

**The role of soil clays and clay-humic complexes in processes controlling
colloidal stability and the sorption and degradation of tetracyclines**

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Co-major: Soil Science (Soil Chemistry); Water Resources

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2005

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DEDICATION

I would like to dedicate this dissertation to my new family all of whom I ‘acquired’ during my Ph.D. program. First of all, I would like to express my deepest affection and gratitude to my husband, Terry Meade, for having the faith in me to finish what I started no matter how unmanageable life sometimes seemed to be. His endless support and sacrifice will not be forgotten. I would like to give a million kisses and hugs to our son Maximilian Pils who simply is the cutest, most adorable happy little boy with the strongest will and temper. The joy and significance he has brought into our lives puts all other obstacles into perspective. Last but not least, I would like to give bear hugs to my dogs, Sierra and Arber, for their love, loyalty, and peace that only animals can give you. Naturally, this dissertation is dedicated to my parents, Brigitte and Rainer Pils, and to my brother, Jochen Pils, for their mental support and their sacrifice to live without me for the past 13 years knowing that it was for the best. Finally, I would like to dedicate this dissertation to my sister, Isabella Walter, who could not live to celebrate my accomplishments with me.

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ACKNOWLEDGEMENTS

Sincere thanks to my advisor Dr. David A. Laird for his guidance throughout my Ph.D. program. His high expectations prepared me tremendously for a career in the professional world.

I thank my committee members, Dr. Dan B. Jaynes, Dr. Thomas E. Fenton, Dr. Robert Horton, Dr. Thomas E. Loynachan, and Dr. Mark D. Porter for their collaboration and service on my committee.

I thank Dr. Tom B. Moorman for his advice during my research. Much thanks to Mr. Pierce Fleming for all his technical and mental support. Our vivid political and scientific discussions will be missed. Lots of thanks to Ms. Beth Douglass for her technical support, her encouragement during the 'darker' times, and her friendship, which I hope will last a lifetime.

I thank Dr. Steve Fales and the Agronomy Department for their financial support.

Last but most importantly I would like to thank Dr. V. P. Evangelou who 'lured' me to Iowa. He had the faith that I could conquer all obstacles in life and complete my Ph.D. successfully. Without him, I would not have met the people I thanked above, all of whom had a tremendous impact on my professional and personal development.

ABSTRACT

The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory has been extensively used to explain dispersion and flocculation in colloidal systems, however little is known about the effect of crystalline swelling on colloidal behavior. The first topic addressed in the dissertation is the effect of monovalent and divalent cations on crystalline swelling and the breakup and formation of quasicrystals (QCs) and how these processes affect flocculation and dispersion of natural soil clay-humic complexes. The results indicate that high Ca^{2+} levels enhance the formation of large QCs, which readily flocculate and settle out of suspension. Increasing the concentration of Na^+ , K^+ , or NH_4^+ results in the breakup of large Ca-QCs, which enhances dispersion. In low ionic strength systems dispersion is caused by both expanded double layers (DLVO) between QCs and by the formation of small QCs. X-ray diffraction analysis of suspended colloids showed that monovalent cations reside primarily on the external surfaces and Ca^{2+} is preferentially retained in the interlayers. In high ionic strength systems increasing concentrations of monovalent cations also decreases the size of QCs but the effect is partially counteracted by compression of the double layers between QCs. X-ray diffraction analysis indicated that monovalent cations are sorbed on both the external surfaces and in the interlayers in high ionic strength systems.

The second part of the dissertation addresses the influence of soil smectites and humic substances and their interactions on sorption and desorption of tetracyclines. Tetracyclines are important in human medicine and are also used extensively in livestock production. Chlortetracycline (CTC) and tetracycline (TC) are routinely used for growth promoting and therapeutic purposes in livestock production. To elucidate the environmental fate of these pharmaceuticals, sorption isotherms were obtained using dilute CaCl_2 or KCl background solutions at different pHs for clays, humic substances (HS), and clay-humic complexes (clay-HC). In all systems, the soil components sorbed > 96% of added tetracyclines. Strongest sorption was observed for clays, followed by HS, and clay-HC adsorbed the least

tetracyclines. Sorption isotherms indicate greater sorption in Ca-systems than K-systems and that CTC is more strongly sorbed than TC. Increasing the pH of the Ca-clay-HC from 5.8 to 7.0 decreased sorption of both CTC and TC. The greater sorption in the Ca-systems and the decreased sorption with increasing pH suggest that charge neutralization and cation bridging contribute to sorption. Furthermore, x-ray diffraction analyses showed that TC and CTC are sorbed in the interlayers of smectites. Desorption studies at pH 7 showed little CTC desorption and only slightly higher desorption for TC, which decreased in the following order: clay-HC > HS > clay. The results indicate that tetracyclines are dominantly sorbed on soil clays and that HS compete with tetracyclines for sorption sites on the clay surfaces.

The third topic addressed in the dissertation is degradation and bioavailability of TC when it is adsorbed to whole soils (WS), soil clay-HC, and soil clays. Specifically studied was the degradation of TC in both aqueous and colloidal systems, the antimicrobial activity of sorbed TC, whether sorbed TC is a long-term reservoir for slow TC release, and the ability of TC resistant bacteria to degrade sorbed TC. Test plate studies indicated that sorbed TC inhibits soil bacteria, however, most likely due to slow TC release from soil components. Tetracycline in the aqueous phase degraded faster for the biotic systems than abiotic systems and in decreasing order for colloidal systems containing soil clay > soil clay-HC > WS. Biotic and abiotic degradation results using ^3H -TC showed increasing tritium and decreasing TC concentrations in the aqueous phase of the colloidal system during a 149-day incubation period. The results indicate that a pool of loosely sorbed (labile) TC (< 16% of added TC) is available for both biotic and abiotic degradation. The labile TC appeared to be associated primarily with the clay fraction as it increases with increasing clay content: clay > clay-HC > WS. Most of the adsorbed TC (84 to 92% of added TC) was retained by the soil and soil components in a form that was not released during the 149-day incubation. This stable pool of adsorbed TC appeared to be primarily associated with the soil organic matter and humic substances.

CHAPTER 1. GENERAL INTRODUCTION – A REVIEW

Introduction

Midwestern soils in the U.S. are predominantly Mollisols characterized by high accumulation and decomposition of soil organic matter (SOM) in the surface horizon and highly active smectitic clays. Mollisols exhibit a mollic epipedon, which is dark in color, humus-rich, relatively fertile, and 40 to 75 cm thick. Additional factors that are associated with the accumulation of organic matter in Mollisols are relatively larger base saturation (> 50%), cation exchange capacity, and water holding capacity. Soil organic matter and clay content have a large impact on the biological, physical, and chemical soil properties.

Soil Organic Matter

Soil organic matter is composed of living organisms (soil biomass), partially decayed plant and animal tissues, and soil humic substances. Partially decomposed plant and animal tissues are the non-humic substances and are composed of enzymes, organic acids, polysaccharides, carbohydrates, proteins, lignins, lipids, pigments, resins, and tannins (Theng, 1979). Humic matter is formed through the chemical and biological humification of plant and animal matter and through the biological activities of micro organisms, and represents the major portion of soil organic matter (Stevenson, 1994; Schnitzer and Schulten 1998). Humic substances are a complex assemblage of organic functional groups, commonly assumed to have an aromatic backbone with many aliphatic substituents (Susic, 2003). A definite structure of humic acids has not been established as the structures of humic substances are highly heterogeneous and may be influenced by the environment in which they form.

Clay

Many Midwestern soils in the U.S. are dominated by smectites, 2:1 phyllosilicates whose individual layers are composed of two Si-O tetrahedral and one Al-O octahedral sheets. In the tetrahedral sheet, four oxygen atoms are coordinated around one Si^{4+} cation, while six oxygens or hydroxyls are coordinated around an Al^{2+} in the octahedral sheet (Dixon and Weed, 1989). The major differentiating characteristic among 2:1 layer silicates is the layer charge, which is a result of isomorphous substitution. Aluminum (Al^{3+}) may substitute for Si^{4+} in the tetrahedral sheet, and Mg^{2+} is most likely to substitute for Al^{3+} in the octahedral sheet. The degree of substitution determines the overall charge of the phyllosilicates. For example, in the case of vermiculite most of the coordinating-cation substitution takes place in the tetrahedral sheet resulting in a layer charge of -0.6 to -0.9 per formula unit (Theng, 1979). In smectites, the layer charge is -0.25 to -0.6 per formula unit (Theng, 1979) and the coordinating-cation substitutions take place mostly in the octahedral sheet.

Layer charge is one of the most significant properties of 2:1 layer silicates. The layer charge affects the mineral's interlayer expansion. For many smectites, octahedral substitution results in a diffuse surface charge which weakens the electrostatic interaction between exchangeable cations and the basal oxygens, leading to a relatively large interlayer expansion and thus influencing the colloidal stability properties of such minerals.

Soil Clay-Humic Complexes

Clays and humic substances most commonly exist in natural soils as clay-humic complexes. Most humic substances in soils are bound to the surface of clay minerals. It has been found that humic acids can solubilize 10 times their own weight of clay particles (Susic, 2003). Based on Theng (1979), there are several bonding mechanisms between the clay mineral surfaces and humic substances. The most important interactions between these two

soil components are complexation reactions, anion and ligand exchange, and interlayer sorption. Secondary mechanisms involve hydrogen bonding and van der Waals interactions. An indirect interaction between clays and humic substances is the entropy effect that arises from the displacement of surface water molecules by a hydrophobic organic moiety.

Polyvalent cations commonly bridge between negative charge sites of clay surfaces and negatively charged organic functional groups on humic substances. The main polyvalent cations responsible for the binding of humic and fulvic acids to soil clays are Ca^{2+} , Fe^{3+} and Al^{3+} . Trivalent cations, Fe^{3+} and Al^{3+} , form much stronger coordination complexes with organic molecules than divalent Ca^{2+} ions. Humic substances are also bound to clay surfaces by Fe- and Al-hydroxides. When clay minerals are coated with hydrous oxides their surface reactions are dominated by these hydrous oxides, which have variable charged surfaces. Under acidic conditions, surfaces of hydrous oxides may become positively charged. Organic anions can be associated with the oxides by simple coulombic attraction. Coordination or ligand exchange may also occur when the anionic group penetrates the coordination shell of aluminum or iron and becomes incorporated with the surface OH layer. The sorption of humic substances on oxide surfaces is accompanied by displacement of OH groups by COO^- ions. The organic ligands are not easily displaced with simple salts, although adsorption is pH sensitive. As was the case with organic cations on clay mineral surfaces, a very strong bond will result if more than one group on the humic molecule participates.

Another important mechanism for retention of proteins and charged organic cations by expandable-layer silicates is through adsorption into interlamellar spaces, yet considerable controversy exists as to whether humic substances are bound this way in natural soils. Schnitzer and Kodama (1967) and Theng (1976) presented evidence for interlamellar adsorption of fulvic acid by montmorillonite at $\text{pH} < 5.5$ and humic complexes have been found to diffuse into the interlayers of 2:1 phyllosilicates (Kulshrestha et al., 2004).

Polar groups of the organic molecules can form H-bonds with adsorbed water molecules or with the oxygens of the silicate surfaces. The strength of a single H-bond is small, but many such interactions may occur and the bond energy is additive, thus total adsorption energy can be appreciable. The sorption of neutral polar and nonpolar molecules with high molecular weight is predominantly influenced by van der Waals forces (Stevenson, 1994).

Flocculation and Dispersion

Clay-humic complexes are subject to dispersion and flocculation phenomena which govern many soil-water processes, including soil crusting (Goldberg and Forster, 1990), infiltration rate (Oster and Schroer, 1979) erosion, hydraulic conductivity, solute transport, soil permeability (Quirk and Shofield, 1955; Gardner et al., 1959; McNeal and Coleman, 1966; Velasco-Molina et al., 1971; Shainberg and Caiserman, 1971; Quirk, 1986; Curtin et al., 1994; Mace and Amrhein, 2001), pollutant transport (McCarthy and Zachara, 1989; Mills et al., 1991), and loss of nutrients (Keren and Bingham, 1985; Goldberg et al., 1993; Gillingham and Thorrold, 2000). Off-site transport of soil colloids (McDowell and Wilcock, 2004) is the major cause of water quality degradation due to suspended solids in lakes and rivers. The transport of nutrient-rich sediments is a key contributor to eutrophication and hypoxia (Turner and Rabalais, 1991; Brunet and Brian-Astin, 1998; Bollinger et al., 2000), and often results in the degradation of aquatic wildlife habitat in streams, rivers, lakes, and reservoirs (Sabo et al., 1999; Aday et al., 2000; Fontenot et al., 2001).

Flocculation and dispersion of soil colloids are complex phenomena and are influenced by the type and amount of clay, its mineralogy (Velasco-Molina et al., 1971; Frenkel et al., 1978; Oster et al., 1980; Das and Datta, 1987; Goldberg et al., 1991), pH (Suarez et al., 1984; Hesterberg and Page, 1990; Goldberg et al., 1991), solution composition (McNeal et al., 1966) organic matter (Shanmuganathan and Oades, 1983; Gupta et al., 1984),

and ionic strength (Gardner et al., 1959; Rowell et al., 1969; Arora and Coleman, 1979; Goldberg et al., 1993; Cione et al., 2000). Ion adsorption on external surfaces and in clay interlayers (Shainberg et al., 1980; Oster et al., 1980; Laird and Shang, 1997), as well as the presence of metal (hydr)oxides, also influence flocculation and dispersion (Goldberg and Glaubig, 1987; Goldberg, 1989). Quirk and Schofield (1955) suggested that the monovalent to divalent cation ratio affects dispersion and flocculation behavior and used the monovalent to divalent cation concentration ratio ($CR_X = [X^+]/[Ca^{2+}]^{1/2}$, where X = Na⁺, K⁺, or NH₄⁺) to describe the solution composition.

McCarthy and Zachara (1989) found that colloids moving through the solid phase enhance the transport of strongly sorbing contaminants. Several column studies have revealed that colloid mobility promotes transport of contaminants in the vadose zone (Vinten et al., 1983; Grolimund et al., 1996; Flury et al., 2002). Other studies have shown that pesticides and heavy metals undergo colloid-facilitated transport (Amrhein et al., 1993; Seta and Karathanasis, 1997; Karathanasis, 1999; Villholth et al., 2000). These studies indicated that compounds strongly sorbed to soil components are subject to colloidal facilitated transport. Although much information has been revealed in recent years concerning pesticide and heavy metal behavior in environmental settings, there is little understanding of fate of antibiotics in soil-water systems.

Introduction to Tetracyclines

Antibiotics, such as tetracycline, penicillin, and erythromycin, are important in human medicine and are also used extensively in livestock production. Healthy cattle, swine, and poultry are routinely given antibiotics throughout their lives to promote growth. Mellon et al. (2001) estimated that antibiotic use for healthy livestock grew from 16.1 million pounds in the mid-1980s to 24.6 million pounds in 2001. Much of the increase can be attributed to poultry production where non-therapeutic antibiotic use increased from 2 million to 10.5

million pounds during that period. Even after considering industrial growth, that increase equates to greater than a 300 percent increase in the amount of antibiotics used per bird.

A wide range of antibiotics have been, and continue to be, introduced to soil through treated and untreated animal waste. Studies have indicated that 80 to 90% of many antibiotics administered to livestock are excreted as the parent compounds or its metabolites in urine and feces (Kroger, 1983; Halling-Sørensen et al., 1998; Jjemba, 2001). Chlortetracycline (CTC) levels reaching 66 mg L⁻¹ in animal manure have been widely documented (Hirsch et al., 1999; Lee et al., 2000; Winckler and Grafe, 2000). Tetracycline (TC) and CTC are excreted with intact antimicrobial activity (Halling-Sørensen, 2000). Soils receiving frequent manure application showed tetracycline concentration of 12 µg kg⁻¹ (Hamscher et al., 2000) to 100 µg kg⁻¹ (Klages and Roth, 2001).

Anthropogenic TET Input

In the US, approximately 50 million pounds of antibiotics are produced annually of which 50% are used by humans and 50% are used in agricultural applications (Levy, 2002). Agricultural applications between 15.4 and 27.6 million pounds per year, mainly penicillins and TET, are used as growth promoters in animal feed, and 99,000 pounds per year of tetracyclines and streptomycines are used as pesticides in orchards (Mellon et al., 2001; Levy, 2002). By contrast, the Animal Health Institute (AHI) (an organization representing 80% of the companies that produce antimicrobial agents for animals in the United States, (Anderson et al., 2003)), reported that only 3.1 million pounds per year of antibiotics (6%) are used as non-therapeutic growth promoters, 14.7 million pounds per year (29.5%) are used for therapeutic and prevention purposes in agriculture and that human use accounts for 32.2 million pounds per year (64.5%) (AHI, 2000).

Natural Production of Tetracyclines

Antibiotics are chemical compounds that are synthesized through a secondary metabolism of living organisms (Thiele-Bruhn, 2003). Tetracyclines are a diverse family of compounds produced primarily by the sequential enzymatic synthesis and biotransformation of polyketide precursors (Nelson et al., 2001), or by various strains of *Streptomyces*. Additionally, TET are biosynthesized by chain cyclization obtained through condensation of acetate and malonate units (Lancini et al., 1995). The most studied bacteria are within the order Actinomycetales, where *Streptomyces aureofaciens* and *Streptomyces rimosus* species, as well as many other soil actinomycetes, have been found to produce structural variants of the TET (Nelson et al., 2001). Natural tetracyclines can be obtained by other antibiotic producers of the Actinomycetales order, whose members number over 3000 distinct species in over 40 genera (Nelson et al., 2001).

Chemistry of Tetracyclines

Tetracyclines are a family of antibiotics that represent a very broad spectrum of action with great therapeutic effectiveness. Tetracyclines are named according to International Union of Applied Chemistry (IUPAC) convention based on the positioning of substituents along the naphthacene ring. Thus, tetracycline is named by convention 4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide and chlortetracycline is named 7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide.

The chemical structure of TC is shown in Figure 1-1. The major part of tetracycline consists of four linearly fused rings. The rings are labeled ABCD starting from the right ring. Carbons of the naphthacene rings are numbered starting at C1 on the A-ring moving

counterclockwise. The “bridgehead tertiary carbon atoms” are designated by a number and a letter (Nelson, 2001).

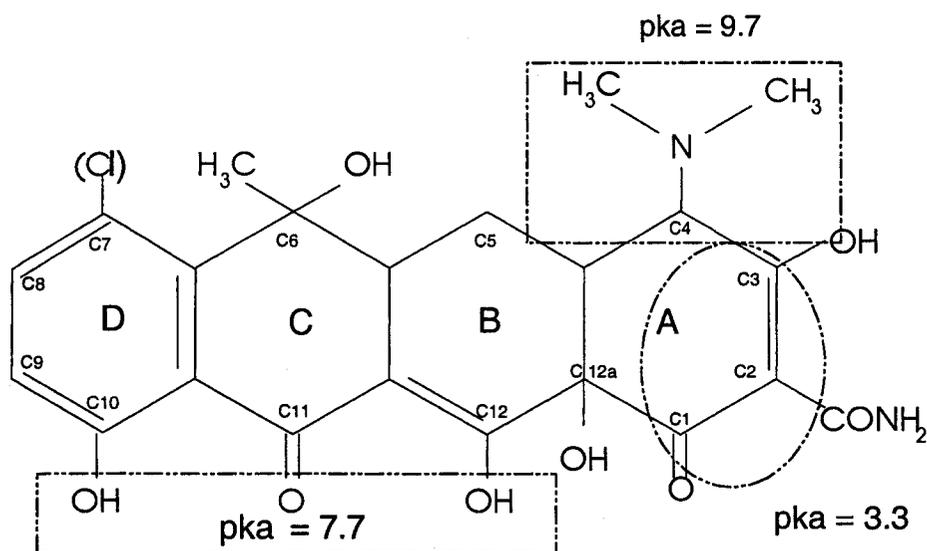


Figure 1-1. Functional groups of tetracycline and chlortetracycline (Cl).

Carbon 1 possesses a carbonyl group, C2 an exocyclic carboxamide group, and C3 a keto-enol functional group (Nelson, 2001). The structure, including C1-C3, is called tricarboxylmethane keto-enol system with a pK_a of 2.8-3.3 (Stephens et al., 1956). The term “tricarboxylamide” can also be used instead of tricarboxylmethane keto-enol system to define the strong coupling of the C1, C2, and C3 complex rather than considering this group as an amide and two independent carbonyls (Myers et al., 1983). The C4 dimethylamino has a weak pK_a of approximately 9.7 (Hochstein et al., 1953; Stephens et al., 1956). The lower peripheral part shows a keto-enol sub-structure including C10, C11, and C12. This region has a pK_a value of 7.7 (Stephens et al., 1956; Nelson et al., 2001). The addition of six oxygen functional groups at C10, C11, C12, C12a, C1 and C3 and the addition of the dimethylamino group to C4 represent the simplest tetracycline molecule with antimicrobial activity.

Chlortetracycline is formed by Cl substitution at the C7 position. The ionization of CTC is very similar to TC; $pK_{\alpha 1} = 3.3$, $pK_{\alpha 2} = 7.4$, and $pK_{\alpha 3} = 9.3$. Table 1-1 shows the charge distribution of the tetracycline compound at various pK_{α} ranges. At a pH below 3.3, tetracycline exhibits an overall positive charge, whereas between pH 3.3 and 7.7 the compound is a zwitterion. Between pH 7.4 and 9.7, tetracycline has one negative charge and above pH 9.7 it exhibits two negative charges.

Table 1-1. Overall charge of TET as a function of pH.

	pH			
<i>Functional Groups</i>	<i>< 3.3</i>	<i>> 3.3 < 7.7</i>	<i>> 7.4 < 9.7</i>	<i>> 9.7</i>
C3-OH	0	-	-	-
C11-O and C12-OH	0	0	-	-
C4-NH ⁺ (CH ₃) ₂	+	+	+	0

Antimicrobial Activity and Development of Tetracycline Resistant Bacteria

Pharmaceutical antibiotics can affect the microbial population of the soil (Ingham and Coleman, 1984; McCracken and Foster, 1993). Antibiotic residue and resistant pathogens affect soil microbial populations and may harm humans and livestock through the food chain (Richter et al., 1996), but most importantly, infections that are caused by resistant bacteria decrease antibiotic efficiency (Richter et al., 1996).

The antimicrobial activity of TET is mainly dependent on functional groups located C10, C11, C12, C12a, C1, C3 and C4 and any changes to these functional groups decreases bioactivity. However, C5, C6, C7, C8, C9, and C2 do not impact antimicrobial activity (Nelson, 2001) and therefore can be modified.

The activity spectrum of TET is particularly broad and is effective against gram-positive and gram-negative bacteria, rickettsiae, chlamydiae, and some protozoa (Lancini et al., 1995). Tetracyclines inhibit the activity of eukaryotic cells, microorganisms, and viruses, and affect the ribosomal binding mechanisms in microbes (Lancini and Parenti, 1982). On a molecular level, the antibiotic molecule is able to bind to a specific site on the target macromolecule forming a molecular complex, which is no longer able to accomplish its original function (Nelson et al., 2001). Inhibiting the action of a macromolecule, such as an enzyme or a nucleic acid which are important for cell multiplication, inhibits the growth of sensitive microorganisms. Specifically, tetracycline inhibits bacterial protein synthesis by preventing aminoacyl-tRNA binding to the A-side of the 30S ribosomal subunit (Chopra et al., 1992). This effect is reversible and, therefore, the family of TET represents bacteriostatic agents.

However, bacteria may be naturally resistant or acquire resistance to TET. Three specific mechanisms of tetracycline resistance have been identified so far: i) TET efflux, ii) ribosome protection, and iii) TET modifications. The first mechanism was observed for Gram-negative bacteria (McMurry et al., 1980) and Gram-positive bacteria that are able to resist TET uptake into their cells, which is achieved by an export protein. The second mechanism was found by Burdett (1996) and involves the production of ribosomal protection proteins. The third mechanism involves a cytoplasmic protein that chemically modifies tetracycline and is linked to direct or indirect transfer of plasmids from non-pathogens to pathogenic microorganisms (Wegener et al., 1998). Tetracycline resistance due to these mechanisms was found in over 20 resistance-determinant classes that represent a different transferable genetic determinant (Levy et al., 1989).

Application of antibiotics to soils has been shown to increase microbial resistance (Levy, 1998; Sengeløv et al., 2003; Witte, 1998) for weeks (Fründ et al., 2000). Huysman et al. (1993) found that TC resistant clostridia significantly accumulated in manure, manure

amended soils, and in ground water. Based on Levy (1998), the frequency of resistance in bacteria and the number of drugs to which they are resistant are increasing. Also microbial resistance to unrelated drugs has been detected when microbes were exposed to tetracycline (Levy, 1982). The core structure of 20 year old antibiotics is very similar to the core structure in newer antibiotics designed for human use (Levy, 1998). This similarity has created antimicrobial resistance of bacteria to antibiotics commonly used in humans (van den Bogarrd et al., 1997). Thiele and Beck (2001) found that even small extractable oxytetracycline (OTC) concentrations produce longer lasting significant effects of the natural microbial population. More important than the development of resistant bacteria in soils is the input of already resistant microorganisms through manure applications (Ali-Shtayeh et al., 1998). Several studies have shown high resistance of microbes against various antibiotics in pig manure (Langlois et al., 1978; van den Bogarrd et al., 2000).

Fate of Tetracyclines in Soil Environment

Sorption

Tetracyclines (TET) are regularly applied to soils, have low mobility within the soil, and are strongly retained by soil components (Pinck et al., 1961; Warman and Thomas, 1981; Sithole and Guy, 1987a,b; Lundestad and Goksøyr, 1990; Stuer-Lauridsen et al., 2000; Rabølle and Spliid 2000; Loke et al., 2002). Sorption of TET in soils highly depends on pH (Holten-Lützhøft et al., 2000; Sithole and Guy, 1987b), ionic strength (Sithole and Guy, 1987a,b), soil organic matter (Langhammer, 1989; Gruber et al., 1990) and soil minerals (Batchelder, 1982). Three sorption mechanisms are thought to control TET retention in the soil environment; ion exchange, complexation by divalent cations, and hydrogen bridging from acidic groups of humic acids to polar groups of the TET (Sithole and Guy, 1987a,b).

The TET interaction with pure clay surfaces is complex and depends on the charge distribution (Table 1-1). At low pH values, the positively charged dimethylammonium group

(C4) interacts with the clay surface by ion exchange. Tetracycline species under environmental pH condition (4 to 8) are zwitterionic in character with increasing negative charge above pH 6. The location of the cationic and anionic sites on the zwitterion is such that ion exchange reaction with clay surfaces is not favored above pH 3.3. All TET can strongly chelate with metal ions, which dramatically influences their antimicrobial properties, at C1, C2, C3, C11, and C12 functional group locations (Blackwood, 1985; Chopra et al., 1992). Sithole and Guy (1987a) found that TET form chelate complexes with divalent metal ions in the clay's double layers and with Al^{3+} ions exposed at the edges of the clay surface. Tetracycline sorption on clay minerals has been studied by Pinck et al. (1961a) and Bewick (1979) who found that TC sorbed stronger to expandable layer clay minerals than to illite or kaolinite. Pinck et al. (1962) first described interlayer sorption of various antibiotics. Kulshrestha et al. (2004) found OTC to sorb within the interlayer of montmorillonites (MM) in a tilted orientation, which was further described by Browne et al. (1980) and Porubcan et al. (1978). To a small extent, functional groups of TC form hydrogen bonds with the silanol groups of the clay or with polar groups of adsorbed organic matter. The acid/base characteristics of the clay surface and tetracycline molecule are such that decreased adsorption occurs in alkaline systems.

The formation of divalent cation complexes is one of the strongest TET-MM sorption mechanisms and sorption can vary greatly between Na- and Ca-MM (Browne et al., 1980). For example, OTC is sorbed to Ca-clay 2.5 times greater than to Na-clays (Sithole and Guy, 1987a). This can be attributed to the formation of reversible complexes with multivalent cations (Wessels et al., 1998). At higher pH values, TET sorb to Ca-MM at the C11 and C12. However as the pH decreases, the dimethylamino group (C4) becomes increasingly involved in TET sorption (Loke et al., 2002). At low pH, the cationic form of TC strongly interacts with the Na-saturated MM whereas the zwitterionic form weakly sorbs to Na-MM.

The strong sorption of TET onto dissolved organic matter was attributed to ionic interactions and hydrogen bond formations (Tolls, 2001). Tetracyclines bind strongly to soil proteins (Oka et al., 2000) and humic acids via anionic functional groups (Loke et al., 2002). Oxytetracycline sorption to the organic soil fraction increased with increasing aromaticity (Suan and Dmitrenko, 1994a). Sorption of TC onto soil organic matter such as peat or humic substances were studied by Sithole and Guy (1987b), who found similar sorption capacities due to the interaction of phenolic and carboxyl group with the polar groups on TC.

Tetracycline sorption onto soils can be further differentiated into two mechanisms with very different kinetics. Fast initial adsorption to the surface and the slower diffusion into interlayers of clay minerals and micropores has been studied by Sithole and Guy (1987b) and Suan and Dmitrenko (1994b). The antibiotic activity of TET decreases upon sorption (Ingerslev and Halling-Sørensen, 2000) to functional groups such as C11, C12, C1, and C4. However, antimicrobial activities are not necessarily eliminated upon TET sorption (Halling-Sørensen, et al., 2003). The antibiotic potency returns once TET are desorbed from soil components as sorption is a reversible process (Samuelsen et al., 1994; Halling-Sørensen et al., 2002).

Degradation

Over the past decade, increasing concern about the development of microbial resistant bacteria has raised questions about the fate and bioavailability of antibiotics in natural soils-water systems. The persistence of antibiotics in the terrestrial environment ranges from less than one day to weeks or even months and is controlled by factors such as temperature, chemical structure of the antibiotic (Gavalchin and Katz, 1994), sensitivity to light (Burhenne et al., 1997; Halling-Sørensen et al., 2003), presence of oxygen, and microbes present. The two major abiotic degradation pathways appear to be photolysis and hydrolysis. Oxytetracycline was photodegraded within 21 days in seawater at a depth of 1 m (Lunestad

et al., 1995). Yet, Thiele-Bruhn (2003) found little evidence of antibiotic photodegradation in land applied sludge and slurry samples. Recent studies have shown that hydrolysis can also transform tetracyclines in soil environments, but work remains to determine the potential of this transformation process (Halling-Sørensen, 2000; Thiele-Bruhn, 2003).

In what is perhaps a strange twist of fate, the best approach for removing antibiotics from the environment may come from the very thing for which they were introduced, microorganisms. Some microbes are able to utilize recalcitrant antibiotics in the environment for energy and are capable of breaking down the antibiotic structures through degradation mechanisms involving enzymatic transformation reactions such as oxidative decarboxylation and hydroxylation (Chen et al., 1997; McGrath et al., 1998). Tetracycline biodegradation up to 100% removal has been demonstrated in a number of manures and manure-amended soils (Galvalchin and Katz, 1994; Jagnow, 1977; Kühne et al., 2000; Runsey et al., 1977; Winckler and Grafe, 2001).

However, antibiotic biodegradation is not without its potential problems. For example, there was little or no biodegradation of TC in a wide range of contaminated soils without the aid of manure amendment (Hamscher et al., 2001; van Gool, 1993). Samuelsen et al. (1994) and Gavalchin and Katz (1994) determined that biodegradation was not effective when antibiotic compounds were fixed to surfaces or in the soil matrix where they can persist for months (van Gool, 1993; Höper et al., 2002). The same has been found in aquatic sediments where TET was only slightly degraded over a period of 180 days (Samuelsen et al., 1994; Hektoen et al., 1995).

One of the major concerns of TET in natural environments is the formation of often toxic degradation products such as anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC) (Kühne et al., 2001). Anhydrotetracycline is formed when $\text{pH} < 7.5$ (Schneider, 2001) and has several deleterious biological effects. In vivo they are phototoxic and hepatotoxic and may cause the development of anemias (Breen et al., 1972; Jones, 1973).

When the soil pH > 7.5, TC is metabolized to iso-tetracyclines (Kühne et al., 2000) and will be rapidly decomposed into smaller fragments (Hlavka and Boothe, 1985). The formation of 4-epitetracycline occurs between pH 3 and 6 and is a reversible process.

Antimicrobial resistant bacteria are a public health problem of growing urgency. Patients once effectively treated for pneumonia, tuberculosis, or ear infections may now have to try three or more antibiotics before they find one that works. As more bacterial strains develop resistance, more people may die because effective antibiotics are not identified quickly enough or because the bacteria causing the disease are resistant to all available antibiotics.

Hypothesis and Objectives

The overall hypotheses of Chapters 2, 3, and 4 are:

- 1) Flocculation and dispersion behavior of smectitic soil colloids as a result of changing CR_X is controlled by two processes; i) osmotic swelling, explained by DLVO theory and ii) the formation and breakup of QC.
- 2) Ionic functional groups of tetracycline and chlortetracycline physically and chemically interact with soil clay minerals and humic substances, thus impacting their fate in soil environments.
- 3) Sorption of tetracycline onto soil components regulates aerobic biotic and abiotic degradation.

The overall objectives of Chapters 2, 3, and 4 are:

- 1) To experimentally i) quantify the influence of CR_{Na} , CR_K , and CR_{NH4} on dispersion of smectitic soil colloids for two ionic strengths, and ii) evaluate interactions between crystalline swelling, cation demixing, the breakup of QC and dispersion.

- 2) To elucidate sorption and desorption behavior of tetracycline and chlortetracycline on K- and Ca-saturated soil clay, humic substances, and clay-humic complexes in dilute aqueous systems at various pH values.
- 2) To evaluate biotic and abiotic degradation of TC sorbed onto whole soils, clays, and clay-humic complexes under aerobic conditions.

Dissertation Organization

This dissertation is organized into 5 chapters and 1 appendix. Chapter 1 contains a general introduction for the main part of the dissertation including a description of soil components and soil clay-humic complexes, flocculation and dispersion processes, the chemistry and biochemistry of TET, and the fate of TET in soil environments. Chapters 2, 3, and 4 were prepared in a format publishable in a scientific journal. Chapter 2 deals with the formation of smectite tactoids as induced by confined and double layer monovalent cations. This research was initiated when working with Dr. Evangelou, Professor of Soil Environmental Chemistry and Mineralogy. After Dr. Evangelou's death in 2002, the work was continued with Dr. Laird. Thereafter the focus of research shifted to elucidating tetracycline interactions in the soil environment. Chapter 3 investigates sorption and desorption of tetracycline and chlortetracycline on K- and Ca-saturated soil clays, humic substances, and soil clay-humic complexes. Chapter 4 presents data on the degradation of tetracycline from Ca-saturated whole soils, soil clays, and soil humic substances. Chapter 5 presents the general conclusions for Chapters 2, 3, and 4.

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CHAPTER 2. ROLE OF CATION DEMIXING AND QUASICRYSTAL FORMATION AND BREAKUP ON THE STABILITY OF SMECTITIC COLLOIDS

A paper to be submitted in Clays and Clay Minerals

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Abstract

The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory has been extensively used to explain dispersed systems, however little is known about the effect of crystalline swelling on colloidal behavior. This study investigated the effect of demixing of monovalent and divalent cations on crystalline swelling and the breakup and formation of quasicrystals (QCs) and how these processes affect flocculation and dispersion of natural soil clay-humic complexes. The results indicated that in a Ca-dominated system the formation of large QCs enhanced flocculation and that increasing the concentration of Na⁺, K⁺, or NH₄⁺ resulted in the breakup of large Ca-QCs, which enhanced dispersion. In low ionic strength systems dispersion was caused by expanded double layers (DLVO) and the formation of small QCs. X-ray diffraction analyses showed that as large Ca-QCs break up, monovalent cations resided primarily on the external surfaces and Ca²⁺ was preferentially retained in the interlayers. In high ionic strength systems increasing concentrations of monovalent cations also decreased the size of QCs but the effect was partially counteracted by compression of double layers between QCs. X-ray diffraction analyses indicated that monovalent cations were sorbed on both the external surfaces and in the interlayers in high ionic strength systems.

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Introduction

Soil dispersion and flocculation phenomena govern many soil-water processes including soil crusting (Goldberg and Forster, 1990), infiltration rate (Oster and Schroer, 1979) erosion, hydraulic conductivity, solute transport, soil permeability (Quirk and Shofield, 1955; Gardner et al., 1959; McNeal et al., 1966; Velasco-Molina et al., 1971; Shainberg and Caiserman, 1971; Quirk, 1986; Curtin et al., 1994; Mace and Amrhein, 2001), pollutant transport (McCarthy and Zachara, 1989; Mills, et al., 1991), and loss of nutrients (Keren and Bingham, 1985; Goldberg et al., 1993; Gillingham and Thorrold, 2000). Off site transport of soil colloids (McDowell and Wilcock, 2004) is the major cause of water quality degradation due to suspended solids in lakes and rivers. The transport of nutrient-rich sediments is a key contributor to eutrophication and hypoxia (Turner and Rabalais, 1991; Brunet and Brian-Astin, 1998; Bollinger et al., 2000), and often results in the degradation of aquatic wildlife habitat in streams, rivers, lakes, and reservoirs (Sabo et al., 1999; Aday et al., 2000; Fontenot et al., 2001).

Flocculation and dispersion of soil colloids are complex phenomena and are influenced by the type and amount of clay, its mineralogy (Velasco-Molina et al., 1971; Frenkel et al., 1978; Oster et al., 1980; Das and Datta, 1987; Goldberg et al., 1991;), pH (Suarez et al., 1984; Hesterberg and Page, 1990; Goldberg et al., 1991), solution composition (McNeal et al., 1966) organic matter (Shanmuganathan and Oades, 1983; Gupta et al., 1984), and ionic strength (Gardner et al., 1959; Rowell et al., 1969; Arora and Coleman, 1979; Goldberg et al., 1993, Cione et al., 2000). Ion adsorption on external surfaces and in the interlayers of clays (Oster et al., 1980; Shainberg et al., 1980; Laird and Shang, 1997) as well as the presence of metal (hydr)oxides influence flocculation and dispersion (Goldberg and Glaubig, 1987; Goldberg, 1989). Goldberg (1989) has shown that aluminum and iron oxides decrease clay dispersion, clay swelling, and critical coagulation concentrations. Exchange

sites of metal hydroxides are highly reactive and interact with carboxylic groups and phenolic-OH groups of humic substances and therefore contribute to large flocc formation (Hayes and Swift, 1990). Furthermore, water stable aggregates are formed when humic substances interact with metal ions, hydroxides, oxides, and minerals (Schnitzer, 1989).

Clay mineralogy plays a critical role in flocculation and dispersion processes. Many Midwestern soils in the U.S. are dominated by smectites, expanding 2:1 phyllosilicates whose layers are comprised of two Si-O tetrahedral and one Al-O octahedral sheets. Smectites have a layer charge of -0.25 to -0.6 per formula unit, which is one of the most significant properties of 2:1 layer silicates (Theng, 1979). Because cation substitution in smectites takes place furthest from the interlayer spacing where most exchangeable cations reside, the electrostatic interaction between exchangeable cations and the two tetrahedral sheets is rather weak. Subsequently, this weak interaction can lead to a relatively large interlayer expansion, which influences the colloidal stability properties of clay minerals.

Smectites are arranged as quasicrystals (QCs), which are sets of 2:1 phyllosilicate layers oriented parallel to each other. Expansion of these layers, crystalline swelling, is a process that occurs within QCs and can be described as the stepwise intercalation of 0 to 4 layers of water molecules between 2:1 phyllosilicate layers (Norrish, 1954). In the process each water layer adds about 0.25 nm to the basal spacings. Attractive van der Waals- and Coloumb and repulsive hydration forces regulate crystalline swelling and are in turn influenced by the interlayer cations' valence and hydration energy, and the clay's negative surface charge (Norrish, 1954; Parker, 1986; Laird, 1996). Laird (1996) describes a macroscopic energy balance model for crystalline swelling of phyllosilicates involving the interaction between cations, solution, and clay surfaces in an open system.

Smectite QCs are dynamic, several small QCs may join together forming one large QC and conversely large QCs may break up forming several smaller QCs. The average number of layers within a smectite QC in an aqueous solution is highly dependent on the

solution's ionic strength and the valence of the interlayer cations. At low electrolyte concentrations large QCs with up to 9 clay platelets develop when divalent cations bridge the smectite layers (Blackmore and Miller, 1961). In contrast, Na-smectite in a dilute suspension may exist as individual platelets (van Olphen, 1977).

The classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948) is commonly used to explain flocculation and dispersion of colloidal systems due to ionic strength effects on the diffuse double layers between colloids. In short, high ionic strength systems compress the double layer and force individual QCs much closer together leading to flocculation. However, in low ionic strength systems, double layer expansion promotes dispersion by increasing the distance between individual QCs. The dispersion of QCs depends on the thickness of the electrical double layer, which is influenced by clay mineralogy, type of exchangeable ions, and electrolyte concentration (Sumner, 1993). The thickness of the double layer itself increases with decreasing exchangeable ion charge, decreasing ionic strength, and increasing ion hydration (Saejiew et al., 2004). These factors also regulate QC settling behavior by controlling the distance between individual QCs.

Monovalent to divalent cation ratio in solution is known to affect clay flocculation and dispersion behavior. Increasing Na^+ concentrations in $\text{Na}^+/\text{Ca}^{2+}$ and $\text{Na}^+/\text{Mg}^{2+}$ systems has been repeatedly shown to disperse smectites and smectitic soils in suspension. Similarly, Bar-On et al. (1970) determined that adding Na^+ to a Ca^{2+} dominated system resulted in considerable increase in electrophoretic mobility of Ca-montmorillonite and the onset of destruction of large QC. Although the effect of the $\text{Na}^+/\text{Ca}^{2+}$ ratio on flocculation and dispersion has been studied extensively, little is understood about the monovalent to divalent cation ratio effect in other systems such as $\text{K}^+/\text{Ca}^{2+}$ and $\text{NH}_4^+/\text{Ca}^{2+}$.

The presence of exchangeable monovalent and divalent cations in distinct regions of clay structures represents the demixing effect, which can affect QC formation and destruction

(Fink et al., 1971). Demixing may occur as monovalent and divalent cations occupying different regions of the same clay interlayer or as separate layers dominated by monovalent and divalent cations. For Na^+ - Ca^{2+} montmorillonite at low electrolyte concentration, Shainberg et al. (1980) and Oster et al. (1980) concluded that Na^+ is preferred on external low electrical potential surfaces of the clay and Ca^{2+} is preferred in the internal surfaces. However, as ionic strength increases so does the affinity for the Na^+ by the high electrical potential internal surfaces. Complex feedback interactions between crystalline swelling and cation exchange selectivity provide a mechanistic explanation for the demixing phenomenon (Laird and Shang, 1997).

Double layer swelling has been widely used to explain colloidal behavior; however the influence of crystalline swelling on colloidal behavior has not been studied. Therefore, this paper is focused on the interactions between double layer swelling and crystalline swelling and their effect on colloidal dispersion. We hypothesize that flocculation and dispersion behavior of smectitic soil colloids as a result of changing monovalent to divalent cation ratio is controlled by two processes; i) osmotic swelling between QC and colloids, explained by DLVO theory and ii) the formation and breakup of QC which is explained by crystalline swelling. To test this hypothesis we experimentally i) quantify the influence of monovalent to divalent cation ratios for Na/Ca -, K/Ca -, and NH_4/Ca -systems on dispersion of smectitic soil colloids for two ionic strengths, and ii) evaluate interactions between crystalline swelling, cation demixing, the breakup of QC, and dispersion.

Materials and Method

Soil

A sample of Zook soil (*fine, smectitic, mesic Cumulic Vertic Endoaquoll*) was collected from the surface horizon (0-40 cm) of a pedon in Story County, Iowa. The soil was air-dried, crushed, sieved through a 2 mm sieve, and stored at 4°C.

Soil Clay-Humic Complexes

Soil clay-humic complexes ($\leq 2 \mu\text{m}$) were collected from the Zook soil by sedimentation. To do so, soil was dispersed in distilled water by shaking and the upper 10 cm of soil suspension was collected by siphoning. The depth of siphoning was determined daily depending on the ambient temperature. Clay-humic complexes were air-dried, crushed in an agate mortar, and stored in a Nalgene™ high-density polyethylene bottle at room temperature until used.

Preparation of Mixed Cation Solutions

Multiple mixed cation solutions were prepared using CaCl_2 in combination with NaCl , KCl , or NH_4Cl . The monovalent concentration ratio (CR_X) is the ratio of monovalent to divalent cations in mmoles ($\text{CR}_X = [\text{X}^+ / [\text{Ca}^{2+}]^{1/2}]$, where $\text{X} = \text{Na}^+$, K^+ , or NH_4^+). Two ionic strengths (I) were chosen for this experiment. Low ($I = 0.004$) and high ionic strength ($I = 0.04$) solutions were prepared by adding appropriate amounts of reagent grade CaCl_2 , NaCl , KCl , and NH_4Cl to deionized water to achieve ionic ratios of 0%, 40%, 90%, 95%, 99%, and 100% (w/w). Concentrations of Ca^{2+} , Na^+ , and K^+ in solutions were confirmed by analysis with ICAP-AES (TJA Model 61E, Thermo Jarrell Ash Corporation, Franklin, MA, USA). Ammonium was analyzed with a Bio-Tek EL X800 Microplate reader (Bio-Tek Instruments, Inc, Winooski, Vermont, USA).

Settling Studies

The clay-humic complexes were treated with various CR_{NH_4} , CR_{K} , and CR_{Na} solutions. To do so, approximately 150 mg of clay-humic complexes were washed with 20 mL of a CR_X solution in Nalgene™ Oak Ridge polypropylene copolymer tubes, vortexed for 30 s and agitated on a flat bed shaker for 24 h at $150 \text{ cycles min}^{-1}$. Samples were centrifuged at $1710 \times g$ for 5 min and the supernatant was decanted. The process was repeated two more

times with an agitation period of 1 h. After the final wash, wet samples were immediately diluted with the same CR_x solution to obtain a 0.2% solid/solution ratio. Suspensions were agitated and a 3 mL sub-sample was transferred to a disposable methacrylate cuvette. Cuvettes were covered with parafilm and agitated for 30 s before measuring absorbance at 450 nm using a Cary 50 Bio UV-Visible Spectrophotometer (Varian Instruments, Walnut Creek, California, USA). The spectrometer was zeroed with the same mixed cation solutions as used for the sample treatment. Absorbance measurements were obtained every 120 s for 150 min. Sample analysis was performed in triplicates and averaged for each sample. Once absorbance (A) was measured, data was normalized by dividing absorbance at time (t) by the absorbance when t = 0 (A₀). The suspension was defined as 100% dispersed at the start of the measurement (t = 0).

XRD Analysis

Preparation of Oriented Specimens for Mineralogical Characterization

Approximately 150 mg of clay-humic complexes were washed with 20 mL of 1 M KCl and 0.5 M MgCl₂ solutions, vortexed for 30 s and agitated for 24 h on a flat bed shaker at 150 cycles min⁻¹. Samples were centrifuged at 1710 x g for 5 min and the supernatant was decanted. This salt-wash was repeated twice with an agitation period of 1 h to ensure complete saturation of the exchange sites by K⁺ or Mg²⁺. After the final wash approximately 15 mL of the various clay suspensions were mounted on ceramic tiles and water was removed by applying a vacuum to the base of the tile. Desired clay orientation was achieved by adding clay suspension in small increments using a Pasteur pipette. Excess salt was removed by using vacuum to pull ~10 mL of Milli-Q water through the tiles. Before the XRD analysis, tiles were stored for 24 h in a dessicator containing saturated Mg(NO)₂ to maintain a relative humidity (R.H.) of approximately 54%. After removal from the dessicator the K⁺ and Mg²⁺ saturated samples were analyzed by XRD at ambient

temperature. The Mg-saturated samples were solvated with ~3 mL of 30% glycerol. Excess glycerol was allowed to drain off, the samples were equilibrated for 24 h above glycerol and then analyzed again by XRD. Potassium saturated samples were analyzed by XRD after heating for 24 h to 105°, 305°, and 545° C. Samples were analyzed with a Siemens D5000 x-ray diffractometer using CuK α radiation. The step size was 0.02 degrees 2-Theta with a speed of 2 degrees/min. X-ray diffraction patterns were collected from 2 to 32 degrees 2-Theta.

XRD Analysis of CR_x-treated Samples Prepared as Oriented Specimens

Samples of the CR_x-treated clay-humic complexes were prepared as oriented specimens on ceramic tiles. After a minimum equilibration time of 24 h at 54% R.H., samples were analyzed by XRD as described above, however, diffractograms were collected from 3 to 12 degrees 2-Theta.

Suspension XRD Analysis of CR_x-treated Clay-Humic Complexes

At low ionic strength, 150 mg clay-humic complexes were suspended in 5 mL of the various CR_x solutions (for CR_x = 0 the samples were suspended in 1 mL solution). In order to ensure stable suspensions for high ionic strength samples, 150 mg clay-humic complexes were suspended in 1 mL of the appropriate CR_x solution. A totally random orientation of the colloids is assumed in the XRD analysis of the clay suspensions. Clay suspensions were analyzed by XRD using a method similar to that used by Shang et al. (1995). Analysis was conducted using a transmission sample cell (30 mm diameter, 0.5 mm thick) with mylar windows. The sample cell was oriented perpendicular to the zero plane of the goniometer such that the x-ray beam passed through the clay suspension. The step size was 0.08 degrees 2-Theta with a speed of 0.02 degrees/min. XRD patterns were collected from 3 to 12 degrees 2-Theta.

All x-ray diffractograms in figures are separated from each other by a constant for better visual presentation, however relative intensity remains the same.

Results and Discussion

The soil used in this study is a typical fine texture Mollisol from Central Iowa. The untreated Zook soil has a pH of 6.8 and a CEC of $36.2 \text{ cmol}_c \text{ kg}^{-1}$. The soil is dominated by clay (46%), followed by silt (41%), and sand (13%).

The mineralogy of the clay separated from the Zook soil was also typical for Midwestern Mollisols. Figure 2-1 shows XRD patterns for Mg- and K-saturated Zook clay-humic complexes. The 1.4 nm peak in the XRD pattern for the Mg-saturated sample expands to 1.8 nm after glycerol salvation indicating the presence of smectite. The sample also contained illite as evidenced by the 1.0, 0.5, and 0.33 nm peaks on the XRD patterns for the Mg-saturated sample, and kaolinite as evidenced by the 0.72 and 0.36 nm peaks that disappeared after heating to 545°C . Smectite in air-dried K-saturated samples at 25°C had one layer of interlayer water molecules as evidenced by the 1.2 nm peak. The unsymmetrical 1.0 nm peaks of XRD patterns for the K- 105°C and K- 305°C treatments suggests the presence of some hydroxy-interlayer material ($\sim 1.15 \text{ nm}$). The shoulder on the XRD patterns indicating hydroxy-interlayer material disappeared when the K-saturated samples were heated to 545°C . The sample also contains quartz (0.43 and 0.34 nm peaks).

The ionic strengths chosen for this study of flocculation and dispersion were based on natural occurrences. Electrical conductivity and monovalent to divalent cation ratio were determined of soils before and after manure applications. Results indicated an ionic strength of approximately 0.04 of soils after manure application and an ionic strength of 0.004 for non-manured soils following a rain event. The combination of settling curves and XRD patterns gives insight into mechanisms contributing to flocculation and dispersion. Settling

curves elucidate the effect of salt concentration and CR_X on settling behavior. The XRD patterns

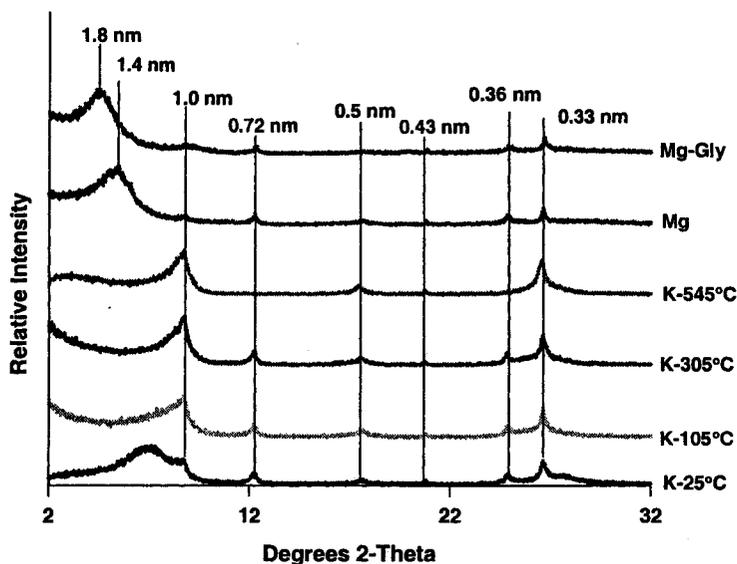


Figure 2-1. Figure 2-1. X-ray diffractograms of Mg- and K-saturated Zook clay-HC [quartz (0.43 and 0.34 nm peaks); kaolinite (0.72 and 0.36 nm peaks); illite (1.0, 0.5, and 0.33 nm peaks); smectite (1.4 nm peak); smectite with expanded interlayer (1.8 nm peak)].

(for oriented specimens and suspensions) elucidate the effects of salt concentration and CR_X on swelling and breakup of QCs and on the distribution of monovalent and divalent cations within QCs.

Settling curves (Figs. 2-2a, 2-2b, 2-2c and 2-5a, 2-5b, 2-5c) are presented as % Dispersion. The absorbance readings were transformed to % dispersion for straightforward interpretation. In the figures, 0% dispersion indicates flocculation and complete settling of the colloids, whereas 100% dispersion represents a stable suspension. The lag time evident in the settling curves indicates the time required for flocculation and settling to occur following shaking of the samples.

Suspension XRD patterns are shown in Figures 3a, 3b, 3c and 6a, 6b, 6c. X-ray analysis revealed broad d_{001} peaks for the Ca-saturated samples ($CR_X = 0$) centered at 1.96 nm for both the low (Figs. 2-3a, 2-3b, 2-3c) and high ionic strength (Figs. 2-6a, 2-6b, 2-6c) suspensions. The peak at 1.96 nm indicates that the Ca-QCs have ~4 layers of water molecules in the interlayers in aqueous suspensions. Increasing CR_X results in loss of intensity and broadening of the 1.96 nm peak. For the K/Ca- and NH_4 /Ca-systems when $I = 0.04$, the 1.96 nm peak shifts to 1.7 nm with higher CR_X . The 1.7 nm peak indicates smectite QCs dominated by three-layer hydrates (1.75 nm) with a few randomly interstratified two-layer hydrates (1.5 nm). The Na/Ca-peak does not shift toward 1.7 nm with increasing CR_{Na} .

XRD patterns of air-dry (54% R.H.) oriented samples prepared from the suspensions are presented in Figures 2-4a, 2-4b, 2-4c and 2-7a, 2-7b, 2-7c. The Ca-saturated samples ($CR_X = 0$) have d_{001} XRD peaks centered on 1.45 nm when $I = 0.04$ and 0.004. These peaks indicate coherent diffracting domains dominated by layers with two layers of interlayer water molecules (1.50 nm) randomly interstratified with a few layers having one layer of interlayer water molecules ($d = 1.25$ nm). The d_{001} smectite peak loses intensity, broadens, and shifts toward 1.23 nm with increasing CR_X . The 1.23 nm peak represents domains dominated by layers with one layer of interlayer water molecules. The Ca-saturated ($CR_X = 0$) samples have small 1.0 nm illite peaks, with increasing CR_X the intensity of the 1.0 nm peaks increases especially for the K/Ca- and NH_4 /Ca-systems. The increase in intensity of the 1.0 nm peaks indicates an increase in the number and/or size of domains with fully collapsed layers. The collapse of the d_{001} smectite peak to 1.23 nm and 1.0 nm indicates that the interlayers are dominated by monovalent cations. Interlayers dominated by weakly hydrated K^+ and NH_4^+ cations will either fully collapse (0-layer-hydrate) or form 1-layer hydrates. Interlayers dominated by more strongly hydrated Na^+ ions will generally form 1-layer-hydrates when air-dried.

Low Ionic Strength System

Settling Curves for $I=0.004$

The settling curves at $I = 0.004$ for the Na/Ca-, K/Ca-, and NH₄/Ca-systems are similar (Figs. 2-2a, 2-2b, 2-2c). After a lag time of about 24 min, the Ca-saturated samples ($CR_X = 0$) rapidly flocculated and settled asymptotically approaching about 20% dispersion after 150 min. With the introduction of a small fraction of monovalent cations ($CR_X = 1$), the lag time was longer (about 68 min) and the transition to the flocculation and settling phase was more gradual. After 150 min the Na/Ca- and NH₄/Ca-systems linearly approached 60% dispersion while the K/Ca-system linearly approached about 48% dispersion. When $CR_{Na, K, NH_4} \geq 7$ the systems remain dispersed (> 90% dispersion) after 150 min. These results indicate that when $CR_X = 0$, Ca²⁺ forms large QCs which readily settle out under the influence of gravity. Medium QCs start to form when $CR_{Na, K, NH_4} \geq 1$ as the introduction of monovalent cations in the systems breaks up the large Ca-QC. This observation is explained by demixing of the adsorbed monovalent cations and Ca²⁺ in a montmorillonite system, with Ca²⁺ sorption in the interlayer and Na⁺ sorption on the external surfaces (Shainberg and Otoh, 1968).

XRD Analysis of Suspensions for $I=0.004$

Suspension XRD patterns give insight to the size of QCs in suspension (Figs. 2-3a, 2-3b, 2-3c) and the nature of the interlayer cations in those QCs. The XRD patterns for the low ionic strength suspensions show 1.96 nm peaks for all of the systems except the $CR_K = 84$, which has a shoulder at approximately 1.7 nm. The presence of the 1.96 nm peaks throughout the CR_X range for all three systems reveal that smectites in suspension exist as quasicrystals rather than random tactoids or delaminated suspensions. The location of the peak at 1.96 nm indicates ~4 layer of water molecules in the interlayer, which is consistent with Ca-dominated QC. At low ionic strength divalent cations are strongly preferred by the

exchange phase. When $CR_{Na} \geq 7$, $CR_K \geq 9$, and $CR_{NH_4} \geq 9$ there is a general decrease in intensity and broadening of the 1.96 nm XRD peak. The broadening of the peak in the suspension XRD patterns suggests the breakup of large Ca-QC to medium Ca-QC and small Ca-QC. However when $CR_K = 84$ the peak shift to 1.7 nm indicates that the interlayers are dominated by three-water layer hydrates (1.75 nm) interstratified with a few two-water layer hydrates (1.50 nm). The peak shift for $CR_K = 84$ indicates the presence of K-QC in suspension.

XRD Analysis of Oriented Specimens for $I=0.004$

The XRD patterns for the oriented-air-dried (54% R.H.) samples prepared from the low ionic strength ($I = 0.004$) suspensions (Figs. 2-4a, 2-4b, 2-4c) provide information on the distribution of the Ca^{2+} and monovalent cations in the clay. Quasicrystals that existed in the clay suspensions during the settling experiments and during the suspension XRD analysis were aggregated together when the clay film (effectively one large QC) was formed during preparation of the air-dried clay specimens. For the Na/Ca-system, the d_{001} peak remains at 1.45 nm for all values of CR_X indicating that Ca^{2+} is the dominant interlayer cation in the clay. If Na^+ was the dominant cation in the interlayers the d_{001} peak would have collapsed to 1.23 nm as the peak did in the XRD patterns for the high ionic strength samples (Fig. 2-7a). Increasing CR_{Na} results in broadening and loss of intensity for the 1.45 nm peak and indicates the formation of small coherent diffracting domains. The results are consistent with the dominance of Ca-domains with two layers of interlayer water molecules (1.50 nm) randomly interstratified with Na-domains having one layer of interlayer water molecules (1.25 nm). For the K/Ca-system, the 1.45 nm peak is clearly evident for $CR_K = 0$ but the peak nearly disappears for $CR_K \geq 1$, and the intensity of the 1.0 nm peak increases substantially for $CR_K \geq 1$. These data suggest for $CR_K > 0$ that any Ca-domains are randomly interstratified with 1.0 and 1.23 nm K-domains. The prominent 1.0 nm peak for $CR_K \geq 1$ indicates that most K-

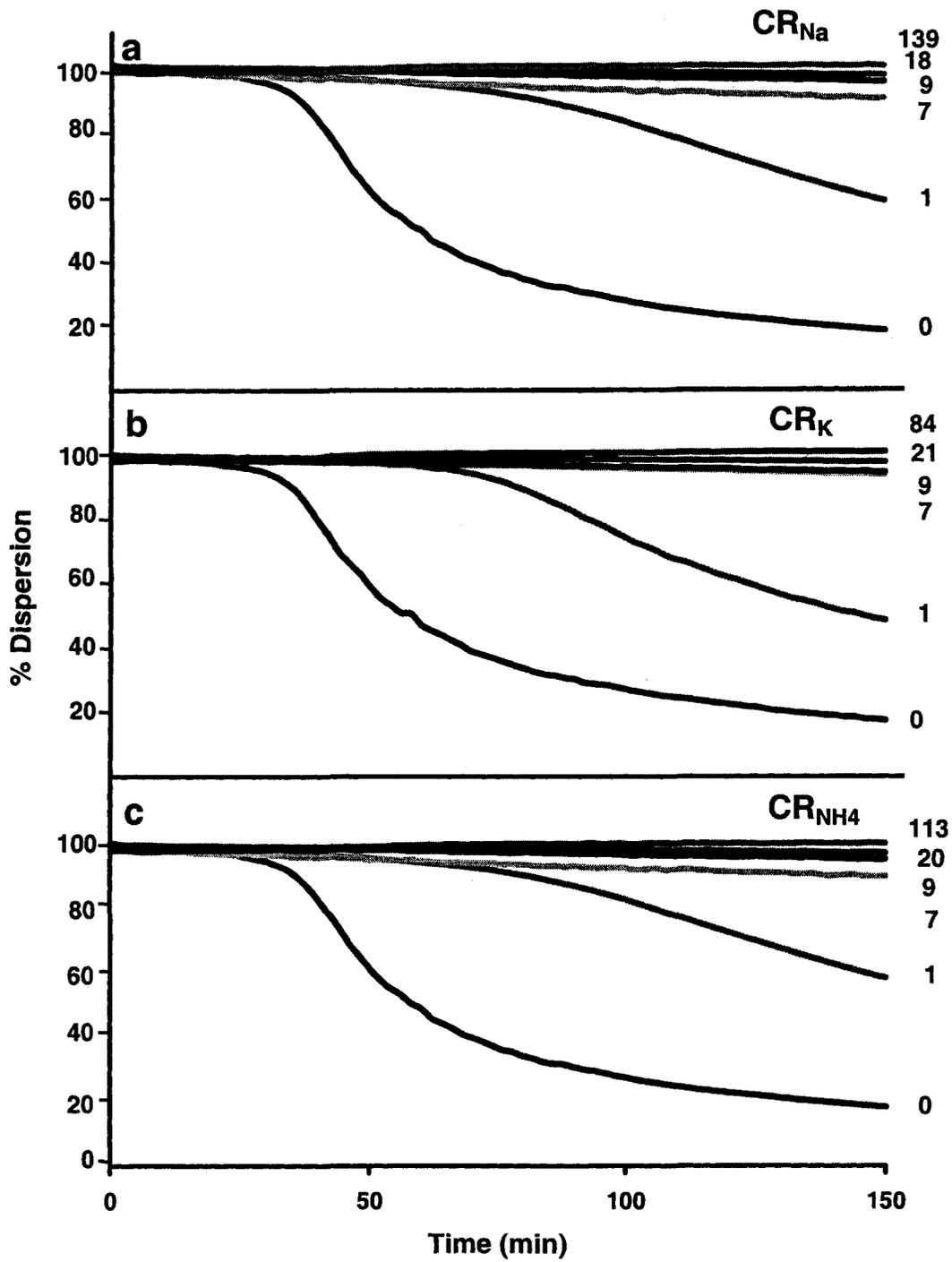


Figure 2-2. Settling curves for low ionic strength ($I = 0.004$) systems as a function of increasing CR_X .

saturated domains fully collapsed when air-dried and that K^+ was the dominant interlayer cation for $CR_K \geq 1$. These results in combination with the distinct 1.96 nm peaks for the $CR_K = 1$ and 7 in the suspension XRD patterns (Fig. 2-3b) suggest that Ca-domains within QCs were able to prop open layers in the aqueous suspensions. The trends in the XRD patterns for the air-dry oriented samples in the NH_4 /Ca-system are similar to those observed for the K/Ca-system. The NH_4 /Ca-system has a more prominent 1.45 nm peak for $CR_{NH_4} = 1$, smaller 1.0 nm peaks for $CR_{NH_4} \geq 1$, and more distinct peaks at 1.23 nm for $CR_{NH_4} \geq 7$ relative to the K/Ca system. These results indicate that the interlayers are dominated by NH_4 for $CR_{NH_4} \geq 7$ and hence suggest that small amounts of interlayer Ca have a large influence on QCs in aqueous suspensions (the 1.96 nm peaks Fig. 2-3c).

High Ionic Strength System

Settling Curves for $I=0.04$

The settling curves for the high ionic strength ($I = 0.04$) Na/Ca- and NH_4 /Ca-systems are similar (Figs. 2-5a, 2-5c). For $CR_X \leq 24$ the systems have a relatively short lag time followed by rapid flocculation and settling phase with dispersion dropping below 20% before 150 min. For $CR_X \geq 24$ the Na/Ca- and NH_4 /Ca-systems remain largely dispersed (> 80% dispersion at 150 min). These observations are similar to the Na/Ca and NH_4 /Ca systems when $I = 0.004$ (Figs. 2-2a, 2-2c). However the critical flocculation CR_X value increased when $I = 0.04$. When $CR_{Na} \leq 17$ and $CR_{NH_4} \leq 18$ the systems are dominated by Ca-domains resulting in large QCs and therefore flocculation occurs. When $CR_{Na, NH_4} \geq 24$ interstratification of monovalent cations results in the effective formation of small QC, hence dispersion. By contrast, there was substantial settling for all samples in the K/Ca-system (Fig. 2-5b) regardless of CR_X (< 33% dispersion at 150 min). At low CR_K , Ca-QC dominated the system resulting in flocculation. At high CR_K , the QCs were undoubtedly dominated by K^+ , but at $I = 0.004$ the K-QCs clearly flocculated and settled.

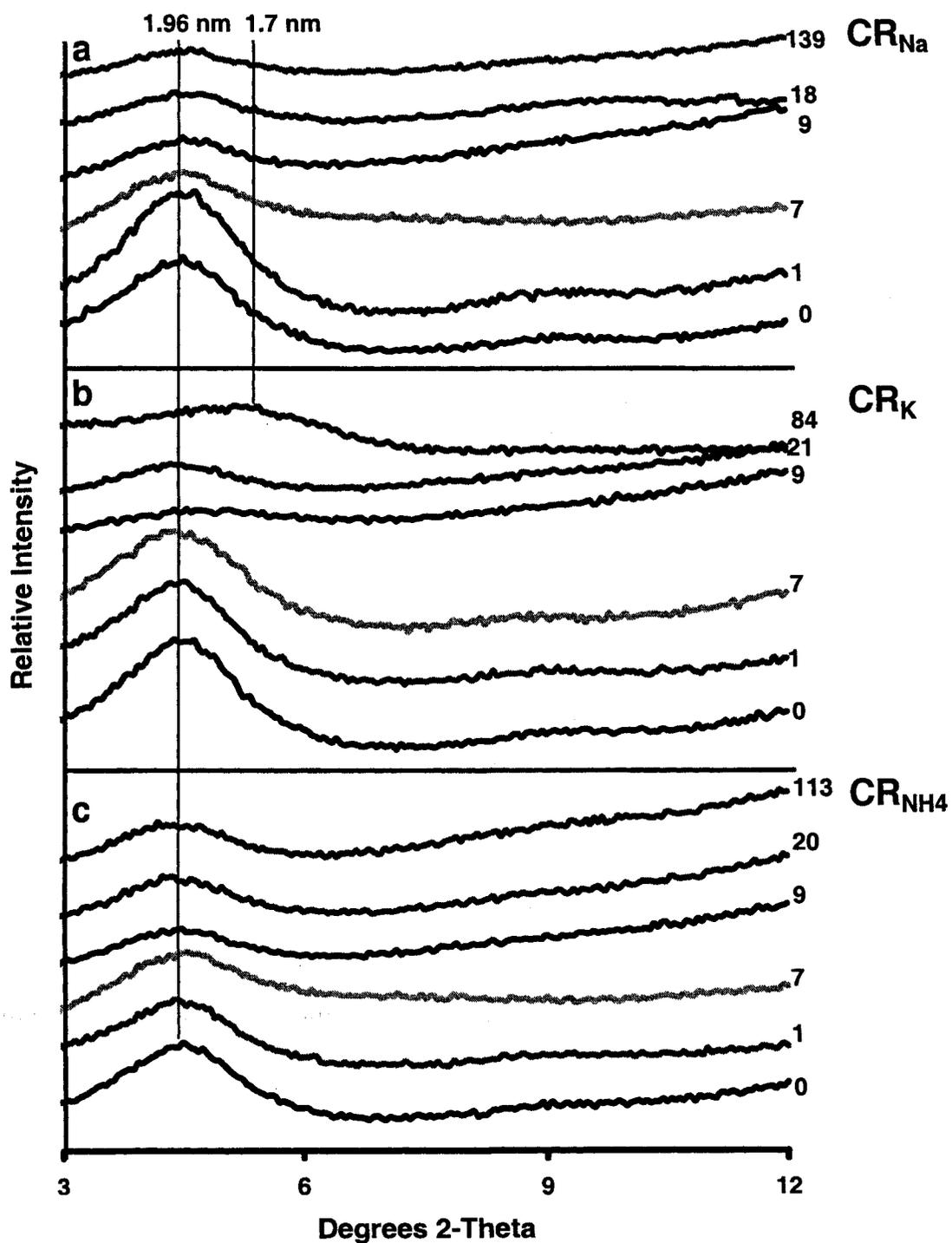


Figure 2-3. Suspension XRD patterns for low ionic strength ($I = 0.004$) systems as a function of increasing CR_x .

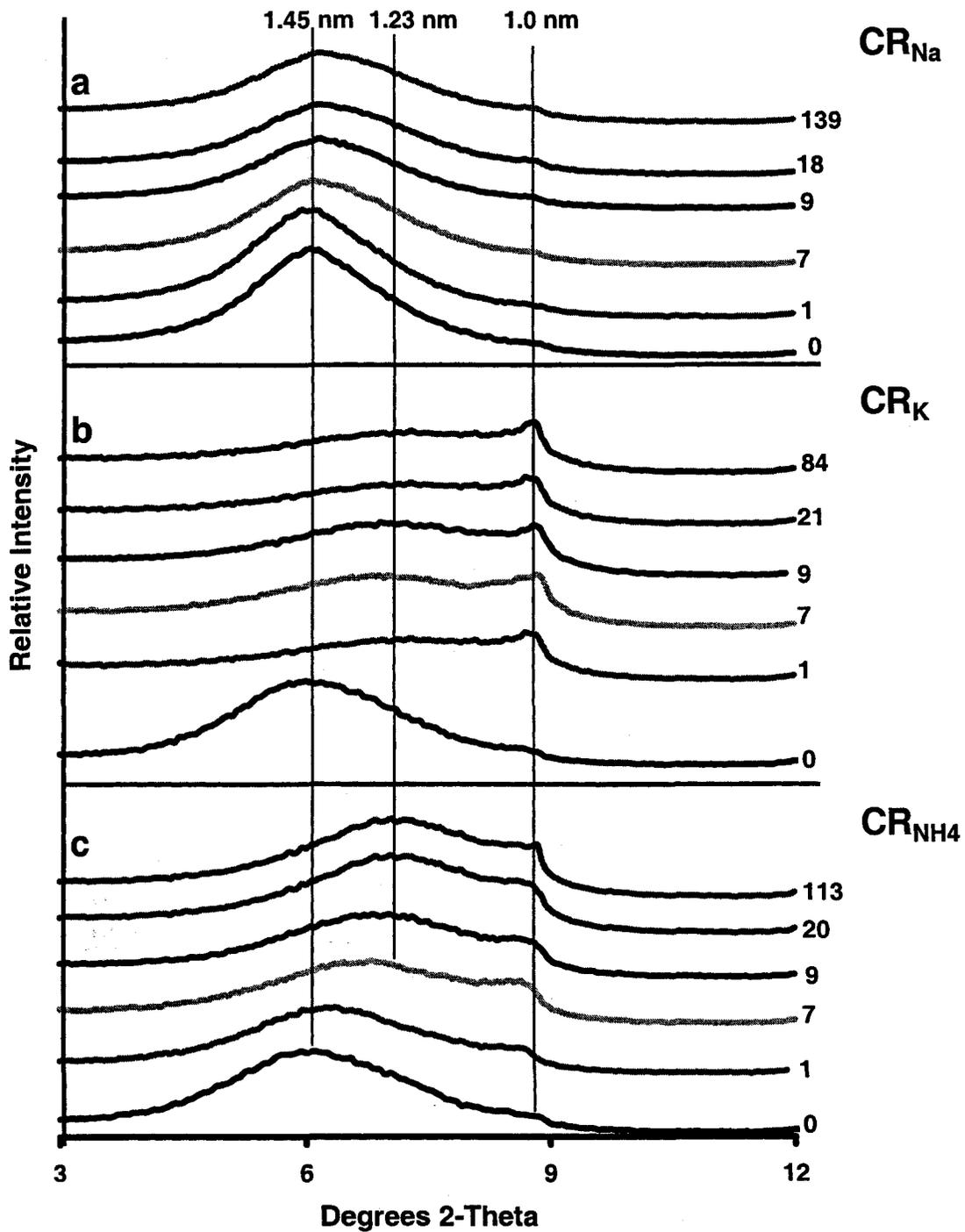


Figure 2-4. Oriented specimen XRD patterns for low ionic strength ($I = 0.004$) systems as a function of increasing CR_X .

XRD Analysis of Suspensions for $I=0.04$

X-ray patterns for the high ionic strength suspensions are shown in Figures 2-5a, 2-5b, and 2-5c for the Na/Ca-, K/Ca-, and NH₄/Ca-systems, respectively. The XRD patterns for all systems show the presence of a peak even at the highest CR_X values, indicating that the clays are organized as QCs in the suspensions. For the Na/Ca-system, the diffractograms show a 1.96 nm peak that broadens as CR_{Na} ≥ 24, but the peak remains at 1.96 nm regardless of CR_{Na}. For CR_K = 0 through CR_K = 3, the peak remained at 1.96 nm with little broadening. The peak broadened when CR_K ≥ 18 and shifted to 1.7 nm when CR_K ≥ 59. Substantial peak broadening was observed when CR_{NH4} ≥ 18 and the peak shifted to 1.7 nm when CR_{NH4} ≥ 51. Broadening of the 1.96 nm peak suggests the break up of large QCs to medium QCs with increasing CR_X for all three systems. The peak shift to 1.7 nm for the K/Ca- and NH₄/Ca-systems, suggests that the QCs are dominated by K⁺ or NH₄⁺. For the Na/Ca and NH₄/Ca systems the peak broadening is generally consistent with increased dispersion, suggesting that the breakup of large QCs into medium and small QCs contributes to dispersion. Although there is both substantial peak broadening and a peak shift for the K/Ca-system, the settling curves (Fig. 3d) indicated that the clay flocculated and settled throughout the CR_K range.

XRD Analysis of Oriented Specimens for $I=0.04$

X-ray patterns of air-dry oriented specimens for the Na/Ca-, K/Ca-, and NH₄/Ca- systems when $I = 0.04$ are in Figures 2-7a, 2-7b, and 2-7c, respectively, and give insight into the distribution of cations in the clays. The Na/Ca-, K/Ca-, and NH₄/Ca-systems behave similarly. When CR_{Na} ≤ 3, CR_K ≤ 3 and CR_{NH4} = 0 the center of the peak is at 1.45 nm. The 1.45 nm peak broadens when CR_{Na} ≥ 17 and CR_{K, NH4} ≥ 3 and shifts to 1.23 nm when CR_{Na} ≥ 17, CR_K ≥ 18, and CR_{NH4} ≥ 3. The collapse of the d_{001} smectite peak to 1.23 nm and 1.0 nm

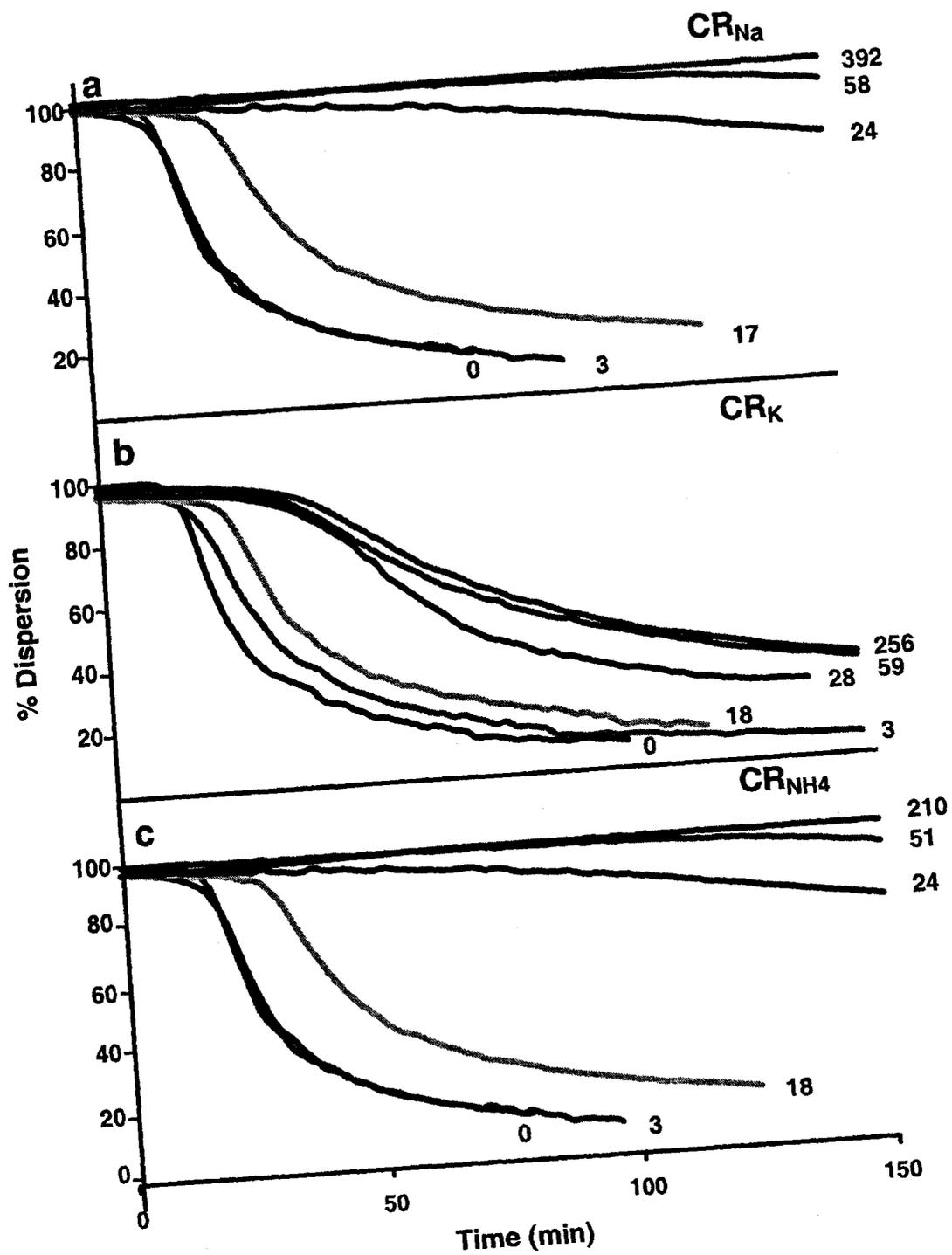


Figure 2-5. Settling curves for high ionic strength ($I = 0.04$) systems as a function of increasing CR_x .

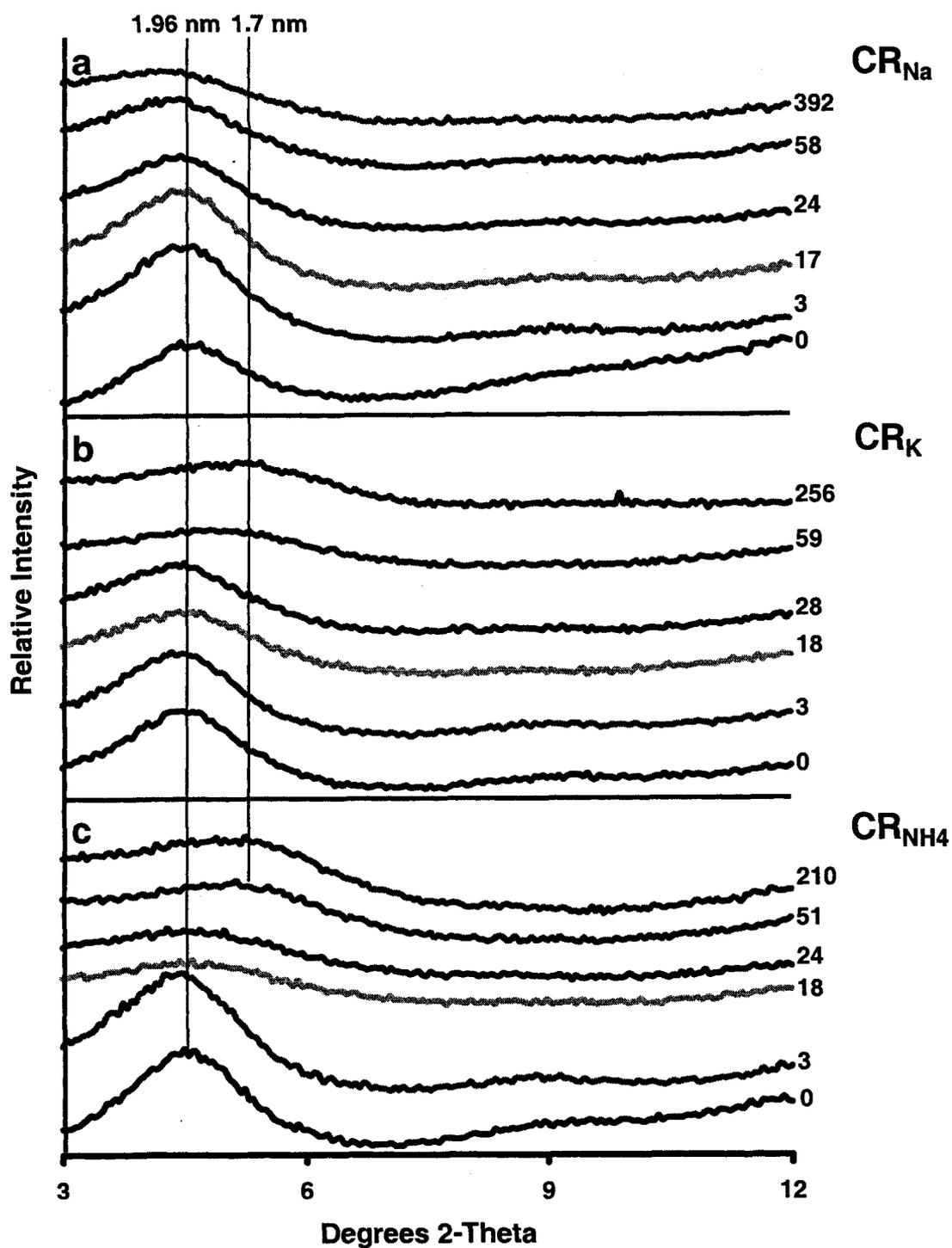


Figure 2-6. Suspension XRD patterns for high ionic strength ($I = 0.04$) systems as a function of increasing CR_X .

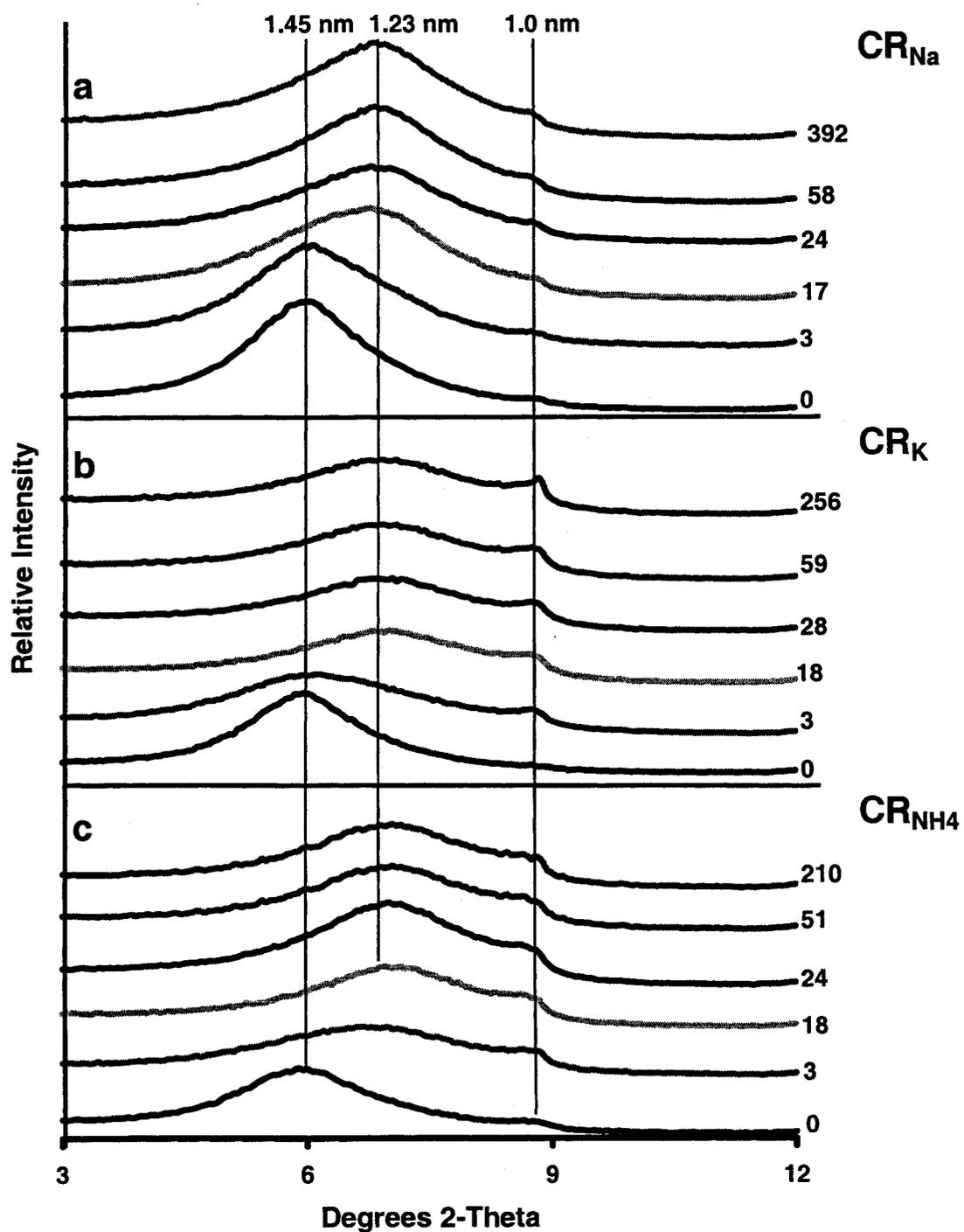


Figure 2-7. Oriented specimen XRD patterns for high ionic strength ($I = 0.04$) systems as a function of increasing CR_x .

indicates that the interlayers are dominated by monovalent cations. When $CR_{K, NH_4} \geq 3$ there is an increase in intensity of the 1.0 nm peak.

Conceptual Model for the Interaction between Crystalline Swelling and Double Layer Swelling

Figures 2-8 and 2-9 present a conceptual model of QC formation and breakup as a function of CR_X for low and high ionic strength systems, respectively. In both figures, 'X' represents Na^+ , K^+ , or NH_4^+ .

In a Ca-dominated system, the formation of large QCs (Figs. 2-8a and 2-9a) is due to the bridging of divalent cations between two adjacent layers. This bridging effect minimizes the separation of charge, greatly enhances the stability of Ca-QCs, and increases the selectivity of interlayer sites for divalent relative to monovalent cations. As CR_X increases, monovalent and divalent cations demix and large Ca-QCs breakup between layers dominated by monovalent cations (Figs. 2-8b and 2-9b). The wide separation of charge sites on external surfaces increases the selectivity of external charge sites for monovalent cations relative to divalent cations. Thus selectivity differences between interlayer sites and external sites are responsible for demixing of monovalent and divalent cations. The resulting smaller QCs are more easily dispersed than large Ca-QCs. Monovalent cations on external surfaces detach from surface sites forming diffuse double layers between QCs.

Figure 2-8 illustrates the formation of smaller QCs with increasing CR_X at low ionic strength ($I=0.004$). As large Ca-QCs breakup at low ionic strength, monovalent cations reside primarily on the external surfaces, whereas Ca^{2+} is preferentially retained in the interlayers (Fig. 2-8b). The break up of large QCs can be observed in the suspension XRD patterns for the Na/Ca-, K/Ca-, and NH_4 /Ca-systems by the peak broadening with increasing CR_X (Fig. 2-3a, 2-3b, 2-3c). When only monovalent cations are present (Fig. 2-8c), much smaller QCs are formed. The 1.45 nm peak for the Na/Ca-system remains with increasing

When a system is completely dominated by monovalent cations, small QCs form as evidenced by the peak shift from 1.45 to 1.23 nm (Fig. 2-7a, 2-7b, 2-7c). However, complete delamination of large QCs does not occur in high ionic strength systems (Fig. 2-9c).

Conclusions

Diffuse double layer swelling between QCs has a large influence on dispersion and flocculation of smectitic colloids. However, in mixed monovalent-divalent cation systems crystalline swelling and the break up and formation of QCs also has a large influence on flocculation and dispersion. In low ionic strength systems, demixing with monovalent cations on the external surfaces and divalent cations on the internal surfaces of QCs, largely controls the average size of QCs in suspensions. In high ionic strength systems, both monovalent and divalent cations are found in the interlayers. The average size of QCs is controlled by the monovalent to divalent cation ratio, the hydration energies of the cations, and the ionic strength of the system.

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CHAPTER 3. SORPTION OF TETRACYCLINE AND CHLORTETRACYCLINE ON K- AND CA-SATURATED SOIL CLAYS, HUMIC SUBSTANCES, AND CLAY-HUMIC COMPLEXES

A paper to be submitted in Chemosphere

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Abstract

Chlortetracycline (CTC) and tetracycline (TC) are used extensively for growth promoting and therapeutic purposes in livestock production. To elucidate the environmental fate of these pharmaceuticals, sorption isotherms were obtained using dilute CaCl₂ or KCl background solutions at different pHs for clays, humic substances (HS), and clay-humic complexes (clay-HC). In all systems, the soil components sorbed > 96% of added tetracyclines. Strongest sorption was observed for clays, followed by HS, and least on clay-HC. Sorption isotherms indicated greater sorption in Ca-systems than K-systems and that CTC was more strongly sorbed than TC. Increasing the pH of the Ca-clay-HC from 5.8 to 7.0 decreased sorption of CTC and TC. The greater sorption in the Ca-systems and the decreased sorption with increasing pH suggested that charge neutralization and cation bridging contribute to sorption. Furthermore, x-ray diffraction analyses showed that TC and CTC were sorbed in the interlayers of smectites. Desorption studies at pH 7 showed little CTC desorption but highest desorption was found for TC in the order of clay-HC > HS > clay. At pH 7 little CTC was desorbed. The results indicated that tetracyclines were dominantly sorbed on soil clays and that HS competed with tetracyclines for sorption sites on the clay surfaces.

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Introduction

In recent years increasing interest has focused on the fate of antibiotics in the environment. Estimates in the literature about the amount of antimicrobial agents used and their applications are variable. In the US, approximately 50 million pounds of antibiotics are produced annually of which 50% are used by humans and 50% are used in agricultural applications (Levy, 2002). Agricultural applications include 15.4 million pounds per year of mainly penicillins and tetracyclines used as growth promoters in animal feed and 99000 pounds per year of tetracyclines and streptomycines used as pesticides in orchards (Levy, 2002). By contrast, the Animal Health Institute (AHI) (an organization representing 80% of the companies that produce antimicrobial agents for animals in the United States, Anderson et al., 2003), reported that only 3.1 million pounds per year of antibiotics (6%) are used as non-therapeutic growth promoters, 14.7 million pounds per year (29.5%) are used for therapeutic and prevention purposes in agriculture and that human use accounts for 32.2 million pounds per year (64.5%) (AHI, 2000). Alternatively, Mellon et al. (2001) reported non-therapeutic use of antibiotics for livestock as 27.6 million pounds per year and therapeutic use as only 2 million pounds per year. Ultimately most of these antibiotics enter the environment in municipal effluents, sewage sludge, solid waste or manure applications. In the present study we will focus on tetracycline (TC) and chlortetracycline (CTC), as they are extensively used in animal husbandry as growth promoters and prophylactics in swine and cattle production.

Halling-Sørensen et al. (1998) states that many antibiotics administered to livestock are excreted as the parent compounds or its metabolites in urine and feces. Jjemba (2002) found insignificant *in vivo* metabolism of all three tetracyclines and excretion rates for parent compounds of 80-90%, >70%, and >80% for TC, CTC, and oxytetracycline (OTC), respectively. Kroker (1983) suggests that up to 90% of an administered dose to humans and

livestock may be excreted through urine and feces. Tetracyclines (TET) are excreted with intact antimicrobial activity (Halling-Sørensen, 2000).

Due to the high excretion rates of bioactive compounds there is growing concern that the non-therapeutic use of antibiotics in livestock production will promote the evolution of microbial populations resistant to the antibiotics used by humans. In the 1970's, legislation in Europe restricted the use of antibiotics as growth promoters after multidrug resistant microbial strains were found in humans (Levy, 1998). The use of antibiotics as growth promoters, which were not designated for human use are still problematic. Witte (1998) and Levy (1998) list a variety of microbes that developed resistance, due to antimicrobial use as growth promoters. The core structure of 20-year old antibiotics is very similar to the core structure in newer antibiotics designed for human use (Levy, 1998). This similarity has created antimicrobial resistance of bacteria to antibiotics used commonly for human use (Welton et al., 1998; van den Bogarrd et al., 1997). These results led to a ban of certain antibiotics in the European Union and the World Health Organization recommended the gradual discontinuation of the use of antibiotics as growth promoters. Denmark prohibited the use of antibiotics as growth promoter by 1999 (Rabølle and Spliid, 2000). However, there has been no change in their use in the US (Levy, 1998).

The continuous application of antibiotics to soils has been shown to increase microbial resistance (Levy, 1998; Sengeløv et al., 2003; Witte, 1998). Based on Levy (1998), the frequency of resistance in bacteria and the number of drugs to which they are resistant are increasing. Resistance of microbes to unrelated drugs has been detected when microbes were exposed to tetracycline (Levy, 1982). Sengeløv et al. (2003) studied bacterial resistance level of streptomycin, macrolides, and tetracycline and found that after a single manure application resistance levels for streptomycin and macrolides did not change, but tetracycline resistant bacteria increased. The increase was temporary as resistance levels returned to original values within several months.

The fate of TET in natural soil-water systems is strongly influenced by sorption and desorption processes depending on the physico-chemical characteristics of the sorbent and sorbate. It has been shown that TC in manure is present up to 20 mg L⁻¹ (Winckler and Grafe, 2000). Other studies reported TC concentrations in pig manure ranging from 0.6 to 66 mg L⁻¹ (Lee et al., 2000). Manure is a very complex and heterogeneous mixture (Tunney and Molley, 1975) with a pH ranging from 7 to 9 (Choudhary et al., 1996; De la Torre et al., 2000). Due to the high amounts of ammonia in manure, manure applications increase the soil pH at least temporarily thus changing the ionization of antibiotics. Boxall et al. (2002) found that manure application increased soil pH and resulted in a decrease in sorption of sulphonamide on a clay loam soil. Loke et al. (2002) reported pig manure to contain NH₄⁺, Na⁺, K⁺, Ca²⁺, Mg²⁺, Cu²⁺, Zn²⁺, Fe³⁺, acetate ions, bicarbonate, fatty acids, phenols, bile acids, amino acids, non-degraded fibers, and proteins. These constituents influence antibiotic behavior once applied to soil. The main conclusion was that OTC was more strongly sorbed to the soil than the manure. Lower metal-ion concentrations in manure and weaker binding of OTC to manure particles compared to soil clays explained the lower OTC sorption on manure. High concentrations of polyvalent cations sorbed onto the clay surface increases OTC sorption onto soil clay (Loke et al., 2002). Overall, sorption of OTC to sediments and soil depends on their physical and chemical characteristics (Pouliquen and Le Bris, 1996) and increased with increasing concentrations of divalent cations due to the formation of complexes (Lundestad and Goksøyr, 1990). Hirsch et al. (1999) studied sewage sludge, surface- and ground waters and found no tetracyclines due to the formation of TET complexes with divalent and trivalent cations in soil systems (Stuer-Lauridsen et al., 2000). However, TET was found in the µg L⁻¹ range in surface waters (Kümmerer, 2001; Lindsey et al., 2001; Kolpin et al., 2002).

Due to the low rate of metabolism and high water solubility, there is increasing interest in the mobility and bioavailability of tetracycline. Although considered a hydrophilic

compound (Kavallaris et al., 1993) TC is strongly adsorbed to soil and leaching of this antibiotic is unlikely (Rabølle and Spliid, 2000). A few studies have been conducted to elucidate the fate of TET in whole soils (Pinck et al., 1961a; Warman and Thomas, 1981; Rabølle and Spliid, 2000) and showed overall high sorption of TET onto various soil types. Soils receiving frequent manure application showed tetracycline concentration of at least $12 \mu\text{g kg}^{-1}$ (Hamscher et al., 2000) to $100 \mu\text{g kg}^{-1}$ (Klages and Roth, 2001). Tetracycline sorbed onto soils showed no antimicrobial activity. Bioassay studies showed no activity of antibiotics sorbed on montmorillonite, however the activity was regained when the antibiotics desorbed from the clay (Pinck et al., 1961b; Soulides et al., 1961). Most research on sorption of antibiotics on natural sorbents use reference clays, humic acids, synthetic organo-clays, whole soils, soil/slurries mixtures, manure, and marine sediments (Pinck et al., 1961a; Browne et al., 1980; Warman and Thomas, 1981; Sithole and Guy, 1987a,b; Gruber et al., 1990; Yeager and Halley, 1990; Pouliquen and Le Bris, 1996; Hirsch et al., 1999; Rabølle and Spliid, 2000; Lindsey et al., 2001; Boxall et al., 2002; Kolpin et al., 2002; Loke et al., 2002; Kulshrestha et al., 2004). To the authors' knowledge no studies have been conducted to elucidate TET sorption among natural soil components such as soil clays, humic substances, and clay-humic complexes.

The chemical structure of TC is shown in Figure 3-1. Tetracycline has three ionizable functional groups that determine the overall charge of the molecule as a function of pH. The tricarbonylamide system has a $\text{pk}_{\alpha 1}$ of 3.3. The term "tricarbonylamide" is used to define the strong coupling of the C1, C2, and C3 complex rather than considering this group as an amide and two independent carbonyls (Myers et al., 1983). Interactions between functional groups located at C1, C2, and C3 influence the pk_{α} with the actual protonation/deprotonation occurring at C3. The second ionizable group ($\text{pk}_{\alpha 2} = 7.7$) is the phenolic diketone moiety (C10, C11, and C12) with protonation and deprotonation occurring predominantly at C11 and C12. The quaternary ammonium has a $\text{pk}_{\alpha 3}$ of 9.7 (C4).

Chlortetracycline is formed by Cl substitution at the C7 position. The ionization of CTC is very similar to TC; $pK_{a1} = 3.3$, $pK_{a2} = 7.4$, and $pK_{a3} = 9.3$.

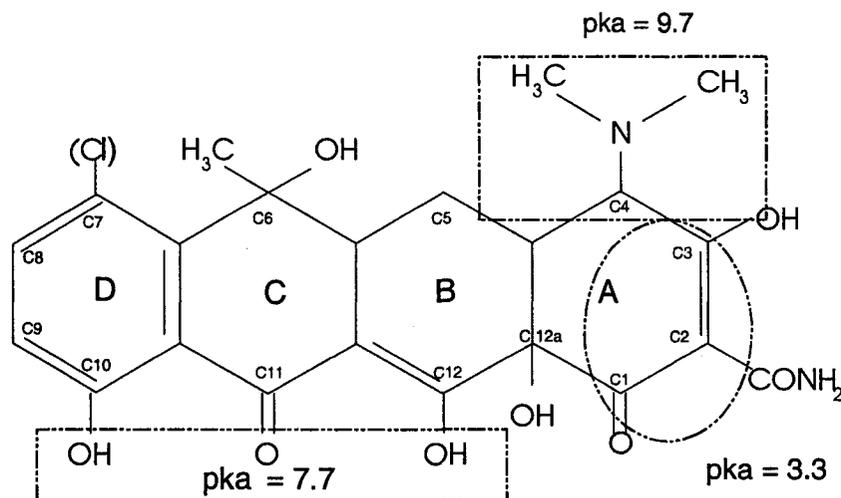


Figure 3-1. Functional groups of tetracycline and chlortetracycline (Cl).

Table 3-1 shows the charge distribution of the tetracycline compound depending on its pH. At a pH below 3.3, tetracycline exhibits an overall positive charge, whereas between pH 3.3 and 7.7 the compound is a zwitterion. Between pH 7.4 and 9.7, tetracycline has one negative charge and above pH 9.7 it has two negative charges.

Table 3-1. Overall charge of tetracyclines as a function of pH.

Functional Groups	pH			
	< 3.3	3.3 - 7.7	7.4 - 9.7	> 9.7
C3-OH	0	-	-	-
C11-O and C12-OH	0	0	-	-
C4-NH ⁺ (CH ₃) ₂	+	+	+	0

Based on above information, it is hypothesized that ionic functional groups of TC and CTC physically and chemically interact with soil clay minerals, humic substances, and clay-

humic complexes, thus impacting their fate in soil environments. The purpose of this study was to elucidate sorption and desorption behavior of tetracycline and chlortetracycline on K- and Ca-saturated soil clay, humic substances, clay humic-complexes, and whole soils in dilute aqueous systems at various pH.

Materials and Methods

Chemicals

Calcium chloride, KCl, HCl, KOH, Ca(OH)₂, and H₂O₂ were of certified A.C.S. grade. Tetracycline hydrochloride salt (Purity > 98%) and chlortetracycline hydrochloride salt (Purity > 98%) were obtained from MP Biomedicals (Irvine, California, USA) and stored at -20°C. Acetonitrile (ACN) and phosphoric acid (85%), both obtained from Fisher, were of HPLC grade. Milli-Q water was used throughout the study.

Whole Soil (WS)

Samples of a Zook soil (*fine, smectitic, mesic Cumulic Vertic Endoaquoll*) and a Nicollet soil (*fine-loamy, mixed, superactive, mesic Aquic Hapludoll*) were collected from the surface horizons (0-40 cm) of pedons in Story County, Iowa. The soils were air-dried, crushed, sieved through a 2 mm sieve, and stored at 4°C.

Soil Clay-Humic Complexes (clay-HC)

Soil clay-HC ($\leq 2 \mu\text{m}$) were separated from the Zook and Nicollet soil by sedimentation. To do so, soil was dispersed in distilled water by shaking and the upper 10 cm of soil suspension was collected by siphoning. The depth of siphoning was determined daily depending on the ambient temperature. Suspensions of clay-HC were centrifuged at $2413 \times g$ for 20 min and immediately K⁺ or Ca²⁺ saturated as described below.

Soil Clay (clay)

The soil clay samples were obtained by oxidizing organic matter associated with clay-HC. Approximately 200 mg of clay-HC were added to 500 mL Pyrex™ glass beakers, treated with 20 mL of 30% H₂O₂ and placed on an unheated sand bath. After the reaction slowed down, the temperature was slowly increased until frothing occurred. Hydrogen peroxide was periodically added until the reaction was complete. After frothing stopped, the temperature was raised to the boiling point to remove excess H₂O₂. Samples were transferred to 250 mL Nalgene™ high-density polyethylene bottles and diluted to 200 mL with water. Samples were placed on a flat bed shaker at 150 cycles min⁻¹. After 24 h, samples were centrifuged at 2413 x g for 20 min and supernatant was decanted. The samples were washed twice with water, placed for 1 h on a flat bed shaker at 150 cycles min⁻¹. After decanting the supernatant, the samples were immediately cation saturated as described below.

Soil Humic Substances (HS)

To extract humic substances, approximately 200 mg of soil was added to 250 mL Nalgene™ high-density polyethylene bottles. The soil was washed with 200 mL of 1 M NaCl. Samples were agitated for 24 h on a flat bed shaker at 150 cycles min⁻¹. After 24 h samples were centrifuged at 6702 x g for 20 minutes and the supernatant was decanted. This process was repeated one more time with a shaking time of 1 h. After decanting the supernatant of last NaCl wash, 200 mL of 0.1 M NaOH were added. The samples were immediately sparged with N₂ for 60 s and sealed with air-tight lids. Samples were then placed on flat bed shaker at 150 cycles min⁻¹. After 24 h the samples were centrifuged at 6702 x g for 20 minutes and supernatant was collected. The pH of the supernatant was adjusted to 7 with 0.01 M HCl. The supernatant was dialyzed against deionized water until excess salts were removed. Samples were then dialyzed against 1 M KCl or 0.5 M CaCl₂ for

several days. To remove excess salts, K-saturated samples were dialyzed against 0.01 M KCl to avoid hydrolysis, whereas Ca-saturated samples were dialyzed against water until the silver nitrate test was negative for chloride.

Cation Saturation of Soil Components

Clay-HS and clay were saturated with K^+ and Ca^{2+} . To do so, the soil components were washed with 200 mL of 1 M KCl and 0.5 M $CaCl_2$. Samples were agitated on a flat bed shaker at 150 cycles min^{-1} . After 24 h the samples were centrifuged at 2413 x g for 20 min and the supernatant was decanted. The process was repeated two more times with an agitation period of 1 h. To remove the excess salts, K-saturated samples were dialyzed against 0.01 M KCl, whereas Ca-saturated samples were dialyzed against water until the silver nitrate test was negative for chloride.

Clay, HS, and clay-HC were adjusted to various pH values. To do so, 0.01 M HCl, KOH, or saturated $Ca(OH)_2$ was added until desired pH was reached. Samples were allowed to equilibrate and the process was repeated until the pH was stable. After pH was adjusted, samples were freeze-dried using a Labconco Freeze Dryer 4.5 (Kansas City, Missouri, USA) and stored in Nalgene™ high-density polyethylene bottles at room temperature until used.

Cation Exchange Capacity (CEC)

After cation saturation, the CECs of Ca- and K-clays, Ca- and K-clay-HC, and WS were measured. To do so, 15 mL of 0.5 M $MgCl_2$ were mixed with 0.2 g of clay and clay-HC from both soils, and 0.43 g of Zook and 0.88 g of Nicollet soil in a 50 mL polypropylene centrifuge tube. Samples were vortexed, placed on a flat bed shaker at 150 cycles min^{-1} and then centrifuged at 1187 x g for 5 min. Supernatant was decanted. This washing process was repeated two more times. Samples were washed with 15 mL of 80% isopropyl alcohol (IPA), vortexed for 1 min and centrifuged at 1187 x g for 5 min. This process was repeated

until electrical conductivity was $< 6 \mu\text{S cm}^{-1}$. Samples were washed with 15 mL of 100% (IPA) until electrical conductivity was $< 1 \mu\text{S cm}^{-1}$. After the final rinse, samples were air-dried in the hood. Fifteen mL of 0.1 M NH_4NO_3 were added and the samples were vortexed and centrifuged. Supernatant was saved in 100 mL volumetric flasks. This process was repeated two more times. The 100 mL volumetric flasks were brought up to volume with Milli Q-water. Solution was analyzed for Mg^{2+} by inductively coupled plasma-atomic emission spectrometer.

Sorption/Desorption of TET onto/from Soil Components

Studies were conducted to quantify sorption and desorption of TET from clay, HS, clay-HC, and WS. To do so, the 200 mg L⁻¹ stock solutions were prepared by dissolving 20 mg of TET in 100 mL 0.005 M CaCl_2 or 0.01 M KCl. Stock and working solutions were prepared fresh within one hour of use.

All sorption and desorption procedures were conducted in 30 mL Nalgene™ Teflon test tubes. For pre-saturation, 0.2 g of clay, HS, and clay-HC from both soils, and 0.43 g of Zook and 0.88 g of Nicollet soil were mixed with background solutions of either 10 mL of 0.01 M KCl or 0.005 M CaCl_2 . The amount of soils used was determined based on their clay content. Suspensions were vortexed for 10 s and then agitated on a flat bed shaker at 150 cycles min⁻¹ for 24 h. Two, 5, or 10 mL of the appropriate 200 mg L⁻¹ TET stock solutions were added and then the samples were diluted to 20 mL with either 0.01 M KCl or 0.005 M CaCl_2 resulting in a final TET concentration of 20, 50, and 100 mg L⁻¹. Blanks were prepared for all three TET concentrations without soil components and controls were prepared for clay, HS, clay-HC, and WS without TET. All samples were triplicated.

The prepared samples were vortexed for 10 s and agitated on a flat bed shaker at 150 cycles min⁻¹ for 24 h. Samples of Ca- and K-Clay, Ca- and K-clay-HC, and Ca-HS were centrifuged at 4747 x g for 10 min. The collected supernatants were passed through a

Fisherbrand™ non-sterile, nylon, 25 mm syringe filter with 0.2 µm pore openings into 1.5 mL amber glass vials and subsequently analyzed by HPLC. Non-salinized amber glass vials were used, as preliminary data did not show significant differences using salinized versus non-salinized vials. Therefore, it was assumed that no TET was sorbed onto the glass surface. Due to increased dispersion of K-HS, supernatants from the K-HS samples were filtered through Waters Oasis® HLB (Waters Corporation, Milford, Massachusetts, USA) solid phase extraction cartridges at a flow rate of 1 mL min⁻¹. The cartridges were pre-treated with 3 x 1 mL of methanol followed by 3 x 1 mL of water. Three x 1 mL of supernatant were added. The cartridges sorbed both TET and organic material; the latter was removed from the cartridge with a 3 x 1 mL 70% methanol. Tetracyclines sorbed onto the cartridges were then eluted with 3 x 1 mL 100% ACN. Throughout the filtration processes the cartridges were never allowed to dry and the flow rate was kept constant at 1 mL min⁻¹. To assess TET recovery, triplicates of 20, 50, and 100 mg L⁻¹ spiked in 0.01 M KCl were passed through the cartridges using above described methodology. The analysis indicated a minimum TET recovery of >99.1 ± 0.36%.

Due to strong sorption of TC and CTC, desorption studies were conducted. Once the supernatant of the sorption experiment was removed, wet samples were brought to volume with 20 mL of the same background solution used in the sorption experiment; either 0.01 M KCl or 0.005 M CaCl₂. Samples were equilibrated for 24 h, and then processed and analyzed for TC and CTC as described above.

Chemical Analysis

Analysis of TC and CTC were performed using HPLC. A Hewlett Packard 1090 Liquid Chromatograph (Palo Alto, California, USA) was used with a 5 µm packing and 100 Å pore size Polymeric Reversed Phase Column (PLRP-S). The column was heated to 40° C during analysis and the flow rate was 1 mL min⁻¹. Method development was based on

findings of White et al. (1993) with modifications based on the amount of sample and the HPLC unit used. Analysis was done with a gradient elution over 12 min. The mobile phase consisted of acetonitrile (Solvent A) and Milli-Q water adjusted to pH 2.5 with H₃PO₄ (Solvent B). The gradient run was 10-100% A from 0-8 min, held constant at 100% A for 2 min and then run back down to 10% A over a 2 min period. The retention times for TC and CTC were 4.4 and 5.2 min, respectively. To test for interference of humic substances on the TC peak, the gradient was changed to elute TC after 8 min. Tetracycline peak areas were identical when the retention times were 4.4 min and 8 min.

Solutes were detected using a diode array detector with a wavelength set at 360 nm and the reference wavelength of 410 nm. Background interfered with peak quality at lower wavelengths. Tetracycline showed good linearity between 500 µg L⁻¹ and 10 mg L⁻¹ in 0.01 M KCl and 0.005 M CaCl₂ (pH 7) with R² = 0.999. Good linearity was established between 500 µg L⁻¹ and 10 mg L⁻¹ for CTC in 0.01 M KCl and 0.005 M CaCl₂ (pH 7) with R² = 1 and R² = 0.999, respectively. Based on a 3:1 signal-to noise ratio, the limits of detection were 500 µg L⁻¹. There was no matrix effect of the background solutions.

XRD Analysis

Preparation of Oriented Specimens for Mineralogical Characterization

Approximately 150 mg of clay-HC of Zook and Nicollet soils were washed with 20 mL of 1 M KCl and 0.5 M MgCl₂ solutions, vortexed for 30 s and agitated for 24 h on a flat bed shaker with 150 cycles min⁻¹. Samples were centrifuged at 1710 x g for 5 min and the supernatant was decanted. This process was repeated two more times with an agitation period of 1 h. After the final wash approximately 15 mL of the various clay suspensions were pipetted onto ceramic tiles and water was removed by applying a vacuum to the base of the tile. Excess salt was removed by using vacuum to pull ~10 mL of Milli-Q water through the tiles. Tiles were equilibrated at 54% R.H. (relative humidity) for 24 h before the XRD

analysis. After removal from the desiccator the K^+ and Mg^{2+} saturated samples were analyzed by XRD at the ambient temperature. The Mg-saturated samples were solvated with ~3 mL glycerol. Excess glycerol was allowed to drain off, the samples were equilibrated for 24 h above glycerol and then analyzed again by XRD. Potassium saturated samples were analyzed by XRD after heating for 24 h to 105°, 305°, and 545° C. Samples were analyzed with a Siemens D5000 x-ray diffractometer using $CuK\alpha$ radiation. The step size was 0.02 degrees 2-Theta with a speed of 2 degrees min^{-1} . X-ray patterns were collected from 2 to 32 degrees 2-Theta.

XRD Analysis of TET treated Samples Prepared as Oriented Specimens

Clay and clay-HC of both soils were treated with 50, 100, and 200 mg TET L^{-1} in 0.005 M $CaCl_2$ and 0.01 M KCl background solutions and prepared as described above. Oriented specimens of these treated samples were prepared for XRD analysis as described above. Tetracycline treated samples were analyzed by XRD after a minimum equilibration time of 24 h at 54% R.H. X-ray patterns were collected as described above from 2 to 14 degrees 2-Theta. After analysis samples were heated to 105° C for 24 h and upon removal from the oven were immediately placed under a constant stream of desiccated air and analyzed again by XRD.

Results and Discussion

Chemical and Mineralogical Analysis

Table 3-2 presents chemical and particle size data for Zook and Nicollet soils. The carbon associated with the clay fraction is 3.2% and 5.2% for the Zook and Nicollet, respectively. The clay content is highest for the Zook, whereas Nicollet is dominated by the sand fraction.

Table 3-2. Characterization of Zook and Nicollet soils.

soil	clay/silt/sand			% total C	pH†	% org. C (clay)
	-----%-----					
Zook	46	41	13	2.5	6.8	3.2
Nicollet	23	29	48	2.7	7.1	5.2

†: 1:1 water:soil

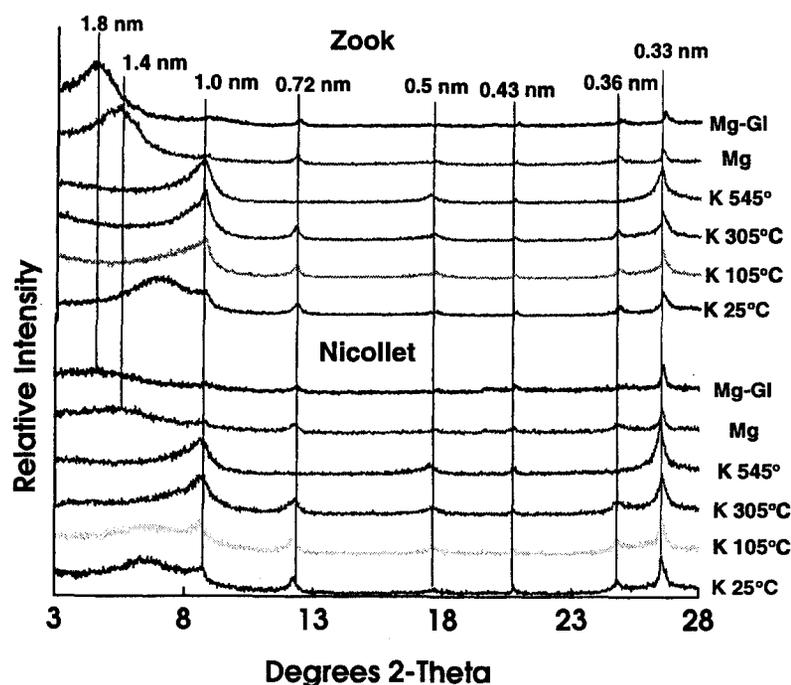


Figure 3-2. X-ray diffractograms of Mg- and K-saturated Zook and Nicollet clay-HC [quartz (0.43 and 0.34 nm peaks); kaolinite (0.72 and 0.36 nm peaks); illite (1.0, 0.5, and 0.33 nm peaks); smectite (1.4 nm peak); smectite with expanded interlayer (1.8 nm peak)].

Figure 3-2 shows x-ray diffraction patterns for the Zook and Nicollet clay-HC. Both samples are dominated by smectite as evidenced by the 1.4 nm XRD peaks for the Mg-saturated samples that expand on glycolation to ~1.8 nm. When the samples were K-saturated, the smectite 001 XRD peaks collapsed to a broad peak centered near 1.3 nm. With increasing intensity of heat treatments for the K-saturated samples, the 001 XRD peaks

progressively collapsed to 1.0 nm. The intensity of the smectite 001 XRD peaks is less for the Nicollet clay-HC sample than for the Zook clay-HC sample, and the 001 peak for the Nicollet sample is more resistant to collapse when heated than is the 001 peak for the Zook samples. These results indicate that the smectite in the Zook clay-HS is truly swelling while the smectite in the Nicollet either has hydroxyl material or organic materials in the interlayer that inhibit both swelling and collapse. Both samples also contain quartz (0.43 and 0.34 nm peaks), kaolinite (0.72 and 0.36 nm peaks), and illite (1.0, 0.5, and 0.33 nm peaks).

Sorption of TET on Soil Components

Sorption isotherms showed that of the total TET added at least 99.6, 98.3, 96.8, and 97.4% of TET were sorbed to clay, HS, clay-HC, and WS, respectively. These results are in close agreement with Rabølle and Spliid (2000) who listed OTC sorption ranging from 95 to 99% in natural soils.

To compare sorption behavior of TET onto soil components, K_d values were obtained. The adsorption distribution coefficient, K_d , is defined as the ratio between the concentrations of TET bound to the soil and the TET concentration in solution following a 24 h equilibration. Tetracycline concentrations bound to the soil is measured indirectly as the difference between the concentration applied at the beginning of the test and the concentration at equilibrium. The K_d sorption values when $\sim 9.75 \text{ mg g}^{-1}$ of TET were sorbed to Zook and Nicollet soil components at pH 7 and 5.8 are shown in Table 3-3. Tables S3-1 and S3-2 list K_d values when ~ 1.75 and $\sim 4.75 \text{ mg g}^{-1}$ of TET were sorbed to soil components, respectively. The 'X' designates complete sorption of TC whereas the dashed lines represents that no data for these systems were obtained. The overall high K_d values indicate high TET sorption ($\geq 96.8\%$). Rabølle and Spliid (2000) established K_d values in the range of 417-1026 for OTC sorption onto natural sandy loam and sandy soils, which are also very high considering the type of soils used in their study.

Table 3-3. K_d values when $\sim 9.75 \text{ mg g}^{-1}$ of TET were sorbed to Zook and Nicollet soil components at pH 7 and pH 5.8.

TC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	6.5×10^4	2.2×10^4	4.9×10^4	4.0×10^4
HS	2.9×10^4	1.0×10^4	1.1×10^4	5.7×10^3
Clay-HC	1.4×10^4	6.2×10^3	4.0×10^3	5.3×10^3
CTC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	5.4×10^4	3.9×10^4	4.4×10^4	1.2×10^4
Clay-HC	1.8×10^4	1.1×10^4	2.3×10^4	1.0×10^4
TC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	----	----	----	----
Clay-HC	1.7×10^4	----	6.6×10^3	----
CTC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	----	----	----	----
Clay-HC	6.4×10^4	----	3.1×10^4	----

X = complete sorption

---- = no data

Based on the K_d values in Table 3-3, several significant trends were established. First, TC and CTC are most strongly sorbed to Zook clays, followed by HS, and least onto clay-HC at pH 7 and 5.8. The same results were found for Nicollet soil components. Second, at pH 7 and 5.8, Ca-saturated soil components favored TET sorption over K-saturated soil components, except TC was preferably sorbed to K-saturated Nicollet soil clay-HC at pH 7. Third, sorption of CTC was generally larger than sorption of TC. Fourth, to compare the pH effect, CTC and TC sorption isotherms were conducted at pH 5.8 for Ca- and K-clays and for Ca-clay-HC. Consistently for all concentrations added, TET sorption was higher at pH 5.8 than pH 7 with stronger preference of soil component for CTC. Last, Zook soil components retained more TET compared to Nicollet soil components.

The degree of TET sorption onto soils and soil components generally increases with their CEC (Table 3-4). The CEC of Zook soil and its components are consistently higher compared to the Nicollet. Calcium-clay has a higher CEC compared to the Ca-clay-HC. Calcium-clays and Ca-clay-HC have a higher CEC compared to the K-clays and K-clay-HC. The trend of CEC for soil components is similar for both soils and decreases in following order; Ca-clay > Ca-clay-HC > K-clay > K-clay-HC > whole soil.

Table 3-4. Cation exchange capacity of soils and soil components.

soil	soil component	CEC‡ ($\text{cmol}_c \text{ kg}^{-1}$)
Zook	Ca-Clay	147.4 ± 1.83
	Ca-Clay-HC	134.8 ± 3.85
	K-Clay	119.2 ± 1.31
	K-Clay-HC	109.6 ± 2.97
	whole soil	36.2 ± 0.4
Nicollet	Ca-Clay	125.2 ± 0.72
	Ca-Clay-HC	94.1 ± 1.70
	K-clay	100.6 ± 0.75
	K-Clay-HC	75.8 ± 2.39
	whole soil	28.7 ± 0.9

Effect of Soil Components on TET Sorption

The overall trend of TET sorption on soil components is the same for Zook and Nicollet, with TET sorption decreasing in the following order; clay > HS > clay-HC. Figure 3-3 shows CTC sorption isotherms for Ca-saturated Zook and TC sorption isotherms for Ca-saturated Nicollet soil components at pH 7. Overall, CTC is strongly sorbed on the Zook soil clay and HS with strongest sorption on the clay fraction. Interactions between clay and HS significantly diminish sorption of CTC on soil clay-HC. The same trend was observed for TC sorption onto Nicollet soil components. The shape of the sorption isotherms indicates a cooperative effect of TC and CTC sorption onto Zook and Nicollet clay-HC.

There are several mechanisms involved in the sorption of TET onto soil components. Lunestad and Goksøyr (1990) showed that OTC binds strongly via complexation with divalent Mg^{2+} and Ca^{2+} ions onto clay minerals. Sorption of TC onto HS was studied by Sithole and Guy (1987b) who found that > 70% of TC was sorbed by divalent cation bridging, ion exchange reactions, and H-bonding between acidic groups of humic acids the polar groups in tetracycline. The significantly diminished sorption of TC on clay-HC relative to clays may be attributed to the structural organization of clays and HS and TET competing with HS for sorption sites. When adding 20 mg L⁻¹ of HS to soil, 100% OTC was sorbed, however, when the HS concentration was increased to 50 and 100 mg L⁻¹, sorption slightly decreased ranging from 98.32 to 99.83% (Sihole and Guy, 1987b). Sorption of OTC to soils decreased by 34% when dissolved humic acids were increased from 1 mg to 10 mg L⁻¹ in soil suspensions (Kulshrestha et al., 2004).

In this study, clay-HC sorbed significantly less TET than either clay or HS indicating that interactions between clay and HS suppress TET sorption. The lesser degree of TET sorption on clay-HC might be due to a decreasing number of sorption sites caused by the interaction of clay with HS. The stripping of HS from the clay might have additionally exposed interlayer sorption sites. The H₂O₂ treatments used to remove the HS from the clays may have created new variable charge sites (silanol or aluminol groups) through precipitation of metal oxyhydroxides in addition to exposing variable charge sites that were masked by the HS. The high sorption of TET to HS is mainly due to the formation of HS-Ca-TET complexes. Above pH 7, HS primarily carry a negative surface charge, the predominant source of which is carboxylate groups, with a lesser contribution from phenol groups. The negative charges of the carboxylate groups ($pK_a \approx 4$) and the phenol groups ($pK_a \approx 9$) are distributed throughout the macromolecular matrix, and are balanced by positively charged quaternary ammonium functional group of TET. Tetracyclines may also sorb to humic

substances through hydrogen bonding between the acidic groups of the HS and the polar groups of tetracyclines.

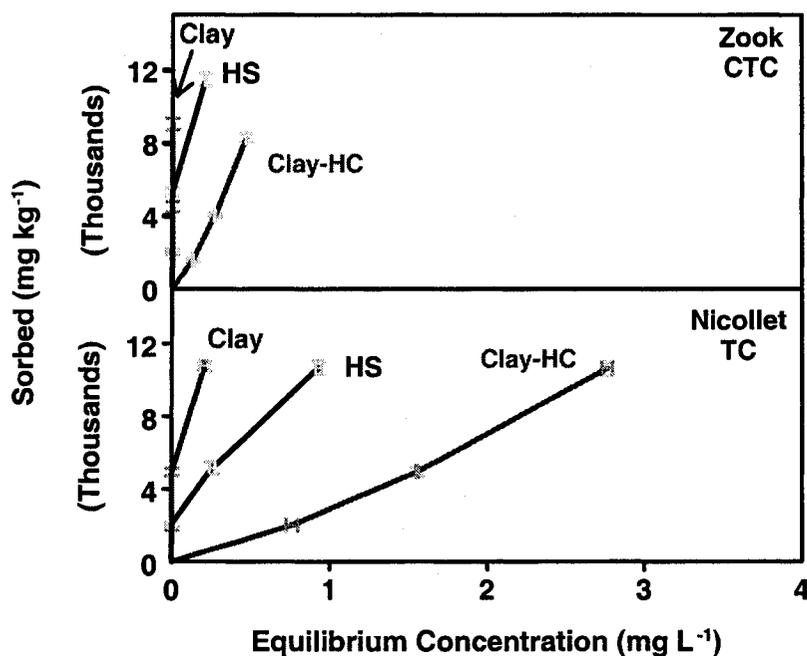


Figure 3-3. Tetracycline and chlortetracycline sorption isotherms for Ca-saturated Zook and Nicollet soil complexes, respectively (pH 7).

In general Nicollet soil components consistently sorb less TET than Zook soil components. Less sorption of TET on Nicollet clays might be due to the lower smectite content in the clay fractions or due to the hydroxyl material in the interlayer which was not removed with H₂O₂ treatments. Less sorption on Nicollet-HS might be due to different humic substance composition. Furthermore, the higher amount of organic carbon (5.2%) associated with Nicollet clay-HC may shield interlayer sorption sites for TET compared to the Zook which has an organic carbon content associated with the clay-HC of only 3.2%.

Effect of Cation Saturation on TET Sorption

Sorption isotherms of TET on Ca- and K-saturated Nicollet HS are shown in Figure 3-4. For both the Ca-HS and K-HS, CTC is sorbed to a greater extent than TC. Greater sorption of CTC may be due to its lower water solubility. The water solubility of CTC is 1 g CTC / 75 mL compared to 1 g TC / 10 mL. These results also suggest the formation of surface-Ca-TET complexes. The almost linear shape of the isotherms indicates strong sorption for both the Ca- and K-HS.

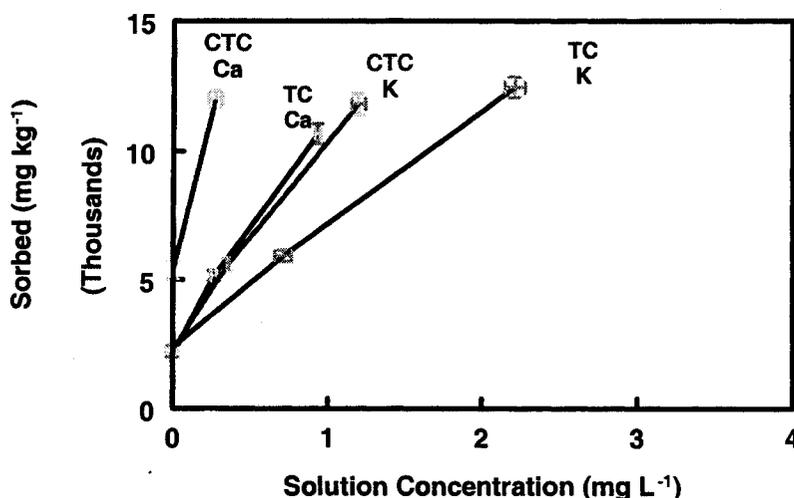


Figure 3-4. Chlortetracycline and tetracycline sorption on Ca- and K-saturated Nicollet humic substances at pH 7.

Effect of pH on TET Sorption

The isotherms for adsorption of TC sorbed on Nicollet Ca-clay and Ca-clay-HC at pH 5.8 and 7 are shown in Figure 3-5. The sorption of TET on soil components increases with increasing acidity. Decreasing the pH increased the cationic character of TC, which may sorbed to soil surfaces by cationic exchange reactions. At pH 5.8, the formation of surface-

Ca-TET complexes is still favored, but stops forming below pH 5 (Myers et al., 1983). Table 3-3 shows that more TET sorbed to Ca-clay-HC at pH 5.8 compared to pH 7 (data not available for K-saturation).

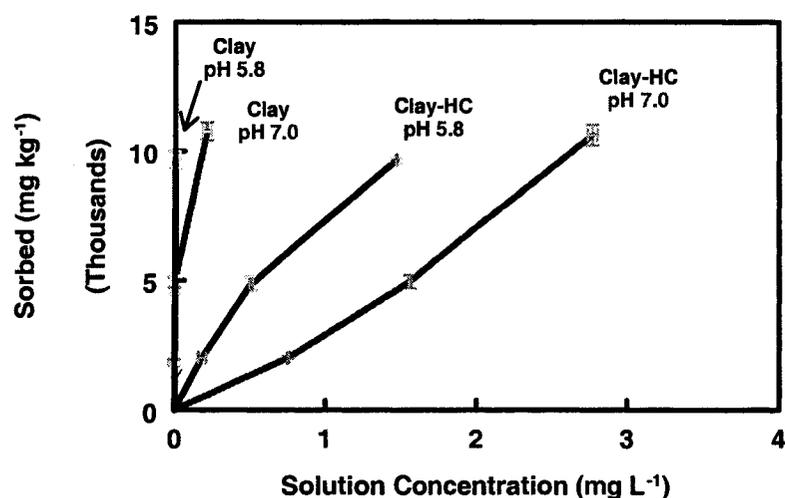


Figure 3-5. Tetracycline sorption isotherms for Nicollet Ca-clay and Ca-clay-HC.

Effect of Soils on TET Sorption

Figure 3-6 shows CTC and TC sorption isotherms on Zook and Nicollet soils at pH 7. The lower TET sorption on Nicollet soil may be due to hydroxyl material or organic carbon content (Table 3-2) in the interlayer of Nicollet smectites. In contrast, the high clay content of the Zook (46%) compared to the Nicollet soil (23%) and the low organic carbon associated with the Zook clay might explain preferential TET sorption to Zook soil. Isotherms are almost linear, suggesting that similar sorption energies are involved. Isotherms from whole soils are most insightful in terms of predicting sorption of TET in natural environments. The results indicated that the Zook soil retains more TET compared to the

Nicollet soil suggesting that TET applied to the Nicollet soil might be more mobile in whole soils.

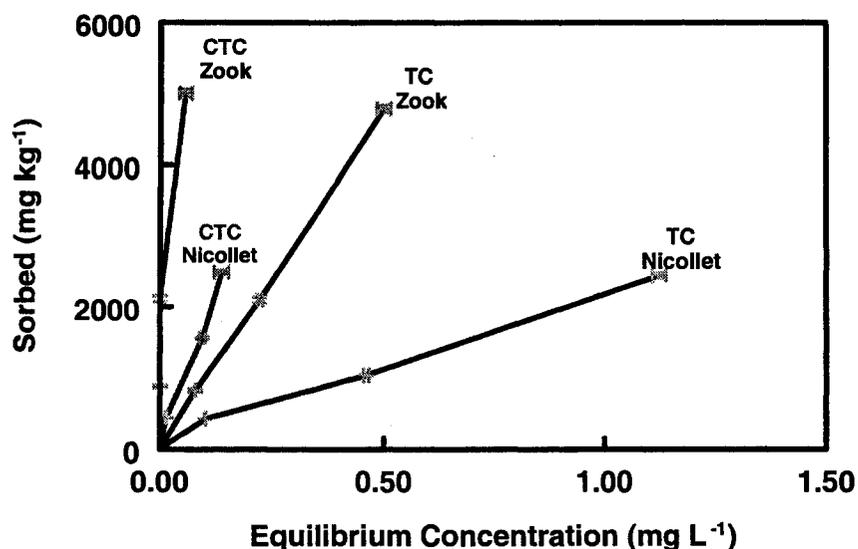


Figure 3-6. Chlortetracycline and tetracycline sorption isotherms for Zook and Nicollet soils.

To elucidate significant interactions effects between soil, soil components, cations, and pH on sorption of TC and CTC, statistical analysis was conducted for all interactions. Table S3-3 lists significant 1- and 2-way interaction effects between variables. Significant 3-way interactions are listed in Table S3-4.

Desorption of TET from Soil Components

Tables 3-5, S3-5, and S3-6 list K_{d-des} values when ~9.75, 4.75, and 1.75 mg g⁻¹ of TET were sorbed to soil components, respectively. Overall, little of the total added TET was desorbed. Desorption trends for Zook and Nicollet soil components decreased in following

order; clay-HC > HS > clay. Tetracycline and CTC were desorbed most from clay-HC, which was consistent throughout the study.

Table 3-5. K_{d-des} values when ~ 9.75 mg g^{-1} TET were sorbed to Zook and Nicollet soil components at pH 7 and pH 5.8.

TC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	7.8×10^4	X
HS	2.2×10^5	1.1×10^4	1.4×10^5	1.2×10^4
Clay-HC	X	2.0×10^4	1.0×10^4	X
CTC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	8.2×10^4	7.5×10^3	9.6×10^4	1.2×10^4
Clay-HC	1.5×10^5	5.6×10^4	X	X
TC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	----	----	----	----
Clay-HC	2.3×10^4	----	1.4×10^4	----
CTC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	---	----	----	----
Clay-HC	X	----	1.0×10^5	----

X = complete sorption

---- = no data

The highest desorption was observed for clay-HC (0.97%), which is crucial as clays and HS are found as complexes in natural soils. To investigate long-term TC release from C-HC, desorption studies were conducted over a period of 36 d (data not shown). When the background solution was replaced every 4 days, desorption of TC from clay-HC ceased after 24 d.

Statistical analysis has been conducted to compare interaction effects between soil, soil components, cations, and pH on CTC and TC desorption and are presented in Tables S3-7 and S3-8.

XRD Analysis of TET treated Soil Components Prepared as Oriented Specimens

Expandable clays, such as smectites, are unique as their d_{001} spacing adjusts depending on the size of intercalated molecules. In order to obtain additional information on the nature of sorption mechanisms, TET treated Zook clay and clay-HC were analyzed by x-ray diffraction. Figure 3-7 shows XRD patterns for TC and CTC sorption onto Zook Ca-clays at pH 7 when equilibrated to 54% R. H. Also shown are XRD patterns after the samples were heated to 105° C for 24 h. This was done to exclude the effect of water on interlayer expansion. The Ca-clay without TC (1A) at 54% R.H. shows a 001 peak at 1.45 nm indicating the presence of approximately two layers of interlayer water molecules. Sorption of 2.1, 5.3, and 10.5 mg g⁻¹ TC to the Ca-clay caused the 001 peak a shift to 1.90 nm (1B, 1C, 1D) and broadening of the peak (1C and 1D). The 1.0 nm illite peak (1A) almost disappeared (1D) due to interlayer expansion. In contrast, after dehydration, the intensity of the 1.0 nm peak increases due to the collapse of interlayers. Upon dehydration, the 1.45 nm peak (1A) shifts to 1.07 nm (2A) due to the removal of interlayer water molecules. Sorption of 5.3 and 10.5 mg g⁻¹ TC (2B and 2C) to the Ca-clay caused the 001 peak to shift to 1.28 nm and to 1.36 nm (2D) when 10.5 mg g⁻¹ TC was sorbed to the clay. In summary, sorption of 10.5 mg g⁻¹ TC, increased the interlayer spacing by 0.45 nm and 0.29 nm under 54% R.H. and dehydrated conditions, respectively. The shift and broadening of the peaks is evidence of TC intercalation. A similar behavior was observed when 1.9 (3B), 4.4 (3C), and 8.9 mg g⁻¹ (3D) of CTC sorbed to the Ca-clay (Figure 3-7). The d_{001} spacing at 54% R. H. increased from 1.45 to 1.95 nm (3A to 3D) and from 1.07 to 1.34 nm for dehydrated samples (4A to 4D). The peak shift of 0.5 nm and 0.27 nm indicated CTC intercalation.

Pinck et al. (1961a) clarified interlayer sorption by applying atom models of several antibiotics and found that amphoteric antibiotics such as oxytetracycline showed a d_{001} spacing of 0.74 nm implying bilayer sorption whereas the dimethylamino group forms

monolayers due to its steric orientation. Interlayer sorption of oxytetracycline (OTC) on Na-montmorillonite at various pHs was studied by Kulshrestha et al. (2004) who found that the montmorillonite expanded from 1.14 to ~ 2.03 nm for pH 1.5 and 5, respectively. Samples were lyophilized before analysis. The authors applied the molecular model HyperChem and obtained the smallest dimension of the OTC to be 0.83 nm. Therefore, the 0.9 nm increase in interlayer spacing was attributed to a tilted intercalation of OTC. Similar results were reported by Porubcan et al. (1978) who studied TC sorption onto montmorillonite at pH 1.5, 5.0, and 8.7. Samples were dehydrated under vacuum for 30 min prior to analysis. The d_{001} spacing without TC measured 0.97 nm and increased to 1.64 nm after TC sorption at pH 1.5 and 5.0. A molecular model that determined the smallest dimension of TC to be 0.63 nm supported tetracycline sorption into the interlayer. However, when increasing the pH to 8.7, the d_{001} spacing increased by only 0.07 nm due to partial interlayer adsorption (Porubcan et al., 1978). In summary, Porubcan et al. (1978) and Kulshrestha et al. (2004) list d_{001} increases upon TC sorption of 0.89 to 0.67 nm at acidic pH and by 0.07 nm at pH 8.7. Our studies show an interlayer expansion of 0.29 nm (2D) for TC and 0.27 nm (4D) for CTC intercalation into Ca-clays under dehydrated conditions at pH 7, which suggests flat lying TET as opposed to the edge on orientation reported previously.

At pH 7, the TET molecule has both a negative and a positive charge at C3 and C4, respectively, which increases the difficulty of adsorbing TET on a predominantly negatively charged clay surface. The molecule has to sterically adjust due the closeness of the positive and negative charges. The neutral area of TET might sorb onto hydrophobic regions within the interlayer. Negatively charged groups will interact with interlayer cations, and positively charged groups may interact with negatively charged surface sites. Further studies have to be conducted to fully elucidate sorption mechanisms upon intercalation of the TET molecules.

The diffusion and sorption of TET into clay interlayer is affected by the presence of HS (Figure 3-8). X-ray patterns for the Zook Ca-clay-HC without TC at 54% R.H. (1A)

shows a d_{001} spacing of 1.96 nm, which remains at 1.96 nm after 1.8 (1B), 4.6 (1C), and 8.0 mg g^{-1} (1D) of TC were sorbed. Similar results were observed after 1.6 (3B), 4.0 (3C), and 8.2 mg g^{-1} (3D) of CTC were sorbed to Zook Ca-clay-HC at 54% R.H. The XRD patterns for Ca-clay-HC without TC (2A) after dehydration show a very broad peak with its center located at 1.13 nm. The interlayer spacing increases to 1.27 nm (2B and 2C) when 1.8 and 4.6 mg g^{-1} TC were sorbed and to 1.40 nm (2D) when 8.0 mg g^{-1} TC were sorbed to Ca-clay-HC. Interlayer water is removed upon heating, thus the increase in d_{001} of 0.27 nm is attributed to the TC diffusion into the interlayer. Calcium-clay-HC without CTC show a broad peak centered at 1.13 nm that, however, only expanded to 1.30 nm after CTC additions (4A to 4D). Overall, the presence of HS did not affect TC sorption, but affected somewhat the intercalation of CTC. Interlayer sorption of TC to Ca-clay (Fig. 3.7; 2D-2A = 0.29 nm) is similar to the Ca-clay-HC systems (Fig. 3.8; 2D-2A = 0.27 nm), whereas CTC sorption increased the d_{001} by 0.27 nm for the clay systems (Fig. 3-7; 4D-4A) and only by 0.17 nm (Fig. 3.8; 4D-4A) for the Ca-clay-HC systems. The 1.0 nm peak is less intense for Ca-clay-HC (Fig. 3-8; 1A to 1D and 3A to 3D) compared to Ca-clay (Fig. 3-7; 1A to 1D and 3A to 3D) under 54% R. H.

To investigate the difference between intercalation of TET as a function of cation saturation, XRD patterns were collected for TC sorption onto Zook K-clays and K-clay-HC (Fig. 3-9). The d_{001} of the K-clay without TC is 1.2 nm (1A) suggesting the presence of one layer of interlayer water molecules. Although the 1.2 nm peak remained at the same position, a slight increase in the shoulder at around 1.4 nm was observed after 1.7 (1B), 5.1 (1C), and 9.8 mg g^{-1} (1D) of TC were sorbed.

Compared to Ca-clay and Ca-clay-HC (Fig. 3-7; 1B to 1D and 3-8; 1B to 1D), when increasing TC concentrations were sorbed, the intensity of the 1.0 nm peak decreases. After dehydration, the 1.2 nm peak disappeared and the 1.0 nm peak increased (Fig. 3-9, 2A). The same trend was observed for TC-K-clays (2B to 2D) however the intensity of the 1.0 nm

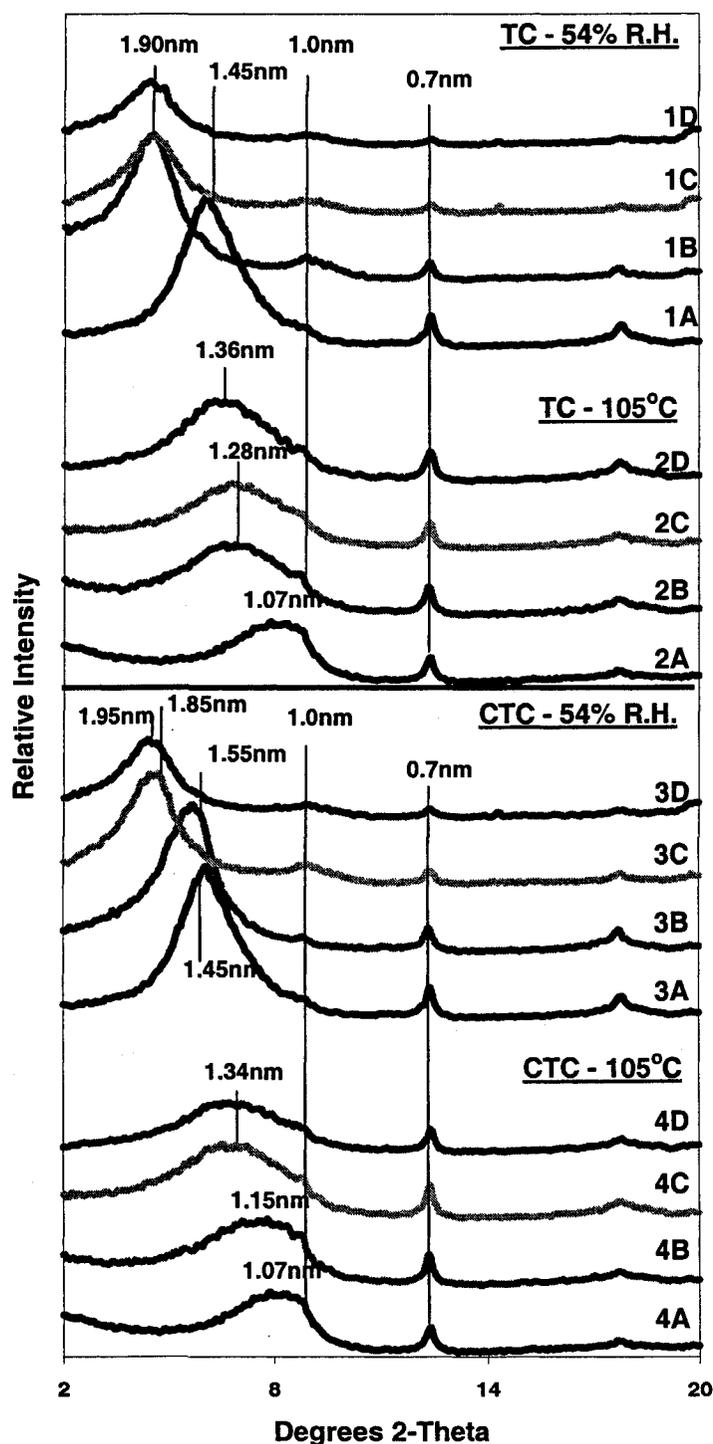


Figure 3-7. X-ray diffractograms of Zook Ca-clays interacted with different concentrations of TET at pH 7 when equilibrated to 54% R. H. and dehydrated (1,2,3,4A, Ca-clay only; 1B-2B, 1C-2C, 1D-2D represent sorption of 2.1, 5.3, and 10.5 mg g⁻¹ TC, respectively; 3B-4B, 3C-4C, and 3D-4D represent sorption of 1.9, 4.4, and 8.9 mg g⁻¹, of CTC, respectively).

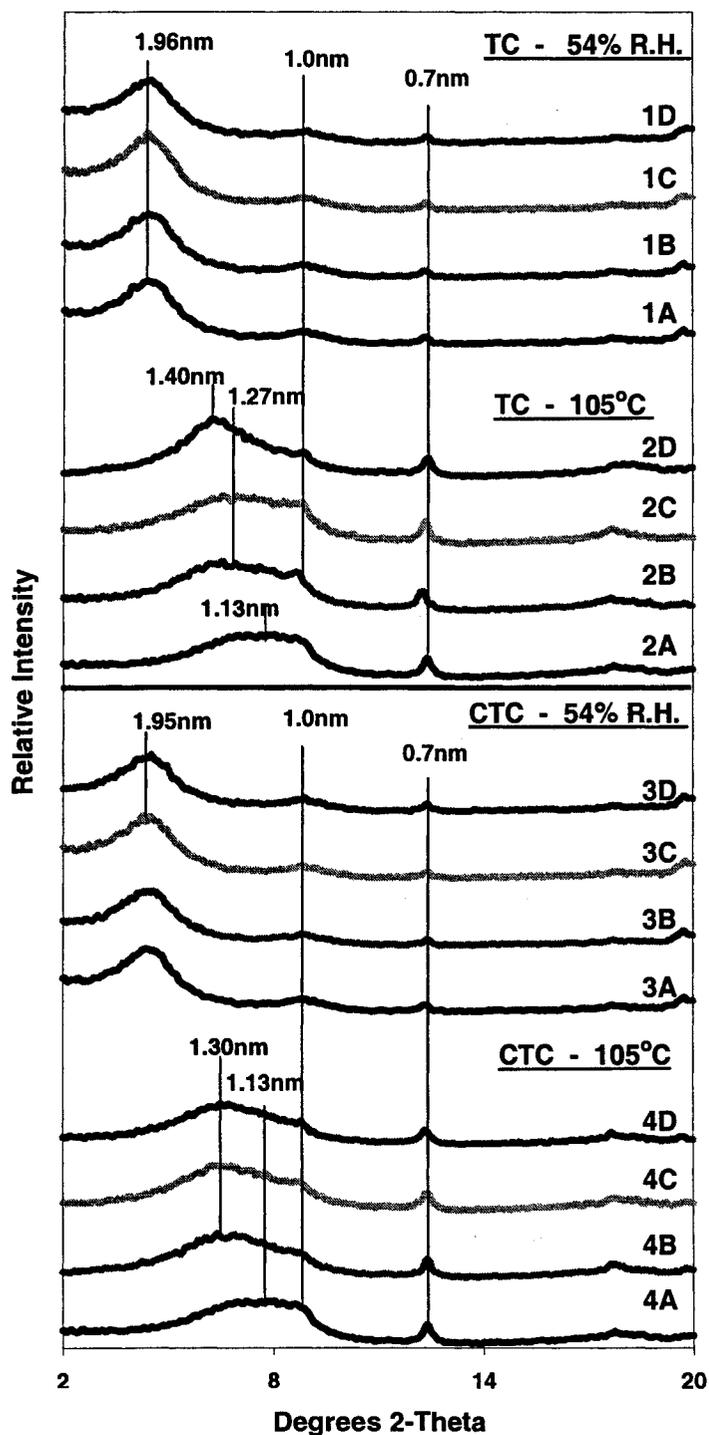


Figure 3-8. X-ray diffractograms of Zook Ca-clay-HC interacted with TET at pH 7 when equilibrated to 54% R. H. and dehydrated (1,2,3,4A, Ca-clay-HC only; 1B-2B, 1C-2C, 1D-2D represent sorption of 1.8, 4.6, and 8.0 mg g⁻¹ TC, respectively; 3B-4B, 3C-4C, and 3D-4D represent sorption of 1.6, 4.0, and 8.2 mg g⁻¹, of CTC, respectively).

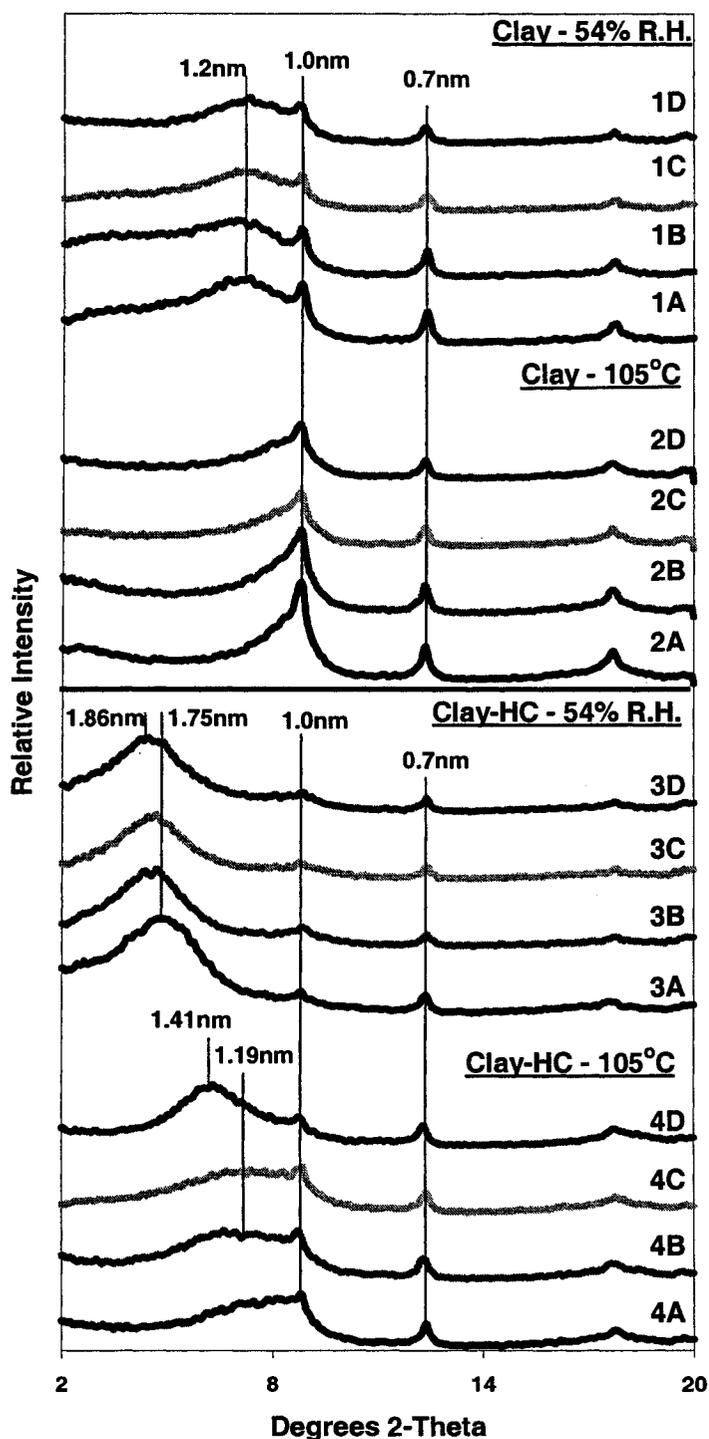


Figure 3-9. X-ray diffactograms of Zook K-clay and K-clay-HC interacted with TC at pH 7 when equilibrated to 54% R. H. and dehydrated (1A-2A, Ca-clay only; 1B-2B, 1C-2C, 1D-2D represent sorption of 1.7, 5.1, and 9.8 mg g⁻¹ TC, respectively; 3A-4B, Ca-clay-HC only; 3B-4B, 3C-4C, and 3D-4D represent sorption of 1.8, 4.6, and 9.4 mg g⁻¹ TC, respectively).

peak was lower due to a shoulder on the left side of the peak. These XRD patterns suggest that although clay is K-saturated, TC was somewhat intercalated.

In contrast to K-clay systems, the XRD pattern of K-clay-HC at 54% R.H. shows a d_{001} of 1.75 nm (Fig. 3-9, 3A), which is slightly less compared to the Ca-Clay-HC (Fig. 3-8; 1A) due to K-saturation. After 9.4 mg g⁻¹ TC was sorbed, the 1.75 nm peak shifted to 1.86 nm (3D). After dehydration, the XRD patterns of the K-clay-HC without TC (4A) shows a small 1.0 nm peak with a broad shoulder which is due to the intercalation of HS. The shoulder remained after 1.8 and 4.6 mg g⁻¹ of TC were sorbed (4B and 4C) and only when 9.4 mg g⁻¹ of TC was sorbed was a distinct 1.41 nm peak observed (4D). Overall, the XRD patterns indicate that the TET were able to diffuse into the interlayer of K-clay and K-clay-HC.

Conclusions

The results of these studies illustrate sorption of CTC and TC on natural soil components. Both TET are highly sorbed onto clay > HS > clay-HC. Highest sorption was observed for CTC at pH 7 and 5.8 and for TC sorption at pH 5.8 onto soil clays. Sorption was mainly attributed to the formation of surface-Ca-TET complexes and interlayer sorption. The largest amount of TC was desorbed from clay-HC followed by HS at pH 7. From an environmental prospective, the significance of TET sorption greatly depends on the persistence and desorption of TET from the soil. The high initial sorption followed by slow desorption especially of TC on and from clay-HC poses a risk for creating a long-term environment for microbes to adjust to sub-therapeutic levels of TET.

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Supplemental Tables

Table S3-1. K_d values after $\sim 4.75 \text{ mg g}^{-1}$ of TET were sorbed to Zook and Nicollet soil components at pH 7 and pH 5.8.

TC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	X	2.6×10^4	2.0×10^4	8.2×10^3
Clay-HC	1.4×10^4	8.5×10^3	3.1×10^3	6.1×10^3
CTC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	X	X	X	1.6×10^4
Clay-HC	1.5×10^4	1.2×10^4	X	X
TC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	----	----	----	----
Clay-HC	1.6×10^3	----	9.4×10^3	----
CTC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	----	----	----	----
Clay-HC	X	----	X	----

X = complete sorption

---- = no data

Table S3-2. K_d values after $\sim 1.75 \text{ mg g}^{-1}$ of TET were sorbed to Zook and Nicollet soil components at pH 7 and pH 5.8.

TC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	X	X	X	X
Clay-HC	1.4×10^4	9.5×10^3	2.6×10^3	6.5×10^3
CTC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	X	X	X	X
Clay-HC	1.2×10^4	1.0×10^4	X	X
TC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	----	----	----	----
Clay-HC	1.3×10^4	----	1.1×10^4	----
CTC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	----	----	----	----
Clay-HC	X	----	X	----

X = complete sorption

---- = no data

Table S3-3. One- and 2-way interaction effects between variables on TET sorption.

No.	Soil	Soil Component	Cation	TET	Soil	Soil Component	Cation	TET	Pr > t
1		Clay-HC				HS			<.0001
2		Clay-HC				Clay			<.0001
3		HS				Clay			<.0001
4			Ca				K		<.0001
5				CTC				TC	0.0014
6	N	Clay-HC			N	HS			0.0021
7	N	Clay-HC			N	Clay			<.0001
8	N	Clay-HC			Z	Clay-HC			<.0001
9	N	HS			N	Clay			<.0001
10	N	HS			Z	HS			<.0001
11	Z	Clay-HC			Z	HS			0.0013
12	Z	Clay-HC			Z	Clay			<.0001
13	N		Ca		N		K		0.0078
14	N		Ca		Z		Ca		0.0006
15	N		K		Z		Ca		<.0001
16	N		K		Z		K		0.0002
17	Z		Ca		Z		K		0.0528
18	N			CTC	N			TC	<.0001
19	N			TC	Z			TC	<.0001
20	Z			CTC	Z			TC	0.0063
21		Clay-HC	Ca			HS	Ca		<.0001
22		Clay-HC	Ca			Clay	Ca		<.0001
23		Clay-HC	K			Clay	K		<.0001
24		HS	Ca			HS	K		<.0001
25		HS	K			Clay	K		<.0001
26		Clay-HC		CTC		Clay-HC		TC	<.0001
27		Clay-HC		CTC		Clay		CTC	<.0001
28		Clay-HC		TC		HS		TC	0.0004
29		Clay-HC		TC		Clay		TC	<.0001
30		HS		CTC		HS		TC	<.0001
31		HS		TC		Clay		TC	<.0001

N = Nicollet soil

Z = Zook soil

Table S3-4. Three-way interaction effects between variables on TET sorption.

No.	Soil	Soil Component	Cation	TET		Soil	Soil Component	Cation	TET	Pr > t
1	N	Clay-HC	Ca		↔	N	HS	Ca		<.0001
2	N	Clay-HC	Ca		↔	N	Clay	Ca		<.0001
3	N	Clay-HC	Ca		↔	Z	Clay-HC	Ca		<.0001
4	N	Clay-HC	K		↔	N	Clay	K		<.0001
5	N	Clay-HC	K		↔	Z	Clay-HC	K		0.0679
6	N	HS	Ca		↔	N	HS	K		<.0001
7	N	HS	K		↔	N	Clay	K		<.0001
8	N	HS	K		↔	Z	HS	K		<.0001
9	Z	Clay-HC	Ca		↔	Z	HS	Ca		0.0092
10	Z	Clay-HC	Ca		↔	Z	Clay	Ca		0.0015
11	Z	Clay-HC	K		↔	Z	HS	K		0.0397
12	Z	Clay-HC	K		↔	Z	Clay	K		0.0002
3	N	Clay-HC		CTC	↔	N	Clay-HC		TC	<.0001
14	N	Clay-HC		CTC	↔	N	Clay		CTC	0.0333
15	N	Clay-HC		TC	↔	N	HS		TC	<.0001
16	N	Clay-HC		TC	↔	N	Clay		TC	<.0001
17	N	Clay-HC		TC	↔	Z	Clay-HC		TC	<.0001
18	N	HS		CTC	↔	N	HS		TC	0.0010
19	N	HS		CTC	↔	N	Clay		CTC	0.0132
20	N	HS		CTC	↔	Z	HS		CTC	0.0419
21	N	HS		TC	↔	N	Clay		TC	<.0001
22	N	HS		TC	↔	Z	HS		TC	0.0004
23	Z	Clay-HC		CTC	↔	Z	HS		CTC	0.0111
24	Z	Clay-HC		CTC	↔	Z	Clay		CTC	0.0030
25	Z	Clay-HC		TC	↔	Z	HS		TC	0.0390
26	Z	Clay-HC		TC	↔	Z	Clay		TC	<.0001
27	Z	HS		CTC	↔	Z	HS		TC	0.0284
28	Z	HS		TC	↔	Z	Clay		TC	0.0448

N = Nicollet soil

Z = Zook soil

Table S3-5. K_{d-des} values after $\sim 4.75 \text{ mg g}^{-1}$ of TET were sorbed to Zook and Nicollet soil components at pH 7 and pH 5.8.

TC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	X	1.7×10^4	X	1.8×10^4
Clay-HC	6.6×10^4	3.1×10^4	1.6×10^4	X
CTC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	X	1.3×10^4	X	1.8×10^4
Clay-HC	X	X	X	X
TC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	----	----	----	----
Clay-HC	3.3×10^4	----	2.0×10^4	----
CTC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	----	----	----	----
Clay-HC	X	----	X	----

X = complete sorption ---- = no data

Table S3-6. K_{d-des} values after $\sim 1.75 \text{ mg g}^{-1}$ TET were sorbed to Zook and Nicollet soil components at pH 7 and pH 5.8.

TC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	X	1.4×10^4	X	1.8×10^4
Clay-HC	X	X	1.8×10^4	X
CTC-pH 7	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	X	1.5×10^4	X	2.0×10^4
Clay-HC	X	X	X	X
TC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	8.3×10^4	X
HS	----	----	----	----
Clay-HC	X	----	2.3×10^4	----
CTC-pH 5.8	Ca-Zook	K-Zook	Ca-Nicollet	K-Nicollet
Clay	X	X	X	X
HS	----	----	----	----
Clay-HC	X	----	X	----

X = complete sorption ---- = no data

Table S3-7. One-, 2-, and 3-way interactions between variables on TET desorption.

No.	Soil	Soil Component	Cation	TET	Soil	Soil Component	Cation	TET	Pr > t
1			Ca				K		<.0001
2				CTC				TC	<.0001
3	N	HS			N	Clay			<.0001
4	N	Clay-HC			N	HS			<.0001
5	N	Clay-HC			N	Clay			<.0001
6	Z	Clay-HC			Z	HS			<.0001
7	Z	Clay-HC			Z	Clay			<.0001
8	Z	HS			Z	Clay			<.0001
9	Z		Ca		Z		K		<.0001
10		Clay-HC	Ca			HS	Ca		0.0054
11		Clay-HC	Ca			Clay	Ca		<.0001
12		Clay-HC	K			HS	K		<.0001
13		HS	Ca			Clay	K		<.0001
14		HS	K			HS	K		<.0001
15		Clay-HC		CTC		HS		CTC	<.0001
16		Clay-HC		CTC		Clay-HC		TC	<.0001
17		Clay-HC		TC		Clay		TC	<.0001
18		HS		CTC		Clay		CTC	<.0001
19		HS		TC		Clay		TC	<.0001
20			Ca	CTC			K	CTC	<.0001
21			Ca	TC			K	TC	<.0001
22	N	Clay-HC	Ca		N	Clay-HC	K		0.0005
23	N	Clay-HC	Ca		N	HS	Ca		0.0097
24	N	Clay-HC	Ca		N	Clay	Ca		<.0001
25	N	Clay-HC	K		N	HS	K		<.0001
26	N	HS	Ca		N	HS	K		<.0001
27	N	HS	K		N	Clay	K		<.0001
28	Z	Clay-HC	K		Z	HS	K		<.0001
29	Z	HS	Ca		Z	HS	K		<.0001
30	Z	HS	K		Z	Clay	K		<.0001
31		Clay-HC	Ca	CTC		Clay-HC	Ca	TC	<.0001
32		Clay-HC	Ca	TC		Clay-HC	K	TC	0.0083
33		Clay-HC	Ca	TC		HS	Ca	TC	<.0001
34		Clay-HC	Ca	TC		Clay	Ca	TC	<.0001
35		Clay-HC	K	CTC		HS	K	CTC	<.0001
36		Clay-HC	K	TC		HS	K	TC	<.0001
37		HS	Ca	CTC		HS	K	CTC	<.0001
38		HS	Ca	TC		HS	K	TC	<.0001
39		HS	K	CTC		Clay	K	CTC	<.0001
40		HS	K	TC		Clay	K	TC	<.0001

N = Nicollet soil

Z = Zook soil

Table S3-8. Four-way interaction effect between variables on TET desorption.

No.	Soil	Soil Component	Cation	TET		Soil	Soil Component	Cation	TET	Pr > t
1	N	Clay-HC	Ca	C	↔	N	Clay-HC	Ca	TC	<.0001
2	N	Clay-HC	Ca	TC	↔	N	Clay-HC	K	TC	<.0001
3	N	Clay-HC	Ca	TC	↔	N	HS	Ca	TC	<.0001
4	N	Clay-HC	Ca	TC	↔	N	Clay	Ca	TC	<.0001
5	N	Clay-HC	Ca	TC	↔	Z	Clay-HC	Ca	TC	<.0001
6	N	Clay-HC	K	CTC	↔	N	HS	K	CTC	<.0001
7	N	Clay-HC	K	TC	↔	N	HS	K	TC	<.0001
8	N	HS	Ca	CTC	↔	N	HS	K	CTC	<.0001
9	N	HS	Ca	TC	↔	N	HS	K	TC	<.0001
10	N	HS	Ca	TC	↔	Z	HS	K	TC	<.0001
11	N	HS	K	CTC	↔	N	HS	Ca	CTC	<.0001
12	N	HS	K	CTC	↔	N	Clay	K	CTC	<.0001
13	N	HS	K	TC	↔	N	Clay	K	TC	<.0001
14	Z	Clay-HC	K	CTC	↔	Z	HS	K	CTC	<.0001
15	Z	HS	Ca	CTC	↔	Z	HS	K	CTC	<.0001
16	Z	HS	Ca	TC	↔	Z	HS	K	TC	<.0001
17	Z	HS	K	CTC	↔	Z	Clay	K	CTC	<.0001
18	Z	HS	K	TC	↔	Z	Clay	K	TC	<.0001
19	Z	HS	K	CTC	↔	Z	Clay	K	CTC	<.0001

N = Nicollet soil

Z = Zook soil

CHAPTER 4. DEGRADATION OF TETRACYCLINE SORBED ON CA-SATURATED WHOLE SOIL, SOIL CLAY-HUMIC COMPLEXES, AND SOIL CLAY

A paper to be submitted in Chemosphere

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Abstract

Tetracycline (TC) used extensively in animal husbandry, poses the threat of breeding antibiotic resistant bacteria. Previous work involving batch equilibrium studies show that > 96% of added TC was sorbed to and less than 1% was desorbed from Ca- and K- saturated soil components. In this study, TC degradation studies were conducted to determine antimicrobial activity of sorbed TC and its ability to inhibit soil bacteria, to investigate whether sorbed TC represents a long term sink for slow TC release, and to elucidate efficiency of resistant bacteria to degrade sorbed TC. Test plate studies indicated that sorbed TC inhibits soil bacteria, most likely due to slow TC release from soil components. Tetracycline in the aqueous phase degraded faster for the biotic systems than the abiotic system and in decreasing order for soil clay > soil clay-humic complexes (clay-HC) > soil (WS). Biotic and abiotic degradation results using ³H-TC showed increasing tritium and decreasing TC concentrations in solution during the 149-day incubation period and indicated that loosely sorbed (labile) TC pool (< 16% of added TC) is available for both biotic and abiotic degradation. The labile TC appeared to be associated with the clay fraction and increased with increasing clay content; clay > clay-HC > WS. Most of the adsorbed TC (84 to 92% of added TC) was retained by the soils and soil components in a form that was not released during the 149 day incubation.

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Introduction

Over the past decade, increasing concern about the development of antibiotic resistant bacteria has raised questions about the fate and bioavailability of antibiotics in natural soils-water systems. In the US, approximately 50 million pounds of antibiotics are produced annually of which 50% are used by humans and 50% are used in agricultural applications (Levy, 2002). Antibiotics such as oxytetracycline (OTC) and chlortetracycline (CTC) have been widely used in animal husbandry for more than 50 years. In animal husbandry TC, OTC and CTC are used predominantly to promote weight gain and to a lesser proportion for disease prevention and control (Mellon et al., 2001).

Whether antibiotics are administered to humans or livestock, large amounts of antibiotics are excreted as parent compounds. The amounts of administered antibiotic excreted as the parent compound varies depending on the chemistry of the compound and the dosage (Kümmerer, 2000). Kroker (1983) suggests that up to 90% of an administered dose of tetracyclines (TET) to humans and livestock may be excreted through urine and feces. Antibiotics given to humans cannot be completely removed through sewage treatment and are often found in sewage sludge and effluent (Stumpf et al., 1999; Ternes et al., 2001). Tetracyclines in surface waters have been detected in levels as high as $1 \mu\text{g L}^{-1}$ (Watts et al., 1983).

Most TET administered to livestock enter the environment through manure applications (Seiler et al., 1999). Halling-Sørensen et al. (1998) stated that many antibiotics administered to livestock are excreted unchanged or as toxic or bioactive metabolites. Overall, residues in feces, urine and food samples consist of at least 80% of the parent drug (Jjemba, 2002) and their metabolites. The amount of TC associated with manure varies greatly. Tetracycline concentrations in manure have been reported in the range from 0.6 to 66 mg L^{-1} (Lee et al., 2000) up to 200 mg L^{-1} (Hirsch et al., 1999). Soils receiving frequent

manure application showed tetracycline concentration of $12 \mu\text{g kg}^{-1}$ (Hamscher et al., 2000) to $100 \mu\text{g kg}^{-1}$ (Klages and Roth, 2001).

Once TET are applied to soil systems, they are subject to degradation processes. The persistence of antibiotics in the terrestrial environment ranges from less than one day to months and is controlled by factors such as temperature, chemical structure of the antibiotic (Gavalchin and Katz, 1994), sensitivity to light (Burhenne et al., 1997; Halling-Sørensen et al., 2003), presence of oxygen, and the microbes present. The two major abiotic degradation pathways appear to be photolysis and hydrolysis. Oxytetracycline was photodegraded within 21 days in seawater at a depth of 1 m (Lunestad et al., 1995). Yet, Thiele-Bruhn (2003) found little evidence of antibiotic photolysis in land applied sludge and slurry samples. Recent studies have shown that hydrolysis can also transform tetracyclines in soil environments, but much work remains in determining the potential of this transformation process (Halling-Sørensen, 2000; Thiele-Bruhn et al., 2003).

The soil pH plays an important role in the stability of antibiotics. Chlortetracycline and TC are unstable in alkaline soils, whereas OTC is stable under these conditions (Pinck et al., 1961). After manure application the pH of soils increases temporarily to 9 (Choudhary et al., 1996; De la Torre et al., 2000), which creates a suitable environment for TC to be transformed to iso-tetracyclines (Kühne et al., 2000).

In a strange twist of fate, the best approach for removing antibiotics from the environment often comes from the very thing they were designed to inhibit: microorganisms. Some microbes are able to utilize recalcitrant antibiotics in the environment for energy and are capable of breaking down the antibiotic structures through degradation mechanisms involving enzymatic transformation reactions such as oxidative decarboxylation and hydroxylation (Chen et al., 1997; McGrath et al., 1998). Tetracycline biodegradation with up to 100% removal have been demonstrated in a number of manures and manure amended soils

(Kühne et al., 2000; Winckler and Grafe, 2001; Jagnow, 1977, Runsey et al., 1977; Galvalchin and Katz, 1994).

However, antibiotic biodegradation is not without its potential problems. For example, there was little or no biodegradation success in a wide range of TC contaminated soils without the aid of manure amendments (Hamscher et al., 2001; van Gool, 1993). Samuelsen et al. (1994) and Gavalchin and Katz (1994) determined that biodegradation was not effective when antibiotic compounds were fixed to surfaces or in the soil matrix where they can persist for months (van Gool, 1993; Höper et al., 2002). The same has been found in aquatic sediments where OTC was only slightly degraded over a period of 180 days (Samuelsen et al., 1994; Hektoen et al., 1995).

One of the major concerns of TET in natural environments is the formation of often toxic degradation products such as anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC) (Kühne et al., 2001). The formation of TC degradation products is mainly influenced by the pH. Anhydrotetracycline is formed when $\text{pH} < 7.5$ (Schneider, 2001) and has several deleterious biological effects. *In vivo* they are phototoxic and hepatotoxic and may cause the development of anemia (Breen et al., 1972; Jones, 1973). The reversible formation of 4-Epi-TC occurs between pH 3 and 6. When the soil $\text{pH} > 7.5$, TC is metabolized to iso-tetracyclines (Kühne et al., 2000) and will be rapidly decomposed into smaller fragments (Hlavka and Boothe, 1985).

The chemical structure of TC is shown in Figure 4-1. Tetracycline has three ionizable functional groups that determine the overall charge of the molecule as a function of pH. The tricarbonylamide system has a $\text{p}K_{\alpha 1}$ of 3.3. The term tricarbonylamide is used to emphasize the strong coupling of the C1, C2, and C3 complex rather than considering this group as an amide and two independent carbonyls (Myers et al., 1983). Interactions between the functional groups located at C1, C2, and C3 influence the $\text{p}K_{\alpha}$ with the actual protonation/deprotonation occurring at C3. The second ionizable group ($\text{p}K_{\alpha 2} = 7.7$), is the

phenolic diketone moiety (C10, C11, and C12) with protonation and deprotonation occurring predominantly at C11 and C12. The quaternary ammonium has a pK_{a3} of 9.7 (C4).

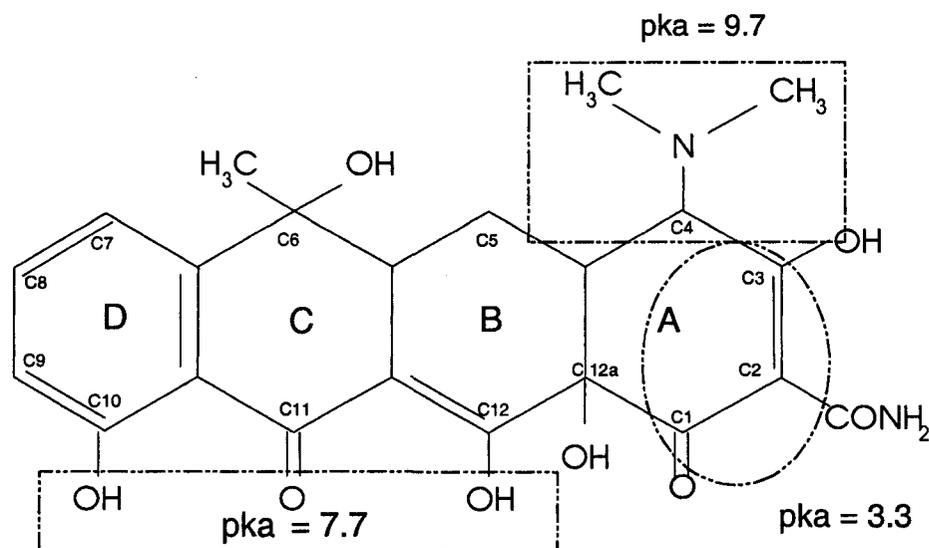


Figure 4-1. Functional groups of tetracycline.

Table 4-1 shows the charge distribution of tetracycline depending on its pH. At a pH below 3.3, tetracycline exhibits an overall positive charge, whereas between pH 3.3 and 7.7 the compound is a zwitterion. Between pH 7.4 and 9.7, tetracycline has a net charge of negative one and above pH 9.7 it has two negative charges.

Table 4-1. Overall charge of tetracycline as a function of pH.

Functional Groups	pH			
	< 3.3	3.3 - 7.7	7.4 - 9.7	> 9.7
C3-OH	0	-	-	-
C11-O and C12-OH	0	0	-	-
C4-NH ⁺ (CH ₃) ₂	+	+	+	0

To the authors' knowledge, little or no scientific work has been conducted focusing on aerobic biodegradation of TC sorbed on whole soils, clays, and clay-humic complexes. Based on the above information we hypothesize that sorption of TC onto soil components regulates aerobic degradation. Therefore we present studies to evaluate biotic and abiotic degradation of TC sorbed onto whole soils, clays, and clay humic complexes under aerobic conditions.

Materials and Methods

Chemicals

Calcium chloride, KCl, HCl, KOH, Ca(OH)₂, H₂O₂, KH₂PO₄, and K₂HPO₄ were of certified A.C.S. grade. Tetracycline-HCl (Purity > 98%), was obtained from MP Biomedicals (Irvine, California, USA) and stored at -20° C. Anhydrotetracycline-HCl (Purity > 97%), 4-epi-anhydrotetracycline-HCl (Purity > 97%), and 4-epi-tetracycline-HCl (Purity > 97%) were obtained from Fisher Scientific and stored at -20° C. Tritium labeled (Figure 4-1, labeled on C7) tetracycline (³H-TC) with a purity of > 95%, stored at 4° C, and Ultima Gold XR Liquid Scintillation Cocktail were obtained from Perkin-Elmer (Boston, Massachusetts, USA). Acetonitrile (ACN) and phosphoric acid (85%), both obtained from Fisher Scientific, were of HPLC grade. BD Difco™ nutrient broth, BD Difco™ nutrient agar and BD Difco™ LB-Broth (Miller, Luria-Bertani) were obtained from Fisher Scientific. Tritium cocktail was obtained from R. J. Harvey Instrument Corporation (Hilldale, New Jersey, USA). Milli-Q water was used throughout the study.

Whole Soil (WS)

A sample of Zook soil (*fine, smectitic, mesic Cumulic Vertic Endoaquoll*) was collected from the surface horizon (0-40 cm) of a pedon in Story County, Iowa. The soil was air-dried, crushed, sieved through a 2 mm sieve, and stored at 4°C.

Soil Clay-Humic Complexes (clay-HC)

Soil clay humic complexes ($\leq 2 \mu\text{m}$) were separated from the Zook soil by sedimentation. To do so, soil was dispersed in distilled water by shaking and the upper 10 cm of soil suspension was collected by siphoning. The depth of siphoning was determined daily depending on the ambient temperature. Suspensions of clay-HC were centrifuged at $2413 \times g$ for 20 min and Ca-saturated as described below.

Soil Clay (clay)

The soil clay sample was obtained by oxidizing organic matter associated with clay-HC. Approximately 200 mg of clay-HC were added to 500 mL Pyrex™ glass beakers, digested with 20 mL of 30% H_2O_2 and placed on an unheated sand bath. After the reaction slowed down, the temperature was slowly increased until frothing occurred. Hydrogen peroxide was periodically added until the reaction was complete. After frothing stopped, the temperature was raised to the boiling point to remove excess H_2O_2 . Samples were transferred to 250 mL Nalgene™ high-density polyethylene bottles and diluted to 200 mL with water. Samples were placed on a flat bed shaker at $150 \text{ cycles min}^{-1}$. After 24 h, samples were centrifuged at $2413 \times g$ for 20 min and supernatant was decanted. The washing process was repeated two more times with 1 h shaking periods. After decanting the supernatant, the samples were immediately cation saturated as described below.

Cation Saturation of Soil Components

Clay-HC and clay were saturated with Ca^{2+} . To do so, the soil components were washed with 200 mL of 0.5 M CaCl_2 . Samples were agitated on a flat bed shaker at $150 \text{ cycles min}^{-1}$. After 24 h samples were centrifuged at $2413 \times g$ for 20 min and the supernatant was decanted. The process was repeated two more times with an agitation period of 1 h. To remove the excess salts, the Ca-saturated samples were dialyzed against water until the silver

nitrate test for chloride was negative. Clay-HC and clay were adjusted to pH 7. To do so, 0.01 M HCl or saturated Ca(OH)₂ was added until desired pH was reached. Samples were allowed to equilibrate and the process was repeated until the pH was stable. All samples were freeze-dried using a Labconco Freeze Dryer 4.5 (Kansas City, Missouri, USA) and stored in Nalgene™ high-density polyethylene bottles at room temperature until used.

Preparation of Oriented Specimens for Mineralogical Characterization by XRD

Approximately 150 mg of clay-HC were washed with 20 mL of 1 M KCl and 20 mL of 0.5 M MgCl₂, vortexed for 30 s and agitated for 24 h on a flat bed shaker at 150 cycles min⁻¹. Samples were centrifuged at 1710 x g for 5 min and the supernatant was decanted. This process was repeated two more times with an agitation period of 1 h. After the final wash approximately 15 mL of the various clay suspensions were pipetted onto ceramic tiles and water was removed by applying a vacuum to the base of the tile. Excess salt was removed by using vacuum to pull ~10 mL of water through the tiles. Tiles were equilibrated at 54% relative humidity (R.H.) for 24 h before the XRD analysis. After removal from the desiccator the K⁺ and Mg²⁺ saturated samples were analyzed by XRD at ambient temperature. The Mg-saturated samples were solvated with ~3 mL glycerol. After excess glycerol drained off, the samples were equilibrated for 24 h above glycerol and then analyzed again by XRD. Potassium saturated samples were analyzed by XRD after heating for 24 h to 105°, 305°, and 545° C. Samples were analyzed with a Siemens D5000 x-ray diffractometer using CuKα radiation. The step size was 0.02 degrees 2-Theta with a speed of 2 degrees/min. X-ray patterns were collected from 2 to 32 degrees 2-Theta.

Procedure for Isolating Microbes from Zook Soil

In order to elucidate degradation potential of sorbed TC, it was necessary to find one microbe that was both tetracycline resistant and a tetracycline degrader. The microbes were

isolated from a sample of field moist Zook soil that had been previously stored in plastic bags at 4° C. An enrichment culture was prepared by adding 5 g of Zook soil to 100 mL of nutrient broth. The flask was incubated for 16 h at 27° C using a Queue orbital shaker (Queue Systems Inc., Asheville, North Carolina, USA) set at 110 rpm. After the incubation, turbidity observed in the flask indicated substantial microbial growth. One mL from the Zook soil enrichment culture was harvested and added to fresh 100 mL nutrient broth, incubated under the same conditions as mentioned above. After the incubation period, cells from the second-generation enrichment culture were harvested as follows. The enrichment culture was transferred to a 25 mL Corex® glass centrifuge tube, centrifuged at 1710 x g for 15 min and the supernatant was decanted. The cell pellet was re-suspended in 25 mL of 0.0125 M potassium phosphate buffer (pH 7.1) and vortexed for 30 s. The potassium phosphate buffer was prepared by adding 8.9 mL of 0.5 M KH₂PO₄ and 16.1 mL of 0.5 M K₂HPO₄ to 1000 mL of water. The cell extracts were centrifuged at 1710 x g for 15 min and the supernatant was decanted. This washing process was repeated two more times. After decanting the supernatant of the last wash, extracts were re-suspended in 25 mL of fresh potassium phosphate buffer and vortexed for 30 s. Before storing cell extracts at 4° C, a 1 mL sub-sample was drawn and serial-diluted to 10⁻⁶ to determine the microbial population of this enrichment culture. Whole spread plates were prepared by spreading 0.1 mL of cell culture on nutrient agar which were incubated as described above. Cells were counted 16 h later and the population of the enrichment culture was approximately 5.3 x 10⁹. Enrichment culture was used within 1 day.

Procedure for Testing Resistance of Microbes to Tetracyclines

All glassware and 0.005 M CaCl₂ solutions were autoclaved at 121° C for 20 min. All lab work related to the incubations was done under aseptic conditions and conducted at 23±1° C.

Procedure to Determine the Effect of TC in Solution on Isolated Soil Microbes

The enrichment culture was serial-diluted to 10^5 cells mL^{-1} . An aliquot (0.1 mL) of the diluted enrichment culture was spread on nutrient agar plates and allowed to dry for 10 min. A cork borer with a diameter of 1 cm was used to remove a plug of nutrient agar in the middle of the plate. Approximately, 1 mL 0.005 M CaCl_2 containing 0.01, 0.05, 0.10, 0.50, and 1.0 mg TC were added to the well of the nutrient agar plate such that the solution was in full contact with the sidewall of the nutrient agar. The plates were prepared in triplicates and incubated at 35°C for ~16 h and then the effect of various TC concentrations on microbial growth was determined by measuring the distance of microbial clearing from the rim of the well.

Procedure to Determine the Effect of TC Sorbed on Clay on Isolated Soil Microbes

Approximately 200 mg of autoclaved clay in a Nalgene™ Oak Ridge polypropylene copolymer tubes were suspended in 10 mL of 0.005 M CaCl_2 . The samples were vortexed for 30 s and agitated on a flat bed shaker for 24 h at $150\text{ cycles min}^{-1}$. One and 5 mL of a 20 mg L^{-1} and 1, 5, and 10 mL of a 200 mg L^{-1} TC stock solutions were added and then the samples were diluted to 20 mL with 0.005 M CaCl_2 resulting in a final TET concentration of 1, 5, 10, 50, and 100 mg L^{-1} TC. To ensure complete sorption, the samples were vortexed for 30 s and agitated on a flat bed shaker at $150\text{ cycles min}^{-1}$ for 24 h. The samples were then centrifuged at 4747 x g for 10 min. HPLC analysis of supernatants showed that $\geq 99\%$ of the added TC were sorbed to the clay. The pelleted clay was re-suspended in 2 mL of 0.005 M CaCl_2 . This sorption process was triplicated for each concentration.

A 0.1 mL aliquot of the enrichment culture as used above was spread on nutrient agar plates and allowed to dry for 10 min. A cork borer with a diameter of 1 cm was used to remove a plug of nutrient agar in the middle of the plate. One mL of clay suspension containing 0.01, 0.05, 0.10, 0.50, and 1.0 mg TC were added to the well of the nutrient agar

plate. The suspension was in full contact with the sidewall of the nutrient agar. The plates were prepared in triplicates and incubated at 35° C for ~16 h to assess the effect of sorbed TC-clay on microbial growth.

Colonies growing close to the source of TC-clay were picked, inoculated and allowed to grow for 16 h in 100 mL nutrient broth placed on an orbital shaker at 110 rpm. The turbid suspension was centrifuged at 1710 x g for 15 min and the supernatant was decanted. The cell extracts were prepared by adding 25 mL of 0.0125 M potassium phosphate buffer (pH 7.1) and vortexed for 30 s. This washing process was repeated two more times. After decanting the supernatant of the last wash, extracts were re-suspended in 25 mL of fresh potassium phosphate buffer and vortexed for 30 s. The cell extracts were stored at 4° C and were used within 1 day. This inoculum, labeled as M1103, was tested for its ability to degrade TC. To ensure uniform cells, M1103 was serial-diluted to 10⁵ cells mL⁻¹ and 0.1 mL was spread on nutrient agar plates. After incubation, growing cells were determined to be identical based on visual comparison. The gram-staining test confirmed Gram-negative microbes.

Testing the Ability of M1103 to Degrade Tetracycline in Solution

To test the efficiency of M1103 to degrade TC in solution, M1103 was added to a TC solution. To do so, 2 mL of the 200 mg L⁻¹ TC stock solution was added to 70 mL 0.005 M CaCl₂ and 30 mL LB-Broth (pH 7). The final TC concentration in solution was ~4 mg L⁻¹. This concentration was chosen as approximately 97% of 100 mg L⁻¹ TC added to WS was sorbed and 3% was left in the aqueous phase. The inoculum of M1103 was serial-diluted to 10⁵ cells mL⁻¹ and a 1 mL sub-sample was added to the 4 mg L⁻¹ TC solution. The triplicated samples were placed on a rotary shaker at 110 rpm for the duration of the degradation experiment. Sub-samples were collected on days 1, 2, 5, 9, 15, and 21 and subsequently analyzed for TC by HPLC.

Procedure for Abiotic and Biotic Degradation of Sorbed TC

Studies were conducted to elucidate aerobic degradation of sorbed TC for biotic and abiotic systems. To do so, a stock solution was prepared by adding 250 μCi ^3H -TC to 0.005 M CaCl_2 and made up to 400 mg L^{-1} with unlabeled TC. This stock solution was filter-sterilized. Analyzing the stock solution by a Liquid Scintillation Analyzer gave a tritium counting efficiency of 75%. Reported experiment results are based on the actual counting efficiency.

All tests were carried out using 100 mL of suspension in 250-mL Erlenmeyer flasks. Approximately 2.15 g of WS and 1 g of clay and clay-HC were added to Pyrex™ Erlenmeyer flasks. The amount of soil used was based on the clay content of the Zook soil. Flasks were plugged with a foam stopper and covered with aluminum foil to prevent light penetration. Samples were autoclaved at 121° C for 20 min. This autoclave procedure was repeated on 4 consecutive days. For pre-saturation of soil components, 45 mL of 0.005 M CaCl_2 was added to each flask. Flasks were placed on a rotary shaker for 24 h at 110 rpm. After 24 h, 30 mL of CaCl_2 and 25 mL of the ^3H -TC stock solution were added to the abiotic pre-saturated samples. Thirty mL of LB-Broth and 25 mL of the ^3H -TC stock solution were added to the biotic samples. LB-Broth was not added to the abiotic samples to prevent contamination. The final volume for all samples was 100 mL containing 100 mg L^{-1} TC and 6.22 μCi ^3H -TC. Biotic and abiotic blanks without soil components were prepared with 50 mg L^{-1} and 100 mg L^{-1} TC, respectively. Controls without TC were prepared for WS, clay-HC, and clay. All samples were triplicated.

Samples were allowed to equilibrate on an InnOva™ Model 2300 Platform shaker (New Brunswick Scientific, Edison, New Jersey, USA) at 110 rpm. After a sorption equilibration time of 24 h, 5 mL aliquots samples were removed from each flask, centrifuged at 4747 x g for 10 min, prepared as described below, and subsequently analyzed by HPLC to

determine the amount of TC sorbed (Day 1). Following the sampling procedure, a M1103 population of 8.0×10^8 cells was added to the biotic samples.

Sampling Procedure

The samples were incubated at $23 \pm 1^\circ \text{C}$ on a rotary shaker at 110 rpm. Flasks were plugged with a foam stopper and covered with aluminum foil that allowed aeration of the samples. Sampling was conducted on day 1, 4, 8, 12, 18, 25, 30, 45, 85, and 149. Five mL of suspension was removed with a sterile BD 20G 1½ Precision Glide® needle attached to BD 5 mL disposable syringe with Luer-Lock™ Tip. The samples were centrifuged at $4747 \times g$ for 10 min. Collected supernatants were passed through a Fisherbrand™ non-sterile, nylon, 25 mm syringe filter with $0.2 \mu\text{m}$ pore openings. The supernatants were additionally cleaned by filtering samples through Waters Oasis® HLB (Waters Corporation, Milford, Massachusetts, USA) solid phase extraction cartridges. Throughout the filtration processes the cartridge was never allowed to dry and the flow rate was kept constant at 1 mL min^{-1} . The cartridges were conditioned with $3 \times 1 \text{ mL}$ of methanol followed by $3 \times 1 \text{ mL}$ of water. Four $\times 1 \text{ mL}$ of supernatant were added. Tetracycline sorbed onto the cartridge was eluted with $3 \times 1 \text{ mL}$ 100% ACN. The eluted ACN containing TC was allowed to evaporate in a water bath heated to $\sim 50^\circ \text{C}$ under a constant stream of argon. Tetracycline was re-suspended in 1 mL of starting solution for HPLC analysis (10% solvent A and 90% solvent B, described below). To provide a guideline of TC recovery, triplicates of controls with 100 mg L^{-1} TC in 0.005 M CaCl_2 were passed through the cartridges using the above described methodology. The analysis indicated a TC recovery of $99.4 \pm 0.45\%$.

Chemical Analysis

Tetracycline and TC-metabolites were determined by HPLC and ^3H -TC was counted with a LSC.

HPLC Analysis

TC Analysis

For HPLC analysis, 0.5 mL of the sample was added to 1.5 mL amber glass vials. Non-salinized amber glass vials were used, as preliminary data showed no significant differences using salinized versus non-salinized vials. Therefore, it was assumed that an insignificant amount of TC was sorbed onto the glass surface. An Agilent 1100 Liquid Chromatograph (Agilent Technologies, Inc, Palo Alto, California, USA) was used with a 5 μm packing and 100 \AA pore size Polymeric Reversed Phase Column (PLRP-S). The column was heated to 40° C during analysis and the flow rate was of 0.5 mL min^{-1} . Method development was based on findings of White et al. (1993) with modifications based on the amount of sample and the HPLC unit used. Analysis was done with a gradient elution over 16 min. The mobile phase consisted of acetonitrile (Solvent A) and Milli-Q water adjusted to pH 2.5 with H_3PO_4 (Solvent B). The gradient run was 10-100% A from 0-12 min, held constant at 100% A for 2 min and then run back down to 10% A over a 2 min period. Using this procedure TC eluted after 8.1-8.2 min. Solutes were detected using a diode array detector with the analytical wavelength set at 360 nm and the reference wavelength at 410 nm. Background interfered with peak quality at shorter wavelengths. Tetracycline showed good linearity between 200 $\mu\text{g L}^{-1}$ and 10 mg L^{-1} in 0.005 M CaCl_2 (pH 7) with a $R^2 = 0.999$. Based on a 3:1 signal-to noise ratio, the limit of detection was 200 $\mu\text{g L}^{-1}$.

Metabolite Analysis

Tetracycline metabolites were analyzed using the same HPLC, mobile phases, and gradient run as described above. Anhydrotetracycline ($R^2 = 0.999$), 4-epi-anhydrotetracycline ($R^2 = 0.999$), and 4-epi-tetracycline ($R^2 = 0.999$) were linear between 100 $\mu\text{g L}^{-1}$ and 10 mg L^{-1} in 0.005 M CaCl_2 (pH 7).

Liquid Scintillation Analysis

For analyzing ^3H -TC concentration in solution, 8 mL of Ultima XR cocktail were added to 0.5 mL of sample and analyzed using a Packard Tri-Carb Liquid Scintillation Analyzer Model 1900TR (Packard Instrument Company (now Perkin Elmer), Boston, Massachusetts, USA).

Oxidation of Soil Components

To account for the tritium sorbed on soil components, air-dried duplicate samples were combusted within an OX-500 biological material oxidizer (R. J. Harvey Instrument Corporation, Hillsdale, New Jersey). The tritiated water vapor was trapped directly in scintillation vials containing the ^3H -cocktail trapping solution. Samples were immediately analyzed with LSC.

Results and Discussion

Chemical and Mineralogical Analysis

The soil used in this study is a fine texture Mollisol from Central Iowa. The untreated Zook soil has a pH of 6.8 and a CEC of $36.2 \text{ cmol}_c \text{ kg}^{-1}$. The soil is dominated by clay (46%), followed by silt (41.4%), and sand (12.6%).

The mineralogy of the clay separated from the Zook soil was also typical for Midwestern Mollisols. Figure 4-2 shows XRD patterns for Mg- and K-saturated Zook clay-HC. The 1.4 nm peak in the XRD pattern for the Mg-saturated sample expands to 1.8 nm after glycerol salvation indicating the presence of smectite. The sample also contained illite as evidenced by the 1.0 nm peak on the XRD patterns for the Mg-saturated sample, and kaolinite as evidenced by the 0.72 nm peak that disappeared after heating to 545° C . Smectite in air-dried K-saturated samples at 25° C had one layer

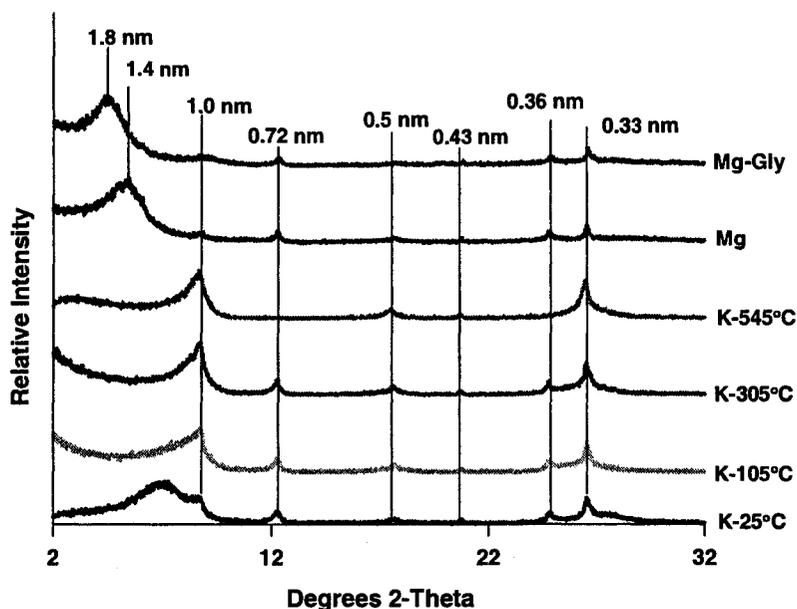


Figure 4-2. X-ray diffractograms of Mg- and K-saturated Zook clay-HC [quartz (0.43 and 0.34 nm peaks); kaolinite (0.72 and 0.36 nm peaks); illite (1.0, 0.5, and 0.33 nm peaks); smectite (1.4 nm peak); smectite with expanded interlayer (1.8 nm peak)].

of interlayer water molecules as evidenced by the 1.2 nm peak. The unsymmetrical 1.0 nm peaks of XRD patterns for the K-105° C and K-305° C treatments suggests the presence of hydroxy-interlayer material. The shoulder on the XRD patterns indicating hydroxy-interlayer material disappeared when the K-saturated sample was heated to 545° C. The sample also contains quartz (0.43 and 0.34 nm peaks), kaolinite (0.72 and 0.36 nm peaks), and illite (1.0, 0.5, 0.33 nm peaks).

Effect of Free and Bound Tetracycline on Microbial Growth

To test the effectiveness of TC on the growth of isolated soil microbes, testplates were prepared by streaking the serial diluted enrichment culture on nutrient plates and placing TC samples in the middle of the plate where previously a plug of nutrient agar had been removed. The effect of various TC concentrations on microbial growth was determined by measuring the inhibition zone from the rim of the well. Table 4-2 lists the inhibition

zones for several TC concentrations in solution (0.005 M CaCl₂) and sorbed onto Zook clay. The first column shows the total TC concentration added to the solution or suspension.

Inhibition of bacterial growth was observed when TC concentrations in 0.005 M CaCl₂ were ≥ 0.001 mg. The clearing ranged from 1 mm for the lowest concentration to 10 mm for 0.1 mg mL⁻¹ TC. Tetracycline sorbed onto clay did not affect bacterial growth below a concentration of 5.0 mg g⁻¹ for which there was a 0.5 mm clearing. A 1 mm clearing and limited growth up to 4 mm was observed when ~ 10 mg g⁻¹ TC was sorbed.

Table 4-2. Inhibition zones of TC in aqueous and sorbed phases.

Total TC added	TC in 0.005 M CaCl ₂	Clearing (mm)	TC in 0.005 M CaCl ₂ + clay	Clearing (mm)
Blank	0.005 M CaCl ₂	0	clay + CaCl ₂	0
0.001 mg	0.001 mg mL ⁻¹	1	0.1 mg g ⁻¹	0
0.005 mg	0.005 mg mL ⁻¹	3	0.5 mg g ⁻¹	0
0.010 mg	0.010 mg mL ⁻¹	5	1.0 mg g ⁻¹	0
0.050 mg	0.050 mg mL ⁻¹	10	5.0 mg g ⁻¹	0.5
0.1 mg	0.1 mg mL ⁻¹	10	10 mg g ⁻¹	1

Figure 4-3 shows the effect of TC on microbial growth when in free and bound state. These results suggest that TC in solution was readily available to inhibit microbial growth, whereas TC sorbed to the clay (10 mg g⁻¹) showed minimal bioavailability. The low TC concentrations in the inhibition zone allowed the growth of TC resistant bacteria (Fig. 4-3b).

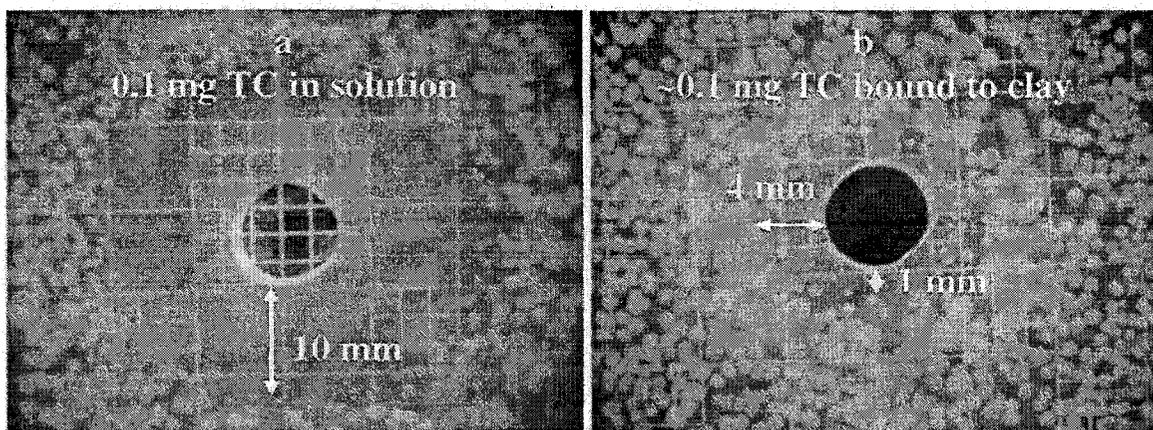


Figure 4-3. Pictures presenting TC effect on microbial growth when in free and bound state.

Ability of Isolated Bacteria to Degrade Tetracycline

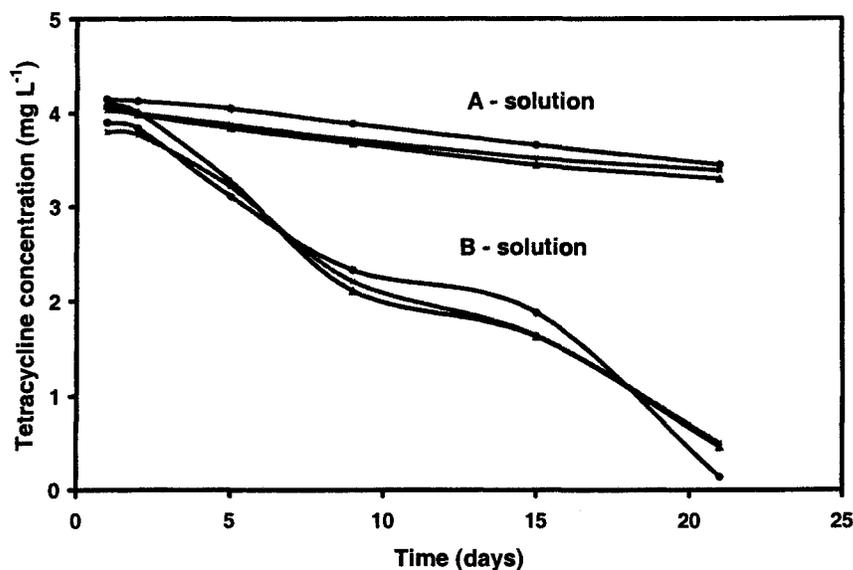


Figure 4-4. Degradation of TC in the abiotic (A) and the biotic (B) solution.

Colonies growing close to the source of the TC-clay (Fig. 4-3b) were isolated and inoculated. This inoculum, labeled as M1103, was tested for its ability to degrade TC. Figure 4-4 shows the disappearance of TC from solution under biotic and abiotic systems. “Blanks + M1103” contain TC, LB-Broth, 0.005 M CaCl₂, and M1103 whereas “Blanks - M1103” represent TC in 0.005 M CaCl₂ without M1103 or LB-Broth. Within 21 d, M1103 was able to degrade TC in solution to ~ 0.4 mg L⁻¹. Blanks without M1103 showed a decline in TC concentration of approximately 1 mg L⁻¹ due to the abiotic transformation of TC to 4-Epi-TC (Fig. 4-5). When the transformation of TC to 4-Epi-TC was accounted for, TC concentrations for the abiotic system remained consistent over the 21-day period (Fig. 4-5).

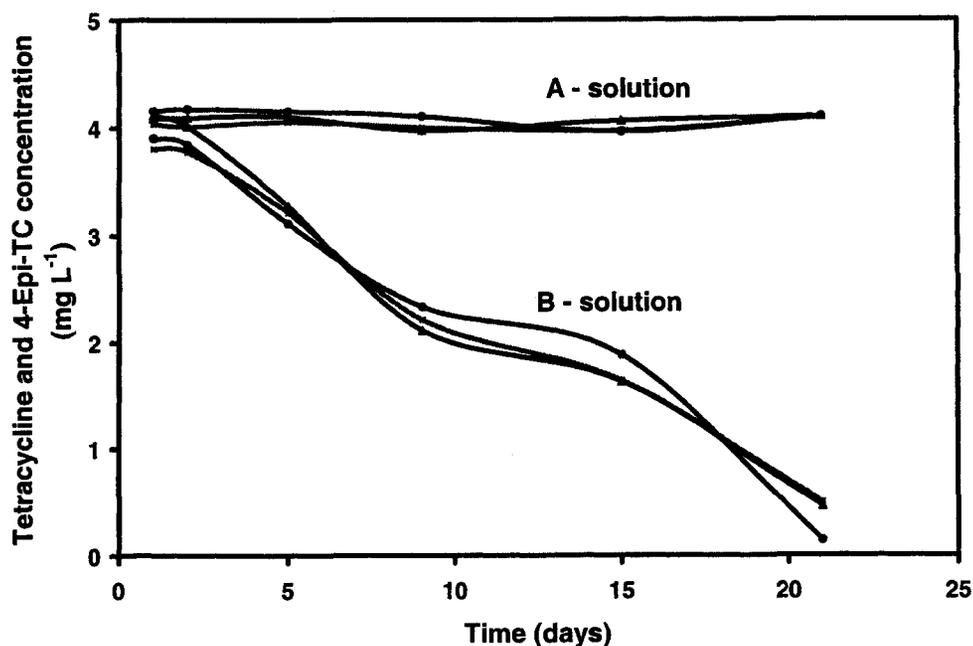


Figure 4-5. Tetracycline and 4-Epi-TC concentrations in the abiotic (A) and the biotic (B) solution.

Figure 4-6 shows chromatographs illustrating the degradation of 50 mg L⁻¹ under biotic and 100 mg L⁻¹ under abiotic conditions. Concentrations larger than 50 mg L⁻¹ inhibited bacterial growth. For the biotic system the initial concentrations for TC (eluted at 8.2 min) and 4-Epi-TC (eluted at 8.0 min) were 47.85 and 4.83 mg L⁻¹, respectively. After 149 d, the concentration of TC and 4-Epi-TC decreased to 4.2 and 0.5 mg L⁻¹, respectively. The initial TC and 4-Epi-TC concentrations for the abiotic system were 98.2 and 8.55 mg L⁻¹, respectively. After the incubation period, TC concentration decreased to 90.9 mg L⁻¹ and 4-Epi-TC increased to 15.4 mg L⁻¹ (Fig. 4-5). The pH decreased from 7 to 5.9, which favors the reversible formation of 4-Epi-TC. No other degradation products were found by HPLC analysis.

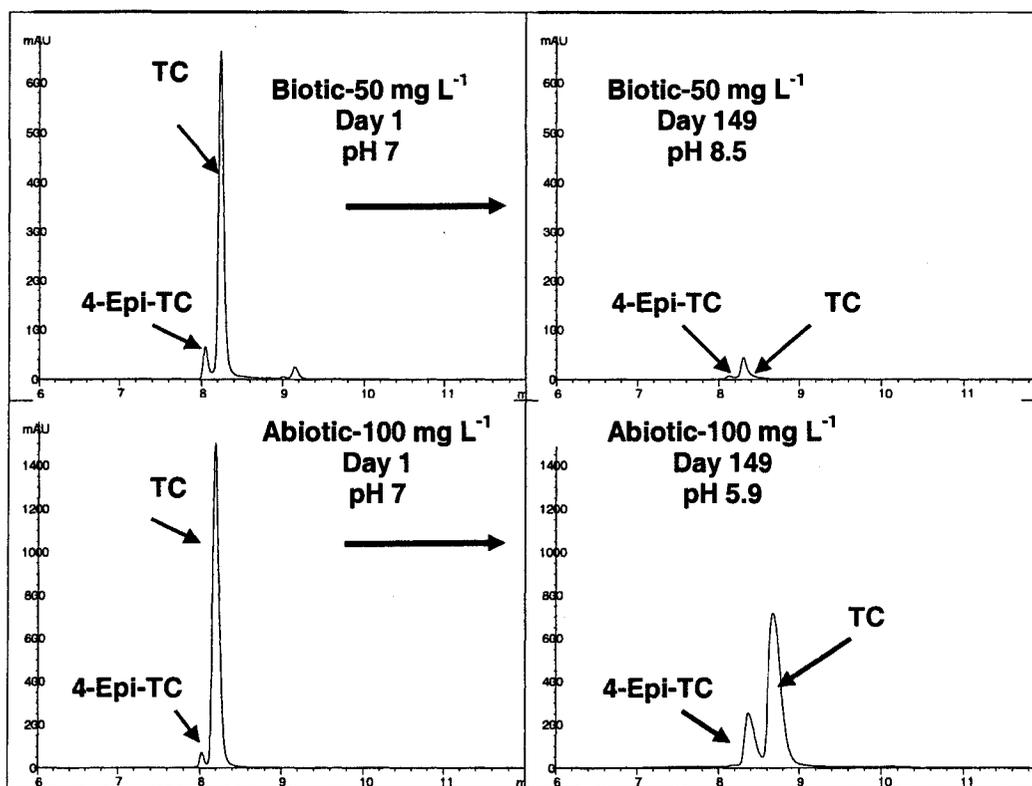


Figure 4-6. Degradation of 50 mg L^{-1} and 100 mg L^{-1} under biotic (B) and abiotic (A) conditions, respectively.

Degradation of TC in the Whole Soil, Clay-HC, and Clay Systems

After TC sorption onto soil components reached equilibrium (24 h), initial TC concentrations were measured for all systems. For the biotic systems, TC concentrations in the aqueous phase for WS, clay-HC, and clay were 9.0 , 7.6 , and 6.7 , mg L^{-1} , respectively. The disappearance of TC in solution was monitored over 149 days (Figure 4-7) and showed half-lives for B-WS, B-clay-HC, and B-clay of 9.7 d, 8.0 d, and 7.4 d, respectively. For the biotic systems, rapid degradation initiated immediately followed by slower degradation starting after 3 to 7 d. The TC in the biotic samples degraded to an undetectable level within 80 d (B-WS and B-clay-HC) and 45 d (B-clay). Lai et al. (1995) presented similar unsteady trends for oxytetracycline (OTC) transformation in aquaculture pond sediments. In their study, approximately 65 mg L^{-1} OTC in sediment slurries showed an initial rapid degradation

with no lag time followed by a slower degradation after approximately 5 d and complete disappearance after 47 d.

In our study, for the abiotic systems at equilibrium, 8.7, 8.1, and 5.8, mg L⁻¹ TC remained in the aqueous phase for the WS, clay-HC, and clay, respectively. Half-lives for A-WS, A-clay-HC, and A-clay were 67.3 d, 60.3 d, and 26.3 d, respectively. Disappearance of TC in solution for the abiotic samples was 7 times slower for the WS, 7.5 times slower for the clay-HC, and 3.75 times slower for the clay compared to the biotic systems. Overall, rate of TC disappearance in solution was fastest for the clay, followed by clay-HC, and slowest for the WS.

The difference in the onset of degradation for WS, clay-HC, and clay under biotic and abiotic systems was dictated by the initial TC concentration in the aqueous phase. For both systems, clays sorbed most and therefore less TC was found in the aqueous phase at the start of the degradation process, followed by clay-HC, and WS sorbed the least with most TC in the aqueous phase.

Tetracycline disappearance curves for the solutions are quite different (Fig. 4-4 and 4-5) compared to TC disappearance curves for the suspensions (Fig. 4-7). In the aqueous system shown in Figure 4-4 and 4-5, the decrease in TC with time is nearly linear for the abiotic and biotic systems, which indicates zero order kinetics. In the colloidal systems (Fig. 4-7), disappearance curves for the abiotic and biotic systems follow an exponential decay, which may indicate the slow desorption of TC from the solid re-supplying TC to the aqueous phase of the colloidal systems.

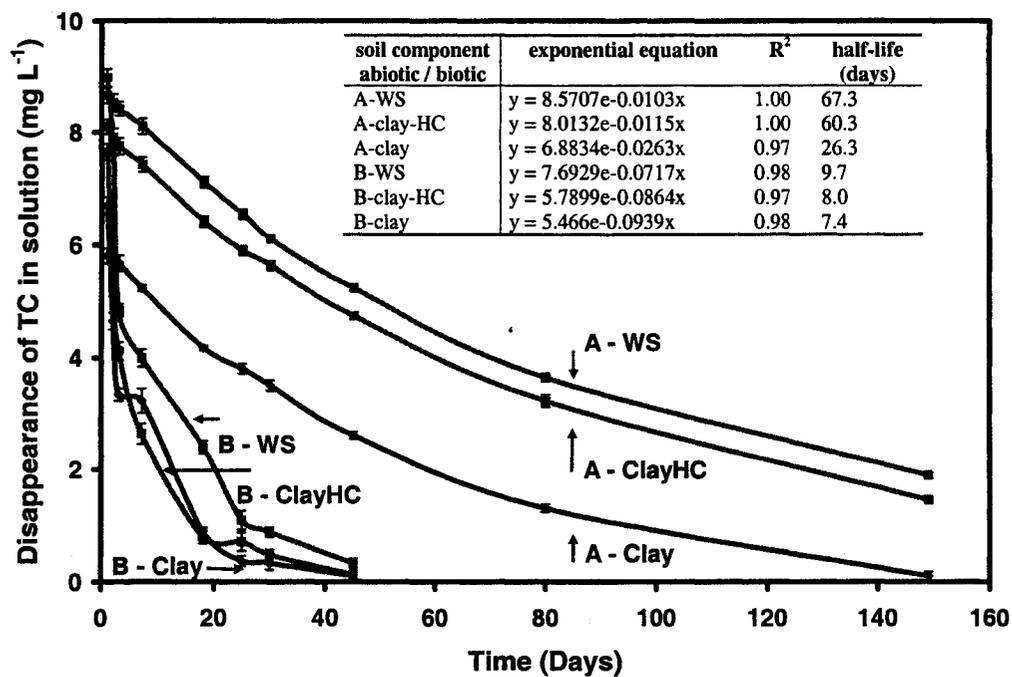


Figure 4-7. Disappearance of TC in aqueous solution for abiotic (A) and biotic (B) systems.

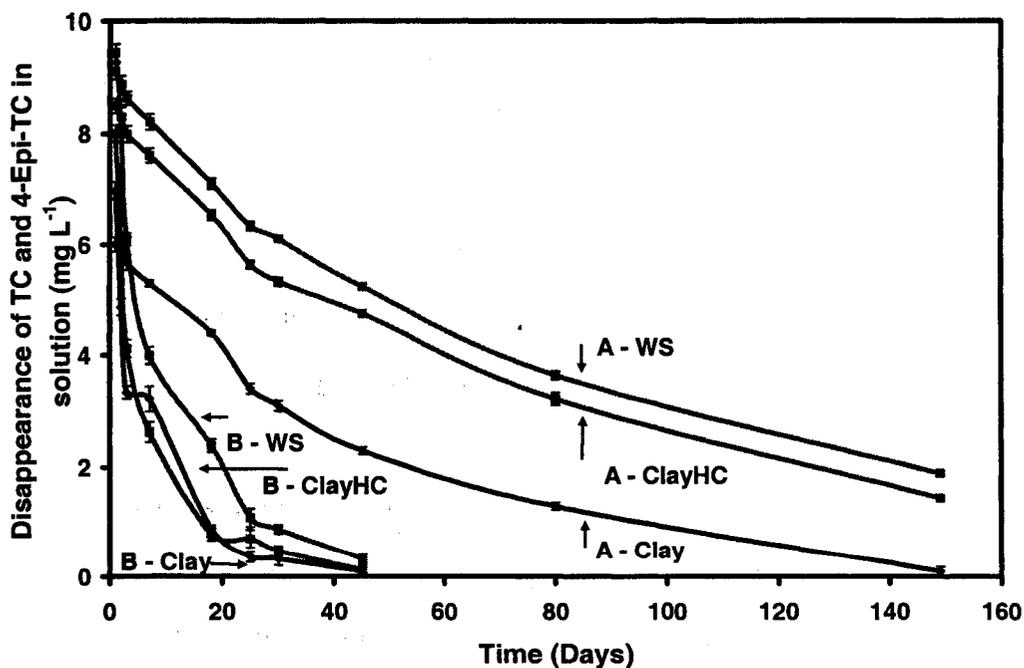


Figure 4-8. Disappearance of TC and 4-Epi-TC in aqueous solution for abiotic (A) and biotic (B) systems.

Figure 4-8 shows the sum of concentrations of TC and 4-Epi-TC in the aqueous phase during the incubation. 4-Epi-TC concentrations increased until day 18 but declined thereafter. The plots showing the sum of TC and 4-Epi-TC concentrations have a small inflection point around day 25 for both the abiotic and biotic systems.

Abiotic and Biotic Degradation Results

To quantify degradation of TC tritium in the aqueous phase was measured during the 149-d incubation period. Figure 4-9 shows tritium concentrations as a percentage of the total tritium added as a function of time for both the biotic and abiotic systems.

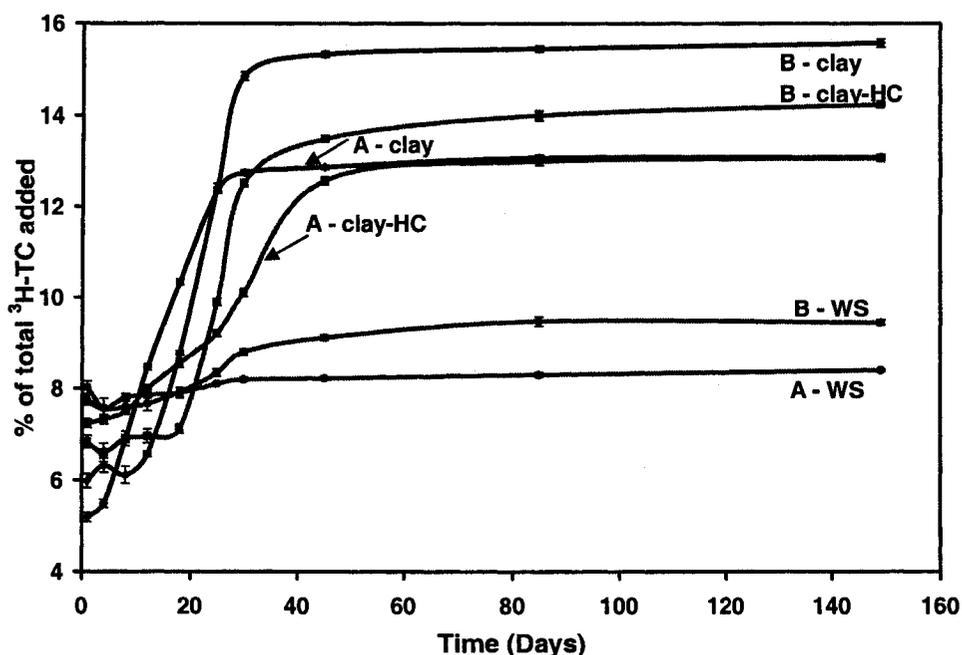


Figure 4-9. Tritium concentration in solution for abiotic (A) and biotic (B) systems.

The initial aqueous tritium concentrations relative to the total amount added for B-WS, B-clay-HC, and B-clay were 8.0, 6.8 and 6.0%, respectively and increased during the 145 d incubation period to 9.4, 14.2, and 15.6%, respectively. The tritium concentration for

A-WS, A-clay-HC, and A-clay initially were 7.7, 7.2, and 5.2%, with final concentrations of 8.4, 13.1, and 13.1%, respectively.

To compare TC release of soil components under biotic and abiotic systems, data presented in Figure 4-9 were normalized relative to the initial tritium concentrations. Through normalization, the LB-Broth effect and the differences in affinities of the soil components for TC were eliminated. The trends of tritium released for soil components under biotic and abiotic systems are presented in Figure 4-10. After 149 d, tritium concentration in solution decreased in following order; B-clay > A-clay > B-clay-HC > A-clay-HC > B-WS > A-WS. Soil components for the biotic systems released consistently more TC compared to soil components under abiotic conditions.

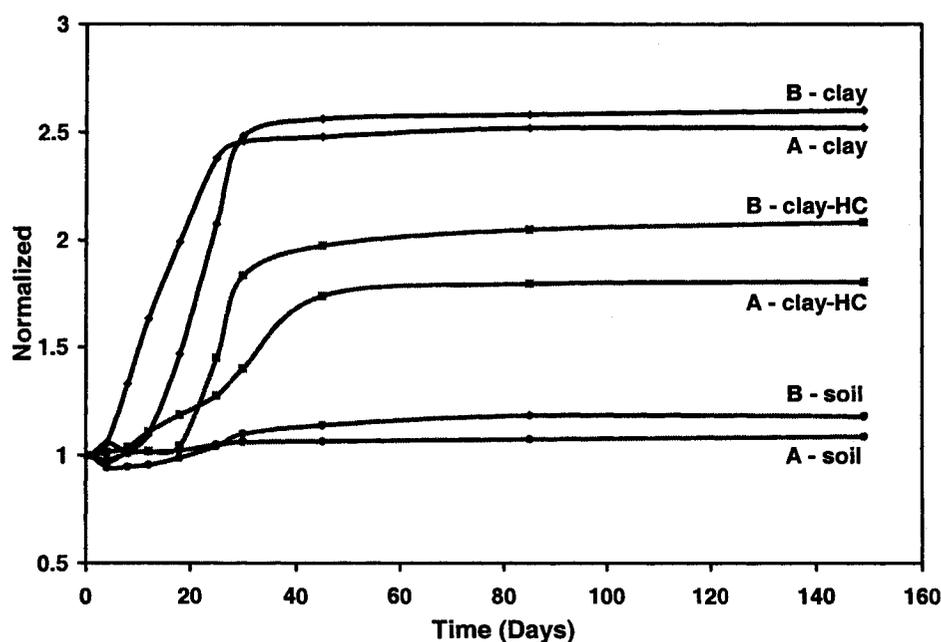


Figure 4-10. Normalized tritium concentration in solution for abiotic (A) and biotic (B) systems.

Tritium concentrations increased during the first 30 to 50 d but remained constant thereafter. The initial tritium release for the abiotic systems is higher than for the biotic systems. This release might be due to a delay in the desorption processes for the biotic

systems caused by interactions between sorbed LB-Broth and TC. LB-Broth contains peptone, which are amino acid polymers linked by amide bonds. Peptide sorption onto soil component surfaces may promote TC sorption through H-bonding and dipole-dipole interactions. When these large molecules are sorbed onto the soil component surfaces, one mechanism of TC release might be the consumption of the sorbed peptone as a carbon source by microbes. Overall tritium concentrations in the aqueous phase increase between day 0 and day 30. After 30 d there is no change in tritium concentrations for the biotic and abiotic systems. These results suggest that some TC is adsorbed in a 'labile' pool, which is subject to both biotic and abiotic degradation during the first 30 d. However, after 30 d most of the initially sorbed TC has become permanently sequestered and is not available for either biotic or abiotic degradation.

The pH for the abiotic system decreased from 7 to 5.9. The epimerization of TC occurs in acid solutions (pH 2-6) leading to 4-Epi-TC (Fig. 4-6) as confirmed by HPLC analysis. By contrast, during the incubation period, the pH values for the biotic systems increased from 7 to 8.5. Tetracycline is very unstable in alkaline soils (Pinck et al., 1961). When pH > 7.5, TC is metabolized to iso-tetracyclines (Kühne et al., 2000) and rapidly decomposes into smaller fragments (Hlavka and Boothe, 1985) which might have been the reason why no iso-tetracycline peaks were detected in our study. Kühne et al. (2000) found TC disappearance from liquid pig manure to follow exponential decay. Tetracycline in liquid pig manure (pH = ~8.5) was present in concentrations > 100 $\mu\text{g kg}^{-1}$ of which 30% to 50% disappeared within 8 days for ventilated and non-ventilated systems, respectively. Simultaneously, 4-Epi-TC concentrations increased by approximately 14% and no other degradation products were found.

Sorption studies (Figs. 4-7 and 4-8) show solution TC concentrations in the order of; WS > clay-HC > clay, but the rate of TC disappearance is in the order of; clay > clay-HC > WS. Tritium is released (Figs. 4-9 and 4-10) in the following order; clay > clay-HC > WS

which is opposite from the order expected based on the sorption studies but is in agreement with the rate of disappearance. Our studies indicate that the clay has the highest tritium release suggesting a larger labile pool. Tetracycline is sorbed to the clay fraction primarily through the formation of TC-Ca-surface complexes and through sorption in the interlayer (Chapter 3). In contrast to the clay system, less TC is initially sorbed to clay-HC and least to WS, but the sorption mechanisms are such that less TC becomes available for degradation. Less TC release for clay-HC and WS is therefore attributed to the presence of humic substances and organic matter, respectively. Possible interactions of TC with humic substances might be the formation of nonhydrolyzable humic substance-like macromolecules formed by oxidative coupling. Significant sorption of sulfonamide with surrogate humic constituents in the presence of enzymes has been attributed to cross-coupling reactions (Pedersen et al., 2004). Higher sorption of TC to clay-HC compared to WS may be due to the Ca-saturation adding more sites for TC sorption.

Figures 4-4 and 4-7 clearly show that TC is degraded by M1103. The fact that tritium in the aqueous solution increases within the first 30 d is clear evidence that some of the initially adsorbed TC is subject to degradation. But it is not clear whether the adsorbed TC is first desorbed to the aqueous phase and then degraded or whether it is degraded while adsorbed. The higher tritium release from the biotic systems compared to the abiotic systems after 149 d does not support higher TC release into the aqueous phase due to microbial activity. The LB-Broth may have created more “labile” sorption sites and TC associated with the adsorbed LB-Broth was released as the adsorbed LB-Broth was consumed.

The disappearance of tetracycline from the aqueous-biotic and aqueous-abiotic systems was approximately linear with time (Figs. 4-4 and 4-10). By contrast, the non-linear disappearance of TC from the aqueous phase of the colloidal systems (Figs. 4-7 and 4-8) suggests that the solid phase in the colloidal systems was slowly re-supplying TC to the aqueous phase. The disappearance of TC from both the aqueous-biotic and aqueous-abiotic

systems (Figure 4-4) and the appearance of 4-Epi-TC in the aqueous-abiotic systems (Figure 4-6) is clear evidence for the degradation and/or transformation of TC when it is in aqueous phase. However, there is no direct evidence that TC was degraded or transformed when sorbed to soil surfaces. After the 149-day incubation most (> 84%, Figure 4-10) of the added tritium remained firmly bound to the solid phase and it is not known whether the adsorbed tritium was still associated with TC or with a degradation product.

After the incubation period, the samples were air-dried and oxidized to determine the amount of tritium left on the sorbed phase. Table 4-3 lists tritium concentration (μCi) in the aqueous and sorbed phase. A total of $\sim 6.22 \mu\text{Ci}$ were added at the beginning of the experiment. The total tritium concentration for all systems ranges from 6.45 to 6.97 μCi . The difference between final and initial concentrations is attributed to the tritium associated with soil component matrix, released upon oxidation. These results indicate that all of the initially added 6.22 μCi were accounted for and no significant evaporation or other losses occurred during the study.

Table 4-3. Tritium concentrations in sorbed and aqueous phases after incubation period.

Soil Component	^3H in Sorbed Phase (μCi)	^3H in Aqueous Phase (μCi)	Total ^3H (μCi)
B-clay	5.39 ± 0.19	1.03 ± 0.040	6.42
B-clay-Control	$0.021 \pm 5.2 \times 10^{-4}$	$0.004 \pm 4.4 \times 10^{-5}$	0.025
A-clay	5.46 ± 0.11	0.82 ± 0.005	6.28
A-clay-Control	$9.2\text{E-}4 \pm 4.5 \times 10^{-5}$	$1.4\text{E-}4 \pm 4.0 \times 10^{-6}$	0.001
B-clay-HC	5.53 ± 0.14	0.94 ± 0.18	6.47
B-clay-HC-Control	$0.01 \pm 2.7 \times 10^{-4}$	$0.002 \pm 6.0 \times 10^{-5}$	0.01
A-clay-HC	5.48 ± 0.22	0.8 ± 0.032	6.28
A-clay-HC-Control	$0.001 \pm 4.1 \times 10^{-5}$	$9.5\text{E-}5 \pm 2.6 \times 10^{-6}$	0.001
B-soil	5.6 ± 0.23	0.64 ± 0.02	6.24
B-soil-Control	$0.002 \pm 5.0 \times 10^{-5}$	$1.9\text{E-}4 \pm 7.0 \times 10^{-6}$	0.003
A-soil	5.94 ± 0.22	0.52 ± 0.004	6.46
A-soil-Control	$6.4\text{E-}4 \pm 2.0 \times 10^{-5}$	$5.5\text{E-}5 \pm 1.8 \times 10^{-6}$	7.2E-4

Conclusions

The inoculum M1103 isolated from the Zook soil is able to degrade TC in the aqueous phase. Tetracycline was shown to transform to 4-Epi-TC under abiotic conditions. Tetracycline disappearance from the solutions for the abiotic and biotic systems decreased in following order; clay > clay-HC > WS, which is in agreement with tritium release for the soil components. Biotic and abiotic degradation results suggest that TC is sorbed into two different pools. The labile pool allows TC to be desorbed back into the aqueous phase and degraded (biotically or abiotically). In contrast, the stable pool permanently binds TC such that it is not readily released back to the aqueous phase. The labile pool is highest for the clay followed by the clay-HC and least for the WS. Clay-HC and WS release the least amount of tritium for both the abiotic and biotic systems. The major reason for the low labile pool for clay-HC and WS is the presence of HS and organic matter, respectively. Apparently, TC is bound to the organic fraction in such a manner that it is not subsequently released and is independent of pH.

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CHAPTER 5. GENERAL CONCLUSIONS OF THE DISSERTATION

The studies presented for Chapter 2 contribute greatly to a more complete understanding of flocculation and dispersion of smectitic colloids in mixed ionic solutions. The diffuse double layer, explained by the DLVO theory, has a major impact on dispersion and flocculation of smectitic colloids and has predominantly been applied to explain the stability of suspensions. The major finding of this study was that cation demixing and the formation and breakup of quasicrystals, which occur in mixed monovalent-divalent cation systems, also affects flocculation and dispersion. In low ionic strength systems, demixing with monovalent cations on the external surfaces and divalent cations on the internal surfaces of quasicrystals largely controls the average size of quasicrystals in suspensions. In high ionic strength systems, both monovalent and divalent cations are found in the clay interlayers. When Ca^{2+} dominates a system, independent of ionic strength, large quasicrystals form and breakup with increasing monovalent cation concentrations. Therefore, the average size of quasicrystals is controlled by the monovalent to divalent cation ratio, by the hydration energies of the cations, and by the ionic strength of the system.

Chapter 3 contains studies targeting the sorption and desorption of tetracycline and chlortetracycline onto and from natural soil components such as clays, humic substances, clay humic substances, and whole soils. Both tetracyclines were preferentially sorbed to clays, and humic substances followed by whole soils and least sorption was observed for clay-humic complexes. Sorption isotherms indicate stronger TET sorption to Ca-saturated soil components than to K-saturated soil components. Chlortetracycline is more strongly sorbed than TC, and both TET are more sorbed at lower pH. The greater sorption in the Ca-systems and the decreased sorption with increasing pH suggest that both charge

neutralization and cation bridging contribute to TET sorption. X-ray analyses showed that TC and CTC are sorbed in the interlayers of smectites. Desorption studies show some CTC desorption from soil components at pH 7, but highest desorption was observed for TC at pH 7 and the extent of desorption decreased in following order; clay-HC > HS > clay. These results indicate that tetracyclines are dominantly sorbed on soil clays and that HS compete with tetracyclines for sorption sites on the clay surfaces.

Chapter 4 investigates antimicrobial activity of sorbed TC and the ability of sorbed TC to act as a growth inhibitor for soil bacteria. This chapter also examined whether sorbed TC represents a long-term reservoir for slow TC release and the ability of resistant bacteria to degrade sorbed TC. Results indicate that sorbed TC does inhibit soil bacteria, most likely due to slow TC release from soil components. The inoculum M1103 isolated from the Zook soil was found to degrade TC in the aqueous phase. Tetracycline disappearance from the aqueous phase for abiotic and biotic systems decreased in following order; clay > clay-HC > WS, which is in agreement with the order of tritium release from ^3H -TC-treated soil components. Biotic and abiotic degradation studies showed increasing tritium concentrations in solution during the 149-day incubation period in decreasing order, soil clay > soil clay-humic complexes > whole soil. These data suggest that TC is sorbed into two different pools, stable and labile. The stable pool permanently binds TC such that it is not readily released back to the aqueous phase. The labile pool allows TC to be desorbed back into the aqueous phase and degraded (biotically or abiotically). The labile pool is highest in the clay followed by the clay-HC and least in the WS. Clay-HC and WS release the least amount of tritium for both the abiotic and biotic systems. Humic substances and organic matter in the clay-HC and WS, respectively, adsorb less TC than soil clay. However, the TC that is adsorbed by organic matter and HS is permanently bound as it is not released as a function of time regardless of pH in either the abiotic or biotic systems.

Unanswered questions concerning TC degradation suggest the need for future research to further investigate sorption/desorption kinetics as well as the effect of microbes on sorbed TC. Studies in Chapter 4 were conducted using complex heterogeneous systems that need to be simplified to basic components to better elucidate sorption and degradation mechanisms. Future studies should begin with TC sorption onto well defined and characterized surfaces such as specifically functionalized resins. As TC has three pka's, either strong or weak acidic and basic resins should be used to elucidate sorption strength as a function of pH. Ion exchange resins which are cross-linked polymers should be used to investigate the role of different cations and anions on tetracycline sorption. The progression of the study should include the addition of one variable at a time including pH, ionic strength, and mixed electrolyte concentrations. Finally, the complexity of the sorption surface should increase to the point that sorption and desorption of TC onto more natural surfaces can be predicted. Investigations should involve spectroscopic studies to help elucidate bonding mechanisms. Studies of microbial degradation of TC in the bound and free state should involve various enzymes and microbes starting with a simplified system. Results from sorption and degradation studies should be coupled to define the "labile" pool, identified in Chapter 4.