al., 2006).

Amoxicillin and cefadroxil which are β -lactam antibiotics have been completely degraded in water at pH 8 by the application of ClO_2 within less than 4 min (Navalon et al., 2008). For sulfonamide antibiotics ClO_2 oxidation with a dose of 0.1 mg/l resulted in more than 95 % removal 30 min, while for macrolides it was more than 80 % (Huber et al., 2005a).

4.1.4. Ozonation

Ozonation is another chemical oxidation method applied to water and wastewater for the removal of several classes of antibacterial agents. The effectiveness of ozone treatment is pH dependent due to changes in the antibiotic moieties related with pKa values (e.g. protonation, deprotonation) as well as oxidation reaction mechanism. Dodd et al. (2006) performed the ozonation of roxithromycin at different pH values in the presence of radical scavenger and the rate of reaction was found strongly dependent on the pH of process. It was suggested that molecular ozone could attack to the neutral tertiary amine group of this macrolide group antibiotic and decreasing pH could result in the protonation of this group thereafter, it caused the reduction of oxidation reaction rate.

In a similar manner, the studies related with the degradation of roxithromycin and lincomycin by ozone showed that the reaction was pH dependent and second order rate

constants for both antibiotics were higher than $10^5 \text{ M}^{-1}\text{s}^{-1}$ at $\text{pH} \geq 7$ (Huber et al., 2003; Qiang et al., 2004). Similar rate constants were also reported for the ozonation of lincomycin in water by Andreozzi et al. (2006).

The complete removal of three different macrolide antibiotics, clarithromycin, erythromycin, and its metabolite dehydroerythromycin in biologically pretreated wastewater was attained by the application ozonation process with a ozone dose range of 3.5-5 mg/l at pH 7 (Ternes et al., 2003; Huber et al., 2005b). Similar to the study of Dodd et al. (2006), Huber et al. (2005b) suggested a reaction between tertiary amine nitrogens in azithromycin and molecular ozone for the degradation of macrolide antibiotic.

Adams et al. (2002) investigated the degradation of sulfonamide group of antibiotics, (sulfachloropyridazine, sulfamethazine, sulfadimethoxine, sulfamerazine, sulfathiazole) by ozonation in a pre-filtered river water sample. More than 95 % removal of sulfonamide was achieved by ozonation with an absorbed ozone dose of 0.3 mg/l at pH 7.5. The complete or near complete elimination of sulfamethoxazole in biologically treated municipal wastewater with the applied ozone dose of 5 mg/l was reported by Ternes et al. (2003) and Huber et al. (2005b). The second order rate constant for the ozonation of sulfamethoxazole in natural river water at pH 7 was reported as $2.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ (Huber et al., 2003). In this study, the dissolved organic matter content of water was found to slow down the sulfamethoxazole degradation. It was suggested that aniline nitrogen was the primary target of molecular ozone attack in the sulfamethoxazole molecule. Same reaction pathway was also proposed by Abellan et al., (2008) who obtained the complete degradation of sulfamethoxazole in water with an ozone dose of 0.05 g/min within 10 min. In another study, 0.4 g/l ozone dose provided 98.6 % removal of sulfamethoxazole with 10 % of mineralization (Dantas et al., 2008). In a similar manner, Dalmazio et al. (2007) observed very low TOC removal after 120 min ozonation reaction, where tetracycline was eliminated almost completely through cyclo addition of ozone at carbon-carbon double bond, suggesting the failure of ozone treatment on mineralization of tetracycline. In another recent study, the complete and fast degradation of oxytetracycline in aqueous solution by the application of 36 mg/l ozone dose have been reported with a pseudo first order reaction rate constant of 4.18 min^{-1} (Seven-Yalap and Akmeahmet-Balcioğlu, 2009).

Authors also observed a great inhibition of natural water components on the degradation rate of oxytetracycline.

Although complete antibiotic removal is important for the efficiency of process, one should consider the toxicity of the degradation products of antibiotic, because of limited mineralization of antibiotics (Dalmazio et al., 2007; Dantas et al., 2008). Abellan et al. (2008) investigated the acute toxicity of the degradation products of sulfamethoxazole and found that some of the intermediates are more toxic to *Daphnia magna* than the parent sulfamethoxazole. Similarly, according to study of Dantas et al. (2008), the degradation products of sulfamethoxazole exhibited slightly higher acute toxicity to *V. fischeri* in the initial period of ozonation. In another study, by-products of oxytetracycline produced by partial oxidation within 5-30 min were also found to be more toxic than the parent compound (Li, K et al., 2008).

Another parameter for the evaluation of ozonation performance for antibiotic treatment is their biodegradability in water. In spite of slight increases in bacterial toxicity, ozonation by products of several antibiotics have been reported to be more biodegradable in terms of BOD₅/COD ratio (Balcioğlu and Ötger, 2003; Arslan-Alaton and Dogruel 2004; Balcioğlu and Ötger, 2003; Li, K et al., 2008; Dantas et al., 2008). Ozone treatment at pH 7 has been suggested to be a promising method for the reduction of ciprofloxacin bacterial resistance due its attack on quinolone core, which is essential for antimicrobial activity (Dewitt et al., 2009).

Ozone has also been demonstrated as an efficient treatment method for adsorbed enrofloxacin on zeolite (Ötger and Balcioğlu, 2005).

4.2. Non-Conventional Treatment Methods

Non-conventional treatment methods used for the removal antibiotics in water and wastewater could be classified as advanced oxidation processes (AOPs) and membrane technologies. AOPs based on the generation of reactive radicals having high oxidation potentials via various chemical (O_3/OH^- , $\text{O}_3/\text{H}_2\text{O}_2$, $\text{Fe}^{2+}/\text{H}_2\text{O}_2$), photochemical (UV / H_2O_2 , UV / O_3), and photocatalytic (UV / TiO_2) processes.

4.2.1. O_3/OH^- , $\text{O}_3/\text{H}_2\text{O}_2$ Treatments

As mentioned in previous section one of the reasons for the strong pH dependency of ozonation efficiency is the formation of more reactive and non selective hydroxyl radicals. Higher ozonation efficiency at $\text{pH} \geq 7$ as a consequence of hydroxyl ion accelerated ozone decomposition have been reported for tetracycline (Li, K et al., 2008), fluoroquinolones, cephalosporine and penicillin group antibiotics (Balcioğlu and Ötker, 2003). Moreover, a decrease in the initial pH value (11.5) of penicillin formulation wastewater during ozonation was found to decrease the efficiency presumably due to reduction in hydroxyl radical generation (Arslan-Alaton and Dogruel, 2004). Therefore, the use of phosphate buffer at pH 11.5 increased COD removal through the prevention of pH reduction during ozonation process. In some cases, however, neutral pH was reported to be more efficient than alkaline pH in spite of higher concentrations of hydroxyl ions in the latter case (Balcioğlu and Ötker 2003; Li, K et al., 2008). The radical scavenger effect of antibiotic degradation products or other constituents of water and wastewater at alkaline pH was denoted as a reason of this observation. On the other hand, Dewitte et al. (2009) observed the fastest ciprofloxacin degradation at pH 3 among pH values of 3, 7 and 10. They explained this result by the possible direct reaction of ozone at the quinolone moiety or by the enhanced degradation by the reactive species at low pH.

The combination of hydrogen peroxide with ozone is known to accelerate hydroxyl radical generation. Accordingly, introduction of an optimum dose of hydrogen peroxide (20 mM) to ozonation system at $\text{pH} = 7$ with an applied ozone dose of 2.96 g/l, improved the COD and aromaticity removal of penicillin formulation wastewater from 69 % and 29 % to 95 % and 90 %, respectively (Balcioğlu and Ötker, 2003). However, this contribution

was very low for enrofloxacin containing wastewater which is already very reactive with ozone itself. In a more recent study, the addition of an optimum hydrogen peroxide dose increased ciprofloxacin removal only by 13.5 % compared to ozonation without hydrogen peroxide (Dewitte et al., 2009).

4.2.2. $\text{Fe}^{2+}/\text{H}_2\text{O}_2$, $\text{Fe}^{2+}/\text{H}_2\text{O}_2/\text{UV}$, and $\text{UV}/\text{H}_2\text{O}_2$ Treatments

Catalyzed hydrogen peroxide is extensively used for the treatment of recalcitrant organic compounds including antibiotics in water and wastewater. The decomposition of hydrogen peroxide to produce hydroxyl radicals is generally achieved by the addition of Fe^{2+} (Fenton process), by the introduction of UV light or by a combination of them (Photo-Fenton).

Tünay et al. (2008) applied Fenton oxidation to cefuroxime axetil, ceftriaxone and sulfisoxazole formulation wastewaters (COD = 1000 mg/l for each) and achieved considerably high (80-93 %) COD removal at the optimum conditions (pH=2; $\text{Fe}^{2+}/\text{H}_2\text{O}_2 = 0.49$ g/g; $\text{H}_2\text{O}_2/\text{TOC} = 2-6$ g/g).

Andreozzi et al. (2004b) investigated the efficiency of UV catalyzed hydrogen peroxide treatment of ofloxacin (0.56 mg/l) in aqueous solution. 5-10 mM hydrogen peroxide in combination with low pressure Hg lamp was found to be sufficient for the complete elimination of antibiotic and reduction of its algal toxicity. Same treatment process was used to produce hydroxyl radicals for the degradation of amoxicillin and the second order rate constant was calculated as $3.93 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Andreozzi et al., 2005). Similar rate constant which is $5.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ was determined by Huber et al. (2003) for the oxidation of sulfamethoxazole by hydrogen peroxide/UV treatment at pH 7. The degradation of same antibiotic substance (200 mg/l) in aqueous solution was also conducted by photo Fenton process in which three black light blue lamps (8 W), Fe^{2+} (10 mg/l), H_2O_2 (300 mg/l) were used at pH 2.8 (Gonzalez et al., 2007). The complete elimination of this antibiotic and its toxicity on pure bacteria and activated sludge bacteria were obtained with a considerable increase in biodegradability (increase in BOD_5/COD ratio from 0 to 0.3) at the forementioned optimum conditions (Gonzalez et al., 2007).

Biodegradability improvement in terms of BOD₅/COD ratio (from 0.1 to 0.45) of penicillin containing formulation effluent by the photo Fenton process ($\text{Fe}^{3+} = 1.5 \text{ mM}$ and $\text{H}_2\text{O}_2 = 25 \text{ mM}$, $\text{pH} = 3$ with UV-A light) was also reported by Arslan-Alaton and Gurses (2004). The degradation of amoxicillin, another penicillin group antibiotic, was investigated in deionized water and sewage treatment plant by means of photo Fenton process using different type of iron salts and light source (Trovo et al., 2008). Under black light irradiation and in the presence of 0.2 mM ferrioxalate, the first order amoxicillin degradation was increased from 0.91 to 2 min^{-1} as the hydrogen peroxide concentration increased from 1 to 2 mM. It was found that amoxicillin degradation was not affected by the matrix or by the type of irradiation. However, authors suggested the use of solar irradiation due to its low operating cost. Photo Fenton process with solar irradiation, ferrioxalate (0.2 mM), and hydrogen peroxide (3 mM) was also very efficient in tetracycline removal both in surface water and sewage treatment plant effluent, achieving total degradation of tetracycline after one min of irradiation (Bautitz and Nogueira et al., 2007).

4.2.3. UV / TiO₂ Treatment

Photocatalytic oxidation using a suitable catalyst (e.g. TiO₂) have earned an important place among AOPs due to its specific properties, such as relative inexpensiveness and the possible use of solar light as light source. This heterogeneous system has been recently proposed as an alternative treatment for antibiotics, such as tetracyclines, fluoroquinolones, and sulfonamides.

Photocatalytic degradation of a fluoroquinolone antibiotic, ofloxacin in aqueous solution was investigated by Andreozzi et al. (2004b). TiO₂/h ν process was not as effective as H₂O₂/UV and ozonation processes in the removal of ofloxacin and the toxicity of treated water. The photocatalytic degradation of another fluoroquinolone antibiotic, norfloxacin was investigated in aqueous suspensions of TiO₂ under a variety of conditions. At optimum pH value (10.4), the first order degradation rate constant of 0.25 mM norfloxacin was 0.016 min^{-1} in the presence of 1 g/l TiO₂ and with a 125 W medium pressure Hg lamp (Haque and Muneer, 2007). Palominos et al. (2008) studied the photocatalysed oxidation of the antibiotic oxolinic acid in an annular reactor operated with immobilized TiO₂ on

sintered glass cylinders. The complete elimination of antibiotic together with 54 % of TOC reduction was reported after 100 min reaction period with 36 W black light at pH 9. The antimicrobial activity was also reduced upon decreasing antibiotic concentration.

Photocatalytic oxidation of sulfonamide group antibiotics was also extensively studied as well as ozonation and catalyzed hydrogen peroxide oxidation. Almost complete degradation of sulfadiazine, sulfamerazine, sulfathiazole and sulfadimethoxine was achieved with 200 mg/l TiO_2 and UV irradiation (1500 W Xenon lamp equipped with 340 nm cut off filter) at 50 °C, after 30 min of treatment (Calza et al., 2004). The cleavage of S-N bond of sulfadiazine and sulfamerazine and destruction of heterocyclic ring of sulfathiazole and sulfadimethoxine were proposed during photocatalytic treatment of sulfonamides.

In another study, sulfamethoxazole in aqueous solution (100 mg/l) was treated for 6 h by using TiO_2 (0.5 g/l), and UV light (1000 W Xenon lamp) at pH 5. At a given conditions, 82 % of sulfamethoxazole and 23 % of TOC reduction were reported (Abellan et al., 2007). Although the pH reduction did not influence the extent of antibiotic degradation TOC reduction was greatly decreased at pH 2. Authors also assessed the biodegradability of sulfamethoxazole solution after photocatalytic treatment. However, the BOD_5/COD ratio of solution could not be raised over 0.15 even after 15 h of treatment (Abellan et al., 2007). The first order degradation rate constants of sulfacetamide, sulfamethoxazole, sulfadiazine, and sulfathiazole were reported as 0.0132, 0.0301, 0.0130, and 0.0175 min^{-1} , respectively in the presence of 2.5 g/l TiO_2 and four UV lamps (Baran et al., 2006). Authors investigated that the untreated solutions of sulfonamides (0.1 mM each) were nonbiodegradable and toxic to *C. vulgaris* and the toxicity of the treated water was significantly lower than toxicity of the parent sulfonamides.

The effective degradation of tetracycline by TiO_2 photocatalytic process has been demonstrated by numerous studies (Di Paola et al., 2004; Addamo et al., 2005; Seven-Yalap and Akmeahmet-Balcioğlu, 2009). Almost complete conversion of tetracycline (99 %) was achieved in 1 h by the $\text{TiO}_2/\text{h}\nu$ treatment with 1 g/l of catalyst UV-A lamp (Seven-Yalap and Akmeahmet-Balcioğlu, 2009). On the other hand, direct photolysis was in effective in tetracycline degradation (Seven-Yalap and Akmeahmet-Balcioğlu, 2009). In a

similar study conducted by Reyes et al. (2006), almost complete degradation of tetracycline was achieved by the illumination with UV lamp in the presence of TiO_2 (0.5 g/l). Significant mineralization and reduction in the antibacterial activity of treated water against *S. aureus* bacteria were also reported for the process (Reyes et al., 2006).

4.2.4. Membrane Technologies

In addition to advanced oxidation processes, pressure-driven membrane processes nanofiltration (NF) and reverse osmosis (RO) have been gaining attention in the past few years. These treatments seem to be able to effectively remove antibiotics from water and sewage treatment plant effluents.

Li et al. (2004) investigated the effectiveness of a system including reverse osmosis and ultrafiltration for the treatment of oxytetracycline waste liquor. The use of reverse osmosis resulted in more than 98 % COD reduction (initial COD = 10000 mg/l) and 92 % OTC reduction (initial OTC = 1000 mg/l) accompanying concentration of 3000-4000 mg/l OTC in the retentate. The recovery of OTC from retentate with a ratio of more than 60 % was reported with additional ultrafiltration treatment. They claimed that ultrafiltration removed large biopolymers in waste liquor which would hinder the OTC crystallization and thus its recovery from the waste.

Similarly, another study considering the treatment of wastewater and recovery of tetracycline in waste stream also showed the effective removal of tetracycline in permeate by reverse osmosis and the requirement for ultrafiltration pretreatment for efficient antibiotic recovery (Zhang et al., 2006). Authors showed that the addition of PAC to waste liquor improved the UF-RO treatment performance in terms of antibiotic and COD removal (Zhang et al., 2006).

Snyder et al. (2007) evaluated the efficiency of several membrane types and the combinations of membranes for the removal of a number of pharmaceuticals including antibiotics, sulfamethoxazole, erythromycin- H_2O and trimethoprim. Microfiltration and ultrafiltration were ineffective in antibiotic rejection from secondary effluent of wastewater treatment plant. Similar to previous studies explained before, the combination of

ultrafiltration and reverse osmosis provided ≥ 99 % removal of all antibiotics. In addition to membranes, authors also used GAC for the treatment of surface water in drinking water treatment plant and found it highly effective for the treatment of antibiotics.

The removal of several antibiotics including sulfonamide, quinolone and tetracycline group antibiotics, from a wastewater of pharmaceutical manufacturing plant by reverse osmosis, tight and loose nanofiltration membranes were studied by Kosutic et al. (2007). Rejections of quinolone and tetracycline group antibiotics by loose nanofiltration membranes were higher than those of some sulfonamide group antibiotics due to their larger molecular mass. The main elimination mechanism was the size of the antibiotics while their charge and solute hydrophobicities did not improve the antibiotic rejection.

On the contrary, retention of sulfamethoxazole by the loose nanofiltration membrane was found to decrease from 100 % to 25 % as the pH decreased from 10.5 to 3.5 due to the transformation of sulfamethoxazole from a negatively charged to neutral form, indicating the importance of antibiotic charge on their retention by membrane as well as size exclusion (Nghiem et al., 2005). On the other hand, considerably high rejection (up to 100 %) of sulfamethoxazole from ground water was determined in drinking water treatment plant in which reverse osmosis and nanofiltration were used as a result of the combination of size exclusion mechanism and repellent electric force between the solutes and the membrane (Radjenovic et al., 2008).

5. MATERIALS AND METHODS

5.1. Materials

5.1.1. Chemicals and Antibiotics

The chemical reagents and antibiotic substances used throughout the experiments are listed at Table 5.1.

All solutions were prepared with Milli-Q ultrapure deionized water (Millipore, Milford, MA).

5.1.2. Standard Soil and Field Samples

Three different standard soil samples obtained from LUFA, Speyer, Germany were used in the sorption experiments of tetracyclines and fluoroquinolones. The occurrence of oxytetracycline, enrofloxacin, and ciprofloxacin in sediment and soil was determined on different samples. Soil samples fertilized with poultry manure were collected from two different depths (20 and 40 cm) in Adapazari and Kayseri, in 2004. Sediment samples were collected from two different depths (9 and 15 m) of three sites nearby fish farms situated in the south-west coast of Turkey in 2005.

5.1.3. Cow Manure and Bedding Materials

Cow manure samples were obtained from an ecological farm located at the west part of Turkey in 2006-2008. Bedding materials, zeolite and sawdust were purchased commercially.

5.1.4. Synthetic Wastewater

Synthetic animal feeding operation wastewater was prepared from cow manure to imitate the wastewater produced from the cleaning of animal barns.

Table 5.1. Molecular formula, experimental use, and the supplier of chemicals.

Chemical	Molecular Formula	Experimental Use	Supplier
Tetracycline	C ₂₂ H ₂₄ N ₂ O ₈	Sorption experiments; analysis development	Aldrich
Oxytetracycline	C ₂₂ H ₂₄ N ₂ O ₉	Sorption experiments; analysis development; treatment processes	Sigma
Chlortetracycline	C ₂₂ H ₂₃ ClN ₂ O ₈	Sorption experiments; analysis development	Sigma
Enrofloxacin	C ₁₉ H ₂₂ FN ₃ O ₃	Sorption experiments; analysis development	Fluka
Ciprofloxacin	C ₁₇ H ₁₈ FN ₃ O ₃	Sorption experiments; analysis development	Fluka
Sulfamethazine	C ₁₂ H ₁₄ N ₄ O ₂ S	Treatment processes	Sigma
Oxalic acid dehydrate	C ₂ H ₂ O ₄ · 2H ₂ O	HPLC analysis	Merck
Citric acid monohydrate	C ₆ H ₈ O ₇	Antibiotic extraction	Merck
Disodium ethylene diaminetetraacetic acid	Na ₂ EDTA	Antibiotic extraction	Sigma
Dipotassium hydrogen phosphate	K ₂ HPO ₄	Antibiotic extraction	Merck
85 % ortho-phosphoric acid	H ₃ PO ₄	Antibiotic extraction	Merck
Potassium dihydrogen phosphate	KH ₂ PO ₄	Antibiotic extraction	Merck
Disodium hydrogen phosphate	Na ₂ HPO ₄	Antibiotic extraction	Merck
25 % ammonia solution	NH ₃	Antibiotic extraction	Merck
Hydrogen peroxide (30%)	H ₂ O ₂	Fenton reagent	Riedel-de Haën
Ferrous sulphate	FeSO ₄ · 7 H ₂ O	Fenton reagent	Carlo Elba
Sodium persulfate	Na ₂ S ₂ O ₈	Persulfate oxidation	Sigma-Aldrich
Magnesium nitrate	Mg(NO ₃) ₂	Desorption	Merck
Formic acid	CH ₂ O ₂	HPLC analysis	Sigma-Aldrich
Resazurin	C ₁₂ H ₆ NO ₄ Na	Toxicity test	Riedel-de Haën
Imidazole	C ₃ H ₄ N ₂	Toxicity test	Fluka
Mercuric chloride	HgCl ₂	Toxicity test	Merck
Tryptic soy broth		Toxicity test	Bio Rad
Tryptone Type I		Toxicity test	HiMedia
Yeast extract		Toxicity test	AppliChem
HPLC grade methanol	CH ₃ OH	HPLC analysis	Riedel-de Haën
HPLC grade acetonitrile	C ₂ H ₃ N	HPLC analysis	Riedel-de Haën
Potassium iodide	KI	Oxidant analysis	Riedel-de Haën
Ammonium molybdate	(NH ₄) ₆ Mo ₇ O ₂₄ · 4H ₂ O	Oxidant analysis	Merck
Sodium thiosulfate	Na ₂ S ₂ O ₃	Oxidant analysis	Sigma-Aldrich

5.2. Methods

5.2.1. Sorption of Tetracyclines and Fluoroquinolones on Standard Soils

Batch sorption experiments of tetracycline and fluoroquinolone antibiotics at multiple concentrations on standard soils were performed in the presence of 0.01 M CaCl_2 as suggested by OECD Test Guideline 106 (OECD, 1997).

5.2.2. Pretreatment of Manure

Pretreatment was performed by mixing antibiotic-contaminated manure with either various concentrations of $\text{Mg}(\text{NO}_3)_2$ or deionized water at different solid/liquid ratios. The resulting manure slurry was subjected to chemical oxidation treatments.

5.2.3. Chemical Oxidation of Manure, Manure Slurry, and Wastewater

5.2.3.1. Ozonation of Manure, Manure Slurry and Synthetic Wastewater. Ozone was produced from dry and pure oxygen in a laboratory scale corona discharge ozone generator (Fisher OZ 500). Schematic diagram of reaction set-up used in experiments is shown in Figure 5.1. For the semi-batch treatment of manure and wastewater ozone and oxygen gas mixture was passed through the borosilicate reactor through a sintered glass disc placed at the bottom of the reactor (0.12 l capacity for manure and 1.5 l capacity for synthetic wastewater). The treatment of manure slurry was performed in 10 ml or 100 ml reactors in which ozone and oxygen gas mixture was bubbled into the slurry with a Pasteur pipette.

5.2.3.2. Fenton Oxidation of Manure Slurry. Fenton oxidation experiments were performed in a 10 ml or 100 ml reactor in which Fenton's reagents, hydrogen peroxide and iron (II) sulfate, were added to manure slurry. During the reaction period mixing of the manure slurry was provided by a thermostated shaker.

5.2.3.3. Persulfate Oxidation of Manure Slurry. Persulfate oxidation experiments were carried out in a 100 ml reactor in which sodium persulfate was added to manure slurry.

During the reaction period mixing of the manure slurry was provided by a thermostated shaker at different temperatures.

5.2.3.4. Persulfate Oxidation of Synthetic Wastewater. Persulfate oxidation of synthetic wastewater was implemented by mixing it with sodium persulfate in a 100 ml reactor at 50° C.

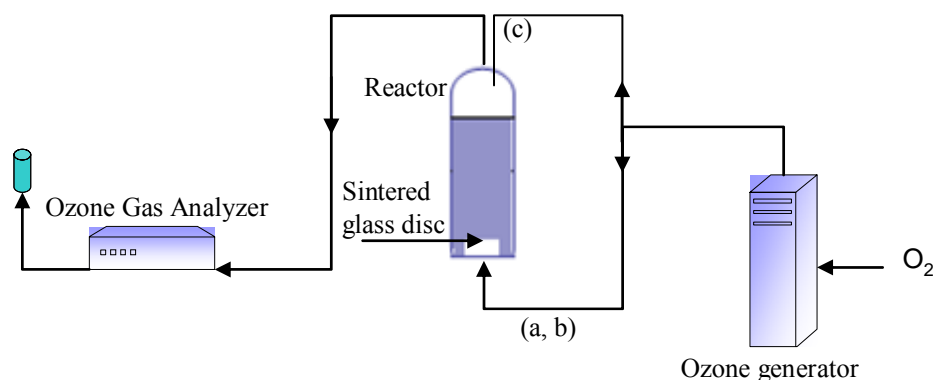


Figure 5.1. Schematic diagram of ozonation experimental set. Treatment of manure and wastewater (a, b); treatment of manure slurry (c).

5.2.4. Antibiotic Analyses in Solid Matrices

For the determination of antibiotics in soil, manure and manure slurry, liquid and solid phase extractions were applied, whereas only the solid phase extraction was implemented for the analysis of antibiotics in the synthetic wastewater.

5.2.4.1. Liquid Extraction. For the analysis of tetracycline, fluoroquinolone and sulfonamide group antibiotics, contaminated manure, manure slurry, standard soils and field samples were first subjected to liquid extraction consisting vortex mixing, sonication and centrifugation. For this purpose, different types of buffer solutions were used depending on matrix and antibiotic substance to be analyzed.

5.2.4.2. Solid Phase Extraction. Solid phase extraction was applied to liquid extracts using SAX (strong anion exchange, 500 mg sorbent, 6 ml capacity; Phenomenex) and HLB (200 mg sorbent, 6 ml capacity; Waters) solid phase extraction (SPE) cartridges in tandem.

Antibiotic substances were eluted through HLB cartridge with an appropriate solvent and filtered through 0.45 μ regenerated cellulose filter.

5.2.4.3. HPLC Analysis. After solid phase extraction, antibiotic analysis in the extracts was performed with an HPLC system (Agilent Technologies 1100 series) equipped with a tertiary pump, a photodiode array, and an autosampler with an automated injection system.

5.2.5. Characterization of Manure

5.2.5.1. Determination of pH. pH of manure was measured in a mixture of manure and water at a ratio of 1:2 (w/v) using a pH probe (WTW pH 330 pH meter).

5.2.5.2. Determination of Organic Carbon. Total organic carbon (OC) of manure was quantified by Walkley-Black method with some modifications (Sanchez-Monedero et al., 1996). 0.02 g of dry manure was weighed in a glass tube and 3 ml of 1 N $K_2Cr_2O_7$ and 3 ml of concentrated H_2SO_4 was added. The mixture was then digested for 2 h at 150 ° C. At the end of digestion, excess Cr^{+3} was determined by a back titration with 0.5 N $Fe(NH_4)_2(SO_4)_2$ solution. Standardization of $Fe(NH_4)_2(SO_4)_2$ solution was performed against the $K_2Cr_2O_7$ solution. Organic carbon content expressed as a percentage in manure can be calculated by the formula;

$$OC(\%) = \frac{(A - B)}{w} \times N \times 0.389 \quad (5.1)$$

where A and B are the volumes (ml) of $Fe(NH_4)_2(SO_4)_2$ used to titrate the excess of $K_2Cr_2O_7$ in the blank and sample analysis, respectively. N is the normality of $Fe(NH_4)_2(SO_4)_2$ solution and w is the weight (g) of manure used.

5.2.5.3. Determination of Humic and Fulvic Acid Carbon. Extraction of the humic substances from the manure samples was performed with 0.1 M NaOH (1:20 w/v ratio) by constantly shaking for 4 h (Sanchez-Monedero et al., 1996). After centrifugation for 15 min at 8000 g, the supernatants were divided into two fractions, one of which was stored for later analysis (sum of humic acids and fulvic acids; C), and the pH of other fraction

adjusted to 1.5 by adding concentrated H_2SO_4 . The precipitates formed at this pH, the humic acid fractions (C_{HA}), were allowed to coagulate for 24 h at 4°C . The dissolved fulvic acids (C_{FA}) were separated by centrifugation and stored for carbon analysis. Organic carbon both in two fractions was determined by oxidizing the organic carbon with $\text{K}_2\text{Cr}_2\text{O}_7$ in H_2SO_4 and subsequently quantifying Cr^{+3} spectrophotometrically (Hach DR/2010, at 600 nm).

5.2.5.4. Determination of Total Nitrogen. 0.2 g of dry manure was digested with 6 ml of concentrated H_2SO_4 and 15 ml of H_2O_2 in a block digester in order to convert all organic nitrogen to ammonia nitrogen. Ammonia nitrogen in the digested sample was then determined by Nessler method.

$$N(\%) = \frac{(A \times 0.0075)}{(B \times C)} \quad (5.2)$$

where A is the absorbance value at 460 nm, B is the weight (g) of manure digested and C is the volume (ml) taken from digest.

5.2.5.5. Determination of Ammonia and Nitrate Nitrogen. 0.5 g of dry manure was extracted with 30 ml of 2 N KCl solution by shaking for about 30 minutes. $\text{NH}_3\text{-N}$ and $\text{NO}_3\text{-N}$ in the extract was determined by Nessler and Cd-reduction methods, respectively (Hach DR/2010).

5.2.5.6. Determination of Iron (Fe) and Manganese (Mn). For the determination of Fe and Mn content of manure, nitric and hydrochloric acid digestion with hydrogen peroxide method was used (Peters et al., 2002). Iron and manganese content of the digest was then analyzed by inductively coupled plasma emission spectroscopy (ICP-OES) (Perkin-Elmer Optima 2100 DV).

5.2.6. Characterization of Synthetic Wastewater

5.2.6.1. Determination of Chemical Oxygen Demand (COD). COD measurements were conducted in accordance with Standard Methods by the closed reflux colorimetric method (APHA/AWWA/WPCF, 1998).

5.2.6.2. Determination of Biochemical Oxygen Demand (BOD₅). BOD₅ measurements were conducted in accordance with Standard Methods by membrane electrode method (APHA/AWWA/WPCF, 1998).

5.2.6.3. Determination of Total Organic Carbon (TOC). TOC of synthetic wastewater was measured by Shimadzu TOC-V CSH TOC Analyzer.

5.2.6.4. Determination of Metals and Anions. Metal (Ca, Mg, K, Cr, Zn, Mn, and Fe) and anion (NO₃⁻, NO₂⁻, Cl⁻, and SO₄⁻) content of synthetic wastewater were analyzed by ICP-OES and ion chromatography (IC) (Dionex ICS-3000), respectively.

5.2.7. Analysis of Oxidants

Persulfate analysis in liquid phase of manure slurry and synthetic wastewater was conducted according to iodometric method (Kolthoff and Carr, 1953). Measured volume (2 ml) of the sample was diluted to 30 ml with water and 4 g of potassium iodide was added. The flask containing the reaction mixture was stoppered and allowed to stand for 15 min. After acidification with 1 ml of 6 N acetic acid, iodine was titrated with 0.1 N thiosulfate solution to the starch indicator end point. Amount of sodium persulfate in aqueous solutions was calculated by the following formula;

$$Na_2S_2O_8 (mg/ml) = \frac{119(A - B)N}{v} \quad (5.3)$$

where A and B are the titration volume for sample and blank, respectively. N is the normality of Na₂S₂O₃ solution and v is the volume of the sample.

Hydrogen peroxide determination in liquid phase of manure slurry was also performed according to iodometric method in the presence of ammonium molybdate catalyst (<http://www.solwaychemicals.us/static/wma/pdf/6/6/2/5/XX-122.pdf>). 2 g of hydrogen peroxide containing solution was weighed into 500 ml flask. After addition of 200 mL of water, 20 mL of potassium iodide solution (10%; w/v), and 25 mL of the acid mixture (0.18 g of ammonium molybdate, 300 ml sulfuric acid, and 700 ml deionized water), flask was stoppered and allowed to stand for 5 min. The mixture was then titrated with 0.1 N thiosulfate solution to the starch indicator end point. The concentration of hydrogen peroxide was calculated by the following formula;

$$H_2O_2 (\%w/w) = \frac{(A - B)}{w} \times N \times 1.7007 \quad (5.4)$$

where A and B are the titration volumes for sample and blank, respectively. N is the normality of $Na_2S_2O_3$ solution and w is the weight (g) of manure used.

Ozone gas concentration in the influent and effluent of ozonation reactor was monitored by using an online ozone analyzer (Fisher Ozotron 23).

5.2.8. Experimental Design

A central composite rotatable experimental design and factorial design were used to investigate the effects of operational parameters in chemical oxidation processes. The data evaluation was performed by Minitab 15 statistical software.

5.2.9. Toxicity Tests

Bacterial toxicity of antibiotics and their oxidation products were assessed by their inhibition on dehydrogenase activity of *Bacillus subtilis* or growth rate of *Staphylococcus aureus* and experimental procedures for toxicity tests are given in Section 8.2.5 and Section 10.2.9.

6. ANALYSIS AND SORPTION OF FLUOROQUINOLONES IN SOLID MATRICES

6.1. Introduction

The fluoroquinolone antibiotics have a wide range of antibacterial activity and are broadly used in treating bacterial infections in both human and veterinary medicine. After administration, fluoroquinolones are excreted in urine and feces as a parent compound or as its active metabolites. The use of animal wastes as a fertilizer in agricultural soils results in a contamination of terrestrial environment with these substances. Strong sorption of fluoroquinolone antibiotics to solid matrices (Nowara et al., 1997; Pouliquen and Le Bris, 1996; Tolls, 2001; Ötöker and Balçioğlu, 2005) may retard their degradation by means of biotic and abiotic processes and make them persistent in feces, soils, sludge and sediments (Hektoen et al., 1995; Golet et al., 2002). Correspondingly, enrofloxacin (a fluoroquinolone antibiotic, primarily used in poultry production), and its main metabolite ciprofloxacin (a fluoroquinolone antibiotic used in human medicine) have been detected in poultry wastes and arable soils up to 8.3 and 0.37 mg/kg, respectively (Martinez-Carballo et al., 2007).

Agricultural soil contaminated with the antibacterial agents is a reservoir of the resistant bacteria which could be transferred to other bacteria living in ground water, drinking water or plants and thus to humans (Kümmerer, 2004). Therefore, it is necessary to monitor fluoroquinolones concentrations in agricultural soils due to their selective pressure on bacteria.

Compared to animal tissue and milk there are only few methods describing the analysis of fluoroquinolones in aquatic systems (Hirsch et al., 1998; Golet et al., 2001; Ternes et al., 2001; Turiel et al., 2003) soils (Golet et al., 2002; Morales-Munoz et al., 2004; Turiel et al., 2006; Martinez-Carballo et al., 2007) and animal feces (Wiuuff et al., 2002; Sunderland et al., 2004). These analytical methods based on the accelerated solvent extraction, microwave or ultrasonic assisted extractions with a number of different aqueous, organic solvents or their combination. The efficiency of analytical methods was

evaluated only for one type of soil and did not consider the potential effect of physico-chemical characteristics of soils on the recovery rates of fluoroquinolones. However, it is known that the soil composition significantly affect the sorption behavior of antibiotics which may influence the efficiency of analytical methods for the determination of fluoroquinolone antibiotics in soil matrices.

The purpose of the present study was to develop an analytical method for detection and quantification of enrofloxacin and ciprofloxacin in different standard soils namely, sandy, loamy sand, and sandy loam, using HPLC-FLD with solid phase extraction (SPE). The sorption of fluoroquinolone group antibiotics on standard soils was also assessed by batch sorption experiments. The efficiency of the developed analytical method for the analysis of these compounds in corresponding soils was correlated in terms of recovery rates and distribution coefficients. Additionally, the developed analytical method was applied to field soil samples collected from two poultry farms located in North West and in Central Turkey to obtain information about the national levels of these substances in arable lands.

6.2. Materials and Methods

6.2.1. Chemicals

Enrofloxacin and ciprofloxacin were obtained from Fluka. Chemical structures of fluoroquinolones are given in Figure 6.1. Individual enrofloxacin (ENR) and ciprofloxacin (CIP) stock solutions were prepared in methanol containing 0.2% 1N HCl in the concentration of 100 mg/l and stored at -20°C in dark for 1 month. 85 % ortho-phosphoric acid (Merck), KH_2PO_4 (Merck), $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ (Merck), 25% NH_3 solution (Merck) and $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (Sigma) were analytical grade. HPLC grade methanol and acetonitrile were purchased from Riedel-de Haën. Ultra pure water was used for the preparation of all solutions.

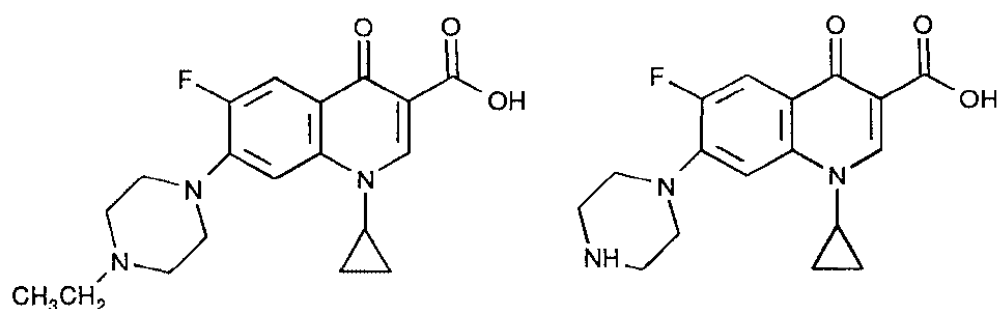


Figure 6.1. Molecular structure of (a) enrofloxacin, (b) ciprofloxacin.

6.2.2. Standard Soils and Field Samples

Three different standard soil samples obtained from LUFA, Speyer, Germany were used for the recovery and sorption experiments of fluoroquinolones. Some properties of soil samples that could affect analysis of fluoroquinolone antibiotics through the adsorption are shown in Table 6.1.

Table 6.1. Physical and chemical characteristics of standard soils.

Soil Type	Composition of soil particles (%)				OC ^a	CEC ^b	pH
	Clay ($< 2\mu\text{m}$)	Silt ($2\text{-}20\mu\text{m}$)	Fine sand ($20\text{-}200\mu\text{m}$)	Coarse sand ($>200\mu\text{m}$)			
Sandy	2.5	4.5	27.8	65.2	0.59	4	6.0
Loamy sand	6.6	7.2	42.5	43.7	2.27	9	6.1
Sandy loam	9.5	12.4	51.0	27.2	1.24	9	6.6

^a Organic carbon

^b Cation exchange capacity(meq/100g)

Farm soils fertilized with manure of two different poultry farms located in North West and Central Turkey were collected from two different depths (20 and 40 cm) to determine enrofloxacin, which is commonly used in poultry farms for therapeutic purposes and its main metabolite ciprofloxacin.

6.2.3. Spiking of Standard Soils

Standard soil samples (1 g) were spiked with 100 μ l mixture of ENR and CIP at two different concentrations (0.2 and 0.5 μ g/g). Spiking solvent acidified methanol was evaporated overnight under the fume board.

6.2.4. Extraction Methods

Four different buffer solutions were used (Table 6.2) during the development of an extraction method for the analysis of ENR and CIP in soil samples as it will be discussed in Section 6.3.1. The most efficient extraction buffer (Buffer C) and method was used for the other parts of the study and explained as follows: 0.2 g of $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ and 5 ml of pH 3 buffer (27.2 g of KH_2PO_4 and 1.35 ml of H_3PO_4 in 1 l water): acetonitrile (1:1) was added to glass centrifuge tubes containing 1 g of spiked or blank soil samples. Each sample were mixed by a vortex (Scientific Industries, Vortex Genie 2; VIRTIS Sentry 5L, USA) for 60 s and placed into an ultrasonic bath for 15 min. The samples were then centrifuged for 5 min at 5000 g and at 20°C. The liquid phase was transferred to another centrifuge tube and extraction procedure was repeated a further three times with additions of 4, 3, and 3 ml of buffers. Extracts were combined and made up to 200 ml with water in order to decrease the acetonitrile content prior to the clean up step by solid phase extraction.

Table 6.2. Effect of extraction solvent and extraction cycle on the recovery rates of fluoroquinolones.

Extraction Solvent Composition	pH	Extraction Cycle	Recovery (%)	
			ENR	CIP
(A) 10 % TCA : ACN (4:1)	1	2	12	19
(B) 0.05 M H_3PO_4 : CAN (1:1)	2	2	37	22
(C) 27.2 g KH_2PO_4 + 1.35 ml H_3PO_4 in 1 l water : CAN (1:1)	3	3	87.5	84.0
(D) 4.64 g KH_2PO_4 + 9.83 g $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ in 1 l water : ACN (1:1)	7	3	56.5	27.0

6.2.5. Solid Phase Extraction (SPE)

For solid phase extraction, SAX (strong anion exchange; 1000 mg sorbent and 6 ml capacity) from Phenomenex, USA and Oasis HLB cartridges (polymeric, 500 mg sorbent, 6 ml capacity) from Waters, Milford, MA, USA were used. They were placed in tandem in order to simultaneously remove negatively charged humic material by SAX and keep the fluoroquinolones on HLB (Jacobsen et al., 2004). The SAX cartridge was placed on the top of the HLB and both columns were conditioned first with 3 ml of MeOH, following 3 ml of pH 3 buffer. 200 ml of extracts were then passed through both SPE-columns at a flow-rate of about 5 ml/min by applying vacuum and both columns were flushed with 10 ml of water. Both columns were first dried for 10 min under vacuum, after that SAX cartridge was removed. Fluoroquinolones were eluted using 4 ml of 6% NH_3 solution containing methanol. Eluates were then evaporated to about 150 μl under gentle N_2 stream and made up to 1 ml by mobile phase.

6.2.6. Sorption Experiments

Batch sorption experiments of fluoroquinolone antibiotics at multiple concentrations on standard soils were performed in the presence of 0.01 M CaCl_2 as suggested by OECD Test Guideline 106 (OECD, 1997) to simulate natural soil water and to minimize the suspension of soil particles. For pre-equilibration, 1 g of each standard soil was mixed with 8 ml of 0.01 M CaCl_2 solution or water in 15 ml polypropylene test tubes and agitated in a rotary shaker for 24 h at 21°C. After the pre-equilibration period, 2 ml mixture of antibiotics was added to reach a final concentration of 2-10 mg/l of each fluoroquinolone compound. Soil free control experiments were also run to check the stability of fluoroquinolones in solution. Duplicate samples were equilibrated with shaking in a rotary shaker for 24 h in a dark room, at room temperature. After equilibration samples were centrifuged at 4000 g for 10 minutes, supernatant was filtered with 0.22 μm regenerated cellulose filter and transferred to HPLC vials.

Sorption data were fit to linear and Freundlich isotherms. Logarithmic form of the Freundlich equation is:

$$\log q_e = n \log C_e + \log K_f \quad (6.1)$$

and the linear sorption isotherm is given by;

$$q_e = K_d C_e \quad (6.2)$$

where;

K_d = sorption distribution coefficient (l/g)

C_e = equilibrium concentration of fluoroquinolones in aqueous phase (mg/l)

K_f = Freundlich coefficient

n = Freundlich linearity parameter ($K_f = K_d$ when $n = 1$)

q_e = concentration of fluoroquinolones in soil (mg/g) and calculated as

$$q_e = \frac{V(C_i - C_e)}{m} \quad (6.3)$$

where;

V = volume of aqueous phase (l)

C_i = concentration of fluoroquinolones in soil free aqueous solutions after 24 h of agitation (mg/l)

m = mass of soil (g)

6.2.7. HPLC Analysis

Concentrations of fluoroquinolones were determined using a 1100 series HPLC chromatograph system (Agilent Technologies, Germany) equipped with a binary pump, fluorescence detector and an auto sampler with an automated injection system. Standard solutions and sample extracts were analyzed on RP Luna C18(2), 5 μ , 150 x 4mm LC column equipped with an ODS, Octadecyl guard column (Phenomenex) at 30°C with an injection volume of 10 μ l. Isocratic elution was carried out with aqueous 0.02 M *o*-H₃PO₄: ACN (80:20) at a flow rate of 0.8 ml/min. The excitation and emission wavelengths for ENR and CIP were 280 and 450 nm, respectively. Standard solutions for the external calibration were made by dilution in mobile phase obtaining final concentrations ranging

from 0.01 to 1 mg/l of ENR and CIP, respectively. Estimates of the amount of analytes were interpolated from these external calibration curves. A representative HPLC chromatogram for the standard ENR and CIP solution and their calibration curve equations are given in Figure 6.2.

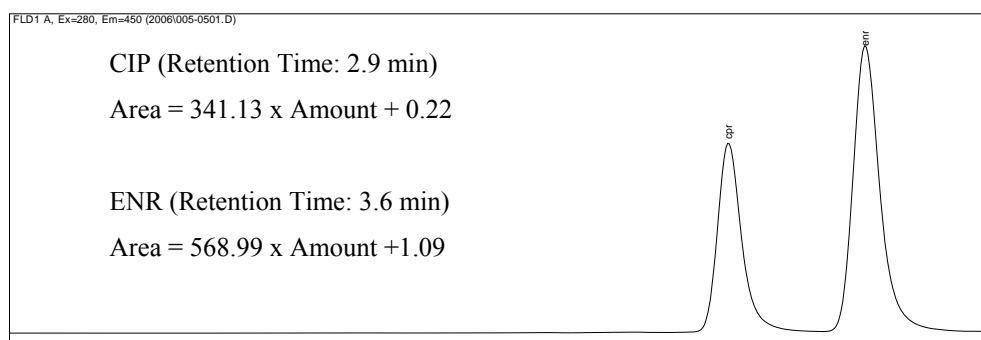


Figure 6.2. Representative chromatogram of enrofloxacin and ciprofloxacin standard solution and calibration parameters.

6.3. Results and Discussion

6.3.1. Selection of Extraction Solvent

The first step during the method development for the analysis of fluoroquinolones was the choice of extraction solvent. The effectiveness of four different extraction solvents (acetonitrile was used as an organic modifier) with the extraction cycle of two were compared for the extraction of fluoroquinolones in sandy soil and presented as recovery rates (Table 6.2).

TCA has been used for the extraction of fluoroquinolones from animal tissues for the purposes of deproteinization of tissue and for the isolation of fluoroquinolones from the matrix (Posyniak et al., 2001). In the present study, two extraction cycles involving vortex, ultrasonication and centrifugation, with the 5 ml of extraction solvent A, followed by HLB clean up resulted in a poor recovery rates of enrofloxacin and ciprofloxacin in sandy soil that was spiked at 0.5 mg/kg fluoroquinolone mixture. Moreover, use of TCA resulted in a variation in the retention times of fluoroquinolones in the HPLC analysis, which might be due to the very low pH of TCA. Same extraction procedure was repeated with solvent B and 25 % of enhancement was obtained in the recovery rate of enrofloxacin. Because of

the drift in retention times of fluoroquinolones and lower efficiency by the use of TCA, method development was carried on with phosphate buffers at different pH values. Three extraction cycles with 5 ml of extraction solvent C or D, was applied to sandy soil in the presence of Na₂EDTA in order to prevent the complexation between fluoroquinolones and divalent metal ions in soil samples. Prior to solid phase extraction with HLB, dilution of the soil extracts was required to prevent the continuous elution of fluoroquinolones by the high content of acetonitrile through the cartridges. For this purpose, extracts were diluted to 50, 100 and 200 ml and a significant loss of fluoroquinolones was observed during the SPE clean up by the dilution of extracts to 50 and 100 ml (data not presented).

Fluoroquinolone antibiotics are amphoteric substances with two pK_a values of approximately 6 and 8. Enrofloxacin and ciprofloxacin are positively charged at acidic medium while at neutral pH values they are in zwitterion form where their solubility is minimum. Therefore, the higher extraction efficiency at pH 3 compared to pH 7 could be attributed to the increased solubility of ENR and CIP in accordance with the literature results (Golet et al., 2002). Moreover, the presence of phosphoric acid in the extraction buffer at pH 3 increases the extraction of humic substances from soil, which can interact with fluoroquinolones by complexation.

6.3.2. Effect of Soil Type on the Analysis of Fluoroquinolones

The further analyses were carried out to extract fluoroquinolone antibiotics from sandy, loamy sand and sandy loam soils that were spiked at a concentration of 0.5 mg/kg with the ENR and CIP mixture. Na₂EDTA and extraction solvent C was used with the extraction cycle of three. By considering the high amount of organic and clay content of sandy loam and loamy sand soils, SAX cartridge was used in solid phase extraction in order to prevent the contamination and overload of the solid phase of the HLB cartridge. Mean recovery values obtained from duplicate analysis are presented in Table 6.3.

Table 6.3. Mean recovery rates (%) for the extraction of ENR and CIP from sandy, loamy sand and sandy loam soils (3 extraction cycles).

Soil Type	ENR (%)	CIP (%)
Sandy	80.0	60.0
Loamy sand	75.5	57.5
Sandy loam	72.0	57.5

Based on the recovery rates of fluoroquinolone antibiotics it can be said that the use of SAX-HLB combination instead of single use of HLB lead to decrease in the extraction efficiency of enrofloxacin and ciprofloxacin from sandy soil (see Table 6.2 and 6.3).

To find out the effect of extraction cycle on the recovery of fluoroquinolone antibiotics from soil samples the extraction cycle was increased to four (without changing the total amount of extracts). Method performance was evaluated with two spike level of enrofloxacin and ciprofloxacin mixture, 0.2 mg/kg and 0.5 mg/kg. Analyses were run in 6 replicates and mean recoveries are presented at Table 6.4 together with the standard deviations.

Table 6.4. Mean recovery rates for the extraction of ENR and CIP from sandy, loamy sand and sandy loam soils together with the standard deviations (n=6) (4 extraction cycles).

Soil Type	ENR (%mean \pm S.D)		CIP (%mean \pm S.D)	
	0.5 mg/kg	0.2 mg/kg	0.5 mg/kg	0.2 mg/kg
Sandy	100 \pm 3.0	100 \pm 3.5	81 \pm 3	89 \pm 4.1
Loamy sand	87 \pm 4.0	71 \pm 2.2	63 \pm 4.4	73 \pm 3.2
Sandy loam	88 \pm 4.3	88 \pm 2.6	61 \pm 5	64 \pm 3.2

Increase of the extraction cycle from three to four resulted in a considerable enhancement of recovery rates of enrofloxacin and ciprofloxacin. This observation is an indication for the strong interaction between the fluoroquinolones and soil matrix which necessitates longer extraction time to recover these substances from soils effectively.

As can be seen from the Table 6.4, the recoveries ranged between 61-89% for ciprofloxacin and 71-100% for enrofloxacin based on the soil type and are comparable with those given by numerous authors provided by other studies in the literature (Golet et al., 2002; Morales-Munoz et al., 2004; Turiel et al., 2006).

6.3.3 Sorption of Fluoroquinolones on Standard Soils

In order to obtain a correlation between the recoveries of fluoroquinolone antibiotics from soils and their sorption behaviors, batch sorption experiments of enrofloxacin and ciprofloxacin at multiple concentrations on standard soils were performed in the presence of 0.01 M CaCl_2 as suggested by OECD Test Guideline 106 (OECD, 1997) to simulate natural soil water and to minimize the suspension of soil particles. Since fluoroquinolones have a strong tendency for sorption, the concentration range of 2-10 mg/l was chosen to obtain adsorption isotherms with high accuracy. Antibiotic solutions were prepared in 0.01M CaCl_2 and the sorption isotherms were given in Figure 6.3. The experimental data were fitted quite well by Freundlich isotherms, as indicated by the very high correlation coefficient values ($r^2 > 0.98$) but they can also be fitted to linear isotherms without significant error since the correlation coefficients were also high ($r^2 > 0.96$) for the linear isotherm case.

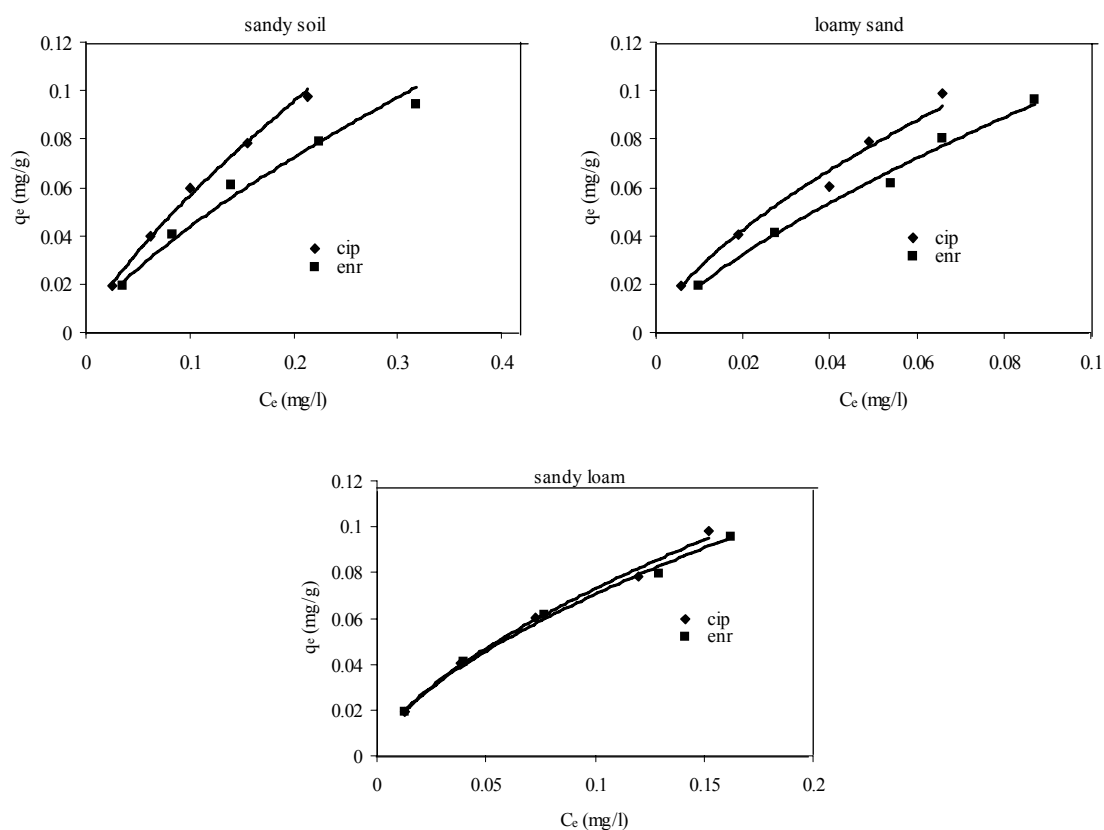


Figure 6.3. Sorption isotherms of fluoroquinolone antibiotics in sandy, loamy sand and sandy loam soils.

The sorption coefficients of fluoroquinolone antibiotics obtained from the present study are summarized at Table 6.5.

Table 6.5. Sorption coefficients of ciprofloxacin and enrofloxacin for different types of standard soils.

Soil Type	Compound	K_d (l/g)	K_f^a	n
Sandy	CIP	0.41	0.32	0.73
	ENR	0.27	0.23	0.72
Loamy sand	CIP	1.29	0.66	0.73
	ENR	0.97	0.56	0.74
Sandy loam	CIP	0.52	0.31	0.63
	ENR	0.46	0.29	0.62

^a The dimension of K_f is l/g only if $n=1$.

As can be seen from Table 6.5, the K_d and K_f values for ciprofloxacin and enrofloxacin were found to be slightly different. The value of the slope n ranged between 0.63-0.74 for all soils, indicating a nonlinear relationship where proportionally more of both antibiotics were sorbed at lower concentrations.

The sorption coefficients of enrofloxacin in standard soils were comparable with literature data, which ranged between 0.26–5.61 l/g for different soil types (Nowara et al., 1997) and 0.11 l/g for dissolved organic carbon (Schmitt-Kopplin et al., 1999). It is suggested that hydrophobicity driven interactions do not explain these high sorption coefficients of fluoroquinolones, which have very low values of $\log K_{ow}$ (Tolls, 2001). Therefore, several mechanisms were proposed like cation bridging, electrostatic interactions, and hydrogen bonding for the sorption of these compounds on organic or clay portions of soils. Based on the sorption coefficients, it seems that the effect of organic portion of soil (hydrophobic interactions) is stronger than that of clay portion on the sorption of fluoroquinolones. This might be due to the type of clay minerals which significantly affect the extent of sorption of fluoroquinolones (Nowara et al., 1997). Competition of fluoroquinolones and Ca^{2+} ions for negatively charged clay minerals could also be a reason for lower sorption coefficients of ENR and CIP in case of sandy loam soil. On the other hand, despite of much higher sorption of fluoroquinolones to loamy sand, their extraction efficiencies are quite similar for both sandy loam and loamy sand soils with high recovery rates. This is indicative for the effectiveness of the developed analytical method for the extraction of antibiotic substances even from the soils having high organic carbon content.

The higher soil distribution coefficients for ciprofloxacin in all types of soils might be due to the stronger and specific adsorption of ciprofloxacin on soil samples compared to that of enrofloxacin and is a probable explanation for the lower recoveries obtained from the extraction experiments.

On the other hand, adsorption is an important phenomenon since it affects the fate and effects of antibiotics in terrestrial environment. Although sorption is known as a process that reduce the antimicrobial activity against bacteria (Thiele Bruhn, 2003), antibiotics could still be effective after sorption and may influence the selection of antibiotic resistant

bacteria in terrestrial environment (Chander et al., 2005). Therefore, further research is required on the antimicrobial activity of these substances to evaluate the importance of sorption extent on their antibiotic potency.

6.3.4. Analysis of Fluoroquinolones in Field Samples

To estimate the exposure of enrofloxacin and accordingly its main metabolite ciprofloxacin in the soil samples taken from North-West and Central Turkey at two different depths. Agricultural soils were fertilized with the poultry manure. Soil samples were analyzed for enrofloxacin which is commonly used in poultry farms for therapeutic purposes and its main metabolite ciprofloxacin and the obtained concentrations are given at Table 6.6.

Table 6.6. Enrofloxacin and ciprofloxacin concentrations in field soil samples (n=2).

Location	ENR (mg/kg)	CIP (mg/kg)
North-West (20 cm)	0.204	0.053
North-West (40 cm)	0.018	n.d
Central (20 cm)	0.035	n.d
Central (40 cm)	0.013	n.d

n.d: not detected

Enrofloxacin was found in soil samples taken from poultry farms in North-West and Central Turkey at two different depths in the concentration range of 0.013-0.204 mg/kg while ciprofloxacin was only detected at the 20 cm of the agricultural soil in North-West Turkey. The decrease in the concentration of enrofloxacin as the soil depth increase is an indicative for the limited mobility of fluoroquinolones to the subsoil. The expected immobility of enrofloxacin and ciprofloxacin were also determined by the high soil distribution coefficients (Table 6.5). In agreement with our results, 0.00-0.35 mg/kg ciprofloxacin and 0.05-0.3 mg/kg another fluoroquinolone antibiotic norfloxacin, which is used in human medicine, were detected in sludge amended soils where decrease in the antibiotic concentration was observed with increasing depth (Golet et al., 2003).

Long term use of sub-MIC (minimum inhibitory concentration) concentrations is one of the major factors responsible for the transfer of resistance genes as well as the already resistant bacteria themselves (Kümmerer, 2004). Sub-therapeutical concentrations of fluoroquinolones continuously added through the sludge or manure application. The combined effect of this continuous input with their relative persistence and immobility result in an accumulation of these substances in agricultural soils. This possibly led to formation of an environmental reservoir of antibiotic resistant bacteria that could transfer resistance back to animals or humans via crops.

6.4. Conclusion

In this study, the effect of soil characteristics on the analysis of two fluoroquinolone antibiotics, enrofloxacin and ciprofloxacin was investigated by means of an HPLC-FLD method including, liquid and solid phase extractions. pH 3 phosphate buffer ($\text{H}_3\text{PO}_4/\text{KH}_2\text{PO}_4$) in combination with acetonitrile (1:1) was found to be most efficient extraction solvent for the recoveries of these compounds from sandy soil due to the high solubility of fluoroquinolones at acidic medium and the ability of phosphoric acid to extract the organic portion of soil which could interfere the extraction via complexation with these compounds. Adsorption coefficients of fluoroquinolone antibiotics were considerably higher for loamy sand soil than the other types of soils despite of the similar recovery rates of fluoroquinolones in loamy sand and sandy loam soils. In the view of these results it can be concluded that the proposed analytical method is very efficient for the extraction of fluoroquinolones from soil samples containing significant portion of organic content. The expected immobility of fluoroquinolones in soils based on the results from sorption experiments was confirmed by the analysis of these compounds in field soil samples at two different depths where very low enrofloxacin concentration was detected at 40 cm than that of 20 cm.

7. ANALYSIS AND SORPTION OF TETRACYCLINES IN SOLID MATRICES

7.1. Introduction

Antibiotics and medicated feeding stuffs are routinely used in high production of agricultural animals. They can be administered for therapeutic or nutritional purposes for a short or extended period. Depending upon the chemical and the animal species, some drugs and additives may be completely metabolized to inactive components. However, some are excreted as active metabolites or parent compounds. All of these residues occur in the animal waste from cattle, swine, poultry, and fish facilities. Runoff and leaching from feedlots or aquaculture activities can carry the residues into surface and ground water. Manure and litter are also used or disposed to the land where it might be incorporated into soil. Accordingly, the presence of trace amounts of antibiotic residues in soil (Hamscher et al., 2002; Jacobsen et al., 2004; Brambilla et al., 2007), surface and ground waters (Richardson and Bowron, 1985; Ternes, 1998; Daughton and Ternes, 1999; Boyd et al., 2003) and sediments (Coyne et al., 1994; Capone et al., 1996) have been detected by numerous scientists. A further problem is the increased use of antibiotics leading to an increase in drug resistant micro flora which is obviously favored by low concentrations of antibiotics (Jorgensen and Halling-Sørensen, 2000; Jacobsen and Guildal, 2000; Iwane et al., 2001; Bryan et al., 2004). Tetracycline group antibiotics, such as oxytetracycline (OTC), tetracycline (TC), and chlorotetracycline (CTC) are extensively used in livestock production and fish farming at therapeutic and sub-therapeutic levels (Sarmah et al., 2006). They are excreted as parent compound in urine and faeces in the range of 40-80 % (Halling-Sørensen, 2000). The ultimate fate of tetracyclines in the terrestrial and aquatic environment significantly depends on the physical and biological conditions and their sorption behaviour (Sithole and Guy, 1987a,b; Pouliquen and Bris, 1996; Rabølle and Spliid, 2000; Jones et al., 2005; Sanderson et al., 2005; Drillia et al., 2005a; Sassman and Lee, 2005). Since sorption may protect them from biodegradation, they tend to persist in the soil and sediments for several months (Samuelson et al., 1994; Hektoen et al., 1995; Hoper et al., 2002). Therefore, analysis of these substances in solid matrices is of vital importance. Previous studies revealed that, depending on the type of soil and utilized

extraction and analysis methods, recoveries varied between 63-119 % for OTC, 33-47 % for TC, and 51-76 % for CTC. Although the analysis and sorption of tetracycline antibiotics have been studied separately, the correlation between the extent of sorption and the efficiency of the tetracycline analysis in different kinds of soils have not been adequately examined.

The aim of this study was to investigate the recoveries of OTC, TC, and CTC spiked on different standard soils by using different extraction buffers and to explain the obtained results with sorption data. The efficiency of the developed method for the analysis of these compounds in corresponding soils was correlated in terms of recoveries and distribution coefficients. The Freundlich model was used to analyze sorption equilibrium of OTC, TC, and CTC mixture. In order to obtain information on the occurrence and persistence of tetracyclines in the environment, an established method for the analysis of three tetracycline antibiotics was applied to sediment and mud samples collected near fish farms and from fish ponds.

7.2. Materials and Methods

7.2.1. Chemicals

Tetracycline (Aldrich), oxytetracycline (Sigma) and chlortetracycline (Sigma) were used as their hydrochloride salts to enhance water solubility. Chemical structures of tetracyclines are given in Figure 7.1. Individual stock solutions (1 mg/ml) were prepared in methanol and stored at 4°C in darkness up to 1 month.

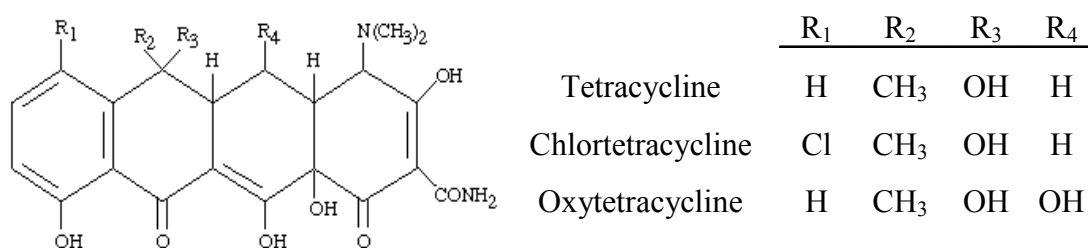


Figure 7.1. Molecular structure of tetracycline antibiotics.

Analytical grade of oxalic acid dihydrate (Merck), citric acid monohydrate (Merck), Na₂EDTA.2H₂O (Sigma), and K₂HPO₄ (Merck) were used for the extraction purposes. HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from Riedel de Haën. CaCl₂.2H₂O was obtained from Merck. Millipore water was used for the preparation of all solutions.

7.2.2. Standard Soils and Field Samples

Three different standard soil samples obtained from LUFA, Speyer, Germany were used for the analysis and sorption of tetracyclines. Detailed information about the properties of soil samples were given in Section 6.2.2.

Sediments were sampled from different depths (9 and 15 m) at three sites (37°12'N 27°34'E; 37°14'N 27°32'E) nearby fish farms situated in the south-west coast of Turkey and named as S1 and S2, respectively. Mud samples have been collected from three different fish farms (M1, M2 and M3) located around the Pearl River Delta (within Guangdong Province), in PR China. In order to avoid interferences caused by moisture content, standard soils, mud and sediment samples were freeze dried (VIRTIS Sentry 5L, USA) prior to analysis.

7.2.3. Spiking of Standard Soils

Standard soil samples (1 g) were spiked with 200 µl mixture of TC, OTC and CTC at two different concentrations (0.2 and 1.0 mg/kg). Spiking solvent, methanol was evaporated overnight under the fume board.

7.2.4. Extraction Methods

Extraction was performed by using three different buffer solutions (Table 7.1). One gram of soil, sediment, or mud sample was weighed into a glass centrifuge tube (Cortex, 30 ml) and 5 ml of extraction buffer was added. 0.2 g of Na₂EDTA was weighed into a centrifuge tube when the extraction was performed with Buffer II and III. Each sample was then vortex mixed (Scientific Industries, Vortex Genie 2) for 30 s and, subsequently,

sonicated in an ultrasonic bath for 10 minutes. The samples were then centrifuged for 10 min at 7000 g and 20°C to separate the solid and liquid phases. 5 ml of supernatant was transferred into another glass centrifuge tube and the extraction procedures were repeated two more times to give a total of 15 ml of supernatant. The joined extracts were then subjected to solid phase extraction.

Table 7.1. Compositions of buffer solutions used for the extraction of tetracyclines.

Buffer Type	Buffer Composition	pH
Buffer I	0.1 M Na ₂ EDTA- McIlvaine Buffer: 61.45 ml of 0.1 M citric acid monohydrate and 38.55 ml of 0.2 M K ₂ HPO ₄	4.0
Buffer II	0.1 M citric acid : methanol (90:10)	4.7
Buffer III	0.5 M citric acid : methanol (90:10)	4.7

7.2.5. Solid Phase Extraction (SPE)

For solid phase extraction, SAX (strong anion exchange; 1000 mg sorbent and 6 ml capacity) from Phenomenex, USA and Oasis HLB cartridges (polymeric, 500 mg sorbent, 6 ml capacity) from Waters, Milford, MA, USA were used. The SAX cartridge was placed on the top of the HLB and both columns were conditioned first with 3 ml of MeOH, following 3 ml 0.05 M citric acid buffer at pH 4.7. Extracts were then passed through both SPE-columns at a flow-rate of about 2 ml/min by applying vacuum and both columns were flashed with 4 ml of 0.05 M citric acid buffer. McIlvaine buffer was used for the conditioning and washing steps of the cartridges when the extraction was carried out by Buffer I. Both columns were first dried for 5 minutes under vacuum, after that SAX cartridge was removed. Tetracyclines were eluted using 10 ml of 0.01 M methanolic oxalic acid. Extracts were evaporated to near dryness using a rotary evaporator at 40° C and 40 mbar and then re-dissolved in 1 ml of mobile phase. Solutions were filtered through a 0.45 µ regenerated cellulose filter (Schleicher&Schuell) and transferred to HPLC amber vials.

7.2.6. Sorption Experiments

Batch sorption experiments of tetracycline antibiotics at multiple concentrations on standard soils were performed in the presence of 0.01 M CaCl_2 as explained in details in Section 6.2.6.

7.2.7. HPLC Analysis

Analyses were carried out with an LC chromatograph system (Agilent Technologies 1100 series, Germany) equipped with a binary pump, a photodiode array, and an autosampler with an automated injection system. For the separation and quantification of the three selected tetracyclines Zorbax SB C_{18} , 5 μ , 150 x 4.6 mm (Agilent) equipped with an ODS, Octadecyl guard column (Phenomenex) was used. Isocratic elution was carried out with aqueous oxalic acid dihydrate 0.01 M: MeOH: ACN (75:12.5:12.5) at a flow rate of 1 ml/min and 26°C with an injection volume of 20 μ l. The acquisition wavelengths were 355 nm for OTC and TC and 375 nm for CTC. Linearity of the detector response was verified with standard solutions in mobile phase over the range 0.01-1 mg/l for oxytetracycline and tetracycline and 0.02-1 mg/l for chlortetracycline. Estimates of the amount of analytes were interpolated from these external calibration curves.

7.3. Results and Discussion

7.3.1. External Calibration of Tetracyclines

External calibration curve parameters, retention times (t_R), and limit of detection (LOD) values of oxytetracycline, tetracycline and chlorotetracycline are presented at Table 7.2.

Table 7.2. Chromatographic and calibration parameters for the separation of tetracyclines.

Compound	t^R (min)	Linearity range(mg/l)	Calibration curve equation ^a	R^{2b}	LOD ^c (mg/l)
mobile phase: 0.01 M oxalic acid : MeOH : ACN (75:12.5:12.5)					
OTC	4.1	0.01-1	$y = 27.6 x + 0.015$	1.000	0.011
TC	4.9	0.01-1	$y = 30.8 x - 0.035$	1.000	0.011
CTC	12.7	0.02-1	$y = 16.8 x + 0.058$	0.999	0.056

^a y = peak area, x = concentration (mg/l)

^b Linear regression coefficient

^c estimated limit of detection using signal-to-noise ratio as $S/N=3$

7.3.2. Comparison of Extraction Buffers

Since TCs have the binding ability for proteins in biological samples and they can be adsorbed on soil via hydrophobic interactions, hydrogen bonding, and complexation with metal divalent and trivalent metal ions (Sithole and Guy, 1987b; Rabølle and Spliid, 2000), strong acid agents are suitable for extracting TCs from solid matrices. However, due to the conversion of tetracyclines to their epi and anhydrous forms under high acidic conditions, mildly acidic solvents and complexation agents (EDTA) were used for the extraction of tetracyclines from biological samples or sediment samples (Capone et al., 1996; Castellari and Garcia-Regueiro, 2003; Yang et al., 2005). On the other hand, combination of high concentration of citric acid buffer and methanol or ethyl acetate were also used as a buffer solution for the extraction of tetracyclines from agricultural soil samples (Hamscher et al., 2002; Jacobsen et al., 2004). In this study, three different buffer solutions (Table 7.1) were used for the simultaneous extraction of tetracycline compounds from loamy sand soil that had been spiked with each tetracycline at a concentration level of 1 mg/kg. The efficiencies

of different buffer solutions were evaluated by the comparison of recoveries which were calculated by comparing individual peak areas of spiked samples with those of corresponding standard solutions (Figure 7.2). Mean recoveries obtained from duplicate analysis of Buffer I extracts were found to be 47 %, 23 %, and 9 % for OTC, TC, and CTC respectively. Hamscher et al. (2002) applied a method to extract the organic fraction of soil (e.g. humic substances) in which ethyl acetate and high molar citric acid buffer at pH 4.7 were used as extraction buffer considering the fact that one of the pathways for the sorption of tetracyclines is the organic constituents of the soil (Sithole and Guy, 1987a). In this study, by using Buffer II, which contains 0.1 M citric acid and methanol in composition, as an extraction solvent, mean recoveries could be increased up to 60 %, 38 % and 25 % for OTC, TC and CTC, respectively. Further increase of the citric acid concentration to 0.5 M in the extraction buffer (Buffer III) resulted in a 10-15 % of enhancement of the recoveries of tetracycline antibiotics in loamy sand.

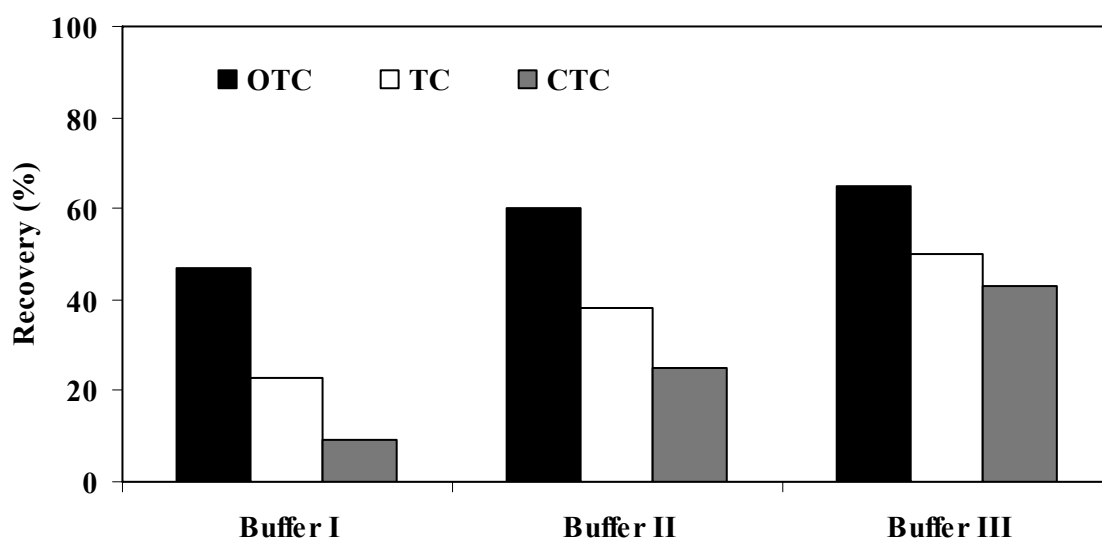


Figure 7.2. Effect of extraction buffer on the recovery of tetracyclines.

7.3.3. Effect of Soil Type on the Analysis of Tetracyclines

Depending on the soil type, performed extraction method, and spiking level, the recoveries of tetracycline antibiotics vary significantly. TC recovery values obtained from various other studies in the literature are listed in Table 7.3 together with the soil characteristics and extraction method used.

Table 7.3. Soil extraction methods and obtained recovery rates for tetracyclines (TCs) in the literature.

Soil Composition (OC % ; clay %)	Extraction Method	Recovery rate (%)	Reference
Sandy (1.8 ; 2.4)	Vortex and shaker (1M citrate buffer + ethyl acetate)	OTC: 73.0	Hamsher et al., 2002
		TC : 41.2	
		CTC: 75.7	
Loamy sand (1.6 ; 11.3)	Pressurized Liquid Extraction (0.2M citric acid + methanol)	OTC: 80.7 CTC: 51.3	Jacobsen et al., 2004
Sandy (1.4 ; 5.2)		OTC: 119.0 CTC: 75.9	
Loamy sand (1.3 ; 8.5)	Vortex and sonication (EDTA+citric acid+Na ₂ HPO ₄ and methanol)	OTC: 63.0	
Sandy loam (1.3 ; 10.3)		OTC: 65.0	Blackwell et al., 2004

In order to evaluate the effect of soil characteristics on the simultaneous analysis of three tetracycline group antibiotics, three standard soils (sandy, loamy sand, and sandy loam) were spiked at two different levels and extracted with Buffer III. Mean recovery values and the relative standard deviations are presented in Table 7.4.

Table 7.4. Effect of soil type on the recovery rates of tetracyclines.

Soil Composition (OC % ; clay %)	Spiking Level (mg/kg)	n	Recovery (% mean \pm S.D)		
			OTC	TC	CTC
Sand (0.59; 2.5)	0.2	5	97 \pm 6	66 \pm 3	69 \pm 4
	1.0	6	85 \pm 4	70 \pm 2	64 \pm 2
Loamy Sand (2.27; 6.6)	0.2	5	65 \pm 3	55 \pm 4	44 \pm 6
	1.0	6	65 \pm 6	50 \pm 3	43 \pm 3
Sandy Loam (1.24; 9.5)	0.2	5	63 \pm 4	41 \pm 5	30 \pm 4
	1.0	6	53 \pm 4	41 \pm 3	30 \pm 3

The recovery values of the three tetracyclines increased parallel to a decline in the organic carbon and clay content of soil in the order of OTC > TC > CTC. The low recovery values for TC and CTC might be a result of a much stronger or more specific sorption of these compounds compared to OTC.

7.3.4. Sorption of Tetracyclines on Standard Soils

In order to see if the recoveries of tetracycline antibiotics from soils correlate with their sorption behavior, a mixture of OTC, TC and CTC solution was shaken with three types of standard soils having different clay and organic carbon contents. Since tetracyclines have a strong tendency for sorption, the concentration range of 2-10 mg/l was chosen to obtain sorption isotherms with high accuracy. Antibiotic solutions were prepared both in the absence and presence of 0.01M CaCl₂ and the sorption isotherms are presented in Figure 7.3. The experimental data fitted quite well to the Freundlich isotherms, as indicated by the high correlation coefficient values ($r^2 > 0.98$). Since the isotherms were close to linearity (n values close to 1), linear partition coefficients were also estimated (Table 7.5).

Table 7.5. Distribution coefficients (K_d) of tetracycline, oxytetracycline and chlorotetracycline for different types of soils.

Soil Type	Compound	K_d (l/g)	K_d (l/g)	K_F	n
		Control ^a	In the presence of 0.01 M CaCl ₂		
Sandy	OTC	0.41	0.21	0.19	0.96
	TC	0.43	0.19	0.18	0.93
	CTC	0.55	0.35	0.40	1.1
Loamy sand	OTC	1.21	0.89	1.00	1.0
	TC	1.34	0.88	1.26	1.1
Sandy loam	OTC	1.14	0.25	0.23	0.78
	TC	1.58	0.29	0.28	0.86

^a K_d values for the control sorption experiments in the absence of 0.01M CaCl₂.

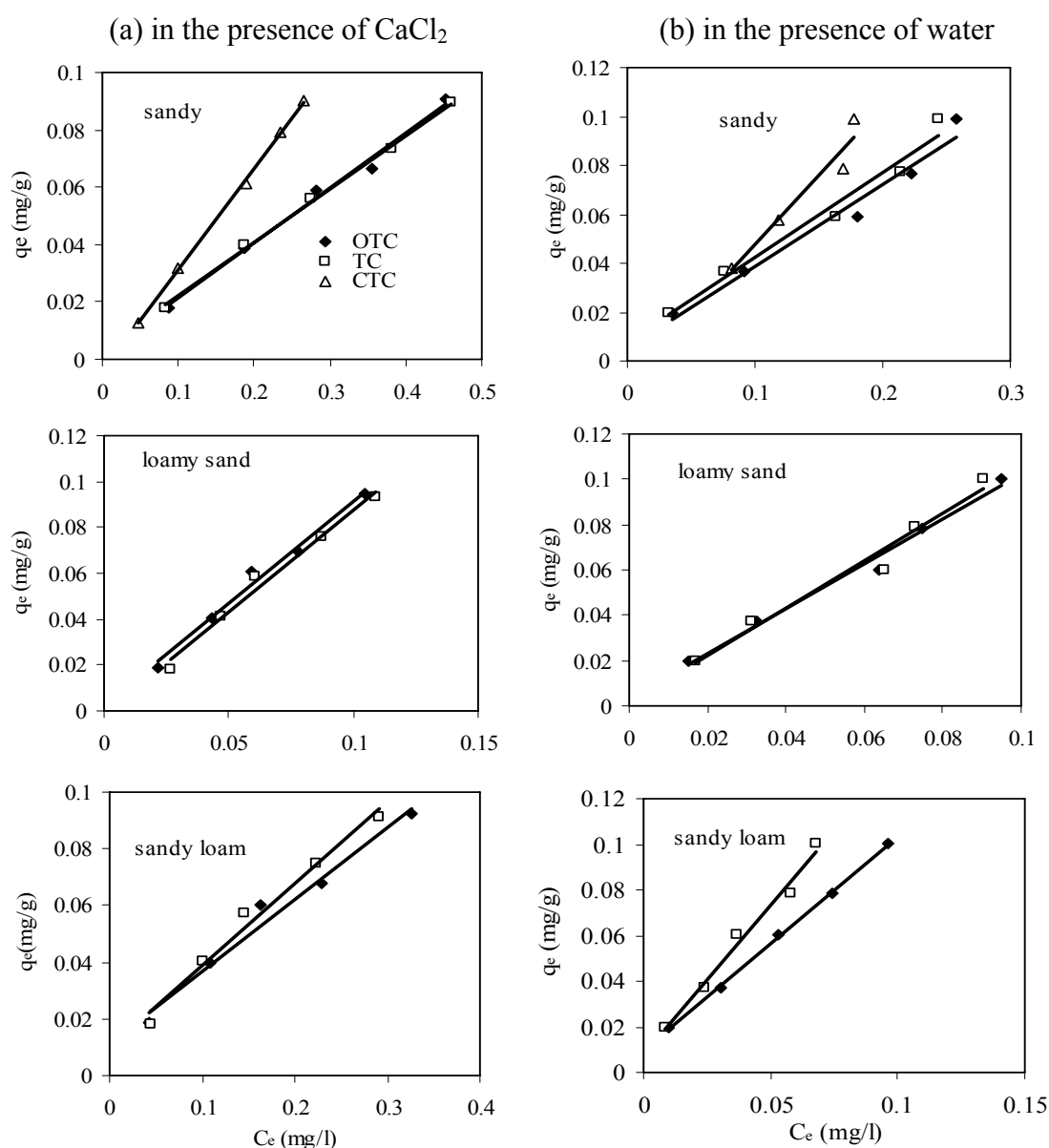


Figure 7.3. Sorption isotherms of tetracycline antibiotics in sandy, loamy sand and sandy loam soils (a) in the presence and (b) absence of 0.01 M CaCl_2 .

Tetracycline and oxytetracycline losses in soil free control bottles were between 0-4 % while chlortetracycline exhibited losses of up to 10 % over the 24 h equilibration time, which might be due to the higher instability of CTC in solution that is also reported by Figueroa et al., (2004). The distribution coefficient of CTC in sandy soil was found to be much higher than that of TC and OTC while it was not possible to estimate K_d and K_f values for CTC in loamy sand and sandy loam soils as the concentrations in aqueous solution for the initial concentrations of 2-4 mg/l were below the detection limit. However, the extent of sorption for CTC at the initial concentration of 10 mg/l was about 99 % in

sandy loam soil, that of TC and OTC were 96.5 and 97 %, respectively. These results could be an explanation for the much lower recovery of CTC in three types of standard soil in comparison to those of TC and OTC.

As listed in Table 7.5, the higher distribution coefficients for tetracycline compounds in loamy sand and sandy loam compared to sandy soil is an obvious evidence of the effect of clay and organic matter content on the sorption behavior of these compounds. The strong sorption of tetracycline and oxytetracycline to negatively charged large clay fractions by ionic interactions or to acid sites in humic substances were also reported in previous studies (Sithole and Guy, 1987a,b; Pouliquen and Bris 1996; Rabølle and Spliid, 2000; Loke et al., 2002; MacKay and Canterbury, 2005). Consequently, the lower recovery values obtained for sandy loam and loamy sand soil compared to sandy soil could be attributed to irreversible sorption of three tetracycline compounds to the clay or organic fraction of soils rendering them non extractable using the chemical methods. On the other hand, very similar K_d values were obtained for sandy and sandy loam soils in the presence of CaCl_2 solution. Although, at pH range of 4.3-4.9 (pH of the antibiotic solutions), the dominant species for tetracycline compounds are zwitterions, cation interaction with the clay surface has a large contribution to overall sorption of these compounds (Figuerola et al., 2004). Therefore, competition between Ca^{2+} and tetracycline compounds for negatively charged mineral surfaces might be a reason for the lower sorption coefficients in sandy loam soils. Considering this fact, it can be concluded that the importance of hydrophobic interactions between soil organic matter and zwitterionic tetracycline compounds becomes higher than electrostatic interactions between clay surface and cationic tetracycline and confirmed by the highest distribution coefficients in loamy sand soil. Additionally, the extraction method used for the analysis of tetracyclines in soil samples was developed to eliminate the interferences originating from humic substances and therefore did not consider the extraction of the compounds sorbed on the clay part of the soil. This could be an explanation for the lower recoveries of tetracyclines in sandy loam than loamy sand, in spite of the corresponding low distribution coefficients.

7.3.5. Analysis of Tetracyclines in Sediment and Mud Samples

Tetracycline group antibiotics, especially tetracycline and oxytetracycline are widely used in fish farming as medicated food pellets. Food surplus not eaten by the fish and medicines excreted with feces will be deposited on the sediment nearby fish cages (Björklund et al., 1991; Coyne et al., 1994; Smith and Samuelsen, 1996;). It was found that OTC was present at a mass ratio range of 0.1 to 11 mg/kg (Björklund et al., 1990; Coyne et al., 1994; Weston et al., 1994; Kerry et al., 1995) and persistent up to 419 days in fish farm sediments (Jacobsen and Berglind, 1988; Samuelsen, 1989; Björklund et al., 1990). Samuelsen et al. (1989, 1992) reported that the persistence of OTC was highly dependent on the sedimentation rate which was confirmed in laboratory experiments. If the OTC containing sediment was covered with a further sediment layer, the half-life of the antibacterial agent increased drastically (>77 days). In this study, collected marine sediment and mud samples were also analyzed for OTC. The concentrations of oxytetracycline in mud and sediment samples and recovery values determined by standard addition are presented at Table 7.6.

Table 7.6. Residual concentrations of oxytetracycline (OTC) in sediment and mud samples.

Sample	OTC (mg/kg) n=2	Mean recovery (%) (n=2, spiked at 1 mg/kg)
<i>Sediment</i>		
S1	0.08-0.10	76.4
S2	0.05-0.02	67.3
<i>Mud</i>		
M1	0.19-0.30	48.2
M2	0.22-0.21	47.4
M3	0.04-0.04	57.2

Residues of OTC were found in the range of 0.02-0.1 mg/kg in sediment samples and 0.04-0.3 mg/kg in mud samples. The fact that even in the depths of between 9-15 m OTC can still be detected indicates that the decomposition of OTC in the fish farm sediments is minimal. The environmental fate of the antibiotics in the bottom sediment is of great

concern. The presence of antibiotics in the sediment may affect the natural bacterial composition and activity, and thereby change the ecological structure of benthic microbial communities. Their accumulation in pond sediment can also lead to decreased or inhibited microbial activity in the sediment, which in turn can cause anaerobic conditions resulting in more toxic by-products such as sulfides and ammonia. Hansen et al. (1992) reported that the total number of bacteria in sediments with added OTC decreased to 50-70% a few days after adding the agent to the sediment leading to an inhibition of sulfate reduction rate to less than 10%. It is described that most of the pathogenic bacteria isolated from the farmed fish and farm sediments were already resistant against oxytetracycline leading to inefficient treatment of farmed animals (Björklund et al., 1991; Husevag et al., 1991; Nygaard et al., 1992). The individual dissipation time (DT_{50}) of four tetracyclines in sediments from outdoor microcosms have been given as 1-4 days, depending on treatment levels up to 100 $\mu\text{l/kg}$, authors assume that at higher drug levels (100-300 $\mu\text{g/l}$) the dissipation may be biphasic with the DT_{50} ranging between 1 and 3 weeks (Sanderson et al., 2005).

7.4. Conclusion

In this study, the effect of soil characteristics on the analysis of three tetracycline antibiotics, oxytetracycline, tetracycline and chlorotetracycline, was investigated by means of an HPLC method including liquid and solid phase extractions. High molar concentration (0.5 M) of citric acid buffer in combination with 10 % methanol was found to be more efficient for the recoveries of tetracyclines from loamy sand soil, compared to McIlvaine buffer and 0.1 M citric acid methanol combination, due to its ability to extract humic substances which play a significant role in the sorption of tetracyclines. Sorption coefficients of tetracycline antibiotics were higher for loamy sand soil in which organic carbon content was the highest. In spite of the lower recoveries of antibiotic compounds from sandy loam, the sorption coefficients were relatively low for this type of soil. The result can be explained by the competition between antibiotics and Ca^{2+} ion for the clay content of sandy loam soil. In addition, the obtained results revealed that the analysis method should be modified to reduce the interferences originating from the sorption of tetracyclines on clay content of the soil as well as its organic content.

The presence of oxytetracycline in marine sediment samples from 9-15 m depths of nearby fish farms indicates its high persistency. Since medical drugs are developed with the intention of performing a biological activity; they are important pollutants in terms of their effects and their fate in the environment. Further research under field conditions is needed addressing the effects of veterinary medicine on the bacterial and macro-fauna near fish farming sites. It is also important to focus on the development and introduction of more suitable drugs and organic feed for use in aquaculture to achieve “best management” practices in the aquaculture industry.

8. OZONATION OF ANIMAL WASTES CONTAINING OXYTETRACYCLINE

8.1. Introduction

Current livestock and poultry productions regularly and widely use antibiotics either directly or as food additives for disease control and prevention as well as for promoting growth, and thus the yield. Tetracyclines are widely used group of veterinary antibiotics and about 50-80 % of the applied dose excreted in urine and feces of animals as a parent compound (Halling-Sørensen et al., 2002). Tetracyclines have been detected at a concentration range of 0.25-1 mg/l in animal waste from swine lagoon (Campagnolo et al., 2002) and up to 46 mg/kg in manure and bedding material (De Liguoro et al., 2003; Balcioğlu et al., 2007; Martinez-Carballo et al., 2007). The fate of tetracyclines in various manure samples has been investigated in numerous studies and the obtained results exhibited wide variation with type of animal, type of feeding operation, treatment practices of manure, and environmental conditions (Kühne et al., 2000; Winckler and Grafe, 2001; De Liguoro et al., 2003; Arıkan et al., 2007). Tetracyclines have a half life ranged from 4.5 days to 105 days, and biodegradation has minor importance on their fate. Land application of animal waste as a fertilizer to improve soil quality, provides routes for tetracyclines to enter the terrestrial environment. Consequently, chlorotetracycline (CTC) and oxytetracycline (OTC) have been found in environmental soil samples at a concentration range of 4-7 µg/kg (Hamsher et al., 2002) and 6-7 µg/kg (De Liguoro et al., 2003), respectively. Tetracycline concentration has been estimated as 232 - 406 µg/l in animal feeding operation flush water (Huang et al., 2001). In spite of their susceptibility to photodegradation (Oka et al., 1989) and affinity to bind solid matrices (Rabølle and Spliid, 2000; Loke et al., 2002; MacKay and Canterbury, 2005), tetracyclines have also been measured in surface waters up to 1.34 µg/l (Lindsey et al., 2001; Kolpin et al., 2002).

A major concern regarding the accumulation of antibiotic residues in the environment is the emergence of more strains of antibiotic-resistant pathogens, along with strains that are becoming more resistant (US EPA, 2001). The bacterial resistance to tetracyclines has been the subject of numerous studies (Halling Sørensen et al., 2002; Sengelov et al., 2003).

Moreover, tetracycline antibiotics are known to be toxic to environmentally relevant bacteria both in manure (Masse et al., 2000; Arıkan et al., 2006) and in soil (Halling Sørensen et al., 2002; Thiele Bruhn and Beck, 2005).

Development of efficient treatment processes is essential to eliminate antibiotics in wastes produced by animal feeding operations. Ozonation is known as an effective process for the degradation of recalcitrant compounds in wastewater (Ikehata et al., 2006) and terrestrial environment (Rivas, 2006). Moreover, the use of ozone at sufficient dose found to be effective in bacterial inactivation (Macauley et al., 2006) and in odor reduction in animal feeding operation wastes (Watkins et al., 1997).

In the light of the facts mentioned above, this research aims to cover the ozonation of oxytetracycline, a tetracycline group antibiotic, both in manure and in wastewater. The effects of antibiotic concentration, ozonation time, humidity of manure, and pH of wastewater on ozonation yield were interrogated. In addition to these, manure characteristics that are important for fertilizer use were evaluated since they show variations upon ozone application.

8.2. Materials and Methods

8.2.1. Chemicals

Oxytetracycline hydrochloride (Sigma, $C_{22}H_{24}N_2O_9 \cdot HCl$, >95% purity) was used for the fortification of manure or wastewater samples. Formic acid and HPLC grade acetonitrile were obtained from Sigma-Aldrich for the HPLC analysis of oxytetracycline. The extraction of oxytetracycline was performed with citric acid monohydrate (Merck), disodium EDTA two hydrates (Fluka), sodium acetate three hydrates (Merck) and HPLC grade methanol (Sigma-Aldrich). Chemicals used for dehydrogenase assay were, resazurin sodium salt redox indicator (Riedel-de Haën), imidazole (Fluka), mercuric chloride (Merck), and tryptic soy broth (Bio-Rad). *Bacillus subtilis* was obtained from Refik Saydam Hygiene Center. All solutions were prepared with Milli-Q ultrapure deionized water (Millipore, Milford, MA).

8.2.2. Preparation of Manure and Wastewater Samples

Cow manure was obtained from an ecological farm located at the west part of Turkey. After drying at 70°C for 24 h, manure was sieved (2 mm) and was subsequently sterilized by autoclave at 120 °C for 15 minutes, to prevent any biological activity. OTC stock solution was prepared in methanol at a concentration of 1 mg/ml. Working solutions of OTC were prepared by dilution of stock solution either with methanol or water for the spiking of manure samples. After mixing thoroughly, it was equilibrated overnight under the hood to evaporate the solvent and to allow the sorption of OTC onto the manure.

Synthetic wastewater was prepared from the manure to imitate the wastewater produced by the cleaning of animal barns. The manure was mixed with deionized water (1:10 w/v) at 150 rpm (Nüve, NM 110) for 24 h. Manure suspension was then centrifuged at 4000 rpm (Hettich Universal 16A) and filtered to obtain synthetic wastewater. Similar to manure samples, synthetic wastewater was sterilized prior to ozonation experiments. Synthetic wastewater was spiked with the OTC stock solution that was prepared in water at a concentration of 1 mg/ml.

8.2.3. Ozonation of Manure and Wastewater Samples

Manure (27 g) was ozonated in a borosilicate glass column (40 cm height; 2 cm diameter) in semi-batch mode with continuous flow of the ozone and oxygen gas mixture (gas phase ozone concentration of 50 g/m³) at a rate of 0.05 m³/h through a sintered disc placed at the bottom of the column. Before entering the reactor, ozone and oxygen gas mixture was moisturized through water (pH=2) in order to maintain desired moisture content in manure. On the other hand, ozonation of 1.5 l synthetic wastewater was performed in another glass ozone bubble column (78 cm height; 6 cm diameter) in semi-batch mode with a gas flow rate of 0.1 m³/h (Arslan and Balçioğlu, 2000). In this reactor, experiments were performed with two different gas phase ozone concentrations; 11.2 and 32.7 g/m³. Wastewater was circulated with a peristaltic pump (Cole Palmer, Vernon Hills, IL) to the ozone bubble column to obtain good mixing conditions. Ozone was produced from dry, pure oxygen in a laboratory scale corona discharge ozone generator (Fisher OZ

500, Pittsburgh, PA) for both reactors. The off-gas ozone concentration from top of the column was monitored by using an online ozone analyzer (Fisher Ozotron 23).

8.2.4. Analysis of Manure and Wastewater Characteristics

pH of manure was measured in a mixture manure and water at a ratio of 1:2 (w/v) using a pH probe (WTW pH 330 pH meter, Weilheim, Germany). Total organic carbon (C_t) of manure was quantified by Walkley-Black method with some modifications (Sanchez-Monedero et al., 1996). The alkaline soluble organic carbon (sum of humic acid and fulvic acid carbon; C) and the fulvic acid carbon (C_{FA}) content of manure were determined spectrophotometrically (Hach DR/2010, at 600 nm, Loveland, CO) in digested manure samples after extraction with 0.1 M NaOH (Sanchez-Monedero et al., 1996). The humic acid carbon (C_{HA}) was calculated by subtracting the C_{FA} from C. Total Kjeldahl Nitrogen (TKN) of manure was analyzed by Kjeldahl method (Peters et al., 2003). NH_4^+ -N and NO_3^- -N were extracted with 2 N KCl and determined by Nessler and Cd-reduction method, respectively (Hach DR/2010 manual). Inorganic nitrogen was defined as the sum of NH_4^+ -N and NO_3^- -N. Selected characteristics of OTC contaminated manure (50 mg/kg) are reported in Table 8.1.

Table 8.1. Some characteristics of manure contaminated with OTC.

Parameter	Value
pH	8.6
Organic carbon (%)	26.5
Total nitrogen (%)	1.42
C_{HA} (%)	0.98
C_{FA} (%)	1.15
NH_4^+ -N (mg/kg)	195
NO_3^- -N (mg/kg)	510

Chemical oxygen demand (COD) and biochemical oxygen demand (BOD_5) of synthetic wastewater were analyzed by using closed reflux and membrane electrode method (APHA/AWWA, 1998), respectively. Total organic carbon (TOC) of synthetic wastewater was measured by TOC Analyzer (Shimadzu TOC-V CSH, Kyoto, Japan) and

total nitrogen was determined according to Kjeldahl method (APHA/AWWA, 1998). Characteristics of OTC contaminated synthetic wastewater (5 mg/L) are presented in Table 8.2.

Table 8.2. Some characteristics of synthetic wastewater contaminated with OTC.

Parameter	Value
pH	8.5
Conductivity ($\mu\text{S}/\text{cm}$)	850
TOC (mg/l)	157.2
COD (mg/l)	465.8
BOD ₅ (mg/l)	25.5

8.2.5. Dehydrogenase Assay for Toxicity

Toxicity of raw and ozonated synthetic wastewater was evaluated by the method described by Brouwer, (1991) with some modifications and *Bacillus subtilis* was used as the bacterial culture due to its abundance in manure and soil. The inhibition of the dehydrogenase activity (DHA) of bacteria in wastewater was determined by measuring the reduction of resazurin to resofurin spectrophotometrically at 600 nm wavelength after about 30 min of incubation. The toxicity was expressed as percent inhibition (%I) of the DHA using the following equation.

$$I(\%) = \frac{(C - B)}{(A - B)} \times 100 \quad (8.1)$$

where

A= absorbance of reagent control (3.75 ml growth medium + 0.25 ml imidazole + 0.5 ml uncontaminated raw wastewater + 1ml rezasurin solution)

B= absorbance of cell control (2.75 ml growth medium + 0.25 ml imidazole + 0.5 ml uncontaminated raw wastewater + 1ml cell culture + 1ml rezasurin solution)

C= absorbance of samples (2.75 ml growth medium + 0.25 ml imidazole + 0.5 ml ozonated or raw wastewater contaminated with OTC+ 1ml cell culture + 1ml rezasurin solution).

8.2.6. Extraction and Determination of Oxytetracycline

Extraction of OTC in manure samples was performed according to the method described by Blackwell et al. (2004) with some modifications. The samples were extracted on a vortex (Nüve NM 110, Ankara, Turkey) for 30 s upon addition of 5 ml of extraction buffer (MeOH: 0.1 M EDTA: McIlvaine buffer (60 ml of 0.2 M citric acid + 40 ml of 0.4 M Na₂HPO₄), 50:25:25) to 1 g of raw or ozonated manure. The extraction was followed by an ultrasonic bath (Sonorex super RK 510, Morfelden-Walldorf, Germany) for 10 min, and then a centrifugation step at 1200 g for 15 min (Selecta Meditronic BL-S, Barcelona, Spain). The supernatant was decanted into a 500 ml glass bottle and the manure residue was extracted two more times. A total of 15 ml supernatant was then combined and diluted to approximately 400 ml with Milli-Q water to reduce the methanol content below 2%, and H₃PO₄ was added to reduce the pH of solution to 2.9. The pH of 20 ml of raw or ozonated wastewater samples was adjusted to 2.9 prior to solid phase extraction for the extraction of OTC from wastewater.

SAX (strong anion exchange, 500 mg sorbent, 6 ml capacity; Phenomenex, Torrance, CA) and HLB (200 mg sorbent, 6 ml capacity; Waters) solid phase extraction (SPE) cartridges were set up in tandem. After conditioning of cartridges with methanol and SPE conditioning buffer, diluted soil extract or wastewater sample was passed through the cartridges. The SAX cartridge was removed and the HLB cartridge was washed sequentially with: SPE washing buffer; 0.1M NaOAc; Milli-Q water; and 20% MeOH. The HLB cartridge was air dried for 10 min and then eluted with 2 ml methanol to produce the extract for analysis which was performed with an HPLC system (Agilent Technologies 1100 series, Santa Clara, CA) equipped with a tertiary pump, a photodiode array and an autosampler with an automated injection system. Gradient elution carried out with acetonitrile (solvent A) and water containing 0.1% formic acid (solvent B) using an analytical column Eclipse XDB C18 (5 μ , 150 x 4 mm) equipped with ODS guard column. Elution started with A:B:10:90 rising linearly to 40:60 from 0 to 5 min and then returning to initial composition from 5 to 8 minutes. OTC was detected at 360 nm with a retention time of 6.0 min. OTC quantification in manure and wastewater extracts was performed by external calibration curve. Oxytetracycline recovery was 93.2 % (\pm 9.4) in synthetic

wastewater samples and 48.8 % (± 5.8) in manure samples. The limits of detection (LOD) values were 1.3 $\mu\text{g/l}$ and 0.22 mg/kg for synthetic wastewater and manure, respectively.

8.2.7. A full 2^3 Factorial Design

The effect of three independent variables (pH, OTC concentration, C_{OTC} and gas phase ozone concentration, $C_{(\text{O}_3)\text{g}}$) to ozonation of synthetic wastewater was tested by a two-levels full factorial design, which involves eight different experimental sets representing all combinations of 3 factors at both high and low levels (Berthouex and Brown, 2002). The high and low levels are denoted by + / –, and design matrix is presented at Table 8.3.

Table 8.3. Design matrix for 2^3 full factorial design.

Run	Factor			Factor	Level (-)	Level (+)
	1	2	3			
I	-	-	-	1. C_{OTC}	5 mg/l	10 mg/l
II	+	-	-	2. pH	8.5	5.0
III	-	+	-	3. $C_{(\text{O}_3)\text{g}}$	11.2 g/m ³	32.7 g/m ³
IV	+	+	-			
V	-	-	+			
VI	+	-	+			
VII	-	+	+			
VIII	+	+	+			

The main effects of three factors and effects of the factor interactions were estimated using model matrix for 2^3 full factorial designs. Resulting effects were then used to develop normal plot with MINITAB software (State College, PA) in order to estimate significance ($\alpha = 0.1$) of effects.

8.3. Results and Discussion

8.3.1. Ozonation of Synthetic Wastewater

In the first part of the study, ozonation of the synthetic wastewater was investigated at two different OTC concentration, pH, and gas phase ozone concentration values. The performance of ozonation process was determined by OTC degradation, TOC, and COD abatement as well as the variations in bacterial toxicity and BOD₅/COD ratio.

8.3.1.1. Effect of Ozonation on OTC Degradation. The degradation of oxytetracycline in synthetic wastewater by means of ozonation was followed at different experimental conditions (Figure 8.1).

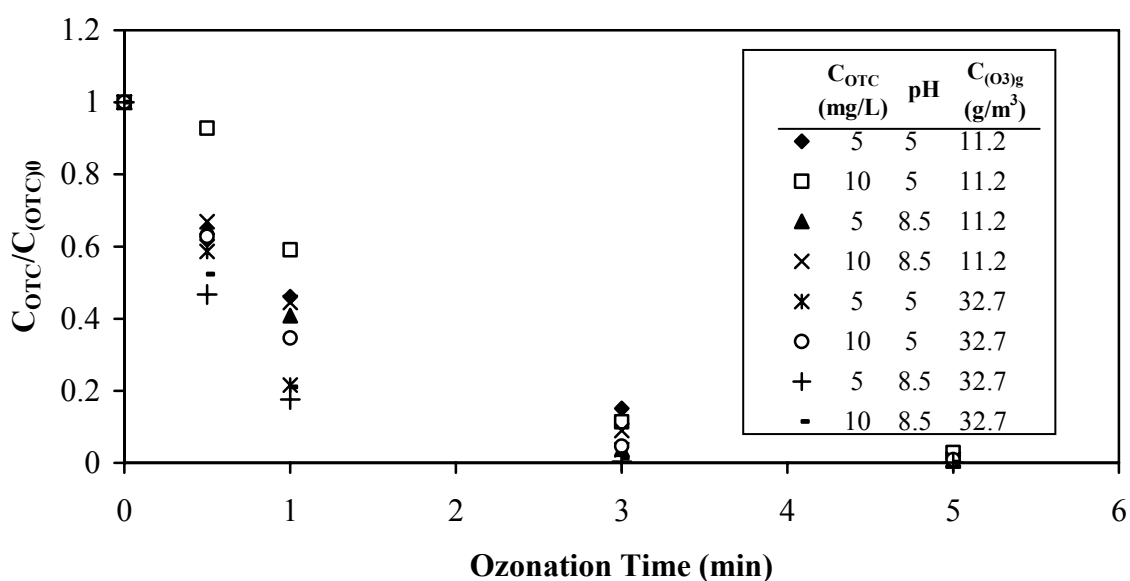


Figure 8.1. Influence of experimental conditions on OTC degradation rate in synthetic wastewater.

As can be seen from Figure 8.1 more than 97 % of OTC was degraded within 5 minutes, regardless of experimental conditions. Our findings are in accordance with previous studies related with the rapid removal of various antibiotics and some other pharmaceuticals treated by ozonation process (Ikehata et al., 2006).

Ozone concentration in the solution is presumably close to constant in our study, since ozone was applied in excess at a continuous mode, so that OTC degradation rate constants were determined based on pseudo first order kinetics. The absorbed ozone concentration by synthetic wastewater, pseudo first order OTC degradation rate constants, and the effects of main factors and their interactions on the OTC degradation rates are depicted in Table 8.4.

Table 8.4. The effects of main factors and their interactions on the OTC degradation rate constants and overall COD removals.

Run	k^a (min^{-1})	Absorbed O_3 (g/l)	% COD Removal	Identity of Effect	Effect, k	Effect, COD Removal
I	0.75	0.031	19.3	C_{OTC}	-0.08	0.57
II	0.75	0.031	22.8	pH	0.3	15
III	1.064	0.035	31.4	$C_{(\text{O}_3)_g}$	0.28	21
IV	0.902	0.035	30.5	$C_{\text{OTC}} \times C_{(\text{O}_3)_g}$	-0.001	0.72
V	0.97	0.078	37.6	$C_{\text{OTC}} \times \text{pH}$	-0.07	- 0.72
VI	0.95	0.080	36.7	$\text{pH} \times C_{(\text{O}_3)_g}$	0.07	5.17
VII	1.409	0.094	57.1	$C_{\text{OTC}} \times C_{(\text{O}_3)_g} \times$ pH	0.008	1.4
VIII	1.26	0.098	57.7			

^a OTC degradation rate constants

The ozone absorption by synthetic wastewater achieved in 5 minutes was considerably increased with increasing pH and influent gas phase ozone concentration. However, increasing OTC concentration did not cause any increase in ozone absorption due to other ozone demanding constituents of synthetic wastewater (Table 8.4).

The evaluation of effects of experimental conditions indicated that OTC degradation rate depended on pH and gas phase ozone concentration, but not initial antibiotic concentration (Table 8.4). Increment of pH value and gas phase ozone concentration from 5 and 11.2 g/m^3 to 8.5 and 32.7 g/m^3 increased the degradation rate of OTC by an average

of 0.3 min^{-1} . It is well expected since at alkaline pH, ozone decomposes into reactive hydroxyl radical which has higher oxidation potential than that of ozone itself. In addition, phenolic groups of OTC (Figure 8.2) are in their deprotonated form at pH 8.5 (Huber et al., 2003), and they react many orders of magnitude faster with electrophilic ozone than their protonated forms since they are stronger nucleophiles.

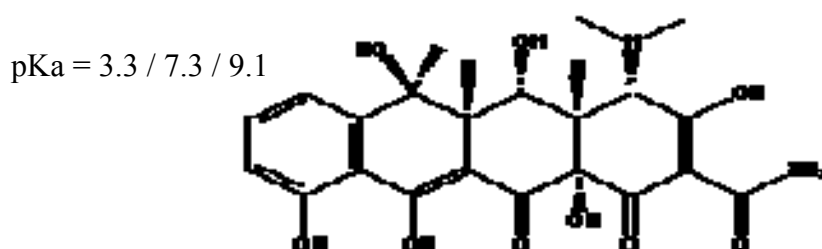


Figure 8.2. Molecular structure of oxytetracycline.

8.3.1.2. Effect of Ozonation on COD of Synthetic Wastewater. Chemical oxygen demand of synthetic wastewater was also determined throughout the ozonation processes. Table 8.4 shows the overall COD removals after 30 minutes of ozonation at different oxidation conditions as well as the effects of main factors and their interactions.

Initial OTC concentration did not affect the overall COD removal (Table 8.4) due to its low contribution (theoretical COD of 5 and 10 mg OTC/l were 9 and 18 mg/l) to the total COD of synthetic wastewater ($465.8 \pm 14.2 \text{ mg/l}$). Similar to OTC degradation, increment of wastewater pH and gas phase ozone concentration increased the COD removal by an average of 15% and 21%, respectively. The effect of pH and gas phase ozone concentration interaction seems to be significant compared to other variables. That means, increment of gas phase ozone concentration at pH 8.5 has more pronounced effect on COD removal than that of at pH 5. Compared to hydroxyl radicals, ozone itself has a limited reactivity with organic compounds, so that increase in the gas phase ozone concentration at low pH did not affect COD removal as much as hydroxyl radicals.

8.3.1.3. Effect of Ozonation on TOC and COD/TOC Ratio of Synthetic Wastewater. Total organic carbon content of synthetic wastewater during ozonation was also monitored to make an evaluation for the fate of organics in wastewater as well as the antibiotic. For the

experimental runs, II, IV, VI and VIII, TOC of synthetic wastewater was measured, to represent effect of pH and gas phase ozone concentration on mineralization of organics in synthetic wastewater, which was contaminated with 10 mg/l OTC. The effect of initial OTC concentration was not considered since its contribution to overall TOC (157.2 ± 4.2 mg/l) of synthetic wastewater was considerably low (theoretical TOC of 5 and 10 mg OTC/l were 2.8 and 5.6 mg/l, respectively). Figure 8.3 represents TOC and COD abatements, and variation in COD/TOC ratio of wastewater at different pH and gas phase ozone concentrations, as well as changes in pH values throughout the ozonation process.

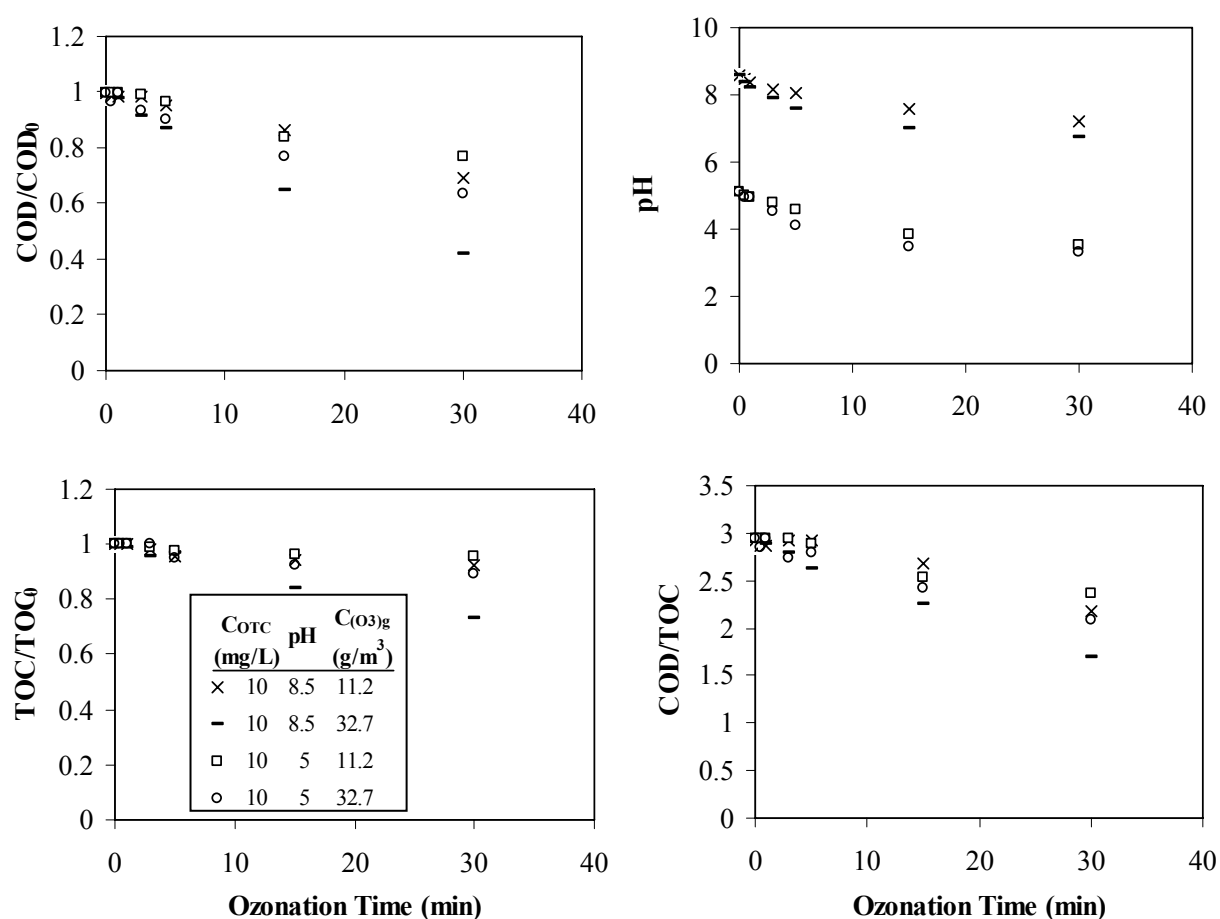


Figure 8.3. Effect of ozonation on COD and TOC abatements, COD/TOC ratio and pH.

Increment of pH from 5 to 8.5 at low and high influent gas phase ozone concentrations enhanced the overall TOC removal by 2 and 15 %, respectively. On the other hand, about 3 fold increment of influent gas phase ozone concentration at low and high pH values increased the overall TOC removal by 6 and 20 %, respectively. The highest TOC removal was 27 % for experimental run VIII (pH = 8.5; $C_{(O_3)g} = 32.7$ g/m³). The abatement in COD

as depicted in Figure 8.3 may be attributed to mineralization, as well as to partial oxidation of organic compounds in synthetic wastewater. Average oxidation state of organic carbon in solution after ozonation was higher than that of untreated wastewater as it appears from decreasing COD/TOC ratio with increasing ozonation time. pH of the synthetic wastewater was not controlled throughout the treatment and 2 unit pH decrease was caused by formation of acidic by-products. However, the reduction in pH did not affect the removal rates of COD and TOC of synthetic wastewater.

8.3.1.4. Effect of Ozonation on Biodegradability and Bacterial Toxicity of Synthetic Wastewater. Degradation of a target compound (OTC) is not sufficient for the evaluation of ozonation process effectiveness. Toxicity and biodegradability of degradation products produced from ozonation of contaminated synthetic wastewater are also important factors for the applicability of the process. Therefore bacterial toxicity and biodegradability were determined for the experimental run VII, in which the fastest degradation of OTC was obtained (Figure 8.4).

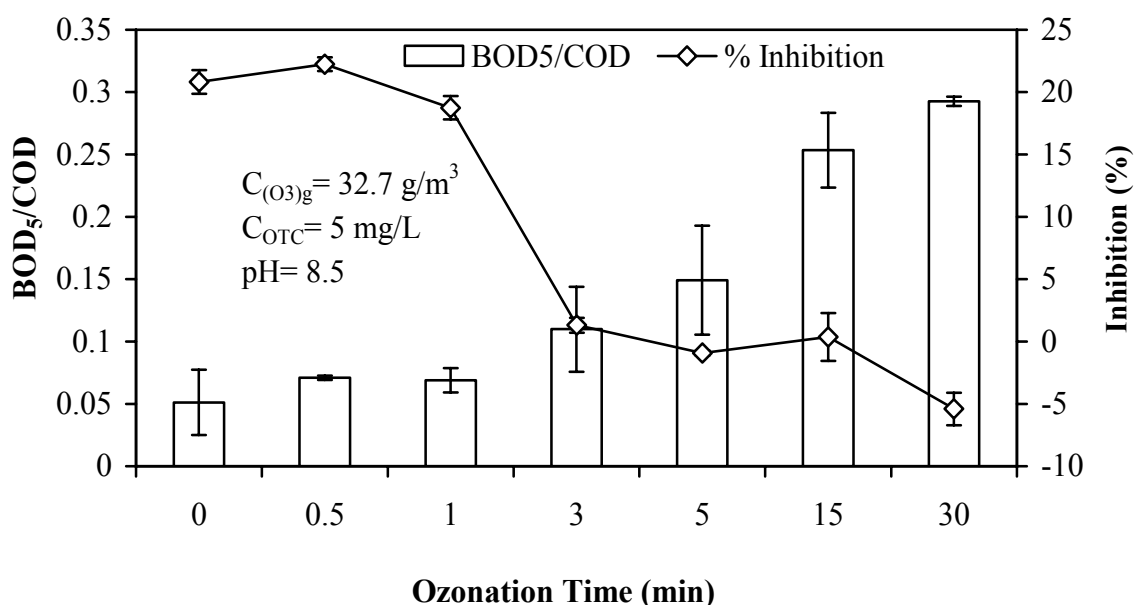


Figure 8.4. Variations in the biodegradability and toxicity of synthetic wastewater during ozonation ($C_{(O_3)g} = 32.7 \text{ g/m}^3$; $C_{OTC} = 5 \text{ mg/l}$; $\text{pH} = 8.5$).

Since several studies have demonstrated (e.g. Brouwer, 1991; Irha et al., 2003) that DHA inhibition assay is a good method for assessing bacterial toxicity, this parameter was used to assess the results of ozonation process. Inhibition on DHA of *Bacillus subtilis* in

synthetic wastewater spiked with 5 mg/l OTC was 21% while it was only 5% in manure contaminated with 50 mg OTC / kg (data not shown). In a previous study (Thiele Bruhn and Beck, 2005), however, any inhibition of OTC on DHA of soil microorganisms was not determined up to antibiotic concentration of 500 mg/kg. The lack of inhibition of OTC in solid matrices could be attributed to the decreased antibiotic potency in soil due to the sorption processes (Thiele Bruhn, 2003). A rapid decrease in the inhibition to almost zero with 99.6 % OTC degradation was attained after 3 minutes' ozonation of synthetic wastewater. This indicates that the degradation products of OTC or other contaminants in wastewater did not exhibit any inhibition on DHA of bacteria. Moreover, extension of ozonation to 30 minutes resulted in a stimulation effect on DHA of *Bacillus subtilis* which could be due to the production of more biodegradable compounds and the release of nutrients.

BOD₅/COD ratio is known as an indicative parameter for biodegradability (Metcalf and Eddy, 1995), and biodegradability of wastewaters containing recalcitrant contaminants can be improved significantly upon ozonation (Balcioglu and Ötöker, 2003). Accordingly, our results indicated a considerable increase in the BOD₅/COD ratio of synthetic wastewater from 0.05 to a value of 0.3 due to the formation of biodegradable by-products (Figure 8.4).

8.3.2. Ozonation of Manure

Preliminary experiments were conducted in order to study the efficiency of ozone on the oxidation of OTC in manure that was contaminated in two different ways; spiking with the OTC solution in water and in methanol. The ozonation yield was compared in terms of OTC degradation rate and the obtained results are presented as function of treatment time (Figure 8.5) for both spiking methods.

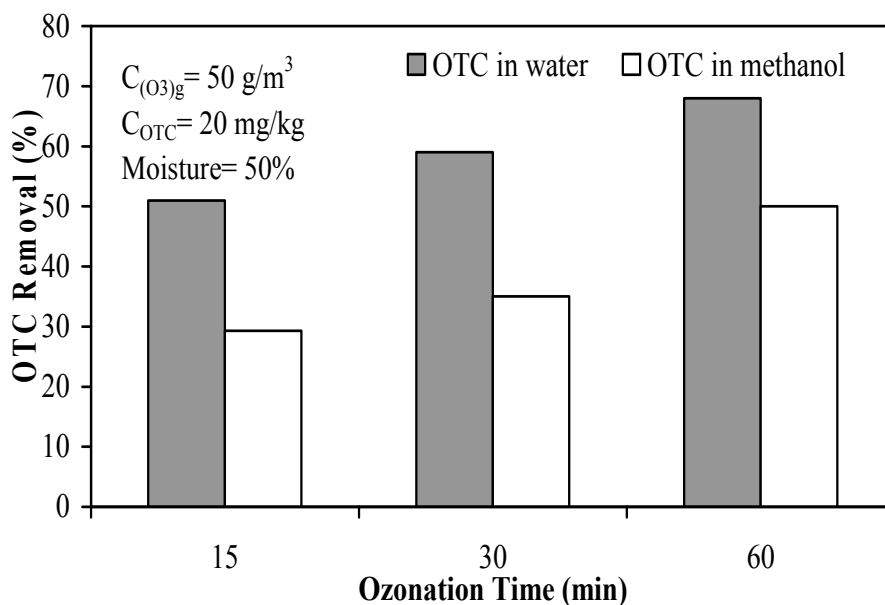


Figure 8.5. Effect of spiking method of manure with OTC on ozonation yield ($C_{(O_3)g} = 50 \text{ g/m}^3$; $C_{OTC} = 20 \text{ mg/kg}$ dry manure; moisture content = 50%;).

The spiking method of manure affected the ozonation yield as seen in Figure 8.5. Almost 20% increase in degradation of antibiotic in manure, which was fortified with OTC solution in water, could be attributed to the fast reaction of ozone with OTC that remained in water phase.

To verify this conclusion, recovery and desorption experiments were conducted and compared for both spiking methods. While the recovery of antibiotic from manure spiked with OTC prepared in methanol was 48.8 % (± 5.8), in case of spiking with OTC in water this ratio increased up to 83.1 % (± 9.7). It is possible that some amount of OTC remains in aqueous phase during the equilibration of dry manure with OTC aqueous solution. This situation might lead to an increase in the extractable OTC concentration. The results of desorption experiments performed with 1 M magnesium nitrate, $\text{Mg}(\text{NO}_3)_2$ solution (1:5 w/v) also supported idea of strong adsorption of OTC on manure in case of methanol spiking. One h equilibration time resulted in 47.6 % of OTC desorption from manure fortified with OTC in methanol while 62 % desorption was attained for manure equilibrated with aqueous OTC solution. These observations all together verify the presence of free OTC in aqueous phase in the latter case.

8.3.2.1. Effect of Initial Antibiotic Concentration. Considering the previous studies related with concentration of OTC in manure (De Liguoro et al., 2003; Martinez-Carballo et al., 2007), fortification levels were chosen between 10 – 50 mg/kg in this study and contaminated manure samples with four different OTC concentrations (10, 20, 35 and 50 mg OTC/kg dry manure) were subjected to ozonation for 15 minutes with the gas phase ozone concentration of 50 g/m³ (Figure 8.6).

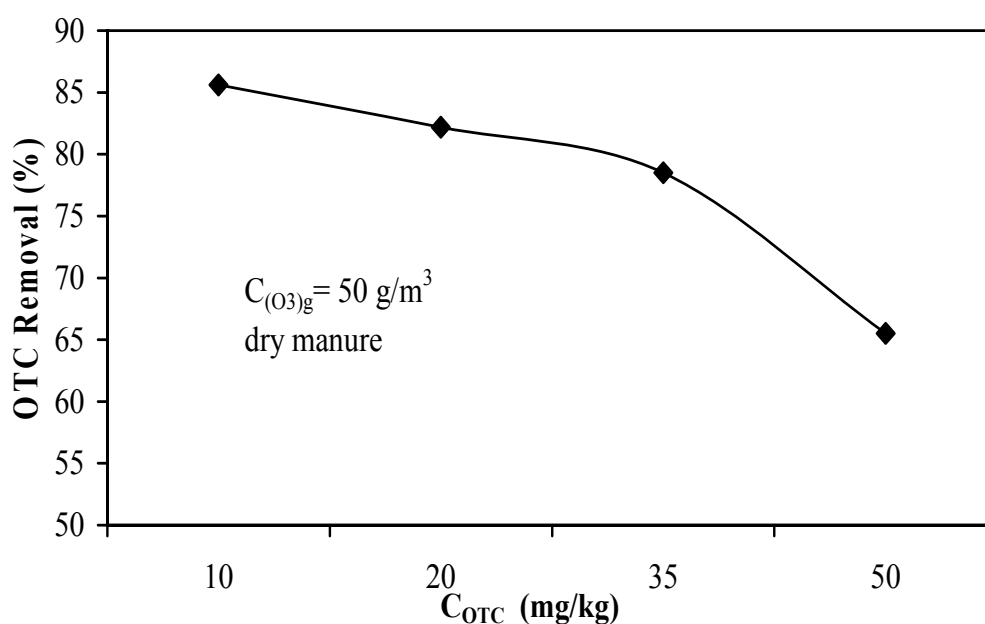


Figure 8.6. Effect of initial antibiotic concentration on the degradation efficiency of OTC.

The amount of absorbed ozone by manure was independent on experimental conditions and was about 0.45 g / 27 g dry manure at the end of 15 minutes of ozone treatment. This is a well expected result since the organic content of manure was comparably high, exerting high ozone demand. However, a five fold increase of OTC concentration in manure resulted in 20% reduction in antibiotic degradation by ozonation as shown in Figure 8.6. Therefore, for the manure contaminated with 10 mg/kg OTC, absorbed amount of ozone per degraded amount of OTC was almost 5 times higher than that obtained with higher OTC containing manure.

8.3.2.2. Effect of Moisture Content. It was reported that moisture content of cow manure can reach up to 87% (Himathongkham et al., 1999). In this part of the study, the manure moisture was provided by the addition of appropriate amount of water to the dry manure. In order to evaluate the effect of manure moisture content on the ozonation process,

contaminated manure samples (20 mg OTC/ kg) with three different moisture contents of 10%, 30% and 50%, was ozonated ($C_{(O_3)g} = 50 \text{ g/m}^3$) for 15 minutes and Figure 8.7 represents OTC degradation as a function of manure moisture content.

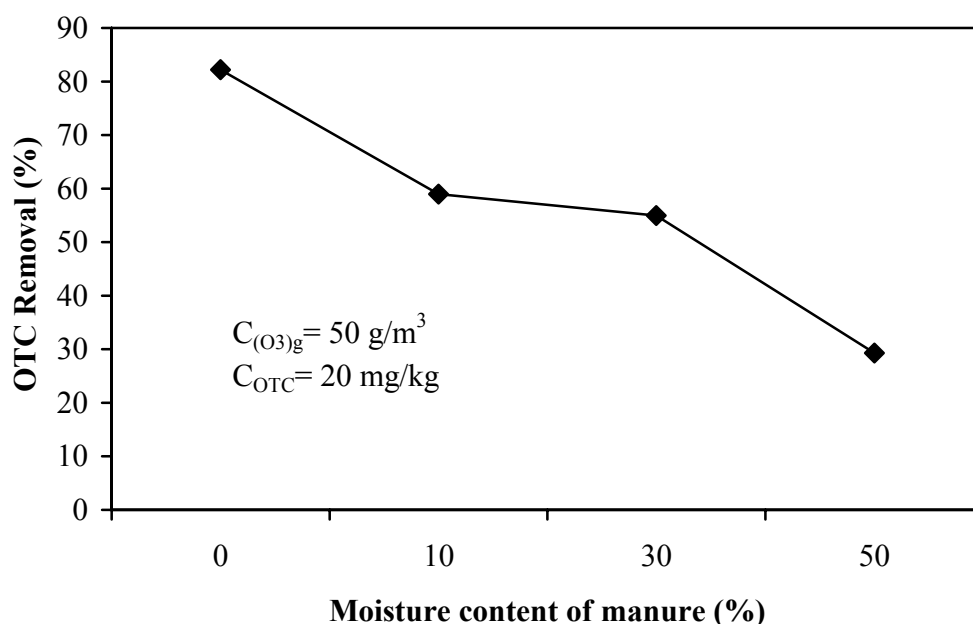


Figure 8.7. Effect of moisture content of manure on the OTC abatement.

Although the increasing moisture content caused an increase in the amount of absorbed ozone (data not shown) Figure 8.7 revealed that increasing moisture content of manure significantly decreased the degradation of OTC. Similar negative effect of soil moisture content was reported for the ozonation of PAHs (polycyclic aromatic hydrocarbons) (Zhang et al., 2005; Kulik et al., 2006; O'Mahony et al., 2006). This result can be explained by two facts. As it is well known that the diffusion coefficient of ozone in gas phase is greater than that in aqueous phase. Therefore, the higher oxidation rate of contaminants will be expected by direct ozonation in the dry manure. Furthermore, the higher concentration of hydroxyl radical that is likely to form heterogeneous reactions between ozone and manure minerals can oxidize the contaminants in the absence of moisture (Beltran, 2004).

8.3.2.3. Effect of Ozonation Time. Time-dependent changes of OTC concentration in dry manure (50 mg OTC/kg dry manure) were investigated for a 90 min ozonation period (Figure 8.8). OTC was used at higher concentration due to its low recovery rate from manure, which might have caused difficulties in the analysis.

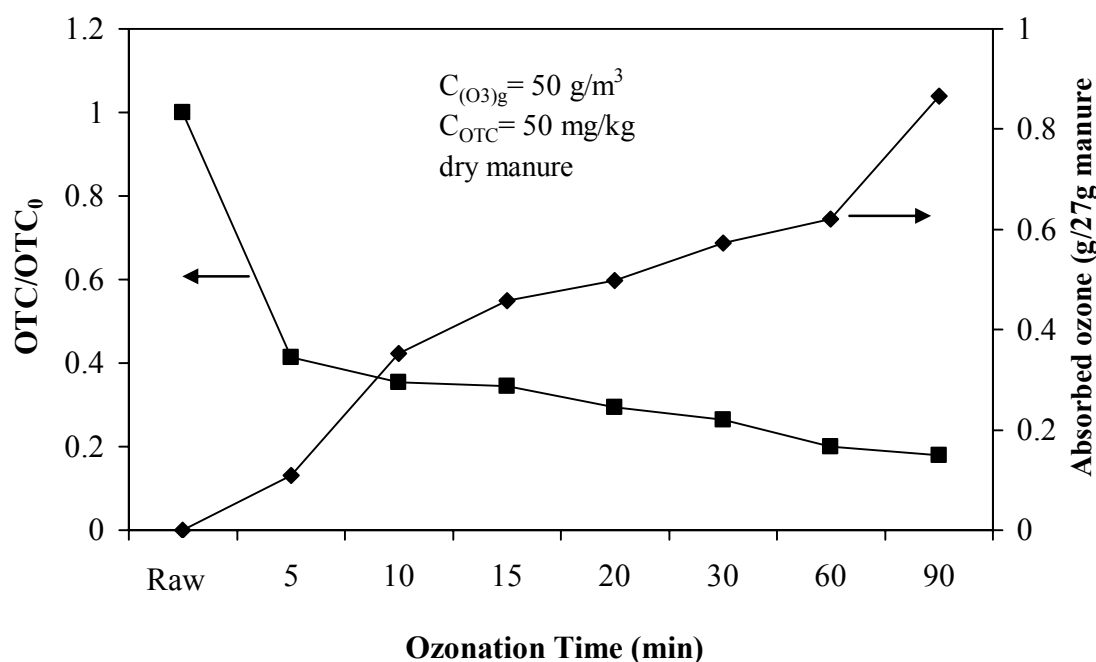


Figure 8.8. Effect of ozonation time on OTC degradation in manure.

A 59% OTC degradation was achieved within five minutes of the ozonation process. Upon the extension of treatment time OTC degradation rate slowed down and additional 23% increase in the antibiotic degradation was achieved up to 90 min. In contrast to the results obtained in the first part of study, complete degradation of OTC was not obtained in manure. This result can be explained by the fact that reactivity of strongly adsorbed organic compounds on solid surfaces is much slower with ozone than the ones in water phase (Choi et al., 2001). At the initial period of ozonation a rapid degradation rate was obtained due to easy contact between ozone and loosely bound OTC. However, the OTC degradation rate would reduce when the loosely bound OTC was being destroyed and the oxidant had to react with OTC that was strongly bound on the solid surface or sorbed in micropores.

8.3.2.4. Effect of Ozonation on Manure Characteristics. The main purpose of the present study was to degrade the oxytetracycline in manure which otherwise could cause antibiotic resistance in the environment by the application of manure on arable lands. However, since manure is used as a fertilizer to improve soil quality, it is also necessary to examine organic carbon content and nutrient content in ozonated manure through out the ozonation process.

It is expected that during oxidation process a series of complex reactions occur between ozone and target compound, OTC, as well as the reactions between ozone and manure organic matter. Variations in organic carbon fractions (C_t , C_{HA} , and C_{FA}) and nitrogen (sum of NH_4^+ -N and NO_3^- -N) content of manure during ozonation are presented in Figure 8.9 and 8.10 respectively.

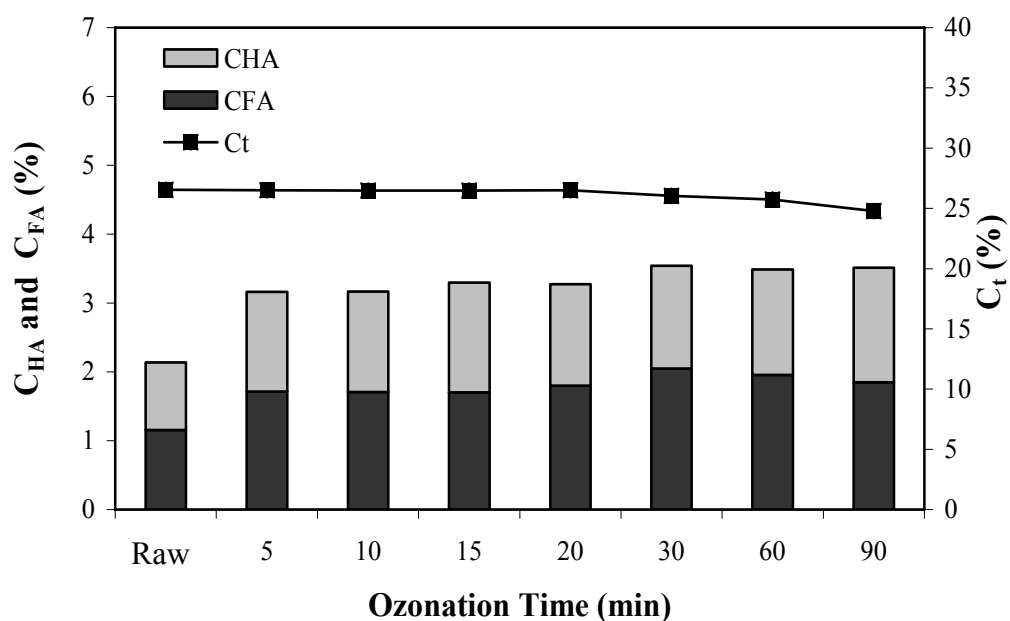


Figure 8.9. Effect of ozonation on total organic carbon, humic and fulvic acid carbon of manure ($C_{(O_3)g} = 50 \text{ g/m}^3$; $C_{OTC} = 50 \text{ mg/kg}$).

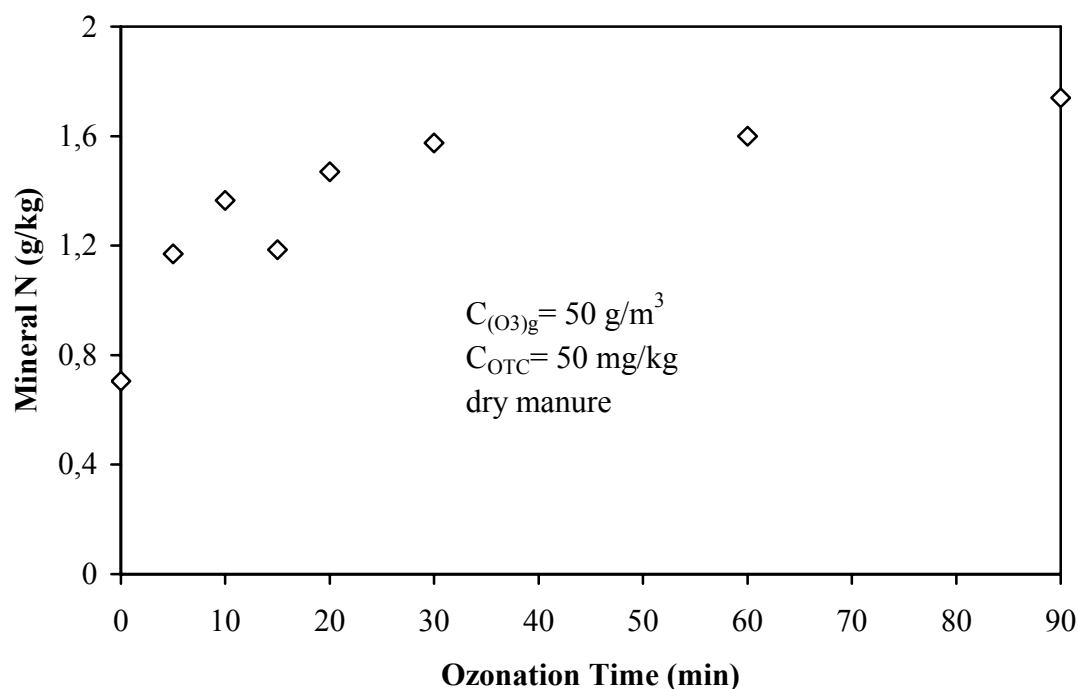


Figure 8.10. Effect of ozonation mineral N content of manure
 $(C_{(O_3)} = 50 \text{ g/m}^3; C_{OTC} = 50 \text{ mg/kg})$.

Total organic carbon of manure (26%) was not significantly affected by ozonation. However, C_{HA} and C_{FA} fractions were greatly increased. Ozone is known to be effective on reducing the average molecular size of organic compounds by oxidation (Ohlenbusch et al., 1998). Degradation of high molecular size humin fraction of manure by ozone could produce smaller humic acid and fulvic acid molecules that have higher fertilizer value and are readily taken into the plant along with trace minerals. The increase in mineral nitrogen ($NH_3\text{-N}$ and $NO_3\text{-N}$) concentration that could be utilized by plants as a nutrient source is another indicative evidence for the improvement of fertilizer value of manure throughout the oxidation process.

8.4. Conclusion

The efficiency of ozonation process for the degradation of OTC in both solid (manure) and liquid (synthetic wastewater) waste generated from animal feeding operations were assessed. Regardless of the experimental conditions application of 5 minutes ozonation resulted in more than 97 % of OTC in synthetic wastewater. The degradation rate of OTC

in synthetic wastewater ranged between 0.75-1.409 min⁻¹, depending on gas phase ozone concentration and pH of synthetic wastewater. Although OTC concentration in synthetic wastewater is much higher than the reported values in real animal farming wastewater, an efficient degradation of OTC would be expected even at low concentrations. Since obtained results revealed that initial antibiotic concentration did not affect the OTC degradation rate constants, significantly. A decrease in COD/TOC ratio of synthetic wastewater was indicative evidence for the increase in average oxidation state of organic carbon in solution after ozonation. The improvement of BOD₅/COD ratio of synthetic wastewater and elimination of bacterial DHA inhibition caused by oxytetracycline were provided by means of ozone treatment. Ozone was not very effective for the degradation of OTC sorbed on manure as it was in synthetic wastewater; i.e. complete degradation of OTC in manure could not be achieved with present conditions and reactor design. Lower ozonation treatment efficiency for manure compared to that of wastewater could be attributed to the high organic matter content of manure, which could act as a strong sorbent for OTC, and could exert high ozone demand, reducing the reaction between ozone and OTC. Mechanical mixing of manure during ozonation process may improve the contact of antibiotic with the oxidant hence overall treatment performance can be increased. The application of ozone increased humic and fulvic acid carbon and mineral nitrogen content and thereby, the fertilizer value of manure.

9. COMPARISON OF OZONATION AND FENTON PROCESSES FOR THE TREATMENT OF ANTIBIOTIC CONTAINING MANURE

9.1. Introduction

Antibiotics, which are classified as emergent pollutants in the environment, are used as therapeutics in human and veterinary medicine and for prophylactic treatment in animal feeding operations. After absorption by the organism, major portions of these substances are excreted via urine or feces and will therefore enter raw sewage or manure. Some of the antibiotics, e.g. tetracyclines, are sorbed to sewage sludge or manure with little or no biodegradation (Loke et al., 2002; Kim et al., 2005). Consequently, use of contaminated sewage sludge or manure as an organic fertilizer results in an emission of these antibiotics into the environment. Complexation with metal ions (Schmitt and Schneider, 2000) in soil can potentially convert tetracyclines into more mobile species that can contaminate local ground and surface water used for drinking water supplies (Lindsey et al., 2001; Campagnolo et al., 2002).

Tetracyclines are one of the most extensively used antibiotics in animal feeding operations. Similar to other antibiotics, they are excreted mostly as the parent compound, representing 50 - 80 % of the applied dose (Halling-Sørensen et al., 2002) and hence, excretion results in a contamination of animal manure with tetracyclines. The degradation studies of tetracyclines in different types of manure reported that the half life of tetracyclines in liquid and solid manure ranged between 3.2 – 105 days depending on the type of manure and manure treatment processes (e.g. anaerobic digestion, composting, storage) (Kühne et al., 2000; Winckler and Grafe, 2001; De Liguoro et al., 2003; Arıkan et al., 2006; Arıkan et al., 2007; Wang and Yates, 2008). The bioavailability of tetracyclines in solid matrices is quite low (Thiele-Bruhn, 2003) due to their strong sorption to solid matrices (Rabølle and Spliid, 2000; Loke et al., 2002; MacKay and Canterbury, 2005). Consequently, tetracyclines have been detected in animal manure and in agricultural soils up to 46 mg/kg and 0.19 mg/kg, respectively (Hamsher et al., 2002; De Liguoro et al., 2003; Martinez-Carballo et al., 2007; Balcioglu et al., 2007).

Sub-therapeutic use of antibiotics in animal feeding operations and their presence in the environment have been proven to cause the development of resistance in both pathogenic and non-pathogenic bacteria (e.g. Halling-Sørensen et al., 2002; Sengelov et al., 2003). The resulting resistant microorganisms can adversely affect the environmental microbial community, animals, and humans via the food chain. Moreover, the toxic effects of antibiotics on the bacterial community and other living organisms in the environment is another problem related to the presence of these substances in the natural environment (Masse et al., 2000; Thiele-Bruhn and Beck, 2005; Arıkan et al., 2006).

Considering the possible adverse effects of antibiotics on the natural environment, there is an obvious need for a suitable treatment process to remove these compounds completely at their source, i.e. in animal manure, to prevent their dissemination into the environment. Chemical oxidation processes have the potential for the treatment of recalcitrant organic compounds. Recent studies revealed that the Fenton and ozonation processes are able to treat water and wastewater containing antibiotics (Balcıoğlu and Ötker, 2003; Ikehata et al., 2006). It was known that these treatment processes can also be used for the remediation of contaminated soil (Zhang et al., 2005; Kulik et al., 2006; O'Mahony et al., 2006; Rivas, 2006). Moreover, the use of ozone at a sufficient dose is found to be effective in bacterial inactivation (Macauley et al., 2006) and for odor control in animal feeding operation waste (Watkins et al., 1997).

Considering these facts, in this study the treatment performances of the Fenton and ozone oxidation processes for the removal of OTC from synthetically contaminated cow manure, and the effect of magnesium nitrate extraction pretreatment on the subsequent oxidation processes were evaluated. To the best of our knowledge, this study is the first to investigate the application potential of an integrated process for the destruction of antibiotics in the manure. In the study, the effects of pH and oxidant doses on the treatment processes were studied in terms of OTC removal. Moreover, variations in soluble chemical oxygen demand (SCOD), organic carbon (OC) content, and the pH of manure were measured throughout the oxidation processes.

9.2. Materials and Methods

9.2.1. Chemicals

Oxytetracycline hydrochloride (Sigma, $C_{22}H_{24}N_2O_9 \cdot HCl$, >95% purity) was used as a model antibiotic substance. Fenton's reagent (H_2O_2 (30%) and $FeSO_4 \cdot 7H_2O$) was obtained from Riedel-de Haën and Carlo Erba. $Mg(NO_3)_2 \cdot 6H_2O$ (Merck) was used in the pretreatment operation for the extraction of OTC from cow manure. Formic acid and HPLC grade acetonitrile were obtained from Sigma-Aldrich for the analysis of OTC. According to the analysis procedure OTC was extracted from the manure by citric acid monohydrate (Merck), $Na_2EDTA \cdot 2H_2O$ (Fluka), Na_2HPO_4 (Merck), and HPLC grade methanol (Sigma-Aldrich). All solutions were prepared with Milli-Q ultrapure deionized water (Millipore, Milford, MA).

9.2.2. Manure

Cow manure was obtained from an ecological farm located at the west part of Turkey. After drying at 70°C for 24 h, manure was passed through a sieve (2mm) and it was subsequently sterilized in an autoclave at 120 °C for 15 min, to prevent any biological activity and finally, it was stored at 4°C until used in the treatment experiments.

Each dry manure sample was synthetically contaminated by spiking it with the OTC stock solution that was prepared in water at a concentration of 1 mg/ml. After mixing thoroughly with a vortex (Nuve NM 110), the contaminated manure was allowed to equilibrate for 2 h.

9.2.3. Pretreatment of Contaminated Manure with Magnesium Nitrate

Prior to the oxidation experiments, a pretreatment operation was applied to the OTC contaminated manure by the addition of 1M $Mg(NO_3)_2$ solution. To obtain consistent results in replicates, extraction, oxidation, and the preparation of samples for the antibiotic analysis were all performed in a 10 ml-Teflon centrifuge tube in which a one -gram portion of the synthetically contaminated manure (20 mg OTC/kg manure) was mixed with 3 ml

Mg (NO₃)₂ solution at 25 °C in a thermostated shaker (Julabo SW 22). After the mixing period, the manure slurry was centrifuged at 5000 rpm (Hettich Universal 16A) for 10 min and then filtered through a 0.45 µm filter (Sartorius, Germany) to analyze the OTC concentration extracted into the solution phase. Control experiments were performed with 3 ml of water instead of magnesium nitrate solution.

9.2.4. The Fenton Process

Acidification of manure slurry to the pH value of 2.6 was carried out by the addition of 10 % H₂SO₄. Moreover, the pH of FeSO₄ and H₂O₂ solutions was adjusted to 2 with dilute H₂SO₄ and H₃PO₄, respectively, since the manure exhibited resistance to the variation of pH. In separate experiments, the manure slurry was treated without any pH adjustment. Different concentrations of 0.2 ml of FeSO₄ and 0.3 ml of H₂O₂ solutions were added to the manure slurry to give a final concentration of FeSO₄ and H₂O₂ in the manure slurry between 5-50 mM and 50-500 mM, respectively. The manure slurry was then mixed in a thermostated shaker (Julabo SW 22) at 150 rpm and 25 °C up to 24 h.

9.2.5. Ozonation Process

Ozone was produced from dry and pure oxygen in a laboratory scale corona discharge ozone generator (Fisher OZ 500). The manure slurry was ozonated in semi-batch mode with a continuous flow of the ozone and oxygen gas mixture (gas phase ozone concentration: 15-130 g/m³) at a rate of 0.01 m³/h. All tubing from the ozone generator to the reactor was made of silicone and the reactor and fittings from Teflon. The mixing of manure slurry was provided by a sonicator (Sonorex Super RK 510) to ensure homogeneity and contact between the manure and oxidants.

9.2.6. Experimental Design

A central composite rotatable experimental design was used to investigate the effects of oxidant doses as well as to characterize potential interactions between the process variables. The central composite design was characterized by the two process variables at five levels with five center points for statistical validity. The regression equation which

describes the system quantitatively and contour plot were obtained using MINITAB software.

9.2.7. Oxytetracycline Analysis

The whole content of manure slurry treated by the ozonation and Fenton oxidation processes was subjected to extraction for OTC analysis in order to ensure homogeneous sampling and to obtain more consistent results between the parallel runs. The extraction of OTC from the manure samples was performed according to the method described by Blackwell et al., (2004). After solid phase extraction, the OTC analysis in the extract was performed with an HPLC system (Agilent Technologies 1100 series) equipped with a tertiary pump, a photodiode array, and an autosampler with an automated injection system. Gradient elution carried out with acetonitrile (solvent A) and water containing 0.1% formic acid (solvent B) using analytical column Eclipse XDB C18 (5 μ , 150 x 4 mm) equipped with an ODS guard column. Elution started with A:B:10:90 rising linearly to 40:60 from 0 to 5 min and then returning to initial composition from 5 to 8 min. OTC was detected at the 360 nm wavelength with a retention time of 5.98 min. OTC quantification in manure extracts was performed by external calibration curve.

The chromatogram of uncontaminated manure was also recorded and compared with that of the OTC - contaminated manure sample (Figure 9.1). As can be seen from Figure 9.1, the chromatogram of uncontaminated manure was remarkably clean and no interference peak was detected at the wavelength that oxytetracycline was determined. The limit of detection for OTC was determined as 0.22 mg/ kg in manure (S/N=3) based on the evaluation of uncontaminated manure chromatogram.

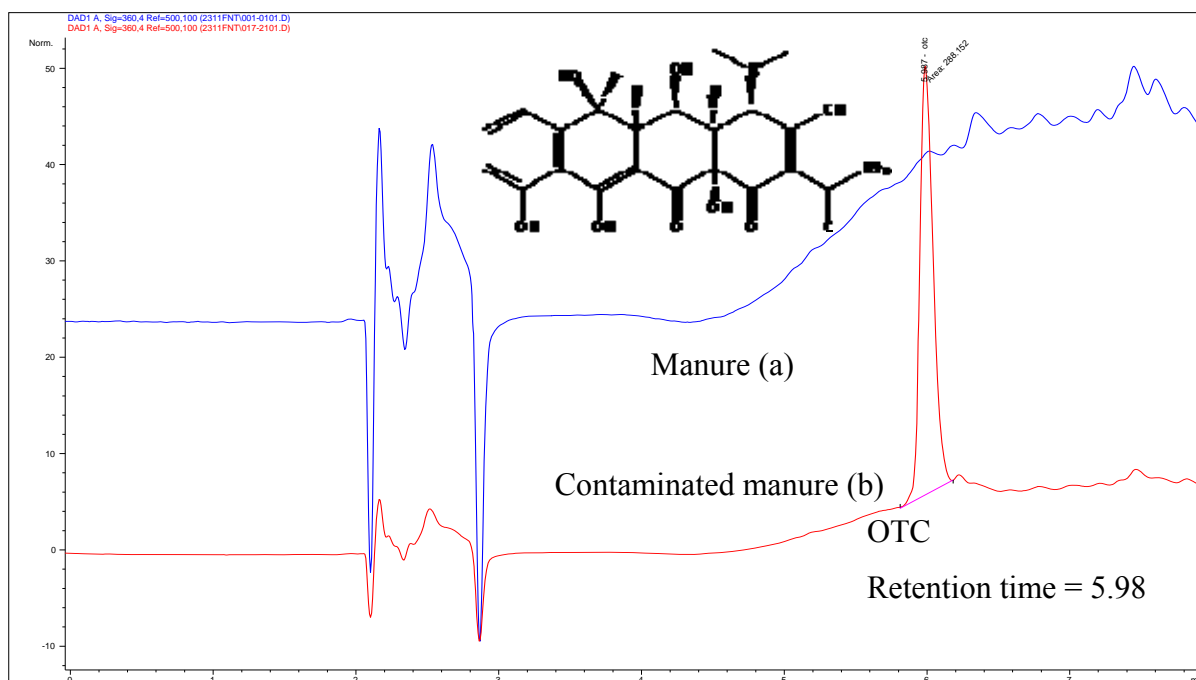


Figure 9.1. Representative chromatogram of manure (a) and OTC-contaminated manure at 20 mg/kg (b).

9.2.8. Other Chemical Analyses

The pH of the manure slurry was measured using a pH probe (WTW pH 330 pH meter). SCOD in the supernatant of the manure slurry and the OC in the precipitated manure were determined according to closed reflux method (APHA/AWWA, 1998) and Walkley Black method (Sanchez-Montero et al., 1996) with some modifications, respectively. Additionally, after the extraction pretreatment process, some metals were analyzed in the liquid phase by ICP-OES (Perkin-Elmer Optima 2100 DV). Hydrogen peroxide was determined by the iodometric method. Inlet and off-gas ozone concentrations were monitored by using an online ozone analyzer (Fisher Ozotron 23). The absorbed ozone dose in the manure slurry was calculated by subtracting the concentration of ozone in the off-gas from the inlet concentration.

9.3. Results and Discussion

9.3.1. Pretreatment of Manure by Extraction

Desorption of contaminants found in sorbed or particulate phases can be a critical step in the overall success of an oxidative treatment process. If the contaminants are not desorbed into the aqueous phase of slurry they could not be efficiently destroyed due to the rapid consumption of oxidants by incidental organic and inorganic constituents of the medium (Sedlak and Andren, 1994; Yeh et al., 2002). In a previous study (Figueroa and MacKay, 2005), magnesium ion has been used to extract OTC from soil samples considering the strong complex forming potential of tetracyclines with divalent or trivalent metal ions. In this study, extraction pretreatment was applied on manure with a 1 M magnesium nitrate solution to keep OTC in the dissolved phase prior to the oxidation processes. Separately, manure was treated with distilled water instead of the magnesium salt solution as a control experiment. The efficiency of pretreatment was evaluated by the OTC analysis performed in the liquid phase after the separation of the manure from the slurry (Table 9.1). The extracted solution was also characterized by SCOD and soluble metals and the results are given as a mean of duplicate analysis together with standard deviations in Table 9.1.

Table 9.1. Characteristics of the liquid phase of manure slurry after desorption at 100 rpm for 30 min.

Parameter	in $\text{Mg}(\text{NO}_3)_2$	in H_2O
OTC (mg/l)	4.2 ± 0.2	0.3 ± 0.1
COD (mg/l)	2433 ± 383	2414 ± 95
Ca (mg/l)	1171 ± 33.9	94.2 ± 6.8
Mg (mg/l)	23705 ± 417	37.8 ± 0.8
Fe (mg/l)	0.7 ± 0.2	0.9 ± 0.0
Cr (mg/l)	0.2 ± 0.0	-
pH	7.2	8.5

While the extraction of OTC into water was about 5 % at the natural pH of the manure and at 100 rpm within a 30 min mixing period, a $\text{Mg}(\text{NO}_3)_2$ solution increased the mean

extraction efficiency of OTC to a value of 63.9 % under the same experimental conditions. The lower extraction efficiency of water indicates a strong sorption of OTC on the manure. This is easily expected since previous studies investigated the sorption of tetracyclines on different soil samples revealed that the organic constituents (e.g humic substances and proteins) of a soil could be responsible for the sorption (Sithole and Guy, 1987a; Loke et al., 2002). Therefore, the high sorption of OTC on the manure in which organic carbon content was 11 % could also be anticipated. The higher extraction efficiency of the $\text{Mg}(\text{NO}_3)_2$ solution is presumably due to the formation of OTC complex with divalent ions in the liquid phase (Schmitt and Schneider, 2000). Furthermore, the addition of magnesium nitrate to the manure resulted in the release of a high concentration of the Ca^{2+} ion (Table 9.1) which can also form strong complexes (Schmitt and Schneider, 2000) with the OTC in the liquid phase of manure slurry. On the other hand, as seen in Table 9.1, the divalent cation concentration in water was not high enough to keep oxytetracycline in solution.

Moreover, extraction pretreatment by a Mg salt solution increased the recovery of OTC in the applied analysis method. While only 68 % of OTC recovery from manure was obtained without any pretreatment, it was increased to 98 % by the application of a Mg salt solution extraction to the manure. It is worthwhile to note that higher recovery could provide higher sensitivity for the OTC analysis.

To delineate the effects of mixing speed, mixing period, and the initial pH on the extraction of OTC by $\text{Mg}(\text{NO}_3)_2$, a series of experiments were performed and the results are summarized in Table 9.2. After the addition of 1 M $\text{Mg}(\text{NO}_3)_2$ to the manure the pH of manure slurry was 7.2 and it was adjusted to 3 and 8 by dilute H_2SO_4 and NaOH solutions, respectively. Since OTC is a zwitterionic compound it is expected to form a stronger complex at alkaline pH depending upon the pK_a values of OTC ($\text{pK}_{a1} = 3.57$, $\text{pK}_{a2} = 7.49$ and $\text{pK}_{a3} = 9.44$) (Schmitt and Schneider, 2000; Figueroa and MacKay, 2005). On the other hand, pretreatment was also performed at acidic pH by considering the subsequent application of the Fenton process.

Table 9.2. Effects of operating parameters on the desorption of OTC from the manure.

Experimental Conditions	Parameter	Desorption (%)
	Reaction period (h)	(mean±SD)
pH = 7.2; Mixing speed = 100 rpm	0.5	63.9 ± 2.9
	1	63.8 ± 1.1
	24	63.2 ± 4.8
	pH	
Mixing period = 0.5 h	3	61.6 ± 2.0
Mixing speed = 150 rpm	8	64.7 ± 0.3
	Mixing Speed (rpm)	
Mixing period = 0.5 h	50	66.9 ± 0.0
pH = 8	100	68.5 ± 3.3
	150	64.7 ± 0.3
	200	67.5 ± 1.9

As can be seen from Table 9.2, the pH did not significantly affect the extraction of OTC ($p > 0.05$). The unexpected high desorption of OTC at pH 3 could be attributed to the increase in the pH value of slurry to 5.83 during extraction pretreatment. This increase in pH value could be explained by the high buffering capacity of manure. The extension of agitation time from 30 min up to 24 h and an increase of mixing strength from 50 rpm to 200 rpm did not significantly enhance the desorption rate of OTC ($p > 0.05$) into the solution. Consequently, it could be concluded that the high desorption percentage of OTC from manure into the liquid phase might be obtained in a broad range of operational conditions.

9.3.2. The Fenton Oxidation Process

9.3.2.1. Effect of Pretreatment on the Fenton Oxidation Process. The Fenton oxidation process was directly applied to manure slurry containing water at a solid to liquid ratio of 1:3 (one step treatment) and separately, it was applied to manure slurry after extraction pretreatment with a magnesium nitrate solution (two-step treatment). Since the manure contained 11.2 g/kg indigenous iron, some additional experiments were performed without

adding iron (the Fenton-like treatment). The efficiencies of one-step and two-step treatment processes were compared in terms of variations in OTC concentration instead of removal rate since the extraction enhanced the recovery of OTC from the manure as mentioned before. The results obtained by the Fenton (50 mM H_2O_2 and 5 mM Fe^{2+}) and Fenton-like (50 mM H_2O_2) processes are presented in Figure 9.2.

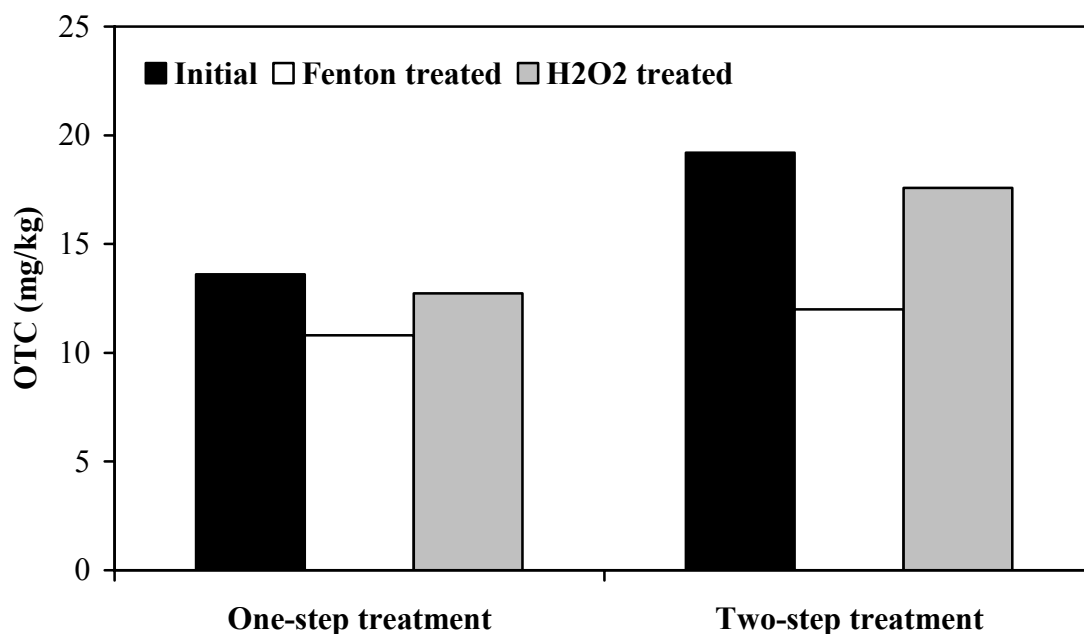


Figure 9.2. Effect of extraction pretreatment on the Fenton oxidation of OTC (pH= natural pH of each manure slurry, $[\text{H}_2\text{O}_2]_i = 50$ mM, $[\text{Fe}^{2+}]_i = 5$ mM, mixing speed=100 rpm).

While the one-step process, by the use of a low dose of Fenton reagent, resulted in a 20 % removal of OTC from the manure slurry the two-step process increased the OTC removal to 38 %. Similar to our results, other investigators have reported that the efficiency of Fenton oxidation was significantly affected by the desorption of polycyclic aromatic compounds from soil using ethanol or vegetable oil (Bogan et al., 2003; Lundstedt et al., 2006). It is obvious that the lower availability of sorbed OTC reduced the performance of the Fenton process and therefore, further experiments were performed as two-step processes.

9.3.2.2. Effects of H_2O_2 and Fe^{2+} Doses. In order to determine the effects of hydrogen peroxide and ferrous ion doses, in accordance with central composite design, 13 sets of experiments were conducted in the concentration range of 5–50 mM Fe^{2+} and 50–500 mM

H₂O₂ and the results are represented in Table 9.3 in terms of mean OTC removal percentage obtained at the end of the 24 h treatment period. Although the initial pH of manure slurry was 7.2, it was reduced to about 4.6 by the addition of H₂O₂ and Fe²⁺ solutions in all experimental sets.

Table 9.3. Effect of H₂O₂ and Fe²⁺ doses on the Fenton oxidation of OTC in manure slurry

Experiment	H ₂ O ₂ (mM)	Fe ²⁺ (mM)	OTC Removal (%)
I	275	27.5	86
II	275	27.5	85
III	50	27.5	65
IV	275	27.5	81
V	434	11.5	92
VI	275	5	70
VII	115	43.4	76
VIII	115	11.5	65
IX	434	43.4	93
X	275	27.5	84
XI	275	50	90
XII	500	27.5	93
XIII	275	27.5	85

The overall mean OTC removal varied between 65 % and 93 %, depending on the Fenton reagent doses. Within the frame of the experimental conditions, the highest OTC removal (93 %) was obtained at 434 mM H₂O₂ and 43.4 mM and Fe²⁺ doses. However, the complete removal of OTC could not be obtained within the present experimental conditions. The higher concentrations of hydrogen peroxide might be required both for the dissolution of strongly adsorbed OTC and to satisfy the oxidant demands of the high organic matter of manure slurry that could compete with OTC for oxidation.

The regression equation based on the statistical analysis of experimental data and its graphical representation is represented in Equation 9.1 and Figure 9.3, respectively.

$$\text{OTC Removal (\%)} = 55.09(\pm 3.25) + 0.065(\pm 0.007) [\text{H}_2\text{O}_2] + 0.316(\pm 0.079) [\text{Fe}^{2+}] \quad (9.1)$$

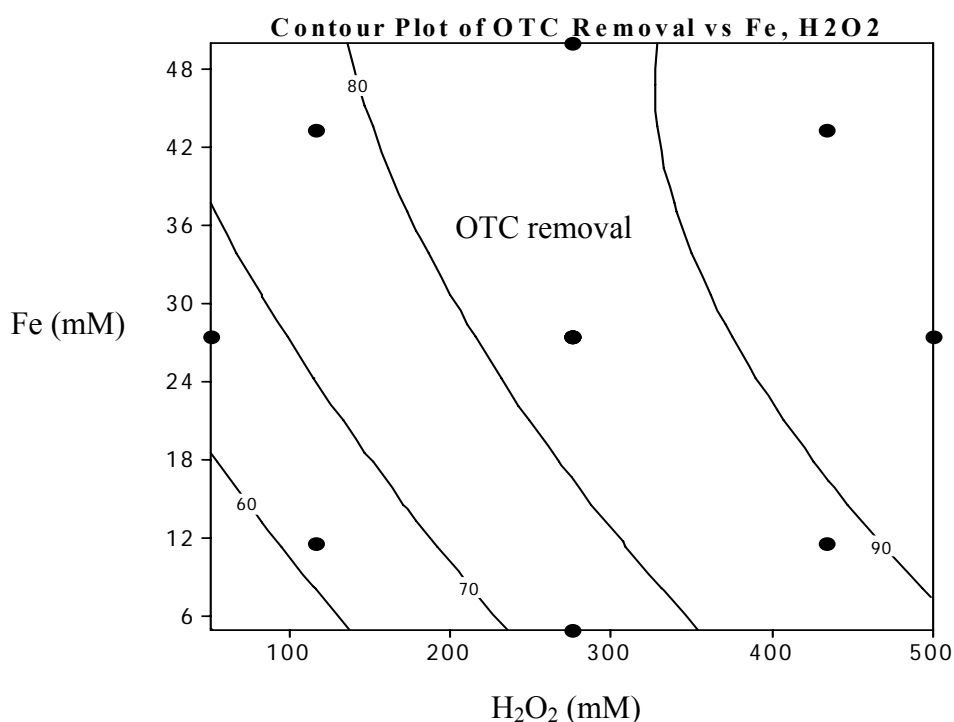


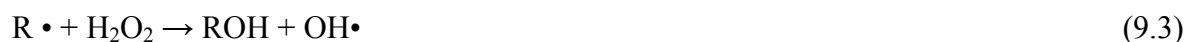
Figure 9.3. Response surface for the removal of OTC as a function of H_2O_2 and Fe^{2+} doses in manure. Solid lines represent the percent OTC removal (pH= natural pH of manure slurry; mixing speed=150 rpm).

Equation 9.1 was characterized by an R^2 of 89 % ($n = 13$; $s = 3.58$; $F = 41.88$) when the experimental data were plotted against values generated by the equations. Inspection of Equation 9.1 and Figure 9.3 reveals that the efficiency of antibiotic removal increased as the concentrations of H_2O_2 and Fe^{2+} increased. While an increase in Fe^{2+} concentration significantly enhanced the removal of OTC in the H_2O_2 concentration range of 50 - 275 mM, beyond a 275 mM H_2O_2 concentration, the effect of Fe^{2+} on the Fenton oxidation process efficiency was less pronounced. In previous studies (e.g. Watts et al., 1999b), the catalytic effect of indigenous iron minerals was observed in the presence of high hydrogen peroxide concentrations.

It is already known that in conventional Fenton's reaction, hydroxyl radical, a non-selective oxidant, is produced by catalytic decomposition of H_2O_2 by Fe^{2+} .



On the other hand, in the presence of high concentrations of H_2O_2 , organic radicals ($\text{R} \cdot$) produced from the reaction between hydroxyl radical and organic molecules could also react with excess hydrogen peroxide to generate additional hydroxyl radicals.



Moreover, hydroxyl radicals could also react with excess hydrogen peroxide to generate perhydroxyl radicals ($\text{HO}_2 \cdot$) and superoxide radical anions ($\text{O}_2 \cdot^-$) which are potential oxidants.



The $\text{HO}_2 \cdot / \text{O}_2 \cdot^-$ generated could also act as a reductant for iron and participate in the iron redox cycle.



Consequently, the effect of the external addition of Fe^{2+} to the manure slurry on the Fenton oxidation efficiency became less significant in the presence of high hydrogen peroxide concentration due to the production of more hydroxyl radicals and other reactive radicals such as $\text{HO}_2 \cdot$ and $\text{O}_2 \cdot^-$, and the possibility for the regeneration of Fe^{2+} .

9.3.2.3. Effect of pH. Although in most studies the Fenton oxidation is applied at acidic pH values due to higher iron solubility, efficient contaminant removal has also been reported at the natural pH of contaminated soil (Yeh et al., 2002). In this study, the effect of pH on the removal kinetics of OTC in manure slurry was evaluated by conducting the Fenton (434 mM H_2O_2 and 43.4 mM Fe^{2+}) and Fenton-like (434 mM H_2O_2) experiments at the natural pH of manure slurry and at pH 2.6. OTC and H_2O_2 abatements throughout the Fenton and Fenton-like treatments are presented in Figure 9.4 together with the variations in the pH of manure slurry, the soluble iron, SCOD, and the OC content in the solid phase.

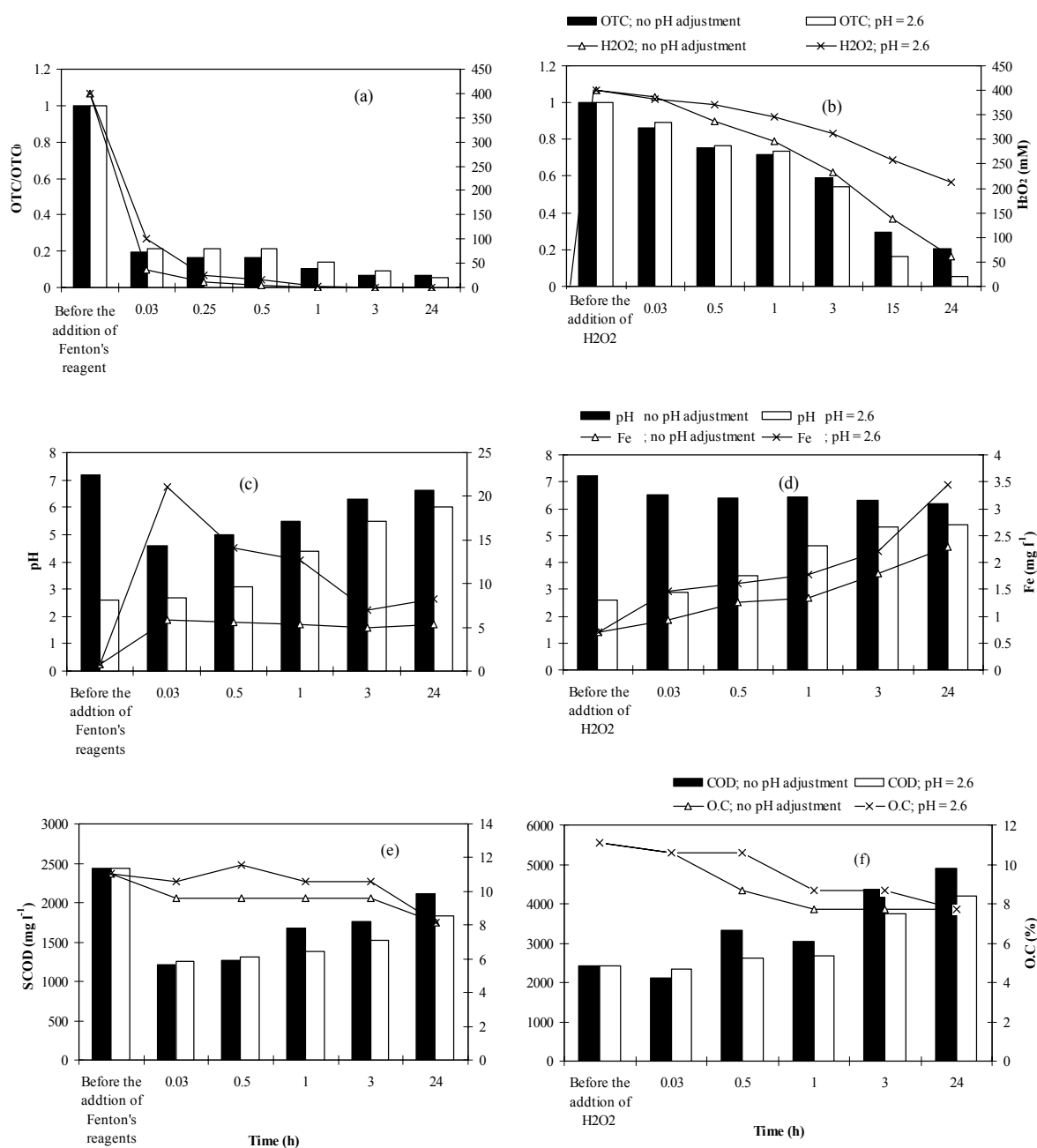


Figure 9.4. Variations in OTC concentration (OTC/OTC₀) and H₂O₂ (mM) (a; b), pH and soluble Fe (mg/l) (c; d), SCOD (mg/l) and OC (%) (e; f) by the application of Fenton and Fenton-like processes ($[\text{H}_2\text{O}_2]_i = 434 \text{ mM}$, $[\text{Fe}^{2+}]_i = 43.4 \text{ mM}$, mixing speed = 150 rpm).

As can be seen from Figure 9.4, there was no significant effect of pH on overall OTC removal in the Fenton oxidation process and efficient removal of antibiotic (93 %) was obtained even in the absence of pH adjustment (Figure 9.4a). On the other hand, in the case of the Fenton-like oxidation process, the adjustment of the initial pH of manure slurry to 2.6 increased the OTC removal from 80 % to 94 % at the end of a 24 h reaction period

(Figure 9.4b). Actually, the addition of Fenton's reagent decreased the initial pH of the manure slurry from 7.2 to 4.6 (Figure 9.4c) whereas the pH of manure slurry was slightly reduced by the addition of hydrogen peroxide in the Fenton-like process (Figure 9.4d). Therefore the pH has a more pronounced effect on the Fenton-like process. Consequently, for both types of Fenton processes acidic pH resulted in the presence of more soluble iron to implement Fenton's reaction (Figure 9.4c and 9.4d). Although the overall OTC removal rate was almost same for Fenton and Fenton-like processes at an acidic pH value, significant variation was observed in the second order OTC removal rate constants that were presented in Table 9.4 together with overall OTC removal percentages.

Table 9.4. Effect of pH on the Fenton and Fenton-like oxidation of OTC in manure slurry.

H ₂ O ₂ (mM)	Fe ²⁺ (mM)	pH	OTC Removal (%)	k _{OTC} (R ²) (M ⁻¹ s ⁻¹)
434	43.4	no adjustment	93	119 (0.87)
434	-	no adjustment	80	4 (0.99)
434	43.4	2.6	94	113 (0.92)
434	-	2.6	95	19 (0.90)

About 80 % of OTC was degraded within 2 min of Fenton oxidation at both pH values and by extension of the oxidation time up to 24 h overall OTC removal reached about 94 %. This result can be explained by slower oxidation of strongly sorbed OTC which could not be extracted from manure. In contrast to the results obtained by Fenton process, OTC gradually decreased by the application of Fenton-like process due to the gradual release of indigenous iron from the manure into the slurry (Figure 9.4d). Furthermore, in Fenton process 72 % and 88 % of the added hydrogen peroxide were consumed within 2 min of the reaction period at acidic and natural manure pH, respectively and after 3 h, the amount of hydrogen peroxide in the manure slurry reached the level that cannot be detected by the iodometric method (0.1 %). Consequently, a remarkably higher removal rate of OTC obtained by Fenton process compared to Fenton-like process could be explained by the rapid production of oxidative radicals leading to a rapid removal of OTC from manure slurry.

Other research has suggested (Bacchiocchi et al., 2003) that the slower rate of hydrogen peroxide decomposition in the Fenton-like process, i.e. longer lifetime of hydrogen peroxide, corresponds to a higher oxidation efficiency of organic contaminants in soil samples. In accordance with this previous study, the higher removal efficiency of OTC at pH 2.6 is in agreement with relatively slow hydrogen peroxide consumption in manure. The considerably higher iron content of manure (11.2 g/kg) could promote radical formation through homogenous and heterogeneous reactions with hydrogen peroxide even in the absence of added soluble iron. Direct oxidation by a high concentration of hydrogen peroxide itself (434 mM) is also a possible pathway for the removal of OTC in manure slurry.

As can be seen from Figure 9.4e, the Fenton oxidation process resulted in a rapid decrease in the initial SCOD value of the solution due to easy contact between the oxidants and the organics in the liquid phase. The subsequent increase in the SCOD value of the solution could be explained by the release of organic matter from the manure into the solution. Simultaneously, the fact that the organic carbon content of manure decreased with treatment time verifies the transfer of organic matter from solid to liquid phase. In the case of the Fenton-like process, due to a lack of a sufficient amount of oxidative radicals SCOD exhibited a remarkable accumulation. It is also expected that during the oxidation process a series of complex reactions occur between the oxidant and the organic matter of the manure and competition between OTC and other reduced chemicals for the oxidants could decrease the removal rate of OTC.

In order to clear up the oxidation mechanism of OTC in manure slurry, parallel experiments were conducted in the presence of a radical scavenger, t-butanol. However, no significant effect of 1 M t-butanol was observed on OTC removal. High organic carbon content and the COD of manure slurry could act as a radical scavenger hiding the scavenger effect of t-butanol. Another possible explanation could be excess formation of reactive radicals in the presence of a high hydrogen peroxide concentration (434 mM) so that the scavenger effect of t-butanol could not be observed. Therefore, experiments with t-butanol were conducted at low hydrogen peroxide and iron doses (Table 9.5).

Table 9.5. Effect of radical scavenger on OTC removal by Fenton oxidation.

H ₂ O ₂ (mM)	Fe ²⁺ (mM)	pH	t-butanol 1 M	OTC Removal (%)
50	5	2.6	-	49
50	-	2.6	-	39
50	5	2.6	+	40
50	-	2.6	+	22

As can be seen from Table 9.5, the addition of a radical scavenger decreased OTC removal by 9 % and 17 % for the Fenton and Fenton-like oxidations, respectively. It can be concluded that both reactive radicals and hydrogen peroxide as an oxidant are responsible for the removal of OTC in manure slurry.

9.3.3. Ozonation Process

9.3.3.1. Effect of Pretreatment on Ozonation Process. The ozonation process with an ozone concentration of 2.5 mg/min was applied to synthetically contaminated manure as both one-step and two-step processes without any pH adjustment for a 1 h treatment period. An additional experiment was performed to assess the effect of mixing on ozonation efficiency. The results revealed that similar to Fenton oxidation, overall OTC removal was enhanced by pretreatment (Figure 9.5).

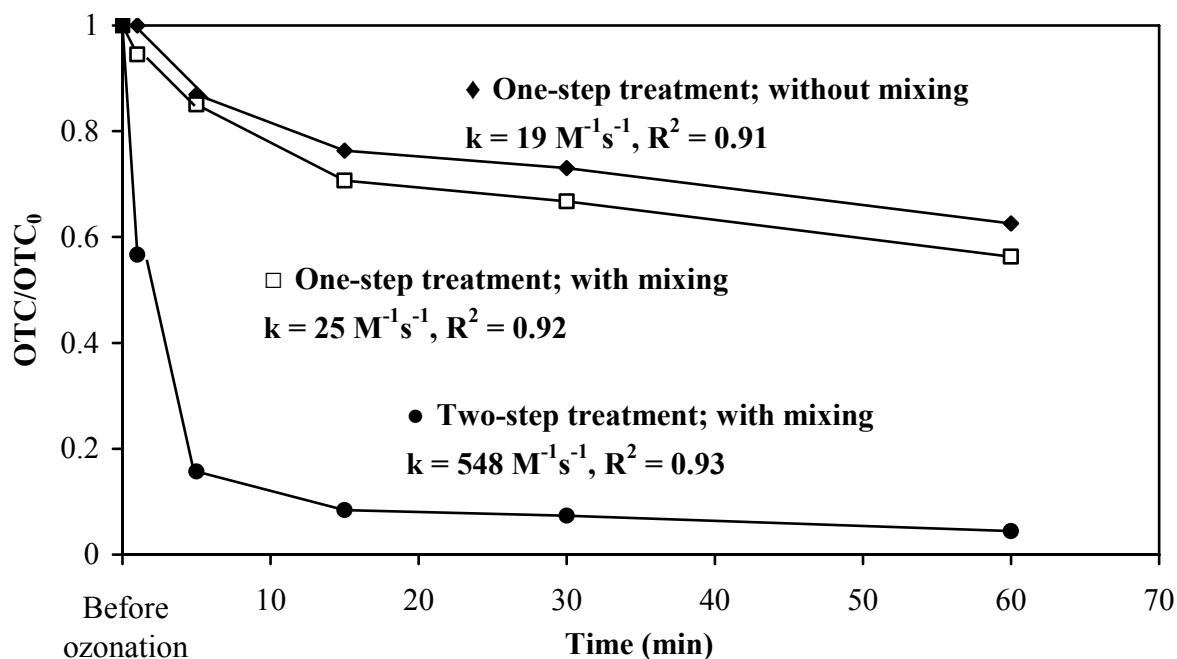


Figure 9.5. Effects of extraction pretreatment and mixing on ozone oxidation of OTC in manure at pH 7.2 ($O_3 = 2.5$ mg/min).

While the one-step treatment resulted in a 44 % overall OTC removal with the second-order removal rate constant of $25 \text{ M}^{-1}\text{s}^{-1}$, in the case of the two-step process, the removal rate and second-order removal rate constant were increased to 96 % and $548 \text{ M}^{-1}\text{s}^{-1}$, respectively. On the other hand, mixing the manure slurry during ozonation had a slightly positive effect on OTC removal from manure and this enhancement could be attributed to a good contact of manure slurry with oxidants. In view of these results, further ozonation experiments were carried out after extraction pretreatment and following mixing.

9.3.3.2. Effects of Hydrogen Peroxide, pH, and Applied Ozone Dose. In order to determine the effects of adding hydrogen peroxide and applying ozone in accordance with central composite design, 13 sets of experiments were conducted in the concentration range of 0–480 mM H_2O_2 and 0–22 mg/min ozone. However, statistical analysis of the results showed that none of the factors were significant for the OTC removal in manure slurry by ozonation process. Additionally, manure slurry was subjected to ozonation at natural pH value (7.2) and pH 8.9 to elucidate the effect of pH on the removal of oxytetracycline. The

results of some selected 5 min-ozonation experiments performed at different experimental conditions are presented in Table 9.6.

Table 9.6. Effects of pH, gas phase ozone concentration and hydrogen peroxide addition on OTC removal by ozonation.

Ozone (mg/g manure slurry)	H ₂ O ₂ (mM)	pH	OTC Removal (%)	Absorbed Ozone (mg/g manure slurry)
3.1	-	7.2	84	1.8
27.5	-	7.2	86	3.8
27.5	-	8.9	73	8.3
3.1	240	7.2	82	n.a
-	240	7.2	6	n.a

It is generally expected that ozone treatment is much more efficient in an alkaline medium due to the production of nonselective hydroxyl radicals through the decomposition of ozone by hydroxyl ions. In this study, however, increments of manure pH from 7.2 to 8.9 lowered the OTC removal from 86 % to 73 %. This could be attributed to an increase in alkali-extracted organic compounds (e.g. humic acids) and carbonate ions in the liquid phase of manure slurry that are known to act as radical scavengers. Relatively higher ozone absorption by manure slurry at pH 8.95 is evidence for the presence of radical scavenging compounds at this pH value (Table 9.6). Alternatively, the increments of applied ozone from 3.1 to 27.5 mg/g manure slurry increased the absorbed amount of ozone per gram manure slurry only from 1.8 mg to 3.8 mg and the increase in the applied ozone dose was not able to increase the overall OTC removal (Table 9.6).

It was shown that the conjugate base of H₂O₂ at mM concentration could initiate the decomposition of ozone much more rapidly into hydroxyl radicals than with the hydroxide ion (Staehelin and Hoigné, 1982). However, in this study, there was no significant enhancement in OTC removal by hydrogen peroxide with the addition of up to a 480 mM concentration. This could be explained by the high metal content of manure (11.2 g/kg Fe

and 0.4 g/kg Mn) that could decompose ozone to produce reactive radicals (Langlais et al., 1991) so that additional effect of hydrogen peroxide could not be observed.

As it is well known, ozone reacts with organic compounds via two different pathways: direct molecular and indirect radical reactions. Therefore in this study experiments were also carried out in the presence of a hydroxyl radical scavenger (1 M t-butanol) in order to elucidate the contribution of either a direct molecular ozone attack or the radical oxidation (Figure 9.6).

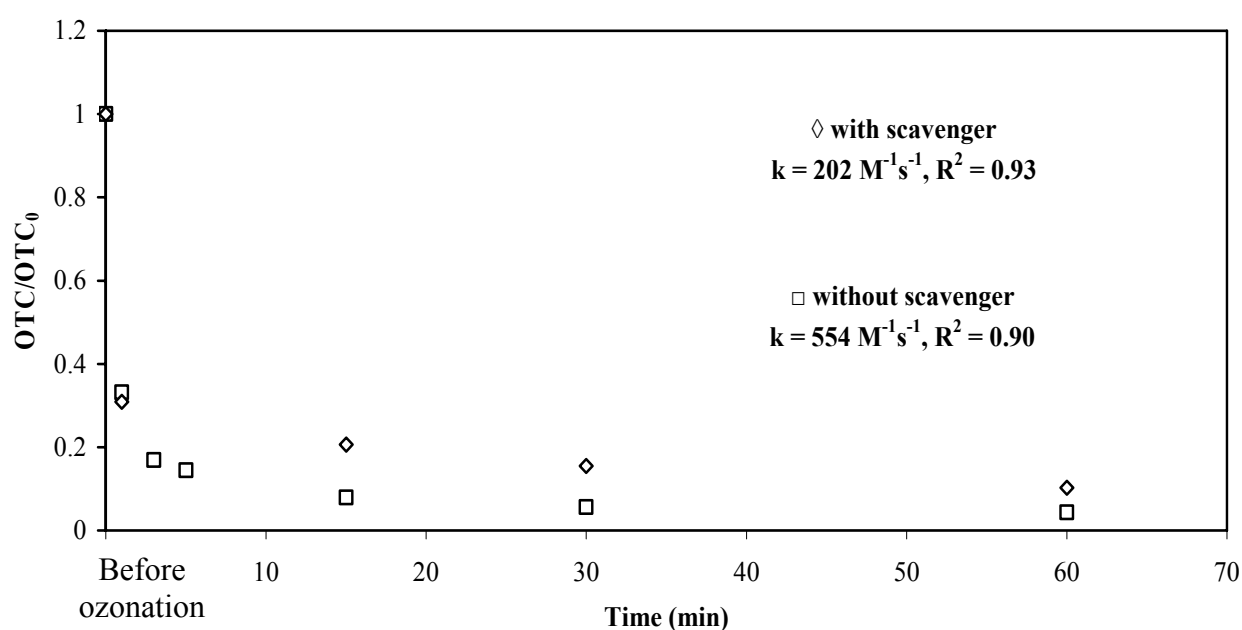


Figure 9.6. Effect of a radical scavenger (1 M t-butanol) on OTC removal kinetics in ozonation process ($O_3 = 22$ mg/min) at pH 7.2.

At the initial stage of the oxidation process, there was no observable effect of a radical scavenger on OTC removal. This can be explained by the rapid reaction between molecular ozone and available OTC in the liquid phase of manure slurry. Upon the extension of treatment time, the role of hydroxyl radicals became important to oxidize the strongly adsorbed OTC in manure. Consequently, the presence of a radical scavenger decreased the second order removal rate constant of OTC from 554 to 202 $M^{-1}s^{-1}$.

The extent of the reaction between ozone and other organic matters in manure slurry was also evaluated by the determination of the SCOD and OC contents of manure throughout the ozonation (Figure 9.7).

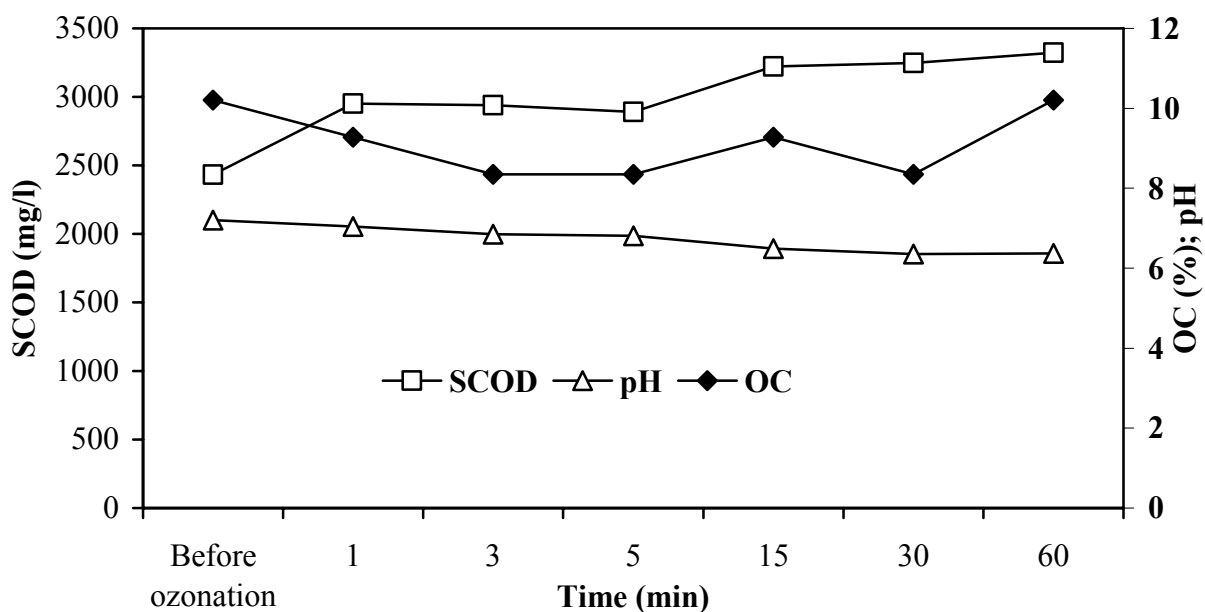


Figure 9.7. Variations in manure slurry characteristics by the application of the ozonation process ($\text{pH}_i=7.2$; $\text{O}_3 = 2.5 \text{ mg/min}$).

The pH of manure slurry decreased from 7.2 to 6.3 by the formation of acidic reaction products and the SCOD and OC did not change as remarkably as in the Fenton oxidation process. From the results it can be deduced that ozone selectively oxidizes OTC in manure slurry rather than other organic constituents.

9.4. Conclusion

In the present study, manure contaminated with a commonly used veterinary antibiotic (oxytetracycline), was treated by an integrated treatment process consisting of extraction pretreatment and chemical oxidation. Pretreatment with a magnesium solution to extract OTC from manure significantly improved the Fenton and ozonation efficiencies in terms of OTC removal. That extraction reached a plateau quickly after 30 min implies that the desorption of OTC from the manure by $\text{Mg}(\text{NO}_3)_2$ is a relatively fast process. The results demonstrated the ability of the investigated oxidation processes to selectively oxidize OTC

while in the presence of a radical scavenger existing naturally in manure slurry. While antibiotic removal was more than 90 % with both oxidation processes, it was much more rapid with ozonation compared to the Fenton oxidation. The Fenton-like oxidation without any added soluble iron also provided high OTC removal as in the case of Fenton oxidation, but at a slower rate. The oxidant dose was a significant factor for the efficiency of Fenton oxidation, but not for the ozonation process. On the other hand, neither of these processes was affected by the pH adjustment of manure. The OTC removal rate would decrease when the loosely bond OTC was being destroyed and the oxidants had to react with OTC that was firmly bound on the solid surface, or sorbed in micropores of manure.

10. SIMULTANEOUS REMOVAL OF OXYTETRACYCLINE AND SULFAMETHAZINE ANTIBACTERIALS FROM ANIMAL WASTE BY CHEMICAL OXIDATION PROCESSES

10.1. Introduction

Tetracyclines (TCs) and sulfonamides (SAs) are commonly used antibacterial agents in livestock production and aquaculture for therapeutic and prophylactic purposes. After administration, up to 90 % of these compounds are excreted as a parent compound through urine and feces. Consequently, both sulfonamides and tetracyclines have been detected in manure samples up to 91 mg/kg and 46 mg/kg, respectively (Martinez-Carballo et al., 2007; Balcioglu et al., 2007). The residues of antibacterial compounds could also be found in wastewater originated from animal feeding operations (Bradford et al., 2008) and subsequent use of manure or wastewater as a fertilizer results in contamination of agricultural soil with the aforementioned substances. TCs adsorb strongly to solid matrices that reduce their bioavailability while SAs have low sorption tendency (Thiele-Bruhn, 2003; Sarmah et al., 2006). As a result of their high mobility, SAs are more frequently detected in surface and groundwater samples compared to TCs (Lindsey et al., 2001).

Use of manure as a fertilizer is a risk factor for public health due to the contamination of terrestrial and aquatic environment with antibacterial resistant pathogens, as well as antibacterial substances. For example, Sengelov et al. (2003) observed increased levels of tetracycline resistance in soil after the application of pig manure slurry containing tetracycline residues. High levels of tetracycline resistance in several bacterial groups were also reported in soil mixed with fresh cow manure and dairy canal water receiving farm run-off (Esiobu et al., 2002). The adverse effect of antibacterials in the environment is not only restricted to resistance development. Methane inhibition in anaerobic swine lagoons due to the presence of SAs and TCs (10 mg/l) was reported up to 54 % and 43 %, respectively (Loftin et al., 2005). The inhibition of sulfonamides on soil respiration in the first two days with EC₁₀ values of 7 mg/kg sulfamethoxazole and 13 mg/kg sulfamethazine was reported by Liu et al. (2009). Having regard to these adverse effects, it is important to

remove antibacterial agents completely at their source (e.g manure) in a short time period, before resistance to the agents can be developed in microorganisms and therefore spread into the environment. Recent studies revealed that the chemical oxidation processes are able to treat water and wastewater containing antibacterials (Balcioğlu and Ötoker, 2003; Ikehata et al., 2006). These processes are also considered as an efficient treatment to degrade recalcitrant compounds in solid matrices. Most commonly used chemical oxidation processes for the treatment of contaminants in soil, sewage sludge, sediments, and manure are iron catalyzed hydrogen peroxide oxidation (Fenton or Fenton-like oxidation), ozonation, and more recently persulfate oxidation (Carrere et al., 2006; Matta et al., 2007; Ferrarese et al., 2008; Uslu and Balcioğlu, 2008; Uslu and Balcioğlu, 2009).

The present study aimed at the complete and simultaneous removal of two veterinary antibacterials, oxytetracycline (OTC) and sulfamethazine (SMZ) from cow manure by means of integrated treatment processes involving magnesium salt pretreatment with subsequent ozone, Fenton or persulfate oxidation. The effects of oxidant doses and bedding materials on the effectiveness of integrated oxidation processes were investigated. This research also aims to remove OTC and SMZ from wastewater produced by animal feeding operations via persulfate oxidation. The effect of persulfate oxidation on macro and micronutrients and the bacterial toxicity of wastewater which can be considered as a fertilizer were also evaluated.

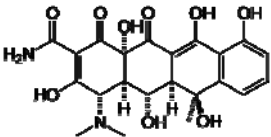
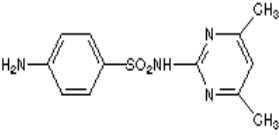
10.2. Materials and Methods

10.2.1. Chemicals

Oxytetracycline (OTC) hydrochloride (Sigma, $C_{22}H_{24}N_2O_9 \cdot HCl$, >95% purity) and sulfamethazine (SMZ) (Sigma, $C_{12}H_{14}N_4O_2S$, 99% purity) were chosen as model antibacterial substances and some of their properties are given in Table 10.1. Fenton's reagent (H_2O_2 (30%) and $FeSO_4 \cdot 7H_2O$) were purchased from Riedel-de Haën and Carlo Erba. Due to its high solubility, $Na_2S_2O_8$ (Sigma-Aldrich) was chosen for persulfate oxidation. $Mg(NO_3)_2 \cdot 6H_2O$ (Merck) was used in the pretreatment operation for the extraction of OTC and SMZ from cow manure. Formic acid and HPLC grade acetonitrile were obtained from Sigma-Aldrich for the analysis of OTC and SMZ. According to the

analytical procedure, antibacterials were extracted from the manure by citric acid monohydrate (Merck), Na₂EDTA.2H₂O (Fluka), Na₂HPO₄ (Merck), and HPLC grade methanol (Sigma-Aldrich). Trypton Type 1 (HiMedia) and yeast extract (AppliChem) were used for the preparation of the growth medium of *Staphylococcus aureus* (*S. aureus*, ATCC 6538; Refik Saydam Hygiene Center) in toxicity assessment tests. All solutions were prepared with Milli-Q ultrapure deionized water (Millipore, Milford, MA) and reagents used for chemical analyses were in analytical grade.

Table 10.1. Selected characteristics of OTC and SMZ.

Antibacterial	Molecular Structure	Molecular Weight (g/mole)	pK _a	K _d soil ^c (l/kg)
Oxytetracycline (OTC)		460.4	3.57;7.49;9.44 ^a	417-1026
Sulfamethazine (SMZ)		278.36	2.07;7.49 ^b	1.0-3.1

^a Figueroa and Mackay, 2005

^b Kurwadkar et al., 2007

^c Thiele-Bruhn, 2003

10.2.2. Manure and Bedding Materials

Cow manure was obtained from an ecological farm located at the west part of Turkey. After drying at 70°C for 24 h, manure was passed through a sieve (2mm) and it was subsequently sterilized in an autoclave at 120 °C for 15 min to prevent any biological activity. Finally, it was stored at 4°C until used in the treatment experiments. Considering the use of bedding material in animal husbandry, sawdust and zeolite were added to manure at a ratio of 20 % (w/w). Bedding materials were also sterilized and stored in a same manner with the manure. Both manure and manure/bedding mixtures were synthetically contaminated by spiking with the OTC and SMZ stock solutions that were prepared in methanol at a concentration of 1 mg/ ml. The initial concentration of OTC and

SMZ in manure was 20 mg/kg of dry manure. After mixing thoroughly with a vortex (Nuve NM 110), the solvent was allowed to evaporate for overnight in the dark.

10.2.3. Synthetic Wastewater

The manure was mixed with deionized water (1:10 w/v) at 150 rpm (Nüve, NM 110) for 24 h to prepare synthetic animal feeding operation wastewater. It was subsequently centrifuged at 4000 rpm (Hettich Universal 16A) and then filtered through an ordinary filter paper. Similar to the manure samples, the synthetic wastewater was sterilized and contaminated with OTC and SMZ stock solutions. The initial concentration of OTC and SMZ in synthetic wastewater was 5 mg/l.

10.2.4. Extraction Pretreatment of Contaminated Manure

Prior to the oxidation experiments, a pretreatment operation was applied to the synthetically contaminated manure by the addition of magnesium salt solution. Five gram of manure or manure bedding mixtures were agitated with 25 ml of 1 M Mg (NO₃)₂ solution at 150 rpm and 25° C for 30 min in a thermostated shaker (Julabo SW 22). After the mixing period, the manure slurry which contained 5 g manure and 25 ml extraction solution, was subjected to ozonation, Fenton, and persulfate oxidation processes. The pH of manure slurry was not adjusted for pretreatment and oxidation processes and all analyses were performed in duplicate.

10.2.5. Ozonation of Manure Slurry

Thirty gram of the manure or manure bedding mixture slurry in a reactor which consisted of a 100 ml gas washing bottle was ozonated for 2 h at room temperature in semi-batch mode with a continuous flow of the ozone and oxygen gas mixture (applied ozone concentration: 15 g/m³) at a rate of 0.01 m³/h. Ozone was produced from dry, pure oxygen in a laboratory scale corona discharge ozone generator (Fisher OZ 500). All tubing from the ozone generator to the reactor was made of silicone and fittings were made of Teflon. Excess ozone gas passed out through the top of the reactor into a ozone gas analyzer (Fisher Ozotron 23) to determine the quantity of ozone generated and applied to

the manure slurry per unit time and the partial pressure of ozone in the feed stream. The mixing of manure slurry was provided by a magnetic stirrer to ensure homogeneity and contact between the manure and oxidants.

10.2.6. Fenton Oxidation of Manure Slurry

Fenton's reagent was slowly added to 100 ml capacity flasks containing 30 g manure or manure and bedding mixture slurry at Fe(II)/ H₂O₂ molar ratio of 1/10 with varying H₂O₂ doses (H₂O₂ = 50-1600 mM). Fenton oxidation was implemented in a thermostated shaker (Julabo SW 22) at 150 rpm and 25 °C for 2 h until all hydrogen peroxide was consumed.

10.2.7. Persulfate Oxidation of Manure Slurry and Synthetic Wastewater

Different concentrations of Na₂S₂O₈ solution were added to a 100 ml capacity flask containing 30 g manure or manure and bedding mixture slurry and then agitated in a thermostated shaker at 150 rpm for 2 and 24 h- treatment periods. The concentration of Na₂S₂O₈ (10-100 mM) and temperature (25-50 °C) were varied in the experiments.

Synthetically prepared contaminated wastewater in a 100 ml capacity flask was treated by the addition of Na₂S₂O₈ solution (2.5-25 mM). The persulfate oxidation of wastewater (50 ml) was performed in a thermostated shaker at 150 rpm and 50 °C. At designated time intervals, 2 ml of samples were taken, filtered (0.45 µm regenerated cellulose, Sartorius) and then immediately analyzed for antibacterials and nutrients.

10.2.8. Antibacterial Analysis

After the application of oxidation processes the treated manure slurry was subjected to the extraction procedure for soil samples as described by Blackwell et al. (2004) with some modifications. The antibacterial analysis in the manure extracts and synthetic wastewater was performed with an HPLC system (Agilent Technologies 1100 series) equipped with a tertiary pump, a photodiode array, and an autosampler with an automated injection system. The recoveries of OTC and SMZ in the manure extracts were determined as 89.5 ± 2.5 %

and 60.3 ± 3.5 %, respectively. When the antibacterial substances in the manure slurry extracts could not be detected by HPLC, they were qualitatively analyzed with LC/MS-MS consisting of Thermo Scientific TSQ Quantum mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled with the company's Surveyor LC pump and autosampler. Table 10.2 provides the operational conditions and used column properties of both systems.

Table 10.2. Operational conditions for HPLC and HPLC/MS-MS analyses.

	HPLC	LC/MS-MS
Mobile Phases	A: ACN (0.1 % formic acid) B: Water (0.1 % formic acid)	A: CAN (0.1 % formic acid) B: Water (0.1 % formic acid)
Flow	0.3 ml/min	0.7 ml/min
Gradient	0 min: 10 % A 0-5 min: linear increase to 40 % A 5-8 min: linear decrease to 10 % A 8-11 min: 10 % A	0 min: 10 % A 0-1 min: linear increase to 90 % A 1-8 min: 90 % A 8-8.5 min: linear decrease to 10 % A 8.5-15 min: 10 % A
Column	Eclipse XDB C ₁₈ (5 μ , 150 x 4 mm) Detection OTC: 360 nm SMZ: 268 nm	Synergy Max RP (4 μ , 150 x 2 mm) Parent Mass OTC : 461 SMZ : 279 Product Mass OTC : 426 – 444 SMZ : 156 – 204

10.2.9. Toxicity of Raw and Treated Synthetic Wastewater

Acute toxicity of contaminated and persulfate treated contaminated wastewater was evaluated by their inhibition effect on the growth of *S. aureus* which was chosen as the test specie due to its abundance in the environment and susceptibility to tetracycline and sulfonamide group antibacterials (Okusa et al., 2007; Genc et al., 2008). In order to overcome the possible interferences of remaining persulfate to the bacterial growth, it was decomposed by the addition of sodium thiosulfate solution prior to administering the bacterial toxicity test.

A colony of *S. aureus* which was plated on Luria-Bertani (LB) agar was inoculated in 25 ml of LB medium ((1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, and 1% (wt/vol)

NaCl)) for 18 h at 37° C and 150 rpm. Three milliter of this culture was transferred to 150 ml of fresh LB medium and agitated at 37° C and 150 rpm until they reached to optical density of 0.12 at 660 nm wavelength. At this time, 20 ml of the culture were added to the flasks containing 5 ml of uncontaminated wastewater, raw, and persulfate treated contaminated wastewater. Incubation of the microorganisms exposed to wastewater samples was continued for 4.5 h. At the end of incubation period the growth inhibition was calculated using the following formula.

$$I(\%) = \frac{(OD_c - OD_f)}{(OD_c - OD_i)} \quad (10.1)$$

where;

OD_c = Optical density (OD₆₆₀) of wastewater at the end of incubation.

OD_i = Optical density (OD₆₆₀) of the contaminated or persulfate treated contaminated wastewater as the culture was added to the flask.

OD_f = Optical density (OD₆₆₀) of the mixture of the culture and contaminated or persulfate treated contaminated wastewater at the end incubation.

10.2.10. Other Chemical Analyses

The organic carbon (O.C) content of the manure and bedding materials were analyzed by using the Walkley-Black method with some modifications (Walkley and Black, 1934). The Cation Exchange Capacity (CEC) of the manure and bedding materials was determined according to EPA Method 9081. Since macro (Ca, Mg, K) and micro (Cr, Zn, Fe) minerals were detected in several types of manure samples at high concentrations (Balcioğlu et al., 2007), their analyses were conducted in filtered (0.45 µm regenerated cellulose, Sartorius) wastewater sample using ICP (Perkin Elmer, Optima 2100 DV). The analysis of NO₃⁻, NO₂⁻, Cl⁻, SO₄⁻² contents were performed by IC (Dionex ICS-3000) with Ionpac AS19 analytical (7.5 µm, 250 x 4 mm) and Ionpac AG19 guard (11 µm, 50 x 4 mm) columns. NH₄⁺-N and reactive PO₄⁻³ contents were determined by Nessler and Ascorbic acid methods using a Hach spectrophotometer.

Persulfate analysis in the liquid phase of manure slurry and synthetic wastewater was conducted according to the iodometric method (Kolthoff and Carr, 1953). Hydrogen peroxide determination in the liquid phase of manure slurry was also performed according to the iodometric method in the presence of an ammonium molybdate catalyst (<http://www.solvaychemicals.us/static/wma/pdf/6/6/2/5/XX-122.pdf>).

10.3. Results and Discussion

10.3.1. Pretreatment of Manure

The dissolution of antibacterial substance from cow manure into the aqueous phase of the slurry by means of complexation with Mg^{2+} ions was proposed as a pretreatment for the enhancement of the oxidation treatment efficiency of OTC (Uslu and Balçioğlu, 2009). In the current study, the effect of this pretreatment on the simultaneous removal of OTC and SMZ was investigated at two different Mg^{2+} ion concentrations and solid/liquid ratios (Figure 10.1). Additionally, extraction pretreatment was performed with deionized water in the absence of Mg^{2+} ion.

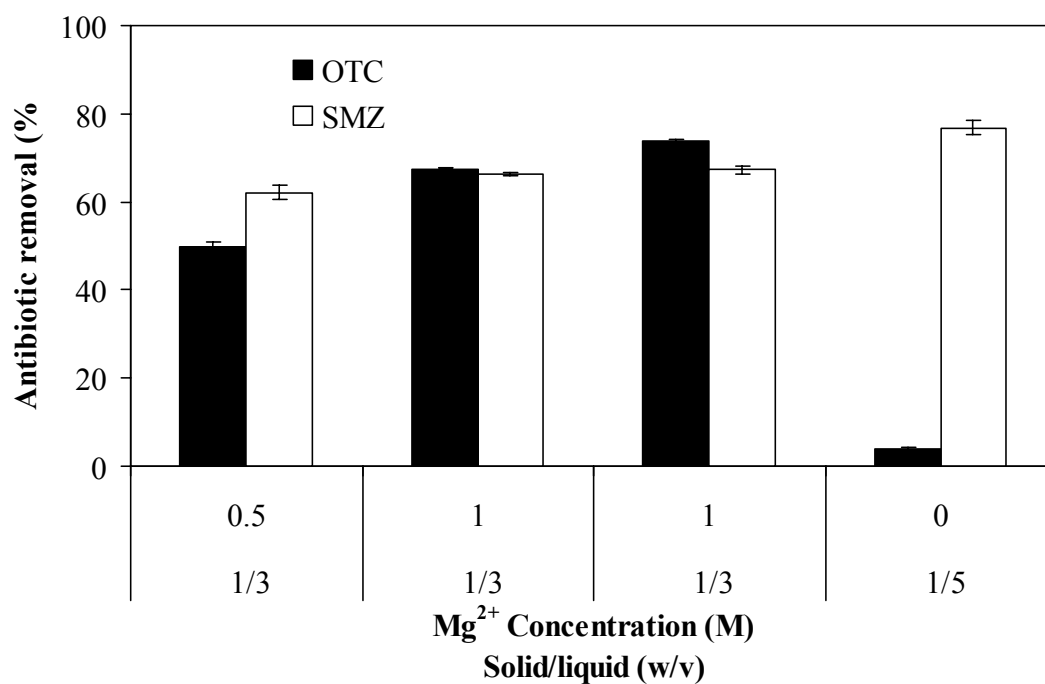


Figure 10.1. Effects of magnesium salt concentration and solid/liquid ratio on manure pretreatment.

As mentioned previously TCs generally exhibit higher sorption tendencies onto soil and clays compared to SAs at environmentally relevant pH values (Thiele Bruhn, 2003; Sarmah et al., 2006). Furthermore, the sorption of sulfonamides has minor importance above their pK_{a2} values when anionic species of sulfonamides dominate (Kurwadkar et al., 2007). Therefore it was not a surprise to obtain more than 75 % desorption of SMZ into the aqueous phase of the manure slurry (solid/liquid=1/5) which has a pH value of 8.6. On the other hand, under the same experimental conditions, the desorbable part of OTC was only 4 %. The addition of Mg^{2+} salt at the 0.5 M concentration greatly increased the extraction of OTC from the manure (50 %) while it reduced the extraction of SMZ about 15%. The reason for the unfavorable effect of extraction with Mg^{2+} salt solution for SMZ can be explained by the decrease of the manure slurry pH to 7.3 at which more sorption of SMZ is anticipated considering the pK_{a2} value (Table 10.1). Further enhancement in the extraction efficiency of OTC was achieved by increasing the concentration and volume of the Mg^{2+} salt solution. They can provide a higher ionic strength in the aqueous phase of manure slurry and metal complexation probability with OTC that lowers the sorption of OTC on the manure. Since manure has high buffering capacity, no effort was spent to adjust the pH of manure slurry. Considering the results obtained, subsequent experiments were performed with the manure pretreated by 1 M Mg^{2+} salt solution and at a solid liquid ratio of 1/5.

10.3.2. Effect of Bedding Materials on the Performance of Pretreatment

Use of bedding in livestock and poultry production is common practice to improve farm animal welfare and the presence of bedding material in manure can affect its treatment. In this study, the effect of bedding material on the treatment of cow manure was investigated by using natural zeolite and sawdust. It was known that OC and CEC are two important parameters affecting the sorption of antibacterials on solid matrices (Jones et al., 2005; Sukul et al., 2008). Therefore, first, these parameters were analyzed in the selected bedding materials and manure (Table 10.3). Thereafter, both beddings were mixed with manure separately at a ratio of 20 % (w/w) (Cusick et al., 2003) and finally, their effects on the extraction of antibacterials were determined (Figure 10.2).

Table 10.3. Organic carbon and cation exchange capacities of manure and bedding materials.

	OC (%)	CEC (m eq /100 g)
Manure	7.3 ± 0.5	28 ± 0.8
Sawdust	58.8 ± 1.25	65 ± 1.0
Zeolite	0.18 ± 0.08	63 ± 1.2

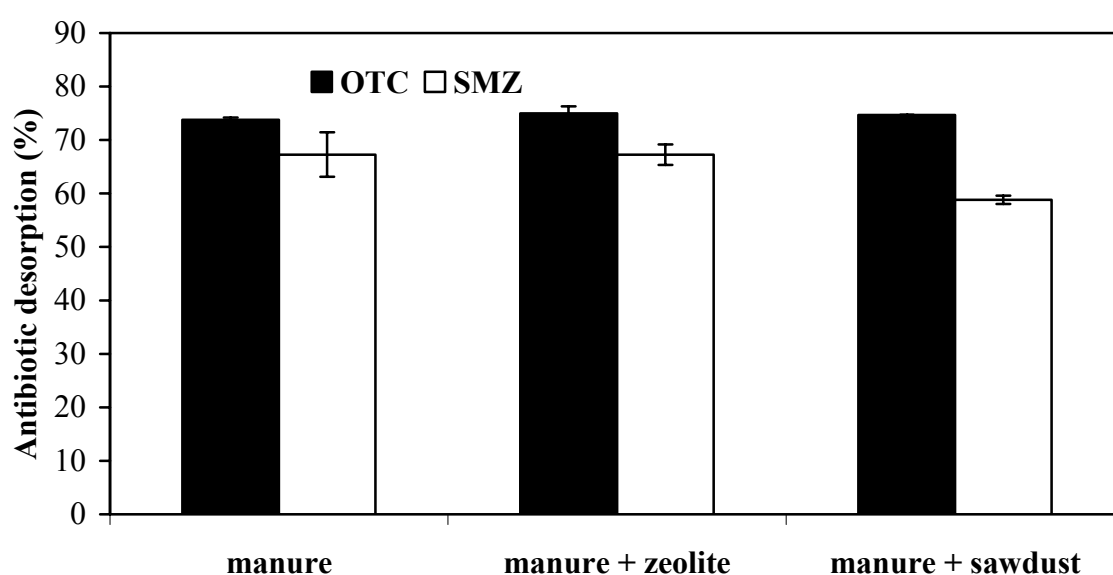


Figure 10.2. Effect of bedding materials on antibacterial extraction from manure.

Although the lower extraction of OTC would be anticipated in manure bedding mixture due to possible stronger sorption there was no observable effect of bedding addition on the extraction of OTC. The excess amount of magnesium ions in the aqueous phase of the manure slurry could sustain the dissolution of OTC. On the other hand, the addition of sawdust to the manure slightly decreased (9 %) the extraction efficiency of SMZ, because sawdust has high amount of organic matter which has been reported to have greatly contributed toward sulfonamide sorption in solid matrices (Sukul et al., 2008). Furthermore, the addition of sawdust resulted in a decrease in the pH value of manure slurry (from 7.3 to 6.8) which may also contribute to the sorption of SMZ on solid mixture.

10.3.3. Ozonation of Manure Slurry

Ozone treatment has been applied to several wastes from animal feeding operations for the purposes of disinfection (Macauley et al., 2006), the removal of natural steroid hormone (Ermawati et al., 2007), and the reduction of malodorous substances (Wu and Masten, 2002), pathogens, and toxic fermentation metabolites (Watkins et al., 2007). Considering the benefits of ozonation on the treatment of animal wastes in the existing study, after the pretreatment, ozonation was applied to the manure for a 2 h treatment period to accomplish the simultaneous removal of OTC and SMZ (Figure 10.3).

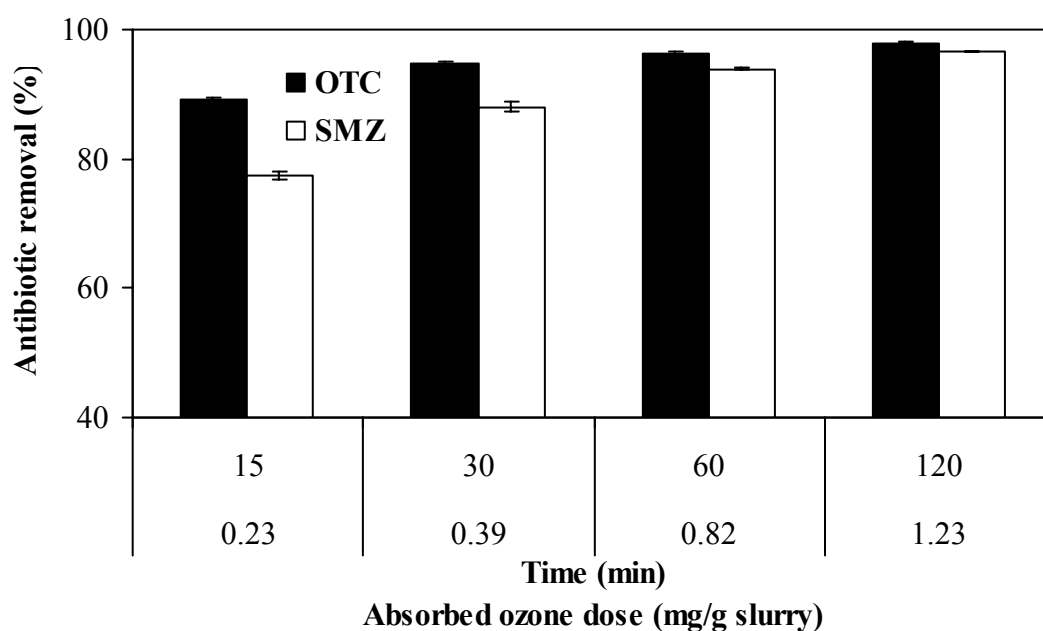


Figure 10.3. Removal of OTC and SMZ as a function of ozonation time and absorbed ozone dose.

As represented in Figure 10.3, in the absorbed ozone dose ranges of 0.23-1.23 mg ozone/g slurry, slightly lower removal rate of SMZ was obtained from the manure slurry compared to that of OTC. Dodd et al. (2006) also reported a lower transformation of sulfonamide antibacterials than tetracyclines in municipal wastewater by ozonation with the ozone reaction rate constants of $5.7 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (sulfamethoxazole) and $3.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ (tetracycline) at pH 7.7, respectively.

10.3.4. Fenton Oxidation of Manure Slurry

Depending upon the results of our previous study related with the abatement of OTC (Uslu and Balçioğlu, 2009), Fenton oxidation was applied to the pretreated manure spiked with OTC and SMZ within a wide hydrogen peroxide dose range ($[H_2O_2]_0=50-1600$ mM; $[H_2O_2]_0/[Fe^{2+}]_0=10$) for a 2 h treatment period. Since the solutions of hydrogen peroxide and iron were acidified before the addition to the manure to increase the stability and solubility, respectively, the initial pH of all manure slurry samples treated with the different doses of oxidant decreased to about 4.5 except for the sample subjected to the addition of 1600 mM H_2O_2 dose. Initial pH descended to the value of 3.6 only at the highest dose of Fenton's reagent. Further pH adjustment was not applied to manure slurry samples in the Fenton oxidation process as in the case of other used oxidation processes. At the end of the 2 h treatment period where added hydrogen peroxide to the manure slurry was completely consumed the pH of manure increased from about 4.5 to 6 (data not shown). The results of oxidation with various doses of Fenton's reagent for the simultaneous degradation of OTC and SMZ are listed in Table 10.4

Table 10.4. Effects of hydrogen peroxide and iron (II) concentrations on Fenton process efficiency.

H_2O_2 (mM)	Fe^{2+} (mM)	Removal (%)	
		OTC	SMZ
50	5	66.7 ± 0.3	75.1 ± 0.1
100	10	90.0 ± 0.5	95.5 ± 0.8
275	27.5	95.9 ± 0.3	94.8 ± 0.3
680	68	96.8 ± 0.2	96.4 ± 1.5
970	97	96.4 ± 0.4	97.4 ± 0.4
1600	160	98.3 ± 0.1	97.8 ± 0.3

Contrary to the results of ozonation, the removal of SMZ was higher (75.1 %) than that of OTC (66.7 %) at the lowest applied dose of Fenton's reagent (50 mM H_2O_2 /5 mM Fe^{2+}). This result is also not consistent with the reported reaction rate constants of the sulfamethoxazole and tetracycline with hydroxyl radicals (Dodd et al., 2006). However,

the degradation reaction pathways for the antibacterials in the manure could be affected by the variation of slurry composition during the oxidation process. Moreover, under the experimental conditions of Fenton oxidation as well as hydroxyl radical hydrogen peroxide could be responsible for the oxidation of target pollutants. Therefore, the abatement rates of antibacterials can be different than those obtained by ozonation.

On the other hand, similar to the results of the ozonation process, the increasing dose of oxidant resulted in a decrease between the removal rates of the two antibacterials and the residuals of both antibacterials remained in the slurry even at excessive dose of Fenton's reagent. As presented in Table 10.4, the rise of hydrogen peroxide concentration in Fenton's reagent from 50 to 275 mM significantly enhanced simultaneous OTC and SMZ removal whereas a further increase in hydrogen peroxide concentration did not cause remarkable improvement for the abatement of antibacterial residuals. The strongly sorbed antibacterials on the manure could not consume the produced radicals because of the rapid decomposition of high amounts of hydrogen peroxide in the presence of high amount of iron through the Fenton's reaction and increase in temperature. Instead reactive radicals oxidized the other organic compounds of the manure released into solution. Since the longer lifetime of hydrogen peroxide in soil slurry systems was reported to enhance contaminant removal (Bacocchi et al., 2003) it would be more efficient to catalyze hydrogen peroxide decomposition by the indigenous iron content of manure at acidic pH. This suggestion is confirmed by the findings of our previous study (Uslu and Balcioglu, 2009) in which a lower amount of oxidant in Fenton-like treatment (434 mM H_2O_2 ; no addition of soluble iron) was able to degrade OTC in the manure compared to Fenton treatment at acidic pH.

At low dose of Fenton's reagent (50 mM H_2O_2 /5 mM Fe^{2+}), magnesium salt pretreatment enhanced the OTC removal by about 10 % while it did not affect overall SMZ removal, since desorption of SMZ was already provided by deionized water. It was reported that high hydrogen peroxide dose (≥ 300 mM) can enhance the efficiency of Fenton oxidation by desorbing the pollutants from soil surface into solution where oxidation takes place (Watts et al., 1999a). By taking into account this property of hydrogen peroxide, contaminated manure was also treated by the highest dose of Fenton's reagent (1600 mM H_2O_2 /160 mM Fe^{2+}) without application of pretreatment. Although it

eliminated the necessity of pretreatment for the more efficient OTC removal, it did not cause the complete degradation of antibacterials (data not shown). Actually, this result is not remarkably different from that obtained by the lower dose of Fenton's reagent.

10.3.5. Persulfate Oxidation of Manure Slurry

Besides the ozone and Fenton oxidation processes, metal ion activated and heat activated persulfate oxidation processes were applied to the contaminated manure slurry. It was reported that sulfate radical ($E^\circ = 2.6$) which has comparable oxidation potential to hydroxyl radical ($E^\circ = 2.7$), is more selective than hydroxyl radical in its reaction with organic compounds and anilinic and phenolic groups exhibit high reactivity to this radical (Neta et al., 1988). Therefore, OTC and SMZ which have forementioned reactive groups in their structure could be efficiently degraded in the manure slurry having high organic matter content by persulfate process. Moreover, its benign end product, sulfate is considered to be a secondary nutrient for plant growth when the treated manure is used as fertilizer.

In this part of the study, Fe (II) activated persulfate oxidation of pretreated manure slurry was implemented with a single dose of persulfate (100 mM) and at an oxidant/catalyst ratio of 10/1. Twenty four h oxidation at 25° C resulted in 93.2 % of OTC and > 99 % of SMZ removal. Although OTC has a structure that could exhibit higher reactivity to the oxidation, SMZ abatement in manure slurry was found higher than that of OTC similar to the results of Fenton oxidation. The variation of the recovery rate of antibacterials from manure slurry by the application of an oxidation process can also be reason for this controversial oxidation yield. Similar to Fenton oxidation, persulfate oxidation resulted in a decrease in the pH value of manure slurry to about 5.2 at which the sorption of SMZ on the manure has been expected. On the other hand, magnesium ion in the slurry could inhibit the sorption of OTC on manure. The recovery rate of sulphonamide antibacterials in soil are strongly correlated with the sorption which is in turn increased by increasing the contact time of an antibacterial with solid matrix (Hamscher et al., 2005; Kahle and Stamm, 2007b). Accordingly, the results of control experiments in the absence of oxidant showed relatively high SMZ abatement (30 %) while that of OTC was negligible within the 24 h whereas no significant variation in the concentration of both

antibacterials was observed within a 2 h treatment period. Although the decrease of OTC and SMZ concentration may be related to the stability of antibacterials in the manure the contribution of this factor could not be predominant at 25° C since the occurrence of sulfonamide antibacterials have been reported in manure and soil samples collected from various fields (e.g. Martinez-Carballo et al., 2007; Karci and Balcioglu, 2009).

Alternatively, the activation of persulfate was performed by increasing the temperature of the medium and this activation method can be more suitable for the treatment of manure. Since elevated temperatures can be obtained by the action of microorganisms during the storage of manure heaps (Hutchison et al., 2005), a thermally activated persulfate process at two different temperatures (40 and 50°C) was applied to pretreated manure at various oxidant doses (10-100 mM Na₂S₂O₈). Furthermore, the effect of the reaction period on antibacterial removal was investigated (Table 10.5).

Table 10.5. Effect of oxidant concentration on thermally activated persulfate process.

Na ₂ S ₂ O ₈ (mM)	Temperature (°C)	Removal (%)			
		OTC	OTC	SMZ	SMZ
		t = 2 h ^a	t = 24 h	t = 2 h	t = 24 h ^b
10	40	73.7 ± 1.1	-	91.2 ± 1.2	nd
10	50	83.8 ± 0.8	98.9 ± 0.1	91.3 ± 0.5	99 ± 0.9
25	40	95.4 ± 0.1	98.0 ± 0.2	92.4 ± 0.4	98 ± 0.4
25	50	-	100	-	100
50	40	98.3 ± 0.2	-	93.0 ± 0.8	nd
100	40	98.7 ± 0.1	> 99	95.5 ± 0.7	> 99
100 + 10 Fe ²⁺	25	-	93.2 ± 0.08	-	> 99

^a Decrease in antibacterial concentrations after 2 h control experiment at 40° C was negligible

^b Decrease in concentration of SMZ after 24 h control experiment at 40° C was 40 %, while that of OTC was negligible.

The removal of OTC was improved by approximately 20 % upon increasing the persulfate dose from 10 to 25 mM at 40°C whereas more than 90 % SMZ was removed even with the 10 mM oxidant dose within a 2 h treatment period. A further increase of the

oxidant dose up to 100 mM resulted in only small enhancement in antibacterial removals within the 2 h oxidation period. As the reaction time was extended to 24 h, thermally activated persulfate oxidation caused more than 99 % removal for both antibacterials which could not be achieved by Fenton and ozone oxidation processes at the described oxidation conditions.

In spite of high organic content of the manure, OTC and SMZ antibacterials were removed by the application of thermally activated persulfate process. More than 90 % removal of antibacterial substances with only 20 % of persulfate decomposition ($[\text{Na}_2\text{S}_2\text{O}_8]_0 = 25 \text{ mM}$) in highly complex manure matrix can be an evidence for the high selectivity of persulfate and sulfate radicals for the studied antibacterial substances compared to the organic matter content of manure.

The increment of the reaction temperature by 10°C and the extension of the reaction period up to 24 h with a 10 mM persulfate concentration increased the OTC and SMZ removals from 74 % and 91 % to approximately 99 % (Table 10.5). Control experiments conducted at 50° C without addition of the oxidant showed 25 % and 50 % decrease in OTC and SMZ concentrations respectively at the end of 24 h. Although with increasing temperature the increased generation of sulfate radicals in the persulfate process (Liang et al., 2003) and accelerated desorption of OTC from animal manure (Wang and Yates, 2008) were reported, the results of the current study revealed that thermal degradation of antibacterials could contribute to their overall removal of them in manure slurry at 50° C. The importance of thermal degradation of OTC in manure during storage or composting process was also investigated by Arıkan et al. (2007) and Wang and Yates (2008).

As the persulfate dose was increased from 10 mM to 25 mM at 50°C, antibacterials in 24 h treated manure extracts were not detected by HPLC analysis. However, LC/MS/MS analysis of the same manure extracts denominates not complete but more than 99 % removal of OTC and SMZ. The complete removal of antibacterial residuals was confirmed by LC/MS/MS analysis in manure extracts that were treated with 100 mM persulfate at 50°C for 24 h.

Consequently, the results obtained revealed that either increasing the dose of persulfate or elevating the temperature can enhance the removal rates of either antibacterials.

The effect of pretreatment on the efficiency of a thermally activated persulfate process performed with 25 mM $\text{Na}_2\text{S}_2\text{O}_8$ at 50° C for 24 h was also investigated. As opposed to the effects on Fenton oxidation carried out with low dose of oxidant, the contribution of Mg salt pretreatment on OTC removal from the manure slurry by persulfate oxidation was only 2 % and it had no effect on SMZ removal. This result could be explained by the thermal desorption and degradation of antibacterials at high temperatures as was discussed previously. Furthermore, it was speculated that persulfate could be responsible for the desorption of organics from the soil surface similar to the effect of excess doses of hydrogen peroxide (Liang et al., 2008b). Hence, this effect also decreased the importance of pretreatment of manure for the degradation of OTC and SMZ. Consequently, persulfate treatment could be proposed for the efficient treatment of contaminated manure at high temperatures because it provides near complete antibacterial removal even at low oxidant doses within only a 2 h treatment period. Moreover its effect on soil organic matter was reported to be analogous to the compositional changes resulting from humification (Cuypers et al., 2002) which is an indicator of the maturity and stability of compost before its application to agricultural areas (Ciavatta et al., 1993).

10.3.6. Effect of Bedding Materials on the Performance of Oxidation Treatment Processes

The effects of bedding material presence in the manure (bedding/manure=20 % (w/w)) on the treatment performance of three different oxidation processes which were applied after the pretreatment were evaluated. Figure 10.4 presents overall antibacterial removals in manure, manure + zeolite and manure + sawdust mixtures obtained by the applications of Fenton (1600 mM H_2O_2 /160 mM Fe (II); 2 h), ozone (2 mg/min ozone dose; 2 h) and persulfate (100 mM S_2O_8 ; 40° C; 2 h) oxidation processes.

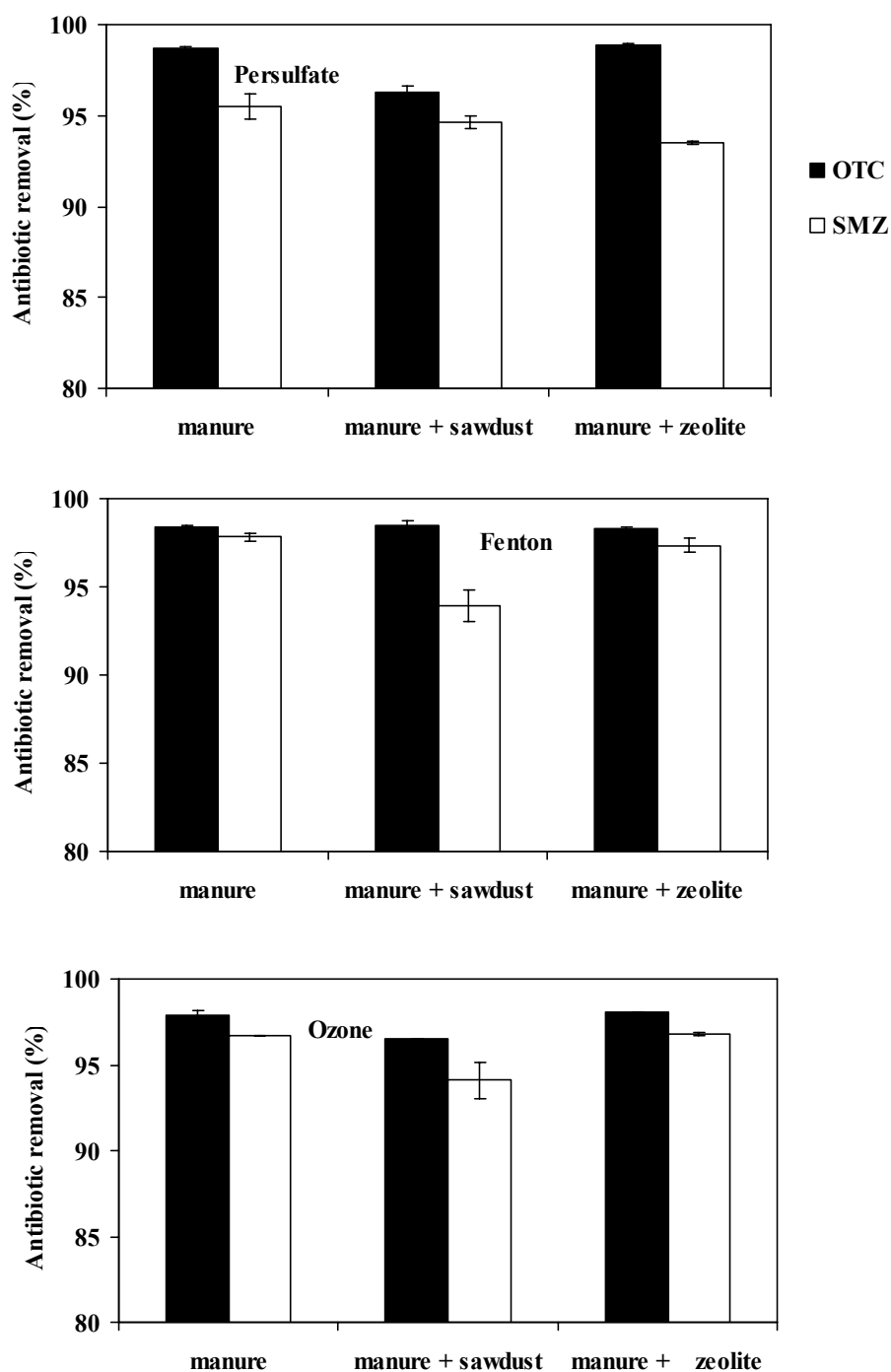


Figure 10.4. Effect of bedding material on the efficiencies of oxidation processes.

As can be seen from Figure 10.4, more than 94 % antibacterial removal was obtained for all matrices. However, the use of sawdust as a bedding material resulted in a minor decrease in OTC and SMZ removal rates probably due to competition of the high organic content of sawdust for reactive radicals generated during oxidation processes.

10.3.7. Persulfate Oxidation of Synthetic Animal Feeding Operation Wastewater

Since the persulfate oxidation was found to be the more effective treatment process on antibacterial removal from the manure, only the thermally activated persulfate oxidation process was applied to the synthetically prepared and contaminated wastewater (OTC and SMZ= 5 mg/l for each) for the simultaneous removal of OTC and SMZ. The effect of the persulfate dose on OTC and SMZ removals at 50° C is presented in Figure 10.5 and also includes the results of control experiments carried out in the absence of oxidant.

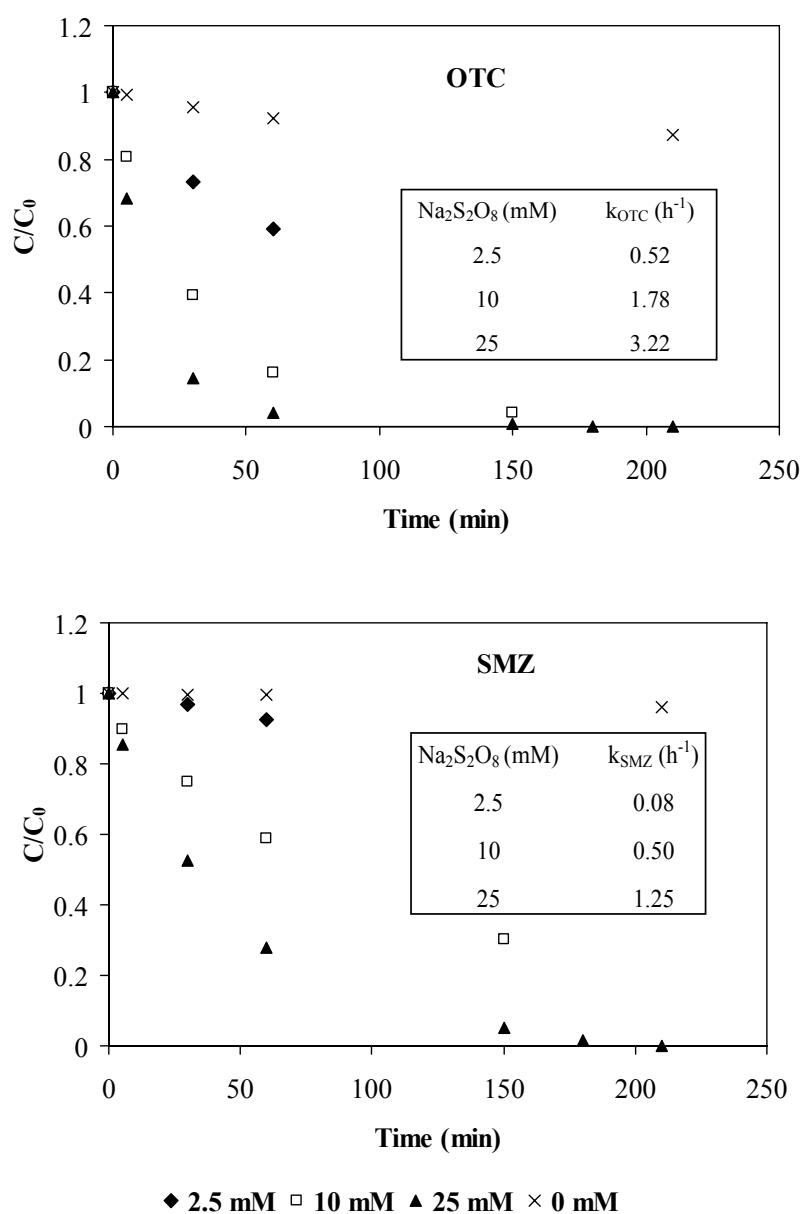


Figure 10.5. Effect of persulfate dose on antibacterial removal (50° C) and pseudo first order removal rate constants.

As can be seen from Figure 10.5, degradation of SMZ and OTC antibacterials in synthetic wastewater follows pseudo first order kinetics and highly depends on the oxidant dose. Contrary to the results obtained in manure treated by persulfate oxidation, a lower degradation rate of SMZ was obtained compare to that of OTC in synthetic wastewater. Actually, this result is consistent with the favorable oxidation tendency of the OTC structure. The main reaction pathway of sulfate radicals with organic compounds occurs by electron transfer mechanism as well as hydrogen abstraction and addition (Neta et al., 1988; Minisci et al., 1983). Therefore, the presence of strong electron withdrawing groups in the structure of organic compounds can reduce their degradation rate (e.g Matta et al., 2007). Accordingly, -SO₂ group in the SMZ structure could explain the slower reaction rate of this antibacterial in this process. However, the pseudo first order removal rate constants of both antibacterials are still remarkably higher than the reported values of some organic compounds treated by persulfate oxidation (Huang et al., 2005).

The complete removal of antibacterials from the wastewater toxicity of reaction products is important for the evaluation of persulfate treatment efficiency. Although the reduction of sulfonamide and tetracycline group antibacterials' toxicity in aqueous solutions treated by different chemical oxidation processes has been investigated (Reyes et al., 2006; Gonzales et al., 2007), their toxicity in wastewater samples after application of chemical oxidation processes has not been studied extensively.

The bacterial toxicity of raw and persulfate (25 mM) treated synthetic wastewater was comparably assessed by determining their inhibition on the growth of *S. aureus* bacteria. The growth curves of *S. aureus* in the presence of untreated and treated contaminated wastewater are presented in Figure 10.6. Experiments were also carried out in water and uncontaminated synthetic wastewater to determine the effects of manure components on the growth of bacteria.

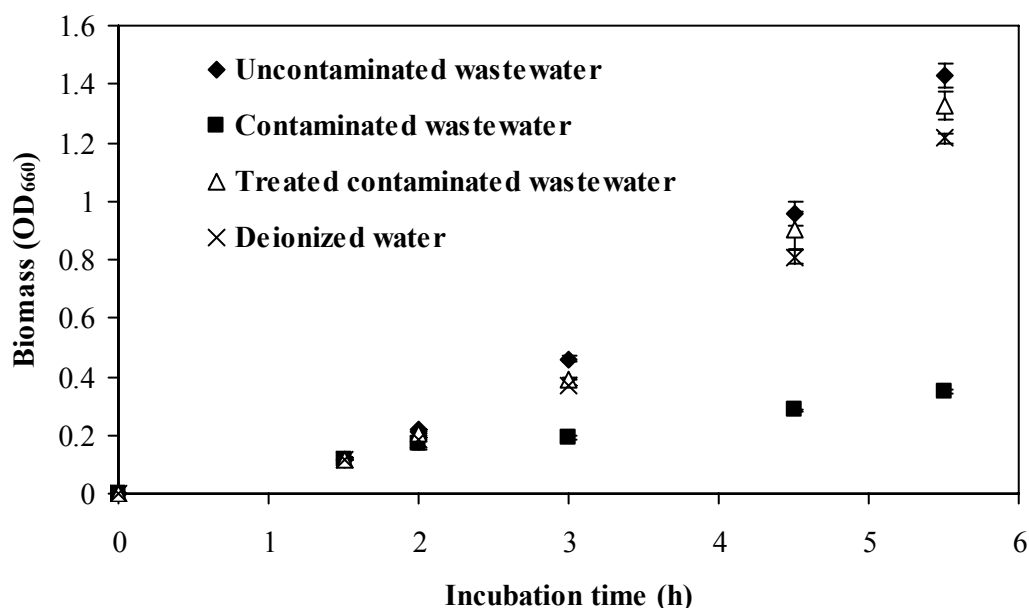


Figure 10.6. Growth curves of *S. aureus* in uncontaminated, contaminated, and treated contaminated wastewater.

As can be seen from Figure 10.6, *S. aureus* exhibited a higher growth rate in wastewater than that obtained in water due to higher nutrient content of synthetic uncontaminated wastewater (Table 10.6). After 4.5 h of exposure the inhibition of untreated contaminated wastewater on *S. aureus* growth was calculated as 82 % with respect to uncontaminated wastewater. The application of the persulfate oxidation process to the wastewater with 25 mM persulfate at 50° C for 3.5 h reduced the inhibition value to 7%.

Since the wastewater generated from animal feeding operations can be used as a valuable fertilizer (Bradford et al., 2009) the variations in the nutrient content of the wastewater were also monitored by the application of the persulfate oxidation process (Table 10.6).

Table 10.6. Some nutrient values of contaminated and treated contaminated wastewater.

Parameter	Concentration (mg/l)	
	Contaminated wastewater	Treated contaminated wastewater
TOC	110	110
NO ₃ ⁻	145	195
NO ₂ ⁻	10	7
NH ₄ ⁺ -N	10	14
PO ₄ ³⁻	28	43
SO ₄ ²⁻	57	270
Ca	36	48
Mg	20	25
K	213	237
Fe	1.3	1.3
Zn	0.07	0.13
Cr	0.03	0.03

Some nutrient values of synthetic wastewater were found to be compatible with the values reported for beef feedlot and dairy lagoon samples (Bradford et al., 2009) and was not changed significantly upon the implementation of persulfate oxidation. While the concentrations of heavy metals were under the maximum limits, the nitrate value of synthetic wastewater was very high relative to irrigation water standards (0-30 mg/l irrigation water) set by Turkish Ministry of Environment and Forestry. However, treated animal feeding wastewater can be used as a fertilizer in agricultural areas as it is applied to soils at agronomic rates using risk management procedures.

10.4. Conclusions

A screening study for the simultaneous removal of TC and SA group antibacterials by means of three different oxidation processes was performed. Although magnesium salt pretreatment was necessary, especially for the removal of OTC from the contaminated manure slurry performed by the ozonation process it was not essential for the enhancement of subsequent Fenton and persulfate oxidation performances. The bedding materials, zeolite and sawdust, had no significant effect on the removal of OTC and SMZ from the manure by means of oxidation processes.

Thermally activated persulfate oxidation was much more effective in antibiotic removal as compared to Fenton oxidation applied at the same oxidant dose. The complete removals of OTC and SMZ in both manure slurry and synthetic wastewater were achieved by persulfate oxidation at 50° C. About 82 % inhibition of antibiotics on *S. aureus* growth in synthetic wastewater was reduced to 7 % upon the complete removal of OTC and SMZ. The nutrient value of synthetic wastewater was not affected by the thermally activated persulfate treatment.

11. CONCLUSIONS

Antibiotic pollution can be considered as a substantial environmental problem with a consequent development of resistance in bacterial communities, which is a growing human health concern. The heavy use of various antibiotics in animal feeding operations may provoke their release into agricultural fields through fertilization with contaminated manure and wastewater. Therefore, it is necessary to apply an appropriate treatment process to manure and wastewater prior to land disposal in order to prevent the spreading of antibiotic residuals and resistant genes into the environment.

The experimental part of this study consisted of five main chapters. In the first two chapters, the analysis and sorption of two antibiotic classes, fluoroquinolones and tetracyclines, were investigated together with their occurrence in different field samples. Remaining three chapters focused on the removal of OTC alone or together with SMZ, from animal waste using three different chemical oxidants, namely Fenton reagent, ozone, and persulfate. The main conclusions drawn from this dissertation are summarized below.

Fluoroquinolones and tetracyclines were detected in different solid field samples at mg/kg ranges. Considerably high concentrations of these antibiotics in the field samples have shown their stability in the terrestrial environment, and can be an indicator of the need for a suitable treatment for their removal from the source of pollution (e.g. animal waste).

Both ozonation and persulfate oxidation processes provided the complete elimination of OTC from the synthetic animal feeding operation wastewater. Although the complete elimination of SMZ was also achieved by persulfate oxidation, the reaction was much slower. The bacterial toxicities of antibiotics were significantly reduced by ozonation and persulfate oxidation processes indicating the non-toxic nature of oxidation products of antibiotics. Ozonation also caused an increase in biodegradability of synthetic wastewater in terms of BOD₅/COD ratio.

The efficiency of ozone treatment for the removal of OTC from synthetically contaminated cow manure was significantly reduced by the presence of moisture probably due to its inhibitory effect on transport of gaseous ozone, and thus prevented the reaction between strongly sorbed OTC in manure and oxidant. Considering the high moisture content of manure in real applications, a pretreatment with magnesium salt solution was suggested for the desorption of OTC from the manure prior to the application of ozone. Application of the pretreatment process greatly enhanced the OTC removal by ozonation process. On the other hand, the effect of magnesium salt pretreatment for Fenton and persulfate oxidation was not significant and near complete antibiotic removals provided with these processes even without a preceding pretreatment. Therefore, it can be concluded that, under applied experimental conditions efficient antibiotic degradation could not be achieved by ozonation due to the ineffective diffusion of ozone gas through contaminated manure, which may cause ineffective ozone utilization.

Due to their high organic matter content and/or high cation exchange capacities, bedding materials, sawdust and zeolite were expected to cause the lower efficacy of applied oxidation processes. However, none of the chemical oxidation processes was affected significantly by the addition of bedding materials to the contaminated manure.

Among all investigated oxidation processes, the only treatment process, which was able to remove antibiotics completely from manure, was the heat activated persulfate oxidation under the applied experimental conditions. Therefore, it can be suggested as an effective process for the control of antibiotic pollution in manure within relatively short time period, which thereby prevented the development of antibiotic resistance and its transfer to the environment.

On the other hand, the high amount of waste generated in animal feeding operations obviously requires higher oxidant doses that can increase the cost of treatment operation. Therefore, in order to reduce the required oxidant dose for the treatment of contaminated animal waste, it can be suggested to segregate sick animals that are treated with antibiotics for therapeutic purposes, which may also provide the welfare of healthy animals.

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