INFLUENCE OF CLAY MINERALS, HUMIC SUBSTANCES, AND SOIL AGGREGATION ON THE FATE AND BIOAVAILABILITY OF 2,4,6-TRINITROTOLUENE (TNT)

by

GREGORY D. PILLAR

(Under the Direction of Miguel Cabrera)

ABSTRACT

Over the last several decades the production, use, and disposal of munitions has resulted in widespread contamination of nitroaromatic explosives, including 2,4,6-trinitrotoluene (TNT). In this dissertation, the environmental fate of TNT in soil, soil aggregates, and primary soil particles was investigated. In the first experiment, the objective was to characterize the influence of humic acid on TNT sorption to clay minerals. TNT sorption to humic acid was significant and increased with a decrease in humic acid concentration. Additionally, humic acid complexed with a high and low charge smectite and kaolinite resulted in greater TNT sorption compared to the corresponding homo-ionic clay minerals. The fractionation of humic acid during adsorption to a low-charge smectite was observed and sequential humic-clay complexation resulted in a reduction in TNT sorption.

The objective of the next study was to characterize how the soil fractionation method used to obtain soil aggregates influences soil properties and processes. Wet- and dry-sieving of three different soils indicated that the particle size distribution, inherent

clay mineralogy, and organic matter content determined to what extent the sieving method altered the aggregate size distribution and aggregate specific soil properties. In all three soils, wet-sieving resulted in a greater distribution of aggregates < 250 μm and a redistribution of organic matter and sand particles from large aggregates to microaggregates. Additionally, wet-sieving significantly altered the microbial community structure of aggregates < 250 μm.

The objective of the third and final study was to determine if earthworms could influence the fate of TNT within soil aggregates and to what extent TNT and earthworms altered the soil microbial community. After 21 d 15.5 to 19.6% of the initial (≈ 100 mg kg^{-1}) TNT was recovered from two soils. After 7 d earthworm activity resulted in a 0 to 45% reduction in TNT depending on the soil type. With time, earthworm influence on TNT disappearance decreased due to the formation of irreversibly bound residues. Additionally, earthworms were able to absorb and transform TNT within their body and alter the distribution of TNT and metabolites among different sized aggregates, and along with TNT alter the microbial community structure.

INDEX WORDS: 2,4,6-trinitrotoluene (TNT), metabolites, clay minerals, humic acid, humic-clay complexes, soil aggregates, soil fractionation, earthworms, *Eisenia fetida*, microbial community structure, FAME

INFLUENCE OF CLAY MINERALS, HUMIC SUBSTANCES, AND SOIL AGGREGATION ON THE FATE AND BIOAVAILABILITY OF 2,4,6-TRINITROTOLUENE (TNT)

by

GREGORY D. PILLAR

B.S., University of Minnesota, 1999

M.S., Kansas State University, 2002

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2006

INFLUENCE OF CLAY MINERALS, HUMIC SUBSTANCES, AND SOIL AGGREGATION ON THE FATE AND BIOAVAILABILITY OF 2,4,6-TRINITROTOLUENE (TNT)

by

GREGORY D. PILLAR

Major Professor: Miguel Cabrera

Committee: Kang Xia
Paul Hendrix
John Seaman
Paul Schroeder

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2006

DEDICATION

This work is dedicated to my parents, Mary and Dennis Pillar. Without their love, guidance, and support from the day I was born I would not be where I am today. Thank you for everything you have sacrificed and given to me to help me get to this point and beyond.

ACKNOWLEDGEMENTS

I would like to thank my major professor Dr. Kang Xia for her support, encouragement, and patience throughout my academic career at Kansas State University and the University of Georgia. I am extremely thankful for the members of my graduate supervisory committee who took the time to advise and mentor me throughout my research: Dr. Miguel Cabrera, Dr. Paul Hendrix, Dr. John Seaman, and Dr. Valentine Nzengung. I am also thankful for the comments and suggestions of Dr. Paul Schroeder who was a welcome addition to my committee.

I owe a debt of gratitude to John Kruse, Karin Lichtenstein, Patrick Davies, and Franta Majs for their friendship, support, and advice over the last four years. I would like to thank Nehru Mantri, Ken Bradshaw, Vickie Hufstettler, Dawit Yifru, Jennifer Yates, Maria Monteros, Patty Hayes, David Butler, Nicolas Vaio, Sarah Schuler, Yabin Zhao and all of the graduate students who have assisted with this research and made graduate school enjoyable and interesting. Thank you to Dr. Bill Miller for his support and encouragement as I worked to finish this dissertation. I owe current and former members of the Department of Crop and Soil Science faculty, specifically Dr. Larry West, Dr. Peter Hartel, Dr. David Radcliffe, and Dr. Mark Williams a world of thanks for sharing their knowledge and skills in soil science. I would also like to thank the department staff, especially Vivienne Sturgill for their help in navigating the ins and outs of Graduate School life

I am very thankful for the help and assistance of my research assistants Amy Thompson, Micah Gardner, Nathan Jensrud, and Jami Schaffner. Their countless hours of hard work were an integral part of the research presented in this dissertation.

Finally, my academic career has been influenced by many, but without a few, I would never be where I am today. I would like to thank Mr. Walter Schumann for believing in me and encouraging me to pursue an education and career in the environmental sciences. I would like to thank my undergraduate advisor, Dr. Jay Bell, who was a major inspiration and empowered me to take control of my education. A special debt of thanks is owed to Dr. Bill Koskinen who provided me my first research opportunity as an undergraduate. The experience I received working for him and the many students, technicians, and post-docs in his lab is the driving force behind my work ethic and desire to pursue a graduate education. I am thankful to Dr. Terry Cooper who was my first soil science instructor and provided me with my first college teaching experience. A special debt of gratitude goes to Dr. Steve Thien, who is the essence and model of a true teaching scholar. I certainly would not be where I am today if it was not for his guidance and friendship over the last 6 years.

Most importantly, the utmost thanks and gratitude goes to my entire family, who has always supported me in everything I do. To my parents Dennis and Mary Pillar, who this dissertation is dedicated to, thank you for your love and for the sacrifices you have made. To the love of my life, Rachel, your love, patience and sacrifice through this challenging time has been amazing. I love you with all of my heart and look forward to whatever the future may hold.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF TABLES x

LIST OF FIGURES xii

CHAPTER

1 INTRODUCTION 1

References 6

2 LITERATURE REVIEW 7

TNT 8

Soil aggregation 29

Effects of earthworms on soil properties and fate of organic contaminants 33

Effects of biotic and abiotic factors and contaminants on the soil microbial community structure 36

Objectives 42

References 43

3 INTERACTION OF 2,4,6-TRINITROTOLUENE WITH HUMIC ACID, CLAY MINERALS, AND HUMIC-CLAY COMPLEXES 57

Abstract 58

Introduction 59

Materials and Methods 62
Results and Discussion 68
Conclusions 80
References 83
4 IMPACT OF SIZE FRACTIONATION METHODS ON PROPERTIES OF SOIL AGGREGATES 100
Abstract 101
Introduction 102
Materials and Methods 104
Results and Discussion 110
Conclusions 120
References 122
5 FATE OF 2,4,6-TRINITROTOLUENE (TNT) AND MICROBIAL COMMUNITY STRUCTURE WITHIN SOIL AGGREGATES IN THE PRESENCE OF EARTHWORMS 134
Abstract 135
Introduction 137
Materials and Methods 140
Results and Discussion 147
Conclusions 166
References 168
6 SUMMARY AND CONCLUSIONS 186
APPENDICES 192

A SUPPLEMENTAL DATA FROM CHAPTER 3 192

B SUPPLEMENTAL DATA FROM CHAPTER 4 198

C SUPPLEMENTAL DATA FROM CHAPTER 5 202

LIST OF TABLES

Page

Table 2.1: Physical and chemical properties of 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (2-ADNT), and 4-amino-2,6-dinitrotoluene (4-ADNT). 11

Table 2.2: TNT sorption isotherm parameters for various sorbents under different environmental conditions 19

Table 2.3: Selected studies showing the relative toxicities of TNT to soil micro- and macro-fauna 24

Table 2.4: Selected studies showing the relative toxicities of TNT to fish, terrestrial plants and mammals 25

Table 2.5: Common fatty acids and corresponding biomarker interpretations.....38

Table 3.1: Properties of reference clay minerals (SWy-2, SAz-1, SHCa-1, and KGa-1b), their K^+- and Ca^{2+} - saturated forms, their humic acid-clay complexes, and sequentially complexed humic acid-clay complexes 87

Table 3.2: Sorption and desorption isotherm parameters for TNT to K^+- and Ca^{2+} - saturated clay minerals and their humic acid (HA)-clay complexes.....88

Table 3.3: Sorption isotherm parameters for TNT to humic acid at different humic acid concentrations.....89

Table 4.1: Physical and chemical characteristics of soils used in this study. Numbers within parentheses reflect the standard deviation (n = 3).....126

Table 4.2: Particle size analysis of soil aggregates collected from dry- and wet-sieving techniques (n = 1). 127

Table 4.3: Cation exchange capacity (CEC) and concentration of total carbon (TC) in soil aggregates collected from dry- and wet-sieving methods as determined on a sand-free basis[†] 128

Table 4.4: Comparison of dry- and wet-sieving methods on the mole percentage of 18 microbial biomarkers in three distinct soils[†] 129

Table 5.1: Physical and chemical soil properties of the Appling (GA) and Theresa (WI) soils 175

Table 5.2: Average mole percentages of individual FAMEs in the whole soil and aggregates of an Appling sandy loam soil exposed to earthworms and/or TNT for 21 d. 176

Table 5.3: Average mole percentage of individual FAMEs in the whole soil and aggregates of a Theresa silt loam soil exposed to earthworms and/or TNT for 21 d 177

Table B.1: Cation exchange capacity (CEC) and concentration of total carbon (TC) in soil aggregates collected from dry- and wet-sieving methods[†] 199

Table C.1: The percentage of TNT, 2-ADNT, and 4-ADNT recovered from soil aggregates compared to the whole soil. 203

Table C.2: Amount of individual FAMEs from 21 d incubation experiment involving an Appling sandy loam soil. 204

Table C.3: Amount of individual FAMEs from 21 d incubation experiment involving a Theresa silt loam soil. 205

LIST OF FIGURES

Page

Figure 2.1: Distribution of 2,307 suspected sites contaminated with military munitions as identified by the U.S. Department of Defense (GAO, 2003). 9

Figure 2.2: Pathways for the aerobic transformation of TNT by microorganisms. Two arrows indicate the conversion of a compound to another is believed to occur through a series of intermediates (modified from Esteve-Nunez et al., 2001)..13

Figure 2.3: Pathways for the anaerobic transformation of TNT by microorganisms. Two arrows indicate the conversion of a compound to another is believed to occur through a series of intermediates (modified from Esteve-Nunez et al., 2001)..15

Figure 2.4: Bioavailability processes in soil (modified from Adriano, 2003; National Research Council, 2003) 27

Figure 2.5: Conceptual model developed by Six et al. (1999) of the aggregate "life cycle" based on aggregate hierarchy models proposed by Tisdall and Oades (1982) and Oades (1984).....31

Figure 3.1: Dialysis unit for the batch equilibrium experiments for TNT and humic acid interactions 90

Figure 3.2: Isotherms describing 2,4,6-trinitrotoluene sorption by K^+- (top) and Ca^{2+}- (bottom) saturated clay minerals. Symbols and error bars represent the mean and ± 1 S.D., respectively (n = 3).....91

Figure 3.3: Sorption/desorption isotherms of TNT to K^+-saturated (K-SWy, K-SHCa, K-SAz) and Ca^{+2}- saturated (Ca-Swy, Ca-SHCa) smectite clay minerals. Symbols and error bars represent sample mean and ± 1 S.D., respectively (*n* = 3).....92

Figure 3.4: Sorption isotherms for TNT sorption to humic acid. Symbols and error bars represent the mean and ± 1 S.D., respectively (*n* = 2). *,*n* = 1. 93

Figure 3.5: Sorption/desorption isotherms of TNT to K^+- saturated (K-HA-SWy, K-HA-SHCa, K-HA-SAz) and Ca^{+2}- saturated (Ca-HA-SWy, Ca-HA-SHCa) humic-clay complexes. Symbols and error bars represent sample mean and ± 1 S.D., respectively (*n* = 3).....94

Figure 3.6: Sorption isotherms representing TNT sorption to K^+- and Ca^{+2}- saturated smectite clay minerals and their corresponding humic-clay complexes; Close and open symbols represent sorption to the clay mineral (-●-) and corresponding humic-clay complex (-○-),, respectively. Symbols and error bars represent sample mean and ± 1 S.D., respectively (*n* = 3) 95

Figure 3.7: Associated *d*-spacings of K-SWy and its corresponding humic-clay complex K-HA-SWy obtained from x-ray diffraction patterns of oriented films as a function of TNT sorption 96

Figure 3.8: Isotherms describing TNT sorption by K^+-saturated kaolinite and humic-kaolinite complexes. Symbols and error bars represent the mean and ± 1 S.D., respectively (*n* = 3).....97

Figure 3.9: Isotherms describing 2,4,6-trinitrotoluene sorption by K^+ -saturated humic-clay complexes sequentially prepared with smectite (K-SWy). Symbols and error bars represent the mean and ± 1 S.D., respectively (n = 3) 98

Figure 3.10: Associated *d*-spacings of sequentially prepared humic-clay complexes obtained from x-ray diffraction patterns of oriented films as a function of TNT sorption 99

Figure 4.1: The amount of soil within each aggregate size fraction as a percentage of total sand-free soil (top-Theresa, middle-Appling, bottom-Marlette-Oshtemo). Significant differences between sieving methods are represented by different letters on the graph ($p < 0.01$, mean ± 1 S.D.) 130

Figure 4.2: Total fatty acid methyl esters (FAME) within each aggregate size fraction obtained by using dry- and wet-sieving techniques (top-Theresa, middle-Appling, bottom-Marlette-Oshtemo). Significant differences between sieving methods are represented by different letters (*p*-value 131

Figure 4.3: Nonmetric multidimensional scaling plots of mole percentages of FAMEs of all aggregate size fractions for both sieving methods and all three soils. The proportion of variance explained by each axis is indicated in parentheses. Ellipses enclose and identify the Theresa, Appling, and Marlette-Oshtemo soils. Multiresponse permutation procedure (MRPP) analysis indicated that the three soils were significantly different from each other. Values of the symbols and error bars represent means and standard error, respectively (n = 3).....132

Figure 4.4: Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs of dry- and wet-sieved Theresa, Appling, and Marlette-Oshtemo soils. The proportion of variance explained by each axis is indicated in parentheses. A one dimensional solution explained the bulk of the variance in the Marlette-Oshtemo soil, but for the sake of clarity a two dimension solution is presented. Multi-response permutation procedure (MRPP) analysis indicated that the sieving method was significantly different from each other within each soil type. Values of the symbols and error bars represent means and standard error, respectively (n = 3). 133

Figure 5.1: Concentrations of 2-ADNT and 4-ADNT in earthworms (A,C) and their biomass (B,D) after exposure to TNT in an Appling sandy loam and a Theresa silt loam loam at initial soil concentrations of 96 mg kg^{-1} and 99 mg kg^{-1}, respectively (C,D). Error bars represent ± 1 S.D., n = 3, * n = 2. 178

Figure 5.2: Aggregate size distribution of an Appling sandy loam and a Theresa silt loam after 21 days of exposure to TNT and earthworms. Values followed by a different lowercase letter within aggregate size class are significantly different between treatments (mean ± 1 S.D., n = 3). 179

Figure 5.3: Concentration of readily extractable (-●- -○-), potentially extractable (-■- -□-), and total extractable (-▲- -Δ-) TNT and its two major metabolites (2-ADNT and 4-ADNT) in the whole soil, 2000 – 4000, 500 – 2000, and 250 - 500μm aggregates of an Appling sandy loam with and without earthworms (open and closed symbols, respectively). Asterisks denote significant differences (using Fishers LSD) between treatments at each time period (p<0.05, n = 3).....180

Figure 5.4: Concentration of readily extractable (-●- -○-), potentially extractable (-■- -□-), and total extractable (-▲- -Δ-) TNT and its two major metabolites (2-ADNT and 4-ADNT) in the whole soil, 2000 – 4000, 500 – 2000, 250 – 500, and 75 – 250 μm aggregates of a Theresa silt loam with and without earthworms (open and closed symbols, respectively). Asterisks denote significant differences (using Fishers LSD) between treatments at each time period (p<0.05, n = 3).....181

Figure 5.5: Concentration of total extractable TNT and its two major metabolites (2-ADNT and 4-ADNT) at 21 d in the different sized aggregates of an Appling sandy loam (left panel) and a Theresa silt loam (right panel) with and without earthworms. Different letters denote significant differences (using Fishers LSD) between aggregate size fractions for each treatment (p < 0.05, n = 3). 182

Figure 5.6: Sorption/desorption isotherms of TNT to an Appling sandy loam (GA) and a Theresa silt loam (WI) soil (mean ± 1 S.D., n = 3).....183

Figure 5.7: Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs in whole soil, 2000 – 4000, 500 – 2000, and 250 – 500 μm aggregates of an Appling sandy loam soil after 21 d of exposure to earthworm and/or TNT. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard errors, respectively (n = 3). 184

Figure 5.8: Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500, and 75-250 μm aggregates of a Theresa silt loam soil after 21 d of exposure to earthworm and/or TNT. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard error, respectively (n = 3). 185

Figure A.1: X-ray diffraction patterns of K-SWy as a function of TNT sorption (Cu-K_α radiation, 0% humidity).....193

Figure A.2: X-ray diffraction patterns of K-HA-SWy as a function of TNT sorption (Cu-K_α radiation, 0% humidity) 194

Figure A.3: X-ray diffraction patterns of K-HA-S2-SWy as a function of TNT sorption (Cu-K_α radiation, 0% humidity).....195

Figure A.4: X-ray diffraction patterns of K-HA-S3-SWy as a function of TNT sorption (Cu-K_α radiation, 0% humidity).....196

Figure A.5: X-ray diffraction patterns of K-HA-S4-SWy as a function of TNT sorption (Cu-K_α radiation, 0% humidity).....197

Figure B.1: The amount of soil within each aggregate size fraction as a percentage of the whole soil (top-Theresa, middle-Appling, bottom-Marlette-Oshtemo). Significant differences between sieving methods are represented by different letters on the graph ($p < 0.01$, mean ± 1 S.D.). 200

Figure B.2: Total fatty acid methyl esters (FAME) within each aggregate size fraction obtained by using dry- and wet-sieving techniques (top-Theresa, middle-Appling, bottom-Marlette-Oshtemo). Significant differences between sieving methods are represented by different letters (p-value ≤ 0.01, mean ± 1 S.D.).201

Figure C.1: Effects of earthworm activity and TNT on aggregate size distribution of an Appling sandy loam (A) and a Theresa silt loam (B) after 14 days. Values followed by a different lowercase letter within aggregate size class are significantly different between treatments (mean ± 1 S.D., $n = 3$). 206

Figure C.2: Effects of earthworm activity and TNT on aggregate size distribution of an Appling sandy loam (A) and a Theresa silt loam (B) after 7 days. Values followed by a different lowercase letter within aggregate size class are significantly different between treatments (mean ± 1 S.D., $n = 3$). 207

Figure C.3: Concentration of acetonitrile extractable TNT and its two major metabolites (2-ADNT and 4-ADNT) at 21 d in the different sized aggregates of an Appling sandy loam (left panel) and a Theresa silt loam (right panel) with and without earthworms. Different letters denote significant differences (using Fishers LSD) between aggregate size fractions for each treatment ($p < 0.05$, $n = 3$)..208

Figure C.4: Concentration of $CaCl_2$ extractable TNT and its two major metabolites (2-ADNT and 4-ADNT) at 21 d in the different sized aggregates of an Appling sandy loam (left panel) and a Theresa silt loam (right panel) with and without earthworms. Different letters denote significant differences (using Fishers LSD) between aggregate size fractions for each treatment ($p < 0.05$, $n = 3$)..209

Figure C.5: Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500 μm aggregates of an Appling sandy loam over time (0, 7, 14, 21 d). The proportion of variance explained by each axis is indicated in parentheses. Values of the symbols and error bars represent means and standard error, respectively ($n = 3$).....210

Figure C.6: Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500 and 75 – 250 μm aggregates of a Theresa silt loam over time (0, 7, 14, 21 d). The proportion of variance explained by each axis is indicated in parentheses. Values of the symbols and error bars represent means and standard error, respectively ($n = 3$).....211

Figure C.7: Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500 μm aggregates of an Appling sandy loam after 14 d. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard error, respectively ($n = 3$).....212

Figure C.8: Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500, and 75 - 250 μm aggregates of a Theresa silt loam after 14 d. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard error, respectively (n = 3).....213

Figure C.9: Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500 μm aggregates of an Appling sandy loam after 7 d. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard error, respectively (n = 3).....214

Figure C.10: Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500, and 75 - 250 μm aggregates of a Theresa silt loam after 7 d. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard error, respectively (n = 3).....215

CHAPTER 1

INTRODUCTION

Since the dawn of the industrial age, the world has seen an untold number of technological advances which have made, and continue to make, our lives easier and more enjoyable. However, with every technological advance comes greater responsibilities and in many cases problems. For example, the creation of every new gadget involves the use of synthetic chemicals during their development, production, storage, and/or disposal in some way. In 2004, more than 23,000 private and federal facilities within the US reported to the Environmental Protection Agency as part of the Toxic Release Inventory Program 4.24 billion pounds of on-site and off-site disposal/release of more than 600 toxic chemicals (USEPA, 2004). Not surprisingly, the general public has become increasingly concerned about the large quantities of toxic substances that have been reported to be in the environment, particularly in soil and water systems. The nitroaromatic explosive 2,4,6-trinitrotoluene (TNT) is just one of these chemicals which has recently posed a ecotoxicological threat, especially with the closing and decommissioning of military institutions in the US and across the world (Spain, 2000).

Over the last 20 years there has been a significant amount of published research on the fate of TNT in the environment. Originally it was reported that TNT is adsorbed by soil with greater affinity for clay minerals than soil organic matter, and can be readily mobilized through desorption processes (Pennington and Patrick, 1990; Haderlein and Schwarzenbach, 1993; Haderlien et al., 1996). Thus, these studies suggested that TNT poses a significant risk to surface and groundwater resources through subsurface transport. However, additional research has shown that TNT is not readily desorbed from soil and may not be easily removed via simple extraction procedures; especially with

prolong exposure (Hundal et al., 1997; Achtnich et al., 1999). There are numerous reasons for these apparent discrepancies in the literature. For example, earlier studies reporting reversible sorption focused on low concentrations of TNT, sorption to individual soil components, and very short exposure/equilibration times. Conversely, research conducted more recently has focused on higher concentrations up to and beyond the solubility of TNT in water, extended exposure of TNT in soil, and fate studies conducted *in-situ* or in the laboratory involving more complex systems. In general, there is still considerable uncertainty with respect to how TNT behaves in the environment. Thus, the research presented in this dissertation is focused on expanding our understanding of how TNT interacts with the environment by looking specifically at interactions with humic-clay complexes and soil systems. With respect to TNT interactions in the soil, this work focused on how the fate of TNT may differ within various soil aggregate size fractions. Overall, the research in this dissertation is presented as three distinct chapters, with each being prepared as separate manuscripts for publication.

The first study (Chapter 3) focuses on TNT sorption and desorption to clay minerals, humic acid, and humic-clay complexes. Clay minerals and components of soil organic matter (such as humic acid) play an important role in the formation of soil aggregates. Although previous work has been conducted examining the sorption of TNT to clay minerals and humic acid individually, little is known on how interactions between humic substances and clay minerals may affect the ability of each to adsorb TNT. Batch sorption experiments were performed to determine the extent and characteristics of TNT sorption to four different clay minerals each saturated with two cations (K^+- and Ca^{2+}-),

humic acid, and the respective humic-acid-clay complexes. This research was presented in Salt Lake City as part of the 2005 Soil Science Society of America Meetings. Additionally this work will be submitted to the *Environmental Science and Technology*, a journal published by the American Chemical Society.

The second study (Chapter 4) deviated from the focus on the fate of TNT in soil and instead focuses on how soil aggregates are obtained for use in *ex-situ* studies. The properties and processes that occur with soil aggregates still remain a mystery because of the lack of *in-situ* methods available to characterize and monitor aggregation. As soon as soil is removed from its natural state and sieved into individual size fractions the complex system is greatly disturbed and thus no longer behaves in the same manner. Although our understanding of processes related to soil aggregates have been greatly improved, there are some who believe that the results of this research is merely an artifact of the sampling, storage, handling, and fractionation methods used. Thus, before examining how TNT may behave in soil aggregates of different size, I tried to characterize how different sieving methods may impact soil properties and processes which are related to the fate of environmental contaminants (i.e. sorption, biotransformation, biodegradation). This included physical soil properties (i.e. clay content, aggregate size distribution), chemical soil properties (i.e. organic carbon, cation exchange capacity), and biological function by characterizing the microbial community structure through the analysis of fatty acid methyl esters (FAME) which can be used as indicators of specific microorganisms (i.e. gram-negative bacteria, fungi, protozoa, etc). This research was presented in Denver as part of the 2003 Soil Science Society of America Meetings.

Additionally this work will be submitted to the *Journal of Soil Biology and Biochemistry*, a journal published by Elsevier Publishing.

Finally, the third study (Chapter 5) focused on the ability of earthworms to mediate and/or accelerate the disappearance and transformation of TNT in soil and soil aggregates. Earthworms are known to contribute to the formation of soil aggregates through incorporation of organic residues, soil mixing, and excretion of soil material as casts which have been characterized as hot beds of microbial activity (Lee, 1985; Edwards and Bohlen, 1996). Thus, they may play a significant role in the distribution and transformation of TNT within soil and soil aggregates. This could come about through direct interaction with TNT or through the alteration of the microbial community, particularly those organisms which are known to breakdown TNT into other compounds or metabolites. Two soils, an alfisol from Wisconsin and an ultisol from Georgia, with contrasting characteristics (i.e. soil organic matter content, clay mineralogy) were used in two 21 d incubation studies involving the earthworm *Eisenia fetida* in small soil microcosms. Over the 21 d the aggregate size distribution, earthworm biomass, amount of TNT disappearance, amount of metabolite formation (2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene) in soil and within earthworms, and the microbial community structure were measured or characterized. The research presented in this chapter was presented in Seattle and Salt Lake City as part of the 2004 and 2005 Soil Science Society of America Meetings, respectively. Additionally this work will be submitted to *Environmental Toxicology and Chemistry*, a journal published by the Society of Environmental Toxicology and Chemistry.

References

Achtnich, C., U. Sieglen, H-J. Knackmuss, and H. Lenke. 1999. Irreversible binding of biologically reduced 2,4,6-trinitrotoluene to soil. Environ. Toxicol. Chem. 18: 2416-2423.

Edwards, C.A., and P.J. Bohlen. 1996. Biology and ecology of earthworms. Chapman and Hall, London.

Haderlein, S.B., and R.P. Schwarzenbach. 1993. Adsorption of substituted nitrobenzenes and nitrophenols to mineral surfaces. Environ. Sci. Technol. 27: 316-326.

Haderlein, S.B., K.H. Weissmahr, and R.P. Schwarzenbach. 1996. Specific adsorption of nitroaromatic explosives and pesticides to clay minerals. Envion. Sci. Tehcnol. 30: 612-622.

Hundal, L., P.J. Shea, S.D. Comfort, W.L. Powers, and J. Singh. 1997. Long-term TNT sorption and bound residue formation in soil. J. Environ. Qual. 26: 896-904.

Lee, K.E., 1985. Earthworms: Their ecology and relationships with soils and land use. Academic Press. Sydney.

Pennington, J.C., and W.H. Patrick. 1990. Adsorption and desorption of 2,4,6-trinitrotoluene by soils. J. Environ. Qual. 19: 559-567.

Spain J.C. 2000. Introduction. p. 1-5, *In* Biodegradation of nitroaromatic compounds and explosives. Spain, J.C. (ed). Lewis Publishers, Boca Raton, FL.

USEPA 2004. Toxics Release Inventory (TRI) Public Data Release Report [Online]. http://www.epa.gov/tri/tridata/tri04/pdfs/2004brochure.pdf (accessed 20 June 2006). USEPA, Washington, DC.

CHAPTER 2

LITERATURE REVIEW

2.1 TNT

Nitroaromatic compounds (NACs) are commonly used as explosives and in munitions by the U.S. Department of Defense (DOD). Over the last several decades, particularly since World War II the closing of U.S. military institutions has brought about concern regarding environmental contamination of chemicals produced, stored, used, and disposed of during the operation of these facilities. A recent report by the General Accountability Office indicated that 2,307 sites consisting of over 15 million acres of land in the U.S. are known to be or suspected of being contaminated with nitroaromatic explosives (Figure 2.1). In addition, the DOD has reported that not all potential sites have been identified and have estimated that the cost to identify, assess, and clean up contaminated land could cost between $8 - $35 billion dollars over 75 years (GAO, 2003). One of the more commonly used NACs that have been identified at these sites is 2,4,6-trinitrotoluene (TNT). As of 2005 the Agency for Toxic Substances and Disease Registry (ATSDR) along with the Environmental Protection Agency (EPA) have listed TNT on the Priority List of Hazardous Substances that pose the most significant potential threat to human health (ATSDR, 2005). Additionally, TNT has been discovered in at least 20 of the 1,430 National Priorities List sites by the EPA. Furthermore, TNT is fairly resistant to degradation but is readily transformed to metabolites such as 2-amino-4,6-dinitrotoluene (2-ADNT), and 4-amino-2,6-dinitrotoluene (4-ADNT) which in some cases pose an even greater risk to human and environmental health as these compounds may be more toxic than TNT.

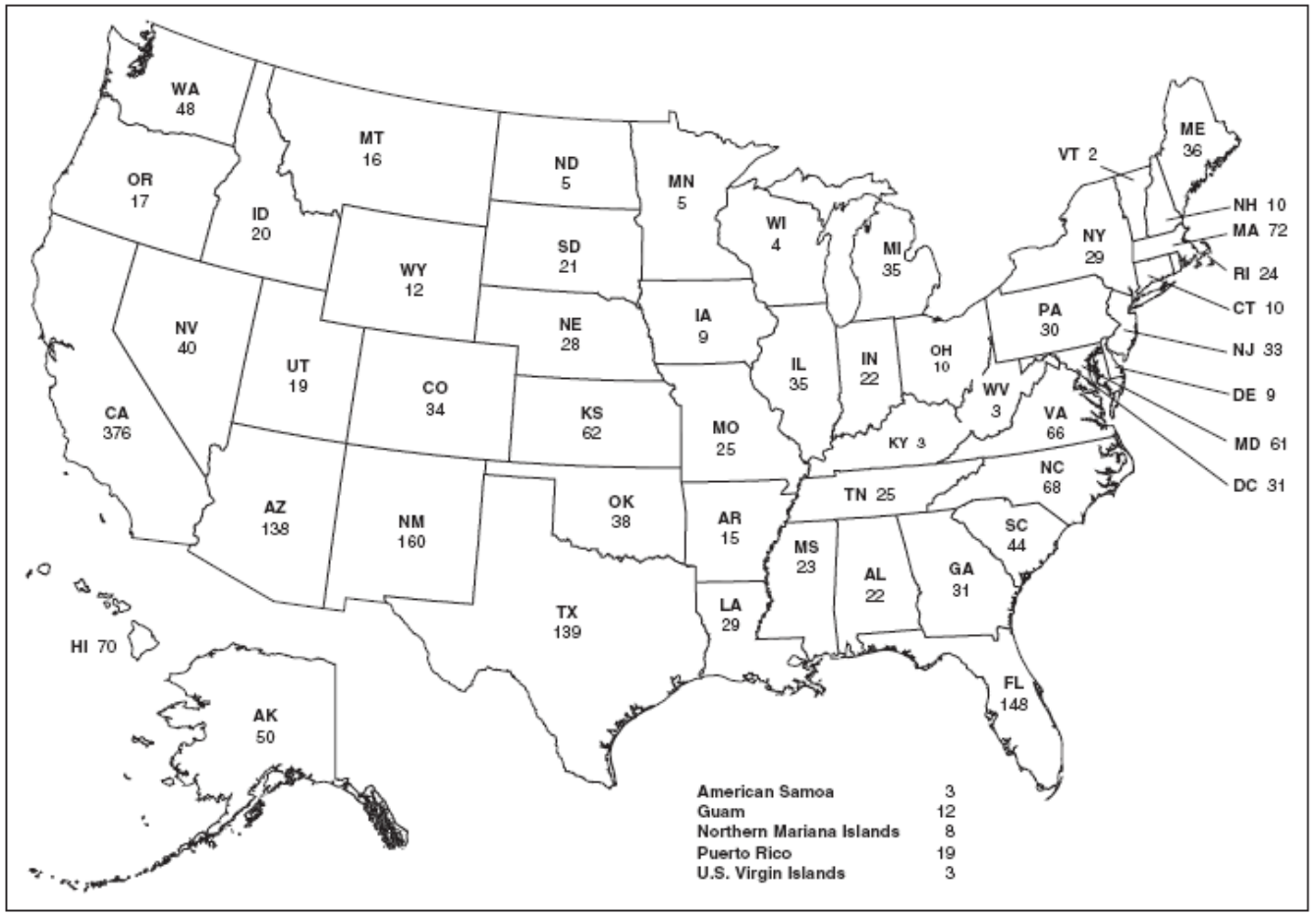


Figure 2.1. Distribution of 2,307 suspected sites contaminated with military munitions as identified by the U.S. Department of Defense. (GAO, 2003)

Physical and chemical properties of TNT and its major metabolites.

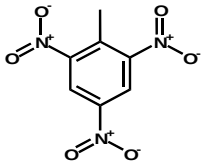
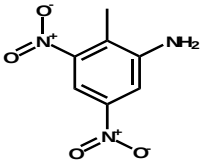
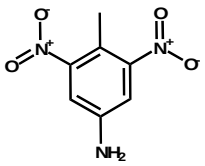
The molecular structure of TNT and closely related metabolites such as 2-ADNT and 4-ADNT dictate their reactivity and overall fate in the environment (Table 2.1). TNT consists of three nitro groups at the 2^{nd}, 4^{th}, and 6^{th} position of a benzene ring, with a methyl group at the 1^{st} position. The presence of the nitro functional groups tends to pull the π electrons of the aromatic ring towards them resulting in strong electron-withdrawing characteristics (Rieger and Knackmuss, 1995). As a result of the electron deficiency of the aromatic ring it is virtually impossible for electrophilic attack to occur by aerobic bacteria. However, the nitro group exists as a resonance hybrid with a partial positive charge on nitrogen and a partial negative charge alternating between the two oxygen atoms. In addition the electronegativity of the oxygen atom is greater than the nitrogen atom resulting in the polarization of the N-O bond (Preuss and Rieger, 1995). These factors allow the nitro groups to be easily reducible to amino groups resulting in metabolites such as 2-ADNT and 4-ADNT.

Fate of TNT in soil-water systems

Transformation of TNT

Transformation is one of the most important factors affecting the fate and transport of TNT and its metabolites in the soil. Numerous reviews have been written covering research focused on the transformation of TNT (Boopathy, 2000; Esteve-Nunez et al., 2001; Pennington and Brannon, 2002; Lewis et al., 2004). Research focused on the biotic transformation of TNT has shown that a variety of microorganisms including gram-negative and gram-positive bacteria, fungi, and actinomycetes are capable of

Table 2.1 Physical and chemical properties of 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (2-ADNT), and 4-amino-2,6-dintirotoleue (4-ADNT).

Name	Structure	Property	Information[†, ‡]
2,4,6-trinitrotoluene TNT		Molecular Weight	227.13
		Color	yellow
		Water solubility at 20 °C	130 mg L^{-1}
		Log K_{ow}	1.6 - 2.0
		Vapor pressure at 20 °C	$2.65x10^{-5}$ kPa
2-amino-4,6-dinitrotoluene 2ADNT		Molecular Weight	197.17
		Color	yellow
		Water solubility at 20 °C	2800 mg L^{-1}
		Log K_{ow}	1.94
		Vapor pressure at 20 °C	$4x10^{-5}$ torr
4-amino-2,6-dinitrotoluene 4ADNT		Molecular Weight	197.17
		Color	yellow
		Water solubility at 20 °C	2800 mg L^{-1}
		Log K_{ow}	1.91
		Vapor pressure at 20 °C	$2x10^{-5}$ torr

[†]Data from ATSDR, 1995

[‡]Data from Walsh et al. 2005

transforming TNT under aerobic, anaerobic, and anoxic conditions (McCormick et al., 1976; Boopathy, 1994; Bruns-Nagel, et al., 1996; Bayman and Radkar, 1997; Ederer et al., 1997; Scheibner et al., 1997; Hawari et al., 1998; Vorbeck et al., 1998; Vasilyeva et al., 2000).

Under normal environmental conditions, aerobic transformation is the most common transformation pathway. For example, Oh et al. (2001) reported that the gram-negative bacteria *Pseudomonas aeruginosa* isolated from TNT-contaminated soil was capable of aerobically transforming TNT (to 2-ADNT and 4-ADNT) through the production of the nitroreductase enzyme. Additionally, *Pseudomonas savastanoi* also isolated from a TNT contaminated soil was capable of denitrifying TNT under aerobic conditions to produce 2,4-dinitrotoluene (2,4-DNT) with 2-ADNT and 4-ADNT as incidental products (Martin et al., 1997). Fungi native to soil (*Trichoderma viride*, *Cladosporium resina*, and *Cunninghamella echinulata* var. *elegans*) and two white-rot basidiomycetes (*Schizophyllum commune* and *Phanerochaete chrysosporium*) have demonstrated the ability to transform TNT via enzymatic reduction (Fernando et al., 1990; Bayman and Radkar, 1997). A study conducted by Bayman and Radkar (1997) demonstrated that *Cunninghamella echinulata* var. *elegans* and *Trichoderma viride* were capable of converting 27 and 19%, respectively, of a 200 ppm TNT solution into aqueous-soluble metabolites and that after three days no TNT was detectible in the cultures. An additional aerobic pathway, although not as likely, is the formation of a Meisenheimer complex (Figure 2.2). Bacteria such as *Mycobacterium sp.* and *Rhodococcus erythropolis* have been shown to produce the hydride-Meisenheimer

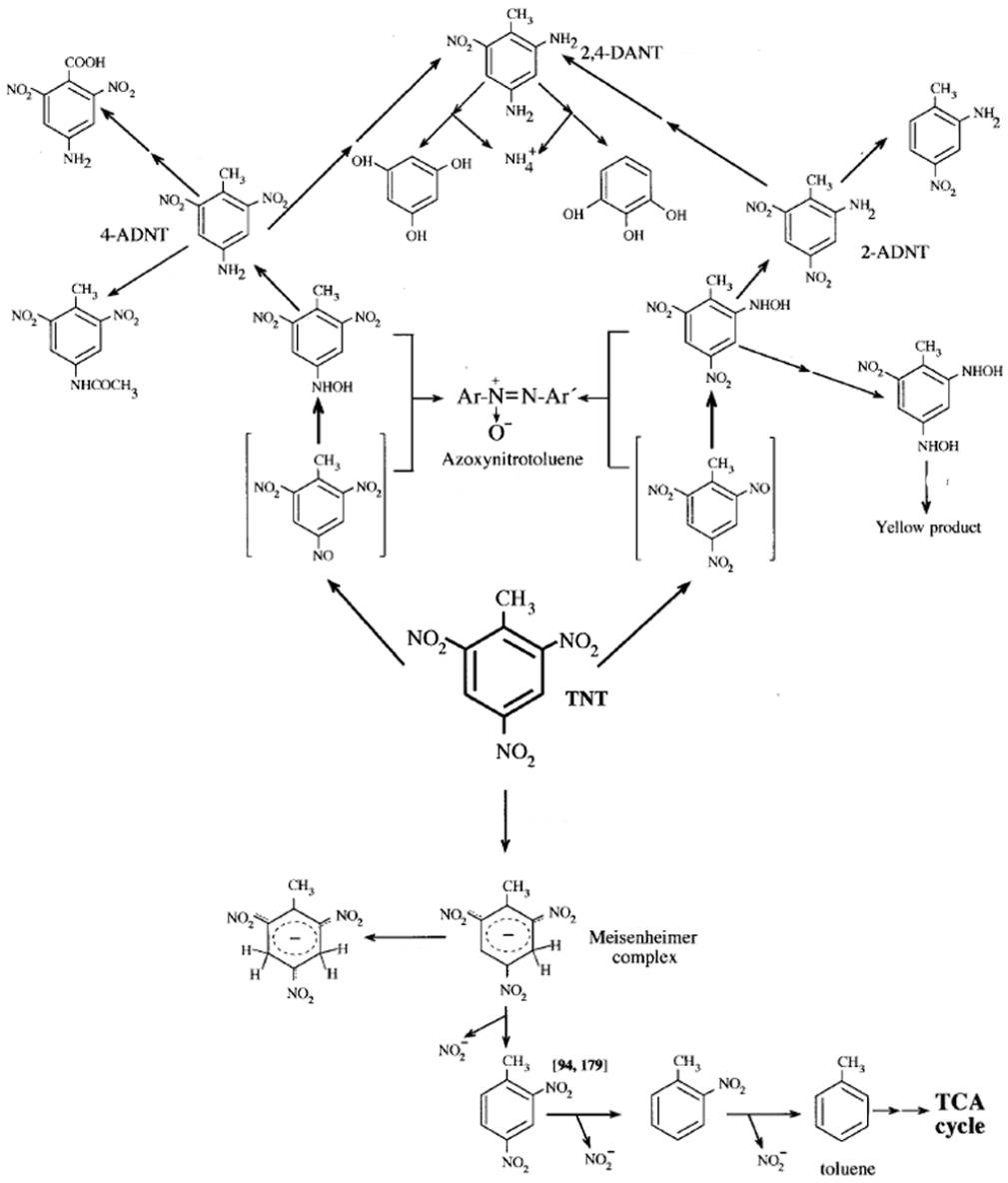


Figure 2.2. Pathways for the aerobic transformation of TNT by microorganisms. Two arrows indicate the conversion of a compound to another is believed to occur through a series of intermediates (modified from Esteve-Nunez et al., 2001)

complex under aerobic conditions (Vorbeck et al., 1994; Vorbeck et al., 1998; Pak et al., 2000). If transformation proceeds via the Meisenheimer complex TNT can potentially be transformed into toluene, a compound that can be incorporated into the trichloroacetic acid (TCA) cycle that is the normal metabolic cycle from which microbes synthesize energy.

The transformation of TNT to 4-ADNT is the energetically favored pathway as it is easier to reduce the nitro- group located at the 4^{th} carbon on the benzene ring than it is to reduce the 2^{nd} carbon due to the close proximity to the methyl- group and the nitro group on the 6^{th} carbon (Elovitz and Weber, 1999). The reduction of two nitro- groups leading to the formation of 2,4- diamino-6-nitrotoluene (2,4-DANT) is energetically more difficult to form and are observed much less frequently then 2-ADNT or 4-ADNT. The reduction of all three nitro- groups leading to the formation of 2,4,6-triaminotoluene (TAT) is rarely observed as it requires strong reducing or anaerobic conditions to reduce all three nitro groups (McCormick et al., 1976; Rieger and Knackmuss, 1995, Lewis et al., 1996; Daun et al., 1998; Hawari et al., 1998). The transformation pathways and products produced under anaerobic conditions are shown in Figure 2.3. Anaerobic transformation of TNT is preferred over aerobic transformation due to the rapid reduction of TNT and absence of azoxy- intermediates that may be more toxic than TNT (Esteve-Nunez et al., 2001; Wang et al., 2003). In general, two genera of bacteria have been identified as reducing TNT under anaerobic conditions: *Clostridium* and *Desulfovibrio*. For example, Lewis et al. (1996) demonstrated that *Clostridium bifermentans* LJP-1 was able to transform TNT into TAT and phenolic compounds capable of being degraded further. Additionally, Boopathy and Kulpa (1994) reported that the sulfate-reducing

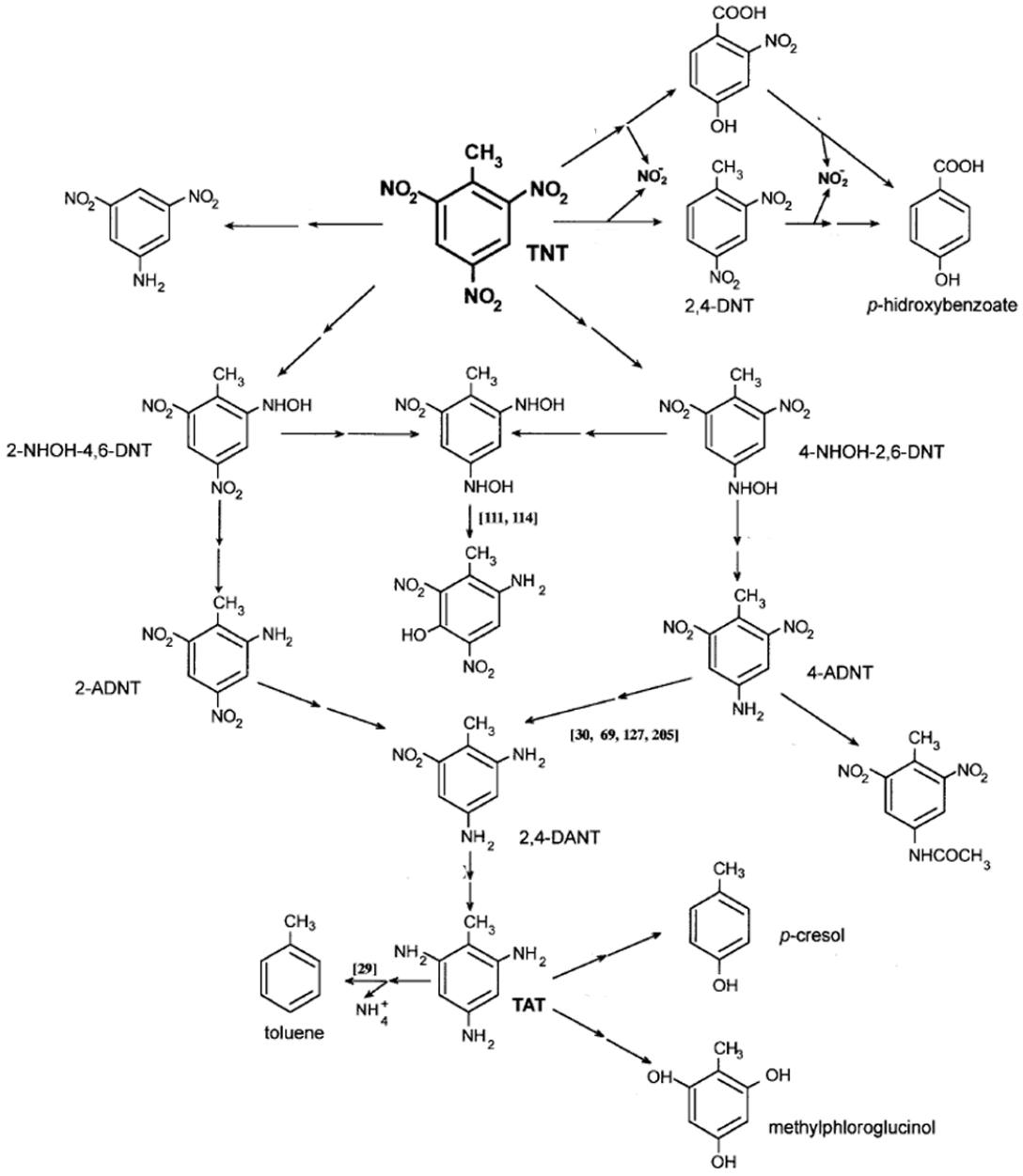


Figure 2.3. Pathways for the anaerobic transformation of TNT by microorganisms. Two arrows indicate the conversion of a compound to another is believed to occur through a series of intermediates (modified from Esteve-Nunez et al., 2001)

bacterium *Desulfovibrio* sp. (B strain) could use TNT as a sole nitrogen source and as an electron acceptor in the absence of sulfate. Under nitrogen-limiting conditions the authors reported 100% removal of TNT after 8 days and detected toluene which formed from the denitration of TAT.

It should be noted that the aerobic progression of TNT to 2-ADNT, 4-ADNT, or 2,4-DANT does not result in metabolizable compounds, however, the anaerobic transformation to TAT does (Figure 2.2). Although a variety of organisms have been identified as being capable of degrading or transforming TNT, in most cases the organisms have required a co-substrate and/or have only been successful in pure cultures without other microorganisms or soil fauna present (Boopathy and Kulpa, 1994; Hawari et al., 2000).

Although there is considerable knowledge regarding biotic transformation, much less is known regarding abiotic transformation of TNT (Haderlein and Schwarzenbach, 1995). Under anaerobic conditions iron (hydr)oxide coatings on mineral surfaces have been shown to promote the reduction of NACs such as TNT (Klausen et al. 1995; Brannon et al., 1998). Brannon and coworkers demonstrated minimal TNT transformation in samples containing only Fe^{+2} and TNT or montmorillonite and TNT. However, in montmorillonite + TNT + Fe^{+2} systems with a pH > 7 the authors reported 80-99% disappearance of the initial TNT concentrations (0.11mmol L^{-1}). Transformation products included 2ADNT, 4ADNT and azoxy compounds. Additionally as much as 50% of the initial TNT was unaccounted for and assumed to be unextractable or converted to unidentifiable transformation products. Nefso et al. (2005) reported insignificant reactions between TNT, Fe^{2+}, and minerals such as magnetite, pyrite, and

goethite in unbuffered systems. However, in the presence of calcite the reduction occurred rapidly to completion. This was attributed to the pH of the calcite system exceeding 7.4. These results indicate that under specific conditions (anaerobic, high pH) the abiotic reduction of TNT on the surfaces of certain minerals may be a significant mechanism for TNT transformation and removal from the soil. Zero-valent iron (Fe^0) has also been used to reduce TNT under anaerobic conditions (Agrawal and Tratnyek, 1996; Hundal et al. 1997; Keum and Li, 2004). Within 8 h a 1% Fe^0 solution was able to remove 100% of a 70 mg TNT L^{-1} solution and after 24 hr a significant amount (98%) of the TNT was adsorbed by the iron within 24 hr and 38% was irreversibly bound (Hundal et al., 1997).

Previously studies have shown that the oxidation of TNT via enzymatic processes is almost nonexistent (Boopathy et al., 1994). This is due to the electron-withdrawing nature of the nitro groups leaving the aromatic structure electron deficient and preventing electrophilic attack my aerobic bacteria (Bruhn et al., 1987; Rieger and Knackmuss, 1995). However, the addition of reduced iron and H_2O_2, which initiates the Fenton reaction, into systems containing TNT has shown promise as a method to remediate TNT contaminated water and soil (Hundal et al., 1997; Li et al., 1997). Fenton oxidation is a strong oxidizing reaction which is capable of oxidizing reduced TNT intermediates and metabolites (Hundal et al., 1997). The products formed from this strong oxidation are readily degradable resulting in the mineralization of TNT. Li et al. (1997) reported 100% destruction of TNT using fenton oxidation and the subsequent exposure to UV-light resulted in > 90% mineralization.

TNT sorption to soil and soil components

Sorption of TNT and its metabolites to soil has been well documented (Pennington and Patrick; 1990; Comfort et al., 1995; Xue et al., 1995; Selim et al., 1995; Pennington and Brannon, 2002). Specifically, clay minerals and soil organic matter are the most common sorbents of these compounds in the environment. Table 2.2 is a summary of TNT sorption parameters for various sorbents under different environmental conditions. Although there have been many studies examining TNT sorption there is some discrepancy as to the extent TNT is reversibly sorbed to soils. Studies performed by Pennington and Patrick (1990) and Haderlein et al. (1996) reported that TNT is readily desorbed from soil and clay minerals. However, others have reported the formation of irreversibly bound residues from the sorption of TNT and related metabolites. One possible explanation for the conflicting results was the length of time TNT was exposed to the sorbent. For example, although equilibration is achieved fairly quickly (< 1 hr), Hederlein et al. (1996) and Pennington and Patrick (1990) used equilibrium times of 30 to 60 min and 2 h, respectively. Alternatively studies involving batch equilibration times of > 24 h and long term aging studies up to 168 d have reported the formation of irreversibly bound residues. These studies reported that 19 to 47 % of the initial concentration of TNT was not removed from the soil after exhaustive extractions using solvents (Hundal et al., 1997; Achtnich et al., 1999; Sheremata et al., 1999). Thus, TNT may be reversibly bound to clay and soil in the short term but with prolong exposure the extractability of TNT becomes more difficult. This also suggests that the sorption mechanism between TNT and these sorbents is complex and may change with time.

Table 2.2. TNT sorption isotherm parameters for various sorbents under different environmental conditions.

	Environmental	Equalibration	Linear		Freundlich			Langmuir			
Sorbent	Conditions	Time	K_d	r^2	K_f	N	r^2	K_L	q_{max}	r^2	Reference
POM	pH = 4.41	22 h	230	0.978	750	0.78	0.998	0.002	150	0.999	Eriksson and
DOM	pH = 4.41	22 h	46	0.346	1460	0.34	0.975	0.034	10	0.943	Skyllberg, 2001
POM	pH = 6.19	22 h	330	0.958	1230	0.72	0.998	0.006	94	0.997	Eriksson and
DOM	pH = 6.19	22 h	110	0.043	2550	0.34	0.899	0.074	14	0.975	Skyllberg, 2001
Humic Acid	pH = 4.6	22 h	0.0516	0.0787[†]	0.0667	0.935	0.0726	0.0034	17	0.0701	Li et al. 1997
Humic Acid	pH = 6.8	22 h	0.0603	0.0144	0.0844	0.962	0.0544	0.0027	29	0.0465	
Humic Acid	IS (mM) = 4 pH = 7.1	22 h	0.088	0.189	0.21	0.752	0.621	0.024	6	0.456	Li et al. 1997
Humic Acid	IS (mM) = 20 pH = 7.1	22 h	0.137	0.241	0.28	0.794	0.467	0.0187	11	0.355	
Cornhusker Topsoil	pH = 7.12	2 h	4.1	0.93	10.2	1.6	0.96	0.16	72	1	Pennington and Patrick, 1990
Crane Topsoil	pH = 4.79	2 h	3.7	0.92	8.8	1.5	0.94	0.12	76	0.99	
Top Soil	Eh = -150, pH = 5.0	2 h	7.1	0.9	18	1.8	0.84	0.55	60	0.75	Pennington and Patrick, 1990
Top Soil	Eh = +450, pH = 5.0	2 h	5.7	0.92	10	1.4	0.91	0.22	63	0.94	
Top Soil	Eh = -150, pH = 8.0	2 h	7.3	0.94	15	1.5	0.95	0.25	83	0.91	Pennington and Patrick, 1990
Top Soil	Eh = +450, pH = 8.0	2 h	5.6	0.98	9.7	1.3	0.98	0.17	73	0.97	
Kaolinite	K+ saturated	30 -60 min	1800								Haderlein et al.,
Illite	K+ saturated	30 -60 min	12500								1996
Montmorillonite	K+ saturated	30 -60 min	21500								
Kaolinite	Ca+ saturated	30 -60 min	0.3								Haderlein et al.,
Illite	Ca+ saturated	30 -60 min	1.2								1996
Montmorillonite	Ca+ saturated	30 -60 min	1.7								
Top Soil	pH = 5.6	22 h			6.38	0.816	0.98				Sheremata et al.,
Illite	pH = 8.0	22 h			223.6	0.469	0.99				1993
Sharpsburg Top Soil	pH = 6.0, O.M. = 3.1%	22 hr			9.5	0.64	0.94				Comfort et al., 1995

[†] The authors used χ^2 instead of r^2 as a measure of goodness of fit, where χ^2 = Sum of $(F_i - f_i)^2$ is the gooness of fit and F_i is the value of the ith data point, and f_i is the value obtained from the fit. The best fit is the one to minimize χ^2

Abbreviations:

K_d - Linear partitioning coefficient, K_f - Freundlich partitioning coefficient, N - Freundlich characteristic constant, K_L - Langmuir constant, q_{max} - Maximum sorption capacity, N - Freundlich characteristic constant, POM - particulate organic matter, DOM - dissolved organic matter

Sorption mechanisms for TNT and other NACs to soil and soil components have been investigated and again, there are conflicting theories reported in the literature. Weissmahr et al. (1997) and Haderlein et al. (1996) using *in-situ* spectroscopic techniques (ATR-FTIR, ^{13}C-NMR, UV/VIS, XRD) proposed a co-planar electron donor-acceptor (EDA) mechanism to explain TNT sorption to clay minerals. Based on this theory, siloxane oxygen sheets with negative charge (arising from isomorphous substitution in the clay structure) donate electrons to NACs, which are e- acceptors due to the electron-deficient π-system as a result of the electron withdrawing properties of the nitro groups. The extent to which EDA complexes are formed was determined to be a function of the type of interlayer exchangeable cation and its corresponding degree of hydration. For example, Haderlein et al. (1996) reported TNT K_d values of 21,500 and 1.7 L kg^{-1} on a K^+- and Ca^{2+}- saturated Arizona smectite (SAz-1), respectively. The weakly hydrated K^+- (-360 kJ mol^{-1} enthalpy of hydration) was easily replaced by TNT where the strongly hydrated Ca^{+2}- (-1669 kJ mol^{-1} enthalpy of hydration) prevented TNT interaction with the clay surface (Haderlein et al. 1996; Johnston et al., 2004). The authors also stated that their results indicated that the EDA complex formation occurred mainly at external and to a lesser degree interlamellar siloxane surfaces inferring that TNT sorption occurs mostly on external surfaces.

Boyd et al. (2001) and Johnston et al. (2001) provided a modified and contrasting mechanism to the EDA complex. Again using *in-situ* spectroscopic methods the authors proposed that sorption of NACs to clay minerals was controlled by the ability of NACs to form inner-sphere complexes directly with exchangeable cations rather than interactions with negatively charged siloxane oxygens. However, similar to the findings of Haderlien

and co-workers the type of cation and the strength of hydration again were important factors involved in NAC sorption. The low hydration energy of K^{+}- would allow for direct inner-sphere coordination with TNT whereas the high hydration energy of Ca^{+2}- inhibits this coordination. Additionally, sorption of TNT to clay minerals increases with an increasing number of nitro- groups and decreases with the presence of electron-releasing groups such as amines. This explains why TNT sorption to clay minerals is greater than its metabolites (2-ADNT, 4-ADNT, 2,4-DANT, etc) as has been reported (Sheremata et al., 1999). Li et al. (2003) examined the sorption of two NAC pesticides to clay minerals and humic-clay complexes. They noted that humic acid, which was associated with the external surface of the clay minerals, had no impact on the sorption of either pesticide by the humic-clay complex. Additionally, XRD analysis indicated intercalation of the smectite clay was no different compared to the humic-clay complex when exposed to the pesticide. Thus, the authors concluded that the interlameller regions were the primary domains for NAC sorption. Furthermore, quantum chemical studies and quantum calculations have provided direct evidence against the EDA mechanism as the significant sorption mechanism for NACs (Pelmenschikov and Leszczynski, 1999; Johnston et al., 2001). In these studies, the high affinity of NACs for the siloxane surface was determined to be the result of nonspecific, short-ranged dispersion interactions otherwise known as van der Waals interactions.

An additional mechanism which has been discussed further elaborates on the nonspecific, short-ranged van der Waals interactions between NACs and clay minerals. Johnston et al. (2004) provides a detailed discussion on the hydrophobic characteristics of clay minerals. Clay minerals without isomorphic substitution or that have low surface

charge densities are known to have hydrophobic characteristics. Hectorite (SHCa-1) is an example of a 2:1 clay mineral that exhibits hydrophobic characteristics (Van Oss and Giese, 1995). Briefly, for clays which have isomorphous substitution, the charged surfaces on the clay mineral are separated by distances of 1 – 2 nm. The space between the charge sites is neutral and has been shown to stabilize NAC sorption due to the neutral and planar characteristics of many NACs. Because TNT has a fairly low solubility in water (90 – 130 mg L^{-1}) and a moderate octanol:water coefficient (K_{ow} = 1.6), these sites are capable of contributing to the sorption of TNT. Studies involving the NAC 4,6-dinitro-o-cresol and non NACs such as atrazine have provided evidence of hydrophobic interactions with clay minerals (Boyd et al., 2001; Sheng et al., 2002). These studies reported that the intercalation of these compounds was due to compound partitioning from the bulk water and into the subaqueous environment of the internal clay surfaces. This also supports the conclusions of Li et al. (2003), discussed above, that the interlayer region provides most of the available sorption sites for NACs.

Overall, these studies do not provide unequivocal evidence of one dominant mechanism for the sorption of NACs and TNT to clay minerals. Additionally, although significant progress has been made to identify TNT sorption mechanisms for clay minerals, little is known as to the mechanism for NAC sorption to organic matter. Studies involving NAC sorption to organic substances such as humic acid, dissolved and particulate organic matter, and natural organic matter have yet to identify anything other than nonspecific interactions (Li et al., 1997; Weissmahr et al. 1999; Eriksson and Skyllberg, 2001). Although there is still much debate, it is obvious that the sorption of

NACs to soil and soil components is complex and involves multiple sorption mechanisms.

Bioavailability and ecotoxicity of TNT in soil-water systems.

Toxicity to humans first became a concern during World War I when > 17,000 people were poisoned and there were ≈475 deaths as a result of TNT contamination (Yinon, 1990). Studies have also determined that TNT is mutagenic, however, since the determination of toxic effects on humans have been limited to experiments with rats, TNT is listed as a possible carcinogen (Won et al., 1976; ATSDR, 1995). Since this time a substantial amount of research has been conducted investigating the toxicity of TNT and other explosives to a variety of organisms including soil bacteria, plants, mammals, and humans. A summary of toxicity research on these organisms is presented in Tables 2.3 and 2.4. Often times the method of exposure or contact is overlooked with respect to interpreting the levels contaminant or TNT toxicity to organisms. For example, Robidoux et al. (1999) exposed the earthworm *Eisenia andrei* to TNT in soil and on filter paper and reported drastically different degrees of toxicity (LC_{50} values). Although the concentrations at which TNT was lethal to 50% of the test population is expressed in different units, if you account for the amount of soil and the area of filter paper; earthworms were much more tolerant of TNT in soil than on filter paper. The differences are due to the methods of exposure and fate of TNT within soil. On filter paper, earthworms are only being exposed to TNT by dermal contact, however, in the soil, they are coming into contact with TNT externally and internally through the ingestion of soil spiked with TNT. This brings up the concept of bioavailability.

Table 2.3 Selected studies showing the relative toxicities of TNT to soil micro- and macro-fauna.

Organism	Species	TNT Concentration	Method of Contact	Result[†]	Reference
		soil microorganisms			
gram negative bacteria	*Pseudomonas* sp.	50 mg L^{-1}	soil	55% decrease in aerobic growth rate	Sciliano et al. 2000
	Pseudomonas sp.	26 mg L^{-1}	soil	50% inhibition of denitrification activity (N_2O - N_2)	Sciliano et al. 2000
gram positive bacteria	determined by PLFA	8-16 mg L^{-1}	culture (agar)	growth inhibition	Fuller and Manning 1998
	determined by PLFA	4,177 mg kg^{-1}	soil	effective concentration causing 50% reduction in the percent of isolates	Fuller and Manning 1998
actinomycetes	determined by PLFA	362 mg kg^{-1}	soil	effective concentration causing 50% reduction in the percent of isolates	Fuller and Manning 1998
fungi	*P. chrysosporium*	10 mg L^{-1}	soil	50% decrease in mycelial dry weight	Spiker et al. 1992
	P. chrysosporium	45 mg L^{-1}	soil	96% decrease in respiration	Stahl and Aust. 1993
	Cunninghamella echinulata var. *elegans*	50 mg L^{-1}	culture (agar)	90% decrease in radial growth after 6 d	Bayman and Radkar 1997
	Trichoderma viride	100 mg L^{-1}	culture (agar)	51% decrease in radial growth after 6 d	Bayman and Radkar 1997
	Cladosporium resinae	100 mg L^{-1}	culture (agar)	61% decrease in radial growth after 6 d	Bayman and Radkar 1997
soil microbial community	NA	50 mg kg^{-1} (DW)[‡], 139 mg kg^{-1} (AE), 314 mg kg^{-1} (IA)	soil	50% inhibition of dehydrogenase activity	Gong et al. 1999a
		soil invertebrates			
Earthworms	*Eisenia andrei*	1.5 - 7.1 μg cm^{-2}	filter paper	LC_{50} after 72 h using 5 trials	Robidoux et al. 1999
		365 mg kg^{-1}	OECD soil	LC_{50} after 14 d	Robidoux et al. 1999
		222 mg kg^{-1}	forest soil	LC_{50} after 14 d	Robidoux et al. 2000
		143 mg kg^{-1}	sandy forest soil	LC_{50} after 14 d	Renoux et al. 2000
		132 mg kg^{-1}	amended sandy forest soil	LC_{50} after 14 d	Lachance et al 2004
	Eisenia fetida	110 mg kg^{-1}	aritifical soil	NOEC (based on change in body weight) after 14 d	Phillips et al. 1993
		< 150 mg kg^{-1}	forest soil	NOEC (based on change in body weight) after 14 d	Phillips et al. 1993
Potworms	*Enchytraeus albidus*	422 mg kg^{-1}	OECD soil	LC_{50} after 21 d	Dodard et al. 2003
	Enchytraus crypticus	1,290 mg kg^{-1}	Lufa 2.2 soil	LC_{50} after 7 d	Schafer and Achazi 1999

[†] Abbrevations used to describe the result:
LC_{50}, concentration at which it is lethal to 50% of the test population
NOEC, no observed effect concentration
LOEC, lowest observed effect concentration
LOAEC, lowest observed adverse effect concentration

[‡] Abreviations used in this study:
DW, toxicity parameter based on the amount of water extractable TNT
AE, toxicity parameter based on the amount of solvent (acetonitrile) extractable TNT
IA, toxicity parameter based on the amount of TNT initially applied to the soil

Table 2.4 Selected studies showing the relative toxicities of TNT to fish, terrestrial plants, and mammals.

Organism	Species	TNT Concentration	Method of Contact	Result[†]	Reference
aquatic invertebrates/fish					
midge	*Tantytarsus dissimillis*	27 mg L^{-1}	water	LC_{50} after 48 hr, static test	Liu et al. 1983b
midge	*Chironomus tentans*	170 mg kg^{-1}	sediment in water	LC_{50} after 10 d	Stevens et al. 2002
rainbow trout	*Oncorhynchus mykiss*	0.8 mg L^{-1}	water	LC_{50} after 96 hr, static test	Liu et al. 1983b
fathead minnow	*Pimephales promelas*	2.6 mg L^{-1}	water	LC_{50} after 96 hr, flow-through test	Smock et al. 1976
terrestrial plants					
Alfalfa	*Medicago sativa* L.	150 mg kg^{-1}	freshly amended soil	LOEC on seedling germination after 7 d	Rocheleau et al. 2006
		215 mg kg^{-1}	aged/wheathered soil	LOEC on seedling germination after 7 d	Rocheleau et al. 2006
Japanese millet	*Echinochloa crusgalli* L.	194 mg kg^{-1}	freshly amended soil	LOEC on seedling germination after 7 d	Rocheleau et al. 2006
		0.1 mg kg^{-1}	aged/wheathered soil	LOAEC on seedling germination after 7 d	Rocheleau et al. 2006
Ryegrass	*Lolium perenne* L.	95 mg kg^{-1}	freshly amended soil	LOEC on seedling germination after 7 d	Rocheleau et al. 2006
		0.2 mg kg^{-1}	aged/wheathered soil	LOAEC on seedling germination after 7 d	Rocheleau et al. 2006
Wheat	*Triticum aestivum* L.	50 mg kg^{-1}	sandy soil	LOAEC on seedling germination after 14 d	Gong et al. 1999 b
Oat	*Acena sativa* L.	50 mg kg^{-1}	sandy soil	LOAEC on seedling germination after 14 d	Gong et al. 1999 b
Turnip	*Brassica rapa*	50 mg kg^{-1}	sandy soil	LOAEC on seedling germination after 14 d	Gong et al. 1999 b
mammals					
Rat		2 mg kg^{-1} d^{-1}	Diet	LOAEL on the kidney, bones, spleen after 24 mon	Furedi et al. 1984
		25 mg kg^{-1} d^{-1}	Diet	LOAEL resulting in testicular atrophy after 13 wk	Levine et al. 1984
Mouse		70 mg kg^{-1} d^{-1}	Diet	LOAEL on the liver, anemia after 24 mon	Furedi et al. 1984
Dog		0.5 mg kg^{-1} d^{-1}	Diet	LOAEL on the liver after 25 wk	Levine et al. 1990
		2 mg kg^{-1} d^{-1}	Diet	LOAEL on the liver, anemia after 13 wk	Dilley et al. 1982

[†] Abbrevations used to describe the results:
LC_{50}, concentration at which it is lethal to 50% of the test population
NOEC, no observed effect concentration
LOEC, lowest observed effect concentration
LOAEC, lowest observed adverse effect concentration
LOAEL, lowest observed adverse effect level

It appears that TNT was more available to earthworms on filter paper than in the soil, possibly due to the sorption of TNT by clay minerals and soil organic matter. So when developing risk assessment of TNT or other contaminants, you need to consider the route of exposure.

The concept of bioavailability with respect to organic and inorganic contaminants in soil-water systems has often produced controversy and historically has been difficult to define. Typically, bioavailability has been defined as the fraction of a contaminant that is not bound to the soil or that can be easily extracted from the soil. Moore (2003) noted that bioavailability is a function of toxicity and the interaction with an ecological receptor needs to be considered when evaluating bioavailability. Thus, bioavailability and toxicity are closely linked. Recently, the Committee on Bioavailability of Contaminants in Soils and Sediments, part of the National Research Council (NRC), defined "bioavailability" as:

> The mass transfer and transport of contaminants to be potentially absorbed, adsorbed or altered by organisms, plants, and animals conditioned by the contaminant properties, soil characteristics, and the biology of organisms under a variety of environmental conditions at a specific time (NRC, 2003).

Their approach was to define bioavailability using a general definition in terms of processes as illustrated by Figure 2.4.

TNT bioavailability in soil has often been determined by using chemical extractions (process A in Figure 2.4). For example, 34,600 and 87,000 mg TNT kg^{-1} were extracted from the Joliet Army Ammunition Plant (JAAP, Joliet Illinois) and the Umatilla Munitions Depot Activity (UMDA, Umatilla, Oregon), respectively using a

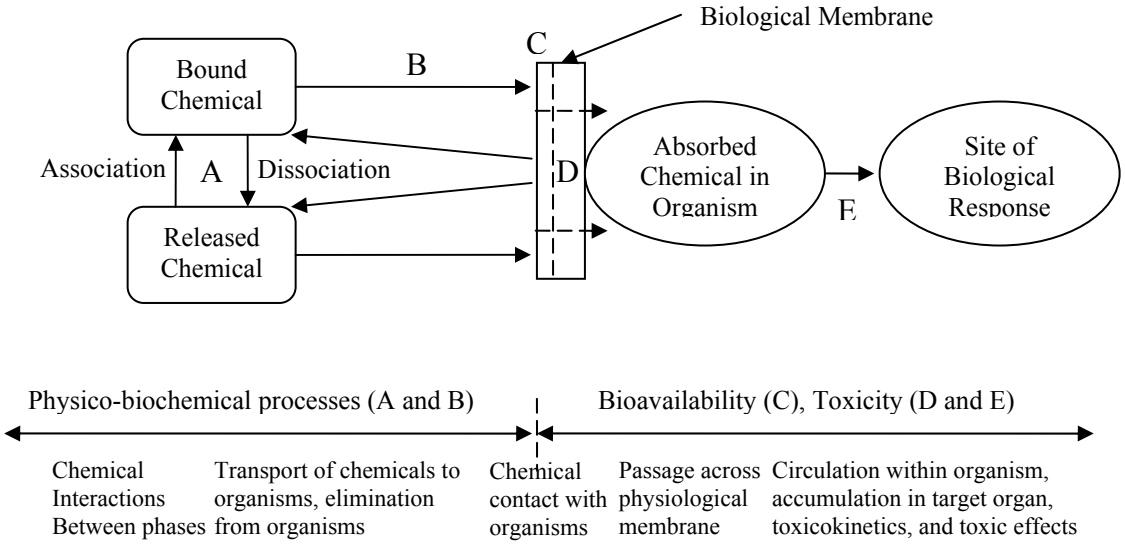


Figure 2.4. Bioavailability processes in soil (modified from Adriano, 2003; National Research Council, 2003).

solvent (acetonitrile) extraction (Pennington et al. 1995; Simini et al., 1995). Additionally it was determined that these values greatly exceed the EPA risk-based concentrations for human exposure of 95 and 21 mg kg^{-1} for industrial and residential areas, respectively (ATSDR, 1995). Although > 34,000 mg TNT kg^{-1} were extracted, this may not be the concentration which is accessible to an organism, plant, or person. Because solvent extractions may remove TNT that under normal conditions is tightly bound to the soil, some researchers have used water or low ionic strength salt solutions to mimic the amount of TNT that could be removed from the soil during a rain or flooding event (Hundal et al., 1997; Gong et al., 1999a). Additionally, sequential extractions using water followed by a solvent have been used to measure the readily and potentially available fractions. For example, Hundal et al. (1997) spiked a Sharpsburg soil (A horizon) with ≈ 210 mg kg^{-1} TNT. Thirty minutes after spiking the soil the authors used a sequential extraction scheme consisting of a 3 mM $CaCl_2$ solution followed by acetonitrile and measured 42.3 and 34.4 mg kg^{-1} TNT, respectively, with a total extracted amount of 76.7 mg kg^{-1} TNT. Thus only 55.1% of the total was removed by water (weak salt solution) and was considered bioavailable.

Although chemical extraction provides a general idea as to the amount of TNT or contaminant that is bioavailable, more direct methods measuring organism exposure have provided a better measure of bioavailability and toxicity (processes B and C, Figure 2.4). Frische (2003) used a battery of five bioassays consisting of plant growth, Collembola reproduction, soil respiration, luminescent bacteria acute toxicity, and a mutagenicity test along with chemical extractions to determine the amount of bioavailable TNT in soil. The advantage of this method is the potential ability to determine the bioavailability and

toxicity of TNT to a variety of receptors (i.e. plants, bacteria, soil fauna, etc). However, the disadvantage is that these multiple methods are more time consuming and may only reflect the bioavailability to a specific receptor. Additionally, the bioavailability of TNT has been measured by assessing plant uptake (Wang et al., 2003; Rocheleau et al., 2006), the absorption of TNT by earthworms (Renoux et al., 2000; Lachance et al., 2004), and fish (Belden et al., 2005; Ownby et al., 2005)

2.2 Soil aggregation and fractionation

Over the past several decades it has been shown that soils can be grouped into distinct aggregate fractions that often show meaningful relationships between ecological function and soil properties such as organic matter dynamics and C and N pool sizes (Tisdall and Oades 1982; Monrozier et al., 1991; Brady and Weil, 1999). Based on a model developed by Tisdall and Oades (1982) these aggregates can be classified into a hierarchical order. Small, primary soil particles (< 53μm) are cemented together into microaggregates (53 – 250 μm) by persistent binding agents (i.e. humified organic material) through various processes (i.e. polyvalent-clay and metal-humic organic matter complexes). These microaggregates are then further bound together by more labile binding agents (i.e. polysaccharides, roots, fungal hyphae) into macroaggregates (> 250 μm).

Oades (1984) proposed a modification to the original model by suggesting that microaggregates could also form within macroaggregates. In this model, macroaggregates could still be derived from microaggregates or from fresh residues (i.e. plant material) that serves as an aggregate "nucleus". Next polysaccharides and other

labile organic compounds are accumulated and deposited around the aggregate nucleus. With time, the nucleus will decompose and break into finer organic particles that interact with clay particles and microbial by-products resulting in the formation of microaggregates within the former aggregate "nucleus" or macroaggregate. Then the cycle can start again as the newly formed microaggregates can then be incorporated into macroaggregates in a similar manner proposed by Tisdall and Oades (1982). A conceptual model of this process was presented by Six et al. (1999) and is shown in Figure 2.5.

Numerous studies and reviews have proposed that earthworms, micro-organisms, binding agents, and land management practices can influence various processes and properties at the aggregate level (Shipitalo and Protz, 1989; Dexter, 1988; Kay, 1990; Oades and Waters, 1991; Six et al., 1998; Six et al., 2000a). However, research focused on the characterization of soil aggregates and the processes associated with them have often produced conflicting results. This is due to the lack of *in-situ* methods to characterize soil aggregate properties and processes and the use of different aggregate separation methods. For example, the rate of denitrification, amount of water soluble carbon, total carbon and overall weight distribution of soil aggregates are different when assessed using dry-sieving compared to wet-sieving procedures (Beauchamp and Seech, 1990; Puget et al., 1995; Angers and Giroux, 1996). As a result, some have suggested that the links between aggregate size and various soil properties and biological processes are merely an artifact of the soil fractionation procedure and not related to intrinsic properties specific to an aggregate size fraction (Ashman, et al., 2003).

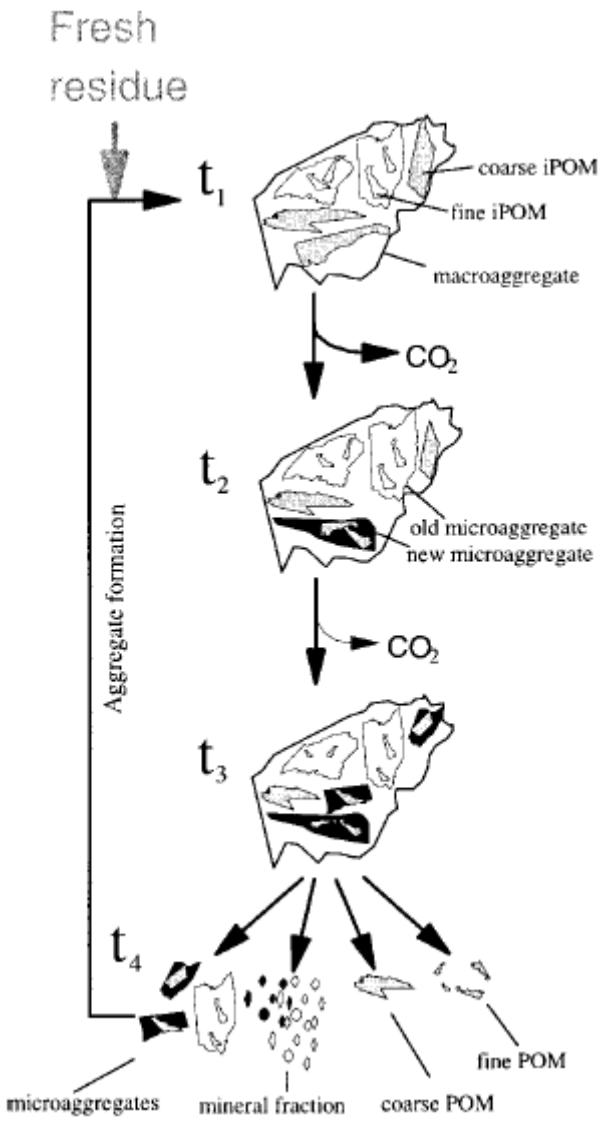


Figure 2.5. Conceptual model developed by Six et al. (1999) of the aggregate "life cycle" based on aggregate hierarchy models proposed by Tisdall and Oades (1982) and Oades (1984).

Typically, the methods used to fractionate soil try to simulate disruptive events that could occur in the field (Young and Ritz, 2000). Wet-sieving is one of the more common fractionation methods which involves the rapid wetting of air-dry samples in water and is supposed to simulate conditions of severe water stress (Nimmo and Perkins, 2002), and thus provide information about the water-stable aggregates and their associated properties/processes. However, there is considerable interest in the characterization of soil aggregates, regardless of stability, to better understand processes related to soil organic matter dynamics (Bossuyt et al., 2005), biological soil processes (Stemmer et. al., 1998; Mummey et al., 2002), and the fate of organic contaminants (Krauss and Wilcke, 2002; DeSutter et. al., 2003). For example, studies on polycyclic aromatic hydrocarbons (PAHs) have shown that the percentage of small molecular weight PAHs (2-3 ring structures) decreased with a decrease in aggregate size fraction (Muller et al., 2000; Krauss and Wilcke, 2002). This indicates that the degradation and/or volatilization of less persistent and more volatile PAHs occurred in larger sized aggregates. Additionally the lack of degradation and/or volatilization of small molecular weight PAHs in small aggregate size fractions were due to the inaccessibility to PAH degrading bacteria or greater retention reducing volatilization (Muller et al., 2000). Additional studies examining the fate of atrazine, polychlorinated biphenyls (PCBs) and PAHs have shown that the sorption and accessibility of contaminant degrading bacteria to the contaminant are dependent on the aggregate size (Muller et al., 2000; Amellal et al., 2001a; Amellal et al., 2001b; DeSutter et al., 2003).

One of the more common concerns surrounding research on these processes is whether wet-sieving methods may create their own artifacts that may misrepresent the

microbial community or the chemical properties of an aggregate fraction (Amato and Ladd, 1980; Ahmed and Oades, 1984; Miller and Dick, 1995). In contrast, dry sieving methods may provide a suitable alternative to overcoming the potential artifacts associated with wet-sieving. There is however limited information on how wet-sieving compares to dry sieving methods for the separation and collection of soil aggregates. Additionally, there is no information on how these methods might influence the apparent distribution of soil properties and their associated microbial communities

2.3 Effect of earthworms on soil properties and fate of contaminants

Earthworms historically are known to play a significant role in the ecological function of soil (Lee, 1985; Coleman et al., 2004). The ingestion and egestion of soil is perhaps one of the most important activities associated with earthworms. Through the production of earthworm casts and mucus excreted on the walls of burrows, earthworms contribute to the cycling of nutrients, formation of soil micro- and macroaggregates, and incorporation of organic materials into soil aggregates (Lee and Foster, 1991; Martin and Marinissen, 1993; Edwards and Bohlen, 1996; Six et al., 2004; Bossuyt et al., 2005). For example, research has shown that the incorporation of organic residues on the soil surface (i.e. alfalfa, plant material) into the soil via earthworm activity increases the carbon content and leads to the formation of stable soil aggregates (Zhang and Hendrix, 1995; Haynes and Fraser, 1998; Bossuyt et al., 2004; Pulleman et al., 2005). This in turn can have a significant impact on physical and biological soil properties such as water retention, solute transport, and microbial activity and biomass (Lee, 1985).

Interestingly, earthworms are known to selectively consume soil particles and organic materials of a specific size (Piearce, 1978; Lowe and Butt, 2004). Specifically, the size of particles they ingest is directly correlated to their body size (Piearce, 1978). During the ingestion of soil material by earthworms, the microaggregates are destroyed when chemical bonds between soil particles are broken (Barois et al., 1993). However, new aggregates are formed and the resulting earthworm cast contains a mixture of new and humified organic substances which promotes formation and strengthening of soil aggregates, and briefly stimulates microbial activity and biomass (Shipitalo and Protz, 1989; Lavelle et al., 1989; Zhang and Schrader, 1993; Brown et al., 2000). The earthworm casts, in a sense, can be compared to the formation of the aggregate nucleus in the aggregate hierarchy models proposed by Tisdall and Oades (1982) and Oades (1984) and discussed previously. However, the type and extent of bonding that occurs between mineral and organic materials during ingestion or after excretion in earthworm casts greatly depends on the properties of the ingested organic debris and chemical properties of the soil materials (Shipitalo and Protz, 1989).

Earthworms can be divided up into three distinct classes; epigeic, anecic, and endogeic species, with each having distinctive impacts on soil aggregates and properties (Edwards and Bohlen, 1996). Epigeic earthworms work the soil litter and contribute very little to soil aggregation and mixing of soil components within the soil. Their casts are often very unstable and don't contribute to the formation of aggregates on the soil surface. Anecic earthworms are mixers who take surface litter and bring it down into their burrows and galleries and incorporate the organic material deeper into the soil allowing for better turnover and decomposition by other soil organisms and fauna (Lee,

1985). They build deep vertical burrows and will consume a mixture of soil and surface litter. Endogeic species are earthworms which feed on the soil and organic matter within the soil. While they do sometimes consume litter material their main function and niche is within the subsoil horizons. More often than not they leave their casts within their burrows which can be quite extensive. They are also known to break down the casts and materials other earthworms have left behind (Edwards and Bohlen, 1996), which can alter the resource availability to other microorganisms and fauna (Jones et al., 1994; Lavelle et al., 1997; Lavelle et al., 1998).

Based on the previous discussion, it is reasonable to assume that earthworm activity can contribute to the translocation, transformation and/or degradation of organic contaminants. Possible examples include the incorporation of contaminants into earthworm casts and microaggregates reducing its availability to contaminant specific degrading bacteria, increasing sorption of contaminants to soil organic matter, and stimulating the microbial community resulting in increased degradation. While there have been a wide range of studies focusing on the toxicity of organic and inorganic contaminants on earthworms, few studies have focused on the ability of earthworms to alter contaminant fate (Robidoux et al., 1999; Conder and Lanno, 2000; Schaefer, 2004). Binet et al. (2006) reported that *Lumbricus terrestris* and *Aporrectodea caliginosa* altered the distribution of atrazine in a soil microcosm leading to greater leaching losses due to deeper incorporation of atrazine into the microcosm. Additionally, the formation of earthworm casts resulted in greater atrazine sorption over the long term (86 d) and thus increased atrazine persistence. Renoux et al. (2000) reported that the earthworm *Eisenia andrei* was capable of facilitating the transformation of TNT at a greater rate than a soil

without earthworms. Overall, there is a considerable need for research that focuses on how soil fauna, such as earthworms can impact the fate of organic and inorganic contaminants in the soil.

2.4 Effects of biotic and abiotic factors and contaminants on soil microbial community structure.

Soil microorganisms play an integral part in the overall health and function of the soil (Brady and Weil, 1999). Although there are a wide range of different species, microorganisms rarely exist as isolated monocultures and instead coexist as part of communities with other soil micro- and macroorganisms (Vestal and White, 1989). Assessment and characterization of these microbial communities can help in our understanding of processes important to the ecological function and health of soil. In the past, microbial cultures have been a common method used to assess microbial growth, activity, and diversity. Pure cultures are good for preliminary studies to determine the growth and function of a specific organism under ideal conditions. Although specific organisms can be examined, this method has major limitations as many organisms require specific conditions for growth and as a result, < 1% of organisms are capable of being cultured (Paul and Clark, 1996). In response to such limitations, of using selectively enriched cultures, scientists have developed methods of assessing microbial biomass, structure, and activity *in situ* to gain a more complete understanding of the ecological function of soil microorganisms (Vestal and White, 1989). Although there are no fool-proof methods, the recent development of lipid analysis has become a popular method to identify microorganisms and characterize their activity and function in soil.

Lipids are commonly found in the cytoplasmic and outer membranes of most microorganisms (Moat and Foster, 1995). In many cases, specific fatty acids are common to a given taxon (Table 2.5). For example, cyclopropane and branched fatty acids are specific to bacteria (Lechevalier, 1989). Additionally, the fatty acid 18:2ω6 makes up ≈40% of the total fatty acids of more than 45 different species of soil fungi (Paul and Clark, 1996). Phospholipid fatty acid analysis (PLFA) has been one of the more common methods to extract and analyze lipids. PLFA analysis is useful in identifying the presence or absence of specific microbial communities and has been used as a measure of microbial biomass (Zelles, 1999). It involves the use of a one phase mixture of chloroform, methanol and water (Paul and Clark, 1996). Lipids which are collected in the organic phase are then further fractionated into neutral, glycol-, and phospholipids. Finally, the phospholipids undergo alkaline methanolysis to obtain fatty acids methyl esters (FAMEs) which are then analyzed by gas chromatography (Schutter and Dick, 2000). Recently, a simpler method to the PLFA method has been developed where microbial cells from soil are saponified by sonication, heat, and the addition of a strong base. After the fatty acids have been removed, they are methylated to form FAMEs and are again analyzed using gas chromatography (Sasser, 1990). One drawback to the FAME method is that it may extract fatty acids from sources other than living organisms, such as humic substances and plant tissue (Schutter and Dick, 2000). As a result, the FAME analysis can not be used as a measure of microbial biomass to the same extent that the PLFA method is.

Table 2.5. Common fatty acids and corresponding biomarker interpretations

Biomarker Interpretation	Fatty Acids	References
gram-positive bacteria	a15:0, i15:0, i16:0, a17:0, i17:0	White et al. 1996 Zelles et al. 1994 Zelles 1997
gram-negative bacteria	16:1ω5, 18:1ω7, cy17:0, cy19:0	Paul & Clark 1996 White et al. 1996 Zelles et al. 1994 Zelles 1997
fungi	18:2ω6, 18:1ω9	Vestel & White 1989 Frostegard et al. 1993
actinomycetes	10Me16:0, 10Me17:0	Zelles et al. 1994
protozoa	20:4ω	White et al. 1996
atress	cy19:0/18:1ω7	Kieft et al. 1997

PLFA and FAME methods have been used to study changes in the microbial community over time due to physical, chemical, and biological perturbations. For example, Butler et al. (2003) used PLFA analysis to characterize soil organic carbon dynamics and flow between the rhizosphere of ryegrass and the bulk soil. The authors reported that gram-positive and gram-negative bacteria utilized ^{13}C-labeled rhizodeposits during two different growing periods and that soil fungi, as indicated by high levels of labeled C in 18:2w6, utilized carbon from the rhizosphere in both growing periods. Olsson et al. (1999) used fatty acid analysis to characterize the bacterial populations found in the rhizosphere of barley. Using multivariate statistics the authors found three distinct groups of bacteria; two different gram-negative populations dominated by *Pseudomonas and Cytophaga* and gram-positive bacteria as indicated by iso-branched fatty acids. The application of dairy manure to a field under corn-clover-ryegrass crop rotation resulted in a 15-27% increase in gram-negative bacteria biomarkers compared to the control, further demonstrating the use PLFAs or FAMEs to assess changes in microbial community structure and composition (Peacock et al., 2001).

Other studies have examined how anthropogenic activity can influence the microbial community structure. Feng et al. (2003) examined the impact of conventional and no-till practices (on soil microbial communities) in a cotton system. The authors noted a shift in the bacterial communities over time as a result of tillage practices, particularly when the field was in fallow or pre-planting. Petersen and Klug (1994) examined how sampling and storage practices may alter the microbial community before the soil is used for other research. After three weeks at elevated temperatures (25°C) there were indications the microbial community was adapting or responding to the

temperature due to an increase in cyclopropyl fatty acids (cy17:0, cy19:0) which have been used as indicators of stress.

FAME analysis has additionally been used to characterize the impact of environmental contamination on soil bacteria, fungi, actinomycetes, and protozoa. The contamination of a forest and arable soil with Cd, Cu, Ni, Pb, or Zn resulted in a decrease in iso-branched fatty acids indicative of gram-positive bacteria and an increase in cy17:0, an indicator for stress (Frostegard et al., 1993). Additionally, metal contamination in the arable soil resulted in a decrease in 10Me16:0 and 10Me18:0 (indicators of actinomycetes), however, in the forest soil there was an increase in these same indicators. This suggests that the actinomycetes were perhaps more tolerable of metal contamination in the forest soil then they were in the arable soil. Gentry et al. (2003) reported an increase in a fatty acid characteristic of protozoa in a soil contaminated with pyrene. The analysis of two different surface mine reclamation sites indicated a significant decrease in FAME biomarkers indicative of fungi and bacteria compared to an undisturbed site (Mummey et al., 2002). The effect of three earthworm species (*Lumbricus terrestris, Allolobophora chlorotica, and Eisenia fetida*) on the microbial community structure of oil-contaminated soil revealed a stimulation in 20:4ω6, an indicator of protozoa, and an increase in the fungal:bacterial ratios (Schaefer et al., 2005). Finally, FAME/PLFA analysis has been used to asses the impact of TNT contamination and remediation on the microbial community. Research has shown that gram-positive bacteria, fungi, and actinomycetes tend to decrease with an increase in TNT contamination (Fuller and Manning, 1998; Wilke et al., 2004). However, the use of molasses addition and humification as bioremediation practices resulted in an increase in microbial biomass,

recovery of the gram-positive bacteria population, and overall the microbial community was altered to a greater degree than contaminated soil (Fuller and Manning, 2004; Wilke et al., 2004).

Overall, lipid extraction followed by PLFA/FAME analysis has significant promise as a tool to monitor and characterize changes in microbial community structure, activity, and biomass as a result of natural or anthropogenic events.

2.5 Objectives

The findings of the research presented in this dissertation address four main objectives:

1. Characterize the impact of wet- and dry-sieving procedures on physical, chemical, and biological properties which may influence the evaluation of chemical and biological processes in soil and soil aggregates (i.e. contaminant fate, sorption, transformation, microbial activity).

2. Investigate the ability of clay minerals and humic substances to sorb TNT and determine to what extent humic-clay complexes may affect the sorption of TNT in soil systems.

3. Investigate the potential of earthworms to facilitate the transformation and distribution of TNT in soil and within soil aggregates.

4. Characterize the impact of earthworms and TNT, both individually and together, on the soil microbial community structure.

References

Achtnich, C., U. Sieglen, H.-J. Knackmuss, and H. Lenke. 1999. Irreversible binding of biologically reduced 2,4,6-trinitrotoluene to soil. Environ. Toxicol. Chem. 18: 2416-2423.

Adriano, D.C. 2003. Biogeochemical processes and abiotic factors controlling bioavailability. Abstract. 2nd International Workshop on Bioavailability of Soil Pollutant and Risk Assessment, Monte Verita Ascona, Switzerland, February 2-7, 2003.

Agrawal, A. and P.G. Tratnyek. 1996. Reduction of nitro aromatic compounds by zero-valent iron metal. 30: 153-160.

Ahmed, M., and J.M. Oades. 1984. Distribution of organic matter and adenosine triphosphate after fractionation of soils by physical procedures. Soil Bio. Biochem. 16: 465-470.

Amato, M., and J.N. Ladd. 1980. Studies of nitrogen mineralization and immobilization in calcareous soil. V. Formation and distribution of isotope-labeled biomass during decomposition of ^{14}C and ^{15}N-labelled plant material. Soil Bio. Biochem. 12: 405-411.

Amellal, N., J.-M. Portal, and J. Berthelin. 2001a. Effect of soil structure on the bioavailability of polycyclic aromatic hydrocarbons within aggregates of a contaminated soil. Appl. Geochem. 16: 1611-1619.

Amellal, N., J.-M. Portal, T. Vogel, and J. Berthelin. 2001b. Distribution and location of polycyclic aromatic hydrocarbons (PAHs) and PAH-degrading bacteria within polluted soil aggregates. Biodeg. 12: 49-57.

Angers, D.A., and M. Giroux. 1996. Recently deposited organic matter in soil water-stable aggregates. Soil Sci. Soc. Am. J. 60: 1547-1551.

Ashman, M.R., P.D. Hallett. and P.C. Brookes. 2003. Are the links between soil aggregate size class, soil organic matter and respiration rate artifacts of the fractionation procedure? Soil Biol. Biochem. 35: 435-444.

ATSDR. 1995. Toxicological profile for 2,4,6-trinitrotoluene (TNT) [Online]. http://www.atsdr.cdc.gov/toxprofiles/tp81.html Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

ATSDR. 2005. 2005 CERCLA priority list of hazardous substances that will be the subject of toxicological profiles and support document [Online]. http://www.atsdr.cdc.gov/cercla/supportdocs/text.pdf, (accessed 15 June, 2006).

Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

Barois, I., G. Villemin, and P. Lavelle. 1993. Transformation of the soil structure through *Pontoscolex corethrurus* (Oligochaeta) intestinal tract. Geoderma, 56: 57-66.

Bayman, P., and G.V. Radkar. 1997. Transformation and tolerance of TNT (2,4,6-trinitrotoluene) by fungi. Int. Biodeter. Biodegr. 39: 45-53.

Beauchamp, E.G., and A.G. Seech. 1990. Denitrification with different sizes of soil aggregates obtained from dry-sieving and from sieving with water. Biol. Fert. Soil 10: 188-193.

Belden, J.B., D.R. Ownby, G.R. Lotufo, M.J. Lydy. 2005. Accumulation of trinitrotoluene (TNT) in aquatic organisms: Part 2 – Bioconcentration in aquatic invertebrates and potential for trophic transfer to channel catfish (*Ictalurus punctatus*). Chemosphere 58: 1161-1168.

Binet, F., A. Kersante, C. Munier-Lamy, R.-C. Le Bayon, M.-J. Belgy, and M.J. Shipitalo. 2006. Lumbricid macrofauna alter atrazine mineralization and sorption in a silt loam soil. Soil Bio. Biochem. 38: 1255-1263.

Boopathy, R. 1994. Transformation of nitroaromatic compounds by a methanogenic bacterium, methanoccoccus SP (strain-B). Arch. Microbial. 162: 167-172.

Boopathy, R., J.F. Manning, C. Montemagno, and C.F. Kulpa. 1994. Metabolism of 2,4,6-trinitrotoluene by a *Pseudomonas consortium* under aerobic conditions. Curr. Microbiol. 28: 131-137.

Boopathy, R. 2000. Bioremediation of explosives contaminated soil. Int. Biodeter. Biodegr. 46: 29-36.

Boopathy, R., and C.F. Kulpa. 1994. Biotransformation of 2,4,6-trinitrotoluene (TNT) by a *Methanococcus* sp. (strain B) isolated from a lake sediment. Can. J. Microbiol. 40: 273-278.

Bossuyt, H., J. Six, and P.F. Hendrix. 2004. Rapid incorporation of carbon from fresh residues into newly formed stable microaggregates within earthworm casts. Eur. J. Soil Sci. 55: 393-399.

Bossuyt, H., J. Six, and P.F. Hendrix. 2005. Protection of soil carbon by microaggregates within earthworm casts. Soil Bio. Biochem. 37: 251-258.

Boyd, S.A., G. Sheng, B.J. Teppen, and C.T. Johnston. 2001. Mechanisms for the adsorption of substituted nitrobenzenes by smectite clays. Environ. Sci. Technol. 35: 4227-4234.

Brady, N.C., and R.R. Weil. 1999. The nature and properties of soils. 12th Ed. Prentice-Hall, New Jersey.

Brannon, J.M., C.B. Price, and C. Hayes. 1998. Abiotic transformation of TNT in montmorillonite and soil suspensions under reducing conditions. Chemosphere 36:1453-1462.

Brown, G.G., I Barois, and P. Lavelle. 2000. Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. Eur. J. Soil Biol. 36: 177-198.

Bruhn, C., H. Lenke, and J.J. Knackmuss. 1987. Nitrosubstituted aromatic compounds as nitrogen sources for bacteria. Appl. Environ. Microbiol. 53:208-210.

Bruns-Nagel, D., J. Bretung, E. von Low, K. Steinbach, T. Borontzy, M. Kahl, K.-H. Blotevogel, and D. Gemsa. 1996. Microbial transformation of 2,4,6-trinitrotoluene in aerobic soil columns. Appl. Environ. Microbiol. 62: 2651-2662.

Butler, J.L., M.A. Williams, P.J. Bottomley, and D.D. Myrold. 2003. Microbial community dynamics associated with rhizosphere carbon flow. Appl. Environ. Microbiol. 69: 6793-6800.

Coleman D.C., Crossley D.A., and P.F. Hendrix. 2004. Fundamentals of soil ecology. 2nd ed. Elsevier, New York.

Comfort, S.D., P.J. Shea, L.S. Hundal, Z. Li, B.L. Woodbury, J.L. Martin, and W.L. Powers. 1995. TNT transport and fate in contaminated soil. J. Environ. Qual. 24:1174-1182.

Conder, J.M., and R.P. Lanno. 2000. Evaluation of surrogate measures of cadmium, lead, and zinc bioavailability to *Eisenia fetida*. Chemosphere 41: 1659-1668.

Daun, G., H. Lenke, M. Reuss, and H.-J. Knackmuss. 1998. Biological treatment of TNT-contaminated soil. 1. Anaerobic cometabolic reduction and interaction of TNT and metabolites with soil components. Environ. Sci. Technol. 32: 1956-1963.

DeSutter, T.M., S.A. Clay, and D.E. Clay. 2003. Atrazine sorption and desorption as affected by aggregate size, particle size, and soil type. Weed Sci. 51: 456-462.

Dexter, A.R., 1988. Advances in characterization of soil structure. Soil Till. Res. 11: 199-238.

Dilley, J.V., C.A. Tyson, R.J. Spanggord, D.P. Sasmore, G.W. Newell, and J.C. Dacre. 1982. Short-term oral toxicity of 2,4,6-trinitrotoluene in mice, rats, and dogs. J. Toxicol. Environ. Health 9: 565-585.

Dodard, S.G., A.Y. Renoux, J. Powlowski, and G.I. Sunahara. 2003. Lethal and subchronic effects of 2,4,6-trinitrotoluene (TNT) on *Enchytraeus albidus* in amended artificial soil. Ecotoxicol. Environ. Saf. 54:131-138.

Ederer, M.M., T.A. Lewis, and R.L. Crawford. 1997. 2,4,6-Trinitrotoluene (TNT) transformation by Clostridia isolated from a munition-fed bioreactor: Comparison with non-adapted bacteria. J. Ind. Microbiol. Biotechnol. 18:82-88.

Edwards, C.A., and P.J. Bohlen. 1996. Biology and ecology of earthworms. Chapman and Hall, London.

Elovitz, M.S., and E.J. Weber. 1999. Sediment-mediated reduction of 2,4,6-trinitrotoluene and fate of the resulting aromatic (poly)amines. Environ. Sci. Technol. 33: 2617-2625.

Eriksson, J., and U. Skyllberg. 2001. Binding of 2,4,6-trinitrotoluene and its degradation products in a soil organic matter two-phase system. J. Environ. Qual. 30: 2053-2061.

Esteve-Nunez, A., A. Caballero, and J.L. Ramos. 2001. Biological degradation of 2,4,6-trinitrotoluene. Microbiol. Mol. Biol. Rev. 65: 335-352.

Feng, Y., A.C. Motta, D.W. Reeves, C.H. Burmester, E. van Santen, and J.A. Osborne. 2003. Soil microbial communities under conventional-till and no-till continuous cotton systems. Soil Biol. Biochem. 35: 1693-1703.

Fernando, T., J.A. Bumpus, and S.D. Aust. 1990. Biodegradation of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysoporium*. Appl. Environ. Microbiol. 56: 1666-1671.

Frische, T. 2003. Ecotoxicological evaluation of in situ bioremediation of soils contaminated by the explosive 2,4,6-trinitrotoluene (TNT). Environ. Pollut. 121: 103-113.

Frostegard, A., A. Tunlid, and E. Baath. 1993. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. App. Environ. Microbiol. 59: 3605-3617.

Fuller, M.E., and J.F. Manning. 1998. Evidence for differential effects of 2,4,6-trinitrotoluene and other munitions compounds on specific subpopulations of soil microbial communities. Environ. Toxicol. Chem. 17: 2185-2195.

Fuller, M.E., and J.F. Manning. 2004. Microbiological changes during bioremediation of

explosives-contaminated soils in laboratory and pilot-scale bioslurry reactors. Bioresource Technol. 91: 123-133.

Furedi, E.M., B.S. Levine, J.W. Sagartz, V.S. Rac, and P.M. Lish. 1984. Determination of the chronic mammalian toxicological effects of TNT: Twenty-four month chronic toxicity/carcinogenicity study of trinitrotoluene (TNT) in the Fischer-344 rat. Final report, phase III. Vol I. AD-A168 637. U.S. Army Medical Research and Development Command, Fort Detrick, MD. ITT Research Institute, Chicago, IL.

GAO 2003. Military munitions: DOD needs to develop a comprehensive approach for cleaning up contaminated sites, Report GAO-04-147, General Accountability Office, Washington, D.C.

Gentry, T.J., D.C. Wolf, C.M. Reynolds, and J.J. Fuhrmann. 2003. Pyrene and phenanthrene influence on soil microbial populations. Biorem. J. 7: 53-68.

Gong, P., S.D. Sicilliano, C.W. Greer, L. Paquet, J. Hawari, and G.I. Sunahara. 1999a. Effects and bioavailability of 2,4,6-trinitrotoluene in spiked and field-contaminated soils to indigenous microorganisms. Environ. Toxicol. Chem. 18: 2681-2688.

Gong, P., B.-M. Wilke, and S. Fleischmann. 1999b. Soil-based phytotoxicity of 2,4,6-trinitrotoluene (TNT) to terrestrial higher plants. Arch. Environ. Contam. Toxicol. 36:152-157.

Haderlein, S.B. and R.P. Schwarzenbach. 1995. Environmental processes influencing the rate of abiotic reduction of nitroaromatic compounds in the subsurface. p. 199-225 *In* Biodegradation of nitroaromatic compounds. J.C. Spain (ed.)., Plenum Press, New York.

Hawari, J., A. Halasz, L. Paquet, E. Zhou, B. Spencer, G. Ampleman, and S. Thiboutot. 1998. Characterization of metabolites in the biotransformation of 2,4,6-trinitrotoluene with anaerobic sludge: Role of triaminotoluene. Appl. Environ. Micro. 64:2200-2206.

Hawari, J., S. Beaudet, A. Halasz, S. Thiboutot, and G. Ampleman. 2000. Microbial degradation of explosives: Biotransformation versus mineralization. Appl. Microbiol. Biotechnol. 54: 605-618.

Haynes, R.J., and P.M. Fraser. 1998. A comparison of aggregate stability and biological activity in earthworm casts and uningested soil as affected by amendment with wheat or Lucerne straw. Eur. J. Soil. Sci. 49: 629-636.

Hundal, L.S., J. Singh, E.L. Bier, P.J. Shea, S.D. Comfort, and W.L. Powers. 1997.

Removal of TNT and RDX from water and soil using iron metal. Environ. Pollut. 97: 55-64.

Johnston, C.T., M.F.D. Oliveira, B.J. Teppen, G. Sheng, and S.A. Boyd. 2001. Spectroscopic study of nitroaromatic-smectite sorption mechanisms. Environ. Sci. Technol. 35: 4767-4772.

Johnston, C.T., S.A. Boyd, B.J. Teppen, and G. Sheng. 2004. Sorption of nitroaromatic compounds on clay surfaces. p. 155-189. *In* Handbook of layered materials for catalytic applications. S.M. Auerbach, K.A. Carrado, P.K. Dutta (eds)., Marcel-Dekker, New York.

Jones, C.G., J.H. Lawton, and M. Shachak. 1994. Organisms as ecosystem engineers. Oikos, 69: 373-386.

Kay, B.D., 1990. Rates of change of soil structure under different cropping systems. Adv. Soil Sci. 12: 1-52.

Keum Y-S., and Q.X. Li. 2004. Reduction of nitroaromatic pesticides with zero-valent iron. Chemosphere. 54: 255-263.

Kieft, T.L., E. Wilch, K. O'Connor, D.B. Ringleberg, and D.C. White. 1997. Survival and phospholipid fatty acid profiles of surface and subsurface bacteria in natural sediment microcosms. App. Environ. Microbiol. 63: 1531-1542.

Klausen, J., S.P. Trober, S.B. Haderlein, and R.P. Schwarzenbach. 1995. Reduction of substituted nitrobenzenes by Fe(II) in aqueous mineral suspensions. Environ. Sci. Technol. 29: 2396-2404.

Krauss, M., and W. Wilcke. 2002. Sorption strength of persistent organic pollutants in particle-size fractions of urban soils. Soil Sci. Soc. Am. J. 66: 430-437.

Lachance, B., A.Y. Renoux, M. Sarrazin, J. Hawari, and G.I. Sunahara. 2004. Toxicity and bioaccumulation of reduced TNT metabolites in the earthworm *Eisenia Andrei* exposed to amended forest soil. Chemosphere 55: 1339-1348.

Lavelle, P., R. Schaefer, and Z. Zaidi. 1989. Soil ingestion and growth in *Millsonia anomala*, a tropical earthworm, as influenced by the quality of the organic matter ingested. Pedobiologia, 33: 379-388.

Lavelle, P., D. Bignell, M. Lepage, V. Wolters, P. Roger, P. Ineson, O.W. Heal, and S. Dhillion. 1997. Soil function in a changing world: The role of invertebrate ecosystem engineers. Eur J. Soil Biol. 33: 159-193.

Lavelle, P., B. Pashani, F. Charpentier, C. Gilot, J.P. Rossi, L.Derouard, J. Andre, J.P.

Ponge, and N. Bernier. 1998. Large-scale effects of earthworms on soil organic matter and nutrient dynamics. p. 103-122. *In* Earthworm ecology. Edwards, C.A. (ed) St. Lucie Press, Boca Raton, FL.

Lechevalier, M.P. 1989. Lipids in bacterial taxonomy. p. 455-560. *In* W.M. O'Leary (ed) Microbial lipids. Vol 1. Academic Press, London.

Lee, K.E., 1985. Earthworms: Their ecology and relationships with soils and land use. Academic Press. Sydney.

Lee, K.E., and R.C. Foster. 1991. Soil fauna and soil structure. Aust. J. Soil. Res. 29: 745-776.

Levine, B.S., E.M. Furedi, D.E. Gordon, P.M. Lish, and J.J. Barkley. 1984. Subchronic toxicity of trinitrotoluene in Fischer-344 rats. Toxicol. 32: 253-265.

Levine, B.S., J.H. Rust, J.J. Barkley, E.M. Furedi, and P.M. Lish. 1990. Six month oral toxicity study of trinitrotoluene in beagle dogs. Toxicol. 63: 233-244.

Lewis, T.A., S. Goszczynski, R.L. Crawford, R.A. Korus, and W. Admassu. 1996. Products of anaerobic 2,4,6-trinitrotoluene (TNT) transformation by *Clostridium bifermentans*. Appl. Environ. Microbiol. 62: 4669-4674.

Lewis, T.A., D.A. Newcombe, and R.L. Crawford. 2004. Bioremediation of soils contaminated with explosives. J. Environ. Manage. 70: 291-307.

Li, A.Z., K.A. Marx, J. Walker, and D.L. Kaplan. 1997. Trinitrotoluene and metabolites binding to humic acid. Environ. Sci. Technol. 31: 584-589.

Li, Z.M., S.D. Comfort, and P.J. Shea. 1997. Destruction of 2,4,6-trinitrotoluene by fenton oxidation. J. Environ. Qual. 26: 480-487.

Li, H., G. Sheng, B.J. Teppen, C.T. Johnston, and S.A. Boyd. 2003. Sorption and desorption of pesticides by clay minerals and humic acid-clay complexes. Soil Sci. Soc. Am. J. 67: 122-131.

Liu, D.H., R.J. Spanggord, H.C. Bailey, H.S. Javitz, and D.C.L. Jones. 1983. Toxicity of TNT wastewaters to aquatic organisms Final report. Vol. I: Acute toxicity of LAP wastewater and 2,4,6-trinitrotoluene. AD A142 144. SRI International, Menlo Park, CA.

Lowe, C.N., and K.R. Butt. 2004. Influence of food particle size on inter- and intra-specific interactions of *Allolobophora chlorotica* (Savigny) and *Lumbricus terrestris*. Pedobiologia 47: 574-577.

Martin, J.L., S.D. Comfort, P.J. Shea, T.A. Kokjohn, and R.A. Drijber. 1997. Denitration

of 2,4,6-trinitrotoluene by *Pseudomonas savastanoi*. Can. J. Microbiol. 43: 447-455.

Martin, A., and J.C.Y. Marinissen. 1993. Biological and physico-chemical processes in excrements of soil animals. Geoderma 56: 331-347.

McCormick, N.G., F.E. Feeherry, and H.S. Levinson. 1976. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. Appl. Environ. Micro. 31: 949-958.

Miller, M., and R.P. Dick. 1995. Dynamics of soil C and microbial biomass in whole soil and aggregates in two cropping systems. Appl. Soil Ecol. 2: 253-261.

Moat A.G., and J.W. Foster. 1995. Microbial physiology. Wiley-Liss, New York.

Monrozier, L.J., J.N. Ladd, A.W. Fitzpatrick, R.C. Foster, and M. Raupach, 1991. Components and microbial biomass content of size fractions in soils of contrasting aggregation. Geoderma 49: 37-62.

Moore, M.R. 2003. Risk assessment in environmental contamination and environmental health. p. 3-19. *In*. Naidu et al. (eds). Bioavailability, toxicity and risk relationships in ecosystems. Science Publishers, Enfield, NH, USA.

Muller, S., W. Wilcke, N. Kanchanakool, and W. Zech. 2000. Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in particle-size separates of urban soils in Bangkok, Thailand. Soil Sci. 165: 412-419.

Mummey, D.L., P.D. Stahl, and J.S. Buyer. 2002. Microbial biomarkers as an indicator of ecosystem recovery following surface mine reclamation. Appl. Soil Ecol. 21: 251-259.

National Research Council. 2003. Bioavailability of contaminants in soils and sediments: Processes, tools and applications. National Academy Press, Washington D.C.

Nimmo, J.R., and K.S. Perkins. 2002. Aggregate stability and size distribution p. 317-328, *In* J.H. Dane and G. Clarke (eds.) Methods of soil analysis. Part 4 Physical Methods. SSSA Book Ser No. 5. SSSA, Madison, WI.

Nefso, E.K., S.E. Burns, and C.J. McGrath. 2005. Degradation kinetics of TNT in the presence of six mineral surfaces and ferrous iron. J. Mazard. Mater. B123: 79-88.

Oades, J.M. 1984. Soil organic matter and structural stability: Mechanisms and implications for management. Plant and Soil 76: 319-337.

Oades, J.M., and A.G. Waters, 1991. Aggregate hierarchy in soils. Aust. J. Soil

Res. 29: 815-828.

Oh, B.-T., G. Sarath, and P.J. Shea. 2001. TNT nitroreductase from a *Pseudomonas aeruginosa* strain isolated from TNT contaminated soil. Soil Biol. Biochem. 33: 875-881.

Olsson, S., S. Alstrom, and P. Persson. 1999. Barley rhizobacterial population characterized by fatty acid profiling. Appl. Soil Ecol. 12: 197-204.

Ownby, D.R., J.B. Belden, G.R. Lotufo, and M.J. Lydy. 2005. Accumulation of trinitrotoluene (TNT) in aquatic organisms: Part 1 – Bioconcentration and distribution in channel catfish (*Ictalurus punctatus*). Chemosphere 58: 1153-1159.

Pak, J.W., K.L. Knoke, D.R. Noguera, B.G. Fox, and G.H. Chambliss. 2000. Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. Appl. Environ. Microbiol. 66: 4742-4750.

Paul, E.A., and F.E. Clark. 1996. Soil microbiology and biochemistry. 2nd ed. Academic Press, New York.

Peacock, A.D., M.D. Mullen, D.B. Ringelberg, D.D. Tyler, D.B. Hedrick, P.M. Gale, and D.C. White. 2001. Soil microbial community responses to dairy manure or ammonium nitrate applications. Soil Biol. Biochem. 33: 1011-1019.

Pelmenschikov, A., and J. Leszczynski. 1999. Adsorption of 1,3,5-trinitrobenzene on the siloxane sites of clay minerals: Ab initio calculations of molecular models. J. Phys. Chem. B. 103: 6886-6890.

Pennington, J.C., and W.H. Patrick. 1990. Adsorption and desorption of 2,4,6-trinitrotoluene by soils. J. Environ. Qual. 19: 559-567.

Pennington, J.C., C.A. Hayes, K.F. Myers, M. Ochman, D. Gunnison, D.R. Felt, and E. F. McCormick. 1995. Fate of 2,4,6-trinitrotoluene in a simulated compost system. Chemosphere 30: 429-438.

Pennington, J.C., and J.M. Brannon. 2002. Environmental fate of explosives. Thermochimica Acta 384: 163-172.

Petersen, S.O., and M.J. Klug. 1994. Effects of sieving, storage, and incubation temperature on the phospholipid fatty acid profile of a soil microbial community. 60: 2421-2430.

Phillips, C.T., R.T. Checkai, and R.S. Wentsel. 1993. Toxicity of selected munitions and munition-contaminated soil on earthworm (*Eisenia foetida*). Edgewood Research Development and Engineering Center. US Army Chemical and Biological Defense Agency, Aberdeen Proving Ground, MD., ERDEC-TR-037.

Piearce, T.G. 1978. Gut content of some lumbricid earthworms. Pedobiologia 18: 153-157.

Preuss, A., and P.G. Rieger. 1995. Anaerobic transformation of 2,4,6-trinitrotoluene (TNT). Arch. Microbiol. 159: 343-353.

Puget, P., C. Chenu, and J. Balesdent. 1995. Total and young organic matter distributions in aggregates of silty cultivated soils. Eur. J. Soil Sci. 46: 449-459.

Pulleman, M.M., J. Six, N. van Breemen, and A.G. Jongmans. 2005. Soil organic matter distribution and microaggregate characteristics as affected by agricultural management and earthworm activity. Eur. J. Soil Sci. 56: 453-467.

Renoux, A.Y., M. Sarrazin, J. Hawari, and G.I. Sunahara. 2000. Transformation of 2,4,6-trinitrotoluene in soil in the presence of the earthworm *Eisenia Andrei*. Environ. Toxicol. Chem. 19: 1473-1480.

Rieger, P.G., and H.J. Knackmuss. 1995. Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil. p. 1-18. *In* Biodegradation of nitroaromatic compounds. Spain J. (ed). Plenum Press, New York.

Robidoux, P.Y., J. Hawari, S. Thiboutot, G. Ampleman, and G.I. Sunahara. 1999. Acute toxicity of 2,4,6-trinitrotoluene in earthworm (*Eisenia andrei*). Ecotox. Environ. Safe 44: 311-321.

Robidoux, P.Y., C. Svendsen, J. Caumartin, J. Hawari, G. Ampleman, S. Thiboutot, J.M. Weeks, and G.I. Sunahara. 2000. Chronic toxicity of energetic compounds in soil determined using the earthworm (*Eisenia andrei*) reproduction test. Environ. Toxicol. Chem. 19:1764-1773.

Rocheleau, S., R.G. Kuperman, M. Martel, L. Paquet, G. Bardai, S. Wong, M. Sarrazin, S. Dodard, P. Gong, J. Hawari, R.T. Checkai, and G.I. Sunahara. 2006. Phytotoxicity of nitroaromatic energetic compounds freshly amended or weathered and aged in sandy loam soil. Chemosphere 62: 545-558.

Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. Tech. Note #101. Microbial ID, Newark, DE.

Schaefer, M. 2004. Assessing 2,4,6-trinitrotoluene (TNT)-contaminated soil using three different earthworm test methods. Ecotox. Environ. Saf. 57: 74-80.

Schaefer, M., S.O. Petterson, and J. Filser. 2005. Effects of *Lumbricus terrestris*, *Allolobophora chlorotica* and *Eisenia fetida* on microbial community dynamics in oil-contaminated soil. Soil Biol. Biochem. 37: 2065-2076.

Schafer, R., and R.K. Achazi. 1999. The toxicity of soil samples containing TNT and other ammunition derived compounds in the enchytraeid and collembolan-biotest. Environ. Sci. Pollut. Res. 6: 213-219.

Scheibner, K., M. Hofrichter, A. Herre, J. Michels, and W. Fritsche. 1997. Screening for fungi intensively mineralizing 2,4,6-trinitrotoluene. Appl. Microbiol. Biotechnol. 47: 452-457.

Schutter, M.E., and R.P. Dick. 2000. Comparison of fatty acid methyl ester (FAME) methods for characterizing microbial communities. Soil Sci. Soc. Am. J. 64: 1659-1668.

Selim, H.M., S.K. Xue, and I.K. Iskandar. 1995. Transport of 2,4,6-trinitrotoluene and hexahydro-1,3,5-trinitro-1,3,5-triazine in soils. Soil. Sci. 160: 328-339.

Sheng, G., C.T. Johnston, B.J. Teppen, and S.A. Boyd. 2002. Adsorption of dinitrophenol herbicides from water by montmorillonites. Clays Clay Miner. 50: 25-34.

Sheremata, T.W., S. Thiboutot, G. Ampleman, L. Paquet, A. Halasz, and J. Hawari. 1999. Fate of 2,4,6-trinitrotoluene and its metabolites in natural and model soil systems. Environ. Sci. Technol. 33: 4002-4008.

Shipitalo, M.J., and R. Protz. 1989. Chemistry and micromorphology of aggregation in earthworm casts. Geoderma 45: 357-374.

Siciliano, S.D., R. Roy, and C.W. Greer. 2000. Reduction in denitrification activity in field soils exposed to long term contamination by 2,4,6-trinitrotoluene (TNT). FEMS Microbiol. Ecol. 32: 61-68.

Simini M., R.S. Wentsel, R.T. Checkai, C.T. Phillips, N.A. Chester, M.A. Major, and J.C. Amos. 1995. Evaluation of soil toxicity at Joliet Army Ammunition plant. Environ. Toxicol. Chem. 14: 623-630.

Six, J., E.T. Elliott, K. Pautsian, and J.W. Doran. 1998. Aggregation and soil organic matter accumulation in cultivated and native grassland soils. Soil Sci. Soc. Am. J. 62: 1367-1377.

Six, J., E.T. Elliott, and K. Paustian. 1999. Aggregate and soil organic matter dynamics under conventional and no-tillage systems. Soil Sci. Soc. Am. J. 63: 1350-1358.

Six, J., E.T. Elliot, and K. Paustian. 2000a. Soil macroaggregate turnover and microaggregate formation: A mechanism for C sequestration under no-tillage agriculture. Soil Biol. Biochem. 32: 2099-2103.

Six, J., H. Bossuyt, S. De Gryze, and K. Denef. 2004. A history of research on the link

between (micro)aggregates, soil biota, and soil organic matter dynamics. Soil Till. Res. 79:7-31.

Spiker, J.K., D.L. Crawford, and R.L. Crawford. 1992. Influence of 2,4,6-trinitrotoluene (TNT) concentration on the degradation of TNT in explosive-contaminated soils by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 58: 3199-3202.

Stahl, J.D., and S.D. Aust. 1993. Metabolism and detoxification of TNT by *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. 192: 477-482.

Steevens, J.A., B.M. Duke, G.R. Lotufo, and T.S. Bridges. 2002. Toxicity of the explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine in sediments to *Chironomus tentans* and *Hyalella azteca*: Low-dose hormesis and high-dose mortality. Environ. Toxicol. Chem. 21:1475-1482.

Stemmer, M., M.H. Gerzabek, and E. Kandeler. 1998. Organic matter and enzyme activity in particle-size fractions of soils obtained after low-energy sonication. Soil Biol. Biochem. 30: 9-17.

Tisdall, J.M., and J.M. Oades, 1982. Organic matter and water-stable aggregates in soils. J. Soil Sci. 62: 141-163.

van Oss, C.J., and R.F. Giese. 1995. The hydrophilicity and hydrophobicity of clay minerals. Clays Clay Miner. 43:474-477.

Vasilyeva, G.K., B.-T. Oh, P.J. Shea, R.A. Drijber, V.D. Kreslavski, R. Minard, and J.-M. Bollag. 2000. Aerobic TNT reduction via 2-hydroxylamino-4,6-dinitrotoluene by *Pseudomonas aeruginosa* strain MX isolated from munitions-contaminated soil. Bioremed. J. *4:111-124.*

Vestal, J.R., and D.C. White. 1989. Lipid analysis in microbial ecology: Quantitative approaches to the study of microbial communities. Biosci. 39: 535-541.

Vorbeck, C., H. Lenke, P. Fischer, and H.-J. Knackmuss. 1994. Identification of a hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene by a *Mycobacterium* strain. J. Bacteriol. 176: 932-934.

Vorbeck, C., H. Lenke, P. Fischer, J.C. Spain, and H.-J. Knackmuss. 1998. Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. Appl. Environ. Microbiol. 64: 246-252.

Wang, C., D.Y. Lyon, J.B. Hughes, and G.N. Bennett. 2003. Role of hydroxylamine intermediates in the phytotransformation of 2,4,6-trinitrotoluene by *Myriophyllum aquaticum*. Environ. Sci. Technol. 37:3595-3600.

Weissmahr, K.W., S.B. Haderlein, and R.P. Schwarzenbach. 1997. In situ spectroscopic investigations of adsorption mechanisms of nitroaromatic compounds on clay minerals. Environ. Sci. Technol. 31: 240-247.

Weissmahr, K.W., M. Hildenbrand, R.P. Schwarzenbach, and S.B. Haderlein. 1999. Laboratory and field scale evaluation of geochemical controls on groundwater transport of nitroaromatic ammunition residues. Environ. Sci. Technol. 33:2593-2600.

White, D.C., J.O. Stair, and D.B. Ringelberg. 1996. Quantitative comparisons of in situ microbial biodiversity by signature biomarker analysis. J. Indus. Microbiol. 17: 185-196.

Wilke B.-M., A. Gattinger, E. Frohlich. L. Zelles, and P. Gong. 2004. Phospholipid fatty acid composition of a 2,4,6-trinitrotoluene contaminated soil and an uncontaminated soil as affected by a humification remediation process. Soil Bio. Biochem 36: 725-729.

Won, W.D., L.H. DiSalvo, and J. Ng. 1976. Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. Appl. Environ. Microbiol. 31: 576-580.

Xue, S.K., I.K. Iskandar, and H.M. Selim. 1995. Adsorption-desorption of 2,4,6-trinitrotoluene and hexahidro-1,3,5-trinitro-1,3,5-triazine in soils. Soil Sci. 160: 317-327.

Yinon, J. 1990. Toxicity and metabolism of explosives. CRC Press, Boca Raton, FL.

Young, I.M., and K. Ritz. 2000. Tillage, habitat space and function of soil microbes. Soil Till Res 53, 201-213.

Zelles, L., Q.Y. Bai, R.X. Ma, R. Rackwitz, K. Winter, and F. Beese. 1994. Microbial biomass, metabolic activity, and nutritional status determined from fatty acid patterns and poly-hydroxybutyrate in agriculturally-managed soils. Soil Biol. Biochem. 26: 439-446.

Zelles, L. 1997. Phospholipid fatty acid profiles in selected members of soil microbial communities. Chemosphere. 35:275-294.

Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: A review. Biol. Fertil. Soil 29: 111-129.

Zhang, Q.L., and P.F. Hendrix. 1995. Earthworm (*Lumbricus rubellus* and *Aporrectodea caliginosa*) effects on carbon flux in soil. Soil Sci. Soc. Am. J. 59:816-823.

Zhang, H., and S. Schrader. 1993. Earthworm effects on selected physical and chemical properties of soil aggregates. Biol. Fert. Soils 15: 229-234.

CHAPTER 3

INTERACTION OF 2,4,6-TRINITROTOLUENE WITH HUMIC ACID, CLAY MINERALS, AND HUMIC-CLAY COMPLEXES

Pillar, G.D., and K. Xia. To be submitted to *Environmental Science and Technology*

Abstract

Clay minerals have typically been considered as the dominant sorbent for nitroaromatic compounds (NACs) such as 2,4,6-trinitrotoluene (TNT) in natural systems. However, it is well known that interactions between soil organic matter and clay minerals can alter the sorption characteristics of some organic chemicals. To determine how soil organic matter influences the sorptive properties of clay minerals for TNT, we measured the sorption and desorption of TNT to humic acid, four different K^+- and Ca^{2+}-saturated reference clay minerals, and their humic-clay complexes. In addition, we examined how the fractionation of humic acid adsorbed to clay minerals may affect TNT sorption. It was found that clay minerals and humic-clay complexes, saturated with K^+ were more effective sorbents than those saturated with Ca^{2+}. Low-charge smectites (SWy and SHCa) had a greater affinity for TNT compared to high-charge smectites (SAz) and 1:1 clay minerals such as kaolinite. Significant sorption of TNT to humic acid was observed, however, this sorption was negatively correlated with HA concentration. Humic-acid resulted in greater adsorption of TNT to the humic-clay surface for K^+-HA-SWy compared to K-SWy. However, XRD analysis showed similar levels of interlayer sorption of TNT (≈12.0 Å) with or without humic acid, indicating a possible increase in TNT sorbed to the humic-clay surface. The sequential complexation of humic acid to K-SWy indicated fractionation of the humic acid based on size and a decrease in TNT sorption with increasing number of complexation steps. In addition, XRD analysis indicated possible intercalation of humic material during the second, third, and fourth sequential complexation steps. The results of this study demonstrate the significant effects of natural organic matter on TNT sorption to clay minerals.

1. Introduction

Nitroaromatic compounds (NACs) such as 2,4,6-trinitrotoluene (TNT) historically are common explosive compounds used in military munitions (Achtnich et al., 1999; Jerger and Woodhull, 2000; Spain, 2000). Recently, the environmental fate of TNT has become a serious concern because of its widespread existence in soils of former ammunition plants and has been shown to be toxic to a variety of organisms, plants, mammals and humans (Levine et al., 1984; Fuller and Manning, 1998; Robidoux et al., 1999; Steevens et al., 2002; Rocheleau et al. 2006). A recent report by the General Accountability Office indicated that 2,307 sites consisting of over 6 million hectares of land in the U.S. are known to be or are suspected of being contaminated with nitroaromatic explosives (GAO, 2003).

When exposed to the soil, studies have shown that transformation and adsorption rather than mineralization are the significant mechanisms governing the environmental fate of TNT as well as other NACs (Xue et al., 1995; Hundal et al., 1997; Pennington and Brannon, 2002). TNT is moderately polar. Polar to semi-polar compounds in aqueous solution generally have a strong tendency to be sorbed by clay minerals rather then organic matter (Koskinen and Harper, 1990). Numerous studies have shown that NACs are strongly adsorbed by clay minerals, particularly 2:1 minerals such as smectite (Haderlein et al., 1996; Weissmahr et al., 1998; Boyd et al., 2001; Johnston et al., 2001). Those studies have proposed several possible mechanisms for TNT sorption to clay minerals. Haderlein et al. (1996) and Weissmahr et al. (1998) proposed an electron donor-acceptor (EDA) mechanism suggesting that TNT binds to clay minerals as a co-planar molecule. The presence of electron-withdrawing nitro groups allows the electron-

deficient π-system to accept electrons from siloxane surfaces of clay minerals that have a negative charge due to isomorphic substitution. The ability of TNT to form EDA complexes with the siloxane surface depends on the ability of TNT to replace hydrated and bound cations from mineral surfaces. It was noted that this phenomena is only likely to occur when weakly hydrated exchangeable cations such as K^+ or NH_4^+ are present compared to strongly hydrated cations such as Ca^{2+} or Mg^{2+}. It was also noted that TNT sorption via EDA complexes was both reversible and not as favorable in the interlamellar region of 2:1 expandable clay minerals. This would suggest that TNT sorption would be more likely to occur on the external surface rather than the interlamellar region of clay minerals. However, research has shown that sorption of nitroaromatic pesticides such as 4,6-dinitro-o-cresol resulted in the intercalation of clay minerals and that the interlayer region provided a significant portion of the available binding sites (Li et al., 2003). In addition, some studies have noted non-reversible sorption of TNT and other NACs (i.e. hysteresis) to soil components (Comfort et al., 1995; Hundal et al., 1997; Achtnich et al., 1999a). Although these findings do not refute the proposed EDA mechanism they suggest that additional mechanisms may be responsible for sorption of NAC by soil components. Boyd et al. (2001) proposed that NAC sorption was the result of complexation of the $-NO_2$ groups with interlayer K^+ ions as a result of partitioning into the interlayer where the NACs may be less hydrated. This would suggest that TNT prefers a hydrophobic environment and recent studies have noted that the hydrophobic characteristics of some clay minerals may contribute to the sorption of NACs (Johnston et al., 2004). Molecular quantum mechanics calculations have supported the mechanism

proposed by Boyd and co-workers (Pelmenschikov and Leszczynksi, 1999; Johnston et al., 2001).

Several studies have noted that in addition to clay minerals, soil organic matter also plays a significant role in the transformation and sorption of NACs in soil (Li et. al., 1997; Eriksson and Skyllberg, 2001; Sheng et al., 2001; Wang et al, 2002; Li et al., 2003). Li et al. (1997) reported significant adsorption of TNT and two metabolites, 2-amino-4,6-dinitrotoluene (2ADNT) and 2,6-diamino-4-nitrotoluene (2,6DANT) to Aldrich (Milwaukee, WI) humic acid under non-sterile conditions. In addition, Eriksson and Skyllberg (2001) reported that the sorption of TNT to dissolved and particulate organic matter (DOM, POM) was due mostly to the initial transformation of TNT to its reduced metabolites which were subsequently sorbed to soil organic matter. They argued that TNT sorption should be considered as the sorption of TNT and its transformation products. Studies using ^{15}N and ^{14}C nuclear magnetic resonance (NMR) have further supported these findings by demonstrating that hydroxylamino and amino metabolites of TNT can form ionic and covalent interactions with humic acid and other organic fractions of soil organic matter (Achtnich et al., 1999; Bruns-Nagel et al., 2000; Thorn et al., 2002; Eriksson et al., 2004). Wang et al. (2002) also demonstrated the ability of humic acid precursors such as catechol to mediate the transformation and subsequent sorption of the TNT metabolites forming either an anilinoquinone via nucleophilic addition or a benzoquinone-imine through condensation reactions. In two separate studies, Hundal et al. (1997) and Kreslavski et al. (1999) reported that as much as 40% of TNT in TNT-contaminated soil was unextractable after exhaustive extraction techniques and that this was attributed in part to interactions with soil organic matter to form stable complexes.

Although numerous studies have examined NAC sorption to clay and humic substances independently, little research is available on the behavior of TNT in the presence of humic-clay complexes (Haderlein et al., 1996; Boyd et al., 2001; Li et al., 2003). Previous work has shown that humic acid associated with smectitic clays can inhibit, enhance, or have no effect on NAC sorption (Li et al., 2003). Research that characterizes the combined role of organic matter and clay minerals on TNT sorption will lead to a better understanding of the fate and bioavailability of TNT and other NACs in the soil environment. The objective of this study was to determine to what extent humic acid and humic acid-clay interactions would influence the sorption and desorption of TNT to clay minerals.

2. Materials and Methods

2.1. Chemicals.

Granular 2,4,6-trinitrotoluene (TNT) (CAS 118-96-7: MW 227.15 g mol^{-1}) used in batch equilibration experiments was purchased from Holston Army Ammunition Plant (Kingsport, TN USA) with a reported purity > 98% and was used as received. Analytical standards for TNT were obtained from Accustandard (New Haven, CT, USA) with a reported purity of ≥ 99%. Humic acid was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). All solvents used were HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA USA).

2.2. Humic Acid- Clay Complex Preparation.

The reference clay minerals, Kaolinite (KGa-1b), smectite low-charge (SWy-2), smectite low-charge (SHCa-1), and smectite high-charge (SAz-1) were purchased from the Source Clays Repository of the Clay Minerals Society (www.cms.org, West Lafayette, IN, USA). The reference clays were used as delivered with no additional purification. The clays were exchanged (i.e. suspended and centrifuged) with 0.1 M aqueous solutions of KCl or $CaCl_2$ to obtain K^+- or Ca^{2+}-saturated clays, respectively. Excess electrolyte was removed by repeated washes with distilled water until the ionic strength of the extractants were less than 0.01 M, which was used in the batch experiments described later. The clay suspensions were then frozen, freeze-dried, and stored in closed containers. The specific surface areas for the prepared clays were analyzed using N_2 sorption following the BET method (Pennell, 2002).

Humic acid-clay complexes were prepared using a modified method by Li et al., (2003). Briefly, 1.0 gram of humic acid was dissolved in a small volume (< 10 mL) of 0.1 M NaOH, brought up to1 L in 0.1 M KCl or $CaCl_2$ solution, followed by shaking with 10 g of the corresponding previously prepared K^+- or Ca^{2+}- clays for 3 days before centrifuging. The pH of the humic-clay complex was not altered, but was monitored over the course of equilibration. The pH of the humic-acid solution was between 7.0 and 7.5 before, and 7.0 and 9.5 (depending on the clay mineral) after equilibration. To ensure saturation of K^+ and Ca^{2+} ,the prepared humic acid-clay complexes were mixed with either 0.1 M KCl (for K^+- clays) or $CaCl_2$ (for Ca^{2+}- clays) solution and then centrifuged before discarding the supernatants. This process was repeated three times. After the saturation, excess electrolyte and humic acid were removed by repeatedly washing the

prepared humic acid-clay complexes with deionized water until there was no light brown color in the supernatant, no visual humic acid particulates settled on the top of the clay fraction after centrifugation, and the ionic strength was less than 0.01 M, which was used for the later batch experiments.

Additional humic-clay complexes for K^+-saturated smectite (K-SWy) were also prepared by sequentially adsorbing humic acid onto the K^+-saturated clay using the following procedures. After the humic- K-SWy complex was prepared for the batch sorption experiments it was removed from the humic acid solution, 10 g of fresh K-SWy smectite was added to the residual humic acid solution and shaken for 3 d before centrifuging. To saturate and clean the humic-clay complexes with K^+, the method described in the previous paragraph was used. The sequential sorption of humic acid onto the K-SWy smectite was repeated a total of three times after the initial saturation resulting in a total of 4 humic acid-clay complexes designated as K-HA-SWy, K-HA-S2-SWy, K-HA-S3-SWy, and K-HA-S4-Swy following the sequence of humic acid sorption). Humic-clay complexes for K^+-saturated kaolinite (KGa-1b) were also prepared following the same procedure, but under two different pH values, 4.5 (K-HA-KGa-A) and 6.5 (K-HA-KGa-B). All of the humic-clay complexes were frozen, freeze-dried, and stored in sealed containers. The organic C contents were measured using the dry combustion method (Nelson and Sommers, 1996). Additionally, the specific surface areas of the prepared humic-clay complexes were measured using the same method mentioned in the previous section. Selected properties of the clay minerals and humic acid-clay complexes are given in Table 3.1.

2.3. Batch Sorption Experiments: Clay and Humic-Clay Complexes.

Experiments for sorption and desorption of TNT to clays and humic-clay complexes were conducted by using a batch equilibration method. Stock solutions of TNT were prepared in acetonitrile and were kept refrigerated before use. Initial aqueous concentrations of TNT ranged from 2.5 to 60 μg mL^{-1} (2.3 to 55% of aqueous solubility). Clays and humic acid-clay complexes were weighed in triplicate into 50 mL polypropylene tubes followed by the addition of the initial TNT solutions. The samples were mixed on a vortex for 1 minute and then placed on a shaker for 24 h at room temperature (25ºC), followed by centrifugation at 1250 x g for 45 min. Previous experiments (data not shown) indicated that equilibrium was achieved within this time and that there was no adsorption to the polypropylene tubes or caps. A 1 mL aliquot of supernatant was collected, filtered through a 0.45-μm filter, and transferred to a glass GC vial for analysis of TNT and its metabolites on HPLC. The HPLC analysis of all samples indicated no quantifiable levels of TNT metabolites and thus, the difference in the initial amount TNT and the amount of TNT remaining after equilibration was assumed to be adsorbed to the solid phase. Desorption of TNT was conducted by using a decant and refill technique. In situations where TNT sorption was low, desorption for certain clays (i.e. Ca-SAz, Ca-HA-SAz, Ca-KGa) was not conducted because of variable, unreliable data. After the sorption experiments, the remaining solution in each tube was carefully decanted and the residual supernatant was determined gravimetrically. The concentration of TNT in the residual solution was assumed to be the same as that measured in the removed supernatant. An appropriate volume of TNT-free solution with the corresponding electrolyte was added into the centrifuge tubes to achieve the initial

volume used for the sorption experiment. The tubes were placed on the shaker for 24 hours and then centrifuged at 1250 x g for 45 min. After centrifugation a 1 mL aliquot of supernatant was removed, filtered through a 0.45-μm filter, and transferred to a glass GC vial for analysis of TNT and metabolites by HPLC. No quantifiable TNT metabolites were detected and, therefore, the amount of TNT detected in the supernatant was considered as the amount desorbed from the solid phase.

2.4. Batch Sorption Experiments: Humic Acid.

The humic acid was fractionated into molecular weights greater than and less than 3500 D by dialyzing it against deionized water using a dialysis membrane with a 3500 D molecular weight cutoff (Spectrum Co). The humic acid > 3500 D molecular weight was retained and used to prepare stock HA solutions for later batch sorption experiments using custom made two-compartment dialysis cells (Figure 4.1). For all calculations and sorption coefficients based on humic acid concentration it was assumed that the humic acid consisted of 49.01% carbon based on the certificate of analysis provided by Aldrich Chemical Co. Five stock solutions containing 24.3, 39.9, 62.4, 129.2, and 200 μg HA mL^{-1} were prepared in 0.01 M KCl at pH 6.7 ± 0.6. After being conditioned in three changes of deionized water, one piece of the 3500 D cutoff dialysis membrane (Spectrum Co) was tightly secured between the two compartments of each cell. For each cell, one compartment was loaded with 5 mL of humic-acid solution at the desired concentration and the other compartment was loaded with 5 mL of TNT solution in 0.01 M KCl at the same pH. The initial TNT concentrations ranged from 0.44 to 24.4 μg mL^{-1} (0.4 to 22% of aqueous solubility). The two-compartment dialysis cells were placed on a shaker,

covered, and allowed to equilibrate for 23 to 24 h. The equilibration time was based on the findings of Li et al. (1997) and was chosen to minimize the impact of degradation or transformation of TNT due to the presence of humic acid. After equilibrium was achieved the concentration of free TNT was equal on both sides of the membrane with the humic acid and TNT-humic acid complexes being retained in only one compartment. Controls were run before batch equilibrium experiments to ensure that TNT molecule was able to pass through the membrane. Following the sorption experiments, an aliquot of sample from the non-humic acid compartment was transferred to a glass GC vial for analysis of TNT and metabolites by HPLC. For the range of TNT concentrations used in this study only 0.01 to 0.05 % of TNT was adsorbed to the membrane, and thus was ignored in all sorption calculations. Since no TNT metabolites were detected, the differences between the amount of initial TNT added and the amount of free TNT measured in the solution at the end of sorption experiments was considered as sorbed or complexed with humic acid.

2.5. HPLC Analysis.

Aqueous TNT concentrations were determined by injection of filtered aqueous samples on a reversed phase ThermoFinnigan HPLC system (Woburn, MA USA) equipped with a Spectra System P4000 pump, AS3000 Autosampler, UV6000LP UV detector (228 nm peak detection) and a J'sphere ODS-H80 (YMC Co. Ltd., Tokyo, Japan) reversed-phase column (5 μm, 150 by 4.6 mm i.d.). The mobile phase was 82:18 (v:v) water:2-propanol with a flow rate of 1.5 mL min^{-1}, a 10-μL injection volume and a column oven temperature of 40° C. Controls used included clay and humic acid-clay

complexes in electrolyte solution, the stock solution of each initial concentration, and blanks containing only the electrolyte concentration. A standard curve was run every 30 samples, with one standard rechecked every 10 samples. The lowest concentration of aqueous TNT at which reproducible results were obtained on the HPLC was 75 μg L^{-1}. The precision of the HPLC method ranged from 0.5 to 5% RSD.

2.6. X-Ray Diffraction Analysis.

X-Ray diffraction (XRD) analysis was conducted for selected samples after the desorption step was completed. The three replicates for each sorption/desorption treatment were pooled together to ensure a sample containing at least 150 mg of clay or humic acid-clay complex for the XRD analysis. Oriented clay slides were prepared for XRD analysis using the filter-membrane technique and stored at 0% humidity before analysis (Drever, 1973). The XRD patterns of clay and humic-clay films were obtained using a Philips PW 1729 automated x-ray diffractormeter equipped with Cu-Kα radiation and a curved crystal monochromator. The scanning angle (2θ) ranged from 3 to 15 at steps of 0.05º with 2 s per step.

3. Results and Discussion

3.1. TNT sorption and desorption by homoionic K^+- and Ca^{2+}- clay minerals.

Isotherms representing sorption of TNT by K^+- and Ca^{2+}- clay minerals (KGa, SWy, SAz, and SHCa) are shown in Figure 3.2. Sorption isotherms describing TNT sorption were fitted to the Langmuir and Freundlich equations for all clay minerals and to the Linear equation for selected minerals,

$$C_S = \frac{Q * K_L C_w}{1 + K_L C_w} \quad [1]$$

$$C_S = K_f C_w^N \quad [2]$$

$$C_S = K_d C_w \quad [3]$$

where C_S ($\mu g\ g^{-1}$) is the amount of TNT sorbed by the clay or humic-clay complex, C_w ($\mu g\ mL^{-1}$) is the equilibrium aqueous concentration of TNT. In the Langmuir model (equation 1), K_L is the Langmuir constant and is related to entropy and Q is the monolayer sorption capacity parameter. In the Freundlich model (equation 2), K_f (mL μg^{-1}) is the Freundlich sorption coefficient and N (unitless) describes the fitness of the isotherm or its curvature. In the linear model (equation 3), K_d (mL μg^{-1}) is the sorption coefficient. The results of fitting the TNT sorption data to the three models are shown in Table 3.2.

The sorption of TNT to K^+- saturated clay minerals followed the order of SWy > SHCa > SAz > KGa. However, the sorption of TNT to Ca^{2+}- saturated clay minerals followed the order of SHCa > SWy > SAz > KGa (Figure 3.2). For all clay minerals there was greater sorption to K^+- saturated minerals compared to the corresponding Ca^{2+}-saturated clay mineral. These results are in agreement with the findings of Haderlein et al. (1996) and Li et al. (2003), who observed similar trends with TNT and 4,6-dinitro-o-cresol, respectively. The greater affinity for K^+- saturated clay minerals can be attributed to the lower hydration energy associated with K^+ compared to the larger and stronger hydration sphere associated with Ca^{2+} (Haderlein et al., 1996; Sheng et al., 2001). In addition, the larger hydration radius associated with Ca^{2+} can obscure the siloxane surface and result in fewer opportunities for TNT sorption on Ca^{2+}-saturated clays

compared to K^+-saturated clays. Similarly, it has been suggested that TNT is able to interact through inner-sphere cation bridging with the K^+- in the interlayers by replacing the water (Boyd et al., 2001). However, since the hydration energy associated with Ca^{2+}- is greater, the interactions between TNT and Ca^{2+}- saturated clay minerals is a result of outersphere interactions with Ca^{2+}- (Harper, 1994). TNT sorption to K-SAz was lower than K-SHCa and K-SWy due to the larger interlayer charge and stronger binding of interlayer cations and vicinal water which makes it more difficult for TNT to replace them (Boyd et al., 2001; Sheng et al., 2002; Li et al., 2003).

The different sequence of TNT sorption to K^+- and Ca^{2+}- saturated SWy and SHCa (Table 4.3) could be attributed to the surface of hectorite (SHCa) exhibiting hydrophobic characteristics that may allow additional neutral siloxane sites and easier exchange of water and Ca^{2+} by TNT (Van Oss and Giese, 1995; Wu, 2001). Recently it has been suggested that the surface of clay minerals may exhibit hydrophobic characteristics between charged sites (from isomorphic substitution) and that surface hydrophobicity is negatively correlated with interlayer charge (Sposito et al., 1999). Hectorite (SHCa – trioctahedral) has a lower layer charge than smectite (SWy - dioctahedral) and has been characterized as more hydrophobic than hydrophilic (Van Oss and Giese, 1995). Similarly, a study examining the sorption of atrazine to Ca^{2+}- saturated clay minerals also noted the hydrophobic characteristics of hectorite as greater adsorption was observed compared to other smectitic minerals (Laird et al., 1992). However, studies by Weissmahr and coworkers indicated that hydrophobic interactions between TNT and clay minerals were minimal and that strong EDA interactions were responsible for TNT

sorption (Weissmahr, et al., 1997). To date, there is still considerable uncertainty as to the mechanism(s) responsible for sorption of TNT to clay minerals.

Desorption isotherms for the K^{+}- and Ca^{+2}- saturated clay minerals are presented in Figure 3.3. For all clay minerals except Ca-SHCa the desorption isotherms indicate significant hysteresis (Figure 3.3, Table 3.2). This is in disagreement with the findings of Haderlein et al. (1996) and Weissmahr et al. (1997) who reported a high degree of mobility consistent with reversible sorption for a variety of NACs including TNT. However, it should be noted that in the studies performed by Haderlein and coworkers an equilibration time of 30 to 60 min. was used compared to the 24 h equilibration time used in this study for both sorption and desorption experiments. It has been recently demonstrated that with prolonged exposure to TNT, there is an increase in the formation of non-extractable residues and an increase in hysteresis (Hundal et al., 1997). The desorption isotherms for the Ca^{2+}- saturated clays were convex to the abscissa ($N \geq 1.0$) indicating greater hysteresis at higher levels of TNT sorption (Figure 4.4). The lack of hysteresis with Ca-SHCa and the linear nature of both the sorption and desorption isotherms indicates that the binding of TNT on Ca-SHCa may be the result of weak, short range, physical interactions which is typical in the partitioning of neutral organic compounds rather than site specific sorption (Harper, 1994). These results support the discussion in the previous paragraph regarding hydrophobic interactions between TNT and netural siloxane sites on hectorite.

3.2 TNT sorption by humic acid

TNT sorption isotherms for humic acid, normalized to HA concentrations (μg TNT g^{-1} HA) are shown in Figure 3.4 and Table 3.3. For all humic acid concentrations a non-linear isotherm was observed which was well described by both the Langmuir and Freundlich isotherms. Previous research has shown that TNT in the presence of humic substances, especially in non-sterile conditions, can be readily reduced to monoamino (i.e. 2ADNT, 4ADNT) and diamino (2,4DANT) which can also be adsorbed by humic substances (Renoux et al., 2000; Eriksson and Skyllberg, 2001; Eriksson et al., 2004). However, it has been shown that this process may be microbially driven and that under sterile conditions, TNT, 4ADNT or 2ADNT do not adsorb to humic substances (Held et al., 1997). Other studies have shown that the reduction of TNT to 4ADNT via humic acid precursors such as catechol can lead to the formation of an azoxy-dimer that is readily adsorbed by humic substances (Achtnich et al., 1999; Wang et al., 2002). Eriksson and Skyllberg (2001) suggested that when discussing TNT sorption to humic materials it is more appropriate to refer to TNT sorption as the sorption of TNT and the sum of its degradation products. Thus, although no TNT metabolites were measured during this study, it is possible that the disappearance of TNT was due in part to transformation to and sorption of 2-ADNT or 4-ADNT In our study, an increase in TNT sorption was correlated with a decrease in HA concentration (Figure 4.5, Table 4.4). This is in agreement with Li et al. (1997) who noted an increase in TNT and 2,6DANT sorption with a decrease in HA concentration from 5.12 μM to 2.56 μM. At low concentrations, HA exhibits a linear polyelectrolyte behavior with more binding sites

available compared to high HA concentrations when the HA tends to be more coiled and aggregated with fewer sites available for TNT sorption (Schnitzer, 1986).

3.3 TNT sorption and desorption by humic-clay complexes

TNT sorption and desorption isotherms and fitted sorption parameters for the humic-clay complexes of the selected K^+ and Ca^{2+}-saturated are shown in Figure 3.5 and Table 3.2. All TNT sorption isotherms for the K^+- saturated humic-clay complexes were nonlinear with the Langmuir and Freundlich equations providing the best fit (Table 3.2). However, the TNT sorption isotherms for the Ca^{2+}- saturated humic-clay complexes were linear to slightly non-linear and were well described by all three equations (Table 3.2).

Except for the Ca-HA-SHCa, TNT desorption coefficients for all other humic-clay complexes were considerably larger than its sorption coefficients indicating sorption hysteresis (Figure 3.5, and Table 3.2). Larger desorption coefficients were found for K-HA-S1-SWy, K-HA-SAz, and Ca-HA-SHCa compared to that for the corresponding non-humic complexed clays, indicating that the humic acid further hindered the desorption of TNT. This is in line with the findings of Eriksson and Skyllberg (2001) and Held et al. (1997) who observed strong irreversible binding of TNT to DOM and HA, respectively. Contrary to these results, a smaller desorption coefficient was observed for K-HA-SHCa and Ca-HA-SWy compared to K-SHCa and Ca-SWy (Table 3.2). The previously mentioned hydrophobic characteristics of hectorite SHCa, and to a lesser extent low-charge smectite (SWy), could have resulted in greater adsorption of humic acid leading to a reduction in potential binding sites and binding strength.

The TNT sorption isotherms for humic-clay complexes and the corresponding K^+- and Ca^{2+}- saturated clay minerals are shown in Figure 3.6. For the smectitic clays K-HA-SWy and K-HA-SAz, humic acid resulted in greater sorption of TNT compared to the clay mineral. However, for K-HA-SHCa, the presence of humic acid resulted in a decrease in TNT sorption compared to the corresponding clay mineral. This implies that the sorption mechanism or mineral surface conditions are different for K^+-saturated SHCa compared to K^+-saturated SWy and SAz. The nature of the humic acid-clay interactions and/or the presence of impurities (i.e. calcite) could have potentially hindered the availability of sorption sites to TNT. Studies by Arnarson and Keil (2001) have noted that the interaction between humic acid and clay minerals may result in sporadic coverage of the clay surface rather than complete encasing by humic acid. Thus, the surface area covered by humic-acid in SHCa may be greater than that covered by SWy, due to the nature of humic-clay interactions, leading to the observed differences in TNT sorption. The decrease in external surface area of K-HA-SHCa and Ca-HA-SHCa compared to K-SHCa and Ca-SHCa supports the theory of increase surface area coverage by humic acid.

Li et al. (2003) observed no difference in 4,6-dinitro-o-cresol or dichlorbenil sorption between K^+- clay minerals and the corresponding humic-clay complexes. They concluded that humic acid, which was limited to the outer clay surfaces, had no impact on sorption because the interlayer region provides the dominant binding sites for these nitroaromatic pesticides. Their conclusion was further supported by XRD analysis. On the contrary, a substantial increase in TNT sorption was observed in this study for K-HA-SWy compared to K-SWy and a moderate increase was observed in K-HA-SAz

compared to K-SAz (Figure 3.6). The XRD analysis of K-HA-SWy and K-SWy further supports these findings (Figure 3.7; Appendix A, Figures A.1., A.2). The interlayer spacing of K-SWy and K-HA-SWy increased with increasing TNT sorption and was ≈12.0 Å at the highest level of TNT sorption. This supports the suggestion that TNT interacts with the basal surfaces as a planar molecule (Haderlein et al., 1996; Sheng et al., 2001). Previous research has demonstrated that the interlayer spacing representing the interaction between siloxane surfaces is 9.2 Å (i.e. pyrophyllite) and that NACs such as TNT have a planar van der Waals molecular thickness of ≈3.5 Å (Sheng et al., 2001; Johnston et al., 2004). Thus, adding the thickness of TNT to the interlayer spacing of two siloxane surfaces yields a total thickness of roughly 12.7 Å, which is similar to that measured in this study. One possible explanation for the difference between the calculated spacing and our measured spacing is that our XRD peaks were fairly broad which is due to the contributions of expanded and non-expanded layers (Johnston et al., 2004). Furthermore, the measured XRD peaks for the humic-clay complexes were extremely broad compared to those of the corresponding clay mineral indicating possible interference from the humic acid or a greater degree of heterogeneity in the number of layers that were expanded and not expanded.

Our results also indicate the possibility that the surface of the humic-clay complex had a greater affinity for TNT than the corresponding clay mineral. For a given amount of TNT sorbed, K-SWy had a greater basal spacing (d_{001}) compared to K-HA-SWy (Figure 3.7), yet overall K-HA-SWy sorbed TNT to a greater extent. This implies that with humic acid, there was less sorption in the interlayer region and a greater degree of sorption on the humic-clay surface then on the corresponding clay mineral. A possible

explanation for this is that the humic-acid associated with the clay increased the affinity for or ability of TNT to bind with the outer surfaces compared to the clay with no humic acid. Thus, the humic acid associated clay surface was more attractive or accessible to TNT than the clay interlayer, however, with additional TNT loadings eventually TNT sorption in the interlayer reached the same degree as that for the non-humic clay complex (K-SWy, Figure 3.7). Further evidence for this is that every humic-clay complex had a reduction in the exterior surface area compared to the corresponding homoionic clay mineral (Table 3.1), yet there was still an increase in TNT sorption for two of the three humic-clay complexes (Table 3.2). This would indicate possible hydrophobic partitioning was involved despite the fact that previous studies have refuted this as a significant mechanism responsible for TNT sorption (Weissmahr et al., 1998).

The effect of humic acid on the sorption of TNT to Ca^{2+} clay minerals was minimal compared to that on K^{+}- saturated clays (Figure 3.6, Table 3.2). The TNT sorption to these humic-clay complexes and clay minerals were well described by linear isotherms which indicate a portioning mechanism rather than site specific sorption which tends to be more common with non-linear isotherms (Harper, 1994). The larger hydration sphere of Ca^{+2} prevented the sorption of TNT by the humic-clay complexes and corresponding clay minerals by reducing the space between exchangeable cations, which are favorable sorptive domains for TNT (Johnston et al., 2004). In addition, the expansion of the interlayer region to almost 14.0 Å (data not shown) for both Ca-SWy and Ca-HA-SWy may allow for greater hydration of the interlayer region and thus reduce the affinity for TNT compared to the K^{+}- saturated clay minerals and humic-clay complexes (Sheng et al., 2001). Overall, humic acid slightly increase sorption to Ca-HA-

SAz compared to Ca-SAz and slightly reduced sorption to Ca-HA-SWy compared to Ca-SWy (Figure 3.6). Li et al. (2003) noted similar trends with 4,6-dinitro-o-cresol by Ca^{+2} saturated SWy and SAz and the corresponding humic-clay complexes.

Sorption of TNT to kaolinite, the only non 2:1 clay mineral examined in this study was lower than any other clay mineral (Figure 3.8). The isotherm was non-linear indicating site specific interactions with the mineral surface. The lack of TNT sorption can be attributed to the low external and total surface area. Humic-clay complexes were prepared only for the K^{+}- saturated kaolinite and under two different experimental conditions of pH 4.5 and 6.5. The isotherms for the humic-clay complexes (K-HA-KGa-A, K-HA-KGa-B) were similar regardless of the pH with both exhibiting a linear characteristic and had linear sorption coefficients that were almost 3x greater than K-KGa (Figure 4.8, Table 4.3). This implies that the sorption of TNT to the humic-kaolinite complex was due to partitioning rather than site specific sorption. Additionally, the significant increase in sorption with humic acid suggests that in soil systems dominated by 1:1 clay minerals soil organic matter is the dominant sorbent for NACs such as TNT. The overall lack of difference in TNT sorption to kaolinite under different pH conditions is typical for nonionizable compounds, such as NACs including TNT (Haderlein and Schwarzenbach, 1993).

3.4 TNT sorption to sequentially prepared humic-clay complexes.

The sorption of TNT to humic-clay complexes was further examined by preparing four humic-clay complexes with the SWy-2 reference mineral sequentially saturated with the same humic acid solution (K-HA-SWy, K-HA-S2-SWy, K-HA-S3-SWy, K-HA-S4-

SWy). Sorption isotherms for TNT to the sequentially prepared humic-clay complexes are presented in Figure 3.9. All the sorption isotherms were well described with the Langmuir and Freundlich models (Table 3.2). The sorption of TNT on the humic-clay complexes decreased with each sequentially prepared humic-clay complex.

Studies involving solid- and liquid-state ^{13}C NMR have provided evidence for the fractionation of humic acid during its adsorption onto the mineral surface that result in the preferential adsorption of aliphatic humic substances (Wang and Xing, 2005). In addition, their UV-visible spectroscopy analysis showed a decrease of humic acid E_4 to E_6 ratios in the residual humic acid solution with increasing order of sequential sorption, indicating an increase in the aromaticity of the humic acid solution. What we observed in the current study, (Figure 3.9, Table 3.2) that TNT sorption decreased with increasing order of sequential complexation with SWy-2 indirectly supports the findings by Wang and Xing (2005).

The second, third, and fourth HA saturations resulted in carbon contents of 3.6, 2.3, and 3.2 g kg^{-1}, respectively, which was significantly lower than the first HA saturation (K-HA-SWy) with a carbon content of 13.9 g kg^{-1} (Table 3.1). This could be due to a reduction in the ratio of humic acid to clay in the humic acid solution with each sequential humic-clay complexation resulting in a decrease in sorbed humic acid. A second possibility is that the nature of the remaining humic acid was changed (i.e. increase in aromaticity) and resulted in a reduction in affinity for the clay mineral surface. The initial humic acid to clay ratio of the humic acid solution was 1:10 (w/w). The reduction in carbon content for the second and third sequential complexation corresponded with an increase in the specific surface area of the humic-clay complexes

compared to that from the initial humic acid sorption (Table 3.1). Although the humic-clay complex from the fourth sequential complexation had similar carbon content as that of the previous two sequential complexations, its specific surface area is the lowest (Table 3.1). Wang and Xing (2005) noted similar change for surface areas of humic-clay complexes with different humic acid loading levels. The authors suggested that the thickness of the HA covering the clay surface may differ depending on the nature of the humic-clay interaction. Arnarson and Keil (2001) suggested that the sorption of clay minerals by humic acid exists at discrete portions of the mineral surface. This would explain the increase in specific surface area observed in the second and third humic acid sequencial complexation steps, as the humic acid covered less surface area of the clay mineral compared to the first HA saturation. However, after the forth sequential complexation, it is possible that the humic acid sorbed to the clay resulted in a thicker and more sporadically located (i.e hydrophobic portions of SWy) layers of HA compared to the previous two sequential complexation steps, resulting in a decrease in specific surface area (Table 3.1). Typically, the surface area of organic matter has been measured at 1 m^2 g^{-1}, thus, the difference in surface area between the second and fourth sequentially saturated humic-clay complexes may be due to the size/thickness of the humic acid particles or the overall surface area that they cover (Chiou et al., 1990).

The XRD analysis of the sequentially saturated humic-clay complexes provided possible evidence of the intercalation by humic acid or impurities in the humic acid (humic substances) within the interlayer regions (Figure 3.10; Appendix A, Figures A2, A3, A4). The adsorption of fulvic acid in clay interlayers has been previously discussed, however, to our current knowledge there is no evidence of humic acid intercalation in 2:1

clay minerals (Schnitzer, 1986). With a TNT loading of 0 ppm, the basal (d_{001}) spacing for K-HA-S2-SWy, K-HA-S3-SWy, and K-HA-S4-SWy was 12.1, 11.6, and 12.5 Å, respectively, compared to K-HA-SWy with a basal spacing of 10.3 Å. These results would suggest that larger molecular weight humic acids were adsorbed to the clay during the initial complexation, leaving behind smaller aliphatic humic substances for later sequential complexation steps. It is not known if the interlayer humic acid sorbed to the clay was aliphatic or aromatic in nature, but when the humic-clay complexes from the 2^{nd}, 3^{rd}, and 4^{th} complexation steps were exposed to TNT their interlayer spacing dropped to 10 – 10.7 Å which was similar to that for K-HA-SWy. This would suggest that the humic substances in the interlayer were weakly adsorbed, possibly due to van der Waals forces or outersphere complexation. In addition, at the highest TNT level similar interlayer spacings of 12.0 Å was observed for all four sequentially complexed humic-clay complexes. This would suggest that the interlayer sorption of TNT was similar for all four humic-clay complexes and that the overall reduction in TNT sorption observed in the second through fourth sequentially complexes humic-clay complexes was due to a reduction in TNT sorption to the humic-clay complex external surface rather than in the interlayer region. Thus, with sequential complexation between clay minerals and humic acid, the surface characteristics changed resulting in a reduction in surface sorption of TNT.

4. Conclusion

Overall, the observed results of TNT sorption to K^+- and Ca^{2+}- saturated clays agree with previous studies. The TNT sorption to humic acid was significant and may

have been the result of TNT reduction to mono- and di-amino metabolites followed by sorption to the humic acid solutions. TNT sorption to humic acid increased with a decrease in humic acid solution concentration and in part explains the high levels of sorption to certain humic-clay complexes (K-HA-SWy, K-HA-SAz). Humic acid associations with clay minerals, particularly K^+- humic-clay complexes enhanced sorption with kaolinite, two smectite minerals (SWy and SAz), and suppressed sorption to a third smectite mineral (SHCa). Additionally, desorption hysteresis was observed with all clay minerals and the corresponding humic-clay complexes, except for Ca-SHCa, indicating strong binding of TNT to the surfaces and interlayer regions of clay and humic-clay complexes. The TNT sorption to smectite minerals which were sequentially complexed with humic acid provided further evidence for fractionation of humic acid during sorption by clay minerals and the possible intercalation of humic acid in smectite.

These findings have relevant applications in the risk assessment and remediation of contaminated sites. Soil contaminated with TNT and that contain a significant amount of organic matter may not pose a threat to groundwater and transport compared to other sites. This is especially true of soils dominated by 1:1 clays such as kaolinite. Although kaolinite does not readily adsorb TNT, if the contribution for organic matter is not considered site assessment may over estimate the potential risk to humans, wildlife, and plants. Although hectorite is not as common as other smectite minerals, the reduction in TNT sorption with the humic acid-hectorite complex raises the possibility of underestimating the amount of TNT bound to the soil. Therefore, clay mineralogy should not be overlooked during site assessment and characterization.

The affinity of TNT for humic acid also raises the possibility of incorporating organic amendments in existing remediation technologies such as composting, phytoremediation, and biostimulation. Organic amendments may reduce the bioavailability of TNT through sequestration and stimulating biotic and abiotic transformation to metabolites which in turn may be readily adsorbed. Overall, these results indicate the importance of considering organic matter-clay interactions when determining the bioavailability and fate of TNT in the environment.

References

Arnarson, T.S.,and R.G. Keil. 2001. Organic-mineral interactions in marine sediments studied using density fractionation and X-ray photoelectron spectroscopy. Org Geochem 32: 1401-1415.

Achtnich, C., U. Sieglen, H.-J. Knackmuss, and H. Lenke. 1999. Irreversible binding of biologically reduced 2,4,6-trinitrotoluene to soil. Environ. Toxicol. Chem. 18: 2416-2423.

Borden, D., and R.F. Griese. 2001. Baseline studies of the clay minerals society source clays: Cation exchange capacity measurements by the ammonia-electrode method. Clays Clay Miner. 49: 444-445.

Boyd, S.A., G. Sheng, B.J. Teppen, and C.T. Johnston. 2001. Mechanisms for the adsorption of substituted nitrobenzenes by smectite clays. Environ. Sci. Technol. 35: 4227-4234.

Bruns-Nagel, D., H. Knicker, O. Drzyzga, U. Butehorn, K. Steinbach, D. Gemsa, and E.V. Low. 2000. Characterization of ^{15}N NMR spectroscopy. 2. Systematic investigation of whole soil and different humic fractions. Environ. Sci. Technol. 34: 1549-1556.

Chiou, C.T., J.-F. Lee, and S.A. Boyd. 1990. The surface area of soil organic matter. Environ. Sci. Technol. 24: 1164-1166

Chipera, S.J., and D.L. Bish 2001. Baseline studies of the clay minerals society source clays: Powder x-ray diffraction analyses. Clays Clay Miner. 49: 398-409.

Comfort, S.D., P.J. Shea, L.S. Hundal, Z. Li, B.L. Woodbury, and W.L. Powers. 1995. TNT transport and fate in contaminated soil. J. Environ. Qual. 24: 1174-1182.

Drever, J.E. 1973. The preparation of oriented clay mineral specimens for x-ray diffraction analysis by a filter-membrane technique. Am. Mineral. 58: 553-554.

Eriksson, J., and U. Skyllberg. 2001. Binding of 2,4,6-trinitrotoluene and its degradation products in a soil organic matter two-phase system. J. Environ. Qual. 30: 2053-2061.

Eriksson, J., S. Frankki, A. Shchukarev, and U. Skyllberg. 2004. Binding of 2,4,6-trinitrotolune, aniline, and nitrobenzene to dissolved and particulate soil organic matter. Environ. Sci. Technol. 38: 3074-3080.

Fuller, M.E., and J.F. Manning. 1998. Evidence for differential effects of 2,4,6-

trinitrotoluene and other munitions compounds on specific subpopulations of soil microbial communities. Environ. Toxicol. Chem. 17: 2185-2195.

GAO 2003. Military munitions: DOD needs to develop a comprehensive approach for cleaning up contaminated sites, Report GAO-04-147, General Accountability Office, Washington, D.C.

Haderlein, S.B., and R.P. Schwarzenbach. 1993. Adsorption of substituted nitrobenzenes and nitrophenols to mineral surfaces. Environ. Sci. Technol. 27: 316-326.

Haderlein, S.B., K.H. Weissmahr, and R.P. Schwarzenbach. 1996. Specific adsorption of nitroaromatic explosives and pesticides to clay minerals. Environ. Sci. Technol. 30: 612-622.

Haper, S.S. 1994. Sorption-desorption and herbicide behavior in soil. Rev. Weed. Sci. 6: 207-225.

Held, T., G. Draude, F.R.J. Schmidt, A. Brokamp, and K.H. Reis. 1997. Enhanced humification as an in-situ bioremediation technique for 2,4,6-trinitrotoluene (TNT) contaminated soils. Environ. Sci. Technol. 18: 479-487.

Hundal, L., P.J. Shea, S.D. Comfort, W.L. Powers, and J. Singh 1997. Long-term TNT sorption and bound residue formation in soil. J. Environ. Qual. 26: 896-904.

Jerger, D.E., and P.E. Woodhull. 2000. Applications and costs for biological treatment of explosives contaminated soil in the U.S. p. 395-424. *In* Biodegradation of nitroaromatic compounds and explosives. Spain, J.C., Joseph B. Hughes, and Hans-Joachim Knackmuss (eds). CRC Press, Boca Raton.

Johnston, C.T., M.F.D. Oliveira, B.J. Teppen, G. Sheng, and S.A. Boyd. 2001. Spectroscopic study of nitroaromatic-smectite sorption mechanisms. Environ. Sci. Technol. 35: 4767-4772.

Johnston, C.T., S.A. Boyd, B.J. Teppen, and G. Sheng. 2004. Sorption of nitroaromatic compounds on clay surfaces. p. 155-189. *In* Handbook of layered materials. Auerbach S.M., Carrado K.A., Dutta P.K. (eds). Marcel-Dekker, New York.

Koskinen, W.C., and S.S. Harper. 1990. The retention process: Mechanisms. p. 51-78 *In.* Pesticides in the soil environment: Processes, impacts, and modeling. Soil Science Society of America, Inc., Madison, WI.

Laird, D.A., E. Barriuso, R.H. Dowdy, and W.C. Koskinen. 1992. Adsorption of atrazine on smectites. Soil Sci. Soc. Am. J. 56: 62-67.

Levine, B.S., E.M. Furedi, D.E. Gordon, P.M. Lish, and J.J. Barkley. 1984. Subchronic toxicity of trinitrotoluene in Fischer-344 rats. Toxicol. 32: 253-265.

Li, A.Z., K.A. Marx, J. Walker, and D.L. Kaplan. 1997. Trinitrotoluene and metabolites binding to humic acid. Environ. Sci. Technol. 31: 584-589.

Li, H., G. Sheng, B.J. Teppen, C.T. Johnston, and S.A. Boyd. 2003. Sorption and desorption of pesticides by clay minerals and humic acid-clay complexes. Soil. Sci. Soc. Am. J. 67: 122-131.

Mermut, A.R., and G. Lagaly. 2001. Baseline studies of the clay minerals society source clays: Layer-charge determination and characteristics of those minerals containing 2:1 layers. Clays Clay Miner. 49: 393-397.

Nelson, D.W., and L.E. Sommers, 1996. Total carbon, organic carbon, and organic matter. p. 961-1010. *In.* Methods of soil analysis. Part 3. Chemical Methods. Sparks, D.L. (ed). SSSA Book Ser. No. 5. SSSA, Madison, WI.

Pelmenschikov, A., and J. Leszczynski. 1999. Adsorption of 1,3,5-trinitrobenzene on the siloxane sites of clay minerals: Ab initio calculations of molecular models. J. Phys. Chem. B. 103: 6886-6890.

Pennell, K.D., 2002. Specific surface area. p. 295-316. *In.* Methods of soil analysis. Part 4. Physical Methods. Dane J.H. and Topp G.C (eds). SSSA Book Ser. No. 5. SSSA, Madison, WI.

Pennington, J.C., and J.M. Brannon. 2002. Environmental fate of explosives. Thermochimica Acta 384: 163-172.

Renoux, A.Y., M. Sarrazin, J. Hawari, and G.I. Sunahara. 2000. Transformation of 2,4,6-trinitrotoluene in soil in the presence of the earthworm *Eisenia Andrei*. Environ. Toxicol. Chem. 19: 1473-1480.

Robidoux, P.Y., J. Hawari, S. Thiboutot, G. Ampleman, and G.I. Sunahara. 1999. Acute toxicity of 2,4,6-trinitrotoluene in earthworm (*Eisenia andrei*). Ecotox. Environ. Safe 44: 311-321.

Rocheleau, S., R.G. Kuperman, M. Martel, L. Paquet, G. Bardai, S. Wong, M. Sarrazin, S. Dodard, P. Gong, J. Hawari, R.T. Checkai, and G.I. Sunahara. 2006. Phytotoxicity of nitroaromatic energetic compounds freshly amended or weathered and aged in sandy loam soil. Chemosphere 62: 545-558.

Rodgers, J.D., and N.J. Brunce. 2001. Treatment methods for the remediation of nitroaromatic explosives. Water. Res. 35: 2101-2111.

Schnitzer M., 1986. Binding of humic substances by soil mineral colloids. p. 77-101. *In* Interactions of soil minerals with natural organics and microbes. Huang P.M., M. Schnitzer (eds.) Soil Science Society of America Inc., Madison, WI.

Spain J.C. 2000. Introduction. p. 1-5. *In* Biodegradation of nitroaromatic compounds and explosives. Spain, J.C. (ed). Boca Raton: Lewis Publishers, New York.

Sposito, G., N.T. Skipper, R. Sutton, S.-H. Park, A.K. Soper, and J.A. Greathouse. 1999. Surface geochemistry of the clay minerals. Proc. Natl. Acad. Sci. 96: 3358-3364.

Steevens, J.A., B.M. Duke, G.R. Lotufo, and T.S. Bridges. 2002. Toxicity of the explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine in sediments to *Chironomus tentans* and *Hyalella azteca*: Low-dose hormesis and high-dose mortality. Environ. Toxicol. Chem. 21:1475-1482.

Thorn, K.A., J.C. Pennington, and C.A. Hayes. 2002. ^{15}N NMR investigation of the reduction and binding of TNT in an aerobic bench scale reactor simulating windrow composting. Environ. Sci. Technol. 36: 3797-3805.

van Olphen, H., and J.J. Fripiat. 1979. Data handbook for clay minerals and other non-metallic minerals. Pergamon, Oxford, UK.

van Oss, C.J., and R.F. Giese. 1995. The hydrophilicity and hydrophobicity of clay minerals. Clays Clay Miner. 43: 474-477.

Wang, C.J., S. Thiele, and J.-M. Bollag. 2002. Interaction of 2,4,6-trinitrotoluene (TNT) and 4-amino-2,6-dinitrotoluene with humic monomers in the presence of oxidative enzymes. Arch. Environ. Contam. Toxicol. 42: 1-8.

Wang, K., and B. Xing. 2005. Structural and sorption characteristics of adsorbed humic acid on clay minerals. J. Environ. Qual. 34: 342-349.

Weissmahr, K.W., S.B. Haderlein, and R.P. Schwarzenbach. 1998. Complex formation of soil minerals with nitroaromatic explosives and other pi-acceptors. Soil Sci. Soc. Am. J. 62: 369-378.

Wilke, B.M., A. Gattinger, E. Froich, L. Zelles, and P. Gong. 2004. Phospholipid fatty acid composition of a 2,4,6-trinitrotoluene contaminated soil and an uncontaminated soil as affected by a humification remediation process. Soil Biol. Biochem. 36: 725-729.

Wu, W. 2001. Baseline studies of the clay minerals society source clays: Colloid and surface phenomena. Clays Clay Miner. 49: 446-452.

Xue, S.K., I.K. Iskandar, and H.M. Selim. 1995. Adsorption-desorption of 2,4,6-trinitrotoluene and hexahydro-1,3,5-trinitro-1,3,5-triazine in soils. Soil Sci. 160: 317-327.

Table 3.1. Properties of reference clay minerals (SWy-2, SAz-1, SHCa-1, and KGa-1b), their K^+- and Ca^{2+}-saturated forms, their humic acid -clay complexes, and sequentially complexed humic acid-clay complexes.

Reference Clay	Prepared clay or humic-clay complex	CEC[†]	Interlayer Charge[‡]	External Surface Area	Organic Carbon
		cmol(+) kg^{-1}	cmol(c) kg^{-1}	m^2 g^{-1} (± 1 S.D.)	g kg^{-1}
SWy-2		85	0.87		
	K-Swy			24.4 (0.5)	--
	K-HA-SWy			16.6 (0.4)	13.9
	K-HA-S2-SWy			22.4 (0.1)	3.6
	K-HA-S3-SWy			25.2 (0.2)	2.3
	K-HA-S4-SWy			12.5 (0.0)	3.2
	Ca-SWy			26.5 (1.0)	--
	Ca-HA-SWy			14.1 (0.6)	21.8
SAz-1		123	0.97		
	K-SAz			82.2 (0.6)	--
	K-HA-SAz			76.2 (0.6)	5.1
	Ca-SAz			60.7 (1.7)	--
	Ca-HA-SAz			52.1 (0.5)	3.2
SHCa-1		66	0.64		
	K-SHCa			51.9 (1.4)	--
	K-HA-SHCa			23.6 (1.4)	17.1[§]
	Ca-SHCa			47.9 (0.0)	--
	Ca-HA-SHCa			20.0 (0.5)	23.3[§]
KGa-1b		3.0	--		
	K-KGa			12.2 (0.2)	--
	K-HA-KGa-A			11.2 (0.0)	5.2
	K-HA-KGa-B			10.9 (0.4)	5.0
	Ca-KGa			12.7 (0.1)	--

[†] Borden and Giese, 2001

[‡] Mermut and Lagaly, 2001.

[§] Acid pre-treatment was used before analysis as hectorite contains between 27 - 43% calcite impurity ($CaCO_3$) (van Olphen and Fripiat, 1979; Chipera and Bish, 2001)

Table 3.2 Sorption and desorption isotherm parameters for TNT to K^+- and Ca^{2+}- saturated clay minerals and their humic acid (HA)-clay complexes.

		Sorption Model								Desorption Model		
		Langmuir			Linear		Freundlich			Freundlich		
Sorbent	**MV**[†]	**K_L**	**Q_L**	**r^2**	**K_d**	**r^2**	**K_f**	**N**	**r^2**	**$K_{f,d}$**	**N_d**	**r^2**
	g ml^{-1}											
K-SWy	0.05/25	0.3946	25,279	0.997	--[‡]	--	8,640	0.38	0.999	36,541	0.74	0.977
K-HA-SWy	0.05/25	0.8192	31,425	0.999	--	--	14,710	0.31	0.993	98,567	1.64	0.984
K-HA-S2-SWy	0.05/25	0.1991	34,166	0.999	--	--	7,780	0.46	0.999	--	--	--
K-HA-S3-SWy	0.05/25	0.2404	32,632	1.000	--	--	9,322	0.38	0.997	--	--	--
K-HA-S4-SWy	0.05/25	0.1531	31,757	0.997	--	--	6,808	0.43	0.993	--	--	--
K-SHCa	0.05/25	0.2809	18,284	0.999	--	--	6,630	0.30	0.999	12,293	0.50	0.996
K-HA-SHCa	0.05/25	0.0674	20,292	0.999	--	--	2,899	0.45	1.000	8,549	0.61	0.999
K-SAz	0.05/25	0.0731	16,819	0.996	--	--	2,387	0.47	0.996	5,260	0.39	0.998
K-HA-SAz	0.05/25	0.1269	16,346	0.999	--	--	3,748	0.37	0.999	6,172	0.38	0.999
K-KGa	0.2/5.0	0.0191	177.3	0.984	1.2	0.947	5.8	0.7	0.985	--	--	--
K-HA-KGa-A	0.2/4.0	--	--	--	2.9	0.996	--	--	--	--	--	--
K-HA-KGa-B	0.2/4.0	--	--	--	2.8	0.984	--	--	--	--	--	--
Ca-SWy	0.08/10	0.0041	11,484	0.994	42.4	0.994	50.0	0.95	0.994	354.0	1.56	0.979
Ca-HA-SWy	0.1/10	0.0056	6,732	0.995	33.2	0.993	40.7	0.94	0.994	123.8	1.03	0.986
Ca-SHCa	0.1/10	0.0069	8,897	0.998	53.1	0.996	71.1	0.91	0.999	83.6	1.01	0.995
Ca-HA-SHCa	0.1/10	0.0047	11,909	1.000	49.1	0.998	64.2	0.92	1.000	121.2	1.35	0.993
Ca-SAz	0.1/5.0	0.0322	225.2	0.992	3.4	0.951	11.8	0.66	0.988	--	--	--
Ca-HA-SAz	0.1/5.0	0.0224	481.8	0.999	6.2	0.975	16.4	0.73	0.998	--	--	--
Ca-KGa	0.1/3.0	0.0252	145.5	0.990	2.0	0.962	5.4	0.72	0.990	--	--	--

[†] M/V is the mass of sorbent/volume of solution

[‡] Not determined

Table 3.3. Sorption isotherm parameters for TNT to humic acid at different humic acid concentrations.

	Exp. Conditions			Sorption Model							
				Langmuir			Linear		Freundlich		
Sorbent	pH	Carbon Concentration	Humic Acid† Concentration	K_L	Q_L	r^2	K_d	r^2	K_f	N	r^2
	(± 1 S.D.)	mg C L^{-1}	mg HA L^{-1}								
Humic Acid #1	7.0 (0.2)	98	200.0	0.0499	62.5	1.000	1.5	0.961	3.9	0.70	0.996
Humic Acid #2	7.1 (0.2)	63	129.0	0.0361	75.9	0.994	1.5	0.966	3.8	0.71	0.998
Humic Acid #3	6.6 (0.2)	30	62.0	0.0252	218.9	0.997	3.5	0.981	7.2	0.77	0.999
Humic Acid #4	7.1 (0.2)	20	40.0	0.0292	215.5	0.994	3.8	0.974	8.3	0.75	0.996
Humic Acid #5	6.0 (0.1)	10	24.0	0.0582	277.9	0.997	7.2	0.953	20.5	0.68	0.996

† Calculated based on the assumption that the HA contains 49.01% carbon

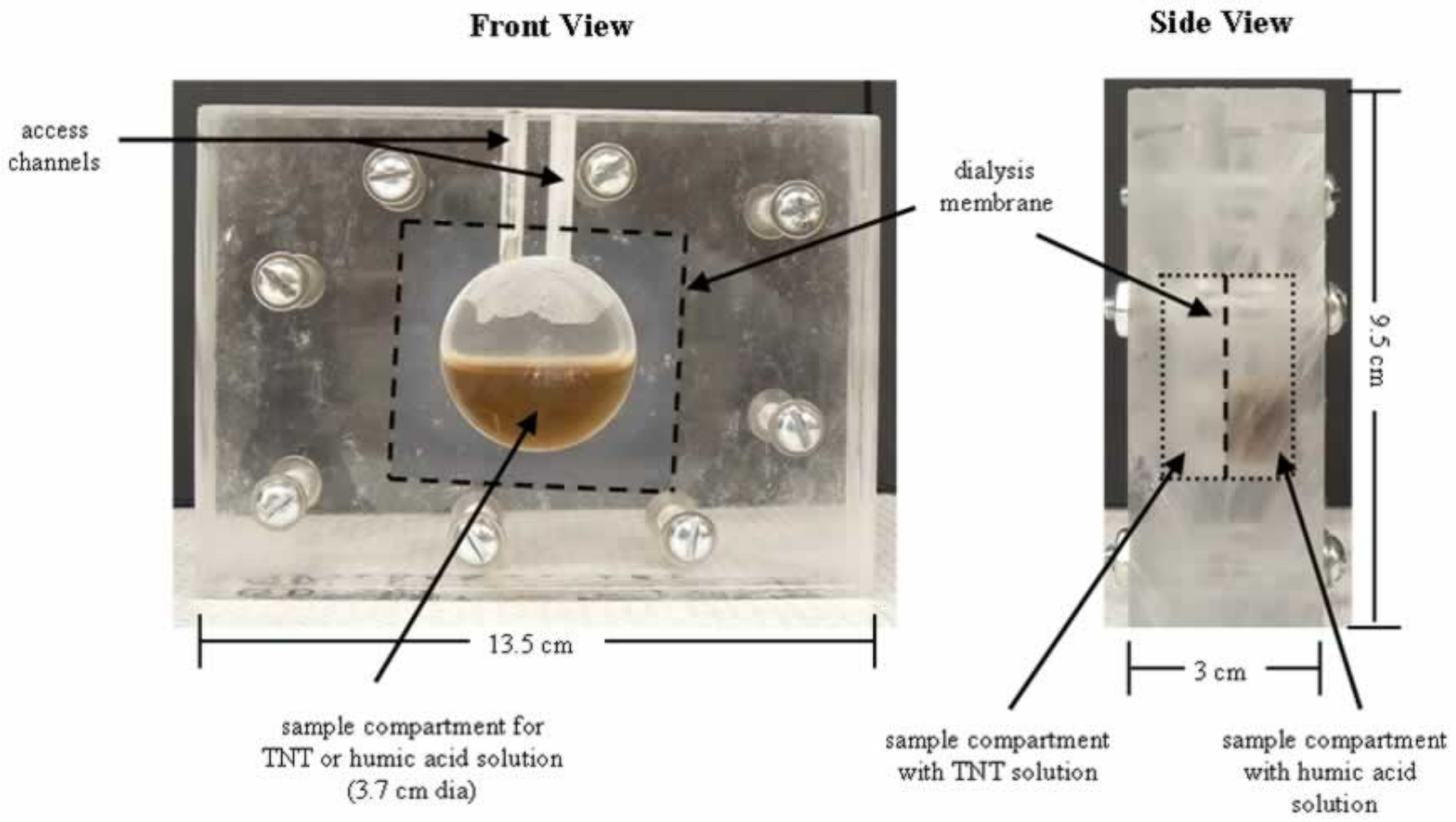


Figure 3.1 Dialysis unit for the batch equilibrium experiments for TNT and humic acid interaction.

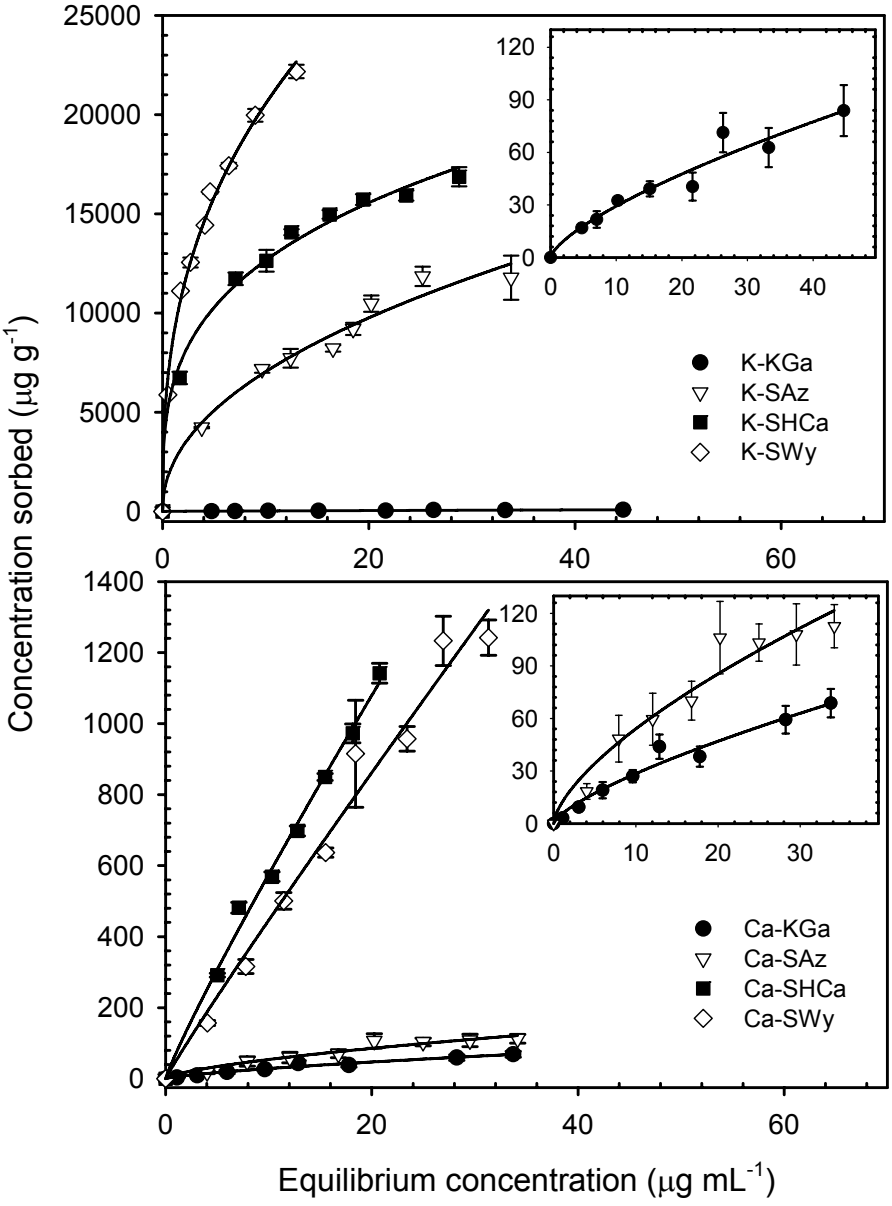


Figure 3.2. Isotherms describing 2,4,6-trinitrotoluene sorption by K^+- (top) and Ca^{2+}- (bottom) saturated clay minerals. Symbols and error bars represent the mean and ± 1 S.D., respectively (n = 3).

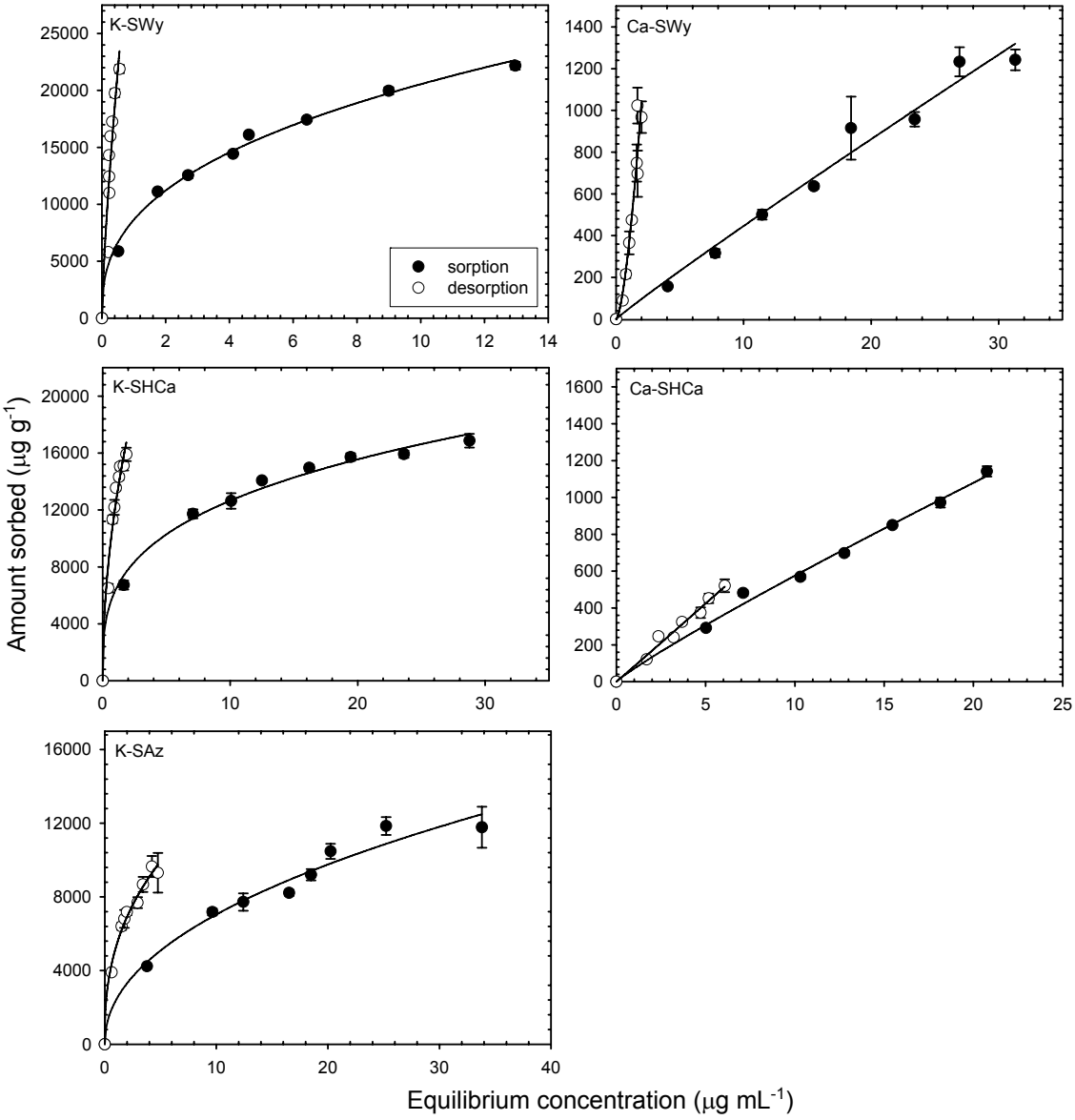


Figure 3.3. Sorption/desorption isotherms of TNT to K^{+}- saturated (K-SWy, K-SHCa, K-SAz) and Ca^{+2}- saturated (Ca-SWy, Ca-SHCa) smectite clay minerals. Symbols and error bars represent sample mean and ± 1 S.D., respectively (n = 3).

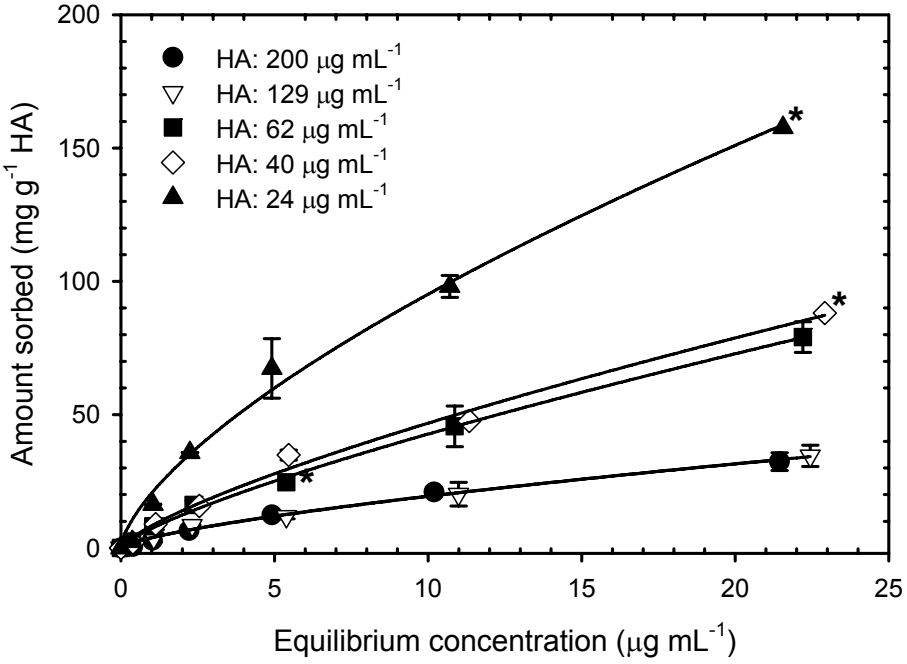


Figure 3.4. Sorption isotherms for TNT sorption to humic acid. Symbols and error bars represent the mean and ± 1 S.D., respectively (n = 2). *, n = 1.

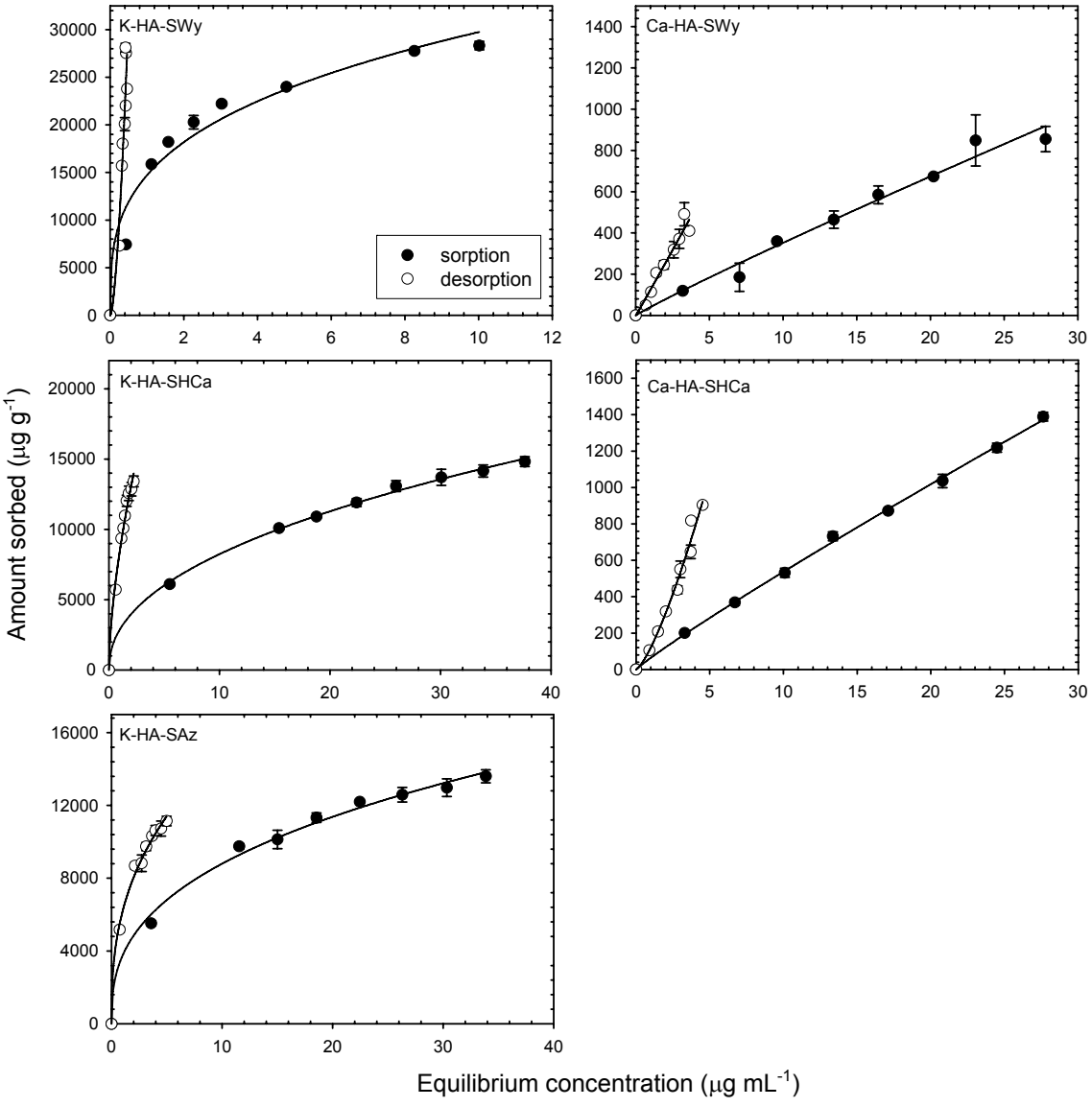


Figure 3.5. Sorption/desorption isotherms of TNT to K^+- saturated (K-HA-SWy, K-HA-SHCa, K-HA-SAz) and Ca^{+2}- saturated (Ca-HA-SWy, Ca-HA-SHCa) humic-clay complexes. Symbols and error bars represent sample mean and ± 1 S.D., respectively (n = 3).

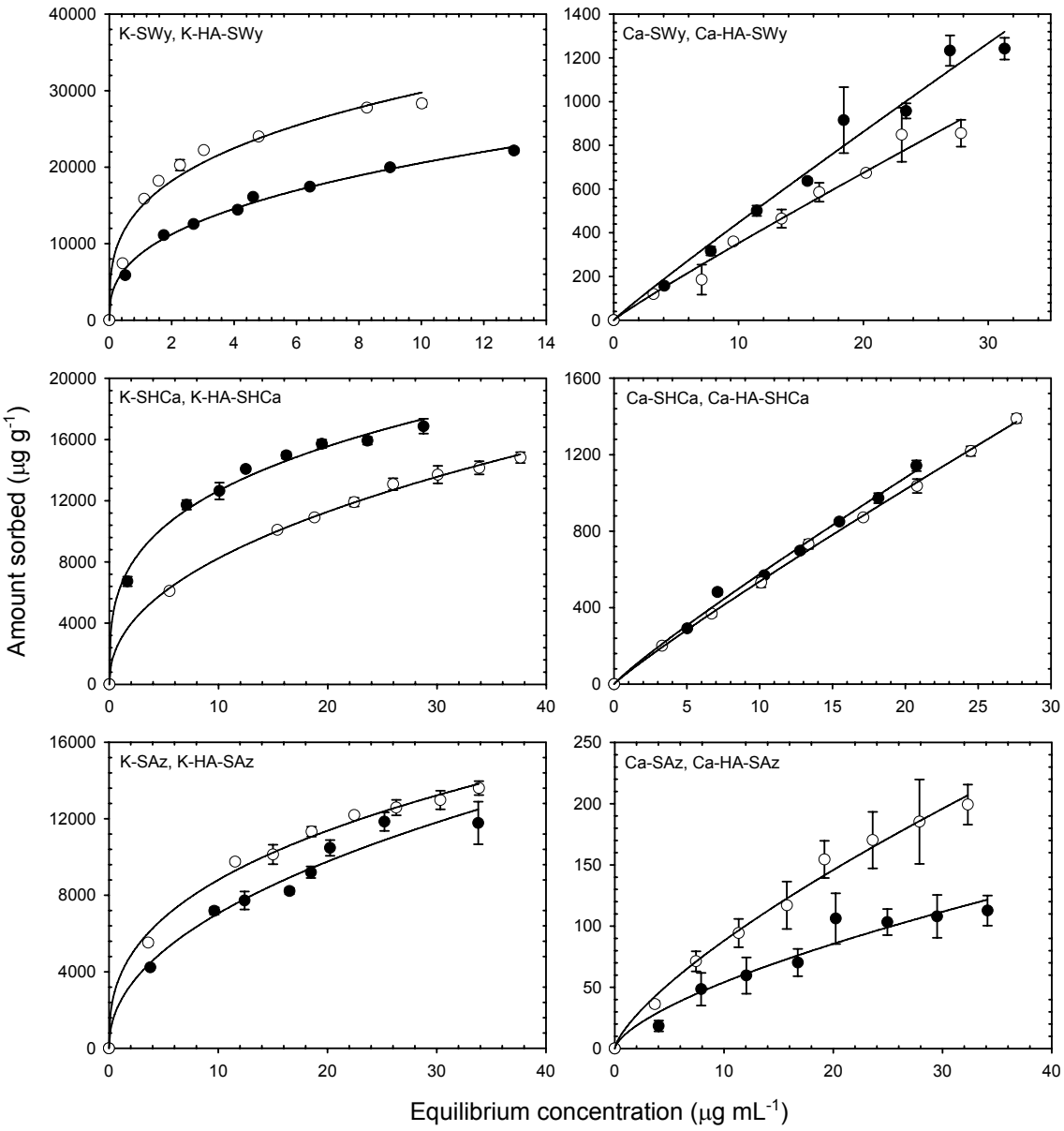


Figure 3.6. Sorption isotherms representing TNT sorption to K^{+}- and Ca^{+2}- saturated smectite clay minerals and their corresponding humic-clay complexes; Closed and open symbols represent sorption to the clay mineral (-●-) and corresponding humic-clay complex (-○-), respectively. Symbols and error bars represent sample mean and ± 1 S.D., respectively (n = 3).

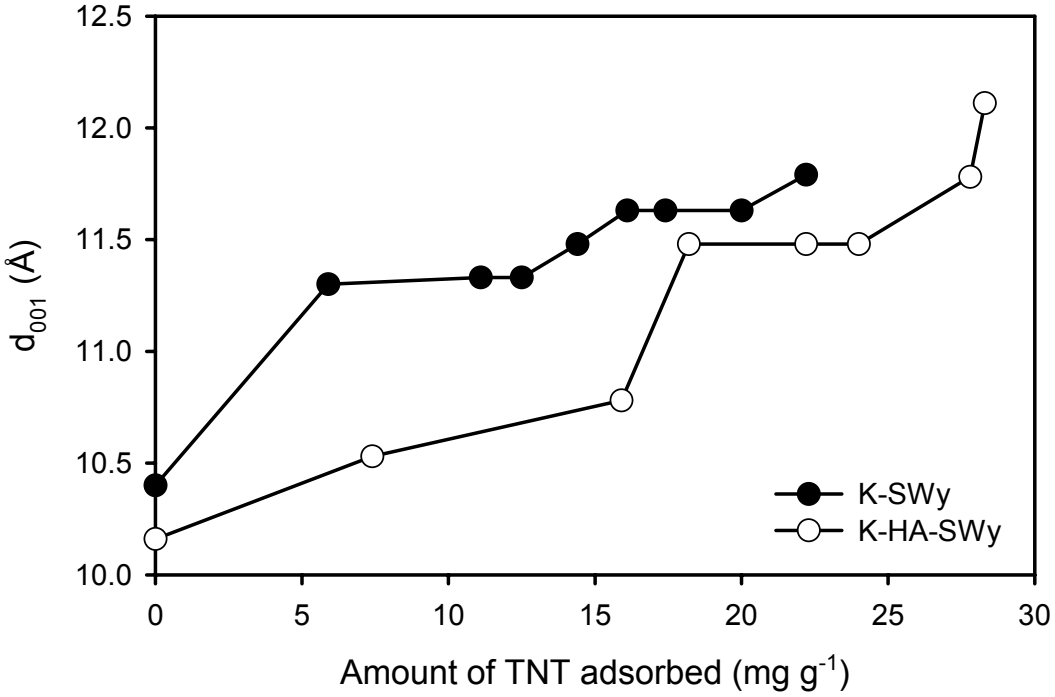


Figure 3.7. Associated *d*-spacings of K-SWy and its corresponding humic-clay complex K-HA-SWy obtained from x-ray diffraction patterns of oriented films as a function of TNT sorption.

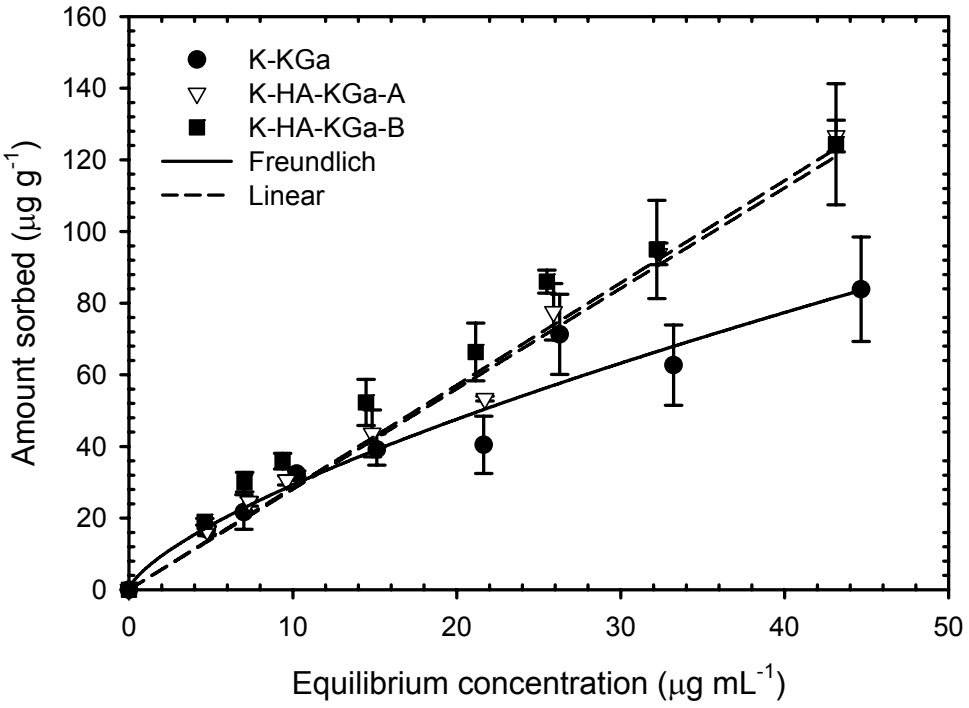


Figure 3.8. Isotherms describing TNT sorption by K^+-saturated kaolinite and humic-kaolinite complexes. Symbols and error bars represent the mean and ± 1 S.D., respectively (n = 3).

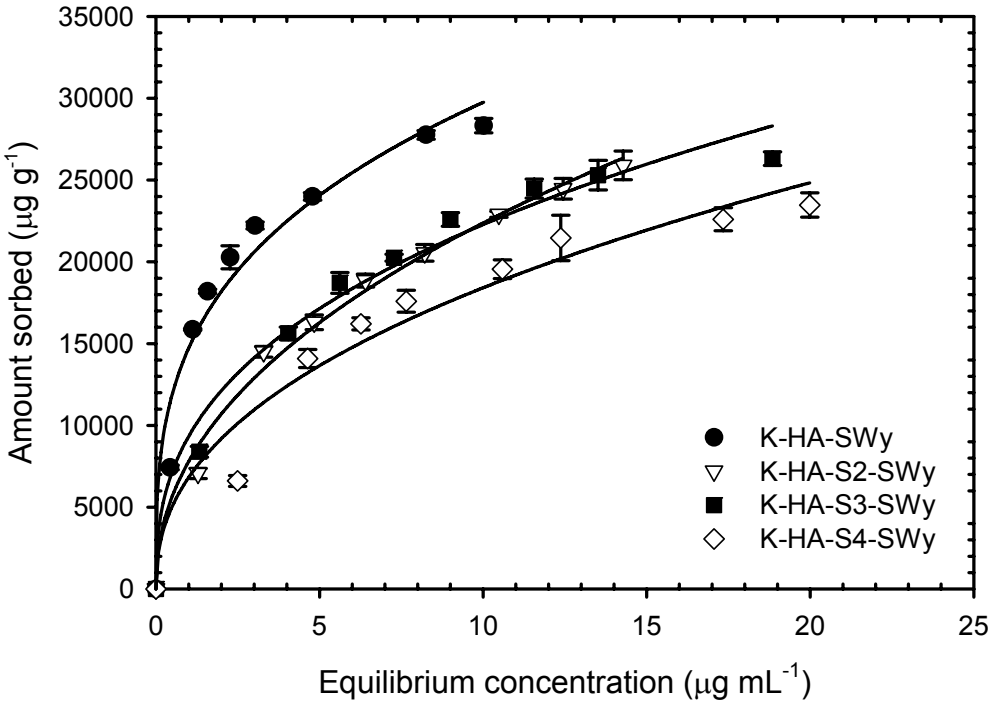


Figure 3.9. Isotherms describing 2,4,6-trinitrotoluene sorption by K^+-saturated humic-clay complexes sequentially prepared with smectite (K-SWy). Symbols and error bars represent the mean and ± 1 S.D., respectively (n = 3).

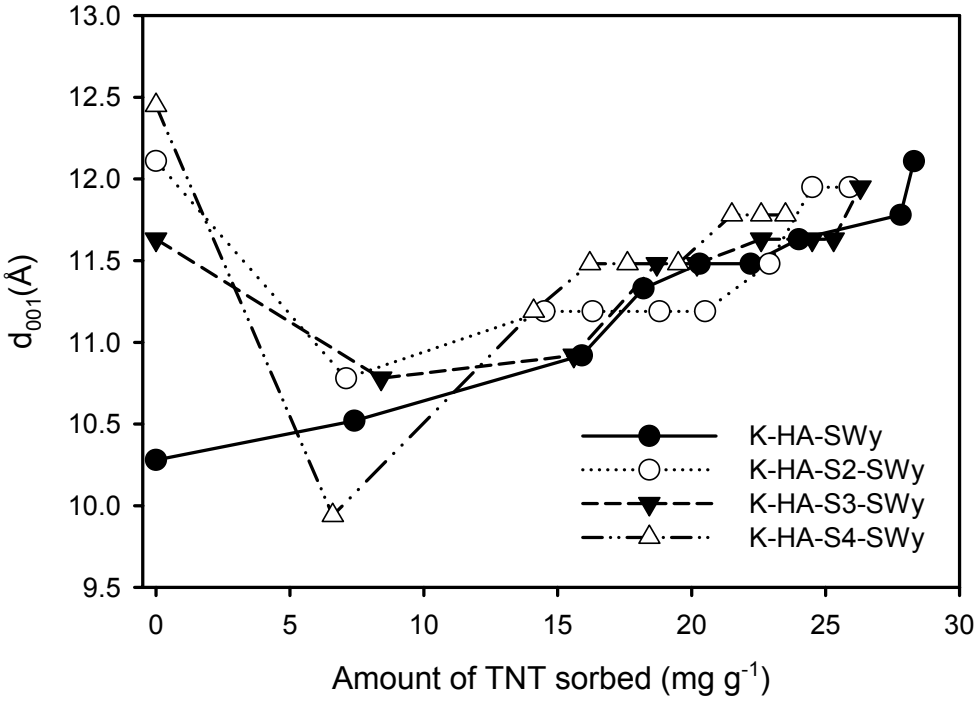


Figure 3.10. Associated *d*-spacings of sequentially prepared humic-clay complexes obtained from x-ray diffraction patterns of oriented films as a function of TNT sorption.

CHAPTER 4

IMPACT OF SIZE FRACTIONATION METHODS ON PROPERTIES OF SOIL AGGREGATES

Pillar, G.D., K. Xia, J. Kim, J. Reeves, and M. Williams. To be submitted to *Soil Biology and Biochemistry.*

Abstract

The effect of wet-sieving and dry-sieving methods on the characterization of soil properties and processes within soil aggregates was evaluated on three soils with contrasting clay mineralogy and organic matter content. The wet-sieving method yielded a greater distribution of soil aggregates less than 250 μm and a significant reduction in the 500 – 2000 μm sized aggregates for all three soils tested. The difference in aggregate size distribution contributed to differences in the physical composition of soil aggregates between the two sieving methods and, therefore resulted in differences in other soil properties. Although differences in total carbon within soil aggregates were not as profound, observed significant differences in the CEC, microbial biomass, and microbial community structure between the two sieving methods could be explained based on the clay mineralogy and dominate aggregate stabilization mechanism for each soil. Specifically, the sieving method used had the most significant impact on the 75 – 250 μm aggregate size fraction regardless of soil type. Our results suggest that wet-sieving is more disruptive than the dry-sieving method and, therefore studies which are designed to characterize microbially mediated functions of soil aggregates should use dry-sieving rather than wet-sieving techniques. Regardless of the analysis being conducted, the inherent clay mineralogy, soil organic matter content, and sand content of the soil should also be considered in the selection of the appropriate sieving technique and the interpretation and extrapolation of the results.

Keywords: soil aggregates; microbial biomass; microbial community structure; aggregate fractionation procedures

1. Introduction

Over the past several decades it has been shown that soils can be grouped into distinct aggregate fractions that often show meaningful relationships between ecological function and soil properties such as organic matter dynamics and C and N pool sizes (Tisdall and Oades 1982; Monrozier et al., 1991; Brady and Weil, 1999). Numerous studies and reviews have proposed that earthworms, micro-organisms, binding agents, and land management practices can influence various processes and properties at the aggregate level (Shipitalo and Protz, 1989; Dexter, 1988; Kay, 1990; Oades and Waters, 1991; Six et al., 1998; Six et al., 2000a). However, research focused on the characterization of soil aggregates and the processes associated with them have often produced conflicting results, possibly a consequence of different aggregate separation methods. For example, the rate of denitrification, amount of water soluble carbon, total carbon and overall weight distribution of soil aggregates are different when assessed using dry-sieving compared to wet-sieving procedures (Beauchamp and Seech, 1990; Puget et al., 1995; Angers and Giroux, 1996). As a result, some have suggested that the links between aggregate size and various soil properties and biological processes are merely an artifact of the soil fractionation procedure and not related to intrinsic properties specific to an aggregate size fraction (Ashman, et al., 2003).

Typically, the methods used to fractionate soil try to simulate disruptive events that could occur in the field (Young and Ritz, 2000). Wet-sieving is one of the more common fractionation methods which involves the rapid wetting of air-dry samples in water and is supposed to simulate conditions of severe water stress (Nimmo and Perkins, 2002), and thus provide information about the water-stable aggregates and their

associated properties/processes. There is also considerable interest in the characterization of soil aggregates, whether relatively stable or unstable, to better understand processes related to soil organic matter dynamics (Bossuyt et al., 2005), environmental fate of contaminants (Krauss and Wilcke, 2002; DeSutter et. al., 2003), and biological soil properties (Stemmer et. al., 1998). One of the more common concerns surrounding research on these processes is whether wet-sieving methods may create their own artifacts that may misrepresent the microbial community or the chemical properties of an aggregate fraction (Amato and Ladd, 1980; Ahmed and Oades, 1984; Miller and Dick, 1995). In contrast, dry sieving methods may provide a suitable alternative to overcoming the potential artifacts associated with wet-sieving. There is however limited information on how wet-sieving compares to dry sieving methods for the separation and collection of soil aggregates, nor information on how these methods might influence the apparent distribution of soil properties and their associated microbial communities.

The objective of this study was to examine how two different soil fractionation techniques, wet- and dry-sieving, may impact the characterization of soil aggregates of various sizes. Specifically, we focused on how the characterization of biological soil properties such as microbial biomass and soil community structure may be effected by the fractionation procedure used. We hypothesized that (1) wet-sieving would result in a decrease in total carbon and cation exchange capacity due to a decrease in macroaggregates relative to dry-sieving and that (2) wet-sieving would result in a decrease in the microbial biomass and significantly alter the microbial community structure as identified by fatty acid methyl-ester (FAME) analysis.

2. Methods and Materials

2.1 Sample collection and preparation

Soil samples were collected from three sites; a Theresa Silt loam (fine-loamy mixed mesic Typic Hapludalf) from New Berlin (Waukesha County), WI, an Appling sandy loam (fine kaolinitic thermic Typic Kanhapludult) from Bogart (Oconee County), GA, and a Marlette-Oshtemo Complex (mesic Typic hapludalf, Alto) from Freeport (Kent County), MI. Throughout the paper, the soils will be referred to as Theresa, Appling, and Marlette-Oshtemo, respectively. Each site was sampled within a 1-m^2 area by carefully loosening the upper 15 cm of soil with a square-bladed spade from below to preserve the soil's natural aggregation. The soil samples were stored at 4ºC until needed for sieving and analysis. General physical and chemical soil data are presented in Table 4.1. The water content of soil at each sampling site was 85%, 82%, and 81% of field capacity (1/3 bar) for the Theresa, Appling, and Marlette-Oshtemo soils, respectively.

2.2 Sieving procedures

To minimize the impact of storage or handling before sieving, all three soils were handled carefully and equally before the experiment. Soils were pre-sieved through a 4-mm mesh. Soil material larger than 4 mm, which consisted primarily of rock debris and plant material, were discarded. This pre-sieving of field-moist soil is considered less disruptive to the relatively 'unstable' aggregates (Denef, et al., 2002).

For the wet-sieving treatment, the Yoder procedure was followed using a mechanical sieving apparatus described by Kemper & Rosenau (1986). Field moist soil was placed on a nest of sieves in order to obtain four aggregate size fractions: 2000 –

4000, 500 – 2000, 250 – 500, and 75 – 250 μm. The nest of sieves was placed in a holder, suspended in a container of water, lowered to the point where the soil sample on the top screen was just covered with water, and allowed to sit for several minutes. The set of sieves were then set into an up and down motion whereby the soils were alternately immersed and removed from the water at a rate of 30 cycles per minute over a 10 min period.

Using the same nest of sieves the dry sieving procedure was conducted with the use of a mechanical shaker (Tyler Ro-Tap© RX-29, Boca Raton, FL, USA) Previous experiments showed no significant difference in the physical weight distribution of dry-sieved aggregates sieved for 3 min. and longer (up to 10 min.) 10 minutes. A 3 minute time was thus chosen to expedite the process of aggregate separation.

After sieving (wet- and dry-sieving), the soil aggregates were frozen and freeze-dried and kept at 4º C until analysis. Freeze drying as opposed to air or oven drying has been proposed as a better method to obtain dry aggregates because freeze drying maintains the intricate structural network which is more representative of field conditions (Chen & Schnitzer, 1989). Freezing and freeze-drying should also help to preserve the biological properties of the soils (Schutter and Dick, 2000).

2.3 Soil characterization

Characterization of physical and chemical soil properties was conducted using methods approved by the Soil Science Society of America (Madison, WI). Total C in the bulk soil and each freeze dried soil fraction was determined with a Shimadzu TOC 5050 total carbon analyzer (Columbia, MD, USA) fitted with an SSM5000A solid sample

module (Nelson and Sommers, 1996). Cation exchange capacity (CEC) was determined using the unbuffered salt extraction method (Sumner & Miller; 1996). Soil pH was measured using the 0.01 M $CaCl_2$ (2:1 ratio) saturated paste method (Thomas, 1996) Particle size distribution (PSD) was determined using the pipette method (Gee & Or, 2002).

2.4 Microbial community characterization

Fatty acid methyl-ester (FAME) extractions were conducted according to the method by Schutter and Dick (2000), with a few modifications. Briefly, three grams of soil from each aggregate size fraction and un-sieved soil were placed in a 40 mL scintillation vial. Fifteen milliliters of 0.2 M KOH dissolved in methanol were added to each sample and the vials were then sonicated for 1 h in a sonicating bath. Three mL of 1 M acetic acid was added in order to neutralize the KOH and the samples were mixed using a vortex for 1 min. Ten milliliters of hexane was added and mixed on a vortex for 1 min. Five milliliters of deionized water was added to each sample to allow for better phase separation of hexane from water. The samples were mixed a third time for 1 min on a vortex. The vials were then placed in a centrifuge at 2500 rpm for 15 min. Five milliliters of the hexane layer was removed using a pipette and placed in a 7-mL disposable glass tube. The samples were dried to complete dryness under a gentle stream of nitrogen gas. The remaining residue was then re-dissolved in 0.5 mL of hexane and placed in a 2-mL GC vial for analysis of FAME on a gas chromatograph-mass spectrometer (GC-MS).

The concentrated hexane extracts were analyzed for FAME on a GC/MS (ThermoFinnigan Polaris Q/Trace GC) using electron impact ionization. An INNOWAX capillary column (30 m, 0.25I.D., 0.25 μm film) from J&W Scientific (Palo Alto, CA, USA) was used. The inlet and transfer line temperatures were maintained at 250º C. A splitless injector was used with an injection volume of 1 μL. Helium was used as the carrier gas at a constant flow rate of 1.2 mL min^{-1}. The initial temperature of each run began at 120º C followed by a 5º C min^{-1} ramp up to 250º C. The temperature was maintained at 250º C for 1 min, then ramped at 10º C min^{-1} to a final temperature of 260º C which was maintained for 3 min. The temperature of the MS ion source was kept at 200º C. Data were collected using a full scan mode with a mass range between 40 to 600 m/z. Fatty acid methyl esters were identified by comparison with 37 components FAME (47885-U) and bacterial acid methyl-ester (BAME, 47080-U) standards purchased from Supelco (Bellefonte, PA, USA).

Fatty acid methyl esters (FAME) are described by standard nomenclature (IUPAC-IUB, 1977). The fatty acids are written as (A:BωC), where "A" refers to the total number of carbon atoms, "B" refers to the number of unsaturated bonds, and "ωC" indicates the number of carbon atoms between the aliphatic end of the molecule and the first unsaturated bond. Methyl branching is designated by the appropriate number from the aliphatic end as a prefix (e.g. 10Me18:0). Cyclopropane fatty acids have the prefix "cy", and finally the prefixes *i* and *a* for *iso* and *anteiso* methyl branching, respectively. Two fatty acids (18:1w7 and 10Me18:0) merged into one peak and is denoted as 18:1+.

2.5 Calculations – sand correction

Typically, the contribution of sand in the formation of soil aggregates and to chemical and biological soil properties is considered to be minimal and passive (Marquez., et al. 2004). The inclusion of free sand or redistributed sand from unstable aggregates can lead to a dilution effect of some properties (e.g. carbon content) in some aggregate size fractions, particularly microaggregates (<250 μm). Since all three soils have a considerable amount of sand (Table 4.1) we concluded that correction for the aggregate-sized sand content would foster a more robust comparison that is more useful for answering our questions regarding effects of sieving methods. All data presented are on a sand-free basis unless otherwise noted.

The percentage of sand-free aggregates (SFA) within each size fraction in a 200 g soil sample was calculated as:

$$SFA_i = \frac{i^{th}\ size\ \ fraction\ (g) - sand_i\ (g)}{200\ g\ -\ sand_t\ (g)}\ x\ 100 \qquad [1]$$

where i = 1 to 4, representing the four aggregate size fractions (4000 – 2000, 2000 – 500, 500 – 250, and 250 to 75 μm) and $sand_t$ represents the total sand in the 200 g soil sample. The percentage of SFA smaller than 75 μm ($SFA_{< 75\ \mu m}$) was calculated as:

$$SFA_{< 75 \mu m} = 100 - \sum SFA_i \qquad [2]$$

where i = 1 to 4, representing the four aggregate size fractions described in Eq. (1), which are subtracted from 100%. Sand-free determinations of total carbon (TC), cation exchange capacity (CEC), and total FAME (fatty acid methyl esters) were calculated as:

$$TC_i\ mg\ kg^{-1} = \frac{TC\ (mg)}{i^{th}\ \ size\ \ fraction\ (kg) - sand_i\ (kg)} \qquad [3]$$

$$CEC_i \ cmol_C \ kg^{-1} = \frac{exchangeable \ cations \ (cmol_C)}{i^{th} \ size \ fraction \ (kg) - sand_i \ (kg)} \quad [4]$$

$$Total \ FAME_i \ nmol \ g^{-1} = \frac{\sum_{k=1}^{18} FAME \ (nmol)}{i^{th} \ size \ fraction \ (g) - sand_i \ (g)} \quad [5]$$

where i = 1 to 4, representing the four aggregate size fractions described in Eq. (1), k = 1 to 18, representing the eighteen different fatty acid methyl esters which can be related to soil bacteria, fungi, actinomycetes and protozoa.

2.6 Statistical analysis

Differences in soil properties between wet- and dry-sieving techniques were evaluated using ANOVA with the GLM procedure of SAS (Statistical Analysis Systems Institute Inc., Cary, NC). Location (WI, GA, and MI), treatment (wet and dry), and particle size were considered the explanatory variables. To satisfy the assumption of constant variance, TC and CEC data were log-transformed. The Tukey multiple comparison method was used within SAS to determine differences between sieving methods for each soil and soil size fraction. Significance for all data, unless otherwise noted, are reported at the p = 0.01 level.

All multivariate and nonparametric data analysis was conducted using PC-ORD v. 4.0 (McCune and Mefford, 1999). Nonmetric multidimensional scaling (NMS) based on Sorensen's distance was used to graphically represent FAME profile relationships. NMS was first performed on combined FAME data of all three sampling locations. Because there were large differences between the three soil types, the NMS analysis was re-run on each individual soil type to better highlight the differences associated with sieving

method and aggregate particle size (see results). The FAME mole percentage data underwent a general relativization before analysis in PC-ORD. Based on an initial Monte Carlo test, the NMS was constrained to two axes using the "slow-and-thorough" autopilot mode of NMS in PC-ORD. The autopilot mode uses the best of 40 runs with a random starting configuration using the real data and 50 runs using randomized data for a Monte Carlo test of significance. Adequate stability of the results was qualified for each run by examining plots of stress versus the number of iterations.

A multi-response permutation procedure (MRPP) using Euclidean distance was used to determine for differences in the mole percentages of FAMEs between the three soil types and two sieving methods. MRPP is another nonparametric procedure which can be used to test group differences and has the advantage of not requiring distributional assumptions such as multivariate normality and homogeneity of variances which are seldom met with ecological community data (McCune and Grace, 2002). 'Indicator species' analysis was conducted to determine which FAMEs were important descriptors of the sieving method and to further support NMS and MRPP analysis.

3. Results and Discussion

3.1 Aggregate size distribution and particle size analysis of aggregate size fractions

There have been numerous attempts to explain the biological activity and structure of physically fractionated soil aggregates (Monrozier et al., 1991; Puget et al., 2000; Six et al., 2000; Bossuyt et al., 2001; Denef et al., 2002), but there have been far fewer studies that have tried to decipher the meaningfulness of biological-soil physical fractionation associations by comparing results from two types of fractionation methods

in three different soils. In confirmation of a variety of other studies, our results demonstrated that the fractionation method had a clear affect on the aggregate size distribution (Beauchamp and Seech, 1990; Ashman et al., 2003; Limon-Ortega et al. 2006). Results from Figure 4.1 suggest that sieving method has a large impact on aggregate size distribution, with wet sieving resulting in a greater mass of soil in the smaller size fractions compared to dry sieving. For example, dry-sieving resulted in 91 to 97% of the sand-free weight distributed into an aggregate fraction that is larger than 250 μm in all three soils. Wet-sieving in contrast, resulted in a more even distribution of soil into all aggregate sizes for all three soils with 46 to 74% of the sand-free soil weight collected as macroaggregates greater than 250 μm (Figure. 4.1). The differences between the weight of aggregates collected in wet compared to dry sieving is most striking in the Marlette-Oshtemo soil. Dry-sieving resulted in 96% while wet-sieving resulted in 46% of the sand-free soil weight collected as aggregates larger than 250 μm (Figure 4.1).

Similar to previous work, the differences observed in the distribution of aggregates between sieving methods were related to aggregate fractions containing inherently different properties (Beauchamp and Seech, 1990; Angers and Giroux, 1996; Ashman et al., 2003). For instance, particle size analysis revealed numerous changes in the percent of sand (coarse and fine) and clay for aggregate size fractions, and a shift in aggregate size distribution (Table 4.2, Figure 4.1). These changes are most likely a result of the disintegration of soil aggregates or the slaking of soil material from sand particles during wet-sieving. The removal of soil material (silt, clay, fine sand) will result in an enrichment of sand, which is very likely to be "loose" sand and not associated with soil aggregates (Cambardella and Elliott, 1993). However, fine sand, which is more likely to

be within and a part of soil aggregates could be released during wet-sieving and redistributed to a smaller size fractions (Elliott et al., 1991). The accumulation of fine-sand in the < 250 μm size fractions would "dilute" the total carbon and microbial biomass measurements possibly leading to a conclusion that wet-sieving had a negative impact on total carbon and microbial biomass. This may be particularly relevant in sandy soils and supports the observed difference in the aggregate size distribution between methods in the Marlette-Oshtemo complex. These factors are especially important to consider when comparing soil aggregate data obtained by using two different sieving methods or comparing the impact of different treatments on properties/process of soil aggregates.

The redistribution of soil material, whether sand or silt and clay from macroaggregates was also evident in the three soils as indicated by the increase in the proportion of micro-aggregates (< 250 μm) obtained by wet- compared to dry-sieving (Figure 4.1). Regardless of soil type, there was considerably less aggregate mass in the two fractions associated with the 250 – 2000 μm size class in wet- compared to dry-sieving. Compared to the larger size fraction (2000-4000 um), the 500 – 2000 μm size fraction was unstable to the effects of wet-sieving, resulting in the re-distribution of soil into smaller aggregate size fractions when compared to dry-sieving. Indeed, it has been previously reported that aggregates larger macroaggregates (i.e. > than 2000 μm) may be more stable than the smaller macroaggregate (i.e. 250 – 2000 μm) classes because of the binding capacity associated with young organic matter (Puget et al., 1995 Angers and Giroux, 1996; Six et al, 2004). However, it is not known if the accumulation of aggregates in the 500-2000 μm size fraction of the dry sieved compared to the wet-sieved soil is a consequence of the more disruptive wet-sieving forces or an artifact of high sand

content clogging the sieve and preventing smaller aggregates from passing through during dry-sieving. In any event, the aggregate size distribution differed considerably between the sieving methods regardless of the sand content of the soil.

Some of the largest effects were concentrated at the smaller aggregate sizes, and in most cases the change in the physical distribution of the aggregates were mirrored by changes in both the chemical and biological properties of the soils. However, our results build upon these previous studies by directly linking the changes associated with the separation methodology with the prevailing physical, chemical, and biological properties of the separated fractions (Ahmed & Oades, 1984; Beauchamp & Seech, 1990; Angers and Giroux, 1996).

3.2 Total carbon and cation exchange capacity

Contrary to other studies, total carbon on a sand-free basis did not differ within soil aggregates between the two sieving methods for any of the soils (Puget et al., 1995; Ashman et al., 2003). These apparent inconsistencies may be a consequence of comparing sieving methods with sand-free rather than whole soil data. On a whole soil basis wet-sieving resulted in a significant decrease ($p < 0.05$) in total carbon in aggregates < 500 μm and an increase in aggregates > 500 μm compared to dry sieving (Appendix B, Table B.1). In addition, contrary to other studies where aggregate hierarchy was evident (Six et al., 2000c) and both dry- and wet-sieving was used to obtain soil aggregates (Puget et al., 1995) we did not detect any significant difference in total carbon between the aggregate size fractions for either sieving method for the Theresa or Marlette-Oshtemo soils.

However, in the Appling soil there were strong differences in the distribution of total carbon among dry- and wet-sieved soil aggregates, respectively (Table 4.3). This result in the Appling but not the Theresa or Marlette-Oshtemo soil may be due inherent differences in clay mineralogy. In Ultisols such as the Appling, oxides and 1:1 clay minerals rather than humic-clay complexes are the dominant stabilizing agent and weak organic matter – clay interactions may allow for the slaking or fractionation of organic matter (Oades and Waters, 1991; Six et al., 2000b; Denef et al., 2002). While Theresa or Marlette-Oshtemo soils mainly contain 2:1 clay minerals, resulting in stronger organic matter-clay complexes.

Although wet-sieving resulted in a significant amount of soil to pass through the < 75 μm, a sub sample from this size fraction was analyzed for total carbon (data not shown). A mass balance of the total carbon within soil aggregates compared with the whole soil measurements was conducted to determine if wet-sieving resulted in a significant loss of water soluble carbon. Mass balance calculations resulted in 94 – 110% recovery from all three soils, indicating most of the carbon was accounted for within the soil aggregates rather than lost in the water used for wet-sieving. However, our results do not support or negate the possibility of a significant loss in water soluble C as a result of wet-sieving.

Cation exchange capacity (CEC) was largely different as a result of sieving method (Table 4.3) among all size fractions. For example, in the Theresa soil, dry-sieving resulted in CEC values which were 20 and 35% lower than wet-sieving for aggregates collected in the 250 and 75 μm size fraction, respectively. In the Marlette-Oshtemo soil the sieving method had a similar effect on CEC values for the same aggregate size classes

as the Theresa soil. In contrast, an opposite trend was observed in the Appling soil which had a 36% reduction in CEC for the 500 μm size fraction compared to dry-sieving. In addition to the differences in CEC between the two sieving methods for a given soil type and size fraction, there were differences in the range of CEC values among aggregate size fractions. Some of the larger differences between the CEC of aggregate size fractions were found associated with the Appling soil, irrespective of sieve method. For the Theresa and Mareltte-Oshtemo soils in contrast, the differences in CEC between aggregate size fractions were confined to the wet-sieving treatment.

While dry sieving tended to have a relatively small influence on the CEC of the aggregate fractions, wet-sieving showed dramatic changes, with CEC's being largest in the smallest size fractions. The decrease in sand content, particularly fine sand (Table 4.2) was greater than the decrease in clay or organic matter and that the loss of sand resulted in a concentrating effect with respect to the silt and clay in the smaller soil aggregates of the wet-sieved soils. This shift in CEC in wet- compared to dry-sieved soils follows the same patterns observed with total carbon and is likely a result of the loss of total carbon in the 500 – 2000 μm size fraction and accumulation in the 75 – 250 μm size fraction. Overall, we suspect that the impact of sieving method on total carbon and CEC was first a consequence of differences in the distribution of aggregate sizes, but also possibly related to the inherent stability of those aggregates that is related to the soil's clay mineralogy and organic-clay complexes.

3.3 Impact of sieving method on soil microbial biomass and community structure as indicated by FAME profiles

The total amount of extractable fatty acids obtained as phospholipid fatty acids (PLFAs), has been used as an estimate of the viable microbial biomass (White et al., 1996). However, in this study the measurement of FAMEs rather than PLFAs was used to characterize the microbial community structure. Thus, caution should be used in the estimate of microbial biomass since FAMEs may include fatty acids from humic substances and plants (White et al., 1996; Zelles, 1999; Schutter and Dick, 2000). Figure 4.2 shows that differences in the total FAMEs are not large, except for the 75 - 250 μm size fraction. In the Theresa soil there was no significant difference in the total FAME content between the two sieving methods (Figure 4.2). In the Appling and Marlette-Oshtemo soils in contrast, there was a 45.1% and a 52.8% decrease, respectively, in total FAME content associated with the 75 - 250 μm size fraction as a result of dry- compared to wet-sieving (Figure 4.2). We hypothesize that differences in the soil biological biomass and microbial community structure between soil aggregates collected from different sieving methods was primarily a result of differences in aggregate size distribution, and not a consequence of losses of microbial community biomass, as is often concluded by other researchers (Amato and Ladd, 1980; Ahmed and Oades, 1984; Chiu et al., 2006). The apparent differences in FAME content found in our work, when comparing the two sieving methods can be accounted for by re-distribution of sand sized particles or organic matter, and not a true loss in the community biomass. For example, the increase in the total amount of extractable fatty acids in the wet-sieved 75-250 μm fraction of the Appling soil coincides with the change in the distribution of total carbon. As discussed above, this shift in the distribution of total carbon within wet-sieved aggregates may be due to the removal of organic matter surrounding sand particles and its

redistribution to the < 250 μm size fraction and thus, may have resulted in a concentration (increase) in microbial biomass. This idea would support the crack hypothesis which Ashman et al. (2003) proposed as an alternative to the aggregate hierarchy model. In this scenario, microaggregates which line pore walls and are enriched in carbon, and microbial biomass, would become dislodged during fractionation and re-deposited in a smaller size fraction, leaving behind an aggregate core or sand particle with lower biological activity.

While inherent properties of soils explained the majority of the variability, there were clear effects exerted by both sieve method and aggregate size that contributed to the variability in structural characteristics of the microbial community (Figure 4.3). An NMS analysis of all three soils clearly showed that the Theresa, Appling, and Marlette-Oshtemo FAME profiles were significantly different from each other (Figure 4.3). An MRPP analysis further indicated significant differences in the distribution of mol % FAME between the three soil types ($p < 0.001$). While indicator species analysis between the soils suggested that almost all of the FAME were important for defining differences, differences as a result of sieving method ($p < 0.05$) were more dependent on specific FAMEs such as the biomarkers for gram positive bacteria (i15:0, i16:0, i17:0, a17:0), actinomycetes (10Me17:0) and fungi (18:2ω6,9).

In many cases there was no clear trend, other than general differences, in how the sieve method influenced the distribution of the microbial communities associated with different aggregate size classes. It is interesting to note however, that microbial community structure of the wet-sieved 75-250 μm size fraction of the Theresa soil was very similar to the dry-sieved 500-2000 and 2000-4000 μm size fractions (Figure 4.3). In

the same light as that mentioned in the previous paragraph, this could be a consequence of the microbial community associated with the larger size fraction have been redistributed into smaller size fractions. Differences between the sieving methods can be identified in the NMS analysis of the soils shown in Figure 4.3. But to more clearly demonstrate the impact of sieving method on the soil microbial community structure both NMS and MRPP analysis were conducted on each soil type separately (Figure 4.4). The separation of clusters indicates the microbial community structure is distinctively different between the two sieving methods. In the all three soils there is a greater degree of separation of data points between wet- and dry-sieving in the fractions < 250 μm compared to the clusters of those > 250 μm. These groups were separated primarily by axis 1 in the Marlette-Oshtemo soil and by both axis 1 and axis 2 in the Appling and Theresa soils. Interestingly, in the Appling and Theresa soils the separation between treatments for the 250-500 μm size fractions was minimal. This is in line with the changes in the aggregate size distribution as this size fraction had smallest reduction as a result of wet-sieving compared to the 500 – 2000 and 2000 – 4000 μm size fractions (Figure 4.1). This may be due to greater aggregate stability and protection of soil organic matter. In summary, the differences among the soils are greater than the effects of sieve method, but sieve method had an important, though not uniform effect on the microbial community.

3.4 Impact of sieving method on the relative abundance of individual FAME biomarkers.

Analysis of individual FAMEs also provided some interesting insights regarding how wet-sieving and dry sieving impact the soil microbial community within different

soil types and soil aggregate size fractions. The results of this study further fuel concern that the choice of method for aggregate separation will determine the relationship of the microbial-aggregate associations. For example, an interesting trend in the Theresa soil was that the increase in gram positive biomarkers i15:0, a15:0, and i17:0 in dry-sieved 75 – 250 μm size fractions corresponded to a decrease of the same biomarkers in the dry-sieved 500 – 2000 μm size fraction (Table 4.4). This further supports the redistribution of soil material from larger to smaller soil aggregates previously mentioned and the similar location of the wet-sieved 75 – 250 μm and dry-sieved 500 – 2000 μm data points on the NMS plot for the Theresa soil (Figure 4.3). Additionally, far fewer differences in mol% FAMEs were observed between sieving methods in the larger size fractions. In particular, no differences were observed between sieving methods among all three soils in 250 – 500 μm size fraction. This follows the NMS analysis where the separation between treatments for this size fraction was minimal (Figure 4.4). Thus, for this particular size fraction sieving method appears to have little if any impact on the microbial community structure.

The mol% distribution of FAMEs shows clearly that certain biomarkers are more important for describing differences between sieving methods (Table 4.4). For example, in all soils, the bulk of the differences we measured could be found in the 75-250 μm size fraction with a preponderance of these differences explainable by an increase in the abundance of gram-positive type biomarkers in dry compared to wet-sieving. In contrast, fungal biomarkers (18:1ω9 and/or 18:2ω6,9) were more abundant in wet- than dry-sieved 75 – 250 μm size fractions. In this context, considerable interest has been focused on examining and explaining the non-random distributions of microbial communities within

soil microsites and associated with soil aggregates (Menendes and Bottomley, 1998; Guggenberger et al.,1999; Vaisanen et al., 2005). More work is certainly needed to explain which microbial community members are associated with particular soil components, why these associations occur, and how they affect the heterogeneous distribution of soil processes, such as microbial activity and N mineralization (Menendes and Bottomley, 1998; Schutter and Dick, 2002). However, our work clearly shows that the choice of method can have a strong influence on microbial-aggregate associations, and until these discrepancies can be explained, conclusions from studies seeking to understand the role of microbial communities in aggregate processes will be limited. This line of thinking is substantiated, for example, by the high abundance of the fungal biomarkers in wet-sieved micoaggregates of the Appling and Marlette-Oshtemo soils that may be a direct result of slaking and disintegration of macroaggregates. This observation follows the statements made by Chiu et al. (2006) that wet sieving might destroy fungal mycelium within soil aggregates, however, this contrasts with the findings of others that assert a strong link between soil fungi and the formation and stabilization of macroaggregates (Tisdall, 1994; Guggenberger et al., 1998).

4. Conclusion

In light of the physical force imparted on soil it is not surprising that dry and wet sieving methods provide different outcomes and interpretations of the aggregate associated biological and chemical properties. This result does not necessarily imply that the search for aggregate-microbial associations in soil is fruitless, however. In instances where inherent aggregate stability is the key question of an experiment, the tendency for

aggregates to break down into smaller sized fractions, as with sieving, might provide consistent data. On the other hand, when researchers intend to study the natural associations found within aggregate hierarchies, or need to extract biological or chemical information that is sensitive to the effects of sieving, then careful consideration of the likelihood of artifacts should be used to select the best aggregate separation method.

References

Ahmed, M., and J.M. Oades, 1984. Distribution of organic matter and adenosine triphosphate after fractionation of soils by physical procedures. Soil Bio. Biochem. 16: 465-470.

Amato, M., and J.N. Ladd, 1980. Studies of nitrogen mineralization and immobilization in calcareous soil. V. Formation and distribution of isotope-labeled biomass during decomposition of ^{14}C and ^{15}N-labelled plant material. Soil Bio. Biochem. 12: 405-411.

Angers, D.A., and M. Giroux, 1996. Recently deposited organic matter in soil water-stable aggregates. Soil Sci. Soc. of Am. J. 60: 1547-1551.

Ashman, M.R., P.D. Hallett, and P.C. Brookes, 2003. Are the links between soil aggregate size class, soil organic matter and respiration rate artifacts of the fractionation procedure? Soil Bio. Biochem. 35: 435-444.

Beauchamp, E.G., and A.G. Seech, 1990. Denitrification with different sizes of soil aggregates obtained from dry-sieving and from sieving with water. Bio. Fert. of Soil 10: 188-193.

Bossuyt, H., K. Denef, J. Six, S.D. Frey, R. Merckx, and K. Paustian. 2001. Influence of microbial populations and residue quality on aggregate stability. Appl. Soil Ecol. 16:195-208.

Bossuyt, H., J. Six, and P.F. Hendrix, 2005. Protection of soil carbon by microaggregates within earthworm casts. Soil Bio. Biochem. 37: 251-258.

Brady, N.C., and R.R.Weil, 1999. The nature and properties of soils. 12th Ed. Prentice-Hall, New Jersey.

Cambardella, C.A., and E.T. Elliott, 1993. Carbon and nitrogen distribution in aggregates from cultivated and native grassland soils. Soil Sci. Soc. Am. J. 57: 1071-1076.

Chen, Y., and M. Schnitzer, 1989. Sizes and shapes of humic substances by electron microscopy p. 622-638 *In* Humic substances: In search of structure. M.B.H. Hayes et al. (ed.) Wiley & Sons, New York, N.Y.

Chiu, C.Y., T.H. Chen, K. Imberger, and G. Tian, 2006. Particle size fractionation of fungal and bacterial biomass in subalpine grassland and forest soils. Geoderma 130: 265-271.

Denef, K., J. Six, R. Merckx, and K. Paustian, 2002. Short-term effects of biological and physical forces on aggregate formation in soils with different clay mineralogy. Plant Soil 246: 185-200.

DeSutter, T.M., S.A. Clay, and D.E. Clay, 2003. Atrazine sorption and desorption as affected by aggregate size, particle size, and soil type. Weed Sci. 51: 456-462.

Dexter, A.R., 1988. Advances in characterization of soil structure. Soil Tillage Res. 11: 199-238.

Elliot, E.T., C.A. Palm, D.E. Reuss, and C.A. Monz, 1991. Organic matter contained in soil aggregates from a tropical chronosequence. Agriculture, Ecosystems & Environment 34: 443-451.

Elliott, E.T. 1986. Aggregate structure and carbon, nitrogen, and phosphorus in native and cultivated soils. Soil Sci. Soc. Am. J. 50: 627-633.

Foster, R.C., 1988. Microenvironments of soil microorganisms. Bio. Fert.Soils 6: 189-203.

Gee, G.W., and D. Or., 2002. Particle-size analysis. p. 255-294. *In.* Methods of soil analysis. Part 4. Physical methods. Dane, J.H., Topp, G.C. (eds) ASA and SSSA, Madison, WI.

Guggenberger, G., E.T. Elliott, S.D. Frey, J. Six, and K. Paustian. 1998. Microbial contributions to the aggregation of a cultivated grassland soil amended with starch. Soil Bio. Biochem. 31: 407-419.

Kay, B.D., 1990. Rates of change of soil structure under different cropping systems. Adv. Soil Sci. 12: 1-52.

Kemper, W.D., and R.C. Rosenau, 1986. Aggregate stability and size distribution. p. 425-442. *In*. Methods of soil analysis. Part 1. Physical and mineralogical methods. Klute, A. (ed.) 2nd ed. ASA and SSSA, Madison, WI.

Krauss, M., and W. Wilcke. 2002. Sorption strength of persistent organic pollutants in particle-size fractions of urban soils. Soil Sci. Soc. Am. J. 66: 430-437.

Limon-Ortega, A., B. Govaerts, J. Deckers, and K.D. Sayre. 2006. Soil aggregate and microbial biomass in a permanent bed wheat-maize planting system after 12 years. Field Crops Res. 97: 302-309.

Marquez, C.O., V.J. Garcia, C.A. Cambardella, R.C. Schultz, and T.M. Isenhart, 2004. Aggregate-size stability distribution and soil stability. Soil Sci. Soc. Am. J. 68: 725-735.

McCune, B., and M.J. Mefford, 1999. PC-ORD Multivariate analysis of ecological data. Version 4.0 MjM Software, Gleneden Beach, Oregon, USA.

McCune, B., and J.B. Grace, 2002. Analysis of ecological communities. MjM Software Design, Gleneden Beach, Oregon, USA.

Mendes, I.C., and P.J. Bottomley. 1998. Distribution of a population of *Rhizobium leguminosarum* bv.*trifolii* among different size classes of soil aggregates. Appl. Environ. Microbiol. 64: 970-975.

Miller, M., and R.P. Dick, 1995. Dynamics of soil C and microbial biomass in whole soil and aggregates in two cropping systems. Appl. Soil Ecol. 2: 253-261.

Monrozier, L.J., J. N. Ladd, A.W. Fitzpatrick, R.C. Foster, and M. Raupach, 1991. Components and microbial biomass content of size fractions in soils of contrasting aggregation. Geoderma 49: 37-62.

Nelson, D.W., and L.E. Sommers, 1996. Total carbon, organic carbon, and organic matter. p. 961-1010. *In.* Methods of soil analysis. Part 3. Chemical Methods. Sparks, D.L. (ed). SSSA Book Ser. No. 5. SSSA, Madison, WI.

Nimmo, J.R., and K.S. Perkins, 2002. Aggregate stability and size distribution p. 317-328, *In* Methods of soil analysis. Part 4 Physical methods. J.H. Dane and G. Clarke (eds.) SSSA Book Ser No. 5. SSSA, Madison, WI.

Oades, J.M., 1984. Soil organic matter and structural stability: Mechanisms and implications for management. Plant Soil 76: 319-337.

Oades, J.M., and A.G. Waters, 1991. Aggregate hierarchy in soils. Aust. J. Soil Res. 29: 815-828.

Puget, P., C. Chenu, and J. Balesdent, 1995. Total and young organic matter distributions in aggregates of silty cultivated soils. Eur. J. Soil Sci. 46: 449-459.

Puget, P., C. Chenu, and J. Balesdent, 2000. Dynamics of soil organic matter associated with particle-size fractions of water-stable aggregates. Eur. J. Soil Sci. 51: 595-605.

Schutter, M.E.,and R. P. Dick, 2000. Comparison of fatty acid methyl ester (FAME) methods for characterizing microbial communities. Soil Sci. Soc. Am. J. 64: 1659-1668.

Shields, J.A., E.A. Paul, W.E. Lowe, and D. Parkinson, 1973. Turnover of microbial tissue in soil under field conditions. Soil Bio. Biochem. 5: 753-764.

Shipitalo, M.J., and R. Protz, 1989. Chemistry and micromorphology of aggregation in earthworm casts. Geoderma 45: 357-374.

Six, J., E.T. Elliott, K. Pautsian, and J.W. Doran. 1998. Aggregation and soil organic

matter accumulation in cultivated and native grassland soils. Soil Sci. Soc. Am. J. 62: 1367-1377.

Six, J., E.T. Elliot, and K. Paustian, 2000a. Soil macroaggregate turnover and microaggregate formation: A mechanism for C sequestration under no-tillage agriculture. Soil Bio.Biochem. 32: 2099-2103.

Six, J., E.T. Elliott, and K. Paustian, 2000b. Soil structure and soil organic matter. II. A normalized stability index and the effect of mineralogy. Soil Sci. Soc. Am. J. 64: 1042-1049.

Six, J., K. Paustian, E.T. Elliott, and C. Combrink, 2000c. Soil structure and soil organic matter. I. Distribution of aggregate-size classes and aggregate-associated carbon Soil Sci. Soc. Am. J. 64: 681-689.

Six, J., H. Bossuyt, S. Degryze, and K. Denef. 2004. A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. Soil Tillage Res. 79: 7-31.

Stemmer, M., M.H. Gerzabek, and E. Kandeler, 1998. Organic matter and enzyme activity in particle-size fractions of soils obtained after low-energy sonication. Soil Bio. Biochem. 30: 9-17.

Sumner, M.E., and W.P. Miller. 1996. Cation exchange capacity and exchange coefficients. p. 1201-1230. *In.* Methods of soil analysis. Part 3. Chemical methods. Sparks, D.L. (ed) SSSA Book Ser. No. 5. SSSA, Madison, WI.

Thomas, G. 1996. Soil pH and soil acidity. p. 475-490. *In.* Methods of soil analysis. Part 3. Chemical methods. Sparks, D.L. (ed) SSSA Book Ser. No. 5. SSSA, Madison, WI.

Tisdall, J.M., and J.M. Oades, 1982. Organic matter and water-stable aggregates in soils. J. Soil Sci. 62, 141-163.

Tisdall, J.M. 1994. Possible role of soil microorganisms in aggregation in soils. J. Soil Sci. 33: 141-163.

Vaisanen, R.K., M.S. Roberts, J.L. Garland, S.D. Frey, and L.A. Dawson. 2005. Physiological and molecular characterization of microbial communities associated with different water-stable aggregate size classes. Soil Bio Biochem. 37: 2007-2016.

Young, I.M., and K. Ritz, 2000. Tillage, habitat space and function of soil microbes. Soil Tillage Res. 53: 201-213.

Table 4.1. Physical and chemical characteristics of soils used in this study. Numbers within parentheses reflect the standard deviation (n = 3)

Soil	TC	CEC	pH	Particle Size Analsyis				
				Coarse Sand	Fine Sand	Total Sand	Total Clay	Total Silt
	mg C g^{-1}	$cmol_c$ kg^{-1}		----- % of total soil----				
Theresa	28.8 (3.2)	19.1 (0.1)	6.5	16.1	23.9	40.0	15.1	45.0
Appling	12.5 (1.1)	5.6 (0.2)	4.2	45.7	20.8	66.5	14.8	18.7
Marlette-Oshtemo	11.4 (1.2)	13.7 (1.0)	6.2	38.2	41.8	80.0	4.9	15.2

Table 4.2. Particle size analysis of soil aggregates collected from dry- and wet-sieving techniques (n = 1).

Soil	Aggregate Diameter μm	Sieving Method	Partical Size Analysis: Coarse Sand	Fine Sand	Total Sand	Total Silt	Total Clay
			----- % of total soil----				
Theresa	4000-2000	Dry	15.2	18.7	33.9	51.7	14.4
		Wet	16.3	17.3	33.5	52.4	14.1
	2000-500	Dry	20.2	14.5	34.6	51.3	14
		Wet	20.1	12.7	32.8	52.6	14.6
	500-250	Dry	20.1	22.5	42.5	45.2	12.2
		Wet	36.7	15.0	51.7	37.3	11
	250-75	Dry	1.7	34.0	35.7	50.1	14.2
		Wet	2.4	51.3	53.6	35.5	10.9
Appling	4000-2000	Dry	33.2	24.6	57.8	19.3	22.9
		Wet	47.9	17.0	64.9	18.2	17
	2000-500	Dry	47.6	23.3	70.9	14.7	14.4
		Wet	58.2	12.6	70.8	14.4	14.8
	500-250	Dry	31.8	32.8	64.6	14.3	21.1
		Wet	53.1	21.7	74.8	13.8	11.4
	250-75	Dry	0.6	62.9	63.5	13.9	22.5
		Wet	1.6	75.0	76.7	14.2	9.1
Marlette-Oshtemo	4000-2000	Dry	32.4	41.4	73.8	19.8	6.4
		Wet	38.0	37.7	75.7	18.5	5.8
	2000-500	Dry	40.3	39.9	80.2	15.4	4.4
		Wet	51.1	31.8	82.9	12.6	4.5
	500-250	Dry	28.6	51.8	80.5	14.7	4.9
		Wet	53.3	36.2	89.5	6.9	3.6
	250-75	Dry	0.7	80.7	81.5	13.7	4.8
		Wet	1.4	89.7	91.1	6.0	2.9

Table 4.3. Cation exchange capacity (CEC) and concentration of total carbon (TC) in soil aggregates collected from dry- and wet-sieving methods as determined on a sand-free basis[†].

Soil	Diameter mm	Dry-sieved aggregates		Wet-sieved aggregates	
		CEC $cmol_c\ kg^{-1}$	TC mg C g-1 soil	CEC $cmol_c\ kg^{-1}$	TC mg C g^{-1} soil
Theresa	4000-2000	27.6 (1.9)	44.6 (4.3)	32.8 (1.5) **A**	49.8 (6.6)
	2000-500	28.5 (2.6)	47.3 (1.5)	36.1 (1.6) **A**	57.6 (4.7)
	500-250	31.6 (3.2) **a**	49.9 (3.5)	39.7 (2.8) **b** **A**	49.0 (3.6)
	250-75	29.2 (0.9) **a**	48.7 (1.9)	44.9 (2.8) **b** **B**	57.1 (4.1)
Appling	4000-2000	12.8 (0.5) **A**	27.0 (3.2) **A**	11.2 (1.3) **A**	28.0 (3.3) **A**
	2000-500	20.0 (2.7) **a** **B**	45.1 (6.0) **B**	12.8 (1.1) **b** **AB**	37.3 (1.6) **A**
	500-250	15.2 (0.2) **A**	38.0 (1.3) **AB**	11.9 (0.5) **AB**	31.6 (4.7) **A**
	250-75	14.9 (0.5) **A**	39.2 (0.4) **B**	15.9 (0.4) **B**	49.3 (3.4) **B**
Marlette-Oshtemo	4000-2000	53.0 (5.6)	75.5 (17.7)	73.7 (3.6) **A**	85.1 (36.2)
	2000-500	67.0 (4.2)	65.0 (4.9)	92.9 (8.1) **b** **A**	102.1 (20.9)
	500-250	68.9 (7.9) **a**	47.6 (8.1)	144.0 (3.2) **b** **B**	56.9 (3.3)
	250-75	68.0 (3.6) **a**	45.5 (3.1)	165.0 (18.0) **b** **B**	68.8 (11.9)

[†] Standard deviations are given in parentheses. Small case letters indicate significant differences between sieving methods for the specific aggregate size fraction and soil type. Large case letters indicate significant differences between aggregate size fractions for a specific soil type (p-value ≤ 0.01, $n = 3$).

Table 4.4. Comparison of dry- and wet-sieving methods on the mole percentage of 18 microbial biomarkers in three distinct soils[†].

		Theresa Size Fraction (μm)				Appling Size Fraction (μm)				Marlette Oshtemo Size Fraction (μm)			
Biomarker Interpretation	**Fatty Acid**	**4000-2000**	**2000-500**	**500-250**	**250-75**	**4000-2000**	**2000-500**	**500-250**	**250-75**	**4000-2000**	**2000-500**	**500-250**	**250-75**
Gram +	i15:0		○***		●***		●*		●***				●***
Gram +	a15:0		○***		●***		●*		●**				
Gram +	i16:0				●***				●***				●***
Common to all	16:0				○***								
Gram -	16:1ω9	●***			●***								
Actinomycetes	10Me16:0				●**				●*				●***
Gram -	16:1ω5												
Gram +	i17:0		○*		●*	●*	●*		●***				●***
Gram +	a17:0								●***				●*
Actinomycetes	10Me17:0	●*							●***	●***	●*		●***
Common to all	18:0				○***					○*			
Fungi	18:1ω9						○*		○***				○***
Gram - / Actinomycetes	18:1ω7+ 10Me18:0	●*			●***								
Fungi	18:2ω6,9				○*								○***
Gram -	cy19:0		●*										
	20:00		○*										●***
Protozoa	20:4ω6								●*				
	22:00												●**

[†] Significant differences between dry- and wet-sieving methods are denoted by solid or open circles (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Solid or open circles indicate that the mole percentage for the particular fatty acid was significantly greater in dry-sieved aggregates or wet-sieved aggregates respectively ($n = 3$).

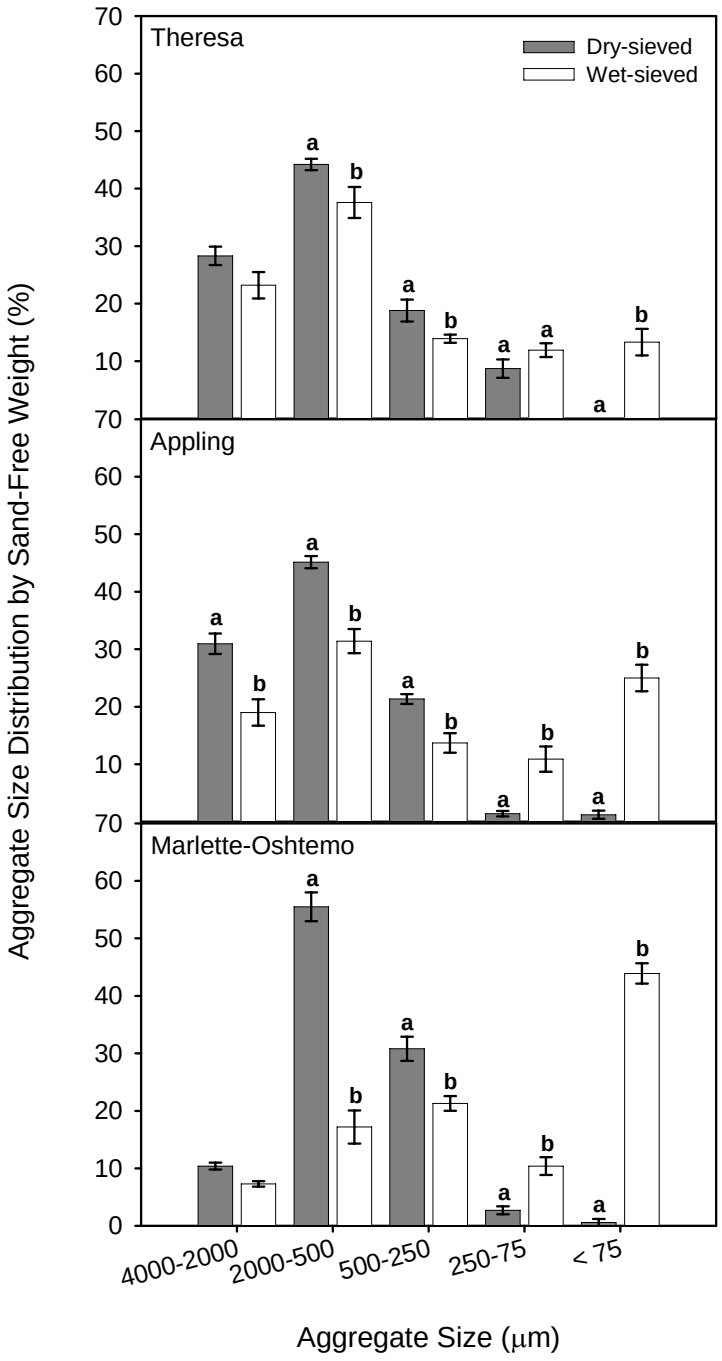


Figure 4.1. The amount of soil within each aggregate size fraction as a percentage of total sand-free soil (top-Theresa, middle-Appling, bottom-Marlette-Oshtemo). Significant differences between sieving methods are represented by different letters on the graph ($p < 0.01$, mean ± 1 S.D.)

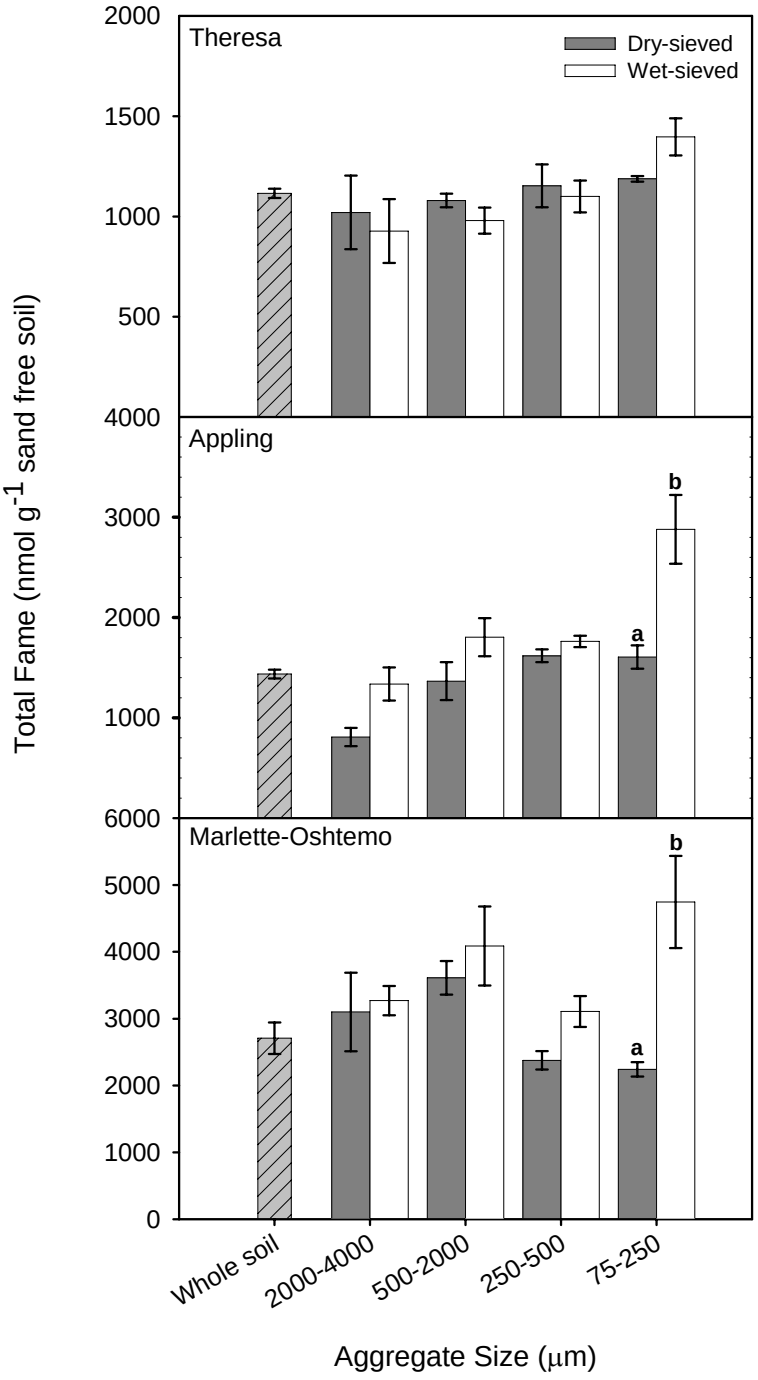


Figure 4.2. Total fatty acid methyl esters (FAME) within each aggregate size fraction obtained by using dry- and wet-sieving techniques (top-Theresa, middle-Appling, bottom-Marlette-Oshtemo). Significant differences between sieving methods are represented by different letters (p-value ≤ 0.01, mean $\pm$ 1 S.D.).

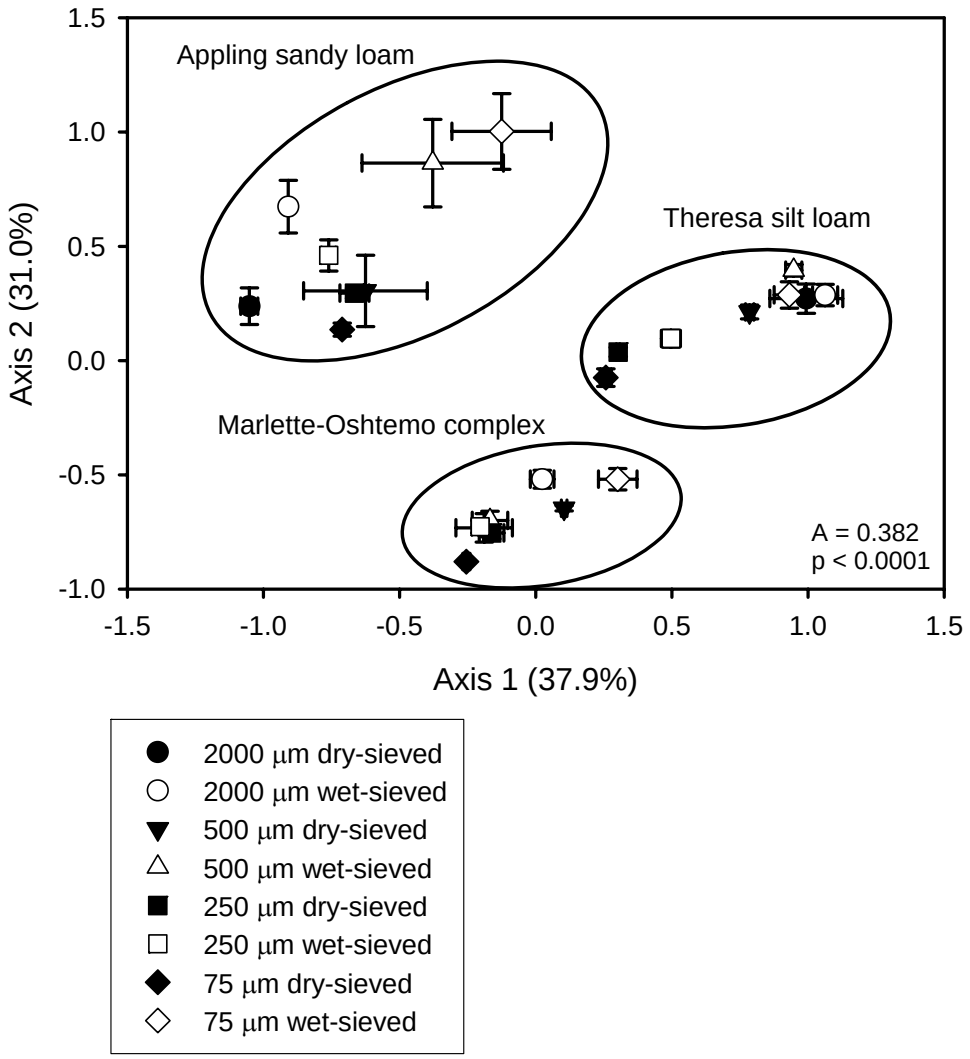


Figure 4.3. Nonmetric multidimensional scaling plots of mole percentages of FAMEs of all aggregate size fractions for both sieving methods and all three soils. The proportion of variance explained by each axis is indicated in parentheses. Ellipses enclose and identify the Theresa, Appling, and Marlette-Oshtemo soils. Multi-response permutation procedure (MRPP) analysis indicated that the three soils were significantly different from each other. Values of the symbols and error bars represent means and standard error, respectively (n = 3).

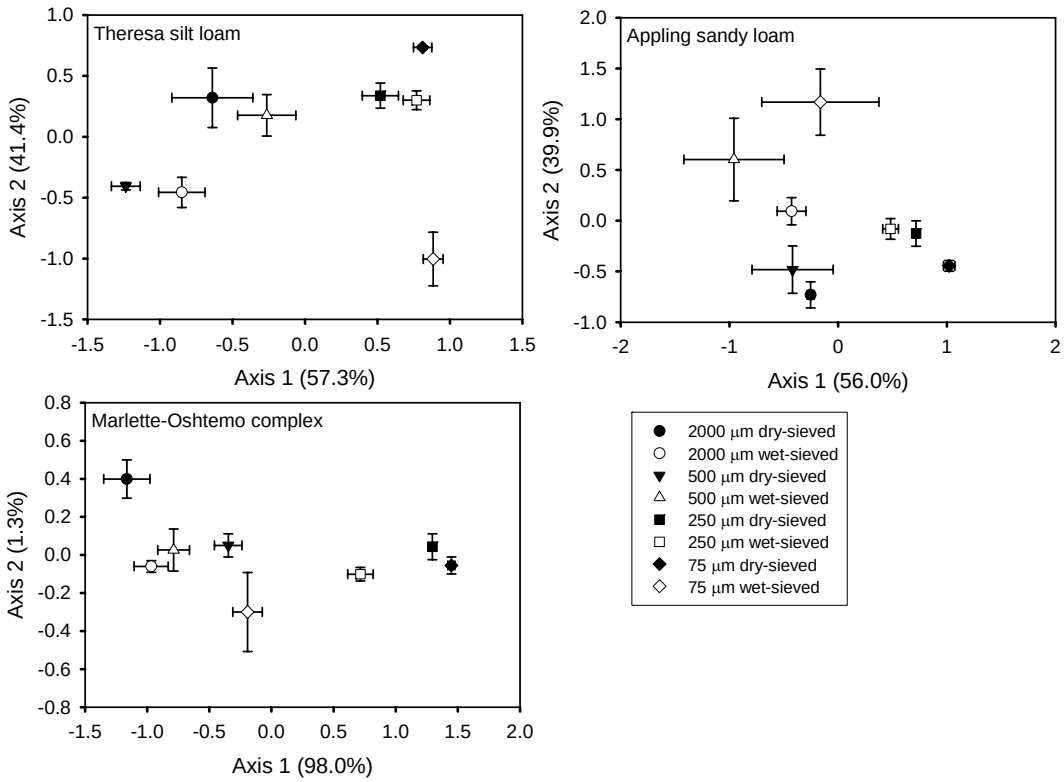


Figure 4.4. Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs of dry- and wet-sieved Theresa, Appling, and Marlette-Oshtemo soils. The proportion of variance explained by each axis is indicated in parentheses. A one dimensional solution explained the bulk of the variance in the Marlette-Oshtemo soil, but for the sake of clarity a two dimension solution is presented. Multi-response permutation procedure (MRPP) analysis indicated that the sieving method was significantly different from each other within each soil type. Values of the symbols and error bars represent means and standard error, respectively (n = 3).

CHAPTER 5

FATE OF 2,4,6-TRINITROTOLUENE (TNT) AND MICROBIAL COMMUNITY STRUCTURE WITHIN SOIL AGGREGATES IN THE PRESENCE OF EARTHWORMS

Pillar, G.D., K. Xia, and P.F. Hendrix. To be submitted to *Environmental Toxicology and Chemistry.*

Abstract

We conducted laboratory studies to determine the ability of the earthworm *Eisenia fetida* to facilitate the transformation of 2,4,6-trinitrotoluene (TNT) in soil and soil aggregates of different size. Soil microcosms consisting of soil with different organic matter contents (2.9 and 1.3 mg kg^{-1}) and clay mineralogy were prepared with and without TNT and earthworms. Over the course of the 21-d study earthworms in the sandy loam soil exposed to TNT accumulated more metabolites such as 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) and had a greater reduction in biomass than those in the silt loam soil. After 21 d 15.5 – 19.6% of the initial (≈ 100 mg kg^{-1}) TNT was recovered from both soils. TNT bioavailability was much less in the sandy loam soil throughout the study as ≈ 10% of the total extractable TNT at 21 d was removed from the whole soil and soil aggregates with a weak salt solution representing readily available TNT. Earthworm activity resulted in an increase in the amount of extractable metabolites 2-ADNT and 4-ADNT after 14 d in both soils despite no impact on the disappearance of TNT in the silt loam soil after 21 d. The presence of earthworms and TNT individually and together had a significant impact on the microbial community structure as determined by fatty acid methyl ester (FAME) analysis. Specifically, individual fatty acids indicative of gram-negative and gram-positive bacteria, actinomycetes, and fungi were impacted. Examination of soil aggregates revealed that earthworms in both soils had the greatest impact on the microbial community structure, TNT disappearance, TNT transformation, and TNT and metabolite distribution in 2000 – 4000 μm soil aggregates. These results provide

evidence of the potential for earthworms to directly and indirectly influence the fate of TNT, 2-ADNT, and 4-ADNT in soil aggregates of different soil systems.

Keywords: TNT, earthworms, *Eisenia fetida*, biotransformation, 2-ADNT, 4-ADNT, metabolites, soil aggregates

1. Introduction

The nitroaromatic explosive 2,4,6-trinitrotoluene (TNT) is a major component of military munitions used throughout the world. Soil and water contamination of TNT and its related transformation products have become a significant concern, particularly at facilities used to manufacture, store, and dispose of munitions (Jerger and Woodhull, 2000; Rodgers and Brunce, 2001). The U.S. Department of Defense has determined that more than 1000 sites have significant contamination by explosives (Rodgers and Brunce, 2001). In addition, > 95% of those sites contained TNT and > 87% exceeded permissible contaminant levels and as a result many of these sites have been placed on the National Priorities List for Superfund cleanup (Talmage et al., 1999). For example, 34,600 and 87,000 mg TNT kg^{-1} were reported in soils from the Joliet Army Ammunition Plant (JAAP, Joliet Illinois) and the Umatilla Munitions Depot Activity (UMDA, Umatilla, Oregon), respectively (Pennington et al. 1995; Simini et al., 1995). These values greatly exceed the EPA risk-based concentrations of 95 and 21 mg kg^{-1} for industrial and residential areas, respectively (USEPA, 2006).

TNT has been characterized as a fairly persistent compound in the environment and has previously been listed by the USEPA as a priority pollutant (Keith and Telliard, 1979; Kaplan and Kaplan, 1982; Vasilyeva et al., 2001). Recently, numerous studies and literature reviews have been conducted examining the factors that influence the environmental fate of TNT (Pennington and Patrick, 1990; Comfort et al., 1995; McGrath, 1995; Haderlein et al., 1996; Pennington and Brannon, 2002). Complete or even partial mineralization of TNT is unlikely due to the difficulty involved in breaking the aromatic structure. Rather, TNT is known to readily undergo reductive

transformation producing nitroso, azoxy, and hydroxylamine intermediates resulting in the formation of aminodinitrotoluenes such as 4-amino-2,6-dinitrotoluene (4ADNT) and 2-amino-4,6-dinitrotoluene (2ADNT) and diaminonitrotoluenes such as 2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene (Thorne and Leggett, 1997; Pennington and Brannon, 2002). In addition, in rare situations involving highly reducing conditions TNT can be transformed into 2,4,6-triaminotoluene (TAT)(Hawari et al., 1998). Although numerous studies have reported high rates of TNT disappearance in soil systems, the formation of transformation products do not completely account for the observed disappearance (Comfort et al., 1995; Pennington et al., 1995). Numerous studies involving the use of radio-labeled TNT compounds and mass balance calculations have shown that with time there is an increase in unextractable TNT and related metabolites due to strong binding to soil components such as organic matter and clay minerals (Comfort et al. 1995; Hundal et al., 1997; Achtnich et al., 1999). This contradicts earlier reports indicating that TNT sorption to soil was readily reversible (Pennington and Patrick, 1990).

Since a significant portion of TNT contamination occurs in soil, particularly near the surface there is avid concern about its possible toxicity to soil-dwelling organisms (Gong, et al., 1999; Renoux et al., 2000; Lachance et al., 2004; Wilke et al., 2004). TNT has been shown to have a negative impact at concentrations as low as 1 mg kg^{-1} on the growth and survival of bacteria, fungi, and actinomycetes in soil (Klausmeier et al., 1973; Bayman and Radkar, 1997; Fuller and Manning, 1998; Gong et al., 1999). TNT contamination has also resulted in adverse effects on microbial processes with a 50% reduction in potential denitrification (PNA) and dehydrogenase activity (DHA) at

concentrations of 50 mg kg^{-1}, and 10 mg kg^{-1} (Gong et al. 1999). In addition to the reported toxicity to soil microorganisms studies have also demonstrated the toxicity of TNT to soil invertebrates and soil fauna (Parmelee et al., 1993; Robidoux et al., 1999; Dodard et al., 2003; Lachance et al., 2004; Schaefer, 2004). However, the levels at which TNT is toxic depends on soil properties such as clay and organic matter content. For example, the reported lethality (LC_{50}) of *Eisenia andrei* in an OECD artificial soil (70% sand, 20% clay, 10% sphagnum peat) and a sandy forest soil (92% sand, 3% clay, 4.2% O.M.) exposed to TNT for 14 d was 365 and 143 mg kg^{-1}, respectively (Renoux et al. 2000; Robidoux et al. 2000). Similarly, the lethality of TNT to another earthworm species *Eisenia fetida* in a forest soil (5.9% O.M.) was reported to be 325 mg kg^{-1} (Phillips et al. 1993).

While the various enzyme systems involved in the biotransformation of TNT may vary among different species, the basic metabolic activity of various organisms supports the hypothesis that earthworms could influence the biotic transformation of TNT (Corbett and Corbett, 1995; Renoux et al., 2000). Micro- and macro-organisms are capable of influencing TNT bioavailability by promoting or inhibiting degradation and transformation processes within soil (Vorbeck et al., 1998; Renoux et al., 2000; Esteve-Nunez et al., 2001; Lewis et al., 2004). However, information on the ability of earthworms to influence the distribution and translocation of TNT and metabolites in soils is lacking (Renoux et al., 2000). Earthworms are known to play a significant role in the formation and incorporation of organic materials into soil aggregates (Lee and Foster, 1991; Martin, 1991; Bossuyt et al., 2005). In addition, earthworms are known to influence soil microbial community dynamics and, therefore, may have additional

indirect impacts on the fate of TNT (Lee, 1985; Postma-Blaauw et al., 2006). Thus, the physical activity of earthworms could have a profound influence on the distribution, transport, and transformation of TNT within the soil.

The overall objective of this study was to determine to what extent earthworms may influence the fate of TNT in soil and within soil aggregates, and in turn, determine how TNT and earthworms may influence the microbial community structure. Experiments were conducted using soil microcosms consisting of two different soils of contrasting soil texture and organic matter content. *Eisenia fetida* was used to characterize the interaction between earthworms and TNT in the soil and within soil aggregates. Finally, the microbial community structure was assessed by measuring fatty acid methyl esters (FAMEs) as indicators of specific microbial populations.

2. Methods and Materials

2.1 Chemicals and equipment

Granular TNT (CAS 118-96-7: MW 227.15 g mol^{-1}) used for spiking soil was purchased from Holston Army Ammunition Plant (Kingsport, TN USA). Analytical standards for TNT, 2-ADNT (CAS 35572-78-2; MW 197.15 g mol^{-1}), 4-ADNT (CAS 19406-51-0; MW 197.15 g mol^{-1}) were obtained from Accustandard (New Haven, CT, USA). The purity of these reagents was ≥ 99%. Acetonitrile (HPLC grade) and calcium chloride ($CaCl_2$) were obtained from Fisher Scientific (Pittsburgh, PA USA). Glassware was washed with phosphate-free detergent, rinsed with methanol, acid washed, and rinsed with deionized water before use.

2.2 Soil preparation

The TNT-uncontaminated soils were collected from the A horizon of a Theresa silt loam (fine-loamy mixed mesic Typic Hapludalf) from New Berlin (Waukesha County), WI, and an Appling sandy loam (fine kaolinitic thermic Typic Kanhapludult) from Bogart (Oconee County), GA. The two soils were chosen for the differences in clay mineralogy, soil texture, and soil organic matter content (Table 5.1).

Soils were spiked with TNT at a target concentration of 100 mg TNT kg^{-1} following a modified procedure described in Renoux et al. (2000). To minimize impact of the solvent on the microbial community, 30 g of air dried soil was mixed with TNT dissolved in 10 mL of acetonitrile, left in the dark under a chemical hood for at least 3 hr to permit acetonitrile evaporation, thoroughly mixed with approximately 270 g of additional soil, and then hydrated to 70% of their water holding capacity (10.3% and 14.7% based on v/w for the Appling soil and Theresa soil, respectively). The prepared TNT-spiked soil was immediately used for the earthworm incubation experiments described in Section 2.3. Controls were established by preparing the soil with the same procedures using 10 mL acetonitrile without TNT.

2.3 TNT degradation and distribution in the presence of earthworm

Earthworms (*Eisenia fetida*) were obtained from a local bait shop (Marion & Buck's Bait & Tackle, Athens, GA) and were maintained at the time of purchase in a newspaper/organic soil mixture. Adult *E. fetida* used for the study had a well-developed clitellum and a wet mass ranging from 250 to 600 mg. Earthworms were placed into non-contaminated soil for a 72-h acclimation period. Six earthworms were then placed

into 1-L glass jars (i.e., test unit), filled with 300 g of the soil (dry weight) prepared following procedures described in Section 2.2, and hydrated back to their 70% of field capacity. Test units were prepared in triplicate for each of four treatments over four time periods. Treatments included soil – earthworm – TNT (- Ew – TNT), soil + earthworm – TNT (+ Ew – TNT), soil – earthworm + TNT (- Ew + TNT), and soil + earthworm + TNT (+ Ew + TNT). The test units were then incubated at 25 ºC in darkness for 0, 7, 14, and 21 d. Soil moisture was monitored on a daily basis by weighing and water was added to maintain the moisture content of the soil. Daily water loss was less than 2.4 mL for both soils over the course of the experiment. For the Appling and Theresa soils, the day 0 treatment corresponded to an exposure time of less than 10 min for the earthworms. After each incubation time period, each glass container was carefully emptied. Surviving earthworms were counted and rinsed with deionized water. The soil particles in their guts were then purged by placing the earthworms from each replicate on a separate moistened (with 5 mL of deionized water) paper towel for 24 h. After 24 h, purged earthworms were counted again, rinsed with deionized water, carefully dried on tissue paper, weighed, and placed into Teflon tubes, and immediately frozen at -80 ºC. Soil from each replicate was placed on a nest of sieves in order to obtain three soil aggregate size fractions: 2000-4000, 500-2000, and 250-500. In the Theresa soil a fourth size fraction was collected and analyzed (75-250 μm). The sieves were then placed on a Tyler Ro-Tap © RX-29 (Boca Raton, FL, USA) mechanical shaker and sieved for 3 min. After sieving, soil aggregates and whole soil samples which were taken before sieving were stored at 5 ºC for less than 5 d before extraction and analysis for TNT and its metabolites and characterization of soil microbial community

2.4 Extraction and measurement of TNT and its metabolites from soil and earthworm tissue.

Extractions of total TNT, 2ADNT, and 4ADNT were performed with modifications to EPA Method 8330 by using a sequential extraction with 0.0033 M $CaCl_2$ followed by acetonitrile. The extraction of TNT and its metabolites using a weak salt solution has been noted as a measure of the readily extractable fraction (also considered bioavailable) and the extraction by a solvent such as acetonitrile has been used as a measure of the potentially extractable fraction (Hundal et al., 1997). The sum of target compound in both extractions was defined as the total extractable. All extractants were filtered through a 0.45-um membrane (Altech) before HPLC analysis for TNT and its metabolites.

The following procedures were used for extraction of TNT and its metabolites in earthworms: frozen whole earthworms were freeze dried and then homogenized in a small amount of liquid nitrogen using a pestle and mortar. The amount of homogenized material was then weighed and mixed with 5 mL of acetonitrile in 50 mL Teflon plastic tubes. The samples were then placed in a sonication bath for 3 h, placed on a shaker for 24 h. A 1.5 mL aliquot was centrifuged for 30 min at 13,000 g to allow for separation of the solvent from the biological tissues. A 0.75 ml aliquot of supernatant was treated with 0.75 mL of 0.0033 M $CaCl_2$ to precipitate any fine particles. Samples were then filtered through a 0.45-um membrane (Altech) for subsequent HPLC analysis.

The concentrations of TNT and its metabolites in filtered soil and earthworm extractants were determined on a ThermoFinnigan HPLC system (Woburn, MA USA)

using a J'sphere ODS-H80 reversed-phase column (5μm, 150 by 4.6 mm i.d.) (YMC Co. Ltd., Tokyo, Japan). The mobile phase was 82:18 (v:v) water:2-propanol with a flow rate of 1.5 mL min^{-1}, a 10 μL injection volume and a column oven temperature of 40° C. A standard curve was run every 30 samples, with one standard rechecked every 10 samples. The lowest concentration of aqueous TNT at which reproducible results were obtained on the HPLC was 75 μg L^{-1}. The precision of the HPLC method was between 0.5 to 5% RSD.

2.5 Microbial Community Characterization

Fatty acid methyl-esters (FAMEs) extractions were conducted according to the method by Schutter and Dick (2000), with a few modifications. Briefly, three grams of soil were placed in a 40-mL scintillation vial. An aliquot of 15 mL 0.2 M KOH dissolved in methanol was added to each vial, and then sonicated for 1 h in a sonicating bath. After the sonication, 3 mL of 1 M acetic acid was added into each vial in order to neutralize the KOH and mixed on a vortex for 1 min. Ten mL of hexane was added into each vial and mixed on a vortex for 1 min. Five milliliters of deionized water was added to each vial and mixed for 1 min on a vortex to allow for better phase separation of hexane from water. After centrifugation at 2500 rpm for 15 min., 5 mL of the hexane layer was removed from each vial using a pipette and placed in a 7 mL disposable glass tube. Each hexane extract was dried to complete dryness under a gentle stream of nitrogen gas. The remaining residue was then re-dissolved in 0.5 mL of hexane and transferred into a 2 mL glass GC vial for analysis of FAMEs on a gas chromatograph-mass spectrometer (GC-MS, ThermoFinnigan Polaris Q/Trace GC) using electron impact ionization. An

INNOWAX capillary column (30 m, 0.25I.D., 0.25 μm film) from J&W Scientific (Palo Alto, CA, USA) was used. The inlet and transfer line temperatures were maintained at 250º C. A splitless injector was used with an injection volume of 1 μL. Helium was used as the carrier gas at a constant flow rate of 1.2 mL min^{-1}. The initial oven temperature of each run began at 120º C, hold for 3 min, followed by a 5º C min^{-1} ramp up to 250º C. The temperature was maintained at 250º C for 1 min and then increased at 10º C min^{-1} to a final temperature of 260º C which was maintained for 3 min. The temperature of the MS ion source was kept at 200º C. Data was collected using a full scan mode with a mass range between 40 to 600 m/z. Fatty acid methyl esters were identified by comparison with 37 components FAME (47885-U) and bacterial acid methyl-ester (BAME, 47080-U) standards purchased from Supelco (Bellefonte, PA, USA).

2.6 Statistical analysis

Differences between treatments in the aggregate size distribution, amount of TNT, amount of metabolites, and individual FAME biomarkers were tested for significance using analysis of variance with soil type, aggregate size fraction, treatment, and time as main effects (ANOVA). Because there were numerous two-way interaction effects involving soil type and time, analyses were run separately for each soil and time period. The Fischer's protected LSD comparison method was used to determine differences between means at $p < 0.05$. All statistics were performed using SPSS 13.0 for Windows.

All multivariate and nonparametric data analysis was conducted using PC-ORD v. 4.0 (McCune and Mefford, 1999). Nonmetric multidimensional scaling (NMS) based on Sorensen's distance was used to graphically represent FAMEs profile relationships. The

NMS was first performed on combined FAMEs data from both soils and all time periods used in this study. Because there were large differences between the two soil types and between the four time periods, the NMS analysis was re-run on each individual soil type and time period to better highlight the differences associated between the four treatments. The FAME mole percentage data underwent a general relativization before analysis in PC-ORD. Based on an initial Monte Carlo test, the NMS was constrained to two axes using the "slow-and-thorough" autopilot mode of NMS in PC-ORD. The autopilot mode uses the best of 40 runs with a random starting configuration using the real data and 50 runs using randomized data for a Monte Carlo test of significance. Adequate stability of the results was qualified for each run by examining plots of stress versus the number of iterations.

A multi-response permutation procedure (MRPP) using Euclidean distance was used to determine for differences in the mole percentages of FAMEs between the four time periods and four treatments. The MRPP is another nonparametric procedure which can be used to test group differences and has the advantage of not requiring distributional assumptions such as multivariate normality and homogeneity of variances which are seldom met with ecological community data (McCune and Grace, 2002). 'Indicator species' analysis was conducted to determine which FAMEs were important descriptors of each treatment and to further support NMS and MRPP analysis.

3. Results and Discussion

3.1 TNT transformation within earthworms and its impact on earthworm activity.

The use of salt or solvent extractions to measure TNT, 2-ADNT, and 4-ADNT is considered an indirect measurement of chemical bioavailability to earthworms whereas the bioaccumulation of these chemicals by earthworms is a direct measure of bioavailability (National Research Council, 2003; Lanno et al., 2004). TNT was not detected in the extracts of earthworms exposed to TNT in either the Appling or Theresa soil at any time period, including 0 d. However, the transformation products 2-ADNT and 4-ADNT were detected in earthworms exposed to TNT in both soils over the entire course of the study (Figure 5.1A,C). Earthworms exposed to TNT in the Appling soil reached a peak accumulation of 2-ADNT and 4-ADNT at 7 d at concentrations of 184.7 and 231.2 $\mu g\ g^{-1}$ dry tissue, respectively. Renoux et al. (2000) and Lachance et al. (2004) reported similar concentrations of 2-ADNT (23 – 107 $\mu g\ g^{-1}$ dry tissue) and 4-ADNT (79 – 180 $\mu g\ g^{-1}$ dry tissue) in the tissue of *Eisenia andrei* in a sandy forest soil exposed to similar initial concentrations of TNT (90-97 mg TNT kg^{-1}) over 7 and 14 d, respectively. Additionally, earthworms in the Appling soil exposed to TNT for < 10 min (0 day) contained 10.3 and 18.2 $\mu g\ g^{-1}$ dry tissue of 2-ADNT and 4-ADNT, respectively (Figure 5. 5.1A,C). Renoux et al. (2000) reported a similar observation with earthworms exposed to TNT for < 5 min. The measurement of metabolites in the tissue of earthworms that were in contact with TNT-spiked soil for such short of time suggests that dermal contact is a significant bioaccumulation mechanism. However, it is not known if TNT transformation occurred via earthworm absorption or if transformation occurred in the soil and the earthworms quickly absorbed 2-ADNT and 4-ADNT. Previous toxicity

studies where earthworms were exposed to filter paper contaminated with TNT suggest that TNT is transformed during absorption by the earthworm. (Robidoux et al., 1999; Renoux et al., 2000). Along with dermal absorption, ingestion is a possible mechanism for transformation of TNT within earthworms and in fact, the authors noted that transfer through the earthworm tissue is only one possible mechanism for TNT transport and transformation in earthworms.

Earthworms from the Appling soil exposed to TNT contained greater amounts of both metabolites compared to those found in the Theresa soil suggesting that TNT and/or the metabolites were more bioavailable in the Appling soil. In addition the decrease in tissue concentrations of both metabolites after 7 d in both soils may be due to several reasons. First, it is possible that the metabolites could be further transformed and/or altered within the earthworm to compounds not extractable or detected by HPLC analysis. Second, the reduction in metabolites after 7 d along with the leveling off of biomass reduction (i.e. in the Appling soil) may be due an unknown detoxification process where earthworms are capable of removing the compound from their system. Finally, the reduction in TNT and metabolite bioavailability with time may have resulted in a reduction in earthworm absorption/transformation after 7 d. It is possible that a combination of these explanations are occurring, however, further investigation using tracers (i.e. C^{14}) or molecular analysis would be needed to further support these findings.

Although the amount of TNT the earthworms were exposed to was not lethal, TNT did have sublethal effects as shown by the loss in biomass, particularly in the Appling soil (Figure 5.2B,D). However, it is not known if these observations were a result of exposure to TNT, 2-ADNT, or 4-ADNT. Lachance et al. (2004) reported the

toxicity of these compounds to *Eisenia Andrei* increased in the order of 2-ADNT < TNT < 4-ADNT. Thus, the significant accumulation of 4-ADNT by earthworms at 7 d in the Appling soil may be partly responsible for the dramatic decrease in earthworm biomass observed over the first 7 d of the experiment (Figure 5.2).

3.2 Impact of earthworm activity and TNT on soil aggregation.

Earthworms are known to promote soil aggregation and the formation of stable macroaggregates. Bossuyt et al. (2005) reported an increase in macroaggregates > 2000 μm due to the presence of the endogeic earthworm *A. caliginosa*. Similarly, other researchers have noted that the presence of earthworms has a positive impact on the formation of water-stable macroaggregates (Martin and Marinissen, 1993; Scullion and Malik, 2000). Thus another potential measurement of the impact of TNT exposure on earthworm activity is to monitor the aggregate size distribution in soil exposed to earthworms and TNT. If earthworm activity (i.e. movement, digestive processes, etc) is impacted by TNT exposure, the formation of macroaggregates may be reduced compared to a soil without TNT, witch in turn may affect the fate and distribution of TNT in soil aggregates. The aggregate size distribution of both soils after 21 d is presented in Figure 5.2 (7 – 14 d presented in Appendix C, Figures C.1, C.2).

Surprisingly, in the Appling soil there was no significant difference in the aggregate size distribution between the treatment with earthworms but no TNT (+Ew – TNT) and the treatment with earthworms and TNT (+Ew +TNT). This infers that although the earthworm biomass dropped significantly due to TNT exposure their activity compared to earthworms in a soil without TNT was similar. The overall lack of

difference in the aggregate size distribution between treatments in the Appling soil may be due in part to unfavorable growth conditions regardless of the presence of TNT. The organic matter content of Appling soil is 12.5 mg C g^{-1}, which is not enough to sustain long term growth of epigenic earthworms such as *Eisenia fetida* (Coleman et al., 2004). Figure 5.1 shows that in the Appling soil after 21 d there was a greater reduction in earthworm biomass in the soil without TNT (+ Ew – TNT) compared to the same treatment in the Theresa soil that has more than twice as much organic matter (28.8 mg C g^{-1}) as the Appling soil.

In the Theresa soil containing earthworms and TNT there was significantly less 2000 – 4000 μm macroaggregates compared to the soil with earthworms and no TNT. Earthworms are known to promote the formation of water-stable macroaggregates and the observed reduction in 2000 – 4000 μm macroaggregates suggests that the incorporation of TNT in the soil may have affected soil aggregation processes *before* and/or *after* ingestion by earthworms. For example, the application of organic compounds such as borate, periodate, and pyrophosphate to soil aggregates can reduce their stability and increase the potential for dispersion when exposed to water (Shipitalo and Protz, 1989). Thus, soil aggregation may have been negatively impacted before earthworm influence. Research has also shown that the incorporation of organic residues (i.e. alfalfa, plant material) in soil via earthworm activity leads to the formation of stable soil aggregates (Zhang and Hendrix, 1995; Bossuyt et al., 2004). In addition, earthworms are known to selectively consume soil particles and organic material of a specific size range (Piearce, 1978; Lowe and Butt, 2004). During the ingestion of soil material by earthworms, chemical bonds between soil particles are broken, and clay particles are

brought into close contact with decomposing, mucilage-coated, organic material which promotes formation and strengthening of soil aggregates (Shipitalo and Protz, 1989; Zhang and Schrader, 1993). However, the type and extent of bonding that occurs between mineral and organic materials during ingestion or after excretion in earthworm casts greatly depends on the properties of the ingested organic debris and chemical properties of the soil materials (Shipitalo and Protz, 1989). Therefore, the incorporation and adsorption of TNT by soil organic matter and clay minerals could have weakened the bonding between organic materials and clay minerals in the excreted earthworm casts, thus leading to a reduction in stable macroaggregates. To test if this theory is true, further research focused on the aggregate stability of earthworm casts and soil aggregates exposed to TNT or other contaminants is needed.

3.3 TNT distribution and disappearance in soil and soil aggregates.

The transformation and bioavailability of TNT in soil have been well characterized by numerous researchers (Comfort et al., 1995; Hundal et al., 1997; Sheremata et al., 1999; Price et al., 2000; Esteve-Nunez et al., 2001). However, little is known regarding the dynamics of TNT fate within soil aggregates. In the present study, TNT disappearance over 21 d in all the whole soil and aggregate fractions of both soils with and without earthworms was rapid, particularly within the first 7 d (Figures 5.3 and 5.4). At the end of the 21 d without exposure to the earthworms only 19.6 and 15.5% of the initial amount of TNT was extractable ($CaCl_2$ extractable + acetonitrile extractable) from the whole soil in the Appling and Theresa soil, respectively (Figures 5.3 and 5.4). In the Theresa soil, after 7 d < 10% of the total extractable TNT was $CaCl_2$-extractable,

indicating a very small amount of readily bioavailable TNT. Because mineralization of TNT to CO_2 is highly unlikely (Hawari, et al., 2000; Esteve-Nunez et al., 2001) and the total measured amount of TNT metabolites did not equal the amount of TNT disappearance, it is likely that some of the TNT and/or metabolites are present in the soil as nonextractable residue (Isbister et al., 1984; Caton et al., 1994; Comfort et al., 1995; Hundal et al., 1997).

In the Appling soil earthworm activity enhanced TNT disappearance over the first 14 d in the whole soil and 2000 – 4000 μm aggregates and the first 7 d in 500 – 2000 μm aggregates (Figure 5.3). At 7 d the soil with earthworms contained 7.2 mg kg^{-1} *readily* extractable $CaCl_2$-extractable TNT from the whole soil compared to 20.3 mg kg^{-1} of TNT in the microcosm without earthworms. However, at 14 d there was no significant difference in *readily* extractable TNT, but the soil with earthworms contained 7.4 mg kg^{-1} of *potentially* extractable TNT from the whole soil compared to 11.6 mg kg^{-1} of TNT in the soil without earthworms. At 21 d there was no difference in TNT disappearance between treatments. This trend of earthworms influencing the disappearance of readily available TNT at 7 d, potentially available TNT at 14 d, and no impact by 21 d suggests that the earthworm assisted disappearance of TNT in the Appling soil decreased with time. Similarly, the diminishing influence of earthworm activity on TNT disappearance with time were observed in 2000 – 4000 and 500 - 200 μm aggregates of the Appling soil (Figure 5.3).

The diminishing impact of earthworms on TNT disappearance with time may be a result of TNT toxicity to earthworms, resulting in a reduction in earthworm activity, as shown by a decrease in earthworm biomass (Figure 5.1). Phillips et al. (2003) reported

that the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for *Eisenia fetida* in a sandy forest soil over 56 d was 55 and 110 mg kg^{-1}, respectively, which is close to the initial concentration of 99 mg kg^{-1} used in this study. Other studies involving *Eisenia andrei* and *Aporrectodea rosea* have reported similar levels of sublethal toxicity (Robidoux et al., 1999; Renoux et al., 2000; Robidoux et al., 2000; Robidoux et al., 2004). Although differences in clay and organic matter content can have a significant impact on the determination of NOEC and LOEC, the dramatic reduction in earthworm biomass and activity during the first 7 d and during the course of the present study (Figure 5.1) certainly indicates a reduction in earthworm activity is at least part of the reduced influence on TNT fate. Another possibility is the formation of irreversibly bound residues to soil components which were not "bioavailable" to earthworms. The decrease in the amount of extractable TNT from surface soil with time has been well documented (Sheremata, et al., 1999; Hundal et al., 1997). Sheremata et al. (1999) reported 19 – 47% of applied TNT was un-extractable using acetonitrile after 44 h and Hundal et al. (1997) reported 30 – 45% of applied TNT was unextractable after 168 days using exhaustive (i.e. solvents, strong alkali and acidic solutions) extractions.

It is interesting to note that earthworms appear to have the most influence on the disappearance of TNT in macroaggregates > 500 μm in the Appling soil. Additionally, although earthworm impact on the disappearance of TNT may diminish with time, earthworms do have a significant impact on the distribution of TNT within the soil. For example, at 21 d the soil with TNT but no earthworms had the highest levels of TNT in 2000 – 4000 μm aggregates, however, the soil with TNT and earthworms had an equal

distribution of TNT across all the aggregate size fractions (Figure 5.5). This is attributed to greater TNT disappearance in the 2000 – 4000 μm size fraction relative to the other size fractions in the soil with earthworms. The disappearance of TNT in the 2000 – 4000 μm size fraction could have been enhanced by transformation of TNT within the earthworms and the resulting cast material containing much lower levels of TNT compared to other aggregates (non-earthworm casts) of the same size, ultimately resulting in a considerable drop in TNT in all aggregates that size. Renoux et al. (2000) conducted experiments exposing earthworms to TNT through direct dermal contact on filter paper. The authors concluded that based on the amount of TNT uptake and transformation through dermal contact on filter paper, the amount of metabolites that accumulated with the earthworms (see section 3.1) exposed to TNT in soil must have been in part due to transformation through soil ingestion. Another possibility is that the earthworms indirectly, through a modification of the soil environment (i.e. increased aeration, exudation of organic mucus, alteration of the soil microbial community), stimulated the transformation of TNT in macroaggregates, including earthworm casts. Finally, the disappearance of TNT in the 2000 – 4000 μm size fraction could be the result of increased sorption due to the organic rich nature of the earthworm casts, thus making it more difficult to extract and giving the appearance that a greater amount of TNT was transformed in the macroaggregates. Further studies using radio labeled compounds and focusing on the fate of TNT within earthworm casts would help to clarify these results.

In the Theresa soil there was no significant difference in TNT disappearance due to earthworm activity in the whole soil or any aggregate size fraction over the entire course of the experiment (Figure 5.4). The lack of earthworm impact on TNT

disappearance in the Theresa soil compared to the Appling soil could be due to differences in the overall bioavailability of TNT. Two observations in our study support this theory. First, the amount of extractable metabolites from earthworms in the Theresa soil was significantly less than the amount extracted from earthworms in the Appling soil ($p < 0.001$) indicating the earthworms in the Theresa soil had less contact with TNT than earthworms from the Appling soil (Figure 5.1, see section 3.1). Second, after 7 d the treatment with no earthworms (- Ew + TNT) in the Appling soil contained 20.3 mg kg^{-1} of readily available TNT ($CaCl_2$ extracted) compared to 2.5 mg kg^{-1} of readily available TNT in the Theresa soil. This may be due to greater sorption and binding of TNT in the Theresa soil. Batch sorption experiments performed indicated that the TNT sorption coefficient (K_F) of the Theresa soil was more than 2x greater than for the Appling soil (Figure 5.8). The differences in the sorption coeffecients can be attributed to the Theresa soil containing more than twice as much organic matter than the Appling soil (Table 5.1). Additionally, XRD analysis (data not shown) indicated the presence of 2:1 clay minerals such as vermiculite and smectite in the Theresa soil. Previous work has provided strong evidence for the abiotic transformation and humification of TNT (and metabolites) in soil as indicated by the significant amount of TNT extracted from humic acid, fulvic acid, and humin (Hundal et al., 1997; Achtnich et al., 1999; Wang et al., 2002). Sorption studies have shown that TNT readily binds with humic substances and TNT metabolites such as 2-ADNT and 4-ADNT have shown to form strong irreversible bonds with organic matter (Achtnich et al., 1999; Eriksson and Skyllberg, 2001). Additionally, under certain environmental conditions smectitic minerals are known to adsorbed greater amounts of TNT than Kaolinite (Haderlein et al., 1996). As a result of the high amount of TNT

sorption and the formation of bound residues it is very unlikely that the earthworms could influence the fate of the bound TNT over time, and thus earthworm influence on the fate of TNT was limited to readily and potentially available TNT during the first 14 d. Gevao et al. (2004) used soil contaminated with atrazine, isoproturon, and dicamba to demonstrate that earthworms had little impact on the fate of bound residues. After 100-d incubation the authors used exhaustive Soxhlet extractions to remove the pesticides and then exposed the earthworm *Aporrectodea longa* to the previously extracted soils for 28 d. They reported that the earthworms absorbed 0.02 – 0.2% of the previously bound chemical into their tissue, thus demonstrating that irreversibly bound residue is not available to earthworms for transformation or degradation.

Although earthworms had little impact on the disappearance of TNT in the Theresa soil, similar to the Appling soil, they did influence the distribution of TNT among soil aggregates (Figure 5.5). For example, after 21 d soil aggregates < 500 μm contained significantly more TNT than aggregates > 500 μm, however, with earthworms the distribution of TNT was more equally distributed. In general there was an increase of extractable TNT (though not statistically significant) in 2000 – 4000 μm aggregates and a decrease in aggregates 250 – 500 μm aggregates. Thus, unlike the Appling soil where earthworms altered the distribution of TNT by enhanced TNT disappearance in larger macroaggregates, in the Theresa soil, earthworms altered the distribution of TNT by moving the TNT from smaller aggregates to the larger macroaggregates. Studies have noted the ability of earthworms to move organic material between aggregates of different size. Bossuyt et al., (2005) reported a shift in radio labeled carbon from micro-aggregates < 250 to macroaggregatses > 500 um as a function of earthworm activity.

Based on this and the previous discussed results of Gevao and co workers, the possibility of bound TNT movement from one size soil aggregate to another is certainly feasible.

3.4 TNT transformation and distribution of metabolites in soil and soil aggregates.

During the course of this study, 2-ADNT and 4-ADNT were the only two metabolites or transformation products of TNT identified in the soil. This observation is in agreement with previous fate studies of TNT (Pennington, et al. 1995; Selim et al., 1995; Comfort et al., 1995; Hundal et al., 1997). Earlier we reported that earthworms had the most influence on the disappearance of TNT within the first 7 – 14 d of exposure to freshly spiked Appling soil and no influence on the disappearance of TNT in the Theresa soil. However, based on the amount of extractable metabolites (2-ADNT and 4-ADNT), earthworms had the greatest influence on the transformation of TNT to metabolites between 14 – 21 d of *both* the Appling and Theresa soils (Figures 5.3, 5.4).

In the Appling soil earthworm activity resulted in a greater amount of $CaCl_2$, solvent, and total extractable 2-ADNT at 14 d in the whole soil and all aggregate size fractions (Figure 5.3). Additionally, earthworm activity resulted in an increase in $CaCl_2$ extractable 2-ADNT at 7 d in 500 – 2000 and 250 - 500 μm aggregates and a decrease in $CaCl_2$ extractable 2-ADNT at 21 d in 250 – 500 μm aggregates. There was also a greater amount of solvent and total extractable 4-ADNT at 21 d in the whole soil and all aggregate size fractions and solvent extractable 4-ADNT at 14 d in the whole soil and 2000 – 4000 μm aggregates. Interestingly, the increase in TNT disappearance at 7 d in the Appling whole soil and 2000 – 4000 μm aggregates due to earthworm activity did not result in a similar increase in 2-ADNT or 4-ADNT at 7 d. As mentioned earlier, the

disappearance of TNT in 2000-4000 μm aggregates could be due to transformation of TNT within the earthworms. However, the bioaccumulation of the metabolites 2-ADNT and 4-ADNT could have resulted in earthworm casts with lower levels of TNT and no significant change in the amount of metabolites. However, after 7 d the amount of 2-ADNT and 4-ADNT within earthworm tissue decreased, possibly due to a detoxification (i.e. adaptation or further transformation) or excretion of the metabolites which could explain the significant increase in these metabolites at 14 and 21 d (Figure 5.3). The gap between the earthworm effect on TNT disappearance at 7 d and the increase in 2-ADNT and 4-ADNT at 14 d could be the time it takes earthworms to remove the metabolites from their systems. Other studies have noted that the bioaccumulation of 4-ADNT and 2-ADNT within the earthworm *Eisenia andrei* as a result of exposure to TNT reaches a maximum level and then begins to drop (Renoux et al., 2000; Lachance et al., 2004). Although the cause of the reduction and disappearance of 2-ADNT and 4-ADNT are not known, these studies note that no further transformation of these compounds likely occurred. Further examination using radiolabeled compounds and molecular level investigation is needed to clarify these results.

Similar to TNT, earthworms influenced the distribution of 2-ADNT and 4-ADNT within soil aggregates. In the Appling soil at 21 d without earthworms, the 250 – 500 μm size fraction contained the most 2-ADNT and 4-ADNT compared to the other size fractions (Figure 5.5). However, in the presence of earthworms their distribution was equal among all size fractions. Interestingly, the biggest change in the concentration of 4-ADNT among the soil aggregates was in the 2000 – 4000 μm fraction. There was a 38.2, 28.8, and 16.9% increase in 4-ADNT in the 2000 – 4000, 500 -2000, and 250 – 500

μm fractions, respectively, compared to the same size fractions not exposed to earthworms. The further supports the theory that with time (> 14 d) earthworms may have excreted or deposited 4-ADNT and 2-ADNT within earthworm casts thus leading to the increases in aggregates exposed to earthworms compared to those that were not.

In the Theresa soil earthworm activity resulted in a greater amount of solvent and total extractable 2-ADNT at 14 and 21 d in the whole soil and 500 – 2000 μm aggregates and 21 d in 2000 – 4000 μm aggregates (Figure 5.4). Additionally, earthworm activity resulted in a greater amount of solvent and total extractable 4-ADNT at 21 d in 2000 – 4000 and 500 – 2000 μm aggregates. For the duration of the study $CaCl_2$ extractable 2-ADNT and 4-ADNT was < 2 mg kg^{-1} in the whole soil and all aggregate size fractions and after 14 d, > 85% of extractable 2-ADNT and 4-ADNT were solvent extractable. The low extractability and bioavailability can be attributed to the high organic matter content and irreversibly bound sorption of 2-ADNT and 4-ADNT (Achtnich et al., 1999). The observed increase in metabolites and the lack of an effect on TNT disappearance in the presence of earthworms in the Theresa soil could be the result of earthworm transformation of TNT and/or excretion of bioaccummulated 2-ADNT and 4-ADNT (as discussed in the previous section) or a possible earthworm effect on the *extractability* of TNT and/or metabolites. For example, the formation of non-extractable residues could have overshadowed the effects of earthworm activity on TNT transformation and thus giving the impression that earthworms had no impact on TNT transformation in the Theresa soil. Alternatively, if the earthworms indeed had no impact on TNT transformation due to low bioavailability of TNT (as previously discussed) earthworm activity could have enhanced the ability to extract TNT metabolites from the soil giving

the impression that more metabolites were being *transformed* from TNT due to earthworm activity than there actually was, particularly in the Theresa soil. The enhanced extractability may be due to changes in the binding strength and/or sorption mechanism for 2-ADNT and 4-ADNT. The increased amount of extractable 2-ADNT and 4-ADNT in the presence of earthworms was limited to aggregates > 500 μm of the Theresa soil. This may be due in part to a shift in the distribution of 2-ADNT and 4-ADNT from < 500 μm to > 500 μm aggregates (Figure 5.5). Although there was not a significant increase in percent of macroaggregates in soil with both TNT and earthworms (Figure 5.2), earthworm activity (excretion of organic mucus, casts, enhanced porosity, etc) may have changed the nature of 2-ADNT and 4-ADNT interactions within the soil and soil particles. To the best of our knowledge, our study is the first to present indirect evidence that suggests earthworm activity may influence the extractability of nitroaromatics such as TNT from the soil.

3.5 Influence of TNT and earthworms on microbial community structure.

The NMS analysis of FAME mol % data was used to determine differences in microbial community structure and differences among fatty acids which are used as microbial indicators. An NMS analysis of both soils clearly showed differences in the microbial community structure between soils (data not shown) and with time (Appendix C, Figures C.5, C.6). These findings were further supported by MRPP analysis which indicated significant differences in the distribution of mol % FAME between time periods ($p < 0.0001$). Indicator species analysis showed that fatty acids i15:0 and a15:0 consistently separated treatments over time ($p < 0.01$) for the Theresa soil and that i15:0,

a15:0, i16:0, 18:2w, 18:1w7, and 10Me18:0 consistently separated treatments over time (p<0.01) for the Appling soil.

The NMS, MRPP, and indicator species analysis was re-run for each soil and time period to determine significant differences between treatments. To simplify the discussion of the effects of earthworms and TNT on the microbial community we focused on the results at 21 d which covers the entire time scale of the study. The NMS results for the Appling and Theresa soil at 7 and 14 d can be found in Appendix C (Figures C.7-C.10). Further NMS analysis performed on both soils at 21 d clearly shows differences in the microbial community structure between treatments for the whole soil and all aggregate size fractions (Figures 5.7., 5.8). An MRPP analysis further indicated significant differences in the distribution of mol % of FAMEs between the treatments for both soils at 21 d ($p < 0.0001$). Indicator species analysis at 21 d showed that cy17:0, 16:1w7, 10Me16:0, 20:0 and 22:0 consistently separated treatments ($p < 0.01$) across all aggregate size fractions and the whole soil in the Theresa soil and 18:2w, i15:0, 18:1w7, and cy19:0 consistently separated treatments ($p < 0.01$) in the Appling soil.

Analysis of individual FAMEs and specific groups which are indicative of certain microbial organisms (i.e. gram-negative bacteria, fungi, etc.) can provide interesting perspective into the impact of earthworms and TNT on the soil microbial community. In addition, the total amount of fatty acids as determined by FAME can provide a relative estimate of the total microbial population represented by the measured fatty acids. However, since the extraction of FAMEs are not as selective as other methods (i.e. phospholipid fatty acids, PLFAs), they do not provide a definite measure of microbial biomass (Vestal and White, 1989; Schutter and Dick 2000; Mummey et al. 2002). In the

Appling soil there were no differences in the amount of total FAMEs between the control and three treatments. In the Theresa whole soil there was a net decrease in total fatty acids in the soil with earthworms (97.5 nmol g^{-1}) and the soil with earthworms and TNT (127.9 nmol g^{-1}) compared to the soil without either (197.9 nmol g^{-1}). Studies have shown that depending on various environmental parameters such as temperature, moisture, soil type, and substrate quality earthworms can have either a positive influence (Parkinson and McLean, 1998; Postma-Blaauw et al., 2006; Binet et al., 2006) or a negative influence on microbial biomass and microbial activity (Saetre et al., 1998; Groffman et al., 2004). Interestingly, in the Theresa whole soil and soil aggregates > 250 μm there was no difference in the amount of fatty acids between the control (- Ew – TNT) and the soil exposed to TNT (- Ew + TNT), thus suggesting that TNT had no impact on the total biomass or any individual indicator (i.e. gram-negative bacteria). This is in contrast to the findings of Wilke et al. (2004) who reported a decrease in the amount of fatty acids due to the presence of TNT. Other studies have also indicated a decrease in microbial activity as a result of TNT, (Fuller and Manning, 1998; Gong et al., 1999; Siciliano et al., 2000), hydrocarbons (Schaefer et al., 2005), and pesticides such as imazethapyr (Perucci and Scarponi, 1994). In the Theresa soil after 7 d there was < 4.2 and after 21 d < 1.1 mg kg^{-1} of readily available TNT in the whole soil or in any of the aggregate size fractions (Figures 5.3, 5.4). This accounted for 8-18% of the total extractable TNT in the soil. Thus, the low bioavailability and high amount of sequestered TNT could explain the lack of an effect on the microbial community.

Examining changes in the mol % of fatty acids rather than the amount (nmol /g) helps to identify changes in the microbial community structure (Vestal and White, 1989).

With respect to the mol % of fatty acids in the whole soil there were occasions where there was contrasting results between the Theresa and Appling soils (Tables 5.2, 5.3). For example, earthworm activity (+ Ew – TNT and + Ew +TNT) in the Theresa whole soil and 2000 – 4000 μm aggregates resulted in a decrease in biomarkers indicative of gram-positive bacteria relative to the treatments without earthworms (- Ew – TNT and – Ew + TNT). Fuller and Manning (1998) reported similar reductions in gram-positive bacteria in soils contaminated with TNT. However, in the Appling soil, the presence of TNT and earthworms (+ Ew + TNT) in the whole soil resulted in no change in the mol % of gram-positive indicators compared to the control (Table 5.2, 5.3). Additionally, in the Appling whole soil and 500 – 2000 μm aggregates with TNT and earthworms (+ Ew + TNT) there was a reduction in the mol % of 18:2ω and 18:1ω9 (fungal biomarkers) compared to the soil without earthworms or TNT (-Ew – TNT). However in the Theresa whole soil and 75 – 250 μm aggregates with TNT and earthworms there was an increase in 18:2ω and 18:1ω9 compared to the soil without earthworms or TNT. Finally, earthworm activity resulted in a decrease in 10Me16:0 and 10Me18:0 (actinomycetes biomarkers) in the Appling whole soil yet there was an increase in these same biomarkers in the whole soil and 2000 – 4000 μm aggregates of the Theresa soil. Frostegard et al. (1993) reported contrasting impacts of metal contamination on actinomycetes biomarkers (10Me16:0, 10Me18:0) in an arable and forest soil. These results suggest that ability of specific organisms to tolerate organic or inorganic contaminates is in part a function of soil characteristics (i.e. organic matter, clay content, pH, etc).

We also found contrasts in the mol % of 18:2ω in various aggregate size fractions. In 250 - 500 μm aggregates of the Theresa soil exposed to TNT and

earthworms (+ Ew + TNT) there were 5.1% of 18:2ω compared to 6.3% in the control (- Ew – TNT). Alternatively, in 75 - 250 μm aggregates exposed to TNT and earthworms there was 12.8% of 18:2ω compared to 11.8% in the control. This same trend, although not significant was observed between the aggregates exposed to TNT and the control. This suggests that fungi associated with the larger 250 – 500 μm size fraction may be more sensitive to TNT and metabolites than those associated with 75 – 250 μm size fraction. Specific species of soil fungi (i.e. such as *Cladosporium resinae*) have demonstrated the ability to transform TNT via azoxy- and hydroxyl intermediates to 2-ADNT and 4-ADNT (Bayman and Radkar, 1997). Although not proven, the relative increase in fungal biomarkers in 75 – 250 μm exposed to TNT may be due to the tolerance of specific fungi associated with that size fraction. The contrasting differences in specific biomarkers response to treatments between soils could be attributed to a number of factors related to the earthworms or TNT including, but not limited to, TNT bioavailability in each soil, earthworm influence on microbial properties (i.e. ingested and excreted substrate quality), and the influence (or lack there of) of earthworms on predatory organisms such as protozoa (Edwards and Bohlen, 1996).

Unique differences in the microbial community composition between the two soils and among treatments were also discovered in various aggregate size fractions. In 75 – 250, 500 – 2000, and 2000 – 4000 μm aggregates of the Theresa soil earthworm activity (+ Ew – TNT) increased the mol % of fatty acids indicative of gram-negative bacteria compared to the control (- Ew – TNT). The same increase due to both earthworms and TNT also occurred in 500 – 2000 and 2000 – 4000 μm aggregates of the Appling soil. Similarly the presence of TNT (- EW + TNT) in 75-250 and 2000 – 4000

μm aggregates of the Theresa soil and 250 – 500 μm aggregates of the Appling soil resulted in an increase in the mol % of gram-negative biomarkers. The increase in gram-negative bacteria relative to other biomarkers suggests that these organisms were able to tolerate or may have experienced favorable conditions as a result of the exposure to TNT and earthworms compared to other organisms. Research has shown that certain gram-negative bacteria such as *Pseudomonas putida*, *Pseudomonas aeruginosa,* and *Pseudomonas fluorescens* can transform TNT via the production of nitroreductase or other enzymatic pathways (Pak et al., 2000; Vasilyeva et al., 2000; Oh et a., 2001; Park et al., 2003). The fact that these organisms can transform TNT under various conditions suggests that they are capable of tolerating exposure to TNT. The increase in gram negative biomarkers in 2000 – 4000 and 500 – 2000 μm aggregates exposed to TNT and earthworms in *both* soils compared to the control provides indirect evidence that earthworm activity may have stimulated soil organisms capable of transforming TNT. This may further explain the differences in TNT disappearance and the amount of 2-ADNT and 4-ADNT extracted from these aggregate size classes in both soils.

Compared to the control, TNT and earthworms resulted in an increase in cy17:0 in 2000 – 4000 μm aggregates and in cy17:0 and cy19:0 in 500 – 2000 μm aggregates of the Appling soil. Increases in the mole % of these fatty acids have previously been an indication of microbial community stress (White, 1993). This would follow with our previous conclusions that TNT was more bioavailable in the Appling soil compared to the Theresa soil. However, there was a greater mol % of cy17:0 in 250 – 500 μm aggregates of the Theresa soil exposed to earthworms and TNT compared to the control, suggesting potential sensitivity to TNT in this specific size fraction.

4. Conclusions

Using soil microcosms and the earthworm *Eisenia fetida* we have demonstrated that earthworms can have a direct impact on the bioavailability of TNT in the soil and soil aggregates. The extent to which earthworms influence the transformation and fate of TNT depends on the sorption and sequestration of TNT within the soil. Earthworm impact on the fate of TNT is greatly diminished when TNT is adsorbed and/or forms irreversibly bound residues. However, earthworm activity can affect the distribution of TNT and metabolites in soil regardless of binding strength and extractability. When TNT and/metabolites are tightly bound in soil similar to the Theresa soil used in this study, earthworms can redistribute these contaminants from small aggregates <500 μm into large aggregates > 2000 μm. TNT also had a negative influence on earthworm activity and soil aggregation compared to soil without TNT. Our results also indicate that earthworms and TNT individually or together can alter the microbial community structure as an increase in gram-negative bacteria biomarkers was observed in both soils, particularly in aggregates > 500 μm in the Appling soil and aggregates > 250 μm in the Theresa soil. To what extent earthworms along with soil organisms are able to facilitate the transformation of TNT into a variety of metabolites remains to be seen. Studies involving the use of tracers (such as ^{14}C) and molecular level approaches (NMR, IR analysis of earthworm casts) will provided further insight into TNT – soil aggregate dynamics. In addition, based on the results of this study, further research is needed to better characterize how earthworms along with other soil organisms can facilitate and/or alter the environmental fate of TNT, particularly how they may affect microorganisms

which are specifically known to transform and cometabolize TNT. This would have wide ranging implications risk assessment and on various remediation technologies such as composting and phytoremediation where earthworms could contribute to the remediation of TNT.

References

Achtnich, C., U. Sieglen, H.-J. Knackmuss, and H. Lenke. 1999. Irreversible binding of biologically reduced 2,4,6-trinitrotoluene to soil. Environ. Toxicol. Chem. 18: 2416-2423.

Bayman, P., and G.V. Radkar. 1997. Transformation and tolerance of TNT (2,4,6-trinitrotoluene) by fungi. Int. Biodeter. Biodegr. 39: 45-53.

Binet,F., A. Kersante, C. Munier-Lamy, R.-C. Le Bayon, M.-J. Belgy, and M.J. Shipitalo. 2006. Lumbricid macrofauna alter atrazine mineralization and sorption in a silt loam soil. Soil Bio. Biochem. 38: 1255-1263.

Boopathy, R. 2000. Bioremediation of explosives contaminated soil. Int. Biodeter. Biodegr. 46: 29-36.

Bossuyt, H., J. Six, and P.F. Hendrix. 2002. Aggregate-protected carbon in no-tillage and conventional tillage agroecosystems using carbon-14 labeled plant residue. Soil Sci. Soc. Am. J. 66: 1965-1973.

Bossuyt, H., J. Six, and P.F. Hendrix. 2004. Rapid incorporation of carbon from fresh residues into newly formed stable microaggregates within earthworm casts. Eur. J. Soil Sci. 55: 393-399.

Bossuyt, H., J. Six, and P.F. Hendrix. 2005. Protection of soil carbon by microaggregates within earthworm casts. Soil Biol. Biochem. 37: 251-258.

Caton, J.E., C.-H. Ho, R.T. Williams, and W.H. Griest. 1994. Characterization of insoluble fractions of TNT transformed by composting. J. Environ. Sci. Health A 29: 659-670.

Coleman, D.C., D.A. Crossley, and P.F. Hendrix. 2004. Fundamentals of soil ecology. 2nd ed. Elsevier, New York.

Comfort, S.D., P.J. Shea, L.S. Hundal, Z. Li, B.L. Woodbury, and W.L. Powers. 1995. TNT transport and fate in contaminated soil. J. Environ. Qual. 24: 1174-1182.

Corbett, M.D., and B.R. Corbett. 1995. Bioorganic chemistry of the arylhydroxylamine and nitrosoarene functional groups. p. 151-182. *In* Biodegradation of nitroaromatic compounds. Spain, J.C. (ed). New York, Plenum Press.

Dodard, S.G., A.Y. Renoux, J. Powlowski, and G.I. Sunahara. 2003. Lethal and subchronic effects of 2,4,6-trinitrotoluene (TNT) on *Enchytraeus albidus* in spiked artificial soil. Ecotox. Environ. Saf. 54: 131-138.

Edwards, C.A., and P.J. Bohlen. 1996. Biology and ecology of earthworms. Chapman &

Hall, London, UK.

Elovitz, M.S., and E.J. Weber. 1999. Sediment-mediated reduction of 2,4,6-trinitrotoluene and fate of the resulting aromatic (poly)amines. Environ. Sci. Technol. 33: 2617-2625.

Eriksson, J., and U. Skyllberg. 2001. Binding of 2,4,6-trinitrotoluene and its degradation products in a soil organic matter two-phase system. J. Environ. Qual. 30: 2053-2061.

Esteve-Nunez A., A. Caballero, and J.L. Ramos. 2001. Biological degradation of 2,4,6-trinitrotoluene. Microbiol. Mol. Biol. Rev. 65: 335-352.

Frostegard, A., A. Tunlid, and E. Baath. 1993. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. App. Environ. Microbiol. 59: 3605-3617.

Fuller, M.E., and J.F. Manning. 1998. Evidence for differential effects of 2,4,6-trinitrotoluene and other munitions compounds on specific subpopulations of soil microbial communities. Environ. Toxicol. Chem. 17: 2185-2195.

Gevao, B., C. Mordaunt, K.T. Semple, T.G. Piearce, and K.C. Jones. 2001. Bioavailability of nonextractable (bound) pesticide residues to earthworms. Environ. Sci. Technol. 35: 501-507.

Gong, P., S.D. Siciliano, C.W. Greer, L. Paquet, and J. Hawari, and G.I. Sunahara. 1999. Effects and bioavailability of 2,4,6-trinitrotoluene in spiked and field-contaminated soils to indigenous microorganisms. Environ. Toxicol. Chem. 18: 2681-2688.

Groffman, P.M., P.J. Bohlen, M.C. Fisk, and T.J. Fahey. 2004. Exotic earthworm invasion and microbial biomass in temperate forest soils. Ecosystems 7: 45-54.

Haderlein, S.B., K.H. Weissmahr, and R.P. Schwarzenbach. 1996. Specific adsorption of nitroaromatic explosives and pesticides to clay minerals. Environ. Sci. Technol. 30: 612-622.

Hawari, J., A. Halasz, L. Paquet, E. Zhou, B. Spencer, G. Ampleman, and S. Thiboutot. 1998. Characterization of metabolites in the biotransformation of 2,4,6-trinitrotoluene with anaerobic sludge: Role of triaminotoluene. Appl. Environ. Micro. 64: 2200-2206.

Hawari, J., S. Beaudet, A. Halasz, S. Thiboutot, and G. Ampleman. 2000. Microbial degradation of explosives: Biotransformation versus mineralization. App. Microbiol. Biotechnol. 54: 605-618.

Hundal, L., P.J. Shea, S.D. Comfort, and W.L. Powers, and J. Singh. 1997. Long-term TNT sorption and bound residue formation in soil. J. Environ. Qual. 26: 896-904

Isbister, J.D., G.L. Anspach, J.F. Kitchens, and R.C. Doyle. 1984. Composting for decontamination of soils containing explosives. Microbiologia. 7: 47-73.

Jerger, D.E., and P.E. Woodhull. 2000. Applications and costs for biological treatment of explosives contaminated soil in the U.S. p. 395-424. *In* Biodegradation of nitroaromatic compounds and explosives. Spain, J.C., Joseph B. Hughes, and Hans-Joachim Knackmuss (eds). Boca Raton: CRC Press.

Kaplan, D.L., and A.M. Kaplan. 1982. Thermophilic biotransformation of 2,4,6-trinitrotoluene under simulated composting conditions. Appl. Environ. Micro. 44: 757-760.

Keith, L.H., and W.A. Telliard. 1979. Priority pollutants. I. A perspective view. Environ. Sci. Technol. 13: 416-423.

Lachance, B., A.Y. Renoux, M. Sarrazin, J. Hawari, and G.I. Sunahara. 2004. Toxicity and bioaccumulation of reduced TNT metabolites in the earthworm *Eisenia andrei* exposed to amended forest soil. Chemosphere 55: 1339-1348.

Lee, K.E., 1985. Earthworms: Their ecology and relationships with soils and land use. Academic Press. Sydney.

Lee, K.E., and R.C. Foster. 1991. Soil fauna and soil structure. Aust. J. Soil Res. 29: 745-776.

Lewis, T.A., D.A. Newcombe, and R.L. Crawford. 2004. Bioremediation of soils contaminated with explosives. J. Environ. Manage. 70: 291-307.

Lowe C.N., and K.R. Butt. 2004. Influence of food particle size on inter- and intra-specific interactions of *Allolobophora chlorotica* (Savigny) and *Lumbricus terrestris*. Pedobiologia 47: 574-577.

Martin, A. 1991. Short- and long-term effects of the endogeic earthworm *Millsonia anomala* (*Omodeo*) (*Megascolecidae Oligochaeta*) of tropical savannas on soil organic matter. Biol. Fert. Soils 11: 234-238.

Martin, A., J. and C.Y. Marinissen, 1993. Biological and physico-chemical processes in excrements of soil animals. Geoderma 56: 331-347.

McCune, B., and M.J. Mefford. 1999. PC-ORD Multivariate analysis of ecological data. Version 4.0 MjM Software, Gleneden Beach, Oregon, USA.

McCune, B., and J.B. Grace. 2002. Analysis of ecological communities. MjM Software

Design, Gleneden Beach, Oregon, USA.

McGrath, C.J. 1995. Review of formulations for processes affecting subsurface transport of explosives. Technical Report IRRP-95-2. U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Mummey, D.L., P.D. Stahl, and J.S. Buyer. 2002. Microbial biomarkers as an indicator of ecosystem recovery following surface mine reclamation. App. Soil Ecol. 21: 251-259.

National Research Council. 2003. Bioavailability of contaminants in soil sand sediments: Processes, tools, and applications. National Academy Press, Washington D.C.

Oh, B.-T., G. Sarath, and P.J. Shea. 2001. TNT nitroreductase from a *Pseudomonas aeruginosa* strain isolated from TNT-contaminated soil. Soil Bio. Biochem. 33: 875-881.

Pak, J.W., K.L. Knoke, D.R. Noguera, B.G. Fox, and G.H. Chambliss. 2000. Transformation of 2,4,6-trinitrotoleune purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. App. Enviro. Microbiol. 66: 4742-4750.

Park, C., T.-H. Kim, S. Kim, S.-W. Kim, J. Lee, and S.-H. Kim. 2003. Optimization for biodegradation of 2,4,6-trinitrotoluene (TNT) by *Pseudomonas putida*. J. Biosci. Bioeng. 95: 567-571.

Parkinson, D. and M.A. McLean. 1998. Impacts of earthworms on the community structure of other biota in forest soils. p. 213-226. *In* Earthworm ecology Edwards C.A. (ed.) CRC Press, Baca Raton, FL.

Parmelee, R.W., R.S. Wentsel, C.T. Philips, M. Simini, and R.T. Checkai. 1993. Soil microcosm for testing the effects of chemical pollutants on soil fauna communities and trophic structure. Environ. Toxicol. Chem. 12: 1477-1486.

Pennington, J.C., and W.H. Patrick. 1990. Adsorption and desorption of 2,4,6-trinitrotoluene by soils. J. Environ. Qual. 19: 559-567.

Pennington J.C., C.A Hayes, K.F. Myers, M. Ochman, D. Gunnison, D.R. Felt D.R, and E.F. McCormick. 1995. Fate of 2,4,6-trinitrotoluene in a simulated compost system. *Chemosphere* 30:429-438.

Pennington, J.C., and J.M. Brannon. 2002. Environmental fate of explosives. Thermochimica Acta 384: 163-172.

Perucci, P., and L. Scarponi. 1994. Effects of the herbicide imazethapyr on soil microbial biomass and various soil enzyme activities. Bio. Fert. Soils 17: 237-240.

Phillips, C.T., R.T. Checkai, and R.S. Wetsel. 1993. Toxicity of selected munitions and munition-contaminated soil on the earthworm (*Eisenia foetida*). Edgewood Research Development and Engineering Center. U.S. Army Chemical and Biological Defense Agency, Aberdeen Proving Ground, MD., ERDEC-TR-037.

Piearce, T.G. 1978. Gut contents of some lumbricid earthworms. Pedobiologia 18: 153-157.

Postma-Blaauw, M.B., J. Bloem, J.H. Faber, J.W. van Groenigen, R.G.M. and de Goede, L. Brussaard. 2006. Earthworm species composition affects the soil bacterial community and net nitrogen mineralization. Pedobiologia 50: 243-256.

Renoux, A.Y., M. Sarrazin, J. Hawari, and G.I. Sunahara. 2000. Transformation of 2,4,6-trinitrotoluene in soil in the presence of the earthworm *Eisenia Andrei*. Environ. Toxicol. Chem. 19: 1473-1480.

Robertson, B.K., and P.K. Jjemba. 2005. Enhanced bioavailability of sorbed 2,4,6-trinitrotoluene (TNT) by a bacterial consortium. Chemosphere 58: 263-270.

Robidoux, P.Y., J. Hawari, S. Thiboutot, G. Ampleman, and G.I. Sunahara. 1999. Acute toxicity of 2,4,6-trinitrotoluene in earthworm (*Eisenia andrei*). Ecotox. Environ. Saf. 44: 311-321.

Robidoux, P.Y., C. Svendsen, J. Caumartin, J. Hawari, G. Ampleman, S. Thiboutot, J.M. Weeks, and G.I. Sunahara. 2000. Chronic toxicity of energetic compounds in soil determined using the earthworm (*Eisenia andrei*) reproduction test. Environ. Toxicol. Chem. 19: 1764-1773.

Robidoux, P.Y., C. Svendsen, M. Sarrazin, S. Thiboutot, G. Ampleman, J. Hawari, J.M. Weeks, and G.I. Sunahara. 2004. Assessment of a 2,4,6-trinitrotoluene-contaminated site using *Aporrectodea rosea* and *Eisenia andrei* in mesocosms. Arch. Environ. Contam. Toxicol. 48: 56-67.

Rodgers, J.D., and N.J. Brunce. 2001. Treatment methods for the remediation of nitroaromatic explosives. Water Res. 35: 2101-2111.

Saetre, P. 1998. Decomposition, microbial community structure, and earthworm effects along a birch-spruce soil gradient. Ecology 79: 834-846.

Schaefer, M. 2004. Assessing 2,4,6-trinitrotoluene (TNT)-contaminated soil using three different earthworm test methods. Ecotox. Environ. Saf. 57: 74-80.

Schaefer, M., S.O. Petersen, and J. Filser. 2005. Effects of *Lumbricus terrestris*, *Allolobophora chlorotica* and *Eisenia fetida* on microbial community dynamics in oil-contaminated soil. Soil Bio. Biochem. 37: 2065-2076.

Schutter, M.E., and R.P. Dick. 2000. Comparison of fatty acid methyl ester (FAME) methods for characterizing microbial communities. Soil Sci. Soc. Am. J. 64:1659-1668.

Scullion J., and A. Malik. 2000. Earthworm activity affecting organic matter, aggregation and microbial activity in soils restored after open case mining for coal. Soil Bio. Biochem. 32: 119-126.

Selim, H.M., S.K. Xue, and I.K. Iskandar. 1995. Transport of 2,4,6-trinitrotoluene and hexahydro-1,3,5-trinitro-1,3,5-triazine in soils. Soil Sci. 160: 328-339.

Sheremata, T.W., S. Thiboutot, G. Ampleman, L. Paquet, A. Halasz, and J. Hawari 1999. Fate of 2,4,6-trinitrotoluene and its metabolites in natural and model soil systems. Environ. Sci. Technol. 33: 4002-4008.

Shipitalo M.J., and R. Protz. 1989. Chemistry and micromorphology of aggregation in earthworm casts. Geoderma 45: 357-374.

Siciliano, S.D., R. Roy, and C.W. Greer. 2000. Reduction in denitrification activity in field soils exposed to long term contamination by 2,4,6-trinitrotoluene (TNT). *FEMS* Microbiol. Ecol. 32: 61-68.

Simini, M., R.W. Wentsel, R. Checkai, C. Phillips, N.A. Chester, M.A. Major, and J.C. Amos.1995. Evaluation of soil toxicity at Joliet army ammunition plant. Environ. Toxicol. Chem. 14: 623-630.

Talmage, S.S., D.M. Opresko, C.J. Maxwell, C.J. Welsh, F.M. Cretella, P.H. Reno, and F.B. Daniel. 1999. Nitroaromatic munition compounds: Environmental effects and screening values. Rev. Environ. Contam. Toxicol. 161: 1-156.

Thorne, P.G., and D.C. Leggett 1997. Hydrolytic release of bound residues from composted soil contaminated with 2,4,6-trinitrotoluene. Environ. Toxicol. Chem. 16: 1132-1134.

USEPA. 2006. EPA Mid-Atlantic Risk Assessment, retrieved June 4th 2006, *http://www.epa.gov/reg3hwmd/risk/index.htm*

Vasilyeva, G.K., B.-T. Oh, P.J. Shea, R.A. Drijber, V.D. Kreslavski, R. Minard, and J.-M. Bollag. 2000. Aerobic TNT reduction via 2-hydroxylamino-4,6-dinitrotoluene by *Pseudomonas aeruginosa* strain MX isolated from munitions-contaminated soil. Bioremed. J 4: 111-124.

Vasilyeva, G.K., V.D. Kreslavski, B.-T. Oh, and P.J. Shea. 2001. Potential of activated carbon to decrease 2,4,6-trinitrotoluene toxicity and accelerate soil decontamination. Environ. Toxicol. Chem. 20: 965-971.

Vestal, J.R., and D.C. White. 1989. Lipid analysis in microbial ecology: Quantitative approaches to the study of microbial communities. Bioscience 39: 535-541.

Vorbeck, C., H. Lenke, P. Fischer, J.C. Spain, and H.-J. Knackmuss. 1998. Initial reductive reactions in aerobic microbial metabolism. App. Environ. Microbiol. 64: 246-252.

Wang, C.-J., S. Thiele , and J.-M. Bollag. 2002. Interaction of 2,4,6-trinitrotoluene (TNT) and 4-amino-2,6-dinitrotoluene with humic monomers in the presence of oxidative enzymes. Arch. Environ. Contam. Toxicol. 42: 1-8.

White, D.C. 1993. In-situ measurement of microbial biomass, community structure and nutritional-status. Philos. T. Roy. Soc. B 344: 59-67.

Wilke, B.-M., A. Gattinger, E. Frohlich, L. Zelles, and P. Gong. 2004. Phospholipid fatty acid composition of a 2,4,6-trinitrotoluene contaminated soil and an uncontaminated soil as affected by a humification remediation process. Soil Bio. Biochem. 36:725-729.

Zhang, H., and S. Schrader. 1993. Earthworm effects on selected physical and chemical properties of soil aggregates. Biol. Fertil. Soils 15: 229-234.

Table 5.1 Physical and chemical soil properties of the Appling (GA) and Theresa (WI) soils.

Property	Appling	Theresa
Physical Soil Properties		
Total sand (%)	66.5	39.9
Total silt (%)	18.7	45
Total clay (%)	14.8	15.1
Water content at 1/3 bar (%, w/v)	15	21
Chemical Soil Properties		
Cation exchange capacity (cmol (+) kg^{-1})	5.6	19.1
Total organic carbon (mg g^{-1})	12.5	28.8
Soil pH	4.2	6.5
Total elemental concentrations[†]		
Al (mg kg^{-1})	14914	10394
Fe (mg kg^{-1})	17914	15366
Ca (mg kg^{-1})	859	21320
K (mg kg^{-1})	1020	1805
Mg (mg kg^{-1})	345	11454
Na (mg kg^{-1})	89	216
P (mg kg^{-1})	277	819
Heavy metals[‡] (mg kg^{-1})	48	193

[†] Analysis conducted by the Soil Testing Laboratory, The University of Georgia, Athens, GA

[‡] Includes Cd, Cr, Cu, Ni, Pb, Zn

Table 5.2. Average mole percentages of individual FAMEs in the whole soil and aggregates of an Appling sandy loam soil exposed to earthworms and/or TNT for 21 d.

		whole soil				2000 - 4000 µm				500 - 2000 µm				250 - 500 µm			
Interpretation	**Fatty Acid**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**
									Mol %								
Gram-positive	i15:0	4.59 ab[†]	4.27 a	4.69 ab	5.03 b	4.6 ab	4.56 a	4.6 ab	5.1 b	4.5	4.6	4.6	4.7	4.5	4.9	4.8	ND[‡]
Gram-positive	a15:0	2.62 a	2.50 a	2.71 ab	2.97 b	2.5 a	2.6 ab	2.7 ab	2.9 b	1.9 a	2.0 ab	2.2 b	2.2 ab	2.2 a	2.4 ab	2.5 b	ND
Gram-positive	i16:0	4.1	3.9	4.0	4.3	4.1	4.2	4.0	4.2	3.8	4.0	3.8	3.9	3.8 a	4.2 b	3.9 ab	ND
Gram-positive	i17:0	2.5 ab	2.4 a	2.5 ab	2.7 b	2.4	2.5	2.4	2.6	1.7	1.8	1.8	1.8	1.9 a	2.2 b	2.0 a	ND
Gram-positive	a17:0	2.5 ab	2.4 a	2.6 ab	2.8 b	2.5	2.6	2.6	2.7	1.8	1.9	2.0	2.0	2.0	2.2	2.2	ND
Total		**16.3 ab**	**15.4 a**	**16.6 ab**	**17.8 b**	**16.0**	**16.5**	**16.3**	**17.6**	**13.7**	**14.3**	**14.5**	**14.6**	**14.3 a**	**16.0 b**	**15.4 ab**	ND
Gram-negative	16:1w9	4.5 ab	4.2 a	4.5 ab	4.8 b	4.5	4.8	4.6	4.6	4.4	4.5	4.1	4.6	4.1 a	4.7 b	4.4 ab	ND
Gram-negative	16:1w5	3.1 a	2.6 b	2.6 ab	3.0 ab	2.8	3.3	2.9	3.0	2.2	2.2	2.1	2.3	2.5	2.1	2.4	ND
Gram-negative	18:1w7	5.4 ab	4.9 b	5.7 ac	6.3 c	5.3 ab	4.8 b	6.0 ac	6.4 c	5.4 a	5.4 a	6.1 ab	6.4 b	4.3 a	5.0 a	5.8 b	ND
Gram-negative	cy17:0	1.8 a	2.0 ab	1.9 a	2.2 b	1.7 a	1.7a	1.8 ab	2.0 b	0.8 a	0.8 a	1.0 ab	1.1 b	1.1	1.3	1.2	ND
Gram-negative	cy19:0	3.8 a	3.5 a	4.2 b	4.5 b	3.8	3.6	3.9	4.0	3.2 a	3.3 a	3.9 b	3.9 b	3.5	3.8	3.8	ND
Total		**18.7 ab**	**17.2 a**	**19.0 b**	**20.8 c**	**18.0 a**	**18.3 ab**	**19.3 ab**	**19.9 b**	**16.2 a**	**16.2 a**	**17.3 ab**	**18.2 b**	**15.6 a**	**16.9 ab**	**17.6 b**	ND
Actinomycetes	10Me16:0	3.7 ab	3.3 a	3.7 ab	4.0 b	3.5	3.6	3.5	3.7	3.3	3.2	3.4	3.3	3.7 a	3.7 b	3.4 ab	ND
Actinomycetes	10Me18:0	3.6 a	3.0 b	3.4 ab	3.4 ab	3.4	3.2	3.2	3.2	2.9	2.9	2.9	2.7	3.0 ab	3.4 a	2.8 b	ND
Total		**7.3 a**	**6.2 b**	**7.1 ab**	**7.4 a**	**6.9**	**6.8**	**6.8**	**6.9**	**6.2**	**6.2**	**6.3**	**6.0**	**6.2 a**	**7.1 b**	**6.2 ab**	ND
Fungi	18:2w	9.6 ab	10.8 b	8.3 ac	6.3 c	9.6 a	9.1 ab	7.4 ab	6.9 b	10.9 a	9.6 ab	8.5 b	7.6 b	10.6 a	8.9 ab	7.1 b	ND
Fungi	18:1w9	13.8 ab	16.2 a	13.2 b	11.8 b	14.2	14.3	13.0	12.5	16.2	16.0	14.6	14.3	16.0	14.3	14.5	ND
Total		**23.3 ab**	**27.1 b**	**21.5 ac**	**18.1 c**	**23.8**	**23.4**	**20.4**	**19.4**	**27.1 a**	**25.6 ab**	**23.2 ab**	**21.9 b**	**26.7 a**	**23.2 ab**	**21.6 b**	ND
Protozoa	20:4w6	1.0	1.0	1.2	1.1	0.9 a	1.0 ab	1.1 b	1.0 a	--[§]	--	--	--	0.4 a	0.2 b	0.1 b	ND
Common to all	16:0	19.4	19.3	19.7	19.7	20.4	19.8	20.1	20.2	23.4 a	23.8 a	24.0 a	24.8 b	21.9 a	22.3 a	23.4 b	ND
Common to all	18:0	5.8 a	6.2 ab	5.9 a	6.3 b	6.1 ab	5.7 a	6.3 b	6.5 b	6.0 a	6.2 ab	6.6 b	6.4 ab	6.3	6.4	6.6	ND
?	20:0	3.9 ab	3.5 a	4.1 b	4.2 b	3.8 a	4.2 ab	4.7 b	4.1 ab	3.54	3.70	3.68	3.82	3.9 ab	3.8 a	4.5 b	ND
?	22:0	4.3 a	4.1 a	5.1 b	4.5 ab	4.2 a	4.3 ab	5.0 b	4.4 ab	3.87	3.95	4.54	4.33	4.9	4.1	4.6	ND

[†] Different letters indicate significant differences ($p < 0.05$) between treatments

[‡] Not determined (inadequate sample amount)

[§] Below detection limit

Table 5.3. Average mole percentages of individual FAMEs in the whole soil and aggregates of a Theresa silt loam soil exposed to earthworms and/or TNT for 21 d.

		whole soil				2000 - 4000 μm				500 - 2000 μm				250 - 500 μm				75 - 250 μm			
Interpretation	Fatty Acid	- EW - TNT	+EW - TNT	- EW + TNT	+ EW + TNT	- EW - TNT	+EW - TNT	- EW + TNT	+ EW + TNT	- EW - TNT	+EW - TNT	- EW + TNT	+ EW + TNT	- EW - TNT	+EW - TNT	- EW + TNT	+ EW + TNT	- EW - TNT	+EW - TNT	- EW + TNT	+ EW + TNT
		Mol %																			
Gram-positive	i15:0	6.2	6.0	6.1	5.7	6.4 a[†]	5.4 b	6.1 a	6.1 a	5.9	6.0	5.9	5.7	6.1	6.0	6.2	6.2	6.5	6.2	6.3	6.7
Gram-positive	a15:0	4.1	4.2	4.2	4.1	4.4 a	3.9 b	4.3 a	4.1 ab	3.9	4.0	3.8	3.9	4.3	4.0	4.3	4.4	4.5	4.5	4.5	4.6
Gram-positive	i16:0	2.7	2.4	2.7	2.5	2.8 a	2.4 b	2.8 a	2.9 a	2.4	2.6	2.3	2.6	2.8	2.8	2.8	3.0	2.4 a	2.7 b	2.9 b	2.8 b
Gram-positive	i17:0	1.8 a	1.2 b	1.8 a	1.4 b	1.9 a	1.7 b	1.9 a	1.7 ab	1.7	1.7	1.5	1.6	2.0	1.8	1.9	2.0	1.3 a	1.8 b	1.7 bc	1.4 ac
Gram-positive	a17:0	1.9 a	1.2 b	1.8 a	1.5 b	1.8 a	1.6 a	2.2 b	1.8 a	1.6	1.6	1.5	1.7	2.0	1.9	2.0	2.0	1.4 ac	1.8 b	1.7 ab	1.2 c
Total		**16.6 a**	**15.0 b**	**16.6 a**	**15.1 b**	**17.5 a**	**14.9 b**	**17.3 a**	**16.7 b**	**15.6**	**15.9**	**15.0**	**15.4**	**17.2**	**16.6**	**17.2**	**17.5**	**16.1**	**17.0**	**17.1**	**16.7**
Gram-negative	16:1w9	4.7 a	6.3 b	5.4 c	5.3 c	4.9 a	5.8 b	5.4 b	5.6 b	4.7 a	5.4 b	5.0 ab	5.2 b	4.9 a	5.2 ab	5.3 ab	5.6 b	5.1 a	5.8 b	5.8 b	5.7 b
Gram-negative	16:1w5	3.2	2.8	3.1	2.4	3.5 ab	3.1 ab	3.8 a	3.0 b	3.3 a	3.3 a	3.2 ab	2.5 b	3.0	3.1	2.9	2.8	3.4 a	2.6 b	2.5 b	2.1 b
Gram-negative	18:1w7	7.9 a	9.7 b	8.7 ab	9.4 b	7.5 a	11.7 b	9.0 c	9.9 c	7.8 a	9.3 b	8.4 ab	9.1 b	7.8	7.9	8.4	8.7	8.4	8.9	9.3	9.1
Gram-negative	cy17:0	1.5 ab	1.4 a	1.8 b	1.3 a	1.6 ac	1.5 a	1.9 b	1.8 bc	1.4	1.6	1.5	1.6	1.6 a	1.7 ab	1.8 ab	1.9 b	1.1 a	1.5 bc	1.6 c	1.2 ab
Gram-negative	cy19:0	4.4 ab	4.2 ab	4.8 a	3.8 b	4.2	4.6	4.7	4.8	4.6	4.8	4.8	4.7	4.9	4.7	4.9	4.8	4.2 ac	4.7 ab	4.8 b	4.0 c
Total		**21.7 a**	**24.5 b**	**23.8 b**	**22.3 a**	**21.7 a**	**26.7 b**	**24.7 c**	**25.1 c**	**21.8 a**	**24.3 b**	**22.7 ac**	**23.0 c**	**22.2 a**	**22.6 a**	**23.4 ab**	**23.9 b**	**22.3 a**	**23.5 b**	**24.0 b**	**22.2 a**
Actinomycetes	10Me16:0	3.9 a	6.1 b	4.1 a	3.5 a	4.4 a	6.5 b	4.3 a	4.3 a	3.8	4.1	3.6	3.7	3.8	3.8	4.0	4.1	4.0	4.3	4.2	3.8
Actinomycetes	10Me18:0	2.5	2.4	2.5	2.1	2.4	2.7	2.6	2.4	1.8 a	2.3 ab	2.3 b	2.0 ab	2.3	2.6	2.6	2.6	2.6 a	2.6 a	2.8 a	1.9 b
Total		**6.4 ad**	**8.5 b**	**6.6 ac**	**5.6 d**	**6.7 a**	**9.2 b**	**7.0 a**	**6.6 a**	**5.6**	**6.3**	**5.9**	**5.7**	**6.1**	**6.4**	**6.6**	**6.7**	**6.6 a**	**7.0 a**	**7.0 a**	**5.6 b**
Fungi	18:2w	5.5 a	3.8 b	5.5 a	5.4 a	11.7 ab	11.2 a	11.7 ab	12.4 b	12.6	13.2	13.1	12.5	6.3 a	6.2 ab	5.6 ab	5.1 b	11.8 a	12.2 ab	12.0 ab	12.8 b
Fungi	18:1w9	11.0 a	12.8 b	11.8 a	13.4 b	4.6	4.8	5.3	4.3	6.7 ac	5.7 ab	7.1 c	5.4 b	11.5	12.4	11.6	11.7	3.5	4.0	4.7	4.6
Total		**16.5 a**	**16.6 a**	**17.3 ab**	**18.8 b**	**16.3**	**15.9**	**17.0**	**16.7**	**19.2 ab**	**18.9 ab**	**20.2 a**	**17.9 b**	**17.9 ab**	**18.6 a**	**17.2 ab**	**16.8 b**	**15.3 a**	**16.2 a**	**16.7 ab**	**17.4 b**
Protozoa	20:4w6	4.1 a	1.1 b	1.8 b	1.6 b	1.7	2.0	1.4	1.4	1.9 ab	1.1 a	2.7 ab	2.8 b	2.3	2.3	2.1	2.1	0.4	1.1	1.3	1.3
Common to all	16:0	23.2 a	24.7 ab	23.7 a	26.3 b	25.2 a	20.4 b	24.0 a	23.9 a	24.0	24.0	23.4	24.3	22.5	22.2	22.8	21.8	25.0 a	23.7 a	23.1 a	27.5 b
Common to all	18:0	5.8	6.4	5.6	5.5	5.8	6.4	5.3	5.5	5.9	5.5	5.3	6.0	6.2	5.9	5.8	5.7	6.9 a	6.6 ab	6.2 ab	5.5 b
?	20:0	1.7	0.8	1.4	1.2	1.6	1.5	1.2	1.1	1.9	0.9	1.7	1.4	2.1 a	1.8 b	1.6 b	1.7 b	3.4	1.7	1.4	0.8
?	22:0	4.1 ac	2.4 b	3.2 bc	3.7 c	3.4 a	3.0 a	2.2 b	3.1 a	4.1 a	3.0 b	3.2 b	3.4 ab	3.5	3.5	3.3	3.7	4.0 a	3.3 ab	3.2 b	3.0 b

[†] Different letters indicate significant differences (p <0.05) between treatments

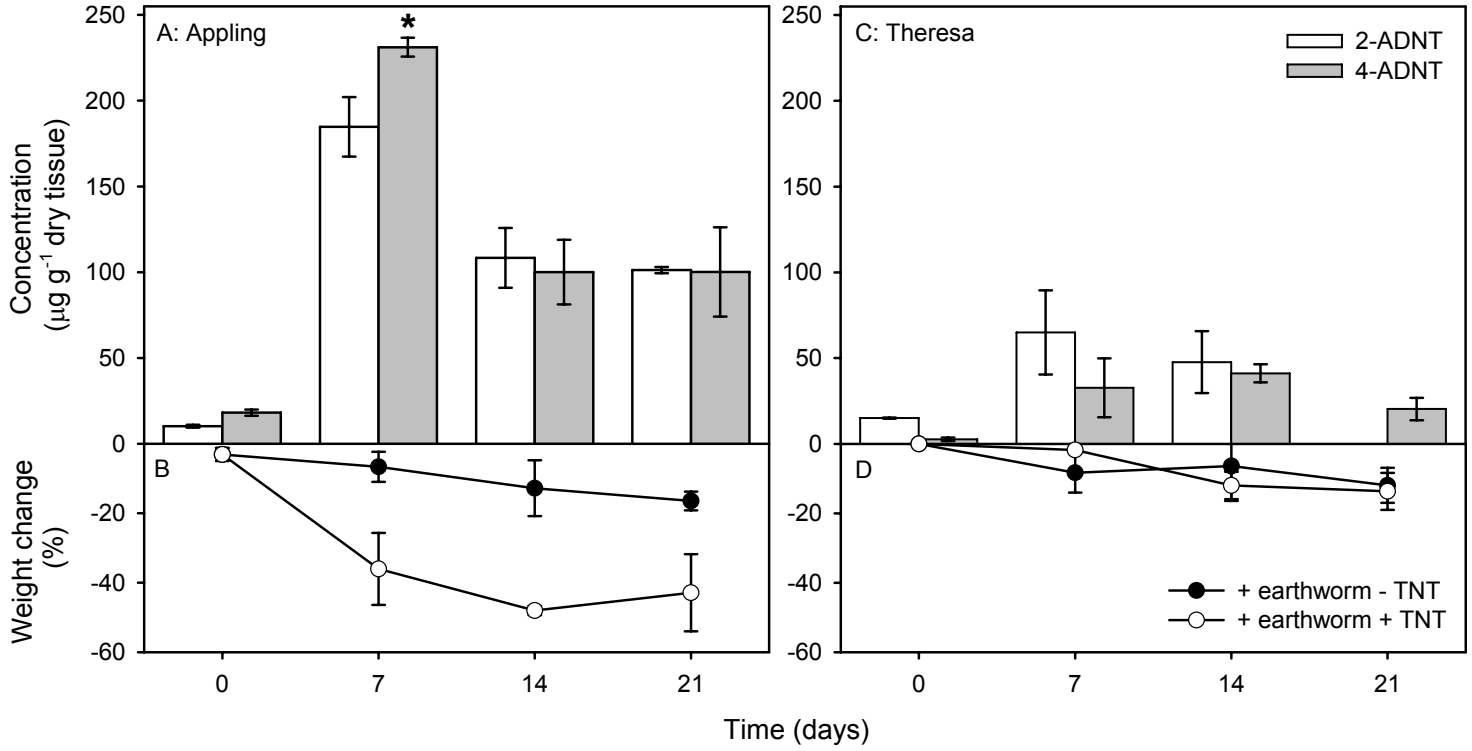


Figure 5.1. Concentrations of 2-ADNT and 4-ADNT in earthworms (A, C) and their biomass (B,D) after exposure to TNT in an Appling sandy loam and a Theresa silt loam at initial soil concentrations of 96 mg kg^{-1} and 99 mg kg^{-1}, respectively (C,D). Error bars represent ± 1 S.D., $n = 3$, * $n = 2$.

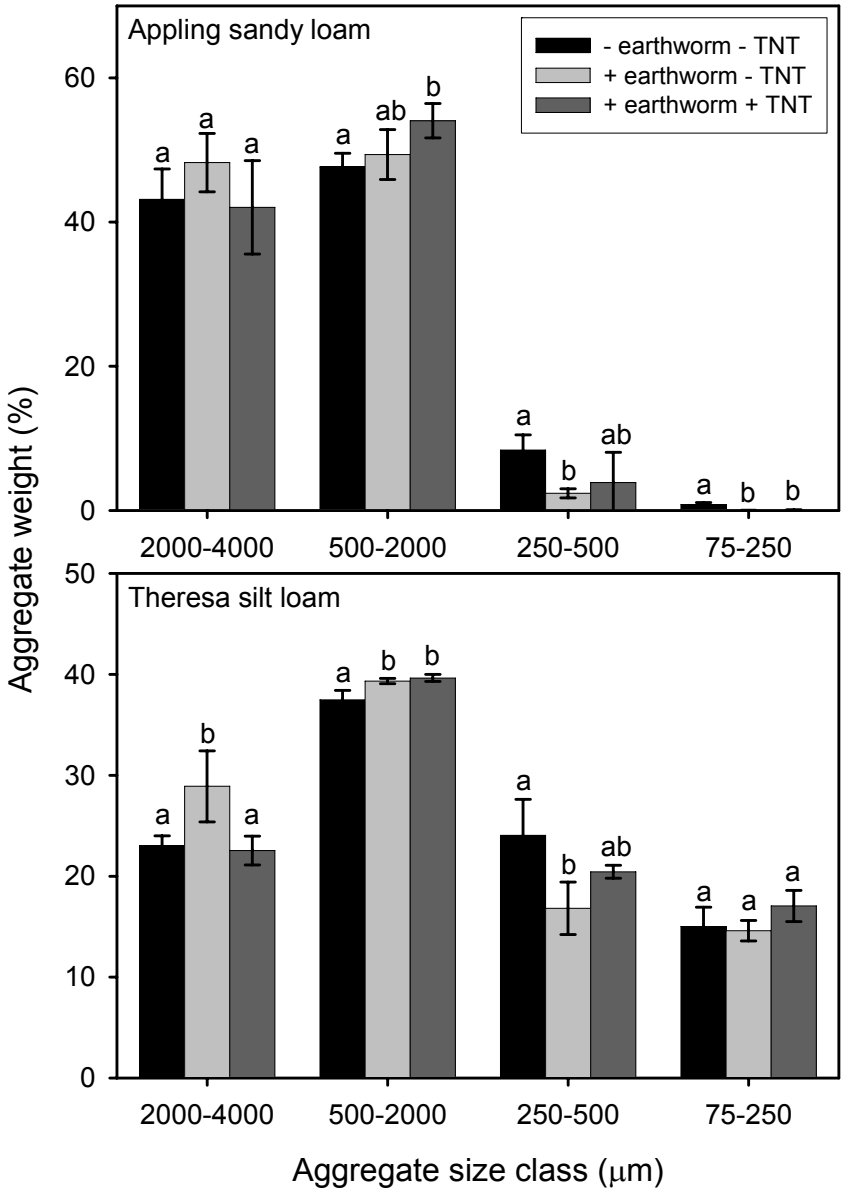


Figure 5.2. Aggregate size distribution of an Appling sandy loam and a Theresa silt loam after 21 days of exposure to TNT and earthworms. Values followed by a different lowercase letter within aggregate size class are significantly different between treatments (mean ± 1 S.D., $n = 3$).

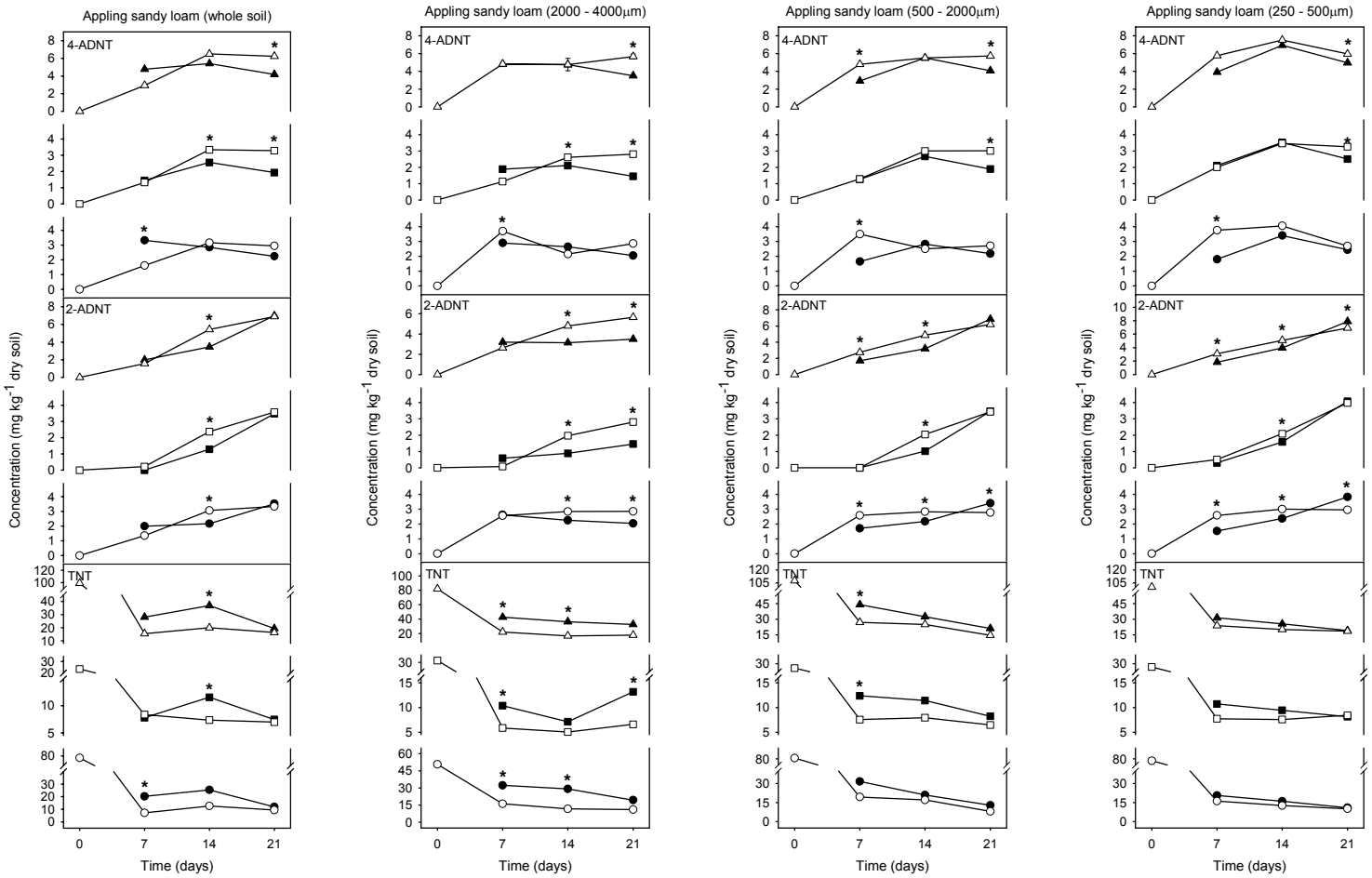


Figure 5.3. Concentration of readily extractable (-●- -○-), potentially extractable (-■- -□-), and total extractable (-▲- -Δ-) TNT and its two major metabolites (2-ADNT and 4-ADNT) in the whole soil, 2000 – 4000, 500 – 2000, and 250 – 500 μm aggregates of an Appling sandy loam with and without earthworms (open and closed symbols, respectively). Asterisks denote significant differences (using Fishers LSD) between treatments at each time period ($p < 0.05$, $n = 3$).

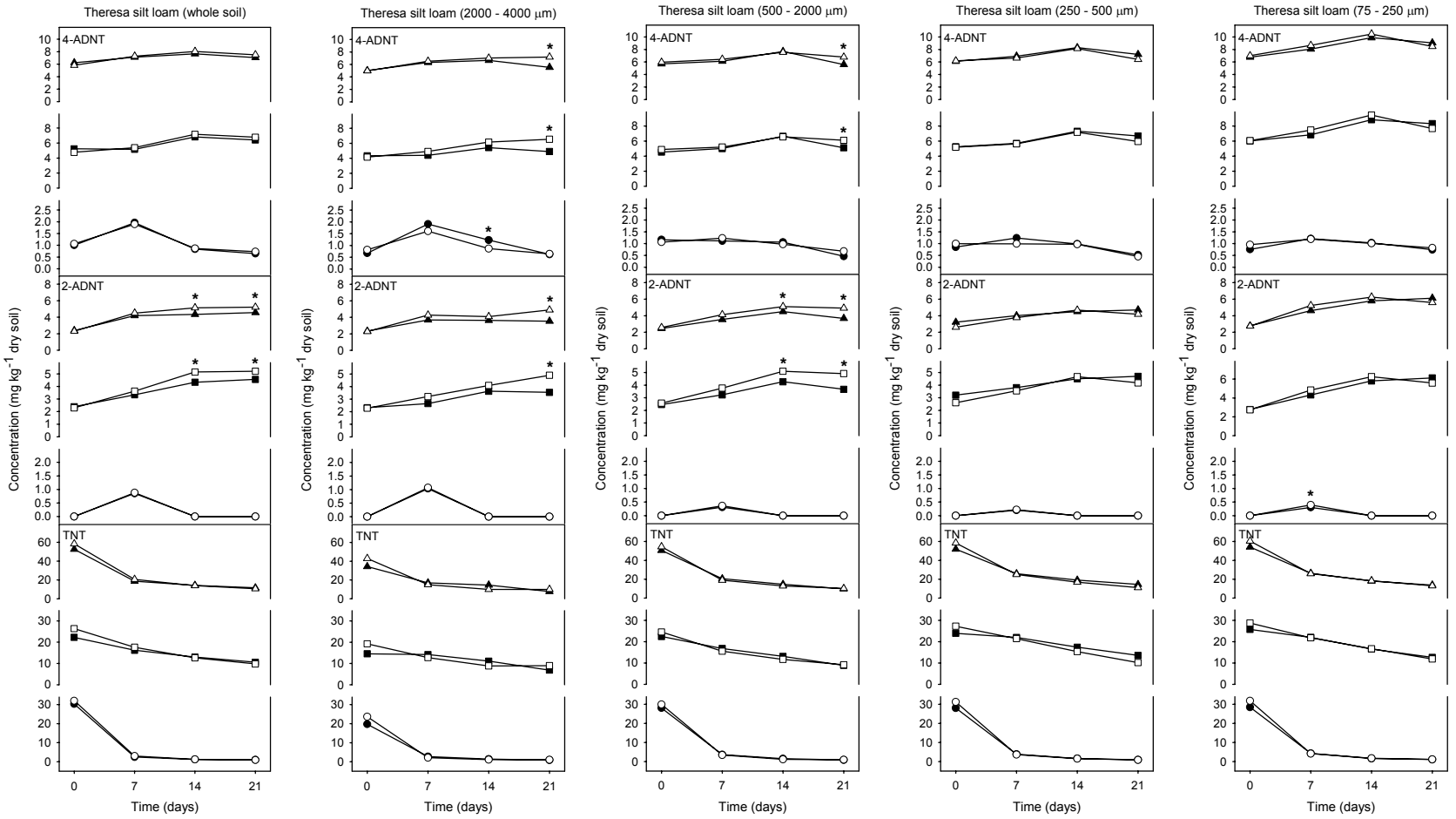


Figure 5.4. Concentration of readily extractable (-●- -○-), potentially extractable (-■- -□-), and total extractable (-▲- -Δ-) TNT and its two major metabolites (2-ADNT and 4-ADNT) in the whole soil, 2000 – 4000, 500 – 2000, 250 – 500, and 75 – 250 μm aggregates of a Theresa silt loam with and without earthworms (open and closed symbols, respectively). Asterisks denote significant differences (using Fishers LSD) between treatments at each time period ($p < 0.05$, $n = 3$).

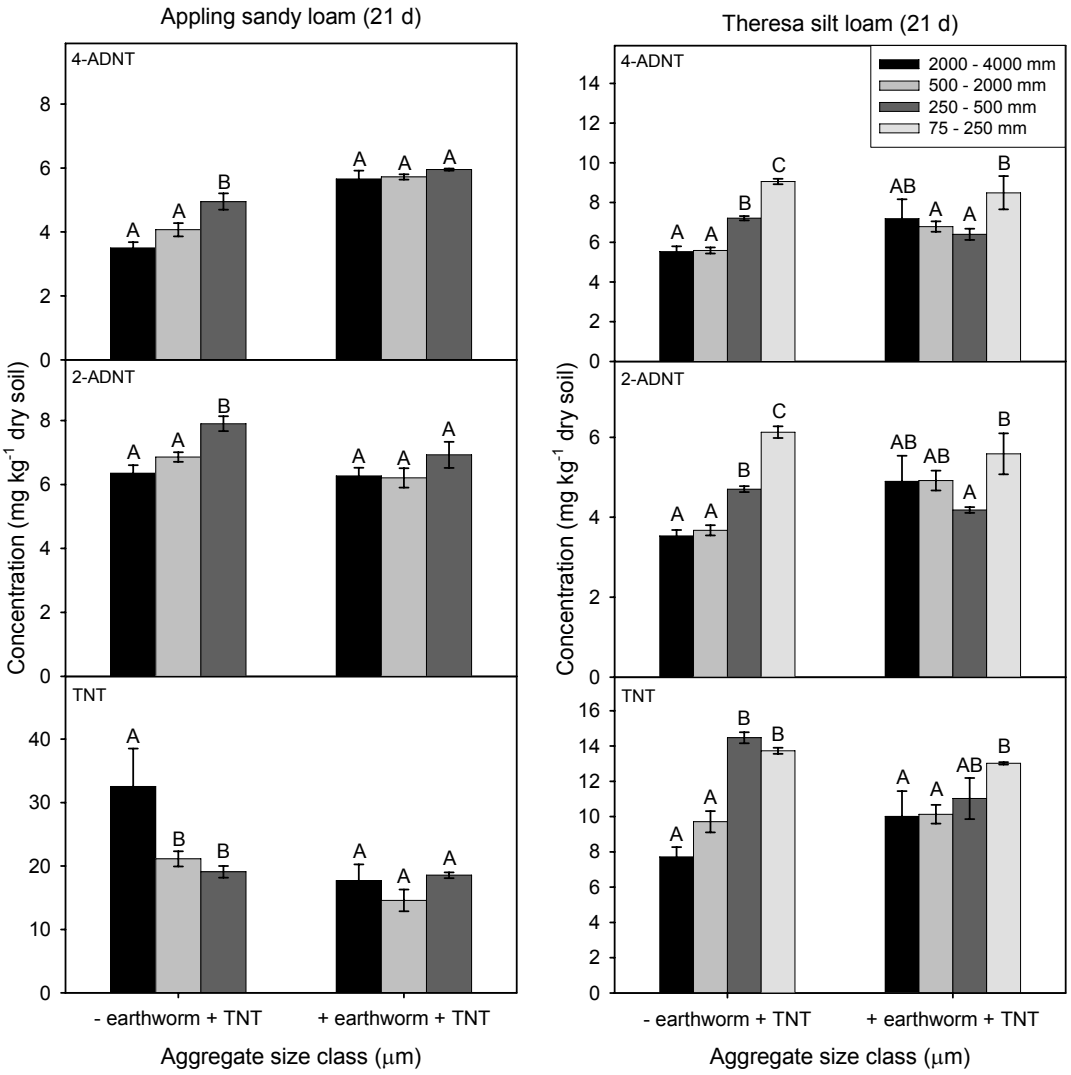


Figure 5.5. Concentration of total extractable TNT and its two major metabolites (2-ADNT and 4-ADNT) at 21 d in the different sized aggregates of an Appling sandy loam (left panel) and a Theresa silt loam (right panel) with and without earthworms. Different letters denote significant differences (using Fishers LSD) between aggregate size fractions for each treatment ($p < 0.05$, $n = 3$).

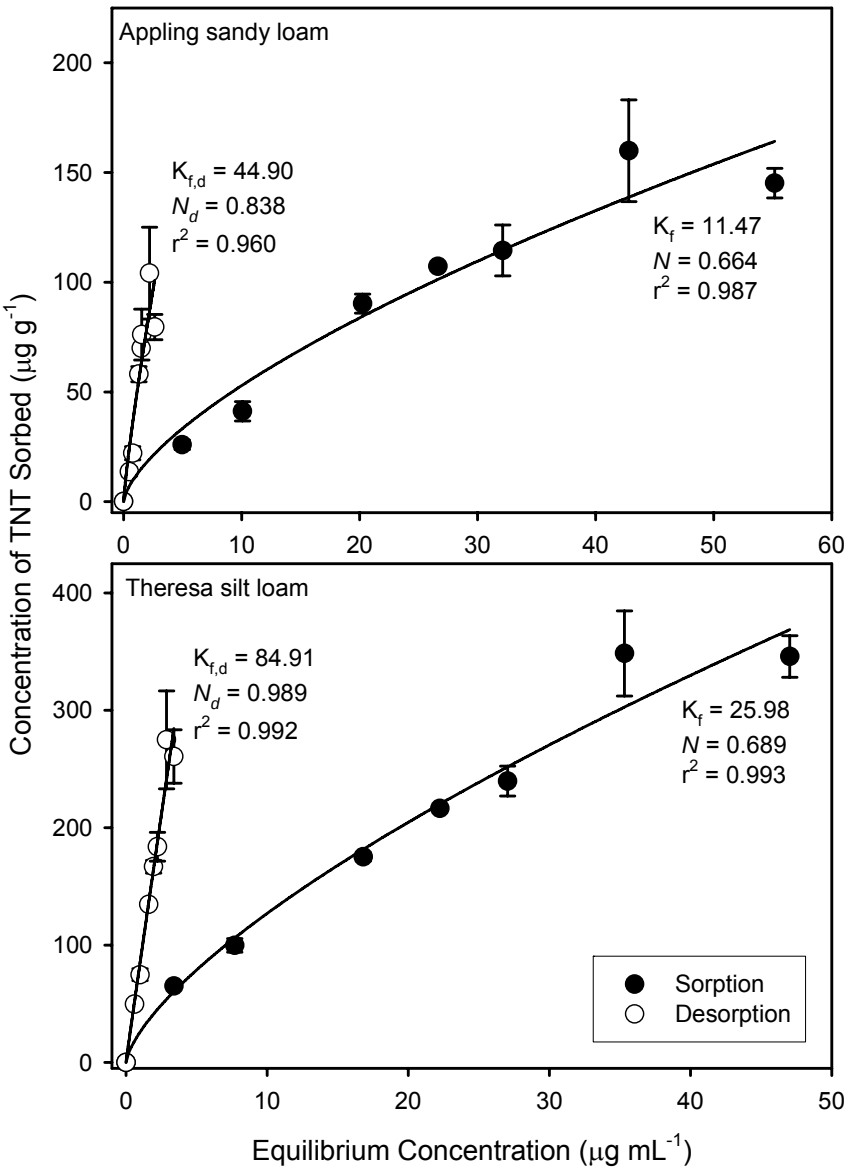


Figure 5.6. Sorption/desorption isotherms of TNT to an Appling sandy loam (GA) and a Theresa silt loam (WI) soil (mean ± 1 S.D., n = 3).

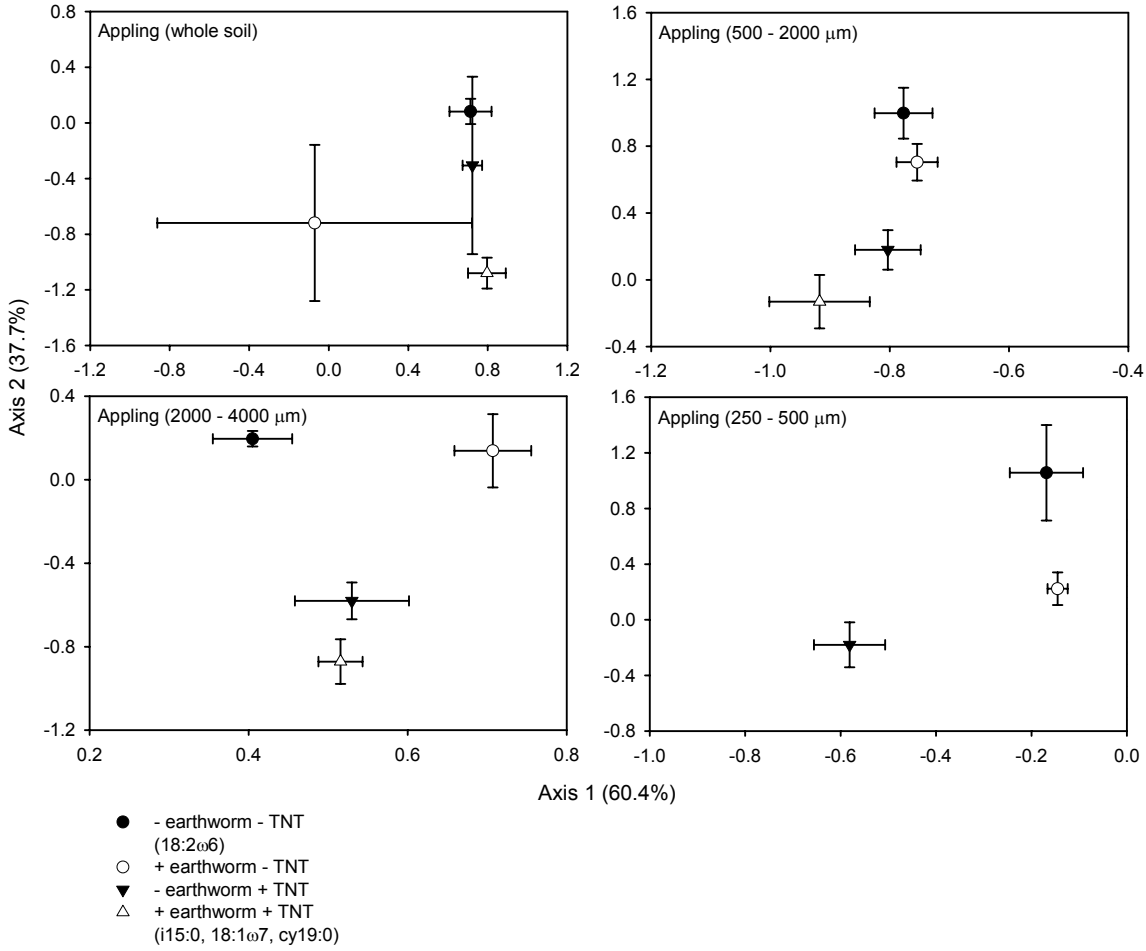


Figure 5.7. Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs in whole soil, 2000 – 4000, 500 – 2000, and 250 – 500 μm aggregates of an Appling sandy loam soil after 21 d of exposure to earthworm and/or TNT. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard error, respectively (n = 3).

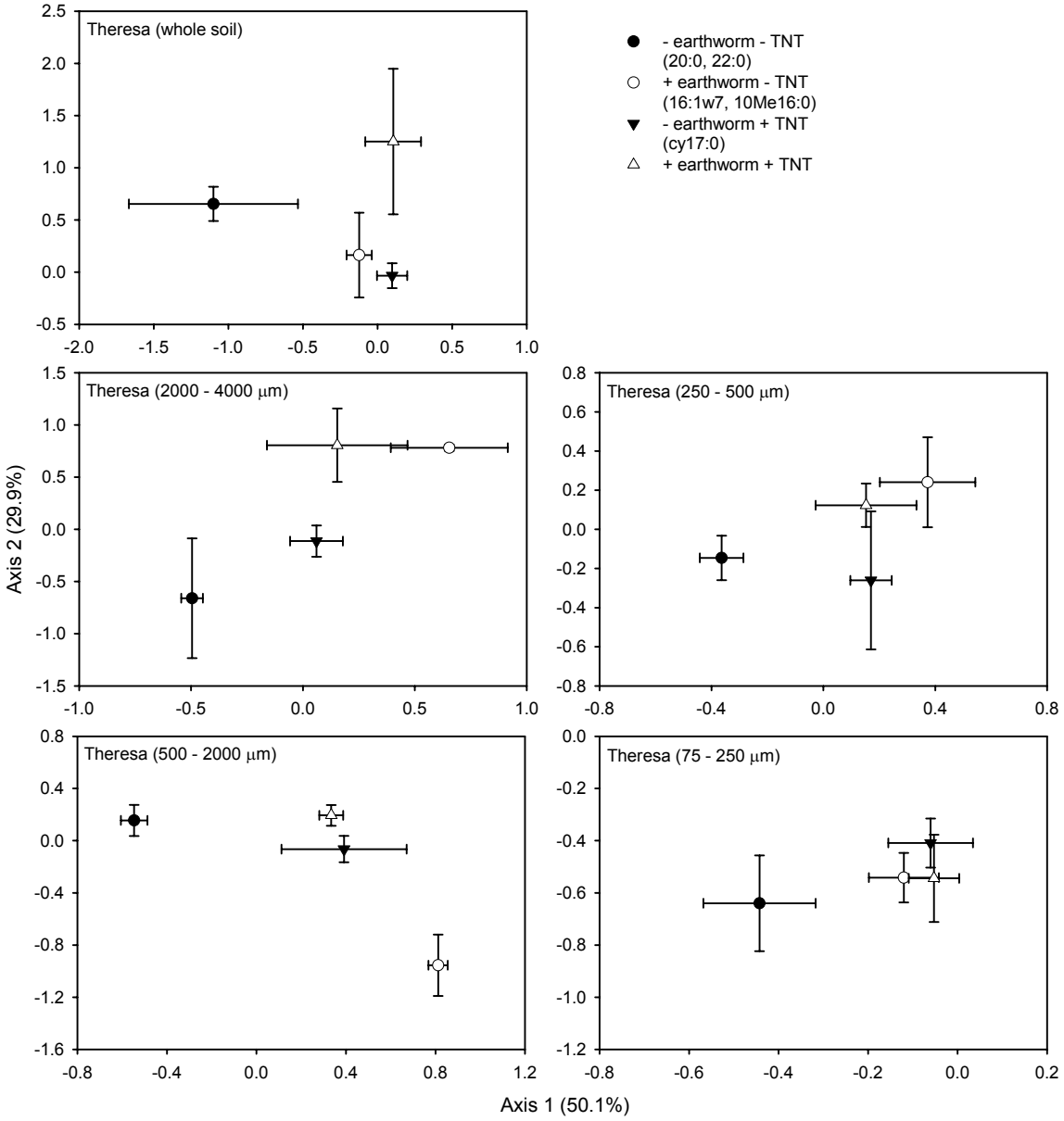


Figure 5.8. Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500, and 75 – 250 μm aggregates of a Theresa silt loam soil after 21 d of exposure to earthworm and/or TNT. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard error, respectively (n = 3).

CHAPTER 6

SUMMARY AND CONCLUSION

Across the US and around the world there are many examples and situations where our natural resources are being polluted and require urgent attention in order to maintain and sustain all forms of life. Every day we are faced with a need to develop innovative and effective methods to handle environmental issues whether they are accidental or natural disasters. A typical approach to assess and remediate contaminated soil is to look at general processes and interactions of the soil as a whole. However, taking a scaled-down approach and focusing on specific soil components may provide a better understanding of what has happened and what can be done. The research presented in this dissertation demonstrated that focusing on the interactions between organic contaminants and soil aggregates or soil particles (i.e. clay, humic-clay complexes) is an effective method to characterize environmental fate processes (i.e. sorption, desorption, transformation, etc). Additionally, it is important to consider the method used to obtain soil aggregates as it may influence measurements and assessment of processes and properties specific to soil aggregates.

In chapter 3, experiments performed clearly demonstrated the role of natural organic matter in the retention of TNT by clay minerals. In general TNT sorption to clay minerals and humic-clay complexes was dependent on the prevailing exchangeable cation and the associated energy of hydration. Sorption to K-saturated clays and humic-clay complexes were greater than to Ca-saturated minerals because K^+ has a low energy of hydration, thus making it easier for TNT to form inner-sphere complexes and/or replace K on the clay surface, depending on which sorption mechanism is taking place (i.e. EDA complex vs inner-sphere interactions). TNT sorption to humic acid was significant and increased with a decrease in humic acid concentration. Additionally, humic acid

associated with prepared minerals such as smectite (Ca-HA-SAz, K-HA-SAz, K-HA-SWy) and kaolinite (K-HA-KGa) enhanced TNT sorption compared to their corresponding homoionic clay, while TNT sorption to hectorite (K-HA-SHCa) was inhibited. Previous studies have noted the possibility of TNT, which has a moderately low solubility, participating in hydrophobic interactions with neutral siloxane sites on clay minerals such as kaolinite and some smectites. Although not confirmed, humic acid associated with K-SHCa may have prevented TNT from interacting with neutral siloxane sites on K-SHCa which has been characterized as exhibiting hydrophobic characteristics. Humic acid increased sorption to K-HA-SWy compared to K-SWy, however, based on XRD analysis, both had similar *d*-spacings when exposed to 60 $\mu g\ mL^{-1}$ TNT. Thus, the increase in TNT sorption to K-HA-SWy over K-SWy may be due to enhanced sorption to the clay mineral surface, as the degree of interlayer association between the two (sorption) are similar. Finally, the sequential preparation of humic-clay complexes using K-SWy and the same humic acid solution supported the findings of previous studies indicating the fractionation and preferential sorption of humic acid with clay minerals. TNT sorption decreased with each additional sequentially prepared humic-clay complex, thus indicating that the properties and nature of the humic-acid associated with the clay mineral had changed.

Clay minerals, soil organic matter, and their interactions are not only important in the retention of contaminants such as TNT, but they are also the main factors governing soil aggregation. In chapter 4, experiments focused on how soil fractionation methods may influence characterization of properties and processes associated with soil aggregates. Three different soils with contrasting clay mineralogy and soil organic

matter were exposed to wet- and dry-sieving methods. As was expected, dry- and wet-sieving resulted in different aggregate size distributions for all three soils. Wet-sieving yielded a greater distribution of soil aggregates less than 250 μm and a significant reduction in the 500 – 2000 μm sized aggregates for all three soils tested. Although differences in total carbon within soil aggregates were not as profound, observed significant differences in the CEC, microbial biomass, and microbial community structure between the two sieving methods could be explained based on the clay mineralogy and dominant aggregate stabilization mechanism for each soil. FAME analysis used to characterize the microbial community structure indicated that wet sieving had the most significant impact on the 75 – 250 μm aggregate size fraction regardless of soil type. This was due largely to the redistribution of soil particles including fine sand from large aggregates (i.e. > 500 μm) into smaller size fractions (< 250 μm). These results do not necessarily imply that research focused on aggregate specific interactions or associations in soil is fruitless, however. In instances where inherent aggregate stability is the key question of an experiment, the tendency for aggregates to break down into smaller sized fractions, as with wet-sieving, might provide consistent data. On the other hand, when researchers intend to study the natural associations found within aggregate hierarchies, or need to extract biological or chemical information that is sensitive to the effects of sieving, then careful consideration of the likelihood of artifacts should be used to select the best aggregate separation method.

The first two studies build up to the third and final study presented in chapter 5. This study focused on how earthworms could influence the fate of TNT within soil and soil aggregates of two contrasting soil types. Additionally, the impact of earthworms and

TNT on the soil microbial community structure was characterized by FAME analysis. Although both soils were exposed to similar levels of TNT, the inherent bioavailability of TNT in each soil was different. Analysis of earthworm tissue and soil extractions indicated that TNT was more bioavailable in the Appling sandy loam (GA) compared to the Theresa silt loam (WI). Earthworms in the Appling soil experienced a reduction in biomass as a result of TNT exposure and accumulated high levels of the metabolites 2-ADNT and 4-ADNT. In contrast, earthworms in the Theresa soil accumulated much lower amounts of 2-ADNT and 4-ADNT and had no significant loss in biomass compared to earthworms not exposed to TNT. Additionally, in the Appling soil, exposure to TNT reduced the impact earthworms had on soil aggregation. However, a similar observation was observed in the Theresa soil indicating that TNT may influence soil aggregation by altering the nature of humic-clay complexes. This effect may be more profound due to the breakdown of ingested soil material by earthworms which is then excreted as a cast and contributes to the formation of stable aggregates. Earthworms demonstrated the ability to enhance TNT disappearance, particularly during the first 7 d and the extraction of metabolites after 14 d in the Appling soil. However, the low bioavailability of TNT in the Theresa soil resulted in earthworms having no effect on the disappearance of TNT. However, similarly to the Appling soil, earthworms did result in greater amounts of extracted 2-ADNT and 4-ADNT. Furthermore, the ability of earthworms to influence the disappearance of TNT diminishes with time due to the formation of irreversibly bound residues. Regardless of soil type, however, earthworms demonstrated the ability to influence the distribution of TNT and metabolites within soil aggregates. For example, earthworms reduced the levels of TNT in the 2000 – 4000 μm

size fraction of the Appling soil compared to smaller size fractions. However, in the Theresa soil, it appears earthworms redistributed TNT from smaller soil aggregates into larger soil aggregates by ingestion of soil material and release in earthworm casts. Earthworms and TNT influenced the microbial community structure in both soils. In particular the presence of TNT and earthworms increased the mol % of fatty acids indicative of gram-negative bacteria, indicating that favorable conditions or the ability to tolerate these factors to a better extent then other soil organisms (such as actinomycetes or gram-positive bacteria). Overall, earthworms had the biggest impact on TNT disappearance, metabolite extraction, and microbial community structure of soil macroaggregates > 2000 μm.

In the end, the work presented in this dissertation resulted in some interesting discoveries and theories related to the dynamics of TNT fate in soil aggregates. However, many of them require further validation to prove if they are true. For example, without using some form of a tracer we do not know for sure to what extent earthworms are redistributing TNT in the Theresa soil. Based on the changes in the amount of TNT extracted from each size fraction between treatments, we have indirect rather than direct evidence. In a situation where resources are not limited I feel that closer examination and revisiting of these studies using radio-labeled compounds (TNT, 2-ADNT, 4-ADNT) and use of molecular chemistry and instrumentation (FTIR, NMR, IR, etc) would be useful.

APPENDIX A

SUPPLEMENTAL DATA FROM CHAPTER 3

INTERACTION OF 2,4,6-TRINITROTOLUENE WITH HUMIC ACID, CLAY MINERALS, AND HUMIC-CLAY COMPLEXES

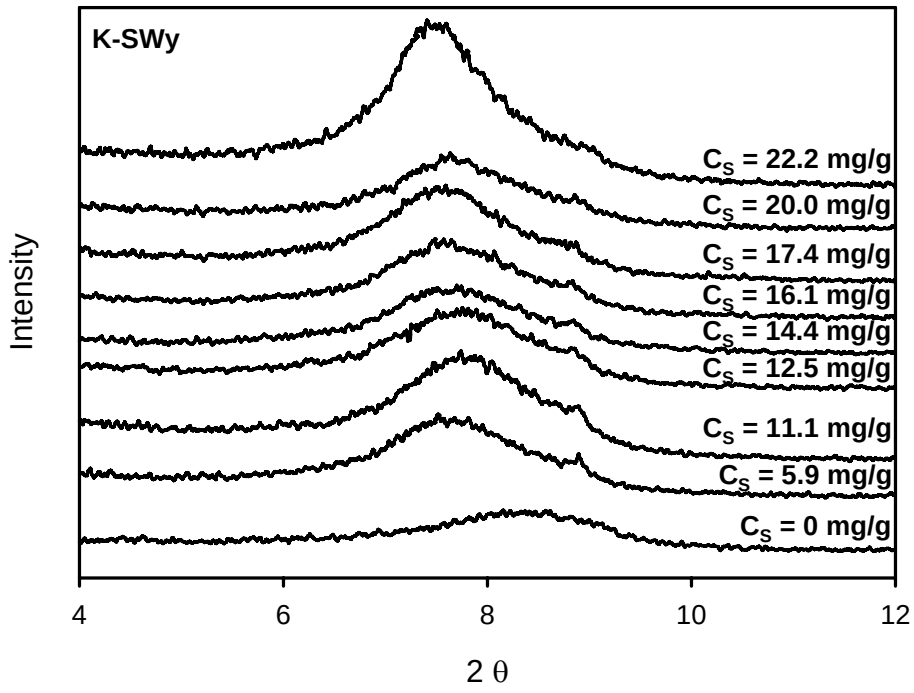


Figure A.1 X-ray diffraction patterns of K-SWy as a function of TNT sorption (Cu-K_α radiation, 0% humidity).

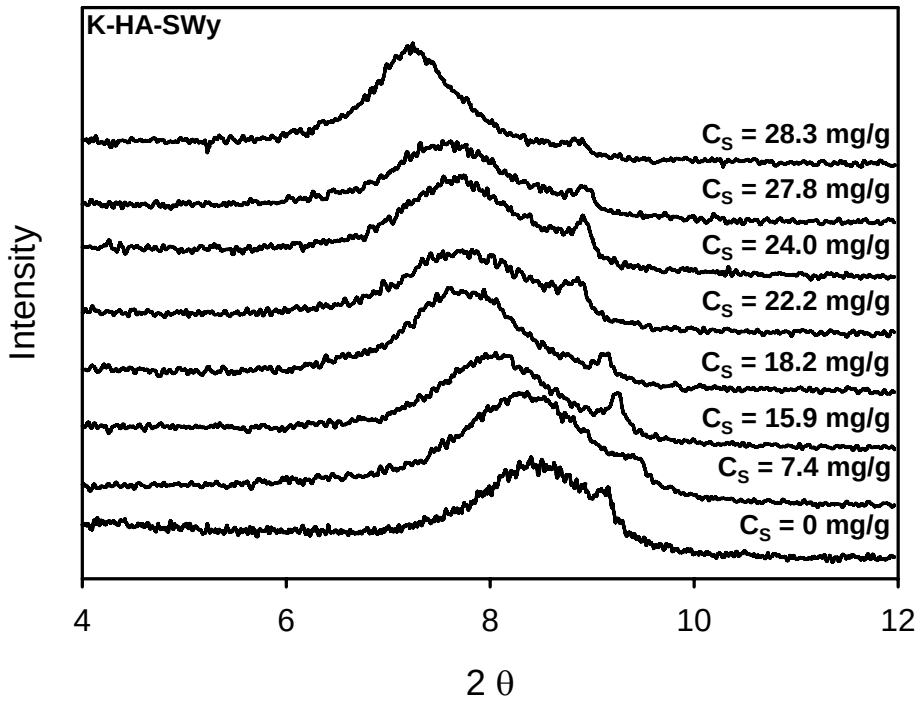


Figure A.2 X-ray diffraction patterns of K-HA-SWy as a function of TNT sorption (Cu-K_α radiation, 0% humidity)..

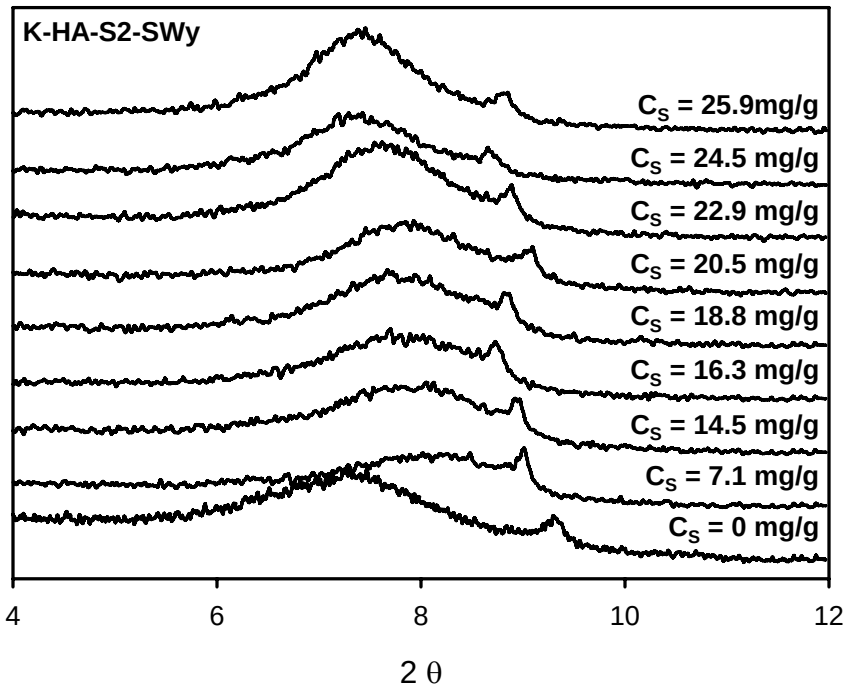


Figure A.3 X-ray diffraction patterns of K-HA-S2-SWy as a function of TNT sorption (Cu-K_α radiation, 0% humidity)..

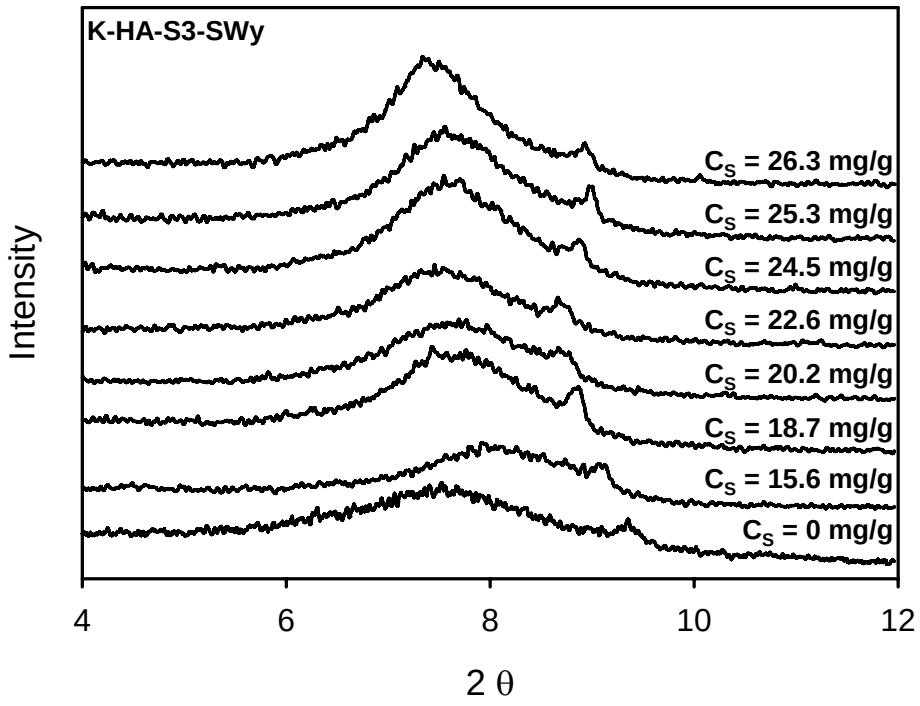


Figure A.4 X-ray diffraction patterns of K-HA-S3-SWy as a function of TNT sorption (Cu-K_α radiation, 0% humidity)..

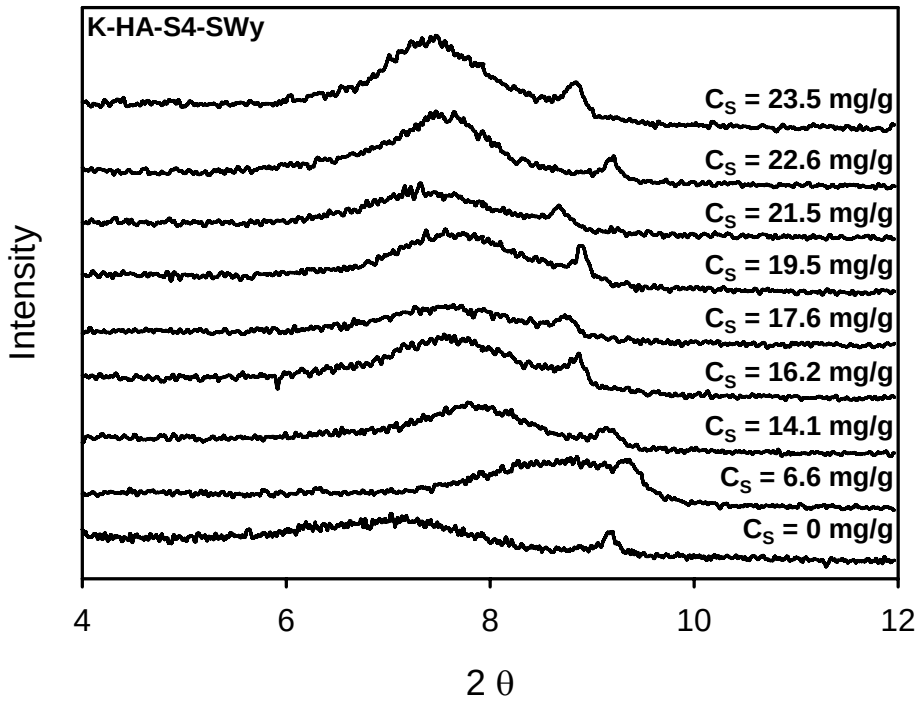


Figure A.4 X-ray diffraction patterns of K-HA-S4-SWy as a function of TNT sorption (Cu-K_α radiation, 0% humidity).

APPENDIX B

SUPPLEMENTAL DATA FROM CHAPTER 4

IMPACT OF SIZE FRACTIONATION METHODS ON PROPERTIES OF SOIL AGGREGATES

Table B.1. Cation exchange capacity (CEC) and concentration of total carbon (TC) in soil aggregates collected from dry- and wet-sieving methods[†].

Soil	Diameter mm	Dry-sieved aggregates CEC $cmol_c\ kg^{-1}$		Dry-sieved aggregates TC mg C g-1 soil		Wet-sieved aggregates CEC $cmol_c\ kg^{-1}$		Wet-sieved aggregates TC mg C g^{-1} soil	
Theresa	4000-2000	18.2 (13)		29.5 (2.9)		21.8 (1.0)	**AB**	33.1 (4.4)	**AB**
	2000-500	18.7 (1.7)	**a**	31.0 (0.9)		24.3 (1.1)	**b A**	38.7 (3.2)	**A**
	500-250	18.2 (1.8)		28.7 (2.0)		19.2 (1.4)	**B**	23.7 (1.8)	**B**
	250-75	18.8 (0.6)		31.3 (1.2)		20.9 (1.3)	**AB**	26.5 (1.9)	**B**
Appling	4000-2000	5.4 (0.2)		11.4 (1.3)		3.9 (0.5)		9.8 (1.2)	
	2000-500	5.8 (0.8)		13.2 (1.7)		3.7 (0.3)		10.9 (0.5)	
	500-250	5.4 (0.1)		13.5 (0.5)		3.0 (0.1)		8.0 (1.2)	
	250-75	5.4 (0.2)		14.3 (0.2)		3.7 (0.1)		11.5 (0.8)	
Marlette-Oshtemo	4000-2000	13.9 (1.5)	**a**	19.8 (4.6)	**A**	17.9 (0.9)	**b**	20.7 (8.8)	**A**
	2000-500	13.3 (0.8)		12.9 (1.0)	**AB**	15.9 (1.4)		17.5 (3.6)	**A**
	500-250	13.4 (1.5)		9.3 (1.6)	**B**	15.1 (0.3)		6.0 (0.4)	**B**
	250-75	12.6 (0.7)		8.4 (0.6)	**B**	14.8 (1.6)		6.1(1.1)	**B**

[†] CEC and TC data are presented based on the whole soil (sand fraction not excluded). Standard deviations are given in parentheses. Small case letters indicate significant differences between sieving methods for the specific aggregate size fraction and soil type. Large case letters indicate significant differences between aggregate size fractions for a specific soil type (p-value ≤ 0.01, $n = 3$).

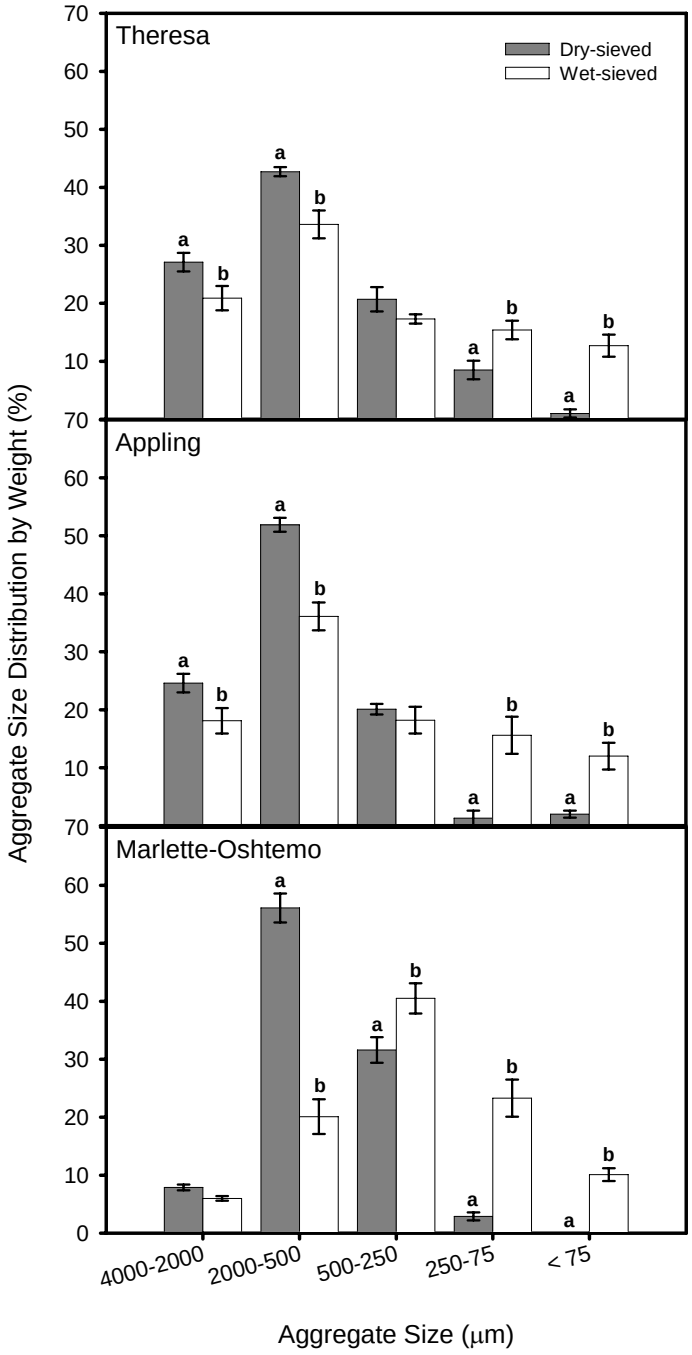


Figure B.1. The amount of soil within each aggregate size fraction as a percentage of the whole soil (top-Theresa, middle-Appling, bottom-Marlette-Oshtemo). Significant differences between sieving methods are represented by different letters on the graph ($p < 0.01$, mean ± 1 S.D.).

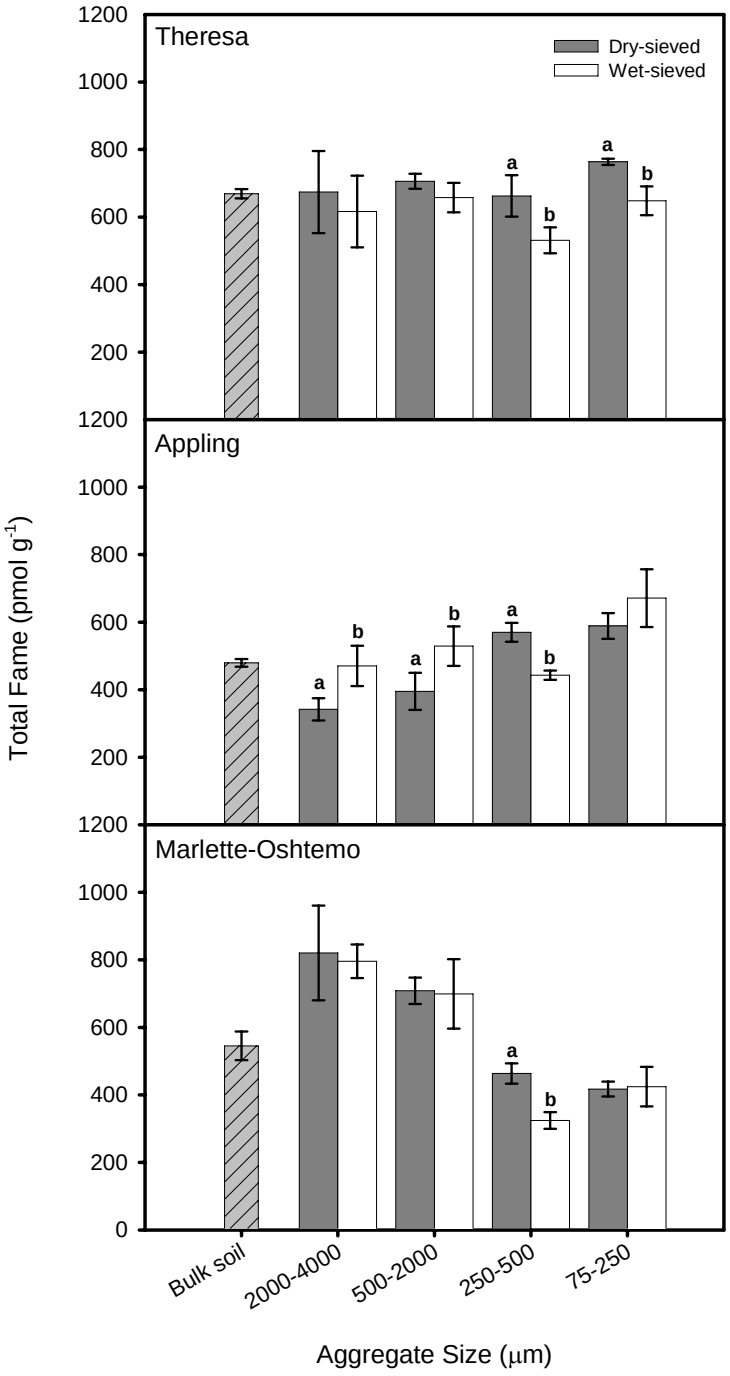


Figure B.2. Total fatty acid methyl esters (FAME) within each aggregate size fraction obtained by using dry- and wet-sieving techniques (top-Theresa, middle-Appling, bottom-Marlette-Oshtemo). Significant differences between sieving methods are represented by different letters (p-value ≤ 0.01, mean $\pm$ 1 S.D.).

APPENDIX C

SUPPLEMENTAL DATA FROM CHAPTER 5

FATE OF 2,4,6-TRINITROTOLUENE (TNT) AND MICROBIAL COMMUNITY STRUCTURE WITHIN SOIL AGGREGATES IN THE PRESENCE OF EARTHWORMS

Table C.1. The percentage of TNT, 2-ADNT, and 4-ADNT recovered from soil aggregates compared to the whole soil.

Soil	Treatment	Time			
		Day 0	Day 7	Day 14	Day 21
			TNT		
			% Recovery[†] (± 1 S.D.)		
Appling	- Ew + TNT	ND[‡]	158.2 (54.6)[§]	92.8 (24.2)	132.7 (38.9)
	+ Ew + TNT	107.6 (41.7)	191.1 (101.5)	117.6 (45.3)	99.1 (24.0)
Theresa	- Ew + TNT	86.9 (4.2)	123.0 (3.6)	111.3 (7.8)	94.3 (5.3)
	+ Ew + TNT	92.6 (24.8)	109.4 (3.2)	100.0 (0.6)	102.4 (8.5)
			2-ADNT		
			% Recovery (± 1 S.D.)		
Appling	- Ew + TNT	--[§]	112.6 (20.5)	94.7 (4.0)	96.7 (3.9)
	+ Ew + TNT	--	199.2 (86.7)	89.5 (1.8)	90.6 (1.2)
Theresa	- Ew + TNT	104.5 (13.3)	92.7 (1.3)	101.5 (1.0)	96.0 (3.1)
	+ Ew + TNT	111.1 (14.0)	95.6 (1.8)	96.7 (3.2)	94.8 (4.2)
			4-ADNT		
			% Recovery (± 1 S.D.)		
Appling	- Ew + TNT	--	77.9 (17.5)	100.2 (5.6)	94.3 (8.1)
	+ Ew + TNT	--	173.7 (41.6)	83.0 (15.6)	92.8 (13.2)
Theresa	- Ew + TNT	92.2 (1.7)	93.4 (1.2)	104.9 (3.9)	92.8 (1.2)
	+ Ew + TNT	99.5 (5.1)	95.2 (0.2)	99.8 (1.2)	95.4 (1.4)

[†] The percent recovery is calculated as the sum of total (CaCl2 + acetonitrile extraction) TNT, 2-ADNT, or 4-ADNT in all size fractions divided by the amount in the whole soil.

[‡] ND, not determined

[§] values were below the detection limits

Table C.2. Amount of individual FAMEs from 21 d incubation experiment involving an Appling sandy loam soil.

		whole soil				2000 - 4000 μm				500 - 2000 μm				250 - 500 μm			
Interpretation	**Fatty Acid**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**
									nmol/g dw								
Gram-positive	i15:0	12.1	11.7	12.2	11.2	11.5 a	9.2 b	9.2 b	10.6 ab	9.0	8.0	9.2	9.1	10.3	10.4	9.6	ND[‡]
Gram-positive	a15:0	6.9	6.8	7.1	6.6	6.2	5.3	5.4	6.1	3.8	3.5	4.4	4.1	5.0	5.0	5.0	ND
Gram-positive	i16:0	10.8	10.4	10.5	9.6	10.2 a	8.4 b	8.0 b	8.8 ab	7.6	6.9	7.7	7.6	8.6	8.9	7.8	ND
Gram-positive	i17:0	6.5	6.4	6.5	6.0	5.9 a	4.9 b	4.9 b	5.5 ab	3.5	3.2	3.6	3.4	4.3	4.7	4.0	ND
Gram-positive	a17:0	6.7	6.5	6.9	6.2	6.2 a	5.2 b	5.1 b	5.6 a	3.7	3.3	4.1	3.8	4.6	4.7	4.3	ND
Total		**43.1**	**41.8**	**43.2**	**39.5**	**39.9 a**	**33.0 b**	**32.6 b**	**36.7 ab**	**27.5**	**24.9**	**28.9**	**28.0**	**32.9**	**33.7**	**30.6**	**ND**
Gram-negative	16:1w9	12.0	11.3	11.9	10.6	11.1 a	9.7 ab	9.3 b	9.6 ab	9.0	7.9	8.3	8.8	9.5	9.9	8.6	ND
Gram-negative	16:1w5	8.4 a	7.1 ab	6.9 ab	6.7 b	7.0	6.7	5.8	6.1	4.5	3.8	4.2	4.5	5.7	4.5	4.8	ND
Gram-negative	18:1w7	14.4	13.6	14.9	13.9	13.1 a	9.7 b	11.9 ab	13.2 a	11.0 ab	9.4 a	12.2 ab	12.2 b	9.9	10.5	11.5	ND
Gram-negative	cy17:0	4.8	4.8	5.9	4.9	4.1	4.1	3.4	3.6	1.7	1.7	1.5	2.0	2.6	2.6	2.7	ND
Gram-negative	cy19:0	10.0	9.7	11.0	10.0	9.5 a	7.3 b	7.8 b	8.2 ab	6.5 ab	5.6 a	7.8 b	7.5 b	8.0	8.0	7.5	ND
Total		**49.5**	**46.5**	**50.5**	**46.1**	**44.9 a**	**37.5 b**	**38.2 ab**	**40.8 ab**	**32.7**	**28.4**	**34.0**	**35.0**	**35.6**	**35.4**	**35.1**	**ND**
Actinomycetes	10Me16:0	9.8	8.9	9.6	9.0	8.7 a	7.1 b	7.0 b	7.7 ab	6.6	5.6	6.7	6.4	7.3	7.8	6.8	ND
Actinomycetes	10Me18:0	9.6 a	8.0 b	8.8 ab	7.5 b	8.5 a	6.4 b	6.5 b	6.6 b	5.9	5.1	5.7	5.1	6.8	7.1	5.6	ND
Total		**19.4**	**16.8**	**18.4**	**16.5**	**17.3 a**	**13.6 b**	**13.5 b**	**14.3 ab**	**12.5**	**10.7**	**12.5**	**11.6**	**14.1**	**14.9**	**12.4**	**ND**
Fungi	18:2w	25.4 ab	33.3 a	22.1 ab	14.0 b	23.9	18.1	14.8	14.2	22.0	16.7	17.1	14.5	24.4	18.9	13.9	ND
Fungi	18:1w9	36.5 ab	49.0 a	35.0 ab	26.3 b	35.4	28.5	25.9	25.9	32.5	27.8	29.3	27.5	36.8	30.1	28.5	ND
Total		**61.9 ab**	**82.2 a**	**57.1 ab**	**40.3 b**	**59.3**	**46.6**	**40.7**	**40.2**	**54.5**	**44.5**	**46.4**	**42.1**	**61.2**	**49.0**	**42.3**	**ND**
Protozoa	20:4w6	2.7 ab	2.6 a	3.1 b	2.5 a	2.2	2.0	2.2	2.1	--[§]	--	--	--	1.0 a	0.4 b	0.3 b	ND
Common to all	16:0	51.3	54.8	51.5	43.7	50.7	39.7	40.2	41.8	47.1	41.3	47.8	47.6	50.3	47.0	46.2	ND
Common to all	18:0	15.4	17.9	15.3	14.0	15.1	11.5	12.5	13.5	12.2	10.8	13.1	12.3	14.4	13.5	13.0	ND
?	20:0	10.4 ab	9.5 a	10.8 b	9.3 a	9.3	8.4	9.2	8.5	7.1	6.4	7.3	7.3	8.9	7.9	8.7	ND
?	22:0	11.4 ab	11.2 a	13.3 b	10.0 a	10.5	8.6	9.9	9.2	7.8 ab	6.9 a	9.1 b	8.3 a	11.1 a	8.7 b	9.2 ab	ND
TOTAL FAME		**265.1 ab**	**284.6 a**	**262.1 ab**	**221.1 b**	**249.1**	**200.2**	**199.2**	**207.4**	**201.4**	**173.7**	**199.7**	**192.1**	**229.6**	**210.6**	**197.5**	**ND**

[†] Different letters indicate significant differences (p <0.05) between treatments

[‡] Not determined (inadequate sample amount)

[§] Below detection limit

Table C.3. Amount of individual FAMEs from 21 d incubation experiment involving a Theresa silt loam soil.

		whole soil				2000 - 4000 μm				500 - 2000 μm				250 - 500 μm				75 - 250 μm			
Interpretation	**Fatty Acid**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**	**- EW - TNT**	**+EW - TNT**	**- EW + TNT**	**+ EW + TNT**
		nmol/g dw																			
Gram-positive	i15:0	12.2 a[†]	5.9 b	11.6 a	7.2 b	9.0 ab	7.9 ab	9.4 a	7.0 b	9.6	8.3	9.6	9.5	10.0	10.6	10.5	11.6	6.7 a	9.2 bc	10.1 b	7.5 ac
Gram-positive	a15:0	8.1 a	4.1 b	8.0 a	5.2 b	6.2 ab	7.9 ab	6.7 a	4.7 b	6.4	5.5	6.3	6.5	6.9	7.2	7.3	8.3	4.7 a	6.7 bc	7.3 b	5.2 ac
Gram-positive	i16:0	5.3 a	2.4 b	5.1 a	3.2 b	4.0	3.4	4.4	3.3	3.9	3.6	3.8	4.3	4.5	4.9	4.8	5.6	2.5 a	4.2 bc	4.7 b	3.2 ac
Gram-positive	i17:0	3.5 a	1.2 b	3.4 a	1.9 a	2.7 ab	2.4 ab	3.0 a	2.0 b	2.7	2.3	2.5	2.7	3.2	3.2	3.1	3.7	1.4 a	2.7 b	2.7 b	1.7 a
Gram-positive	a17:0	3.7 a	1.2 b	3.4 a	2.0 a	2.5 ab	2.3 a	3.5 b	2.1 a	2.6	2.3	2.4	2.8	3.3	3.4	3.3	3.9	1.4 a	2.7 b	2.8 b	1.5 a
Total		**32.8 a**	**14.8 b**	**31.7 a**	**19.5 b**	**24.4 ab**	**23.9 ab**	**26.9 a**	**19.2 b**	**25.2**	**21.9**	**24.6**	**25.8**	**28.0**	**29.4**	**29.1**	**33.2**	**16.7 a**	**25.5 b**	**27.7 b**	**19.0 a**
Gram-negative	16:1w9	9.3 a	6.1 b	10.2 a	6.7 b	6.8 ab	8.3 ab	8.4 a	6.4 b	7.6	7.4	8.1	8.7	8.0 a	9.3 ab	9.0 ab	10.6 b	5.4 a	8.5 b	9.4 b	6.5 a
Gram-negative	16:1w5	6.3 a	2.8 b	5.9 a	3.1 b	4.9 ab	4.5 ab	6.0 a	3.5 b	5.4	4.6	5.2	4.2	4.9	5.5	4.9	5.2	3.6	3.9	4.0	2.5
Gram-negative	18:1w7	15.5 a	9.4 b	16.6 a	11.7 b	10.5 a	17.1 b	14.0 c	11.3 a	12.6 a	12.7 a	13.7 ab	15.3 b	12.7 a	13.9 a	14.2 ab	16.5 b	8.8 a	12.9 b	15.0 b	10.1 a
Gram-negative	cy17:0	3.1 a	1.4 b	3.4 a	1.8 b	2.2 ab	2.2 a	3.0 b	2.1 a	2.3	2.1	2.4	2.6	2.6 a	3.0 ab	3.1 ab	3.5 b	1.1 a	2.3 bc	2.7 c	1.5 ab
Gram-negative	cy19:0	8.7 a	4.1 b	9.0 a	5.0 b	5.9	6.7	7.3	5.6	7.5	6.6	7.9	7.8	7.9	8.4	8.2	9.2	4.4 a	7.0 b	7.8 b	4.7 a
Total		**42.9 a**	**23.8 b**	**45.1 a**	**28.3 b**	**30.4 ab**	**38.8 ab**	**38.7 a**	**28.9 b**	**35.4**	**33.4**	**37.3**	**38.6**	**36.2**	**40.1**	**39.5**	**45.0**	**23.3 ac**	**34.6 bc**	**38.9 b**	**25.3 c**
Actinomycetes	10Me16:0	7.6 a	5.8 b	7.7 a	4.5 b	6.1 ac	9.4 b	6.8 a	4.9 c	6.2	5.6	5.9	6.1	6.2 a	6.8 ab	6.7 ab	7.9 b	4.2 a	6.4 b	6.8 b	4.3 a
Actinomycetes	10Me18:0	5.0 a	2.3 b	4.8 a	2.7 b	3.3	3.9	4.1	2.7	2.8	3.1	3.9	3.4	3.7	4.6	4.5	5.0	2.7 ac	4.0 ab	4.5 b	2.3 c
Total		**12.6 a**	**8.1 b**	**12.5 a**	**7.2 b**	**9.4 ac**	**13.4 b**	**10.8 a**	**7.7 c**	**9.0**	**8.7**	**9.8**	**9.5**	**9.9 a**	**11.4 ab**	**11.2 ab**	**12.9 b**	**6.9 a**	**10.4 b**	**11.4 b**	**6.6 a**
Fungi	18:2w	10.9 a	3.8 b	10.5 a	6.9 b	16.3	16.3	18.3	14.2	20.4	18.2	21.6	21.0	10.3	11.0	9.4	9.7	12.3 a	18.0 bc	19.5 c	14.2 ab
Fungi	18:1w9	21.8 a	12.4 b	22.4 a	16.8 c	6.5 ab	7.0 ab	8.3 a	4.9 b	10.8 ab	8.0 a	12.0 b	9.1 ab	18.8	21.9	19.6	22.2	3.7 a	6.2 ab	7.6 b	5.2 ab
Total		**32.7 a**	**16.2 b**	**32.8 a**	**23.7 c**	**22.8 ab**	**23.2 ab**	**26.6 a**	**19.1 b**	**31.2 ab**	**26.2 a**	**33.6 b**	**30.1 ab**	**29.2**	**32.9**	**28.9**	**31.8**	**16.0 a**	**24.2 bc**	**27.1 c**	**19.5 ab**
Protozoa	20:4w6	8.6 a	1.1 b	3.5 b	2.2 b	2.4	2.9	2.2	1.6	3.1	1.7	4.5	4.8	3.7	4.1	3.5	4.0	0.4	1.8	2.2	1.6
Common to all	16:0	45.5 a	24.2 b	45.0 a	33.4 c	35.3 ac	29.6 ab	37.4 c	27.5 b	38.9	33.1	38.2	40.7	36.6	39.3	38.4	40.9	26.0 ad	34.4 bc	37.4 c	30.3 bd
Common to all	18:0	11.4 a	6.2 b	10.7 a	7.1 b	8.2 ab	9.3 a	8.3 ab	6.3 b	9.6	7.8	8.7	10.1	10.1	10.5	9.9	10.8	7.3 ac	9.6 ab	10.1 b	6.4 c
?	20:0	3.3 a	0.8 b	2.7 ac	1.6 bc	2.2	2.2	1.9	1.3	3.1 a	1.4 b	2.7 ab	2.4 ab	3.5	3.1	2.7	3.2	3.6 a	2.5 ab	2.3 ab	1.0 b
?	22:0	8.1 a	2.4 b	6.1 c	4.8 c	4.8	4.4	3.4	3.5	6.7 a	4.1 b	5.2 ab	5.7 ab	5.9	6.3	5.6	7.0	4.2	4.9	5.1	3.6
TOTAL FAME		**197.9 a**	**97.5 b**	**190.0 a**	**127.9 b**	**139.9 ab**	**147.8 ab**	**156.2 a**	**114.9 b**	**162.3**	**138.3**	**164.6**	**167.7**	**162.9**	**176.9**	**168.6**	**188.7**	**104.4 a**	**147.9 bc**	**162.2 c**	**113.2 ab**

[†] Different letters indicate significant differences (p <0.05) between treatments

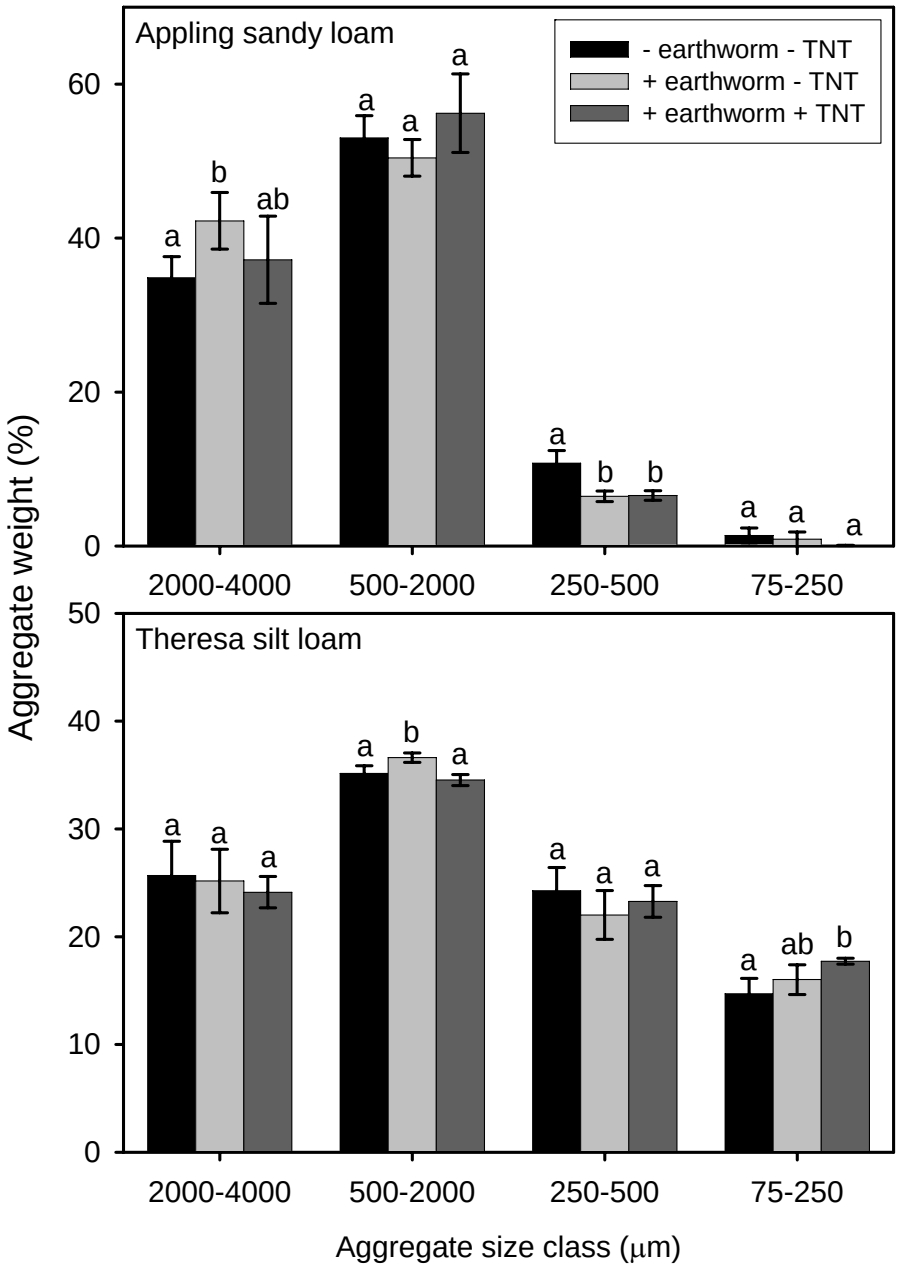


Figure C.1. Effects of earthworm activity and TNT on aggregate size distribution of an Appling sandy loam (A) and a Theresa silt loam (B) after 14 days. Values followed by a different lowercase letter within aggregate size class are significantly different between treatments (mean ± 1 S.D., n = 3).

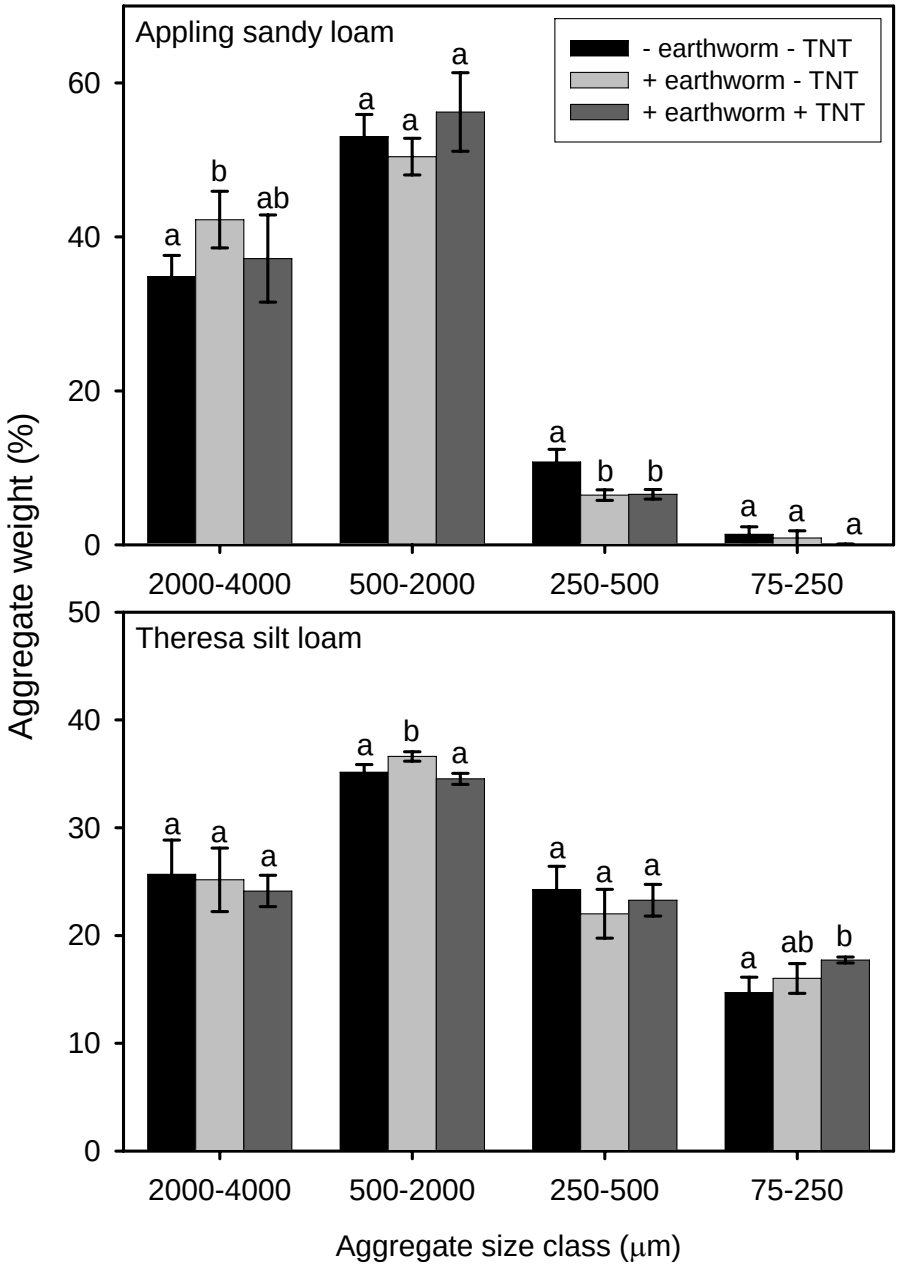


Figure C.2. Effects of earthworm activity and TNT on aggregate size distribution of an Appling sandy loam (A) and a Theresa silt loam (B) after 7 days. Values followed by a different lowercase letter within aggregate size class are significantly different between treatments (mean ± 1 S.D., n = 3).

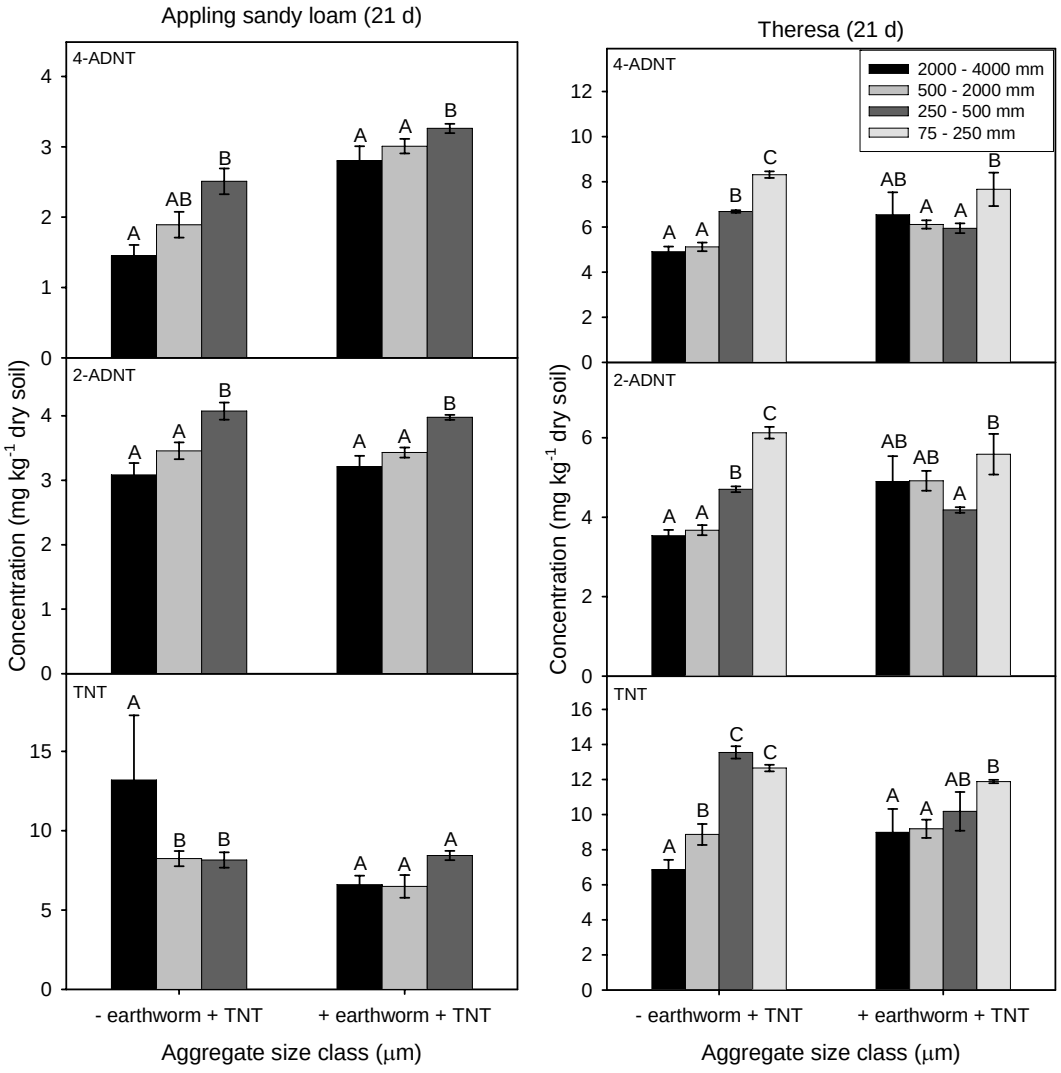


Figure C.3. Concentration of acetonitrile extractable TNT and its two major metabolites (2-ADNT and 4-ADNT) at 21 d in the different sized aggregates of an Appling sandy loam (left panel) and a Theresa silt loam (right panel) with and without earthworms. Different letters denote significant differences (using Fishers LSD) between aggregate size fractions for each treatment ($p < 0.05$, $n = 3$).

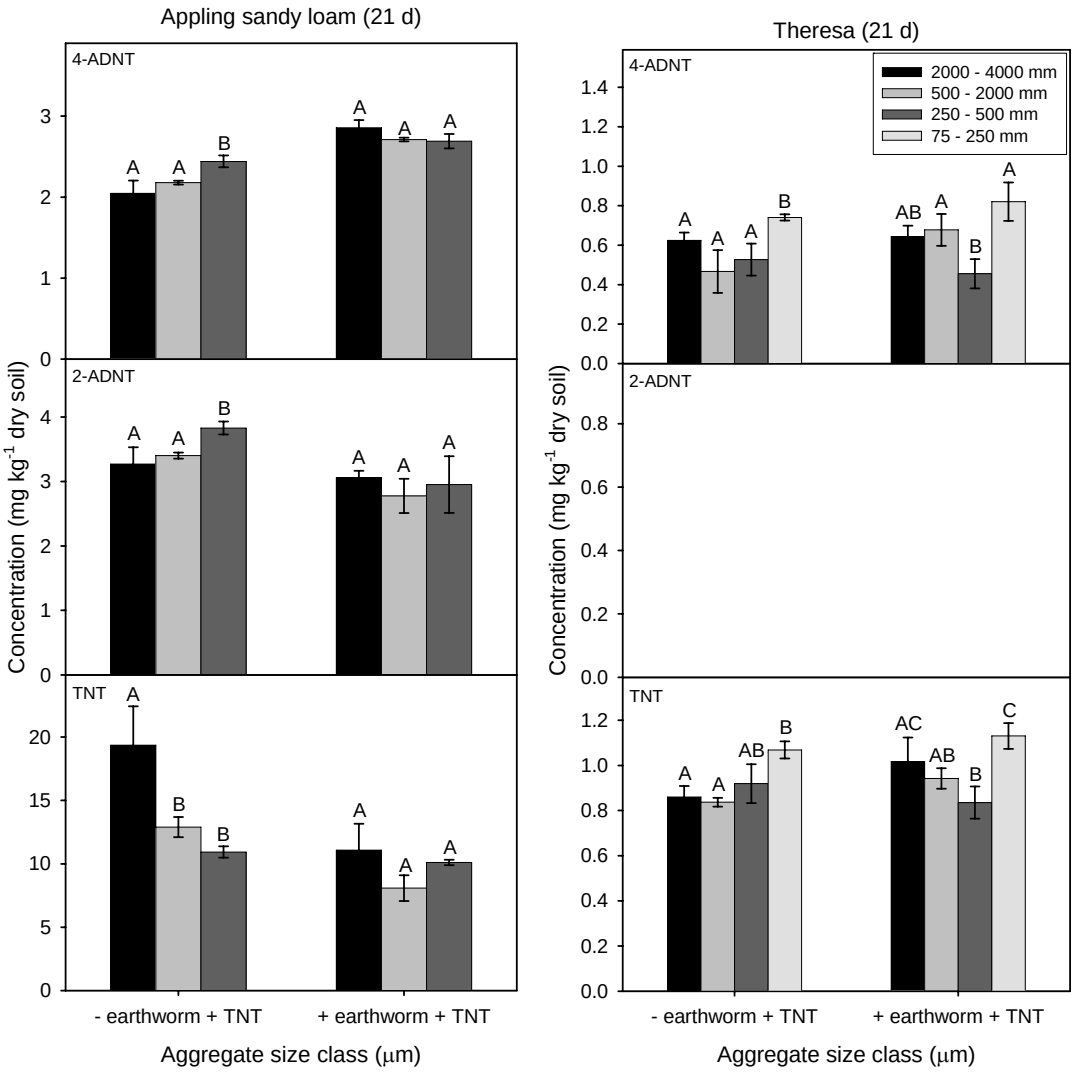


Figure C.4. Concentration of $CaCl_2$ extractable TNT and its two major metabolites (2-ADNT and 4-ADNT) at 21 d in the different sized aggregates of an Appling sandy loam (left panel) and a Theresa silt loam (right panel) with and without earthworms. The concentration of $CaCl_2$ extractable 2-ADNT in the Theresa soil were below the detection limits. Different letters denote significant differences (using Fishers LSD) between aggregate size fractions for each treatment ($p < 0.05$, $n = 3$).

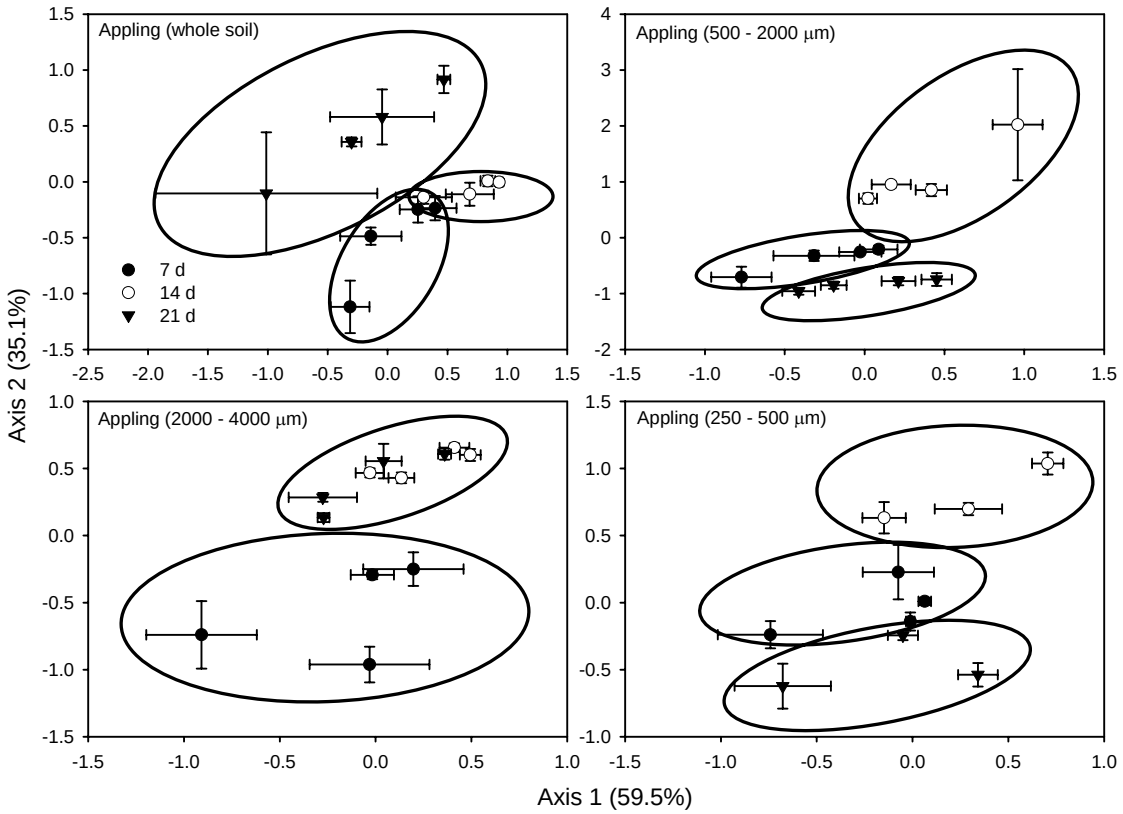


Figure C.5. Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, and 250 – 500 μm aggregates of an Appling sandy loam over time (0, 7, 14, 21 d). The proportion of variance explained by each axis is indicated in parentheses. Values of the symbols and error bars represent means and standard error, respectively (n = 3).

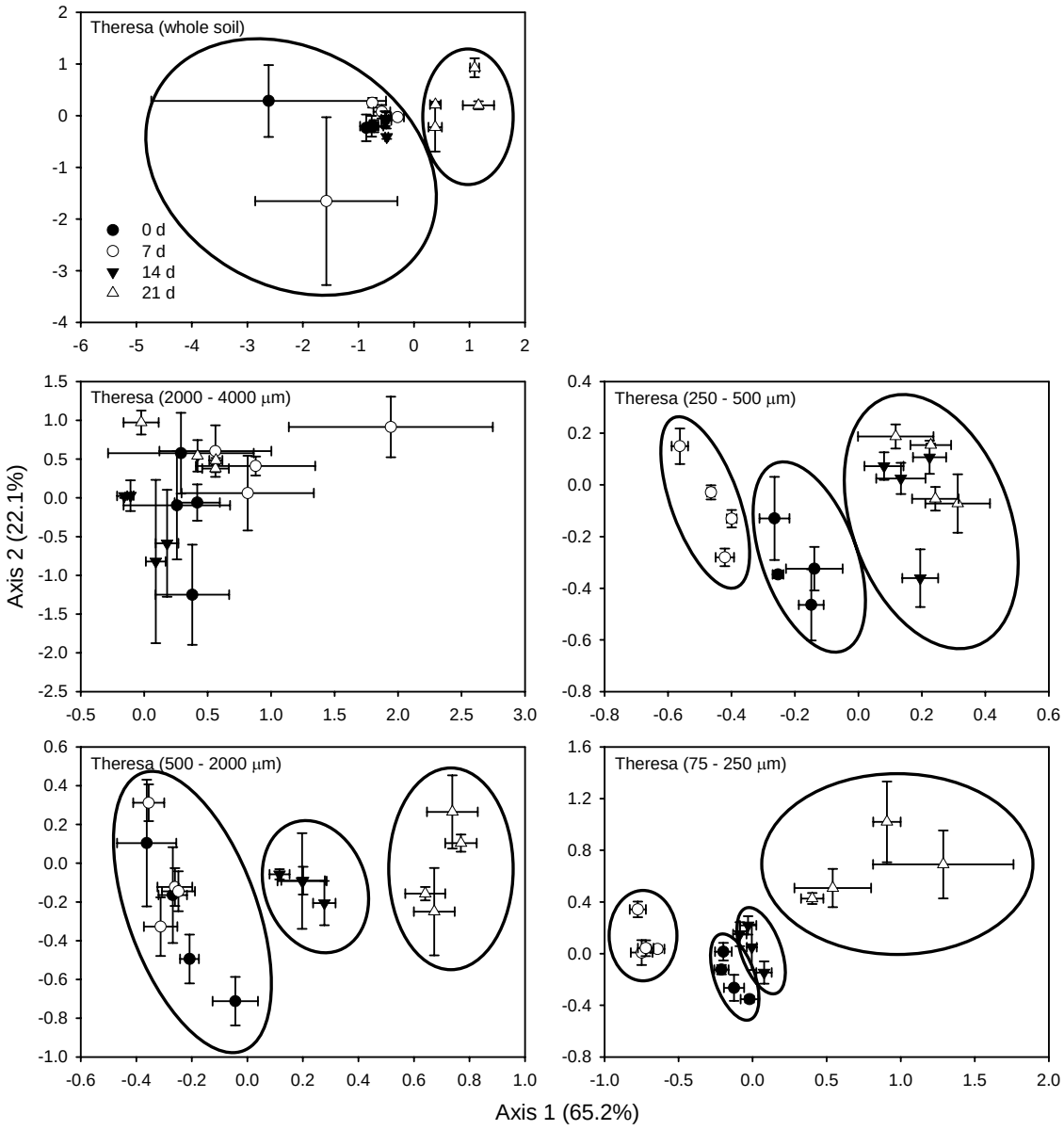


Figure C.6. Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500, and 75 – 250 μm aggregates of a Theresa silt loam over time (0, 7, 14, 21 d). The proportion of variance explained by each axis is indicated in parentheses. Values of the symbols and error bars represent means and standard error, respectively (*n* = 3).

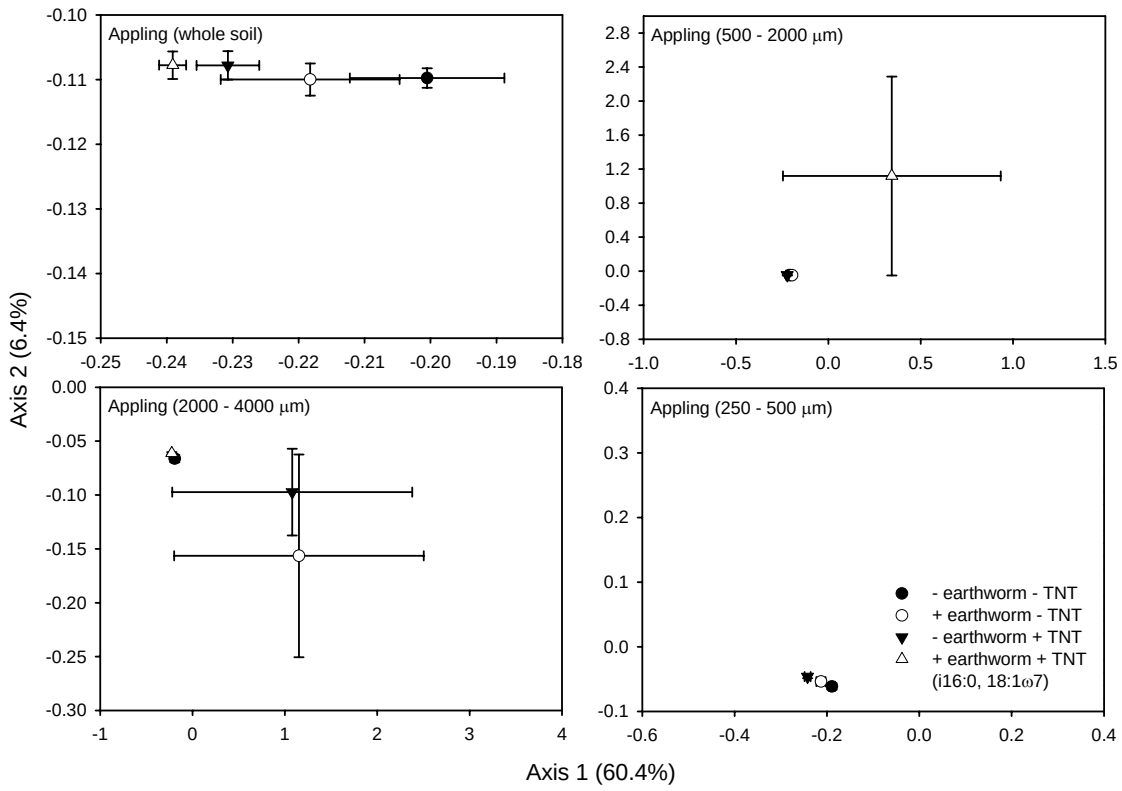


Figure C.7. Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, and 250 – 500 μm aggregates of an Appling sandy loam soil after 14d. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard error, respectively (n = 3).

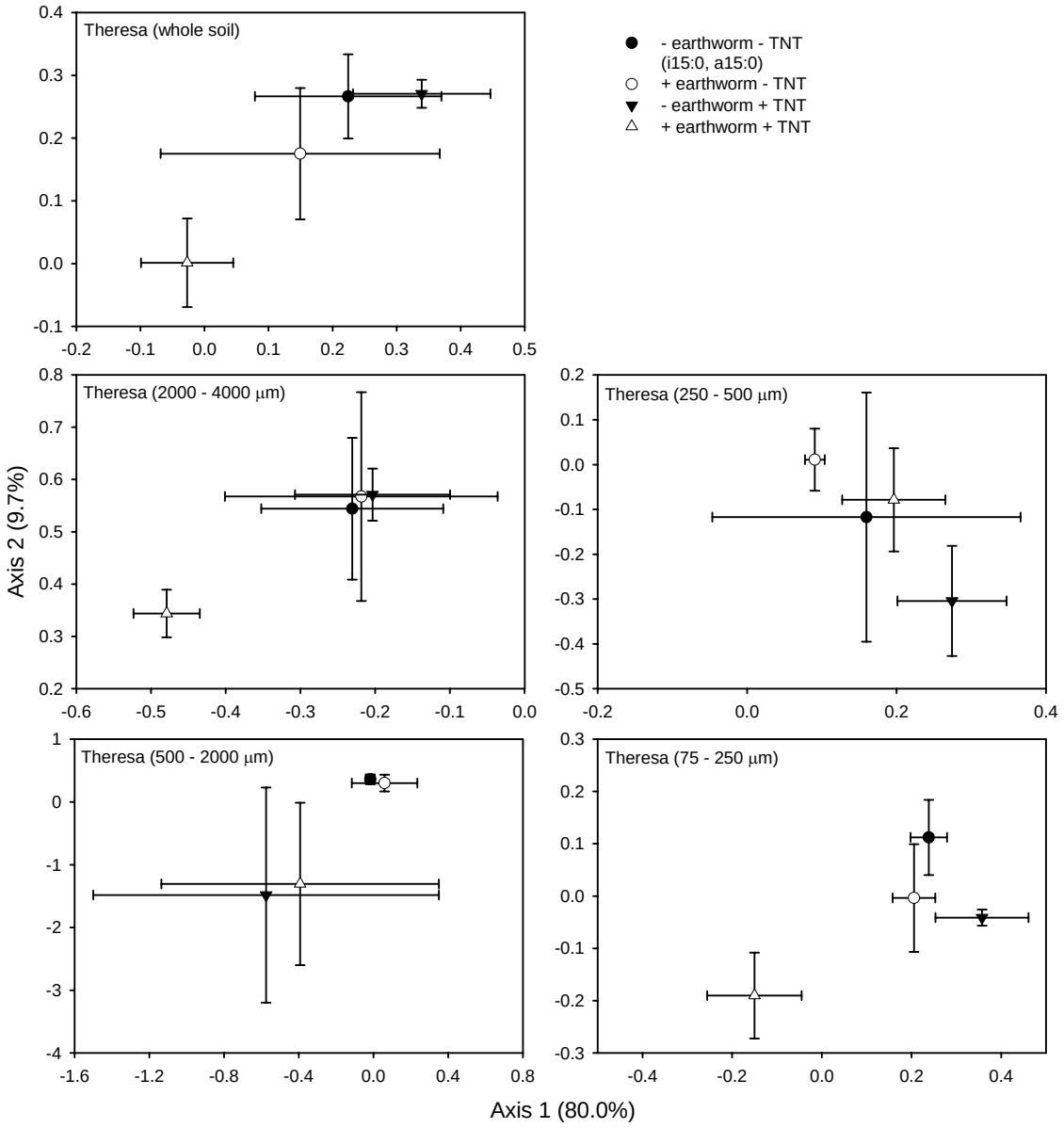


Figure C.8. Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500, and 75 – 250 μm aggregates of a Theresa silt loam soil after 14d. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard error, respectively (n = 3)

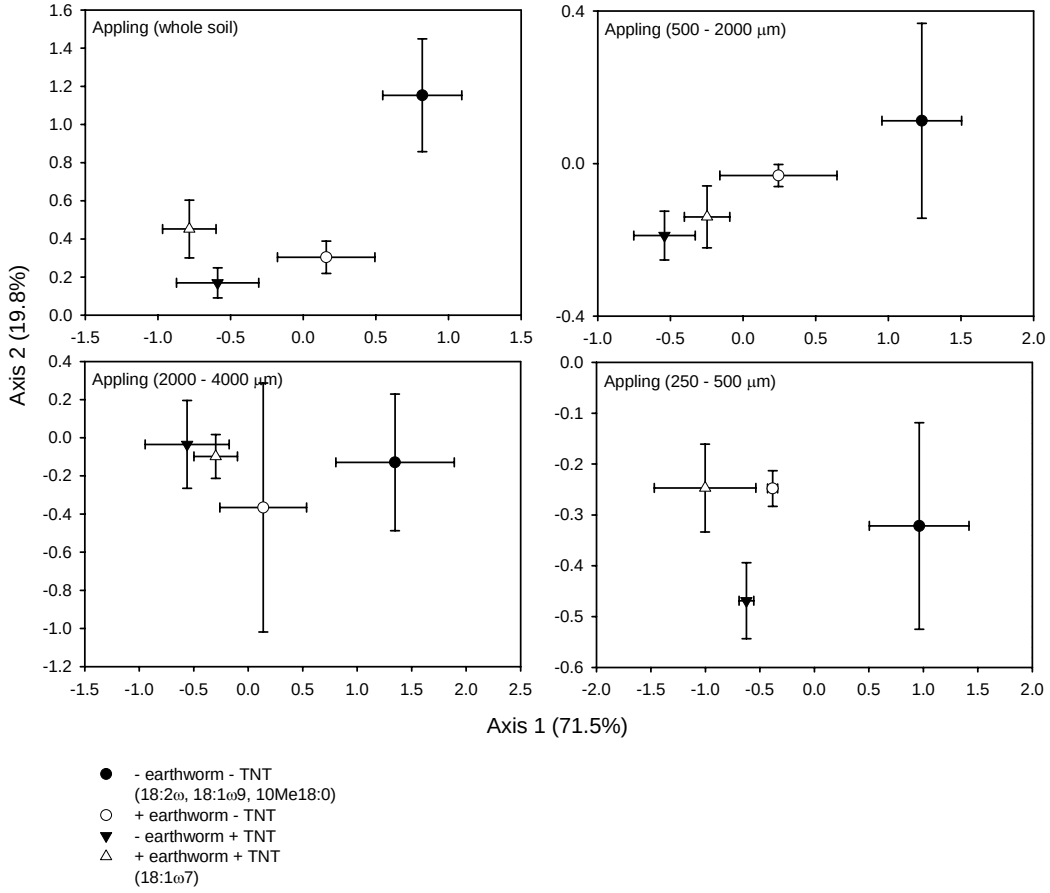


Figure C.9. Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, and 250 – 500 μm aggregates of an Appling sandy loam soil after 7 d. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error barsrepresent means and standard error, respectively (n = 3).

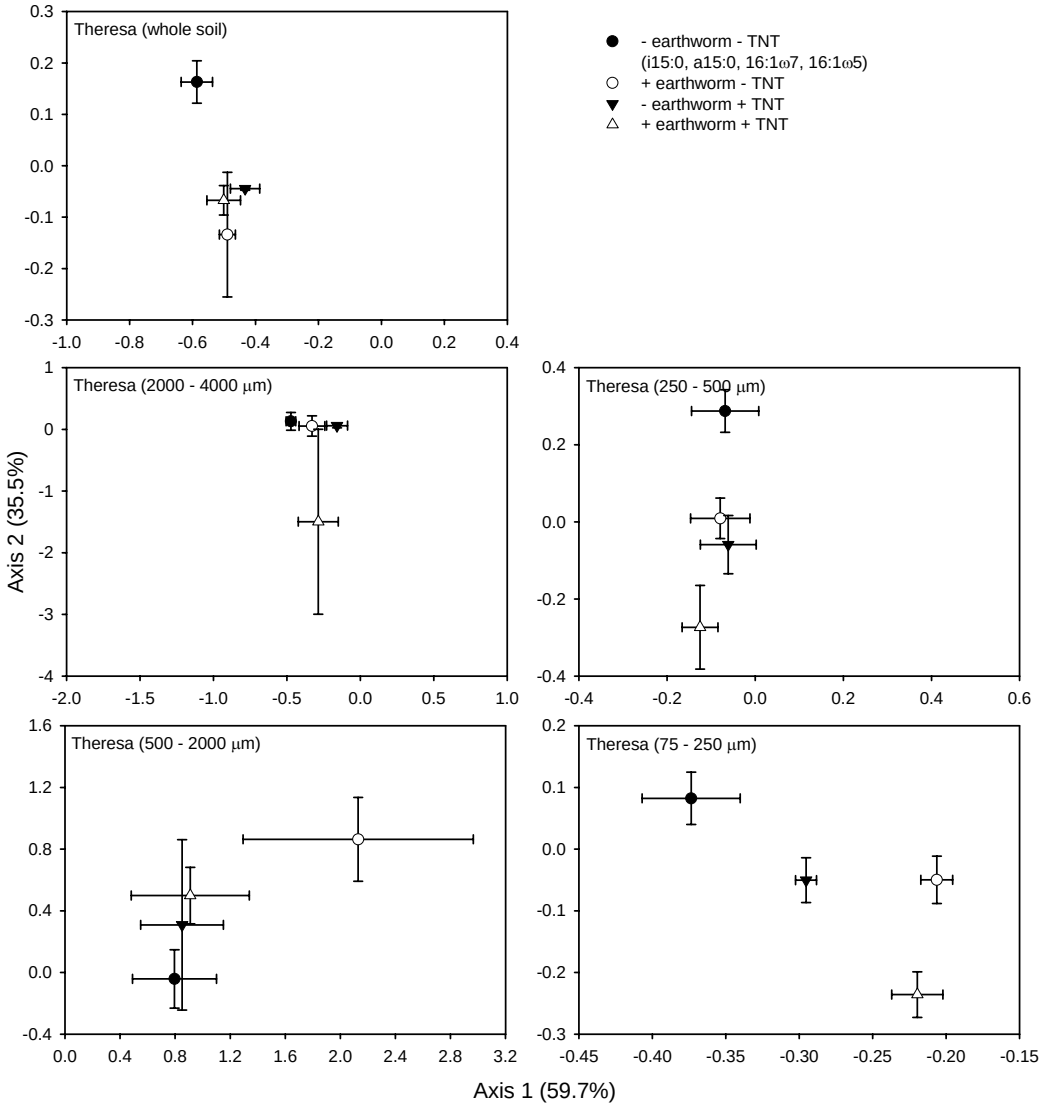


Figure C.10. Nonmetric multidimensional scaling plots of mole percentages of all individual FAMEs from whole soil, 2000 – 4000, 500 – 2000, 250 – 500, and 75 – 250 μm aggregates of a Theresa silt loam soil after 7d. The proportion of variance explained by each axis is indicated in parentheses. Indicator FAME biomarkers for each treatment are listed in the legend. Values of the symbols and error bars represent means and standard error, respectively (n = 3).