Fate of Estrogenic Compounds in Agricultural Soils and Development of an Immunoassay for their Environmental Detection

By

Emmanuelle Caron

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Soil Science
University of Manitoba

Winnipeg

ABSTRACT

Caron, Emmanuelle. Ph.D., The University of Manitoba, April 2011. Fate of Estrogenic Compounds in Agricultural Soils and Development of an Immunoassay for their Environmental Detection. Major professor: Annemieke Farenhorst.

Estrogens produced by livestock can be released into soils when their manure is spread onto agricultural land. This is the first study to determine the sorption of a range of estrogens in a wide range of soils at the regional scale, including the sorption of the phytoestrogen equol which had never been previously studied. Sorption increased in the order of 17β-estradiol=estriol <estrone<equol in surface soils collected from 41 agricultural fields in Alberta and was significantly positively correlated with soil organic carbon content (SOC) for all estrogens. 17β-estradiol was further investigated and its mineralization in non-amended and manure-amended soils never exceeded 30% at 90 days, which suggest that even under optimum environmental conditions for mineralization, 17β-estradiol or its metabolites estrone and/or estriol appear to have a relatively long persistence in Alberta soils. Maximum 17β-estradiol mineralization was significantly positively correlated with sorption and hence increased in soils with greater SOC such as those used in this study with a long-term history of solid beef manure applications. Two ELISAs were developed using rabbit polyclonal antibodies for future field experiments and environmental monitoring. Of these, a developed 17β-

estradiol+estrone+estriol ELISA could detect estriol in water from an edge of field experiment at concentrations as low as 1 ng mL^{-1}.

ACKNOWLEDGEMENTS

During my four year study, I had the chance to work with many great people. First, my co-supervisors, Dr. Annemieke Farenhorst and Dr. Claudia Sheedy are not only great scientists but also they are exceptionally good persons. I would like to thank them for helping me become a better scientist and a better person. The members of my advisory committe, Dr. Don Flaten and Dr. Mark Hanson, have been of great help and I would like to thank them for their support and guidance. I had the chance to work with technicians and students from the University of Manitoba and Agriculture and Agri-Food Canada, Lethbridge. I would like to thank Mr. Rob Ellis at the University of Manitoba for being patient with answering all my questions. Thanks to Michael Mendenhall for his help in Lethbridge. Thanks to Courtney Lamb and Paul Dawson for their help, for making me laugh and relax and for welcoming me in Lethbridge. Thanks to Brenda Pink for her tremendous help with the animals. Thanks to the late Mr. Paul Panich for his help with the radioisotope laboratory in Lethbridge. Thanks to Brett Hill, Pamela Caffin and Clarence Gilbertson for their help. Special thanks go to Mr. Dan Inaba for knowing everything and everyone, for being my adoptive dad while I was in Lethbridge, for taking care of me when I was sick and for being my friend. Thanks to my mom and dad for being so supportive and understanding without being too invasive. Finally, last but not least, thanks to Mr. Steven Charest for his support and comprehension, for his love, for always being there for me, for cheering me up, for giving me confidence in the future and for being the man of my life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table 1.1: List of abbreviations (in the order in which they appear)

Abbreviation		Abbreviation		Abbreviation		Abbreviation	
GC	Gas chromatography	MBU	Mixed Boreal Uplands	pKa	Acidic dissociation constant	I	Irrigated
HPLC	High pressure liquid chromatography	AP	Aspen Parkland	Kow	Octanol-water partition coefficient	R	rainfed
MS	Mass spectrometry	MMG	Moist Mixed Grassland	SOM	Soil organic matter	CV	Coefficient of variation
ELISA	Enzyme Linked ImmunoSorbent Assay	FG	Fescue grassland	Max	Maximum mineralization at infinite	IL	Illinois
Kd	Soil sorption coefficient	MG	Mixed Grassland	HL	Mineralization half-life	E2	17β-estradiol
Koc	Sorption coefficient per unit organic carbon	CEC	Cation exchange capacity	MO	Missouri	E1	Estrone
USA	United States of America	EC	Electrical conductivity	M	Mol	E3	Estriol
IUPAC	International Union on Pure an Applied Chemistry	Rpm	Revolutions per minute	FDA	Fluorescein diacetate hydrolysis assay	IC_{50}	Concentration inhibiting 50% of signal
SOC	Soil organic carbon	Cs	Concentration sorbed	MD	Maryland	OVA	Ovalbumin
ANOVA	Analysis of variance	Ce	Concentration in equilibrium	ON	Ontario	MES	Morpholino ethanesulfonic acid

			solution				
PLS	Partial least square	HSD	Honestly significant difference	CA	California	PBS	Phosphate buffered saline
2,4-D	2,4-dichloro phenoxyacetic acid	MDA	Multiple discriminant analyses	foc	Organic carbon fraction	BSA	Bovine serum albumin
3H-N	nominally	Br.C	Brown chernozem	NJ	New Jersey	B	Optical density
G	generally	DBr.C	Dark Brown chernozem	t	time	HRPO	Horseradish peroxidase
3H	tritium	Bl.C	Black chernozem	Ns	Not significant	ABTS	2,2’azino-di(3-ethylbenzthiazoline-6-sulphate
LSC	Liquid scintillation counting	GL	Gray luvisol	RMSE	Root mean square error	KPL	Kirkegaard and Perry laboatories
N	North	DGL	Dark gray luvisol	EXP	Explained variance	MI	Michigan
W	West	U	Upper slope	NA	Not available	HLB	Hydropphilic-lipophilic balance
PL	Peace Lowland	M	Mid slope	Total C	Total carbon	MA	Massachusetts
BT	Boreal Transition	L	Lower slope	Total N	Total nitrogen	C18	Octadecilsilane

1. INTRODUCTION

Since the early 1990's, the scientific community and broader public are increasingly concerned about the presence and potential impacts of estrogens in the environment. This is well illustrated by a key publication by Purdom et al. (1994) suggesting that estrogens present at the part per trillion concentration in effluent from waste treatment plants can induce increased concentrations of blood vitellogenin (egg protein) in male rainbow trout.

Estrogens are part of a group of compounds described as endocrine disrupting chemicals. Endocrine disrupting chemicals can alter or disrupt the endocrine system by interfering with the synthesis or degradation of hormones and by antagonizing or mimicking these hormones (World Health Organization, 2002).

This chapter consists of an introduction on the main theme of this Ph.D. research, namely the study of the fate and detection of natural estrogens in agricultural soils. In addition, the four objectives carried out as part of this project are presented.

1.1 Properties of Natural Estrogens

Estrogens are synthesized from androgens, which are themselves synthesized from cholesterol. This reaction takes place mainly in the ovaries but can also occur in fat tissues and in the placenta. The synthesis of estrogens is governed by two other

hormones: the follicle-stimulating hormone and the luteinizing hormone (Nussey and Whitehead, 2001).

Estrogens are molecules of low molecular weight and low solubility. Natural steroidal estrogens include 17β-estradiol, estrone, estriol and 17α-estradiol, the first three being included in this research. The molecular weights of 17β-estradiol, estrone and estriol are respectively 272.4, 270.4 and 288 g mol^{-1} and their solubility is 13 mg L^{-1} in water at 20^{o}C (the same for all three compounds) (Ying and Kookana 2005). Their vapour pressure is low at $3x10^{-8}$ Pa for 17β-estradiol and estrone and even lower for estriol at $9x10^{-13}$ Pa (Hanselman et al., 2003); they therefore have a relatively low volatility. Estrogens are not considered to be easily ionizables in acidic or neutral environments since their acid dissociation constant is high, ranging from 10.3 to 10.8 (Hurwitz and Liu, 1977; Lewis and Archer 1979).

1.2 Sources

Natural estrogens are produced and excreted by all vertebrates, including humans and livestock. Estrogens produced and excreted by humans can still be present when treated wastewater is released in the environment. Johnson et al. (2000) estimated that a pregnant woman can excrete 259 μg per day of 17β-estradiol. Males also excrete estrogens but in lesser amounts (Johnson et al., 2000). In effluents from wastewater treatment plants in Canada, Ternes et al., (1999) measured concentrations up to 48 ng L^{-1} and 64 ng L^{-1}, of 17β-estradiol and estrone respectively. In sediments from the Tasman Sea (between

Australia and New Zealand), estrogen concentrations reached 2.48 ng g^{-1}(17β-estradiol), 1.17 ng g^{-1}(estrone) and 0.5 ng g^{-1}(17α-ethinylestradiol) at a sampling site 7 km away from a sewage treatment discharge point (Braga et al., 2005). Although wastewater effluent can be applied to increase the productivity of agricultural soils, a more common soil amendment is that of livestock manure. In swine lagoons from farrowing sow operations, Fine et al., (2003) measured concentrations ranging from 2,200 to 3,000 ng L^{-1}, 5,000 to 10,400 ng L^{-1}, and 9,600 to 24,900 ng L^{-1}, of 17β-estradiol, estriol and estrone respectively. Other studies showed concentrations of 113,000 ng kg^{-1} and 203,000 ng kg^{-1} for 17β-estradiol and estrone, respectively, in dairy manure (Williams, 2002), and 1,215 μg kg^{-1} and 4,728 μg kg^{-1} of 17β-estradiol and estrone, respectively, in farrowing sow pits (Williams, 2002).

The proportion of estrogens excreted in feces and urine differs among species. Cattle will excrete 58% of estrogens in feces, while poultry will excrete 69 % of estrogens in urine (Ivie et al., 1986; Palme et al., 1996). Urinary estrogens are mostly conjugated to glucuronides and/or sulfates, whereas in feces, estrogens are mainly present in their free form (Palme et al., 1996). Huang and Sedlak (2001) observed that conjugated estrogens accounted for less than 2% of the hormones in water samples from municipal waste water treatment effluents, a wetland, the Colorado River and the Sacramento River Delta.

Estrogens from livestock can reach surface waters through runoff from manure-applied land. For example, when broiler litter was applied to grasslands, concentrations of 17β-estradiol in soil and runoff reached 675 ng kg^{-1} and 2,530 ng L^{-1}, respectively (Finlay-

Moore et al., 2000). Background concentrations of 17β-estradiol of 55 ng kg^{-1} in soils and of 50 to 150 ng L^{-1} in runoff were measured prior to broiler litter application. In creeks where cattle had access to water, 86% of samples contained estrogens, with maximum concentrations reaching 44 ng L^{-1} after rain events, suggesting a contribution of runoff to surface water contamination (Kolodziej and Sedlak, 2007). In 10 to 20% of those samples, estrogens concentrations exceeded the predicted no-effect concentration for fish (Kolodziej and Sedlak, 2007). In a monitoring study conducted in the United Kingdom (Matthiessen et al., 2006), the 17β-estradiol equivalent concentrations ranged from 0.04 to 3.6 ng L^{-1} in upstream and downstream sampling locations livestock farms (mainly dairy but including cattle, swine and sheep). Downstream locations showed on average 17β-estradiol equivalent concentrations 16 times greater than those detected in upstream locations while the remaining 40% of the locations appeared to have contributed low estrogen concentrations to the stream.

Phytoestrogens are estrogenic molecules derived from plants. Equol, which is studied along with three other estrogens in Chapter 2, is a phytoestrogen metabolized by bacteria in the intestines from the isoflavones daidzein and formononetin (Mustonen et al., 2006). Equol was found to be the major source of estrogenic activity in manure (Lorenzen et al., 2006). It is also known that exposure to equol reduces aggressive behaviour in male fighting fish (*Betta splendens*) (Clotfelter and Rodriguez, 2006) and can lead to reproductive disorders in ewes (Mustonen et al., 2006).

1.3 Ecotoxicology

Literature regarding the ecotoxicological impacts of estrogens is abundant. Several literature reviews on the ecotoxicology of estrogens were published prior to 2006 (Lai et al., 2002; Mills and Chichester, 2005), therefore, the present section will summarize in a table the work published after 2005. The synthetic estrogen 17α-ethinylestradiol has been most studied, followed by 17β-estradiol. However, very few studies were conducted on estrone, estriol or phytoestrogens such as equol.

Few studies have assessed the impact of mixtures of estrogenic compounds on organisms or ecosystem health. Stumper and Jobling (1995) reported that mixtures of estrogenic chemicals are more potent than single chemicals. Brian et al. (2007) also found that 17β-estradiol, 17α-ethinylestradiol, nonylphenol, octylphenol and bisphenol A acted additively to induce vitellogenesis in male fathead minnow. Another study showed that 17β-estradiol and 17α-ethinylestradiol acted additively to induce the production of vitellogenin in male crucian carp (Zhang et al., 2004).

Animals other than vertebrates have estrogen receptors and can be affected by estrogenic compounds, these include invertebrates such as bivalves and crusteceans and it has also been shown that estrogencic compounds can affect plant growth and development (Lai et al., 2002).

Table 1.2: Work published after 2005 related to the ecotoxicology of estrogens.

Compound	Species	Lowest concentration to see effect	Total duration of exposure	Effects observed	Reference
17α-ethinylestradiol	Adult male zebrafish (*Danio rerio)*	2 ng L^{-1}	14 days	Reduction of social dominance	Coe et al., 2009
17β-estradiol, estrone	Japanese Medaka (*Oryzias latipes*)	5 ng L^{-1}	14 days	Induction of vitellogenin	Kitamura et al., 2009
17β-estradiol	Rare minnow (*Gobiocypris rarus*)	25 ng L^{-1} adult stage and 100 ng L^{-1} in larval and juvenile	21 days	Induction of vitellogenin	Liao et al., 2009
17α-ethinylestradiol	Sand gobies (*Pomatoschis-tus minutus*)	5 ng L^{-1}	1 to 4 weeks	Reduction of the effect of male size on mating success	Saaristo et al., 2009
17α-ethinylestradiol	Male fathead minnow (*Pimephales promelas*)	40 ng L^{-1}	21 days	Decreased ability to compete for spawning substrate, reduced frequency of substrate cleaning and chasing, increased vitellogenin, reduced ketotestosterone, 17β-estradiol, testosterone, tubercles; gonadosomatic index, gonadal maturity ranks, and number of resorbed tubercles, presence of an ovipositor	Salierno and Kane, 2009

17β-estradiol	Japanese sea bass (*Lateolabrax japonicus*)	200 ng L^{-1}	30 days	Increased respiratory burst activity, myeloperoxidase, immunoglobulin concentrations differential leukocyte counts, reduction of serum lysozyme, bactericidal activity	Thilagram et al., 2009
17α-ethinylestradiol	Tadpoles (*Rana temporaria)*	77 ng L^{-1}	From hatch to metamor-phosis	Induction of vitellogenin	Brande-Lavridsen et al., 2008
17α-ethinylestradiol	*Chironomus tentans, Hyalella azteca*	1.5 mg L-1 0.36 mg L	42 days	Reduction in emergence Reduction in reproduction	Dussault et al., 2008
17α-ethinylestradiol	Male female zebrafish (*Danio rerio*)	2 ng L^{-1}	3 months	Reduces fecundity, adversely affects sex differentiation, gametes development	Xu et al., 2008
17β-estradiol	African clawed frog (*Xenopus laevis)*	1μg L^{-1}	From larva to meta-morphosis	Accelerated spermatogenesis	Hu et al., 2007
17α-ethinylestradiol	Fathead minnow (*Pimephales promelas*)	5-6 ng L^{-1}	7 years	Production of vitellogenin intersex in males, altered oogenesis in females, near extinction	Kidd et al., 2007

17β-estradiol	Chinese loach (*Misgurnus anguillicau-datus*)	0.5 ng L^{-1}	42 days	Induction of vitellogenin	Lv et al., 2007
17β-estradiol	Sand goby (*Pomatoschis-tus minutus*)	97, 669 ng L^{-1}	8 months	Inhibited male sexual maturation, induced vitellogenin, delayed spawning, production of fertile eggs at a slower rate in the 97 ng L^{-1} group. Increased mortality, affected haematological parameters, almost total lack of reproductive activity, both sexes failing to mature in the 669 ng L^{-1} group.	Robinson et al., 2007
17α-ethinylestradiol	Juvenile male turbot (*Psetta maxima*)	3.5 ng L^{-1}	15 days	Ratio of androgens to estrogens similar to female in plasma and testis	Labadie and Budzinski, 2006
17β-estradiol	Adult male Rainbow trout (*Oncorhyn-chus mykiss)*, Grayling (*Thymallus thymallus)*	0.5 ng L^{-1}	50 days pre-spawning season	At ≥ 1.0 ng/L impact on sperm motility patern	Lahnsteiner et al., 2006
17β-estradiol 17α-ethinylestradiol	Guppy (*Poecilia reticulata*)	200 ng L^{-1}	3.5 months	Decreased sperm quantity and body coloration, increased body weight in the 200 ng/L group	Nielsen and Baatrup, 2006

17α-ethinylestradiol	Pearl dace (*Margariscus margarita*)	4.5-8.1 ng L^{-1}	3 years	Increased vitellogenin, edema to ovaries, inhibited development of testicules, intersex, kidney lesions	Palace et al., 2006
17β-estradiol	Male mosquitofish, (*Gambusia holbrooki*)	500 ng L	8-12 weeks	Decreased number of phenotypic males	Rawson et al., 2006
17α-ethinylestradiol	Lake trout *(Salvelinus namaycush)*	5 ng L^{-1}	3 years	No impact	Werner et al., 2006
17α-ethinylestradiol	Green frog *(Rana clamitans)*	<10 ng L^{-1}	3 years	Reduction of hatchling success, no impact on mass or sex ratio	Park and Kidd, 2005

1.4 Quantification of Estrogens in Environmental Matrices

Estrogens can be quantified using conventional chemistry techniques such as gas chromatography (GC) or high pressure liquid chromatography (HPLC) coupled to mass spectrometry (MS). These detection methods require extraction of the estrogens from the samples using techniques such as solid phase extraction (for liquid samples) or accelerated-solvent extraction (for solid samples), followed by clean-up of the extracts prior to injection into the instrument. Some examples of extraction methods for solid samples were presented by Ternes et al. (2002) (17β-estradiol, estrone and 17α-ethinylestradiol), Hanselman et al. (2004) (17β-estradiol), Hájková et al. (2007) (17β-estradiol, estrone and 17α-ethinylestradiol) and Hutchins et al., (2007) (17β-estradiol, 17α-estradiol, estrone, estriol, estrone-3-sulfate, 17β-estradiol-3-sulfate,17α-estradiol-3-sulfate and 17β-estradiol-17-sulfate). Extraction methods for liquid samples are numerous and examples of a few recent ones include (Kumar et al., 2009, Kuster et al., 2009, Lien et al., 2009, Miege et al., 2009, Pedrouzo et al., 2009, Yan et al., 2009, Zhou et al., 2009, Chang and Huang, 2010 and Lui, 2010).

Prior to quantifying estrogens by GC, samples must be derivatized (chemical reaction by which estrogens are made more volatile by their conjugation to a more volatile chemical moiety). Diverse techniques for derivatization can be found in Huang and Sedlak (2001) (17β-estriol and 17α-ethinylestradiol), Mouatassim-Souali et al. (2003) (17β-estradiol, estrone, estriol and 17α-ethinylestradiol), Quintana et al., (2004) (17β-estradiol, estrone,

estriol and 17α-ethinylestradiol) and Sarmah et al. (2006) (17β-estradiol, estrone, estriol, 17α-estradiol). Examples of detection methods by GC can be found in Huang and Sedlak (2001) (17β-estriol and 17α-ethinylestradiol), Ternes et al. (2002) (17β-estradiol, estrone and 17α-ethinylestradiol), Mouatassim-Souali et al. (2003) (17β-estradiol, estrone, estriol and 17α-ethinylestradiol), Quintana et al. (2004) (17β-estradiol, estrone, estriol and 17α-ethinylestradiol), Labadie and Budzinsky (2005) (17β-estradiol, estrone, estriol and 17α-estradiol) and Sarmah et al. (2006) (17β-estradiol, estrone, estriol and 17α-estradiol).

HPLC detection methods are described in Vanderford et al. (2003) (17β-estradiol, 17α-ethinylestradiol), Rodriguez-Mozaz et al., (2004) (17β-estradiol, estrone, estriol, estradiol-17-glucuronide, estradiol-17-acetate, estrone-3-sulfate, 17α-ethinylestradiol), Hu et al., (2005) (17β-estradiol, estrone, estriol, and 17α-ethinylestradiol) and Labadie and Hill (2007) (17β-estradiol, estrone and 17α-ethinylestradiol) among others.

Other methods of detection for estrogens include immunoassays based on the use of antibodies able to specifically bind estrogens. There are two basic types of antibodies: 1) polyclonal antibodies, which are produced by different lymphocytes and which recognize several epitopes (immunogenic regions of an antigen) and 2) monoclonal antibodies, which are produced by a single lymphocyte and which are able to recognize one unique epitope of the antigen (Kuby, 1997).

The ability of a given compound to induce an immune response in animals injected with this compound is dependent upon its molecular size (larger molecules induce stronger

immune responses than smaller ones), its foreignness (molecules distant or foreign in origin in relation to the animal immunized tend to induce stronger immune responses) and its heterogeneity (complex molecules induce stronger immune responses) (Kuby, 1997).

The most common antibody-based technique is the Enzyme-Linked ImmunoSorbent Assay (ELISA). In a comparison of two ELISA kits (polyclonal antibodies from Neogen and Biopharm) and GC-MS/MS for the detection of estrogenic compounds in wastewater effluent and surface water after extraction by solid phase extraction on C-18 (Octadecylsilane), both techniques gave comparable results but the ELISA kits were faster, had lower detection limits and were less susceptible to matrix interference than GC-MS/MS (Huang and Sedlak, 2001).

1.5 Sorption

Sorption is defined as the adherence of a chemical to a solid particle, either internally or at its surface. Sorption sites are not all equally available but sorption proceeds from the most available to the least available sorption site. Factors that can influence sorption are: the molecular structure of the chemical (the type and number of functional groups, the polarity of the compound, the distribution of its charges and the solubility of the compound), the chemical characteristics of the sorbent and environmental characteristics such as the amount of water, pH, temperature, ionic strength and redox potential (Loffredo and Senesi, 2006).

Sorption is usually determined at equilibrium (when the rate of sorption and desorption are equals). Diverse methods can be used to determine sorption. One of them is the batch equilibrium experiment where soil and an aqueous solution in which the chemical of interest is dissolved are shaken together until equilibrium is reached. The chemical concentration of the equilibrium solution is then quantified to calculate the soil sorption coefficient. As another example, soil-column experiments provide for a measure of the retention of a contaminant along the soil profile. In these experiments, there is lower water to soil ratio which is closer to the natural state of the soil compared to the slurry used in batch equilibrium experiments. In addition, column studies can provide information on desorption more readily than batch equilibrium experiments (Bi et al., 2010). However, relative to batch equilibrium experiment, column experiments require more material and experimental preparation. For batch equilibrium experiments, sorption can be described by isotherms representing the relationship between the concentration sorbed by soil and the concentration left in the equilibrium solution. Different types of isotherms have been proposed including the linear isotherm, the Freundlich isotherm (Freunlich, 1909), and the Langmuir isotherm (Langmuir, 1916).

Studies that have investigated the sorption of estrogens by soils are presented in Table 1.2. Previous studies (Yu et al., 2004; Hildebrand et al., 2006; Loffredo and Senesi, 2006) have showed that the sorption of estrogens is influenced to a great extent by soil organic carbon content. For most organic contaminants, sorption and mineralization are negatively correlated since sorption reduces bioavailability (Ogram et al., 1985; Greer and Sheldon, 1992; Gaultier et al., 2008). In contrast, Casey et al., (2003) and Layton et

al. (2000) concluded that transformations of 17β-estradiol could occur in the sorbed phase for estrogens.

Table 1.3: Work published on the sorption of estrogens by soils.

Texture	**Organic content (g C kg^{-1})**	**Soil sorption coefficient (Kd L kg^{-1})**	**Number of soils**	**Reference**
Silty clay loam, clay loam, sandy clay loam, silt loam and loam	19.1-53.3	17β-estradiol Kd:86-6,670	5	Casey et al., 2003
Heavy clay, loamy sand, silt loam, freshwater sediment	2.2 -29.1	17β-estradiol Kd: 3.6 -83.2	6	Lee et al., 2003
Soil minerals	-	goethite ≤ illite < kaolinite ≤ montmorillonite.	4	Van Emmerick et al., 2003
Silty clay loam,fresh water sediment	2.2-29.1	17β-estradiol Kd:3.4-83.2 estrone Kd:3.4-48.1	2	Das et al., 2004
Agricultural silt loam	53.3	17β-estradiol Kd: 18.0-89.5, estrone Kd: 30.4-95.2 depending on time	1	Casey et al., 2005
Agricultural soils	54 -74	17β-estradiol: Kd: 31-123, estrone Kd: 26-108, estriol Kd: 9-68	4	Ying and Kookana 2005
Sand, silt loam, clay loam and silty clay	7.9-24.5	17β-estradiol Kd:48-158 Lkg^{-1}	4	Hildebrand et al., 2006
Acidic sandy	1.1-9.3	17β-estradiol Kd:1-3.5	2	Loffredo and Senesi, 2006
Sandy loam	-	17β-estradiol Kd 9.56-21.90 [1]	1	Sangsupan et al., 2006

1.6 Mineralization

Unless otherwise noted, mineralization is defined in this thesis as the degradation of a compound to CO_2 and inorganic compounds. 17β-estradiol was the only estrogen included in this research for studies on mineralization. The reported 17β-estradiol half-lives are mineralization half-lives. A table presenting results from previous studies on the mineralization of 17β-estradiol in soils is presented in Chapter 3 (Table 3.7).

In agricultural soils of eastern Canada (Colucci et al., 2001), Australia (Ying and Kookana, 2005) and the USA (Fan et al., 2007; Xuan et al., 2008) it was observed that mineralization of 17β-estradiol was biological. Many bacteria, including *Pseudomonas* spp. which is a common bacterium in agricultural soils (Stumm-Zollinger and Fair, 1965), are able to participate in the biodegradation of estrogens (Hanselman et al., 2003). The first order kinetics is the most frequently used equation to describe 17β-estradiol mineralization (Colucci et al., 2001; Ying and Kookana, 2005; Fan et al., 2007; Xuan et al., 2008). The first-order kinetics occurs when the rate of the reaction depends on the concentration of the chemical only (IUPAC, 1997). In four agricultural soils from Israel and Germany, Stumpe and Marschner (2007) observed that the mineralization of 17β-estradiol occurred co-metabolically. One definition of co-metabolism is by Horvath (1972) which implies that co-metabolism is the oxidation of a chemical by microorganisms without them utilizing the energy derived from the oxidation for their growth.

Under aerobic conditions in sediment and groundwater from an aquifer, 17β-estradiol had half-lives ranging from 2 to 7 days while under anaerobic conditions the estimated half-lives were 107 days (Ying et al., 2003). Similar results were found for an American agricultural soil where 6% of 17β-estradiol was mineralized under aerobic conditions but no mineralization was observed under anaerobic conditions after 132 hours (Fan et al., 2007). In an American silt loam, Xuan et al (2008) identified estrone as a degradation product of 17β-estradiol while estriol was that of estrone. Estrone is the most persistent among these three estrogens (Colucci et al., 2001). It has also been found that white root fungi produce an enzyme (lignin peroxydase) which is able to transform 17β-estradiol and eliminate estrogenic activity (as measured by the E-screen assay) (Mao et al., 2010).

1.7 Gaps in Science

Most of the research on endocrine disrupters and estrogens has been conducted with regards to their possible physiological and toxicological impacts. There is a general lack of scientific knowledge on estrogen environmental fate, particularly in agricultural soils even though they are exposed to estrogens because of sewage-sludge or livestock manure applications. Where the sorption and mineralization of estrogens in soils has been studied, research predominantly focused on the fate of 17β-estradiol (Table 1.3). These studies were also conducted only for a few soils (generally less than ten) and have not provided information at a broader scale (e.g., field and regional scales). The limited number of samples used in previous studies not only restricts the type of statistical

analysis that can be conducted but also impairs generalization of the obtained results to a larger scale and therefore, such studies do not provide enough information to allow for the development of prediction equations that could potentially be used by modellers and/or decision makers.

In addition, the impact of repetitive manure applications over time as well as fresh manure application on estrogen fate in soils has not been studied to a great extent. It is of primary importance to understand the role manure plays in the fate of natural estrogens since estrogens will most often be released in combination with manure in the agricultural ecosystem, but also because manure can alter soil characteristics such as soil organic content (SOC) (Gami et al., 2009; Huang et al., 2009; Mugwe et al., 2009; Huang et al., 2010). To my knowledge, the only publication examining at the impact of repetitive manure applications on the fate of estrogens was recently published in 2010 by Stumpe and Marschner.

1.8 Objectives

The first objective of the research was to better understand the relationship between soil properties and sorption of four estrogens (17ß-estradiol, estrone, estriol and equol) at a field and regional scale (Chapter 2). The second objective was to better understand the relationship between soil properties, sorption and mineralization at a provincial and field scale (Chapter 3). The third objective was to determine the impact of fresh and a history of manure application on sorption and mineralization of 17β-estradiol (Chapter 4).

Finally, the fourth objective was to develop an Enzyme-Linked ImmunoSorbent Assay for the determination of estrogen concentrations in environmental samples (Chapter 5).

Therefore, the present thesis aims at a comprehensive understanding of the fate (sorption and mineralization) of estrogens at the regional and field scales as influenced by soil properties and manure application as well as their potential detection in environmental samples by ELISA. Both elements (fate and detection) are essential in advancing scientific understanding of the potential impacts estrogens of agricultural origin might have on ecosystems.

1.9 Hypotheses

The hypotheses are: (1) Sorption of estrogens is positively related to soil organic content (SOC), therefore, soils with a greater SOC have greater sorption. (2) Sorption and mineralization are negatively correlated because sorption reduces bioavailability, therefore, soils with a greater SOC have greater 17β-estradiol sorption but lesser 17β-estradiol mineralization. (3) Manure application to soil increases SOC, therefore increasing sorption and reducing mineralization of 17β-estradiol in soil.

1.10 Outline of the Thesis

This is a sandwich-style thesis in which four chapters will be presented following this introduction. Chapter 2. Sorption of Four Estrogens by Surface Soils from 41 Cultivated Fields in Alberta, Canada was published in Geoderma (Caron, E., Farenhorst, A.,

Gaultier, J., Rank, N., Goddard, T. and Sheedy, C. 2010. Sorption of Four Estrogens by Surface Soils from 41 Cultivated Fields in Alberta, Canada. Geoderma 155:19-30). Chapter 3. Mineralization of 17ß-estradiol in 36 Surface Soils from Alberta, Canada has been published by Agriculture, Ecosystems and the Environment (Caron, E., Farenhorst, A., Mc Queen, R., Sheedy, C., Goddard, T.and Gaultier, J. 2010. Mineralization of 17ß-estradiol in 36 Surface Soils from Alberta, Canada. Agriculture, Ecosystems and Environment, 139(4):534-545. Chapter 4. Manure Application Effects on Soil Properties and 17ß-Estradiol Sorption and Mineralization Parameters is going through internal review before being submitted to Journal of Environmental Science and Health Part B, Pesticides, Food Contaminants, and Agricultural Wastes. Chapter 5. Development of Competitive ELISAs for 17β-Estradiol and 17β-Estradiol +Estrone+Estriol Using Rabbit Polyclonal Antibodies was published in Journal of Environmental Science and Health Part B, Pesticides, Food Contaminants, and Agricultural Wastes (Caron E., Sheedy, C. and Farenhorst, A. 2010. Development of Competitive ELISAs for 17β-Estradiol and 17β-Estradiol +Estrone+Estriol using rabbit polyclonal antibodies. Journal of Environmental Science and Health Part B: Pesticides, food contaminants and agricultural wastes 45(2):145-151). These four chapters are followed by a general discussion on the main results of this thesis as well as the implications and impacts of the four studies on current scientific knowledge. Suggestions to improve future studies on estrogen's fate and risk assessments are also discussed.

My contribution to each of Chapter 2 to 4 of the thesis is as follows: conducting field and laboratory experiments, analyzing results including calculation in Excel spreadsheets and

performing additional statistical analyses in various software packages, writing drafts and performing subsequent corrections to the manuscripts, finalizing and submitting manuscripts to refereed international journals, and addressing reviewers and editorial comments prior to publication and in some cases (Chapter 2) supervising undergraduate students who facilitated laboratory work. With respect to Chapter 5, this included the above, as well as assisting under the leadership of Dr. Claudia Sheedy with the immunizations and care of animals (rabbits).

1.11 Rationale

The Province of Alberta was chosen as a case study because Alberta is the most important cattle producing province in Canada (Alberta Agriculture and Food, 2006). Estrogens have been detected in rivers in the southern part of Alberta, with some indication that fish populations have been impacted by these chemicals (Jeffries et al. 2008). For example, Jeffries et al. (2008) found longnose dace with high vitellogenin concentrations in the Bow and Oldman rivers and this fish species also showed female-biaised sex-ratios in the Oldman River.

Soil samples were collected from 41 fields in Alberta. These sites were subsets of the well-documented Alberta benchmark sites program (Leskiw et al., 2000, Cathcart et al., 2008). The benchmark sites are distributed across ecodistricts in Alberta, hence reflective of the diversity of soil-landscapes and agricultural management systems found in Alberta. In general, the agricultural production systems included in the benchmark sites program

are majoritarily annual cropping systems and also pasture lands. Three of the agricultural production systems use irrigation. 20 sites were classified as undulating and hummocky soil-landscapes, whereas 21 additional sites either had more leveled soil-landscapes or were described as rolling with a slope of less than 5% (Cathcart et al., 2008). Within each site, landscape position along a catena was determined by Can-Ag Enterprises using information from the Agricultural Region of Alberta Soil Inventory Database (AGRASID) (CAESA–Soil Inventory Project Working Group, 1998) (Cathcart et al., 2008).

Four estrogens were chosen for the sorption study: 17β-estradiol, estrone, estriol and equol. 17β-estradiol is the most potent of natural estrogens and estrone and estriol are its degradation products while equol has been found to be the dominant source of estrogenic-activity in swine manure (Lorenzen et al., 2004).

The research described in this thesis is based on a much larger soil sample size that was included in previous studies. Because the number of samples was relatively large (121 for sorption and 36 for mineralization), stronger statistical test could be utilized, which in turn provided for stronger analyses of the differences in estrogen fate at the regional and field-scale levels, as well as that it provided for opportunities to develop regression equations. These equations may be used by modelers and decision makers when conducting environmental risk studies in order, for example, to determine agricultural systems or regions that could potentially result in a higher risk of estrogen movement from agricultural land to surface waters. If these agricultural systems or regions are

identified, there could be a greater focus on environmental monitoring in these regions, and the development of best management practices for these agricultural systems.

In Chapter 2 and 3, the soil sorption coefficient, Kd, is used to express the extent of sorption by soil. For practical purposes, equilibrium time was established using a very small subset of soil samples, rather than the 121 soil samples that were included in the overall study. In contrast, in Chapter 4, the sorption of estrogens in the soil at equilibrium, Cs, is used to express the extent of sorption by soil because this study included only 24 soil samples and hence a more comprehensive investigation of the impact of rotation time on estrogen sorption by soil was investigated. Such investigation requires the sorption of the chemical by soil to be determined as Cs.

Detection methods using enzyme-linked immunosobent assay already exist for the detection of estrogen. However, immunoassays for estrogens are most often developed for physiological matrices (blood or urine), while this research focused on the development of techniques that use antibodies for the detection of estrogens in environmental matrices. In addition, developing an in-house ELISA is a good tool for cost-reduction in research activities.

1.12 References

Alberta Agriculture and Food, Economics and Competitiveness Division, Statistics and Data Development Unit. 2006. Census of Agriculture for Alberta, Provincial Highlights. AGDEX 852-5.

Bi, E., Schmidt, T.C. and Haderlein, S.B. 2010. Practical issues relating to soil column chromatography for sorption parameter determination. Chemosphere 80:787:793.

Braga, O., Smythe, G.A., Schäfer, A.I. and Feitz, A.J. 2005. Steroid estrogens in ocean sediments. Chemosphere 61:827-833.

Brande-Lavridsen, N., Christensen-Dalsgaard, J. and Korsgaard, B. 2008. Effects of prochloraz and ethinylestradiol on sexual development in *Rana temporaria*. J. Exp.Zool., 309A:389-398.

Brian, J.V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G., Jonker, N., Runnals, T., Bonfà, A., Marcomini, A. and Sumpter, J.P. 2005. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environ. Health Perspect., 113(6):721-728.

CAESA-Soil Inventory Project Working Group. 1998. AGRASID: Agricultural Region of Alberta Soil Inventory Database (Version 1.0). Edited by J.A. Brierley, B.D. Walker, P.E. Smith, and W.L. Nikiforuk. Alberta Agriculture, Food and Rural Development. Publications. CD-ROM.

Casey, F.X.M., Larsen, G.L., Hakk, H. and Šimůnek, J. 2003. Fate and transport of 17β-estradiol in soil-water systems. Environ. Sci. Technol., 37:2400-2409.

Casey, F.X.M., Šimůnek, J., Lee. J., Larsen, G.L. and Hakk, H. 2005. Sorption, mobility, and transformation of estrogenic hormones in natural soils. J. Environ. Qual. 34:1372-1379.

Cathcart, J., Cannon, K., and Heinz, J. 2008. Selection and establishment of Alberta agricultural soil quality benchmark sites. Can. J. Soil Sci., 88:399-408.

Chang, C.C. and Huang, S.D. 2010. Determination of the steroid hormone levels in water samples by dispersive liquid-liquid microextraction with solidification of a floating organic drop followed by high-performance liquid chromatography. Anal. Chim. Acta, 62(1):39-43.

Clotfelter, E.D. and Rogriguez, A.C. 2006. Behavioral changes in fish exposed to phytoestrogens. Environ. Pollution, 144:833-839.

Coe, T., Hamilton, P.B., Hodgson, D., Paull, G.C. and Tyler, C.R. 2009. Parental outcomes in response to estrogen exposure are modified by social grouping in zebrafish. Environ. Sci. Tot., 43:8400-8405.

Colucci, M.S., Bork, H. and Topp, E. 2001. Persistence of estrogenic hormones in agricultural soils: I. 17β-estradiol and estrone. J. Environ. Qual., 30:2070-2076.

Das, B.S., Lee, L.S., Rao, P.S. and Hultgren, R.P. 2004. Sorption and degradation of steroid hormones in soils during transport: Column studies and model evaluation. Environ. Sci. Technol., 38:1460-1470.

Dussault, E.B., Balakrishnan, V.K., Solomon, K.R. and Sibley, P.K. 2008. Chronic toxicity of the synthetic hormone 17α-ethinylestradiol to Chironomus tentans and Hyalella azteca. Environ. Toxicol. Chem. 27(12):2521-2529.

Fan, Z., Casey, F.X.M., Hakk, H. and Larsen, G.L. 2007. Persistence and fate of 17ß-estradiol and testosterone in agricultural soils. Chemosphere, 67:886-895.

Fine, D.D., Breidenbach, G.P., Price, T.L. and Hutchins, S.R. 2003. Quantification of estrogens in ground water and swine lagoon samples using solid-phase extraction, pentafluorobenzyl/trimethylsilyl derivatizations and gas chromatography-negative ion chemical ionization tandem mass spectrometry. J. Chromatogr. A, 1017:167-185.

Finlay-Moore, O., Hartel, P.G. and Cabrera, M.L. 2000. 17β-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. J. Environ. Qual., 29:1604-1611.

Freundlich, H. 1909. Kapillarchemie: eine Darstellung der Chemie der Kolloide und Verwandter Gebiete, Leipzig, Akademische Verlagsgesellschaft, 591 p

Gami, S.K., Lauren, J.G. and Duxbury, J.M. 2009. Soil organic carbon and nitrogen stocks in Nepal long-term soil fertility experiments, Soil Tillage Res., 106(1):95-103.

Gaultier, J., Farenhorst, A., Cathcart, J. and Goddard, T. 2008. Regional assessment of herbicide sorption and degradation in two sampling years. J. Environ. Qual., 37:1825-1836.

Greer, L.E. and Sheldon, D.R. 1992. Effect of inoculant strain and organic matter content on kinetics of 2,4-Dichlorophenoxacetic acid degradation in soils. App. Environ. Microbiol., 58:1459-1465.

Hanselman, T.A., Graetz, D.A. and Wilkie, A.C. 2003. Manure-borne estrogens as potential environmental contaminants: A review. Environ. Sci. Technol., 37(4):5471-5478.

Hanselman, T.J., Graetz, D.A. and Wilkie, A.C. 2004. Comparison of three enzyme immunoassays for measuring 17-ß-estradiol in flushed dairy manure wastewater. J. Environ. Qual., 33:1919-1923.

Hájková. K., Pulkrabová, J., Schůrek, J., Hajšlová, J., Poutska, J., Nápravniková. M. and Kocourek, V. 2007. Novel approaches to the analysis of steroid estrogens in river sediments. Anal. Bioanal. Chem., 387:1351-1363.

Hildebrand, C., Londry, K.L. and Farenhorst, A. **2006**. Sorption and desorption of three endocrine disrupters in soils. J. Environ. Sci. Heal. B, 41:907-921.

Horvath, R.S. 1972. Microbial co-metabolism and the degradation of organic compounds in nature. Bacteriological Rev. 36(2):146-155.

Hu, J., Zhang, H. and Chang, H. 2005. Improved method for analyzing estrogens in water by liquid chromatography-electrospray mass spectrometry. J. Chromatogr. A, 1070:221-224.

Hu, F., Smith, E.E., and Carr, J.A. 2008. Effects of larval exposure to estradiol on spermatogenesis and in vitro gonadal steroid secretion in African clawed frogs, Xenopus laevis. Gen. Comp. Endocr., 155:190-200.

Huang, C.-H. and Sedlak, D.L. 2001. Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. Env. Toxicol. Chem., 20(1):133-139.

Huang, Q.R., Hu, F., Huang, S., Li, H.-X., Yuan, Y.-H., Pan, G.X. and Zhand, W.J. 2009. Effect of long-term fertilization on organic carbon and nitrogen in a subtropical paddy soil. Pedosphere, 19(6):727-734.

Huang, S., Peng, X, Huang, Q and Zhang, W. 2010. Soil aggregation and organic carbon fractions affected by long-term fertilization in a red soil of subtropical China. Geoderma, 154(3-4):364-369.

Hurwitz, A.R., and Liu, S.T. 1977. Determination of aqueous solubility and pka values of estrogens. J. Pharm. Sci., 66(5):624-627.

Hutchins, S.R., White, M.V., Hudson, F.M. and Fine, D.D. 2007. Analysis of lagoon sample from different concentrated animal feedings operations for estrogens and estrogen conjugates. Env. Sci. Technol., 41(3):738-744.

Ivie, G.W., Christopher, R.J., Munger, C.E. and Coppock, C.E.J. 1986. Fate and Residues of [4-C-14] Estradiol-17-beta after intramuscular injection into Holstein steer calves. J.Anim. Sci., 62:681-690.

IUPAC. 1997 Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997). XML on-line corrected version: http://goldbook.iupac.org (2006-) created

by M. Nic, J. Jirat, B. Kosata; updates compiled by A. Jenkins. ISBN 0-9678550-9-8. doi:10.1351/goldbook.

Jeffries, K.M., Nelson, E.R., Jackson, L.J. and Habibi, H.R. 2008. Basin-wide impacts of compounds with estrogen-like activity on longnose dace (Rhinichthys Cataractae) in two prairie rivers of Alberta, Canada. Environ. Toxicol. Chem. 27(10):2042-2052.

Johnson, A.C., Belfroid, A. and Di Corcia, A. 2000. Estimating steroid estrogens inputs into activated sludge treatment works and observations on their removal from the effluent. Sci. Tot. Environ., 256:163-173.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M. and Flick, R.W. **2007**. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci., 104(21):8897-8901.

Kitamura, K., Miyajima, K., Higashitani, T., Nadaka, N., Komori, K and Suzuki, Y. 2009. The effect of estrogens, river water, and treated wastewater on vitellogenin induction of Japanese medaka. J. Amer. Water Resour.Assoc., 45(1):22-34.

Kolodziej, E.P. and Sedlak, D.L. 2007. Rangeland grazing as a source of steroid hormones to surface water. Environ. Sci. Technol., 41:3514-3520.

Kuby, J. 1997. Immunology 3rd ed. W.H. Freeman and Company, N.-Y., 664 pages.

Kumar, V., Nadaka, N., Yasojima, M., Yamashita, N., Johnson, A.C. and Tanaka, H. 2009. Rapid determination of free and conjugated estrogen in different water matrices by liquid chromatography-tandem mass spectrometry. Chemosphere, 77(10):1440-1446.

Kuster, M., Azevedo, D.A., de Alda, M.J.L., Neto, F.R.A. and Barcelo, D. 2009. Analysis of phytoestrogens, progestogens and estrogens in environmental waters from Rio de Janeiro (Brazil). Environ. Int., 35(7):997-1003.

Labadie, P., and Budzinski, H. 2005. Development of an analytical procedure for determination of selected estrogens and progestagens in water samples. Anal. Bioanal. Chem., 381:1199-1205

Labadie, P. and Budzinski, H. 2006. Alteration of hormone profile in juvenile turbot (*Psetta maxima*) as a consequence of short-term exposure to 17α-ethynylestradiol. Chemosphere, 64:1274-1286.

Labadie, P. and Hill, E.M. 2007. Analysis of estrogens in river sediments by liquid chromatography-electrospray ionization mass spectrometry Comparison of tandem mass spectrometry and time-of-flight mass spectrometry. J. Chromatogr. A, 1141:174-181.

Lahnsteiner, F., Berger, B, Kletzl, M. and Weismann, T. 2006. Effect of 17ß-estradiol on gamete quality and maturation in two salmonid species. Aquat. Toxicol., 79:124-131.

Lai, K.M., Scrimshaw, M.D. and Lester, J.N. 2002. The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit. Rev. Toxicol., 32 (2):113-132.

Langmuir, I. 1916. The constitution and fundamental properties of solids and liquids. part I. solids. J. Am. Chem. Soc. 38: 2221-95.

Layton, A.C., Gregory, B.W., Seward, J.R., Schultz, T.W. and Sayler, G.S. 2000. Mineralization of steroidal hormones by biosolids in wastewater treatment systems in Tennessee U.S.A. Environ. Sci. Technol., 34:3925-3931.

Lee, L.S., Strock, T.J. Sarmah, A.K. and Rao, P.S.C. 2003. Sorption and dissipation of testosterone, estrogens and their transformation products in soil and sediment. Environ. Sci. Technol., 37:4098-4105.

Leskiw, L.A., Yarmuch, M.S., Waterman, L.P. and Sansom, J.J. 2000. Baseline soil physical and chemical properties of forty-three soil quality benchmark sites in Alberta. File #99-042, Can-Ag Enterprises Ltd., Edmonton, AB., 175pp.

Lewis, K.M. and Archer, R.D. 1979. pKa values of estrone, 17 beta-estradiol and 2-methoxyestrone. Steroids, 34(5):485-499.

Liao, T., Guo, Q.L., Jin, S.W., Cheng, W., and Xu, Y. 2009. Comparative responses in rare minnow exposed to 17*b*-estradiol during different life stages. Fish Physiol. Biochem., 35:341-349.

Lien, G.W., Chen., C.Y., Wang, G.S. 2009. Comparison of electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization for determining estrogenic chemicals in water by liquid chromatography tandem mass spectrometry with chemical derivatizations J. Chromatogr. A, 1216(6):956-966.

Liu, Z.H., Hashimoto, T., Okumura, Y., Kanjo, Y. and Mizutani, S. 2010. Simultaneous analysis of natural free estrogens and their conjugates in wastewater by GC-MS. Clean-Soil Air Water, 38(2):181-188.

Loffredo, E and Senesi, N. 2006. Fate of organic pollutants in soils with emphasis on adsorption/desorption processes of endocrine disruptor compounds. Pure Appl. Chem., 78(5):947-961.

Lorenzen, A., Burnison, K., Servos, M. and Topp, E. 2006. Persistence of endocrine-disrupting chemicals in agricultural soils. J. Environ. Eng. Sci., 5:211-219.

Lv, X., Zhou, Q., Song, M., Jiang, G and Shao, J. 2007. Vitellogenic responses of 17β-estradiol and bisphenol A in male Chinese loach (*Misgurnus anguillicaudatus*). Environ.Toxicol. Pharmacol., 24:155-159.

Mao, L., Lu, J., Habteselassie, M., Luo, Q., Gao, S., Cabrera. M. and Huang, Q. 2010. Ligninase-mediated removal of natural and sybthetic estrogens from water: Reactions with 17β-estradiol. Environ. Sci. Technol., 44(7):2599-2604

Matthiessen, P., Arnold, D., Johnson, A.C., Pepper, T.J., Pottinger, T.G. and Pulmam, K.G.T. 2006. Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. Sci. Tot. Environ., 367:616-630.

Miege, C., Bados, P., Brosse, C. and Coquery, M. 2009. Method validation for the analysis of estrogens (including conjugated compounds) in aqueous matrices. TrAC-Trends. Anal. Chem., 28(2):237-244.

Mills, L.J. and Chichester, C. 2005. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Sci. Tot. Environ., 343:1-34.

Mouatassim-Souali, A., Tamisier-Karolak, S.L., Perdiz, D., Cargouet, M. and Levi, Y. 2003. Validation of a quantitative assay using GC/MS for the trace determination of free and conjugated estrogens in environmental waters amples. J. Sep. Sci., 26:105-111.

Mugwe, J., Mugendi, D., Mucheru-Muna, M., Odee, D. and Mairura, F. 2009. Effect of selected organic materials and inorganic fertilizer on the soil fertility of a Humic Nitisol in the central highlands of Kenya. Soil Use Manage., 25(4):434-440.

Mustonen, E.A., Jokela, T., Saastomoinen, I., Taponen, J., Taponen, S., Saloniemi, H. and Wähälä, K. 2006. High serum S-equol content in red clover fed ewes: the classical endocrine disruptor is a single enantiomer. Environ. Chem. Lett., 3:154-159.

Nielsen, L. and Baatrup, E. 2006. Quantitative studies on the effects of environmental estrogens on the testis of the guppy, *Poecilia reticulata*. Aquat. Toxicol., 80:140-148.

Nussey, S.S. and Whitehead, S.A. **2001**. Endocrinology, an integrated approach. Taylor and Francis
[On Line] http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=endocrin.section.1022

Ogram, A.V., Jessup, R.E., Ou, L.-T. and Rao, P.S.C. 1985. Effect of sorption on biological degradation rates of 2,4-dichlorophenoxyacetic acid in soils. App. Environ. Microbiol., 49:582-587.

Palace, V.P., Wautier, K.G., Evans, R.E., Blanchfield, P.J., Mills, K.H., Chalanchuk, S.M., Godard, D., McMaster, M.E.,Tetreault, G.R., Peters, L.E., Vandenbyllardt, L and Kidd, K.A. 2006. Biochemical and histopathological effects in Pearl dace

(*Margariscus margarita*) chronically exposed to a synthetic estrogen in a whole lake experiment. Environ. Toxicol. Chem. 25(4):1114-1125.

Palme, R., Fischer, P., Schildorfer, H., and Ismail, M.N. 1996. Excretion of infused C-14-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci., 43:43-63.

Park, B.J. and Kidd, K. 2005. Effects of the synthetic estrogens ethinylestradiol on early life stages of mink frogs and green frogs in the wild and in situ. Environ. Toxicol. Chem. 24(8):2027-2036.

Pedrouzo, M., Borull, F., Pocurull, E. and Marce, R.M. 2009. Estrogens and their conjugates: Determination in water samples by solid-phase extraction and liquid chromatography-tandem mass spectrometry. Talanta, 78(4-5):1327-1331.

Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler, C.R. and Sumpter JP. 1994. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol., 8:275-285.

Quintana, J.B., Carpinteiro, J., Rodriguez, I., Lorenzo, R.A., Carro, A.M. and Cela, R. 2004. Determination of natural and synthetic estrogens in water by gas chromatography with mass spectrometric detection. J. Chromatogr. A, 1024:177-185.

Rawson, C.A., Lim, R.P., Warne, M.,St. J. and Doyle, C. 2006. The Effect of 17β-Estradiol on the development of modified hemal spines in early–life stage *Gambusia holbrooki*. Arch. Environ. Contam. Toxicol., 51:253-262.

Robinson, C.D., Brown, E., Craft, J.A., Davies, I.M., Megginson, C., Miller, C. and Moffat, C.F. 2007. Bioindicators and reproductive effects of prolonged 17β-oestradiol exposure in a marine fish, the sand goby (*Pomatoschistus minutus*). Aquatic Toxicol.,81:397-408.

Rodriguez-Mozaz, S., Lopez de Alda, M.J. and Barceló, D. 2004. Picogram per liter level determination of estrogens in natural waters and waterworks by a fully automated on-line solid-phase extraction-liquid chromatography-electrospray tandem mass spectrometry method. Anal. Chem.,76:6998-7006.

Saaristo, M, Craft, J.A., Lehtonen, K.K., Brörk, Lindström, K. 2009. Disruption of sexual selection in sand gobies (*Pomatoschistus minutus*) by 17α-ethinylestradiol, an endocrine disruptor. Hormones and Behavior, 55:530-537.

Salierno, J.D. and Kane, A.S. 2009. 17α-ethinylestradiol alters reproductive behaviours, circulating hormones and sexual morphology in male fathead minnows (*Pimephales promelas*). Environ. Toxicol. Chem., 28(5):953-961.

Sangsupan, H.A., Radcliffe, D.E., Hartel, P.G., Jenkins, M. B., Vencil, W.K. and Cabrera, M.L. 2006. Sorption and transport of 17β-estradiol and testosterone in undisturbed soil columns. J. Environ. Qual. 35:2261-2272.

Sarmah, A.K., Northcott, G.L., Leusch, F.D.L. and Tremblay, L.A. 2006. A survey of endocrine disrupting chemicals (EDCs) in municipal sewage an animal waste effluents in the Waikito region of New Zealand. Sci. Tot. Environ., 355:135-144.

Stumm-Zollinger, E. and Fair, G.M. 1965. Biodegradation of steroids hormones. Steroid Hormones, 37(11):1506-1511.

Stumpe, B. and Marschner, B. 2007. Long-term sewage sludge application and wastewater irrigation on the mineralization and sorption of 17β-estradiol and testosterone in soils. Sci. Total Environ., 374:282-291.

Stumpe, B. and Marschner, B. 2010. Organic waste effects on the behavior of 17β-estradiol, estrone and 17α-ethinylestradiol in agricultural soils in long-and short-term setups. J. Environ. Qual., 39:907-916.

Stumper, J.P. and Jobling, S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health. Perspect., 103, Supplement 7:173-178.

Ternes, T.A., Stumph, M., Mueller, J., Haberer, K., Wilken, R.D. and Servos, M. 1999. Behaviour and occurence of estrogens in municipal sewage treatment plants-I. Investigations in Germany, Canada and Brazil, Sci. Tot. Environ., 225:81-90.

Ternes, T.A., Anderson, H., Gilberg, D. and Bonerz, M. 2002. Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. Anal. Chem., 74 (14):3498-3504.

Thilagram, H., Gopalakrishan, S. Bo, J. and Wang, K.-J. 2009. Effect of 17β-estradiol on the immunocompetence of Japanese bass (*Lateolabrax japonicus*). Environ. Toxicol. Chem., 28(8):1722-1731.

Vanderford, B.J., Pearson, R.A., Rexing, D.J. and Snyder, S.A. 2003. Analysis of endocrine disruptors, pharmaceuticals, and personal care products in water using liquid chromatography/tandem mass spectrometry, Anal. Chem., 75:6265-6274.

Van Emmerik, T., Angove, M.J., Johnson, B.B., Wells, J.D. and Fernandes, M.B. 2003. Sorption of 17β-estradiol onto selected soil minerals. J. Colloid Interface Sci., 266:33-39.

Werner, J., Palace, V.P., Wautier, K.G., Mills, K.H., Chalanchuk, S.M. and Kidd, K.A. 2006. Reproductive fitness of lake trout (*Salvelinus namaycush*) exposed to

environmentally relevant concentrations of the potent estrogen ethinylestradiol (EE2) in a whole lake exposure experiment. Sci. Mar., 70,S2, October:56-66.

Williams, E.L. 2002. Survey of estrogen concentrations in dairy and swine waste storage and treatment facilities in and around Tennessee M.S. Thesis, University of Tennessee, Knoxville, 94 pp.

World Health Organization. 2002. Global assessment of the state-of-the-science of endocrine disruptors, Edited by Damstra, T., Barlow, S., Bergman, A., Kavlock, R. and Van Der Kraak, G., WHO/PCS/EDC/02.2, 180 pages.

Xu, H., Yang, J., Wang, Y., Jiang, Q., Cheng, H. and Song, H. 2008. Exposure to 17α-ethynylestradiol impairs reproductive functions of both male and female zebrafish (*Danio rerio*). Aquatic Toxicol., 88:1-8.

Xuan, R., Blassengale, A.A. and Qiquan, W. 2008. Degradation of estrogenic hormones in a silt loam soil. J. Agric. Food Chem., 56:9152-9158.

Yan, W., Zhao, L.X., Feng, Q,Z., Wei, Y.L., and Lin, J.M. 2009. Simultaneous determination of ten estrogens and their metabolites in waters by improved two-step SPE followed by LC-MS. Chromatographia, 69(7-8):621-628.

Ying, G.G., Kookana, R.S. and Dillon, P. 2003. Sorption and degradation of selected five endocrine disrupting chemicals in aquifer material. Water Res., 37:3785-3791.

Ying, G.-G. and Kookana, R.S. 2005. Sorption and degradation of estrogen-like – endocrine disrupting chemicals in soil. Environ. Toxicol. Chem., 24(10):2640-2645.

Yu, Z., Xiao, B., Huang, W. and Peng, P. 2004. Sorption of steroid estrogens to soils and sediments. Environ. Toxicol. Chem., 23(3):531-539.

Zhang, H., Kong, F.-X., Wang, S.-H., Yu, Y. and Zhang, M. 2009. Vitellogenin induction by a mixture of steroidal estrogens in freshwater fishes and relevant risk assessment. Environ. Toxicol., 24(5):484–491.

Zhou, Y.Q., Zhou, J., Xu, Y.P., Zha, J.M., Ma, M. and Wang, Z.J. **2009**. An alternative method for the determination of estrogens in surface water and wastewater treatment plant effluent using pre-column trimethylsilyl derivatization and gas chromatography/mass spectrometry. Environ. Monit. Assess. 158(1-4):35-49.

2. SORPTION OF FOUR ESTROGENS BY SURFACE SOILS FROM 41 CULTIVATED FIELDS IN ALBERTA, CANADA.

Caron, E., Farenhorst, A., Gaultier, J., Rank, N., Goddard, T. and Sheedy, C. 2010. Sorption of Four Estrogens by Surface Soils from 41 Cultivated Fields in Alberta, Canada. Geoderma 155:19-30. Reproduced with permission from the editor.

2.1 Abstract

Estrogenic compounds in livestock manure are also present in soils because manure is land applied on account of its value as an important nutrient source in agricultural production. This is the first study to compare the sorption of 17β-estradiol, estrone, estriol and equol in a wide range of soils. Specifically, for each of these four estrogens, the soil sorption coefficient (Kd) and the sorption coefficient per unit organic carbon (Koc) were determined in 121 surface soils (0-15 cm) obtained from upper-slopes, mid-slopes, and lower-slopes in agricultural fields across seven ecoregions in the Province of Alberta, Canada. Soil organic carbon content (SOC), texture, pH, carbonate content, electrical conductivity and cation exchange capacity were also determined on the air-dried and sieved (2 mm) soil. Both Kd and Koc values significantly increased in the order of Kd-estriol (23 L kg^{-1} soil and 1,059 L kg^{-1}, respectively) = Kd-17β-estradiol (23 L kg^{-1} soil and 1,082 L kg^{-1}, respectively) < Kd-estrone (33 L kg^{-1} soil and 1,557 L kg^{-1}, respectively) < Kd-equol (42 L kg^{-1} soil and 2,080 L kg^{-1}, respectively). For each

estrogen, SOC was the strongest significant factor explaining variations in Kd values among soils and particularly small Kd values were observed when SOC was below a threshold value of 10 g C kg^{-1}. Regardless of the estrogen, the Mixed Grassland ecoregion characterized by Brown Chernozem soils, as well as upper-slopes in general demonstrated lesser Kd values because of reduced SOC. However, regardless of the estrogen, the soils with reduced SOC displayed greater Koc values. Equations to predict 17β-estradiol, estriol, estrone or equol Kd or Koc values at the regional level were established using either soil properties (Partial Least Squares (PLS) regression) or one single estrogen (ordinary least squares regression). Regardless of the estrogen or the regression used, the strength of the prediction model, as determined by the coefficient of determination (r^2) and other factors, was always better for Kd than Koc values. Regardless of the regression used, the r^2 of the prediction models exceeded 0.70 for Kd-17β-estradiol and Kd-estriol, but r^2 was below 0.52 for Kd-equol. For both Kd and Koc values, the prediction using soil properties (ranging from a r^2 of 0.51 to 0.87 for Kd and from a r^2 of 0.32 to 0.44 for Koc) always provided better prediction models than using a single estrogen (ranging from a r^2 of 0.38 to 0.71 for Kd and from a r^2 of 0.18 to 0.40 for Koc). We conclude that data on basic soil properties are good tools for estimating Kd values of 17β-estradiol and estriol in western Canadian soils. Additional studies are required to seek better prediction models at the regional scale for estimating Kd-estrone and Kd-equol and for estimating Koc values of estrogens, particularly because such information could be important for agri-environmental policy analyses in Canada and elsewhere.

2.2 Introduction

Livestock excrete a considerable amount of estrogens (Lange et al., 2002) and elevated concentrations of estrogens have been detected in soils following the application of manure onto agricultural land (Shore et al., 1995). Estrogens may move from agricultural fields to the broader environment by processes such as runoff and leaching (Nichols et al., 1997; Finlay-Moore et al., 2000; Kjær et al., 2007). Estrogens in stream water and lakes affect aquatic organisms by reducing their sperm count, elevating their blood vitellogenin, as well as inducing male feminization, poor offspring development and unusual behavioural effects (Purdom et al., 1994; Lai et al., 2002). Most environmental and toxicological studies have focused on steroidal compounds such as 17ß-estradiol, estrone and estriol.

Equol is a nonsteroidal compound produced by gastrointestinal bacteria in mammals from isoflavones such as formononetin and daidzein. Isoflavones are present in a range of crops and food products. Equol has been associated with reproductive disorders in ewes feeding on clover (Mustonen et al., 2006) but also to lower incidences of prostate cancer in humans favouring a diet of soybean based products (Akaza et al., 2004). Equol is excreted in the urine and can dominate estrogenic activity in manure (Lorenzen et al., 2006). To our knowledge, there have been no studies on the fate of equol in agricultural soils.

Sorption often controls the fate of organic compounds in agricultural soils. Sorption is generally defined by sorption coefficients, which are measured using batch equilibrium experiments. For most estrogens, equilibrium is usually reached within 24 h (Lai et al., 2000; Ying and Kookana, 2005; Hildebrand et al., 2006). However, equilibrium times vary among estrogens (Lee et al., 2003) with reported equilibrium times ranging from 1 h for 17ß-estradiol, estrone and estriol (Lai et al., 2000) to 168 h for 17ß-estradiol (Casey et al., 2003). Equilibrium times in the same soil ranged from 42 h for 17β-estradiol to 72 h for 17α-ethinyl estradiol. (Lee et al., 2003). Using 17ß-estradiol, Yu et al. (2004) reported that the equilibrium time of an estrogen in soil is also dependent on the estrogen concentration used.

Batch-equilibrium studies using estrogens have been conducted on a limited number of soils and no information on soil-landscape or regional-scale variations have been reported. Previous studies demonstrate that estrogen sorption is predominantly influenced by soil organic carbon content (SOC) (Yu et al., 2004; Hildebrand et al., 2006; Loffredo and Senesi, 2006), but specific surface area and hence soil texture can also affect estrogen sorption (Yu et al., 2004; Loffredo and Senesi, 2006).

At a regional scale spanning the provinces of Alberta, Saskatchewan and Manitoba, Canada, Anderson (1979) reported that SOC increases in the order of Dark Brown < Black < Dark Gray Chernozem soil great groups. In the Province of Alberta, Gaultier et al. (2008) quantified that SOC and 2,4-dichlorophenoxyacetic acid (a herbicide) sorption was significantly lesser in the Mixed Grassland ecoregion that contains Brown

Chernozems than in five other ecoregions with different soil great groups. Farenhorst et al. (2008) concluded that SOC and 2,4-D sorption typically increase in the order of upper < mid < lower slope positions in agricultural fields. There are no data on spatial variations of estrogen sorption within and between fields, soil great groups or ecoregions. Such information can be useful for defining beneficial land management practices for manure disposal and for agri-environmental policy analyses in Canada (Lefebvre et al., 2008).

The Province of Alberta is an important contributor to agricultural production in Canada with more than 96,000 km^2 devoted to annual cropping (Alberta Agriculture and Food 2006). The Alberta livestock industry accounts for more than 6 million cattle and calves and more than 2 million hogs, directly contributing to the Province of Alberta economy and generating manure to enhance the fertility of Alberta's crop land (Alberta Agriculture and Food, 2006). Manure applications introduce estrogens in agricultural soils but there have been no studies on the fate of estrogens in the Province of Alberta agricultural soils.

The objectives of this study were to determine Kd and Koc values of 17β-estradiol, estrone, estriol and equol in 121 soil samples collected throughout the Province of Alberta and relate these values to variations in soil properties, soil-landscape position, soil great groups and ecoregions.

2.3 Materials and Methods

2.3.1 Estrogens and Analytical Techniques

Analytical grade 17β-estradiol, estrone, estriol and equol with a purity of 98% or higher were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO. Estradiol [6,7-3H(N)] (99% radiochemical purity, specific activity $1.48x10^{12}$-$2.22x10^{12}$ Becquerels $mmol^{-1}$) (N: nominally labeled), estrone [6,7-3H (N)] (99% radiochemical purity, specific activity $1.48x10^{12}$-$2.22x10^{12}$ Becquerels $mmol^{-1}$) and equol [3H(G)] (99% radiochemical purity, specific activity $3.7x10^{10}$-$1.85x10^{11}$ Becquerels $mmol^{-1}$) (G: generally labeled, not nominally but at a stable non-exchangeable site, definition from American Radiolabeled Chemicals) were purchased from American Radiolabeled Chemicals, St-Louis, MO. Estriol [2,4]3H (99.5 % radiochemical purity, specific activity $7.4x10^{11}$-$1.48x10^{12}$ Becquerels $mmol^{-1}$) was purchased from Moravek Biochemicals and Radiochemicals, Brea, CA (Figure 2.1). All radiochemicals were delivered in ethanol. The amount of radioactivity in estrogen stock solutions and samples from experiments was determined using Liquid Scintillation Counting (LSC) with automated quench correction (#H method) (the H method calculates "the difference (in channel) between the inflection points of the unquenched standard and the sample" in the Compton spectrum of an element. Compton electrons behave like beta particles and are "generated by automatically positioning a gamma emitting isotope near the sample vial while it is in the counting chamber" (Steiner, 1996)) (LS 7500 Beckman Instruments, Fullerton, CA). Radioactivity was measured using 5 mL of Scintisafe scintillation cocktail (Beckman) and a maximum counting time of 10 minutes.

2.3.2 Study Area

Alberta Agriculture and Rural Development established a benchmark program in 1997 to monitor soil quality in agricultural fields throughout the Province of Alberta. Detailed site descriptions are given in Leskiw et al. (2000) and Cathcart et al. (2008). The benchmark sites were established with cropping systems being in most cases annual cereal or oilseed crops in an area spanning 49–60^{o}N longitude and 110–120^{o}W latitude thereby including a range of ecoregions.

Forty-one agricultural fields located across seven ecoregions were considered in the present study (Figure 2.2). The locations of these sampling locations are described in Gaultier et al. (2008) and Cathcart et al. (2008). Ecoregions constitute units with distinct regional ecological factors (i.e., climate, physiography, vegetation, soil, water, and fauna) and are an entity within a national hierarchy of ecostratification (Ecological Stratification Working Group, 1995). The seven ecoregions in our study are the Peace Lowland (PL), Boreal Transition (BT), Mixed Boreal Uplands (MBU), Aspen Parkland (AP), Moist Mixed Grassland (MMG), Fescue Grassland (FG) and Mixed Grassland (MG) (Table 2.1). The AP, MMG, and MG also constitute a large part of the agricultural area of the provinces Manitoba and Saskatchewan, Canada and roll up to be a major part of the Prairie Ecozone. The Prairie Ecozone contributes to the majority of the crop production in Canada. Soil great groups in the seven ecoregions included Brown Chernozem, Dark Brown Chernozem, Black Chernozem, Dark Gray Chernozem, Gray Luvisol and Dark

Gray Luvisol soils (Soil Classification Working Group, 1998, Carthcart et 2008) (Table 2.1).

Figure 2.1 A

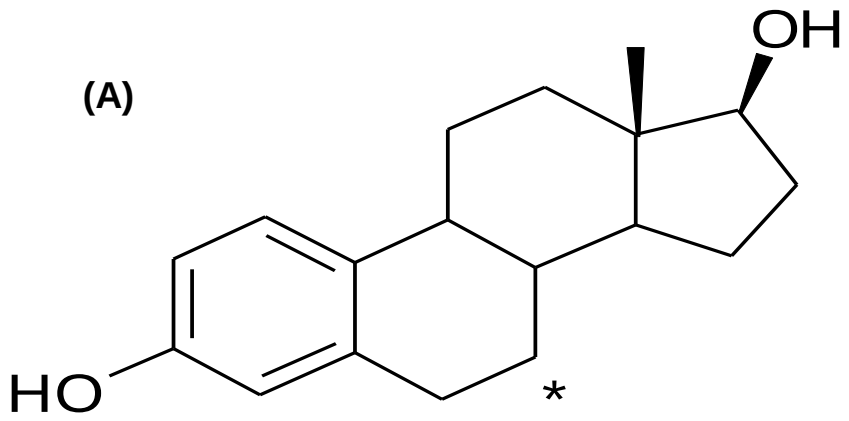


Figure 2.1 B

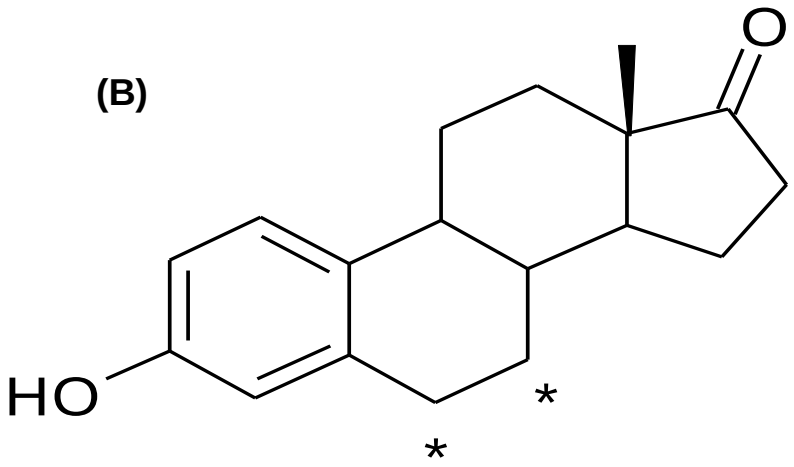


Figure 2.1 C

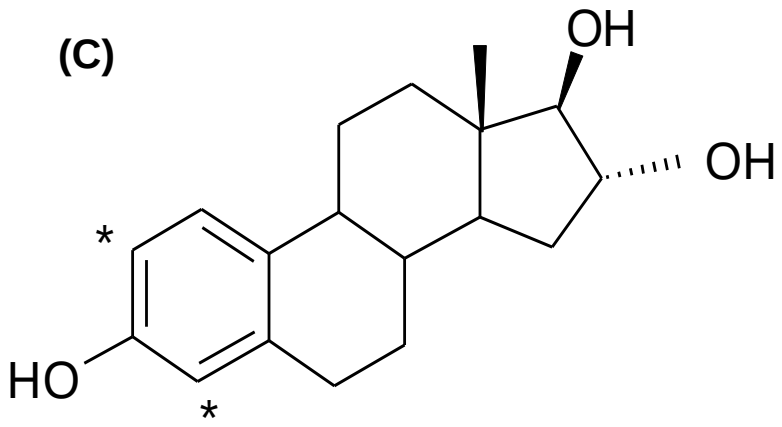


Figure 2.1 D

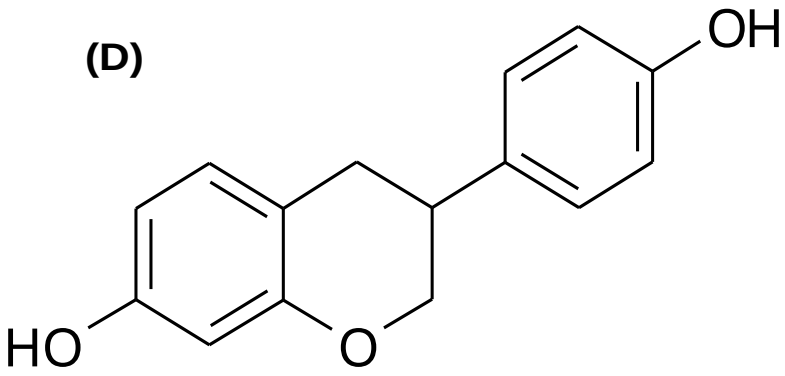
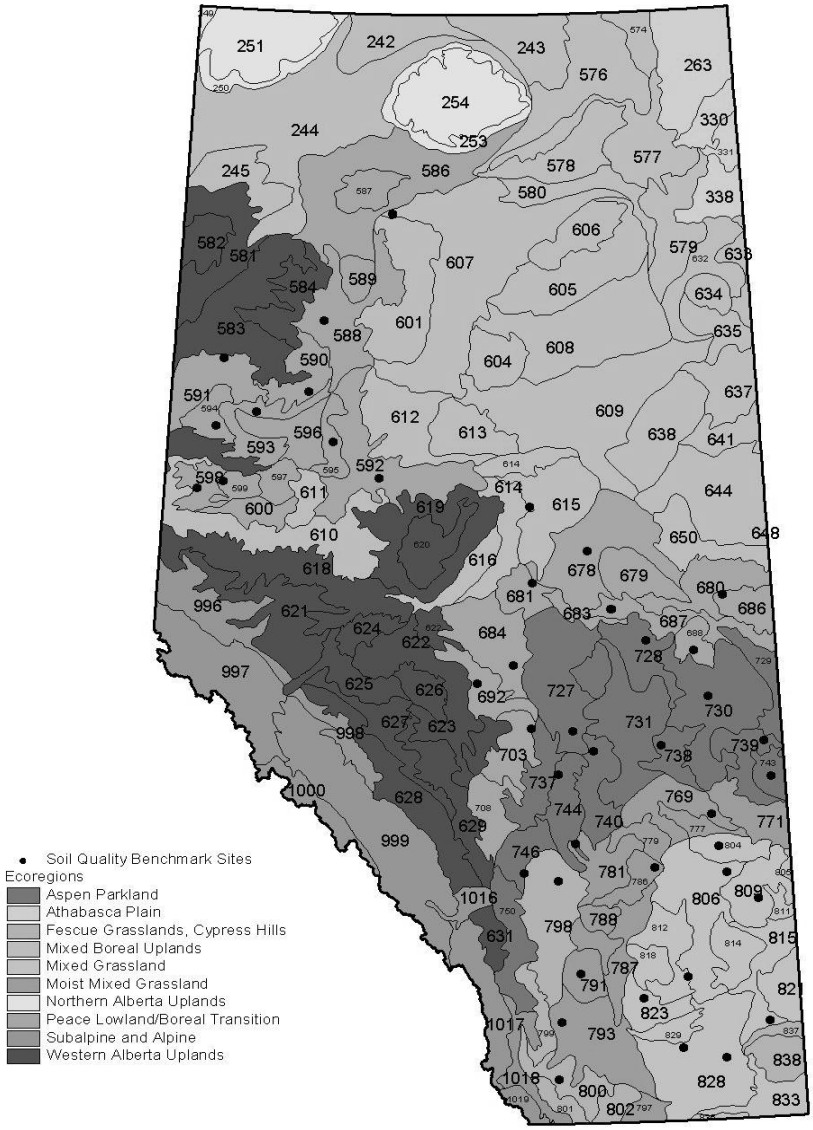


Figure 2.1: Structure of the compounds including labeling sites (*): A) Estradiol [6,7- H^3], B) estrone (6,7-H^3), C) estriol (2,4-H^3) and D) equol H^3 (Unspecified).



Location of Benchmark Sites, Ecoregions and Ecodistricts in Alberta.

Figure 2.2: Map of the study area (Ecological Stratification Working Group, 1995; Leskiw et al., 2000; Cathcart et al., 2008; Gaultier et al., 2008)

Table2.1: Characteristics of the sampled ecoregions (adapted from Gaultier et al., 2008).

Ecoregion	**Soil Great group**	**n (total)**	**Mean temperature July (^{o}C)**	**Precipitation (mm)**
Peace Lowland	Gray Luvisol (n=11) Dark Gray Luvisol (n=4) Black Chernozem (n=6) Dark Gray Chernozem (n=6)	27	13.3	435-517
Mid boreal Upland	Gray Luvisol (n=3)	3	15.5	508
Boreal Transition	Gray Luvisol (n=9) Dark Gray Luvisol (n=9) Black Chernozem (n=3) Dark Gray Chernozem (n=3)	24	15.9	428-535
Aspen Parkland	Black Chernozem (n=23) Dark Brown Chernozem (3)	26	16.4	391-478
Moist Mixed Grassland	Dark Brown Chernozem (n=12) Black Chernozem (n=3)	15	17.0	368-422
Fescue Grassland	Black Chernozem (n=6)	6	15.6	427-537
Mixed Grassland	Dark Brown Chernozem (n=3) Brown Chernozem (n=17)	20	17.9	314-363

* 791 lower slope, 800 upper slope, 804 lower slope and 815 mid slope were not collected.

2.3.3 Soil Sampling and Characterization

Surface soil samples (0-15 cm) were taken from each of three soil-landscape positions (upper-slopes, mid-slopes, lower-slopes) in each of the 41 agricultural fields. SOC, % sand, % clay, pH, $CaCO_3$, CEC and EC were determined on air-dried and sieved (2 mm) soil. SOC was determined by a Leco model CHN 600 C and N combustion analyser (Leco Instruments LTD., Missisauga, ON), after removal of inorganic carbon by digestion with 6 M HCl (Nelson and Sommers, 1982). Soil texture was determined using the hydrometer method (Gee and Bauder, 1986). Soil pH was determined as described in McKeague (1978) using 10 ml of $CaCl_2$ and 5 g of soil. Carbonate content was determined using a volumetric calcimeter following addition of 6 M HCl $\cdot FeCl_2$ as described in Loeppert and Suarez (1996). Cation exchange capacity (CEC) and electrical conductivity (saturated paste) were determined as in McKeague (1978).

2.3.4 Sorption of Estrogens

Batch-equilibrium experiments were used to measure the sorption of estrogens by soil. Estrogen biodegradation during the experiment leads to underestimating Kd values and hence the sterilization of laboratory equipment and soils is desired (Wolf et al., 1989). We utilized autoclaving, which is the single most commonly-used sterilization technique in batch-equilibrium sorption studies (e.g., see recent studies by Shaw and Hooker, 2008; Yang et al., 2008; Burns et al., 2009; Lee et al., 2009; Hong et al., 2008; Ren et al.,

2009). It has been demonstrated that autoclaving has no altering effect on soil properties such as SOC, pH, CEC and soil surface area (Wolf et al., 1989; Lotrario et al., 1995), nor Kd values of some chlorinated hydrocarbons (Lotrario et al., 1995) and pesticides (Benoit et al., 1996). In contrast, the use of gamma-sterilization caused a significant decrease in CEC and altered the reactivity of both the organic and inorganic fractions (Bank et al., 2008). Chemical sterilization involving the use of methyl bromide or other chemicals induced a significant decrease in herbicide Kd values due to increased competition for sorption site (Stephens et al., 2002). No significant degradation of 17β-estradiol was observed in autoclaved soils samples collected in agricultural soils from South Australia (Ying and Kookana, 2005) and North Dakota (Fan et al., 2007) and from a grassland in Pennsylvania (Xuan et al., 2008), which suggest that chemical degradation of 17β-estradiol is a negligible process.

The equilibrium time is an important consideration in batch-equilibrium experiments (Wauchope et al., 2002), but difficult to generalize when working with four estrogens and 121 soils because previous studies report that equilibrium times are dependent on the molecular structure of the chemical of interest (Lee et al., 2003; Flogeac et al., 2005), the properties of the soil (Qualls, 2000) and environmental parameters (Renaud et al., 2004). For determining an appropriate equilibrium time, preliminary batch-equilibrium runs were conducted in duplicates. Using soil samples containing about 80 g C kg^{-1} SOC, we tested a series of batch-equilibrium times for each estrogen ranging from 0.5 to 96 hours (Figure 2.3). In evaluating these data we considered the most appropriate equilibrium times to be 2 h for 17β-estradiol, 24 h for equol, 72 h for estrone and 96 h for estriol

because these points reflect the maximum sorbed concentration or when the sorbed concentration is relatively stable between two or three consecutive measurements (Figure 2.3).

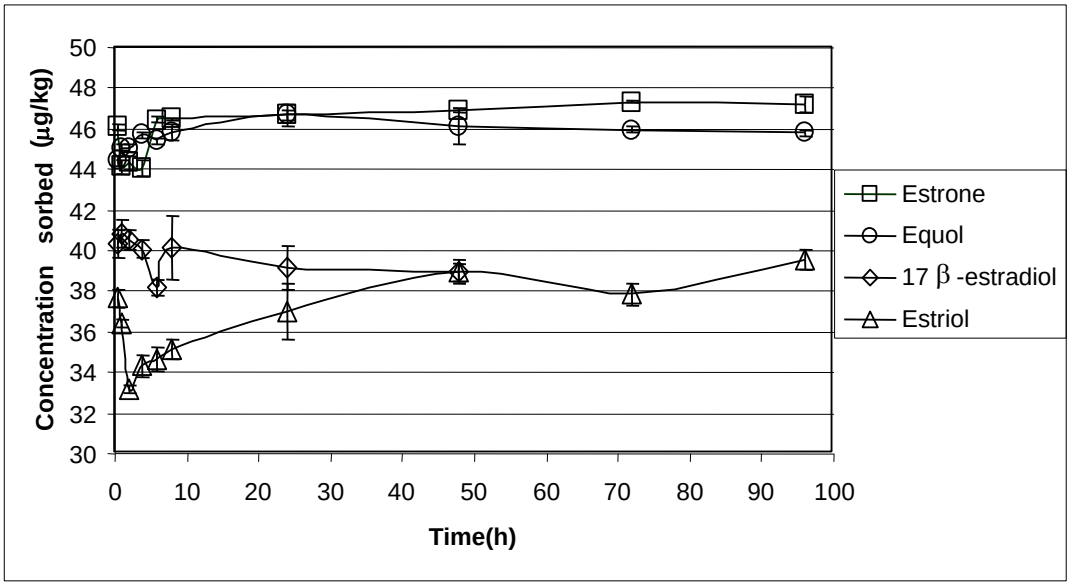


Figure 2.3: Time-dependent sorption for each of the four estrogens.

Additional details on the batch-equilibrium protocols are as follows. Pipette tips, bottles, water and glassware involved in preparing estrogen solutions were autoclaved for 30 minutes at 121^{o}C and 21 psi. The stock solution was a mixture of non-labeled and labeled compounds in water, kept at 4^{o}C in glass amber bottles in the dark when not in use and was never kept for more than a week. The percentage of ethanol contained in the stock solution was always less than 0.6 %, therefore the impact on sorption of such a low percentage of ethanol is minimal. For each estrogen, the concentration of the stock solution had a specific activity of 16.67 Becquerels mL^{-1} and was prepared such that 10 mL of solution added to 5 g of soil would be equivalent to an estrogen concentration of

50 μg $kg^{-1}$of soil (the concentration of the stock solution was 25 μg L^{-1} and 50 μg kg^{-1} refers to 100% sorption). This concentration is within the mid range of what was used by Casey et al. (2003) for sorption experiments but are higher than what is typically found in soils following manure application. Finlay-Moore et al. (2000) measured 675 ng kg^{-1} of 17β-estradiol in soil after application of poultry manure. The concentration used was within the linear range of the sorption isotherm for 17β-estradiol and estrone as determined by Casey et al. (2005). Casey et al. (2005) defined sorption isotherms for 17β-estradiol and estrone at 0.5 h, 1h, 5h, 24h and 48h. The isotherm for 1h for 17β-estradiol was linear from 0 to 1000 μg kg^{-1} of sorbed concentration. For estrone, the 24 h isotherm was linear from 0 to 700 μg kg^{-1} of sorbed concentration.

Soil slurries were rotated at 5^{o}C in the dark as a supplementary precautionary step to avoid biodegradation and then centrifuged for 30 min at 7,000 rpm. (6000g) Supernatant (1 mL) was added to a scintillation vial (in duplicate) containing 5 mL of scintillation cocktail to determine radioactivity remaining in the equilibrium solution. The average % sorbed was 90% for 17β-estradiol, 92% for estrone, 89% for estriol and 94% for equol. Controls (autoclaved glass tubes with solution only) were also included. The controls showed no loss of radioactivity in solution.

Amounts of radioactivity in the initial solution and remaining in the equilibrium solution were used to calculate Kd by Cs Ce^{-1} where Cs = the amount of estrogen sorbed by the soil (μg kg^{-1}) and Ce = the estrogen concentration of the equilibrium solution (μg L^{-1}). The sorption of estrogen per unit organic carbon, Koc (L kg^{-1}), was calculated by

dividing Kd values by foc, where foc is the fraction of soil organic carbon in the soil sample. This way of calculating Koc is the most appropriate when wanting to take into account the variability of the nature of the organic matter (Wauchope et al., 2002).

Prior to statistical analyses, the four Kd values determined for the same soil (i.e., duplicated soil samples and duplicated supernatant samples for each soil sample) were averaged. The standard deviation between replicates for the same sample, as averaged for the 121 samples, was 4.1 L kg^{-1} for 17β-estradiol, 6.3 L kg^{-1} for estrone, 2.0 L kg^{-1} for estriol and 8.4 L kg^{-1} for equol.

2.3.5 Data Analysis

Frequency distribution curves and box plots were used to visualize and examine data of soil properties (SOC, % sand, % clay, pH, $CaCO_3$, EC and CEC) and sorption values (17β-estradiol, estrone, estriol and equol Kd or Koc values) (1989-2002, JMP version 5.0, SAS Institute).

Pearson correlation coefficients ($P < 0.05$) were determined among soil properties (SOC, % sand, % clay, pH, EC and CEC) and sorption values (17β-estradiol, estrone, estriol and equol Kd or Koc values) (1989-2002, JMP version 5.0, SAS Institute). Visual observations confirmed that when the correlations were significants, they were linear. These parametric correlation analyses were applied to untransformed data due to the robust nature of these analyses (Legendre and Legendre, 1998). In subsequent statistical analyses, variables, except for soil pH, were log transformed to achieve normality.

In order to determine if there were significant differences among the four estrogens in either Kd or Koc values, the one-way Analysis of Variance (ANOVA; $P < 0.05$) and Tukey-Kraemer HSD multiple comparison test ($P < 0.05$) was applied on the log transformed data (1989-2002, JMP version 5.0, SAS Institute). When the Kolmogorov-Smirnov test for normality failed (in case of Kd values) ANOVA on Ranks was performed with the Dunn`s test for pairwise comparison (SigmaStat for Windows version 3.1 Systat Software Inc.).

For each estrogen (17β-estradiol, estrone, estriol or equol), multiple discriminant analysis (MDA) was performed on both log Kd and log Koc values with discriminating factors being the (1) seven ecoregions (PL, BT, MBU, AP, MMG, FG and MG), (2) six soil great groups (Brown Chernozem, Dark Brown Chernozem, Black Chernozem, Dark Gray Chernozem, Gray Luvisol and Dark Gray Luvisol soils) or (3) three soil-landscape positions (upper-slopes, mid-slopes, lower-slopes) (1989-2002, JMP version 5.0, SAS Institute).

For selected soil properties (i.e., those that showed significant associations with estrogen Kd or Koc values: SOC, % sand, % clay and CEC), the one-way ANOVA ($P <0.05$) and Tukey-Kraemer HSD multiple comparison test ($P < 0.05$) were applied (1989-2002, JMP version 5.0, SAS Institute) to further explore differences among the seven ecoregions, six soil great groups or three soil-landscape positions. In cases where the Kolmogorov-Smirnov test failed (% sand, Kd estrone and Kd equol) even on the log transformed data,

ANOVA on Ranks was performed followed by the Dunn`s test for pairwise comparison (SigmaStat for Windows version 3.1 Systat Software Inc.).

In addition, two types of regression analysis were applied to develop predictive models that could be used for estimating Kd and Koc values at a regional scale. In the first approach, data on soil properties were used to predict Kd and Koc values of each estrogen. The predictive models and their strengths based on their coefficients of determination (r^2) and other factors were established using Partial Least Squares (PLS) regression (2002-2009, Unscrambler version 8.0, CAMO PROCESS ASA). We chose to use PLS regression because this technique is able to deal with collinearity (Martens and Martens, 1986) such as that observed for our soil properties data set. For the PLS, significant factors were determined using the Marten's Uncertainty test (Martens and Martens, 2000); the number of principal components was optimized when the variation of residual variance was minimized; and outliers were detected using the automatic outlier detection provided with the software. In the case of the prediction of Koc, SOC was not included as a factor since it is used in the calculation of Koc.

In the second approach, ordinary least squares regression (1989-2002, JMP version 5.0, SAS Institute) was used to develop predictive models for Kd and Koc values of each estrogen based on the Kd or Koc value of another estrogen. The model had to be significant at the 0.05 level in order to be included.

2.4 Results

The SOC ranged from 6 to 138 g C kg $^{-1}$, but most samples had a SOC less than 60 g C kg $^{-1}$ (Figure 2.4A). Considering all soil samples (n=121), soil pH ranged from extremely acidic (4.2) to slightly alkaline (7.5) (Figure 2.4B) and the percentages of clay (Figure 2.4C) and sand (Figure 2.4D) also demonstrated a wide range. In contrast, $CaCO_3$ was usually below 0.1 mol kg^{-1} (Figure 2.4E). The EC measurements were always below 2.8 dS m^{-1}, therefore soils were non-saline to slightly-saline (Figure 2.4F). CEC ranged from 2 to 65 cmol kg $^{-1}$ soil (Figure 2.4G), which is expected for soil textures ranging from sandy to clay with the largest CEC numbers representing organic soils.

Kd-17β-estradiol values ranged from 7 to 65 L kg $^{-1}$ soil (Figure 2.4H), Kd-estrone values ranged from 11 to 119 L kg $^{-1}$ soil (Figure 2.4I) and Kd-estriol values ranged from 5 to 62 L kg $^{-1}$ soil (Figure 2.4J). Equol demonstrated a wider range in Kd values, 14 to 177 L kg $^{-1}$ soil (Figure 2.4K). Average Kd values significantly increased in the order of Kd-17β-estradiol (23 L kg $^{-1}$ soil) = Kd-estriol (23 L kg $^{-1}$ soil) < Kd-estrone (33 L kg $^{-1}$ soil) < Kd-equol (42 L kg $^{-1}$ soil). Based on visual comparison on individual soil samples, around 90% of the samples followed the main trend for Kd and 95% of the samples followed the main trend for Koc. For the remaining 10% and 5% of the samples that did not follow the main trend for Kd and Koc respectively, soil properties could not explain their deviation from the main trend. Stronger significant positive associations were observed between Kd-estriol and Kd-17β-estradiol (r=0.84, $P < 0.001$), than between Kd-

Figure 2.4A

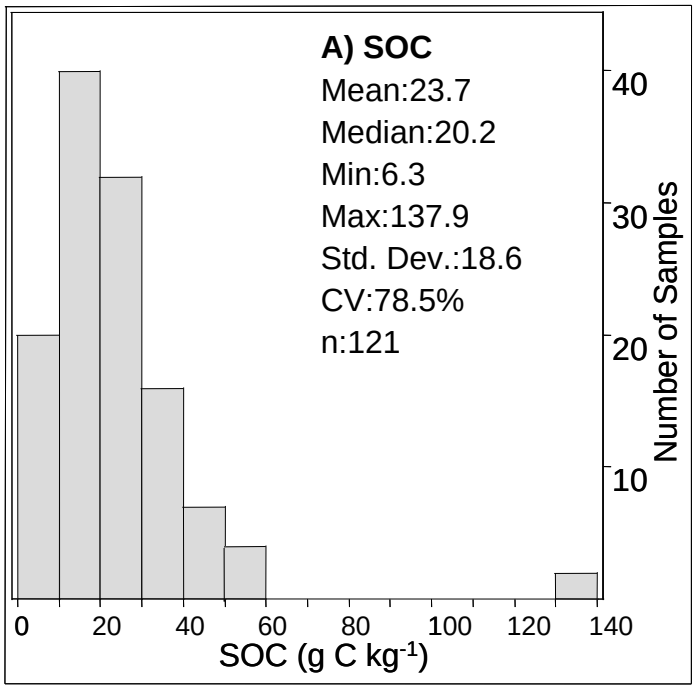


Figure 2.4B

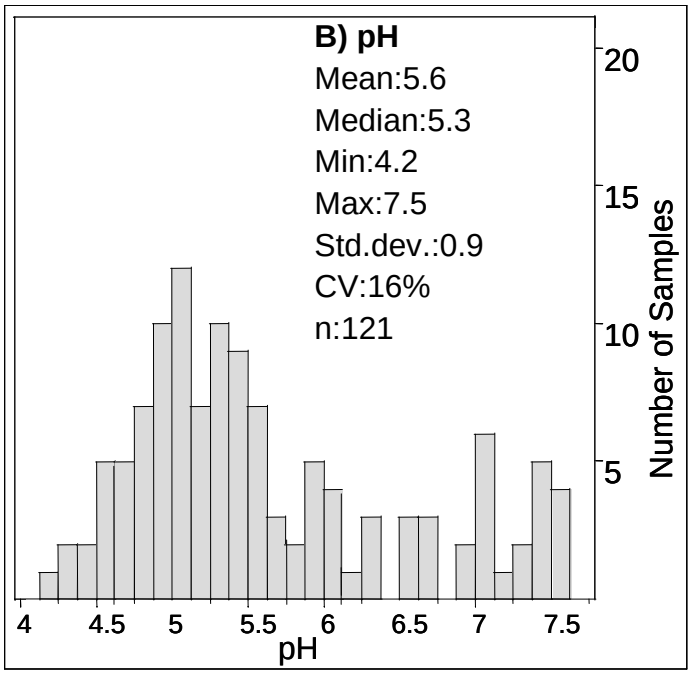


Figure 2.4C

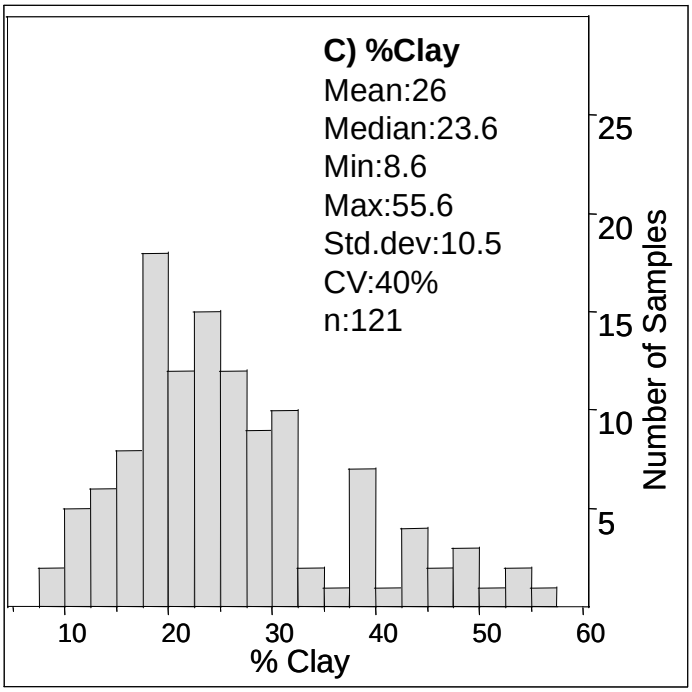


Figure 2.4D

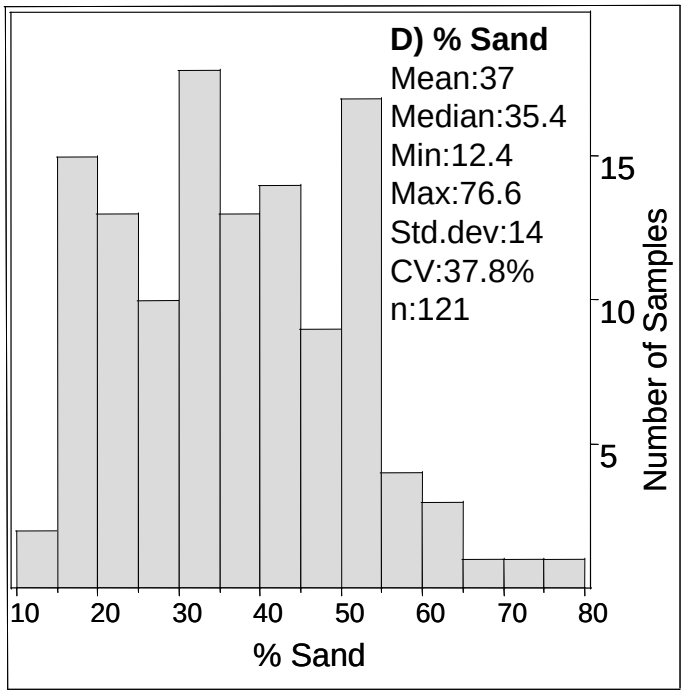


Figure 2.4E

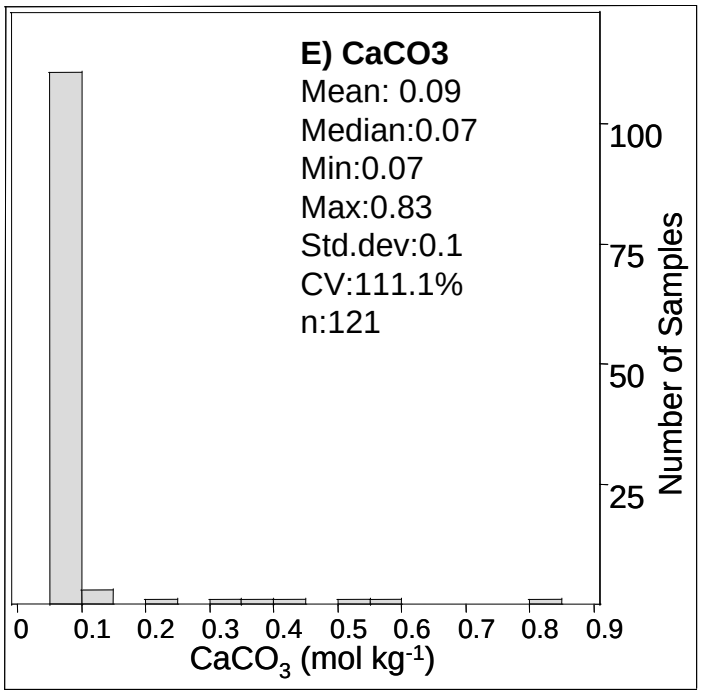


Figure 2.4F

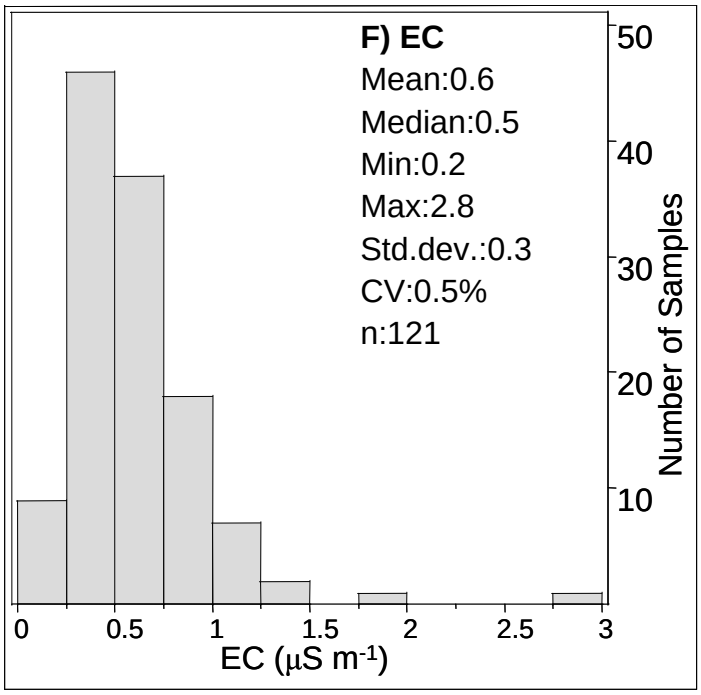


Figure 2.4G

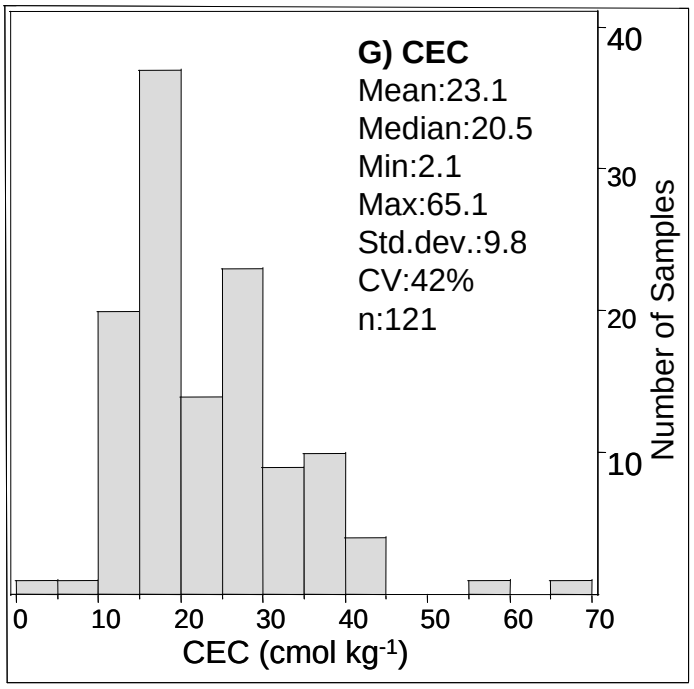


Figure 2.4H

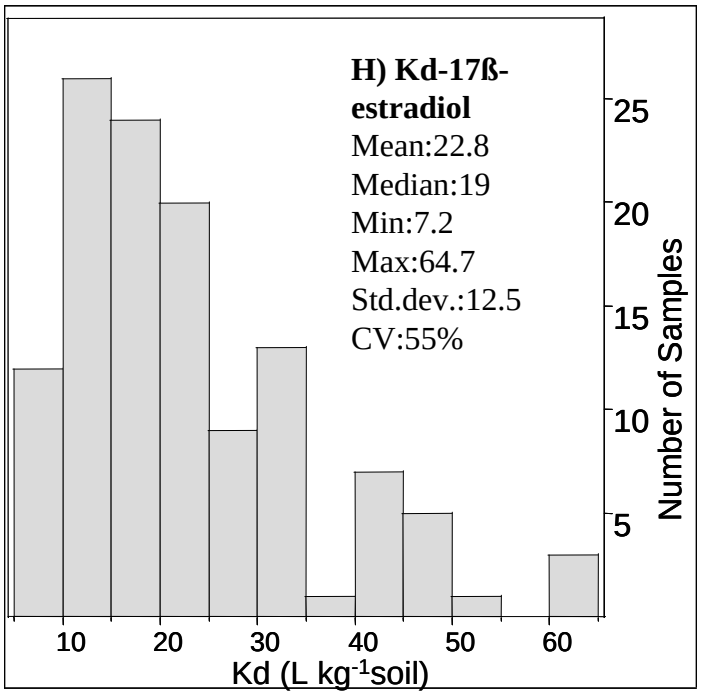


Figure 2.4I

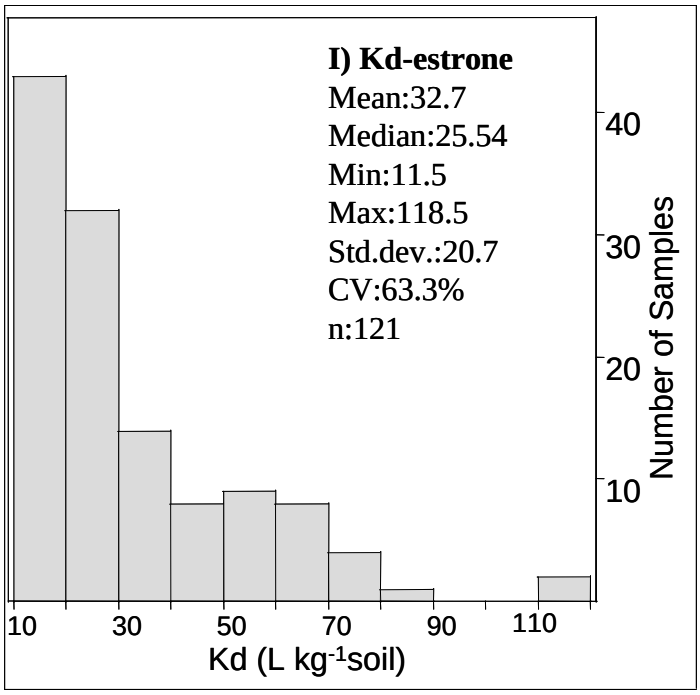


Figure 2.4J

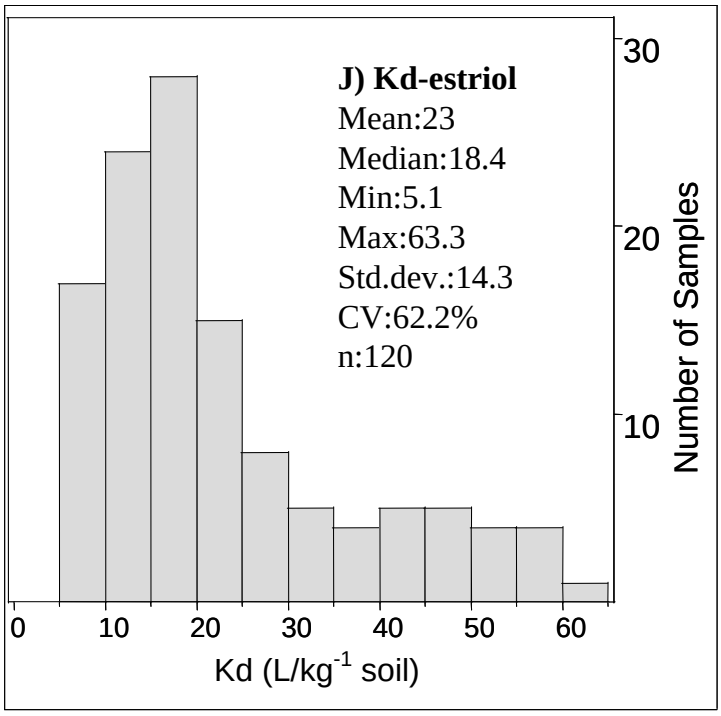


Figure 2.4K

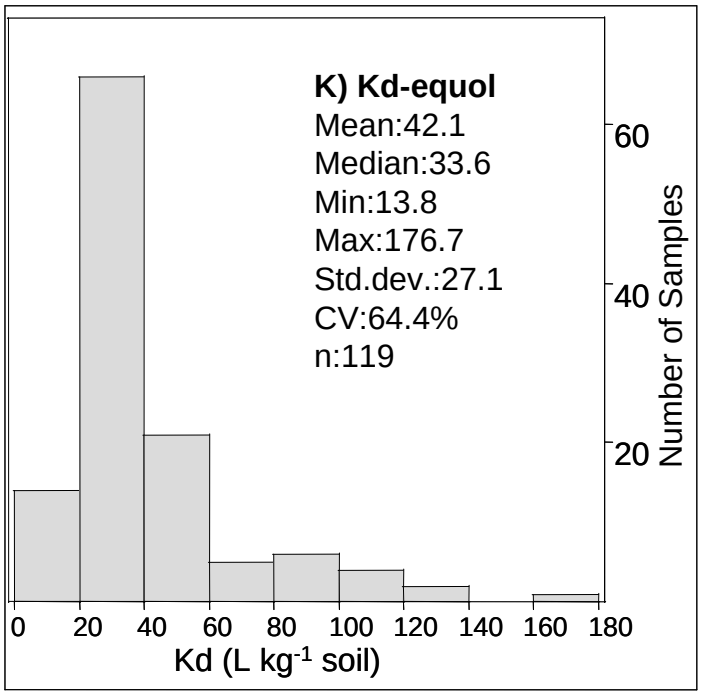


Figure 2.4L

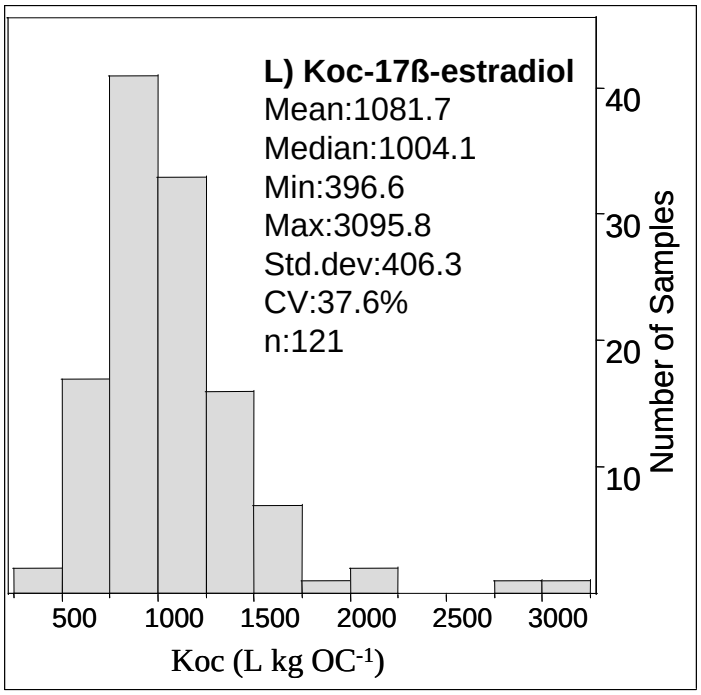


Figure 2.4M

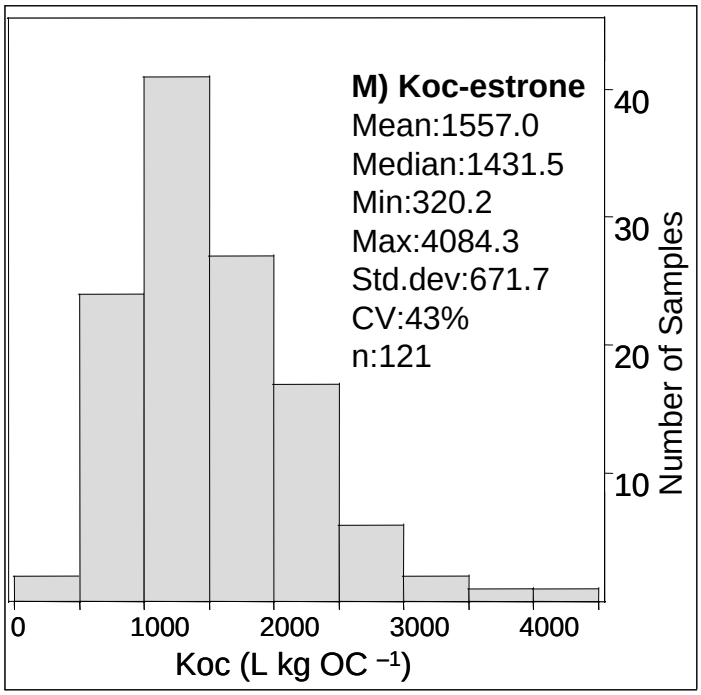


Figure 2.4N

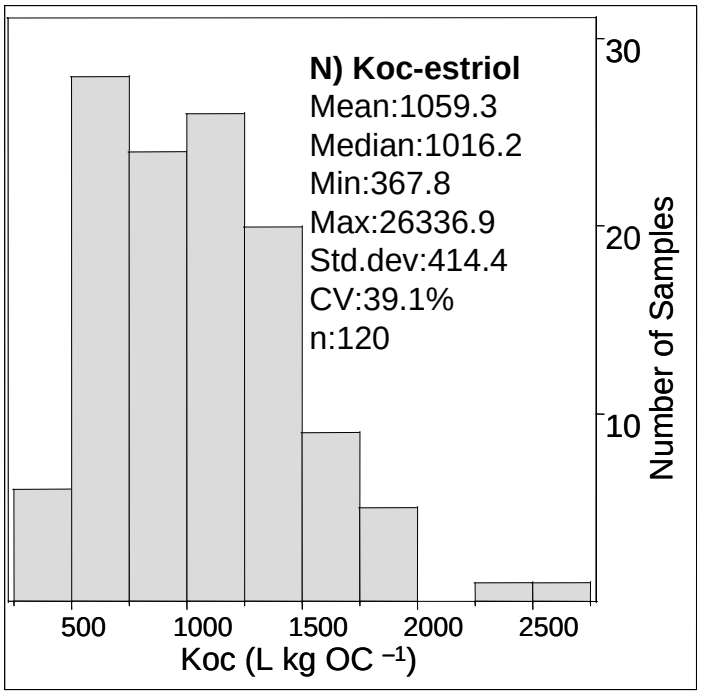


Figure 2.4O

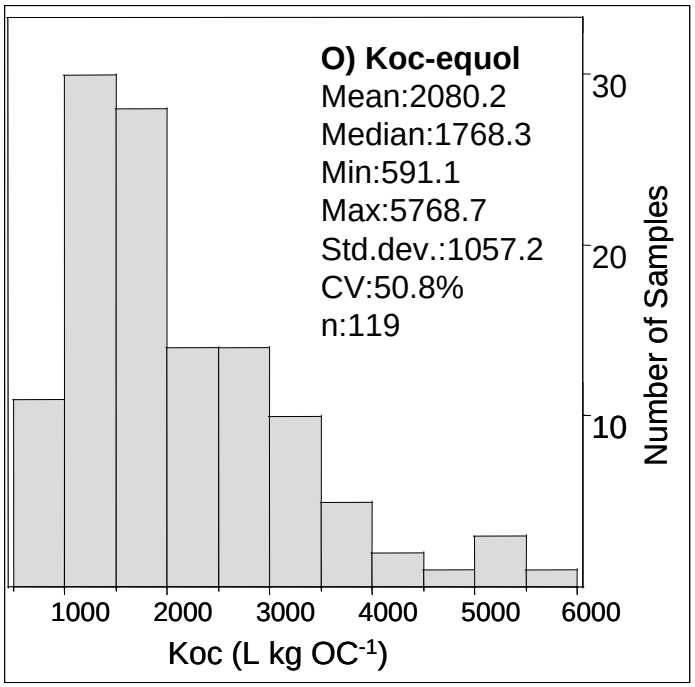


Figure 2.4: Frequency distribution, mean, median, deviation, minimum, maximum, standard deviation, coefficient of variation and n on the entire dataset for A) SOC, B) pH, C) % clay, D) % sand, E) $CaCO_3$, F) EC, G) CEC, H) Kd estradiol, I) Koc estradiol, J) Kd estrone, K) Koc estrone, L) Kd estriol, M) Koc estriol, N) Kd equol, O) Koc equol

estriol and Kd-equol (r=0.70, $P < 0.001$), Kd-estriol and Kd-estrone (r=0.62, $P < 0.001$), Kd-17β-estradiol and Kd-equol (r=0.69, $P < 0.001$) and Kd-17β-estradiol and Kd-estrone (r=0.61, $P < 0.001$). There was a relatively weak significant positive association between Kd-estrone and Kd-equol (r=0.54, $P < 0.001$). Kd values of all estrogens were positively associated with SOC, with correlation coefficients ranging from 0.75 to 0.50 and being significant at the 0.001 level (Table 2.2). Given the positive association between SOC and CEC (r=0.67, $P < 0.001$), values of Kd-17β-estradiol (r=0.73, $P < 0.001$), Kd-estriol (r=0.63, $P < 0.001$) and Kd-equol (r=0.47, $P < 0.001$) were also significantly positively

associated with CEC, but the association was not significant for Kd-estrone (Table 2.2). Values of both Kd-17β-estradiol (r=-0.26, $P < 0.01$) and Kd-estrone (r=-0.19, $P < 0.05$) were significantly negatively associated with % sand, but the associations were not significant for Kd-estriol and Kd-equol (Table 2.2). The negative association between % sand and estrogen sorption is most likely the effect of the decreasing SOC in soils with increasing sand content (Table 2.2). However, Kd values of estrogens were not significantly associated with % clay, nor with soil pH, $CaCO_3$ or EC (Table 2.2).

Koc-17β-estradiol values ranged from 397 to 3,096 L kg^{-1} (Figure 2.4L), Koc-estrone values ranged from 320 to 4,084 L kg^{-1} (Figure 2.4M), and Koc-estriol values ranged from 368 to 2,637 L kg^{-1} (Figure 2.4M, 2.4L and 2.4N). Equol demonstrated a smaller range in Koc values, 591 to 5,769 L kg^{-1} (Figure 2.4O). Average Koc values significantly increased in the order of estriol (1059 L kg^{-1}) = 17β-estradiol (1082 L kg^{-1}) < estrone (1557 L kg^{-1}) < equol (2080 L kg^{-1}). Stronger significant positive associations were observed between Koc-equol and Koc-estriol (r=0.64, $P < 0.001$) and Koc-equol and Koc-17β-estradiol (r=0.59, $P < 0.001$) than between Koc-equol and Koc-estrone (r=0.42, $P < 0.001$). There were stronger significant positive associations between Koc-17β-estradiol and Koc-estriol (r=0.50, $P < 0.001$) than between Koc-17β-estradiol and Koc-estrone (r=0.38, $P < 0.001$). There was a relatively weak significant positive association between Koc-estrone and Koc-estriol (r=0.25, $P < 0.01$)

.

Table 2.2: Correlations between soil properties and selected parameters using all soil samples (n=120).

	SOC (g C kg^{-1})	pH	% clay	% sand	$CaCO_3$ (mol kg^{-1})	EC (µS cm^{-1})	CEC (cmol kg^{-1})
Soil properties							
%SOC	1						
pH	-	1					
%clay	-	-	1				
%sand	-0.29**[a]	-	-0.79 ***	1			
$CaCO_3$	-	0.46 ***	-	-	1		
EC (µS/cm)	-	0.28 *	-	-	-	1	
CEC (meq/100g)	0.67***	-	0.41***	-0.45***	-	-	1
Sorption coefficient, Kd [L kg^{-1} of soil]							
17β-Estradiol	0.75***	-	-	-0.26**	-	-	0.73***
Estrone	0.50***	-	-	-0.19*	-	-	-
Estriol	0.69***	-	-	-	-	-	0.63***
Equol	0.54***	-	-	-	-	-	0.47***
Sorption per unit organic carbon, Koc [L/kg]							
17β-Estradiol	-0.38***	-	-	0.28**	-	-	-
Estrone	-0.35***	-	-	0.29*	-	-	-0.21*
Estriol	-0.29**	-	-0.44***	0.48***	-	-	-
Equol	-0.38***	-	-0.30***	0.39***	-	-	-0.30***

[a] * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). *** Correlation is significant at the 0.001 level (2-tailed). - Correlation is not significant at the 0.05 level.

Koc values of all estrogens were significantly positively associated with % sand, with correlation coefficients ranging from 0.48 to 0.28 (Table 2.2). Koc-estriol (r=-0.44, $P < 0.001$) and Koc-equol (r=-0.30, $P < 0.001$) were significantly negatively associated with %clay, but the associations were not significant for Koc-17β-estradiol and Koc-estrone (Table 2.2). Koc-equol (r=-0.30, $P < 0.001$) and Koc-estrone (r=-0.21, $P < 0.001$) were significantly negatively associated with CEC, but the associations were not significant for Koc-17β-estradiol and Koc-estriol (Table 2.2). Koc values of estrogens were not significantly associated with soil pH, EC or $CaCO_3$ (Table 2.2).

For all estrogens, MDA results for Kd or Koc values at the ecoregion level demonstrated two distinct (significant at $P < 0.05$) groupings: (a) MG and (b) all other ecoregions (not shown). Based on the ANOVA analyses, Kd-17-ß-estradiol, Kd-estrone, Kd estriol and Kd-equol values were significantly smaller in MG than other ecoregions (Table 2.3). Koc-17β-estradiol values were significantly larger in MG than PL and BT (Table 2.3). Koc-estrone and Koc-equol values were significantly larger in MG than PL. Koc estriol was significantly larger in AP than MBU, MG and PL. For soil properties significantly associated with Kd or Koc values (Table 2.2), SOC was significantly smaller in MG than FG, AP, MBU, PL and BT. CEC was significantly smaller in MG than PL and AP. % sand was significantly smaller in PL than BT, AP, MMG and MG (Table 2.3). % clay was significantly smaller in MG than the other ecoregions (Table 2.3).

Soils in the MG are Brown Chernozems (n=17) and Dark Brown Chernozems (n=3) (Table 2.1). Soils in other ecoregions are Dark Brown Chernozems (n=15), Black

Table 2.3: Mean (coefficient of variation %) of estrogen Kd and Koc values in 7 ecoregions, 3 soil-landscape positions and 6 soil great orders. For ecoregions, soil great orders or landscape positions, group means in columns with the same letter are not significantly different from each other (One-way ANOVA performed on log-transformed data with $P< 0.05$. Multiple comparison using the tukey-Kramer HSD test with $P< 0.05$.For Kd estrone, Kd equol and %sand, ANOVA on ranks was performed because of the non-normal distribution). Groups identified by different letters are statistically differents.

	Sorption coefficient, Kd (L kg $^{-1}$ of soil)				Sorption per unit organic carbon, Koc (L kg^{-1})				Soil properties significantly associated with Kd or Koc (see Table 2.2)			
Unit (n)	17β-estradiol	Estriol	Estrone	Equol	17β-estradiol	Estriol	Estrone	Equol	SOC (g C kg^{-1})	CEC (cmol kg^{-1})	%Sand	%Clay
					Ecoregions							
PL (27)	28.1(49.5) a	25.5(52.0) ab	39.9(48.6) a	51.6(74.4) ab	873(26.3) c	794(35.5) c	1273 (39.0) b	1526(47.6) b	31.1 (28.0) a	29.4(29.3) a	23 (26.1) b	37 (24.3) a
MBU (3)	33.8(71.6) ab	33.3(78.4) ab	36.4(45.1) ab	58.4(74.4) a	761(35.6) abc	731(39.5) bc	977(39.5) ab	1300(34.5) ab	59.9 (110.4) ab	20.6 (35.9) abc	33(9.1) ab	16 (25.0) b
BT (24)	20.9(64.4) bc	22.8(57.0) b	30.2(58.9) ab	37.3(40.5) a	996(51.5) bc	1114 (38.0) ab	1548 (38.0) ab	2053(49.1) ab	24.9 (105.2) bc	24.1(57.3) abc	38 (44.7) a	26 (42.3) b
AP (26)	27.0(43.7) ab	33.6(47.0) a	41.3(69.5) ab	54.4(52.6) a	1153 (31.5) ab	1390 (34.2) a	1679 (34.2) ab	2413(50.3) a	25.4 (53.1) abc	24.1(31.1) ab	42 (31.0) a	21 (33.3) b
MMG (15)	19.0(25.3) abc	16.0 (17.5) b	24.2(39.3) ab	32.7(47.7) a	1242 (41.5) ab	1055 (33.1) abc	1578 (33.1) ab	2167(53.4) ab	16.3 (27.0) cd	18.0(36.1) bc	49 (24.5) a	19 (31.6) b
FG (6)	27.9(21.5) ab	25.4(32.3) ab	40.9(38.4) a	44.7(28.6) ab	1183 (21.6) abc	1055 (28.2) abc	1697 (28.2) ab	1907(33.1) ab	23.8 (12.6) abc	26.1(23.0) abc	33 (27.3) ab	29 (55.2) ab
MG (20)	12.4(28.2) c	9.8 (34.7) c	18.0(46.1) b	24.1(37.3) b	1273 (25.0) a	993(27.5) bc	1821 (27.5) a	2520(42.7) a	10.1 (32.7) d	15.7(15.9) c	41 (19.5) a	24 (16.7) b

	Sorption coefficient			Sorption per unit organic carbon				Soil properties			
	Estriol	Estrone	Equol	17β-estradiol	Estriol	Estrone	Equol	SOC	CEC	%Sand	%Clay
Soil Great Groups											
Br.C (17)	11.8(22.8) b	9.2(35.9) d	16.5(55.8) ab	22.9(39.7) b	1292 (26.1) a	985(28.2) ab	1803 (28.2) a	2538(45.1) a	9.5 (27.4) c	15.9(16.4) b	40 (20.0) a	25 (12.0) abc
DBr.C (18)	18.3(29.5) ab	15.3(18.3) c	25.7(47.1) b	34.3(45.4) a	1273 (37.7) ab	1088 (30.1) ab	1761 (30.1) a	2383(43.8) ab	15.1 (29.1) bc	19.0(28.4) ab	49 (22.4) ab	19 (31.6) c
Bl.C (41)	28.5(47.0) a	32.2(49.1) a	40.3(59.8) a	54.0(62.4) ab	1101 (29.6) ab	1239 (37.0) a	1581 (37.0) a	2143(51.3) abc	26.8 (45.5) a	25.1(37.1) a	39 (33.3) ab	24 (45.8) bc
DGC (9)	27.2(32.0) a	30.0(44.7) ab	40.3(54.6) a	44.5(44.9) ab	867(20.4) bc	957(39.3) ab	1266 (39.3) a	1432(37.1) bc	31.3 (22.4) a	31.9(18.2) a	25 (36.0) b	34 (32.4) ab
GL (23)	22.6(54.9) a	20.6(52.4) bc	34.1(54.5) a	38.8(53.3) ab	876(58.2) c	795(37.9) b	1309 (37.9) a	1528(44.4) c	29.2 (84.2) a	24.2(33.2) a	27 (51.9) b	33 (39.4) a
DGL (13)	23.2(69.4) a	23.1(59.3) abc	31.9(65.8) a	46.2(67.7) ab	994(28.2) abc	1102 (45.1) ab	1519 (45.1) a	2312(52.2) abc	29.6 (118.6) ab	24.2(71.8) ab	36 (33.3) ab	25 (24.0) abc
Landscape -positions											
U (41)	21.0(59.5) b	19.6(65.3) b	28.1(59.4) a	37.0(56.6) a	1187 (41.6) a	1081 (40.5) a	1625 (40.5) a	2210(48.1) a	18.7 (56.8) b	22.8(39.0) a	38 (36.8) a	27 (40.7) a
M (39)	20.8(48.6) ab	22.2(58.1) ab	32.6(69.0) a	40.9(58.7) a	1071 (38.5) ab	1093 (33.9) a	1618 (34.0) a	2207(51.9) a	20.9 (48.0) b	22.1(43.4) a	38 (39.5) a	25 (40.0) a
L (41)	26.9(51.7) a	27.6(57.2) a	37.7(57.6) a	49.1(68.8) a	981(27.1) b	1013 (43.1) a	1420 (43.1) a	1831(52.2) a	31.4 (88.2) a	24.6(43.9) a	35 (40.0) a	26 (42.3) a

Soil properties: SOC=Soil organic carbon content, CEC=Cation Exchange Capacity; ***Ecoregions:*** PL= Peace Lowland, BT=Boreal Transition, MBU=Mixed Boreal Uplands, AP=Aspen Parkland, MMG=Moist Mixed Grassland, FG=Fescue Grassland, MG=Mixed Grassland; ***Soil great groups:*** Br.C=Brown Chernozem, DBr.C=Dark Brown Chernozem, Bl.C=Black Chernozem, DGC=Dark Gray Chernozem, GL=Gray Luvisol and DGL=Dark Gray Luvisol soils; ***Slope-positions:*** U=upper-slopes, M-mid-slopes and L=lower-slopes.

Chernozems (n=41), Dark Gray Chernozems (n=9), Gray Luvisols (n=23), and Dark Gray Luvisols (n=13) (Table 2.1). For all estrogens, MDA analyses for Kd or Koc values at the soil great group level demonstrated two distinct (significant at $P < 0.05$) groupings: (a) Brown Chernozems and (b) all other soil great groups. Based on the ANOVA analyses, Kd-estriol values were significantly smaller in Brown Chernozems than other soil great groups (Table 2.3). Kd-17β-estradiol values were significantly smaller in Brown Chernozems than most soil great groups (Table 2.3). Kd equol was larger in the Brown Chernozems than in the Dark Brown Chernozems. Kd-estrone values were smaller in the Dark Brown Chernozems than other soil great groups except the Brown Chernozems. Koc-17β-estradiol and Koc-equol values were significantly larger in Brown Chernozems than Dark Gray Chernozems and Gray Luvisols (Table 2.3). Koc-estriol values were significantly larger in Black Chernozems than Gray Luvisols (Table 2.3). Koc-estrone values did not show significant differences among soil great groups (Table 2.3). SOC and CEC were significantly smaller in Brown Chernozems than in Dark Gray Chernozems, Black Chernozems and Gray Luvisols (Table 2.3). Percent clay was significantly higher in Gray Luvisols than Dark Brown Chernozems (Table 2.3). Percent sand was significantly lower in Gray Luvisols than in Dark Gray Chernozems and Gray Luvisols (Table 2.3).

Regardless of the estrogen considered, MDA analyses for Kd or Koc values at the soil-landscape level also demonstrated two distinct (significant at $P < 0.05$) groupings: (a) upper-slopes and (b) lower-slopes, while mid-slopes were not significantly different from either upper- or lower-slopes. Based on the ANOVA analyses, Kd-17β-estradiol and Kd-

estriol values were significantly smaller in upper-slopes than lower-slopes (Table 2.3). Kd-estrone and Kd-equol values were numerically smaller in upper-slopes than lower-slopes but differences were not significant (Table 2.3). For all estrogens, Koc values were numerically larger in upper-slopes than lower-slopes but differences were not significant (Table 2.3). SOC was significantly smaller in upper-slopes than lower-slopes, but landscape position had no significant impact on CEC, %sand and %clay (Table 2.3).

SOC and CEC were factors that were consistently included in the PLS regression models for predicting Kd (Table 2.4). The best model was obtained for Kd-17β-estradiol because it had a higher explained variance (~ 76%) and r^2 (0.87) than other models, while its Root Mean Squared Error (RMSE) was only 4.98 L kg^{-1}. Based on these criteria, the predictive models for Kd-estriol and Kd-estrone were also strong, while the predictive model for Kd-equol was weaker (Table 2.4). For all estrogens, models for Koc had relatively low predictive power with r^2 values ranging from 0.32 for Koc-estrone to 0.44 for Koc-estriol.

Given the relatively strong correlation between Kd-17β-estradiol and Kd-estriol values, the ordinary least squares regression models for these estrogens provide stronger predictions (r^2=0.71) compared with predictive models developed for Kd-equol (r^2=0.49) and Kd-estrone (r^2=0.38) (Table 2.5). Data on Kd-estriol and Kd-17β-estradiol values were important in establishing predictive models for Kd-equol and Kd-estrone, respectively (Table 2.5). Ordinary least squares regression provided very weak predictions of Koc-estriol (r^2=0.40), Koc-equol (r^2=0.40), Koc-17β-estradiol (r^2=0.35) and particularly Koc-estrone (r^2=0.18) (Table 2.5), with the first three estrogens having

Table 2.4: Models for the prediction of Kd and Koc values of estrogens at the regional scale. Models were established using PLS regression. Root Mean Squared Error, explained variance and r^2 are presented.

	n	RMSE	EXPVAR	Model
				Prediction of Kd
17β-estradiol	113	4.98	75.94	Kd 17β-estradiol= -7.32 +0.66 SOC +0.36 CEC +0.18 % sand (r^2=0.87)
Estrone	111	12.53	48.86	Kd estrone=1.15+0.84 SOC +0.52 CEC (r^2=0.71)
Estriol	110	8.56	59.03	Kd estriol=-0.72 +1.35 CEC -0.66% clay+0.27 % silt (r^2=0.76)
Equol	112	18.78	26.59	Kd equol=8.62 +0.67 SOC +0.46 CEC +0.16 % silt (r^2=0.51)
				Prediction of Koc
17β-estradiol	116	301.11	13.42	Koc 17β-estradiol=883.6 –3.20 %silt +7.96 %sand (r^2=0.36)
Estrone	115	566.99	10.72	Koc estrone=1353.19 –7.16 %silt +11.77 %sand (r^2=0.32)
Estriol	115	328.81	20.24	Koc estriol=969.62 –6.34%clay -1.75 %silt+ 8.09 %sand (r^2=0.44)
Equol	112	833.17	18.77	Koc equol= 2093.98- -7.57 CEC- 10.39 %clay- 7.23 %silt+ 17.62 %sand (r^2=0.42)

stronger associations with each other than either of them with estrone. The optimum models established for Koc-17β-estradiol, Koc-estriol and Koc-estrone all relied on Koc-equol values (Table 2.5). Regardless of the estrogen, the predictive models established for Kd or Koc values using a single estrogen (Table 2.5) always yielded numerically smaller coefficients of determination than the predictive models using soil properties (Table 2.4). However, the differences in the strength of the predictive models using single estrogens or soil properties were extremely small for Kd or Koc values for estriol and equol, and hence using soil properties had only a significant advantage over using a single estrogen in cases of predicting Kd-estrone and 17β-estradiol or Koc estrone (Tables 2.4 and 2.5).

Table 2.5: The most suitable models to predict estrogen sorption coefficients using data on other estrogen sorption coefficients. Models were established using simple linear regression. Models were only included if they were significant at the 0.05 level.

Estrogen	n	Prediction model (coefficient of determination)
		Sorption coefficient, Kd (L $kg^{-1}$of soil)
17β-estradiol	120	Kd 17β-estradiol = 5.85 +0.74 Kd estriol (r^2=0.71)
Estriol	120	Kd estriol=1.22+0.953 Kd 17β-estradiol (r^2=0.71)
Estrone	121	Kd estrone= 9.44+1.018 Kd 17β-estradiol (r^2=0.38)
	120	Kd estrone=12.138+0.899 Kd estriol (r^2=0.38)
Equol	119	Kd equol=11.204+1.335 Kd estriol (r^2=0.49)
		Sorption per unit organic carbon, Koc (L kg^{-1})
17β-estradiol	119	Koc 17β-estradiol = 605.61+0.229 Koc equol (r^2=0.35)
Estriol	119	Koc estriol=548.774+0.248 Koc equol (r^2=0.40)
Estrone	119	Koc estrone=1007.436+0.268 Koc equol (r^2=0.18)
Equol	119	Koc equol = 347.392+1.628 Koc estriol (r^2=0.40)

2.5 Discussion

The pKa values of 17β-estradiol, estrone and estriol range from 10.3 to 10.8 (Hurwitz and Liu, 1977; Lewis and Archer 1979) and the pH of the Alberta soils ranges from 4.2 to 7.5. As such, most of the estrogen molecules were non-ionized with SOC being the most important soil characteristic explaining the variation in Kd-17β-estradiol, Kd-estriol, Kd-estrone and Kd-equol values across the 121 soil samples. Kd values of 17β-estradiol, estriol and equol showed equally strong positive associations with CEC perhaps because of the positive association between SOC and CEC. Using a smaller range of soil samples, other studies have reported on the positive associations between SOC and 17β-estradiol or estrone sorption (Yu et al., 2004; Hildebrand et al., 2006; Khanal et al., 2006; Loffredo and Senesi, 2006) and between CEC and 17β-estradiol sorption (Yu et al., 2004; Khanal et al., 2006; Loffredo and Senesi, 2006). In our studies, the associations between estrogen sorption and SOC or CEC were stronger for Kd-17β-estradiol than for Kd-estriol, Kd-estone and Kd-equol.

Log_{10} Koc values ranged from 2.56 to 4.42 for estriol, from 2.60 to 3.49 for 17β-estradiol, from 2.51 to 3.61 for estrone and from 2.77 to 3.76 for equol. These Log_{10} Koc values are within the ranges of previously reported Log_{10} Koc values, which varied from 3.21 to 3.46 for 17β-estradiol and from 3.19 to 3.22 for estrone in Lee et al. (2003), from 3.14 to 5.38 for 17β-estradiol and from 3.3 to 5.25 for estrone in Yu et al. (2004), and were 3.2 for 17β-estradiol and 3.3 for estrone in Casey et al. (2005). Based on previous reports (Ying and Kookana, 2005; Rothwell et al., 2005), the octanol-water partition

coefficient (Kow) increases in the order of estriol (Log Kow of 2.81) < equol (Log Kow of 3.2) < estrone (Log Kow of 3.9) = 17β-estradiol (Log Kow of 3.9). Although Kow is being used in fugacity models to estimate the partition of organic molecules into soil organic matter (Clark et al., 1995), average Koc values in Alberta soils increased in a different order of estriol (1,059 L kg^{-1}) = 17β-estradiol (1,082 L kg^{-1}) < estrone (1,557 L kg^{-1}) < equol (2,080 L kg^{-1}). A poor association between Log Kow and Log Koc values has been previously reported for sorption of 17ß-estradiol on sediments and dissolved organic matter surrogates (Lai et al., 2000; Holthaus et al., 2002) possibly indicating that hydrophobic partitioning is not the only mechanism of estrogen sorption (Yamamoto et al. 2003).

Considering all soil samples (n=121), the Koc values of the studied compounds were always greater than 320 L kg^{-1} with a maximum of 5,770 L kg^{-1} for Koc-equol. The Koc values of the estrogens (320 to 5,770 L kg^{-1}) were thus similar to the Koc values of a range of agricultural pesticides used in the Province of Alberta such as mancozeb (998 L kg^{-1}), clodinafop-p-propargyl (1466 L kg^{-1}), trifloxystrobin (2,377 L kg^{-1}), and triallate (4,301 L kg^{-1}) (FOOTPRINT Consortium 2006).

Recent studies describe the application of pesticide fate models for determining estrogen fate in soil (Casey et al., 2003; 2005). These studies focused on estrogen leaching through column experiments in the laboratory but additional work could lead to the use of pesticide fate models for assessments of the risk of estrogen transport at the large-scale. Estrogens are organic molecules like most pesticides and sorption coefficients are among

the most sensitive input parameters in pesticide fate models (Boesten and van der Linden, 1991; Dubus et al., 2003). In fact, pesticide fate models at the large-scale have indicated that site-specific data on sorption parameters are more important than the choice of the pesticide fate model itself (Dann et al., 2006). Several approaches have been proposed to obtaining data on pesticide sorption parameters at the large-scale. These approaches include direct measurements, which are costly particularly when multiple pesticides are involved, or by indirect measures such as regression equations based on soil properties data (Dann et al., 2006; Gaultier et al., 2008). Such indirect approaches are also valid for estrogens but, based on r^2 values, the strength of the predictions were relatively poor for Kd-estrone, Koc-17β-estradiol, Koc-equol Koc-estriol and Koc-estrone.

Another approach to improve on large scale analyses of the risk of off-site estrogen movement would be to divide the region into units with distinct sorption coefficients so that sorption input parameters in models could vary by ecoregion, soil great groups and/or soil-landscape position. Our studies demonstrated that Brown Chernozems were on average low in SOC (10 g C kg^{-1}) and CEC (15.7 cmol kg^{-1}) and therefore demonstrated on average 1.5 to 3.5 times smaller estrogen Kd values than other ecoregions. Brown Chernozems are in the MG ecoregion and therefore MG demonstrated on average 1.3 to 3.4 times smaller estrogen Kd values than other ecoregions. Such differences in sorption coefficients are expected to influence the persistence and transport of organic chemicals in soil (Boesten and van der Linden, 1991; Farenhorst et al., 2008). The weaker Kd values for soils with SOC < 10 g C kg^{-1} has been highlighted in pesticide sorption studies (Wauchope et al., 2002; Gaultier et al. 2008). In our studies, estrogen Kd

values also varied significantly within fields, with the estrogen Kd values being on average 1.3 to 1.4 times smaller in upper-slopes than lower-slopes. Soils from upper-slopes generally had a lower SOC than lower-slopes probably because tillage erosion results in the translocation of topsoil from upper-landforms (i.e., knolls, convex elements) to lower-landforms (i.e., toeslopes, concave elements) (Papiernik et al., 2007).

There were variations in the sorption of estrogens per unit organic carbon. Soil organic matter has a wide range of physical, chemical and biological characteristics and this could influence the retention of chemicals by soil (Farenhorst, 2006). For example, the fraction humic acids in dissolved organic matter is more chemically reactive with 17β-estradiol than the fraction fulvic acids (Yamamoto et al., 2003). In studies at the large-scale, the degree of aromaticity of SOM has been used to improve predictions of pesticide Koc values across soil types in Australia and Pakistan (Ahmad et al., 2001). SOM characteristics are influenced by factors such as parent material, pedogenesis, humification and land management practices (Stearman et al., 1989; Grathwohl, 1990; Chen and Pawluck, 1995; Senesi et al., 1996; Cuypers et al., 2002; Ding et al., 2002). As such, additional studies relating SOM characteristics and associated factors to estrogen sorption by soil will further advance large-scale analyses of the risk of off-site estrogen transport.

2.6 Conclusion

This is the first study to compare the sorption of 17β-estradiol, estrone, estriol and equol in a wide range of soils. Both Kd and Koc values significantly increased in the order of estriol = 17β-estradiol < estrone < equol. SOC and Kd values were significantly positively correlated (r ranging from 0.50 to 0.75, $P < 0.001$) and particularly small Kd values were observed when SOC was below a threshold value of 10 g C kg^{-1}. The Mixed Grassland ecoregion (Brown Chernozem soils), as well as upper-slopes in general, demonstrated lesser Kd values because of reduced SOC. Despite the smaller Kd values in the surface soils of the MG ecoregion with Brown Chernozems, these surface soils demonstrated greater sorption per unit organic carbon relative to soils in other regions. The strength of the prediction models, as determined by the coefficient of determination (r^2) and other factors, was always better for Kd than Koc values. Regardless of the regression used, the r^2 of the prediction models exceeded 0.70 for Kd-17β-estradiol and Kd-estriol, but r^2 was below 0.52 for Kd-equol. Since regression models were weak in predicting Koc values for each estrogen, further studies are required to understand the impact of soil properties on the sorption of estrogens per unit organic carbon. Such information could benefit large-scale analyses for defining beneficial land management practices for manure disposal and for agri-environmental policy analyses in Canada.

2.7 Acknowledgments

The authors would like to acknowledge the financial support of the Natural Sciences and Engineering Research Council of Canada. E.Caron would like to thank the University of Manitoba Graduate Felllowship for financial support. The authors also thank the

employees of Alberta Agriculture and Food for sampling the soils. In addition, the authors thank Mr. R. Ellis and Dr. R. McQueen Department of Soil Science, University of Manitoba, for providing assistance to the laboratory experiments and feedback on earlier drafts of the manuscript, respectively.

2.8 References

Ahmad, R., Kookana, R.S., Alston, A.M. and Skjemstad, J.O. 2001. The nature of soil organic matter affects sorption of pesticides. 1. Relationships with carbon chemistry as determined by ^{13}C CPMAS spectroscopy. Environ. Sci. Technol., 35:878-884.

Alberta Agriculture and Food, Economics and Competitiveness Division, Statistics and Data Development Unit. 2006. Census of Agriculture for Alberta, Provincial Highlights. AGDEX 852-5.

Akaza, H., Miyanaga, N., Takashima, N., Naito, S., Hirao, Y., Tsukamoto, T., Fujioka, T., Mori, M., Kim, W.-J., Mann Song J. and Pantuck, A.J. **2004**. Comparisons of percent equol producers between prostate cancer patients and controls: Case-controlled studies of isoflavones in Japanese, Korean and American residents. Jpn. J. Clin. Oncol., 34:86-89.

Anderson, D.W. 1979. Processes of humus formation and transformation in soils of the Canadian Great Plains. Eur. J. Soil. Sci., 30(1):77-84.

Bank, T.L., Kukkadapu, R.K., Madden, A.S., Ginder-Vogel, M.A., Bladwin, M.E. and Jardine, P.M. 2008. Effects of gamma-serilization on the physico-chemical properties of natural sediments. Chem. Geol., 251:1-7.

Benoit, P., Barriuso, E., Houot, S. and Calvet, R. 1996. Influence of the nature of soil organic matter on the sorption desorption of 2,4-dichlorophenol and the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D). Eur. J. Soil Sci., 47:567-578.

Boesten, J.J.T.I. and van der Linden, A.M.A. 1991. Modeling the influence of Sorption and Transformation on Pesticide Leaching and Persistence. J. Environ. Qual. 20 (2):425-435.

Burns, J.M., Hall, S. and Ferry, J.L. 2009. The adsorption of saxitoxin to clays and sediments in fresh and saline waters, Water Res., 43(7):1899:1904.

Casey, F.X.M., Larsen, G.L., Hakk, H. and Šimůnek J. 2003. Fate and transport of 17β-estradiol in soil-water systems. Environ. Sci. Technol., 37:2400-2409.

Casey, F.X.M., Šimůnek, J., Lee, J., Larsen, G.L. and Hakk, H. 2005. Sorption, mobility, and transformation of estrogenic hormones in natural soils. J. Environ. Qual., 34:1372-179.

Cathcart, J., Cannon, K., and Heinz, J. 2008. Selection and establishment of Alberta agricultural soil quality benchmark sites. Can. J. Soil Sci., 88:399-408.

Chen, Z. and Pawluk, S. 1995. Structural variations of humic acids in two Alberta Mollisols. Geoderma, 65:173-193.

Clark, B., Henry, J.G. and Mackay, D. 1995. Fugacity analysis and model of organic chemical fate in a sewage treatment plant. Environ. Sci. Technol., 29:1488-1494.

Cuypers, C., Grotenhuis, T., Nierop, K.G.J., Franco, E.M., de Jager, A. and Rulkens, W. 2002. Amorphous and condensed organic matter domains: The effect of persulfate oxidation on the composition of soil/sediment organic matter. Chemosphere, 48:919-931.

Dann, R.L., Close, M.E., Lee R. and Pang L. 2006. Impact of data quality and model complexity on prediction of pesticide leaching. J. Environ. Qual., 35:628-640.

Ding, G., J.M. Novak, D. Amarasiriwardena, P.G. Hunt, and B. Xing. 2002. Soil Organic Matter Characteristics as Affected by Tillage Management. Soil Sci. Soc. Am. J., 66:421–429.

Dubus, I.G., Brown, C.D. and Beulke, S. 2003. Sensitivity analyses for four pesticide leaching models. Pest Manag. Sci., 59:962–982.

Ecological Stratification Working Group. 1995. *A National Ecological Framework for Canada*. Agriculture and Agri-Food Canada, Research Branch, Centre for Land and Biological Resources Research and Environment Canada, State of the Environment Directorate, Ecozone Analysis Branch, Ottawa/Hull. Report and national map at 1:7 500 000 scale. 125 pp.

Fan, Z., Casey, F.X.M., Hakk, H. and Larsen, G.L. 2007. Persistence and fate of 17β-estradiol and testosterone in agricultural soils. Chemosphere, 67:886-895.

Farenhorst, A. 2006.Importance of soil organic matter fractions in soil-landscape and regional assessments of pesticide sorption and leaching in soil. Soil Sci. Soc. Am. J, 70:1005-1012.

Farenhorst, A., Londry, K., Nahar, N. and Gaultier, J. 2008. In-Field Variation in 2,4-D mineralization in relation to sorption and soil microbial communities. J. Environ. Sci. Heal. B, 43(2):1-7.

Finlay-Moore, O., Hartel P.G. and Cabrera, M.L. 2000. 17β-Estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. J. Environ. Qual., 29:1604-1611.

Flogeac, K., Guillon, E. and Aplincourt M. 2005. Adsorption of several metal ions onto a model soil sample: Equilibrium and EPR studies. J. Colloid Interface Sci., 286:596-601.

FOOTPRINT Consortium 2006. FOOTPRINT creating tools for pesticide risk assessment and management in Europe [Online WWW]. Available URL "http://www.eu-footprint.org/home.html."[Accessed November 2007].

Gaultier, J., Farenhorst, A., Cathcart, J. and Goddard, T. 2008. Regional assessment of herbicide sorption and degradation in two sampling years. J. Environ. Qual., 37:1825-1836.

Gee, G.W. and Bauder, J.W. 1986. Particle size analysis. In A. Klute, ed. Methods of soil Analysis. Part 1. Physical and Mineralogical Methods. ASA/SSSA, Madison, WI. pp 383-412.

Grathwohl, P. 1990. Influence of organic matter from soils and sediments from various origins on the sorption of some chlorinated aliphatic hydrocarbons: Implication on K_{oc} correlation. Environ. Sci.Technol., 24:1687-1693.

Hildebrand, C., Londry, K.L. and Farenhorst, A. 2006. Sorption and desorption of three endocrine disrupters in soils. J. Environ. Sci. Heal. B, 41:907-921.

Holthaus, K.I.E., Johnson, A.C., Jurgens, M.D., Williams, R.J., Smith, J.J.L. and Carter, J.E. 2002. The potential for estradiol and ethinylestradiol to sorb to suspended and bed sediments in some English rivers. Environ. Toxicol. Chemi., 21(12):2526-2535.

Hong, Y., Halasz, A., Zhao, J.S., Monteil-Rivera, F. and Hawari, J. 2008. Experimental Evidence of *insitu* natural attenuation of 2,4- and 2,6-dinitrotoluene in marine sediments. Chemosphere, 70:791-799.

Hurwitz, A.R., and Liu, S.T. 1977. Determination of aqueous solubility and pka values of estrogens. J. Pharm. Sci., 66(5):624-627.

Khanal, K.S., Xie, B., Thompson, M.L., Sung, S., Ong, S.-K. and Van Leeuwen, J.H. 2006. Fate, transport, and biodegradation of natural estrogens in the environment ad engineered systems. Environ. Sci.Technol., 40(21):6537-6546.

Kjær, J., Olsen, P., Bach, K., C. Barlebo, H., Ingerslev, F., Hansen, M. and Halling Sørensen B. 2007. Leaching of estrogenic hormones from manure-treated structured soils. Environ. Sci. Technol., 41(11):3911-3917.

Lai, K.M., Johnson, K.L., Scrimshaw, M.D. and Lester, J.N. 2000. Binding of waterbone steroid estrogens to solid phases in river and estuarine systems. Environ. Sci. Technol., 34:3890-3894.

Lai, K.M., Scrimshaw, M.D. and Lester, J.N. **2002**. The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit. Rev. Toxicol., 32 (2):113-132.

Lange, I.G., Daxenberger, A., Schiffer, B., Witters, H., Ibaretta, D. and Meyer, H.H.D. 2002. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. Anal. Chim. Acta, 473:27-37.

Lee, L.S., Strock, T.J., Sarmah, A.K. and Rao, P.S.C. 2003. Sorption and dissipation of testosterone, estrogens and their primary transformation products in soil and sediments. Environ. Sci. Technol., 37:4098-4105.

Lee, S., Pardue, J.H., Moe, W.M. and Kim, D.J. 2009. Effect of sorption and desorption-resistance on biodegradation of chlorobenzene in two wetland soils. J. Hazard. Mater., 161:492-498.

Lefebvre, A., W. Eilers, and B. Chunn (eds.), 2005. Environmental Sustainability of Canadian Agriculture: Agri-Environmental Indicator Report Series – Report #2. Agriculture and Agri-Food Canada, Ottawa, Ontario. 136-137.

Legendre, P. and L. Legendre. 1998. Numerical Ecology. 2nd Ed. Elsevier B.V., Amsterdam, The Netherlands.

Leskiw, L.A., Yarmuch, M.S., Waterman, L.P. and Sansom, J.J. 2000. Baseline soil physical and chemical properties of forty-three soil quality benchmark sites in Alberta. File #99-042, Can-Ag Enterprises Ltd., Edmonton, AB., 175pp.

Lewis, K.M. and Archer, R.D. 1979. pKa values of estrone, 17 beta-estradiol and 2-methoxyestrone.Steroids, 34(5):485-499

Loeppert, R.H. and Suarez, D.L. 1996. Carbonate and Gypsum. pp 437-475
In: M. Bartels, ed. Methods of Soil Analysis. Part 3. Chemical methods. ASA/SSSA, Madison, WI.

Loffredo, E and Senesi, N. 2006. Fate of organic pollutants in soils with emphasis on adsorption/desorption processes of endocrine disruptor compounds. Pure Appl. Chem., 78 (5):947-961.

Lorenzen, A., Burnison, K., Servos, M. and Topp, E. **2006**. Persistence of endocrine-disrupting chemicals in agricultural soils. J. Environ. Eng. Sci., 5:211-219.

Lotrario, J.B., Stuart, J.B., Lam, T., Arands, R.R., O`Connor, O.A. and Kosson, D.S. 1995. Effects of Sterilization Methods on the Physical Characteristics of soil: Implications for sorption isotherm analyses, Bull. Environ. Contam. Toxicol., 54:668-675.

Martens, M. and Martens, H. 1986. Partial least square regression. In J.R. Piggott, Statistical procedures in food research pp.292-360, London, Elsevier Applied Sciences.

Martens, H. and Martens, M. 2000. Modified Jack-Knife estimation of parameter uncertainty in bilinear modelling by Partial Least Squares regression (PLSR), Food Qual. Prefer., 11:5-16.

McKeague, J.A. 1978. Manual on soil sampling and methods of analysis. Canadian Society of Soil Science, Ottawa, Canada, 212 pp.

Mustonen, E.A., Jokela, T., Saastomoinen, I., Taponen, J., Taponen, S., Saloniemi, H. and Wähälä, K. 2006. High serum S-equol content in red clover fed ewes: the classical endocrine disruptor is a single enantiomer. Environ. Chem. Lett., 3:154-159.

Nelson, D.E. and Sommers, L.E. 1982. Total carbon, organic carbon, and organic matter. In A.L. Page, ed. Methods of Soil Analysis. Part 2. Chemical and microbial properties. ASA/SSSA, Madison, WI. pp. 539-577.

Nichols, D.J., Daniel, T.C., Moore, P.A. Jr, Edwards, D.R. and Pote, D.H. 1997. Runoff of estrogen hormone 17β-Estradiol from poultry litter applied to pasture. J. Environ. Qual., 26:1002-1006.

Papiernik, S.K., Lindstrom, M.J., Schumacher, T.E., Schumacher, J.A., Malo, D.D. and Lobb, D.A. 2007. Characterization of soil profiles in a landscape affected by long-term tillage. Soil Till. Res.,93(2):335-345.

Purdom C.E., Hardiman P.A., Bye V.J., Eno N.C., Tyler C.R. and Sumpter J.P. 1994. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol., 8:275-285.

Qualls, R.G. 2000. Comparison of the behavior of soluble organic and inorganic nutrients in forest soils, Forest Ecolog. Manage.,138:29-50.

Ren, W.-X., Li, P.-J., Geng, Y. and Li, X.J. 2009. Biological leaching of heavy metals from a contaminated soil by Aspergillus Niger. J. Hazard. Mater., 167(1-3):164-169

Renaud, F.G., Leeds-Harrison, P.B., Brown, C.D., and van Beinum, W. 2004. Determination of time-dependent partition coefficients for several pesticides using diffusion theory, Chemosphere 57:1525–1535.

Rothwell, J.A., Day, A.J., Morgan, M.R.A. 2005. Experimental determination of octanol water partition coefficients of quercetin and related flavonoids. J. Agric. Food. Chem., 53:4355-4360.

Senesi, N., Miano, T.M., and Brunetti, G. 1996. Humic-like substances in organic amendments and effects on native soil humic substances. pp 531-593. In: A.Piccolo, ed. Humic substances in terrestrial ecosystems. Elsevier Science, Amsterdam.

Shaw, L.J. and Hooker, J.E. 2008. The fate and toxicity of the flavonoids Naringenin and Formononetin in soil. Soil Biol. Biochem.,40:528-536.

Shore. L.S., Correll, D.L. and Chakraborty, P.K. 1995. pp155-162. In K.F. Steele ed. Animal waste and the land-water interface, Lewis Publihers, Boca Raton, Fl.

Soil Classification Working Group. 1998. The Canadian system of Soil Classification (Third Edition), NRC_CNRC Press, 187 pages.

Stearman, G.K., Lewis, R.J., Tortorelli, L.J. and Tyler, D.D. 1989. Herbicide reactivity of soil organic matter fractions in no-tilled and tilled cotton. Soil Sci. Soc. Am. J., 53:1690-1694.

Steiner, R. 1996. Advanced Technology Guide for LS 6500 Series Scintillation Counters (BR-7885B) Beckman, Fullerton, CA, Page 9.

Stephens, K.D., Farenhorst, A., and Fuller, L.G. 2002. Effect of soil sterilization by mercuric chloride on herbicide sorption by soil. J. Environ. Sci. Heal. B., No. 6, pp. 561–571.

Wauchope, R.D., Yeh, S., Linders, J.B.H.J., Kloskowski, R., Tanaka, K., Rubin, B., Katayama, A., Kordel., W., Gerstl, Z., Lane, M . and Unsworth, J.B. 2002. Review: Pesticide soil sorption parameters: theory, measurements, uses, limitations and reliability. Pest Manag. Sci. 58:419-445.

Wolf, D.C., Hao, T.H., Scott, H.D. and Lavy, T.L. 1989. Influence of sterilization methods on selected soil microbisl, physical and chemical properties. J. Environ.Qual.,18:39-44.

Xuan, R., Blassengale, A.A. and Wang, Q. **2008**. Degradation of estrogenic hormones in a silt loam soil. J.Agric. Food Chem., 56:9152-9158.

Yamamoto, H., Liljestrand, H.M., Shimizu,Y. and Morita, M. 2003. Effects of Physical-Chemical Characteristics on the Sorption of Selected Endocrine Disruptors by Dissolved Organic Matter Surrogates. Environ. Sci. Technol., 37 (12): 2646-2657.

Yang, H., Halasz, A., Zhao, J.-S. Monteil-Rivera, F. and Hawari. J. 2008. Experimental evidence of in situ natural attenuation of 2,4 and 2,6-dinitrotoluene in marine sediment, Chemosphere, 70:791-799.

Ying, G.G. and Kookana, R.S. 2005. Sorption and degradation of estrogen-like-endocrine disrupting chemicals in soil. Environ. Toxicol. Chem., 24 (10):2640-2645.

Yu, Z., Xiao, B., Huang, W. and Peng, P. 2004. Sorption of steroid estrogens to soils and sediments. Environ. Toxicol. Chem., 23 (3):531-539.

3. MINERALIZATION OF 17ß-ESTRADIOL IN 36 SURFACE SOILS FROM ALBERTA, CANADA

Caron, E., Farenhorst, A., Mc Queen, R., Sheedy, C., Goddard, T.and Gaultier, J. 2010. Mineralization of 17ß-estradiol in 36 Surface Soils from Alberta, Canada. Manuscript accepted by Agriculture, Ecosystems and Environment. Reproduced with permission from the editor.

3.1 Abstract

Recent column studies suggest that pesticide fate models could be used to estimate the fate of estrogens in soil. Estrogens are detected in livestock manure which is used as a nutrient source on agricultural land. This is the first study to examine estrogen mineralization in a wide range of agricultural soils at the regional-scale. Soil samples were collected from upper and lower landscape positions of 18 agricultural fields in an area spanning 49–60^{o}N longitude and 110–120^{o}W latitude and these samples were used to determine 17β-estradiol mineralization parameters in microcosm experiments. Maximum 17β-estradiol mineralization (Max) ranged from 5.8 to 19.2% and was, on average, significantly ($P < 0.05$) less than the 47.9 to 61.9% range measured in the same soils for the widely-used herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). Maximum 17β-estradiol mineralization was positively correlated to Kd-17β-estradiol ($r=0.62$, $P < 0.001$) while 2,4-D maximum mineralization was negatively correlated to Kd-2,4-D ($r=-0.52$, $P < 0.01$), even though 17β-estradiol and 2,4-D sorption parameters (Kd) were

positively correlated (r=0.62, P < 0.001), and both Kd-17β-estradiol and Kd-2,4-D values were significantly positively correlated to SOC (r=0.71, P < 0.001; and r=0.67, P < 0.001, respectively). Hence, the mineralization of 2,4-D decreases as its sorption to soils increases while the mineralization of 17β-estradiol increases as its sorption to soil increases. This suggests that some steps in the 17β-estradiol mineralization process are occurring in the sorbed phase. Equations to predict 17β-estradiol and 2,4-D sorption and mineralization parameters were established using Partial Least Squares regression. Significant models for mineralization (r^2 from 0.42 to 0.56) had lower r^2 than significant sorption models (r^2 from 0.78 to 0.85). Given the poor results of the mineralization regression models, we conclude that probability density functions, rather than regression models, are likely to be more useful for describing pesticide or estrogen mineralization parameters at the regional scale. Based on our findings, agri-environmental policy-analysis in Alberta should use the log-logistic probability density function to describe the mineralization rate (k) of either 17ß-estradiol or 2,4-D at the large-scale. Max-17β-estradiol at the large-scale is best described by the extreme values probability density function and Max-2,4-D by the triangular probability density function.

3.2 Introduction

Estrogens are naturally produced and excreted by vertebrates. One way by which enhanced concentrations of estrogens can be released into the environment is through the application of livestock manure onto agricultural lands. 17β-estradiol concentrations in manure can be as high as 115 (±11) μg kg^{-1} (Laegdsman et al. 2009). Estrogens present in

manure-applied soils could potentially reach surface water through runoff. Estrogens at concentrations ranging from 0.8 to 8 ng L^{-1} in surface waters induced the production of vitellogenin in male rainbow trout (Routledge et al., 1998).

The Province of Alberta, Canada has more than 6 million cattle and calves and more than 2 million hogs. A portion of the manure generated by this livestock is applied to Alberta crop land (Alberta Agriculture and Food**,** 2006). Knowledge of factors influencing the persistence and transport of estrogens in soil is becoming increasingly important as this knowledge could be used for the development of beneficial management practices that will reduce the movement of estrogens to surface and ground waters.

Recent laboratory (column) studies suggest that pesticide fate models could be used to estimate the fate of other organic chemical contaminants of interest such as estrogens (Casey et al., 2003; Das et al., 2005). Pesticide sorption and mineralization parameters are among the most sensitive input parameters in pesticide fate models (Boesten and van der Linden, 1991; Dubus and Janssen, 2003; Dann et al., 2006), hence information on estrogen sorption and mineralization parameters will be important if pesticide fate models are going to be effective in the area of policy analysis concerned with manure management.

Soil organic carbon content (SOC) is an important factor in the movement of estrogens in the soil environment. In Alberta, at the regional scale, SOC was the main factor influencing the sorption parameters of four estrogens: 17β-estradiol, estrone, estriol and

equol (Caron et al., 2010). The importance of SOC in the sorption behavior of 17β-estradiol had also been described by Casey et al. (2003), Lee et al. (2003), Yu et al. (2007) and Loffredo and Senesi (2006). Sorption has been correlated with the degradation and mineralization of organic chemicals such as pesticides, probably because sorption influences pesticide bioavailability to microbial degraders (Chamignon et al., 2008; Zablotowicz et al., 2009).

The mineralization of 17ß-estradiol in soils is influenced by soil characteristics and environmental factors. In Ontario, Canada, Colucci et al. (2001) found that between 4 and 15 % of the applied 17β-estradiol in one agricultural loam soil was mineralized after 61 days under temperatures varying between 4 and 37^{o}C and that the higher temperatures resulted in greater mineralization. Fan et al. (2007) found that 6 % of 17β-estradiol in a USA northern prairie sandy soil was mineralized under aerobic conditions while no mineralization occurred under anaerobic conditions.

Using stochastic modeling approaches to predict pesticide fate at the regional scale, modelers have hypothesized that a normal, log-normal or uniform distribution is the best choice for describing sorption input parameters, and that a log-normal or beta distribution is best for describing degradation input parameters (Dubus et al. 2003). The accurate determination of these probability density functions is particularly important in the case of sorption and degradation parameters because those parameters are known to have a significant impact on model output (Dubus and Brown, 2002). For pesticides and estrogens, little is known of the actual distribution of the sorption and degradation

parameters at the soil-landscape scale (Novak et al., 1997; Farenhorst et al. 2008) or regional scale (Ahmad et al., 2001; Gaultier et al., 2008a; Caron et al., 2010). This lack of data creates uncertainty when pesticide fate models are used in regulatory and environmental risk assessments (Dubus et al., 2003). In this study we are investigating these distributions of 17β-estradiol and 2,4-D (a widely applied herbicide) sorption and mineralization parameters at the regional scale in Alberta, Canada.

To our knowledge, the current research is the first comprehensive regional-scale study on the mineralization of 17β-estradiol in soils spanning an area of 49–60^{o}N longitude and 110–120^{o}W latitude. The objectives of this study were to a) determine 17ß-estradiol mineralization parameters in soils from upper and lower landscape positions of 18 agricultural fields in three ecoregions in the province of Alberta, and b) relate these parameters to variations in 17ß-estradiol sorption parameters and soil properties, and to the mineralization and sorption of a common herbicide (2,4-D) in the same soils.

3.3 Material and Methods

3.3.1 Chemicals

Analytical grade 17β-estradiol (99% pure) was purchased from Sigma-Aldrich Chemical Company, St. Louis, MO. 17β-estradiol [6,7-3H(N)] (99% radiochemical purity, specific activity 1.48x10^{12}-2.22x10^{12} Becquerels mmol^{-1}) used in the sorption experiments and 17β-estradiol [4-^{14}C] (99% radiochemical purity, specific activity 1.66x10^{12}-2.257x10^{12}

Becquerels $mmol^{-1}$) (Figure 3.1) used in the mineralization experiment were purchased from American Radiolabeled Chemicals, St-Louis, MO. Ring labelled 2,4-D (ring-U-^{14}C 2,4-dichlorophenoxiacatic acid, 99 % radiochemical purity, specific activity 191.3 MBecquerels $mmol^{-1}$) used for the sorption experiments, carboxyl labelled 2,4-D (99% radiochemical purity; specific activity 632.7 MBq $mmol^{-1}$) used in the mineralization experiments, as well as analytical grade 2,4-D (95% chemical purity), were purchased from Sigma-Aldrich Chemical Company. The solubility of 17β-estradiol is 13 mg L^{-1} in water at 20^{o}C (Ying and Kookana, 2005) and the solubility of 2,4-D in water is 890 mg L^{-1} at 25^{o}C (Vergili and Barlas, 2009).

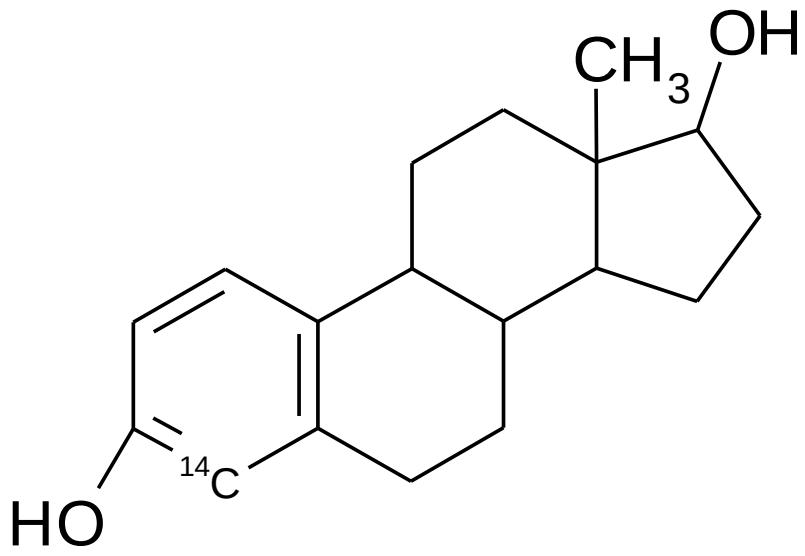


Figure 3.1: Structure of 17β-estradiol [4-^{14}C]

3.3.2 Study Area

The Province of Alberta, Canada initiated a soil quality benchmark program in 1997 to monitor soil quality in fields representative of soil-landscapes and agricultural practices within ecodistricts (i.e., polygons of the Canadian ecostratification system) (Ecological Stratification Working Group, 1996). The program included 43 agricultural fields across the 24 million hectare agricultural area of Alberta. Detailed site descriptions can be found in Leskiw et al. (2000) and Cathcart et al. (2008).

In the current study, thirty-six surface soil samples (0-15 cm) were collected in 2002 from upper and lower slopes in each of 18 sites across three ecoregions (Mixed Grassland, Peace Lowland and Boreal Transition) (Table 3.1). At each of the 36 sampling points, six soil cores were collected with a sterile (rinsed with 10% bleach aqueous solution) auger in an area equivalent to a circle with a 2 m radius, and then composited to yield the soil sample.

3.3.3 Soil Properties

A portion of air-dried and sieved (2 mm) soil was used to determine SOC, texture, pH, carbonate content ($CaCO_3$), electrical conductivity (EC) cation exchange capacity (CEC), and hydrolytic bacterial activity. SOC was determined using a Leco model CHN 600 C and N combustion analyser as in Nelson and Sommers (1982). Soil texture was

Table 3.1: Characteristics of the sampled ecoregions (adapted from Gaultier et al., 2008a and Caron et al., 2010).

Ecoregion	**Soil Great Group**	**n**	**Mean Temperature January (^{o}C)**	**Mean Temperature July (^{o}C)**	**Precipitation (mm)**
Peace Lowland	Gray Luvisol (n=4) Dark Gray Luvisol (n=2) Black Chernozem (n=4) Dark Gray Chernozem (n=2)	12	-17.2	13.3	435-517
Boreal Transition	Gray Luvisol (n=4) Dark Gray Luvisol (n=4) Black Chernozem (n=2) Dark Gray Chernozem (n=2)	12	-15.0	15.9	428-535
Mixed Grassland	Dark Brown Chernozem (n=2) Brown Chernozem (n=10)	12	-12.8	17.9	314-363

determined using a hydrometer (Gee and Bauder, 1986). Soil pH was determined using 5 g of soil and 10ml of 0.01M $CaCl_2$ (McKeague, 1978). $CaCO_3$, was determined by a volumetric calcimeter following the addition of 6 M $HCl{\cdot}FeCl_2$ (Loeppert and Suarez, 1996). EC (saturated paste) and CEC were determined as in McKeague (1978). Hydrolytic bacterial activity was measured by the fluorescein diacetate hydrolysis assay (FDA) (Adam and Duncan, 2001).

3.3.4 17β-estradiol Sorption

Batch equilibrium experiments with air-dried sieved soil were used to determine the sorption of 17β-estradiol in these and other soils from Alberta, as described in Caron et al. (2010). Briefly, the experimental protocol was as follow. The stock solution was prepared using autoclaved ultra-pure water and contained 25 μg L^{-1} analytical grade 17β-estradiol and 16.67 Becquerels mL^{-1} of 17β-estradiol [6,7-3H(N)]. The stock solution was prepared such that 10 mL of solution added to 5 g of soil would be equivalent to a concentration of 50 μg 17β-estradiol $kg^{-1}$of soil (assuming 100% sorption); this concentration is within the linear range of sorption as determined by Casey et al. (2005) and Kozarek et al. (2008). Air-dried soil (5 g) was placed in borosilicate glass tubes (duplicates), capped with aluminum foil, and autoclaved for 30 minutes at 121^{o}C and 21 psi. Sterilization of laboratory equipment and soils is desired because biodegradation leads to an underestimation of Kd values (Wolf et al., 1989). Autoclaving is the most commonly-used sterilization technique in batch-equilibrium sorption studies (e.g., see recent studies by Shaw and Hooker, 2008, Burns et al., 2009, Lee et al., 2009, Riviera

and Hawari, 2008 and Ren et al., 2009). Autoclaving has no altering effect on SOC, pH, CEC and soil surface area (Wolf et al., 1989; Lotrario et al. 1995), nor on the measurement of Kd values of pesticides (Benoit et al., 1996) and chlorinated hydrocarbons (Lotrario et al. 1995). In contrast, Bank et al. (2008) observed that gamma-sterilization significantly decreased CEC and altered the reactivity of both the organic and inorganic fractions, and Stephens et al. (2002) found that chemical sterilization significantly decreased herbicide Kd values because of increased competition for sorption sites. Stock solution (10 mL) was added to each tube and soil slurries were rotated (Rotamix, Appropriate Technical Resources Inc., Laurel, MD) for two hours at 5^{o}C (as an additional step to avoid biodegradation) in the dark to reach equilibrium (as determined in Caron et al., 2010). The soil slurry was then centrifuged for 30 min at 7,000 rpm (6000 g). Supernatant (1 mL) was added to scintillation vials (duplicates) containing 5 mL of scintillation cocktail (Scintisafe, Beckman, Missisauga, ON). The amounts of radioactivity in the initial and equilibrium solutions were determined by Liquid Scintillation Counting (LSC) with automated quench correction (#H method) and a maximum counting time of 10 minutes (LS 7500 Beckman Instruments, Fullerton, CA).

The soil sorption coefficient, Kd [L kg^{-1} soil], was calculated by dividing the concentration of 17β-estradiol in soil at equilibrium, C_s [µg kg^{-1}], by the concentration of 17β-estradiol in solution at equilibrium, C_e [µg L^{-1}]. The organic carbon-normalized sorption coefficient, Koc [L kg^{-1} OC], was calculated by dividing Kd by the fraction of organic carbon in soil (f_{OC}). Wauchope et al., (2002) stressed that the calculation of Koc allows to take into account the variability of the nature of the organic matter.

3.3.5 17β -estradiol Mineralization

Another portion of soil was frozen at –40^{o}C and then thawed for use in mineralization experiments. Mortensen and Jacobsen (2004) have showed that freezing of soils prior to mineralization experiments did not significantly influence mineralization results. Soils were wetted to 80% of their field capacity by adding Milli-Q water (minus 1 mL which was the volume used to apply the 17ß-estradiol solution).

Field capacity was determined by the container method in which a mass of soil is saturated in a known volume, drained under gravitational forces and then oven dried for 24 h at 105^{o}C to determine the mass of water. About 20 g of thawed soil was used to determine the initial moisture content of the soils by comparing the initial weight to the weight after 24 h in the oven at 105^{o}C. Moisture content was then used in calculations to adjust each soil sample in the mineralization experiments to 80% of its field capacity.

Thirty-six surface soils in triplicate were analyzed. Each microcosm consisted of a 1 L Mason jar containing one glass jar (90 mL) with 25 g of soil (oven-dry weight basis). The microcosm also contained a 20 mL scintillation vial with 5 mL of 0.5 M NaOH to trap evolved CO_2. Three mL of acidified water (pH 3) in a test tube were placed in the jar to maintain moisture without trapping CO_2. Soils near 80 % of their field capacity were incubated in microcosms for 7 days in the dark at 20^{o}C. At the same time, six additional

microcosms consisting of two controls in triplicates: a) 25 g of autoclaved silica sand and b) 25 g of non-autoclaved silica sand were set up.

For the 17β-estradiol applications, analytical grade 17β-estradiol and 17β-estradiol [4-^{14}C] were mixed in autoclaved ultra-pure water to prepare a stock solution of 1,250 μg L^{-1} of 17β-estradiol and 1,667 Becquerels mL^{-1} of 17β-estradiol [4-^{14}C]. The solution was stored in a glass amber bottle at 4^{o}C and used the following day.

Soils and silica sand in microcosms were spiked with 1 mL of stock solution to achieve a 17β-estradiol concentration of 50 μg kg^{-1} in soil and 1,667 Becquerels of 17β-estradiol [4-^{14}C] per microcosm. This concentration was chosen in order to be consistent with the concentration used in batch equilibrium experiments. The stock solution was added drop wise in a grid pattern and then thoroughly mixed with the soil to achieve a homogenous distribution. The microcosms were then sealed and incubated in the dark at 20^{o}C. Soil moisture content was gravimetrically maintained weekly by adding Milli-Q water to the microcosms.

The scintillation traps were removed and replaced by new ones on days 3, 6, 10, 13, 19, 25, 33, 46, 63 and 90 days. After adding scintillation cocktail (8 mL, 30% Scintisafe scintillation cocktail; Fisher Scientific, Fairlawn, NJ) to the removed scintillation traps, the amounts of radioactivity in the traps were measured by LSC as described above.

The first-order degradation equation was fitted to the cumulative $^{14}CO_2$ evolved using Sigma Plot 2000 for Windows version 6.00 (SPSS Inc., 1986-2000) to calculate the mineralization rate constant, k (day^{-1}) and the amount of 17β-estradiol [4-^{14}C] mineralized to $^{14}CO_2$ at infinity (Max in %) for each microcosm. k and Max were thus determined by $M_t=Max(1-e^{-kt})$, where M_t is the % of applied 17β-estradiol mineralized at time t (day). Half-lives were calculated by ln (2) divided by k. For k and Max calculations, the three replicates of each of the 36 soil samples were averaged. Coefficients of variation between replicates were on average 37 % for k and 14 % for Max.

3.3.6 2,4-D Experiments

2,4-D mineralization and sorption experiments on these and other soil samples from Alberta had previously been conducted (Gaultier 2008a,b). The experimental protocols were similar to those described above for 17β-estradiol sorption and mineralization except that the equilibrium time for sorption by batch equilibrium experiments was 24 h at room temperature in non-autoclaved teflon tubes and the incubation time for the microcosms was 42 days at 90% field capacity following a pre-incubation period of 14 days. For the 36 surface soils utilized in the present study, the mineralization and sorption parameters for 2,4-D were extracted from our database for use in statistical analyses as described below.

3.3.7 Statistical Analysis

Probability density functions for soil properties, sorption parameters (Kd and Koc for 17β-estradiol or 2,4-D) and mineralization parameters (k and Max for 17β-estradiol or 2,4-D) were determined using @Risk 5.0. (@Risk 5.0, Palisade, 2008). The best fitted probability density function was considered to be the equation (modeled distribution) with the lowest Chi-Square ($P < 0.05$). The goodness of fit by the Chi-Square test was further compared for three functions (best fitted, normal and log-normal) for each soil property, sorption and mineralization parameter. The normal and log-normal probability density functions were selected because these distributions are commonly used in pesticide fate modelling (Nofziger et al., 1994; Wolt et al., 2001; Dubus and Brown; 2002).

Pearson correlation coefficients ($P < 0.05$) were determined among soil properties (SOC, % sand, % clay, pH, EC, CEC and FDA), mineralization parameters (k and Max for 17β-estradiol or 2,4-D) and sorption parameters (Kd and Koc for 17β-estradiol or 2,4-D) (JMP 5.0, SAS Institute, 1989-2002). Pearson correlations were applied to untransformed data due to the robust nature of these analyses (Legendre and Legendre, 1998).

The Paired t-test ($P < 0.05$) (SigmaStat 3.1, Systat Software Inc. 2004) was used to determine significant differences among average values of Kd-17β-estradiol and Kd-2,4-D, Koc-17β-estradiol and Koc-2,4-D, k-17β-estradiol and k-2,4-D, or Max-17β-estradiol

and Max-2,4-D. This was done on log-transformed data in order to respect normality (Shapiro-Wilks K >0.90, Analyse-it for Microsoft Excel, version 2.20).

One-way ANOVA ($P < 0.05$) followed by the Tukey-Kramer HSD multiple comparison test ($P < 0.05$) (SigmaStat 3.1, Systat Software Inc. 2004) were applied to both 17ß-estradiol and 2,4-D mineralization parameters to detect significant differences among ecoregions (Mixed Grassland, Peace Lowland and Boreal Transition), between landscape positions (upper- versus lower-slopes) or between soil orders (Chernozems versus Luvisols). Similar ANOVA and multiple comparison tests were also applied to soil properties (SOC, CEC, FDA, pH, % clay) and sorption parameters (Kd and Koc values for 17ß-estradiol or 2,4-D) that showed significant correlations with 17ß-estradiol or 2,4-D mineralization parameters (k or Max). SOC, FDA, Kd-17β-estradiol, Koc-17β-estradiol, k-17β-estradiol and Kd-2,4-D data were log-transformed in order to respect normality (Shapiro-Wilks K >0.90, Analyse-it for Microsoft Excel version 2.20).

Subsequently, the data set was divided in three groups based on SOC. The division into these groups was justified because of the effect of SOC on pesticide (Wauchope et al., 2002) or estrogen sorption and mineralization in soils (Casey et al., 2003; Lee et al., 2003; Yu et al., 2007; Loffredo and Senesi, 2006; Caron et al., 2010) and on soil bacterial activity (Willems et al., 1996; Bolan and Baskaran, 1997; Nkedi-Kizza and Brown, 1998; Smalling and Aelion, 2004). SOC groups were 0 to 9.9 g C kg^{-1} (n=10), 10.0 to 29.9 g C kg^{-1} (n=13) and $\geq$ 30.0 g C kg^{-1} (n=13). These groups provided distinct ranges of SOC with about the same number of samples in each group. To test for significant differences

between SOC groups, data were then subjected to a one-way ANOVA and Tukey-Kramer HSD multiple comparison test ($\alpha < 0.05$). SOC, FDA, Kd-17β-estradiol, Koc-17β-estradiol, k-17β-estradiol and Kd-2,4-D data were log-transformed in order to respect normality (Shapiro-Wilks K >0.90, Analyse-it for Microsoft Excel, version 2.20).

Predictive models were developed for the sorption (Kd and Koc) and mineralization parameters (k and Max) of both 17β-estradiol and 2,4-D, using soil properties as independent variables. This was done using Partial Least Squares (PLS) regression (The Unscrambler, CAMO ASA, 2002-2009) because this form of regression is able to deal with the multicollinearity (Martens and Martens, 1986) observed in the soil properties dataset. Marten's uncertainty test (Martens and Martens, 2000) was used to determine significant factors in the regression. The number of principal components was optimized when the variation of residual variance was minimized. Outliers were detected using the automatic outlier detection provided in the software and removed from the analysis. SOC was removed from the analyses for obtaining the predictive models for Koc because SOC is used in calculating Koc. PLS regression was again applied to the 17β-estradiol and 2,4-D sorption and degradation parameters which had been grouped according to SOC to test whether the predictions of Kd, Koc, k and Max could be improved (r^2 higher by at least 0.1) by utilizing a narrower SOC range. PLS was performed on centered data.

The results of PLS regression were compared to the results obtained from an ordinary least squares regression (JMP 5.0, SAS Institute, 1989-2002). Least squares regression was used to predict the mineralization parameters (k and Max) of 17β-estradiol and 2,4-D

using either Kd or Koc as an independent variable. Ordinary least squares regression (JMP 5.0, SAS Institute, 1989-2002) was also used to estimate Kd, Koc, k and Max for 17β-estradiol by using the corresponding parameters for 2,4-D and, similarly, to predict 2,4-D parameters by using corresponding 17β-estradiol parameters as independent variables. Models had to be significant at $P < 0.05$. All ordinary least squares regressions were applied to untransformed data due to the robust nature of these analyses (Legendre and Legendre, 1998).

3.4 Results

Mineralization of 17β-estradiol in Alberta soils followed first order kinetics with the goodness of fit (R^2) ranging from 0.80 to 1.00. Mineralization of 2,4-D also follows first-order kinetics (R^2 ranging from 0.95 to 0.99) as reported previously (Gaultier et al. 2008a,b).

The k-17β-estradiol was significantly smaller (0.10 day^{-1} ± 0.075) than k-2,4-D (0.38 day^{-1} ± 0.16) and hence half lives for 17β-estradiol (9.88 days ± 4.48) were significantly longer than those obtained for 2,4-D (2.19 days ± 0.96). Max-17β-estradiol was also significantly smaller (11.32 % ± 3.14) than Max-2,4-D (53.27 % ± 3.26). The Kd-17β-estradiol (24.65 L kg^{-1} soil ± 11.53) and Koc-17β-estradiol (1,079 L kg^{-1} OC ± 452) were significantly greater than the Kd-2,4-D (5.24 L kg^{-1} soil ± 4.87) and Koc-2,4-D (188 L kg^{-1} OC ± 107), respectively. Kd-17β-estradiol and Kd-2,4-D were significantly positively correlated (r=0.62, $P < 0.001$) but not their Koc values (Table 3.2). Their

mineralization parameters were not significantly correlated (Table 3.2). Hence, there were only two significant models: a) 17β-estradiol-Kd=14.25+1.99 2,4-D-Kd (n=36, R^2=0.39, $P < 0.0001$) and b) 2,4-D-Kd=0.43+0.19 17β-estradiol-Kd (n=36, R^2=0.39, $P < 0.0001$), when using one chemical to predict the sorption and mineralization of the other chemical.

Based on the numerical value of the coefficient of variation, k and Max were more variable across the 36 surface soils for 17β-estradiol than for 2,4-D (Table 3.3). In contrast, Kd and Koc were less variable for 17β-estradiol than for 2,4-D (Table 3.3). For both 17β-estradiol and 2,4-D, k was best described by the log-logistic probability density function and Kd was best described by the exponential probability density function (Table 3.4). Both Max-17β-estradiol and Koc-17β-estradiol were best described by the extreme values probability density function. Max-2,4-D and Koc-2,4-D were best described by the triangular probability density function.

The best-fitted probability density functions for soil properties (Figures 3.2A to Q) were the extreme values probability density function (EC, CEC and % silt), exponential probability density function (SOC and pH), inverse Gaussian probability density function (FDA and % clay), logistic probability density function (% sand) or beta general probability density function ($CaCO_3$) (Table 3.4).

Table 3.2: Correlations among soil properties, sorption, and mineralization parameters (n=36 for all parameters except n=34 for FDA)

	SOC	pH	% clay	% sand	% silt	EC	CEC	FDA	Kd 17β-estradiol	Koc 17β-estradiol	k 17β-estradiol	Max 17β-estradiol	Kd 2,4-D	Koc 2,4-D	k 2,4-D	Max 2,4-D
SOC	1															
pH	-	1														
%clay	-	-	1													
%sand	-	-	-0.84***	1												
%silt	-	-	-	-0.44**	1											
EC	0.35*	-	-	-	-	1										
CEC	0.85***	-	0.33*	-0.40*	-	-	1									
FDA	0.37*	-0.34*	-	-	-	-	-	1								
Kd-17β-estradiol	0.71***	-	-	-	-	-	0.80***		1							
Koc-17β-estradiol	-0.35*	-	-	-	-	-0.37*	-	-	-	1						
k-17β-estradiol	-	-	-	-	-	-	-	-	-	-	1					

Max-17β-estradiol	0.63***	-	-	-	-	-	0.60***	-	0.68***	-	-	1				
Kd-2,4D	0.67***	-0.40**	-	-	-	-	0.71***	-	0.62***	-	-	-	1			
Koc-2,4-D	-	-0.69***	-	-	-	-	-	-	-	-	-	-	0.79***	1		
k-2,4-D	-	-	0.40**	-0.42**	-	-	-	-	-	-	-	-	-	-	1	
Max-2,4-D	-0.45***	0.35*	-	-	-	-	-0.60***	-	-0.50***	-	-	-	-0.52**	-0.35*	-	1

[a] * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). *** Correlation is significant at the 0.001 level (2-tailed). - Correlation is not significant at the 0.05 level.

Table 3.3: Mean (standard deviation) of mineralization parameters (Max and k), sorption parameters (Kd and Koc), and selected soil properties (SOC, CEC, and FDA) for all soil samples and for the dataset divided into three ecoregions or three SOC groups.

	SOC (g C kg $^{-1}$)	**CEC (c mol kg^{-1})**	**FDA (µg g^{-1})**	**%Clay**	**pH**	**Kd-17β-estradiol (L kg $^{-1}$)**	**Koc-17β-estradiol (L kg OC^{-1})**	**k-17β-estradiol-(day $^{-1}$)**	**Max-17β-estradiol (%)**	**Kd-2,4-D-(L kg $^{-1}$)**	**Koc-2,4-D (L kg OC^{-1})**	**k-2,4-D (day $^{-1}$)**	**Max-2,4-D-(%)**
All samples n=36	25.8 (23.3)	25.88 (0.23)	0.32 (0.22) n=34	30.72 (11.26)	5.6 (1.0)	24.65 (11.53)	1079.92 (451.76)	0.095 (0.075)	11.32 (3.14)	5.24 4.87	187.73 (107.29)	0.38 (0.16)	0.5357 (3.26)
						Dataset divided by ecoregions							
Peace Lowland n=12	A 34.5 (8.7)	A 31.54 (5.62)	A 0.32 (0.18) n=10	A 40.01 (9.40)	B 5.62 (0.84)	A 33.14 (16.37)	A 926.3 (276.90)	A 0.084 (0.047)	A 12.84 (3.42)	A 7.48 (3.36)	A 217.26 (94.36)	A 0.47 (0.15)	A 53.40 (2.42)
Boreal Transition n=12	A 32.3 (35.4)	A 29.90 (15.77)	A 0.32 (0.19)	B 24.93 (3.93)	A 6.57 (1.14)	AB 26.91 (16.18)	A 1109.70 (698.94)	A 0.087 (0.059)	AB 11.98 (3.40)	A 7.04 (6.01)	A 243.48 (111.76)	B 0.29 (0.09)	A 55.05 (3.45)
Mixed Grassland n=12	B 10.6 (3.4)	B 16.18 (2.93)	A 0.31 (0.28)	B 27.22 (12.46)	AB 5.55 (0.74)	B 13.91 (5.21)	A 1203.68 (201.93)	A 0.11 (0.11)	B 9.51 (1.60)	B 1.21 (1.08)	B 102.46 (54.41)	AB 0.41 (0.18)	A 52.24 (3.29)
						Dataset divided by SOC groups							
SOC 0-9.9	C 10.7	B 15.0	A 0.23	A 24.0	A 6.60	B 12.51	A 1254.01	A 0.12	B 9.42	C 0.94	B 105.20	A 0.39	A 55.08

n=10	(6.8)	(3.1)	(0.14)	(3.44)	(1.07)	(4.61)	(180.20)	(0.12)	(1.68)	(0.53)	(54.87)	(0.17)	(3.74)
SOC	B	B	A	A	AB	B	A	A	B	B	AB	A	AB
10-29.9	19.2	23.7	0.38	32.03	5.67	20.37	1116.83	0.083	10.33	4.18	203.97	0.37	54.34
n=13	(6.6)	(8.8)	(0.27) n=12	(14.36)	(0.99)	(10.31)	(653.35)	(0.045)	(2.18)	(2.74)	(93.64)	(0.19)	(2.80)
SOC	A	A	A	A	B	A	A	A	A	A	A	A	B
≥30	46.7	36.4	0.33	34.59	5.62	38.28	909.10	0.088	13.78	9.61	234.98	0.39	51.55
n=13	(29.7)	(9.9)	(0.20) n=12	(9.95)	(0.74)	(15.40)	(292.72)	(0.057)	(3.35)	(4.90	(119.04)	(0.14)	(2.33)

Note: For ecoregions or SOC groups, group means in columns with the same letter are not significantly different from each other (One-Way ANOVA $P < 0.05$). Multiple comparison was done using the Tukey-Kramer HSD test with $\alpha < 0.05$.)

Table 3.4: Goodness of fit (Chi-Square with $P < 0.05$) for probability distributions (best fitted distribution, normal distribution and log normal distribution) of soil properties, and sorption and mineralization parameters for 17β-estradiol and 2,4-D.

Parameter	**Name of Best-fitted**	**Best fitted**	**Normal**	**Log-Normal**
Soil properties				
SOC	Exponential	6.39	20.00	14.56
FDA	Inverse Gaussian	1.41	18.29	1.41
pH	Exponential	5.61	11.44	11.06
EC	Extreme values	4.44	8.722	6.00
CEC	Extreme values	2.89	6.39	3.28
%sand	Logistic	6.00	8.33	9.11
% clay	Inverse Gaussian	3.67	8.33	3.67
% silt	Extreme values	0.56	2.11	2.11
$CaCO_3$	Beta general	121.10	142.50	-
17β-estradiol				
Kd	Exponential	1.20	15.60	4.40
Koc	Extreme values	3.20	9.60	4.80
k	Log-logistic	6.00	29.33	7.94
MAX	Extreme values	2.89	12.22	2.89
2,4-D				
Kd	Exponential	0.56	22.72	3.28
Koc	Triangular	2.36	4.44	3.67
k	Log-logistic	3.47	5.94	4.71
MAX	Triangular	1.00	1.41	3.06

Figure 3.2 A

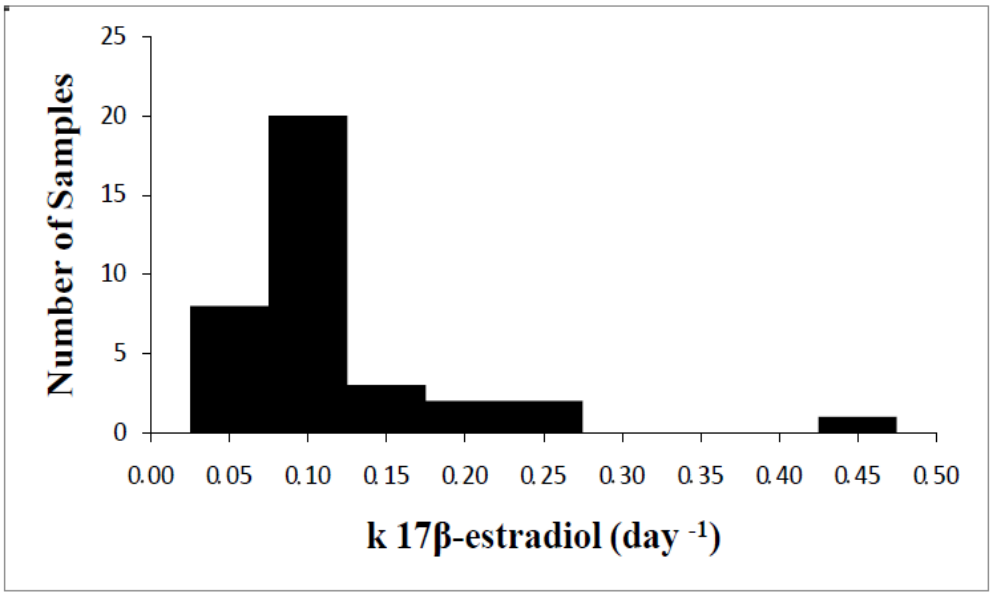


Figure 3.2 B

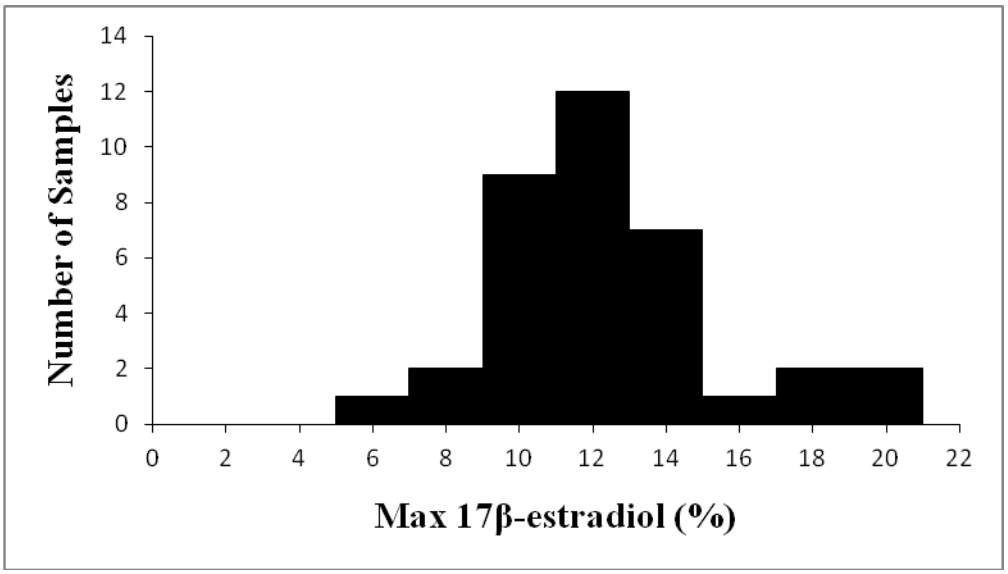


Figure 3.2 C

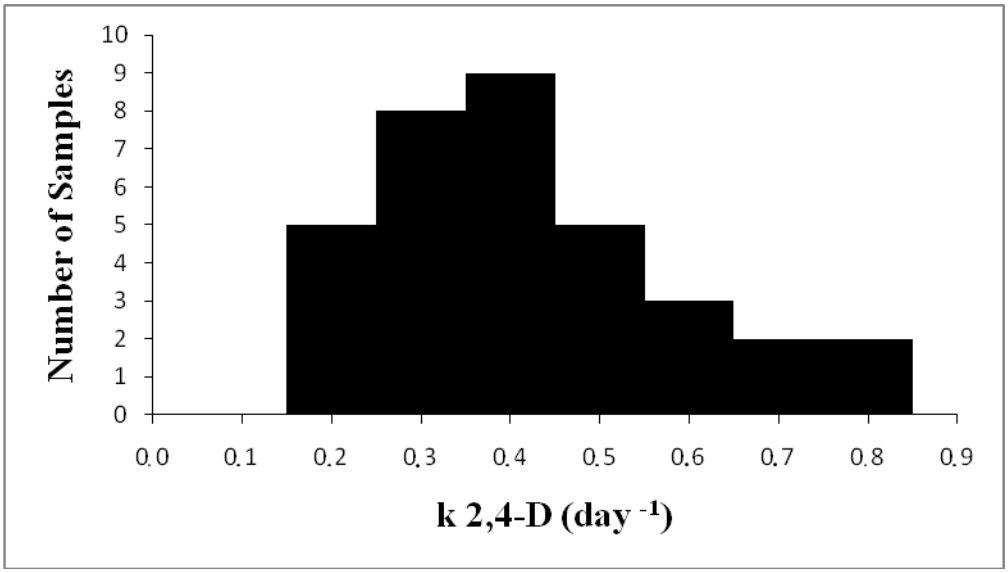


Figure 3.2 D

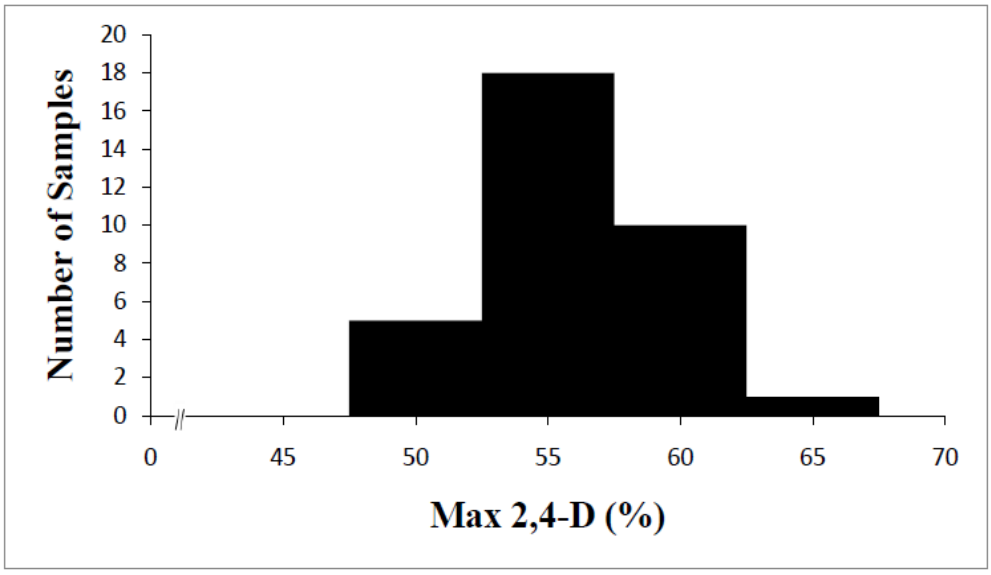


Figure 3.2 E

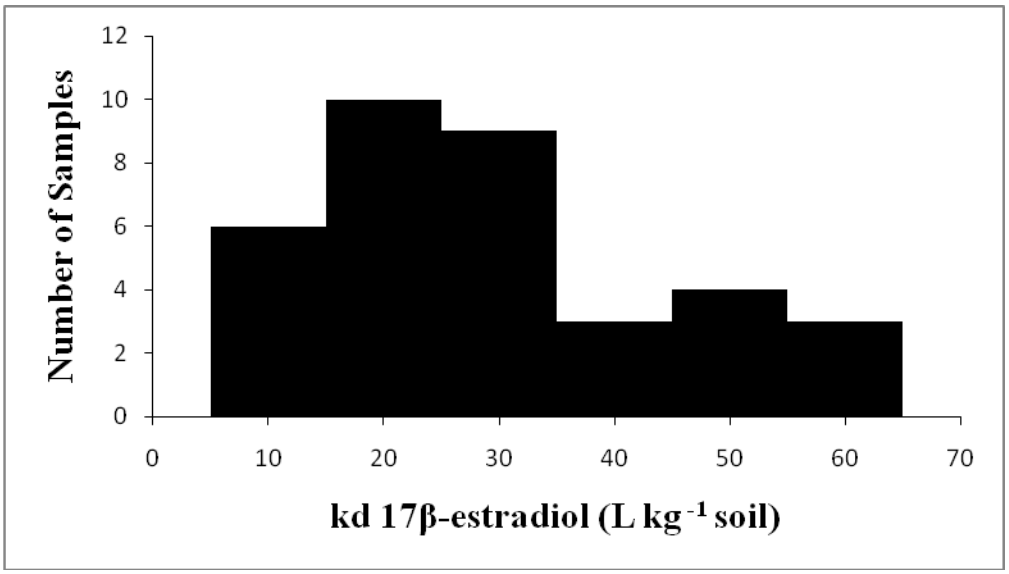


Figure 3.2 F

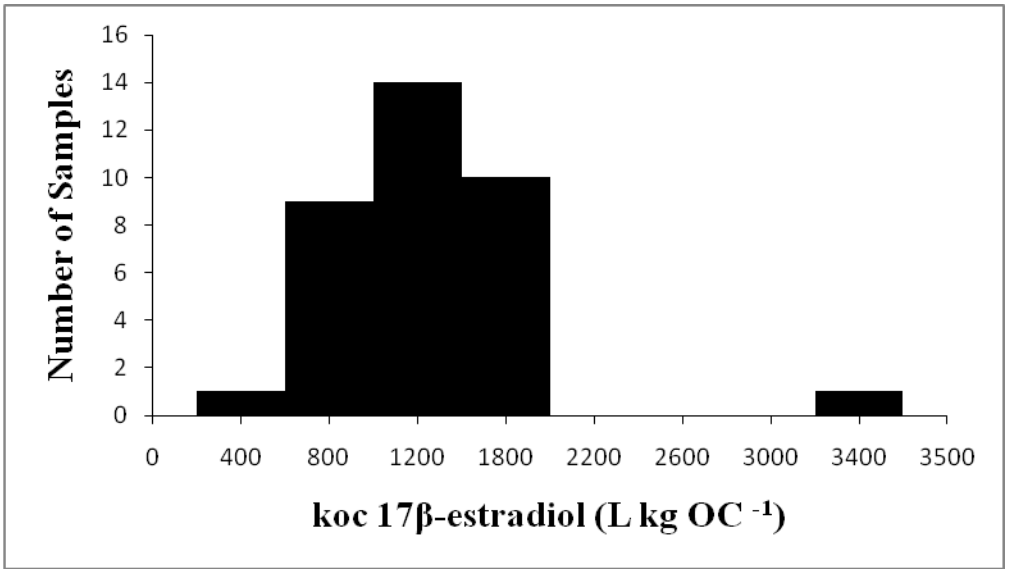


Figure 3.2 G

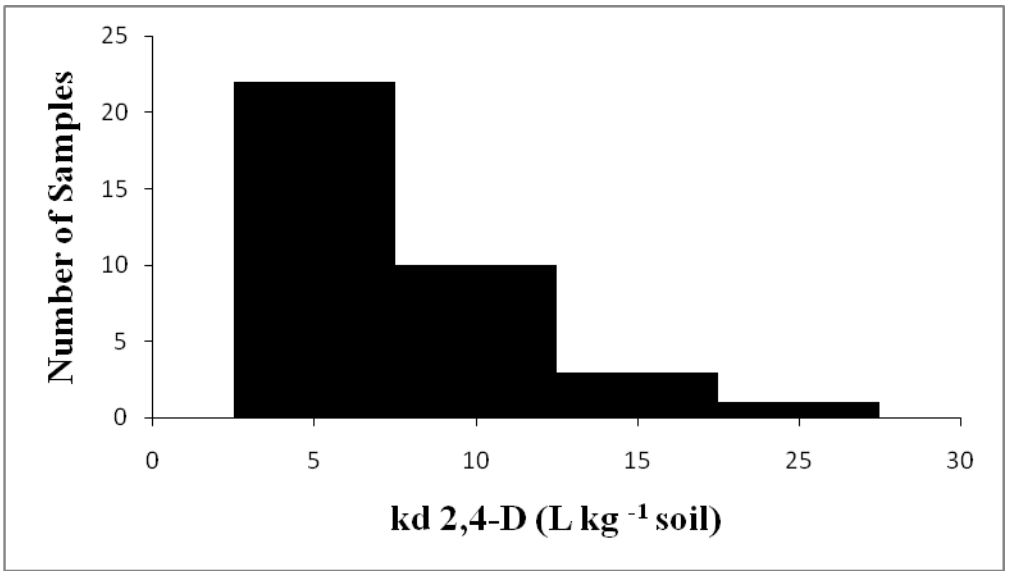


Figure 3.2 H

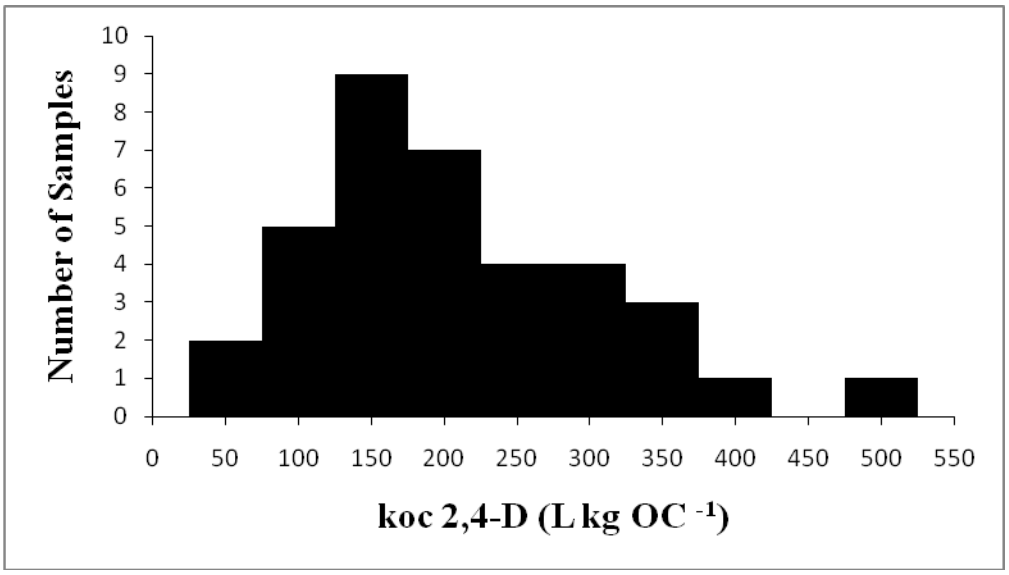


Figure 3.2 I

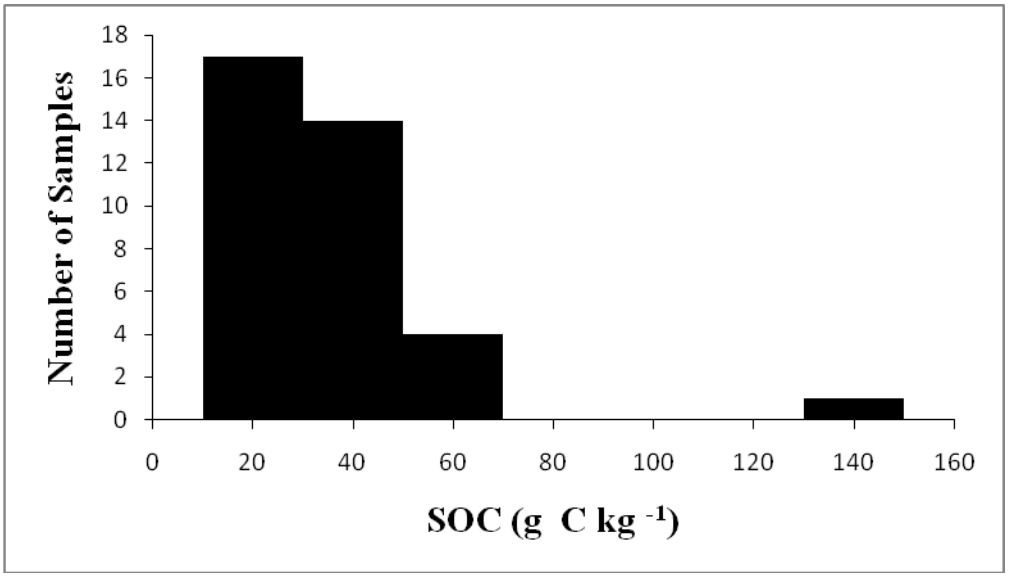


Figure 3.2 J

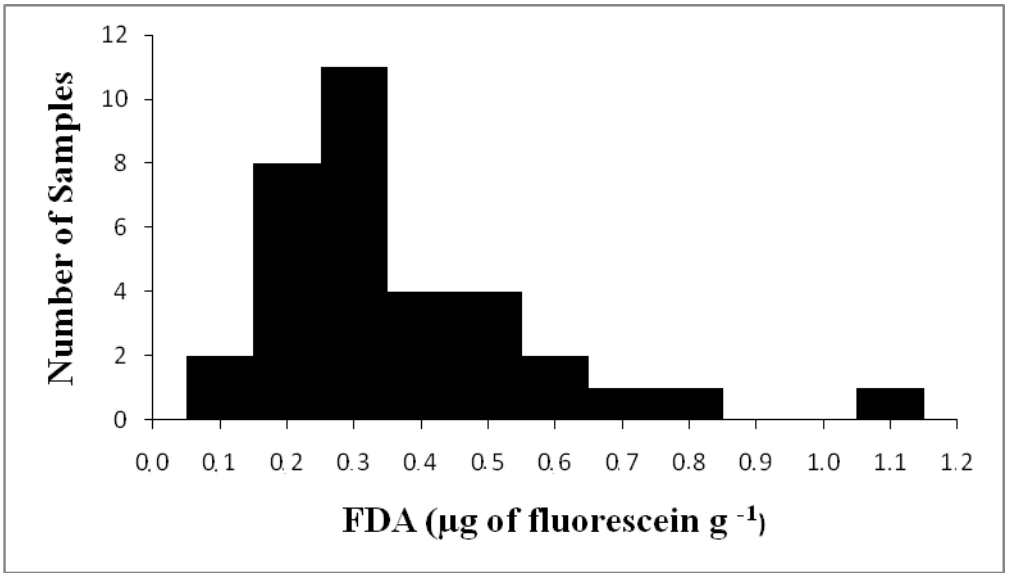


Figure 3.2 K

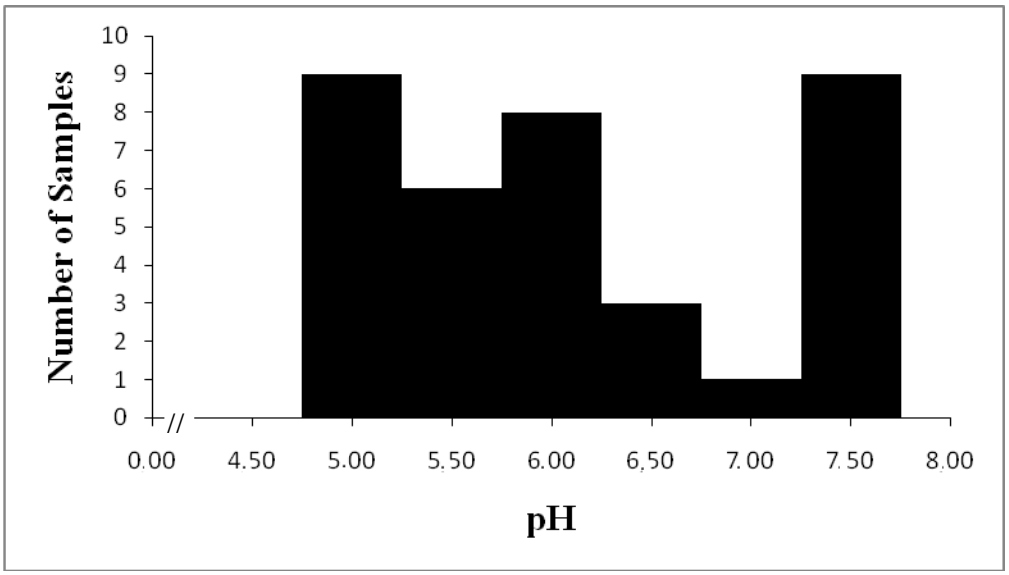


Figure 3.2 L

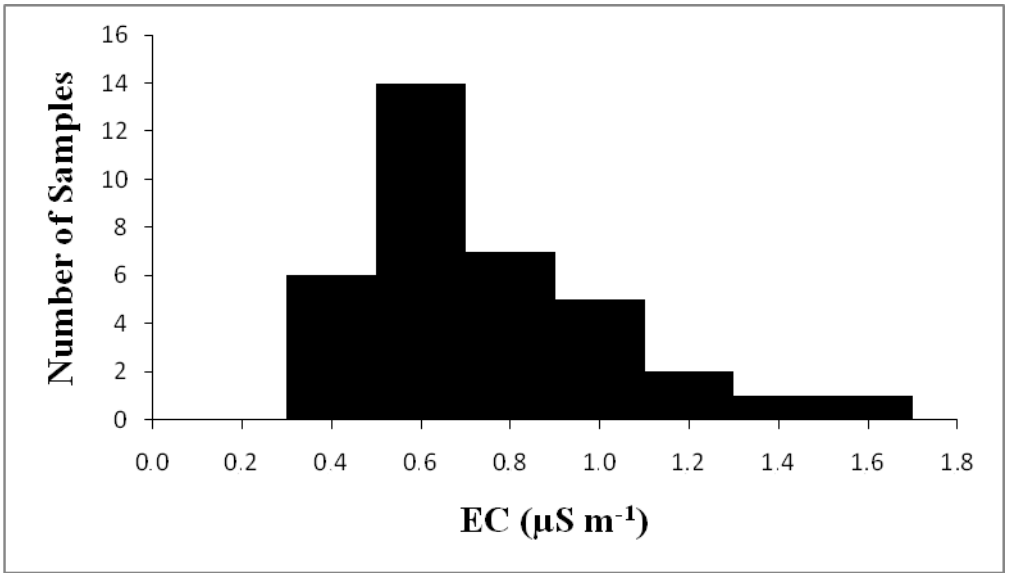


Figure 3.2 M

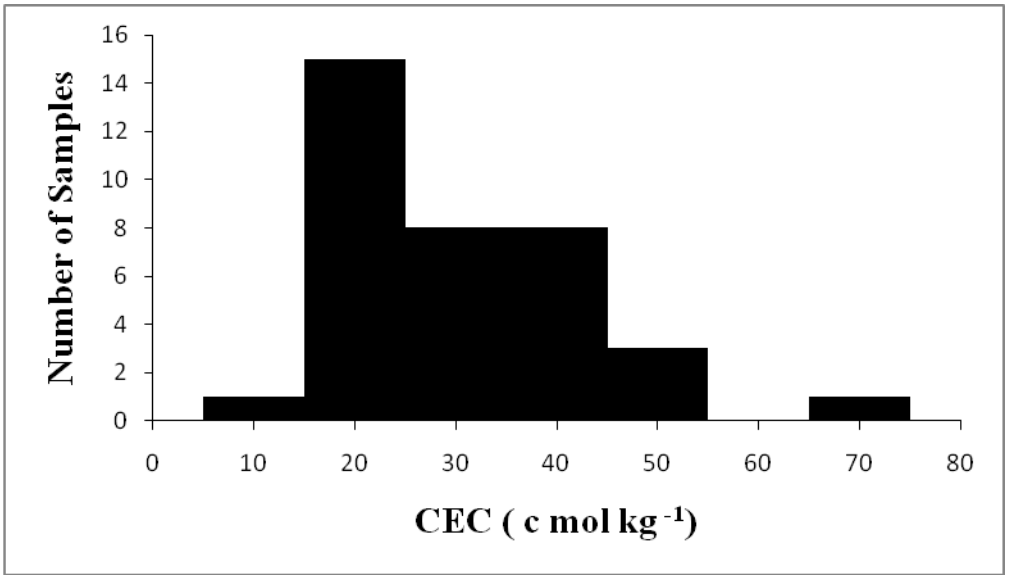


Figure 3.2 N

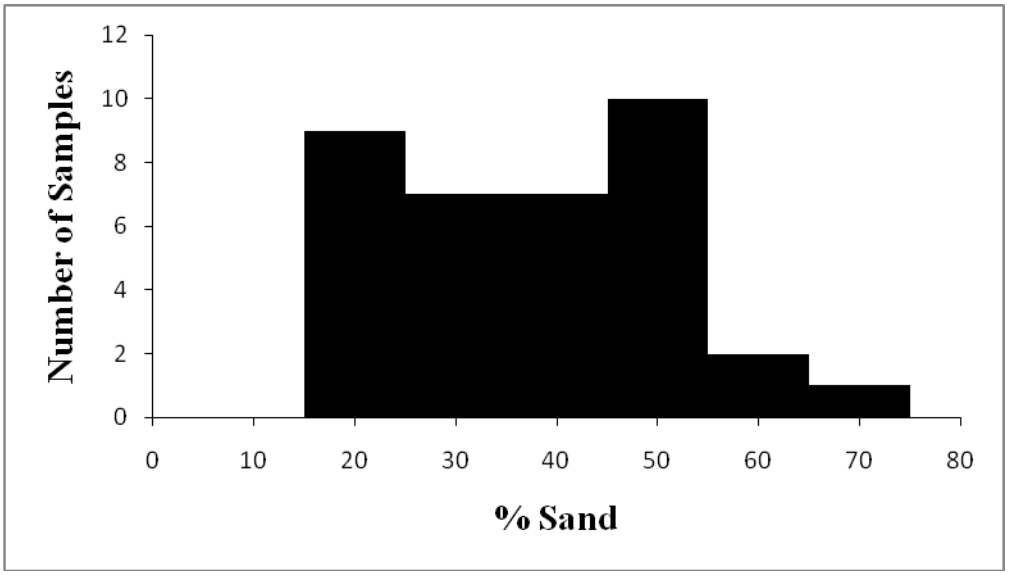


Figure 3.2 O

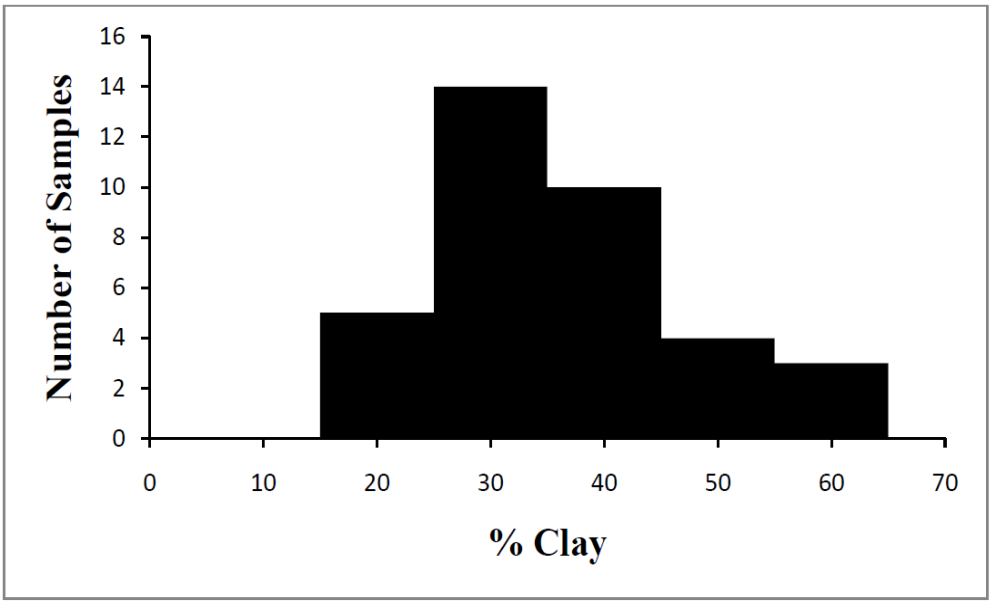


Figure 3.2 P

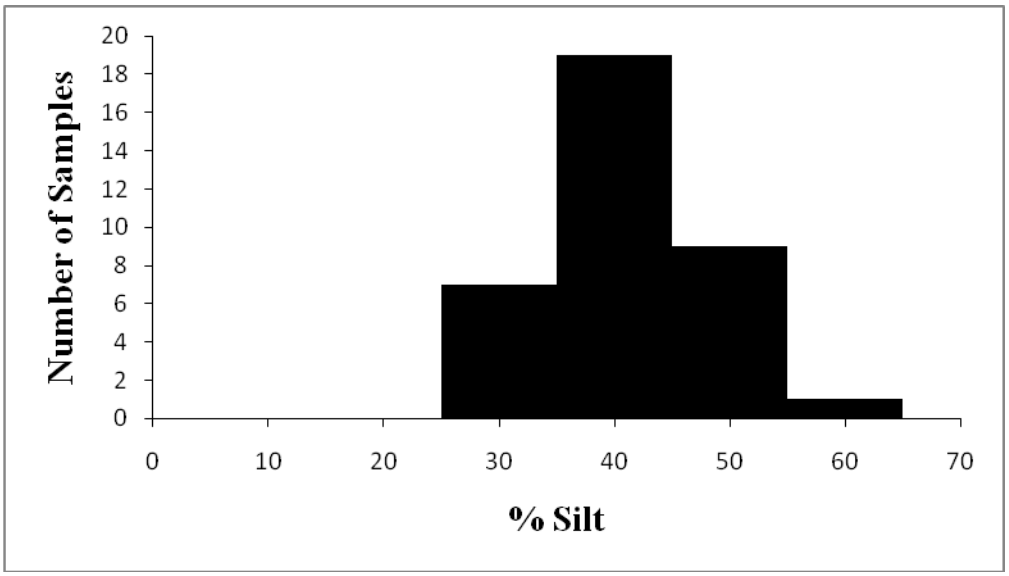


Figure 3.2 Q

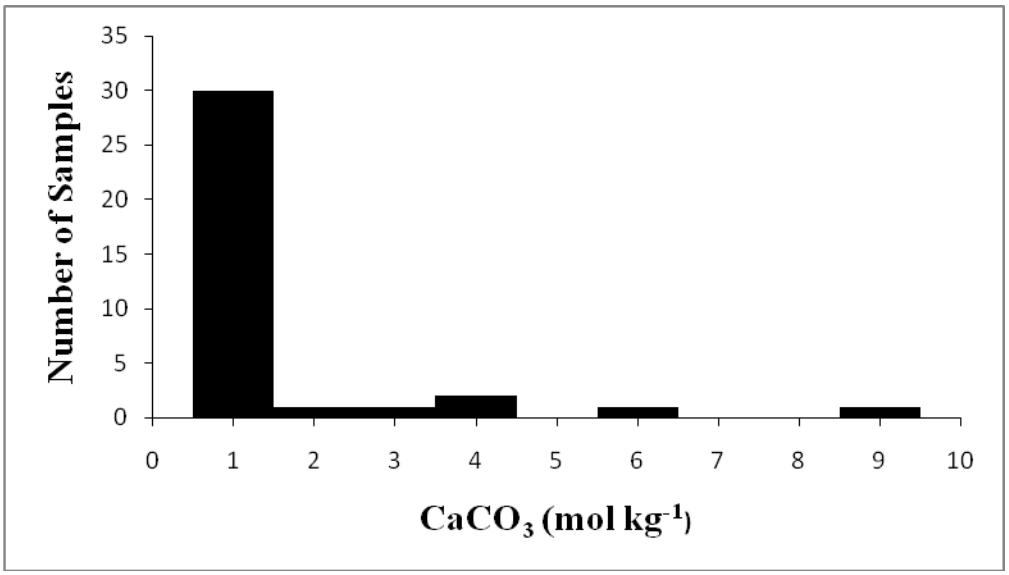


Figure 3.2: Distribution (n=36 unless otherwise noted): A) k-17β-estradiol, B) Max-17β-estradiol, C) Kd-17β-estradiol, D) Koc-17β-estradiol, E) k-2,4-D, F) Max-2,4-D, G) Kd-2,4-D, H) Koc-2,4-D, I) SOC, J) FDA, K) pH, L) EC, M) CEC, N) % sand, O) %clay, P) % silt, Q) $CaCO_3$

None of the mineralization parameters were significantly correlated with FDA (Table 3.2). The significant correlation between SOC and FDA (Table 3.2) was strongly influenced by the sample with the largest SOC (137.9 g C kg^{-1}) and when this sample is removed from the analyses, the correlation is no longer significant for the remaining samples for which the SOC ranged from 7.1 to 51.1 g C kg^{-1}.

Although lower landscape positions had significantly ($P < 0.05$) greater SOC concentrations than upper landscape positions, landscape position had no significant influence on other soil properties, nor on 17β-estradiol or 2,4-D sorption and mineralization parameters. At a broader level, the two soil orders (Chernozems and

Luvisols) were not significantly different with respect to soil properties, 17β-estradiol or 2,4-D sorption and mineralization parameters. At the regional scale, the Mixed Grassland ecoregion had a significantly smaller SOC, CEC, Kd-2,4-D, Koc-2,4-D than both Peace Lowland and Boreal Transition ecoregions (Table 3.3). The Mixed Grassland ecoregion also had significantly smaller Kd-17β-estradiol and Max-17β-estradiol than the Peace Lowland ecoregion (Table 3.3). The Peace Lowland ecoregion had a significantly greater k-2,4-D than the Boreal Transition ecoregion (Table 3.3).

When the dataset was divided according to SOC, the CEC, Kd-17β-estradiol, Kd-2,4-D, Koc-2,4-D, and Max-17β-estradiol were significantly greater in the ≥ 30.0 g C kg^{-1} than in the other groups (Table 3.3). In contrast, Max-2,4-D was significantly less in the ≥ 30.0 g C kg^{-1} SOC group, relative to the other two groups (Table 3.3). Soil pH was significantly greater in the 0 to 9.9 g C kg^{-1} SOC group than in the other groups (Table 3.3).

The regression models for 17ß-estradiol were always stronger (higher explained variance and r^2) for Kd than for Koc (not significant) or for the mineralization parameters Max and k (Table 3.5). For 2,4-D, the models for sorption parameters (Kd and Koc) had a similar r^2 and explained variance and were stronger than the models for mineralization parameters (Table 3.5). The models for predicting Koc and k were stronger for 2,4-D than for 17ß-estradiol (not significant), while the strength of the models for predicting Kd and Max were similar for 2,4-D and 17ß-estradiol (Table 3.5).

Table 3.5: Partial Least Squares regression for sorption and mineralization parameters for 17ß-estradiol and 2,4-D as predicted by soil properties.

Predicted variable	RMSE	EXP VAR	r^2	Model
			17ß-estradiol	
Kd (n=33)	7.07	71.48	0.85	Kd= 3.06+0.87 SOC
Koc				*ns*
k				*ns*
Max (n=33)	2.21	25.92	0.51	Max=8.65+0.10 SOC +0.00024 EC
			2,4-D	
Kd (n=33)	2.13	64.16	0.80	Kd=-0.61+0.23 SOC
Koc (n=34)	54.36	61.40	0.78	Koc=588.47-69.80 pH
k (n=34)	0.14	17.78	0.42	k=0.56- 0.0054 % sand
Max (n=33)	2.57	31.69	0.56	Max=58.10-0.18 CEC

*RMSE: Root Mean Square Error; EXP VAR: explained variance, *ns*:not significant

When data were divided into groups according to SOC, no significant model for predicting Kd-17ß-estradiol was obtained. In addition, grouping according to SOC did not produce significant models for Koc-17ß-estradiol (Table 3.6). Both the 10.0 to 29.9 g C kg^{-1} SOC and ≥ 30 g C kg^{-1} groups improved the prediction of k-17ß-estradiol (Table 3.6) when compared to the analysis using the whole dataset. The 0 to 9.9 g C kg^{-1} SOC group improved the prediction of Max-17ß-estradiol (Table 3.6). For Kd-2,4-D and Koc-2,4-D, grouping by SOC resulted in either similar or poorer (r^2 lower by at least 0.1) prediction models (Table 3.6). The 10.0 to 29.9 g C kg^{-1} and the ≥ 30.0 g C kg^{-1} SOC groups improved the prediction for k-2,4-D (Table 3.6). When using data divided according to SOC groups, no significant models were obtained for Max-2,4-D (Table 3.6).

Table 3.6: Partial Least Squares regressions for 17β-estradiol data divided by SOC groups.

	SOC	n	RMSE	EXP	r^2	Model
				17ß-estradiol		
Kd	0 to 9.9					*ns*
	10.0 to 29.9					*ns*
	≥30.0					*ns*
Koc	0 to 9.9					*ns*
	10.0 to29.9					*ns*
	≥30.0					*ns*
k	0 to 9.9					*ns*
	10.0 to 29.9	12	0.034	40.57	0.51	k=0.15-0.00030 CEC-0.0019 % sand
	≥30.0	11	0.053	1.38	0.12	k=0.050+0.00070 SOC+0.00030 CEC
Max	0 to 9.9	9	0.40	86.95	0.63	Max=5.45-0.16 SOC+0.37 CEC
	10.0 to 29.9	12	1.51	39.34	0.59	Max=8.85+0.10 % sand-0.045 % silt
	≥30.0					*ns*
				2,4-D		
Kd	0 to 9.9	10	0.22	80.48	0.89	Kd=1.50+0.20 SOC -0.35 pH
	10.0 to 29.9	13	1.59	48.97	0.70	Kd=-0.88+0.25 SOC
	≥30.0					*ns*
Koc	0 to 9.9	10	26.20	75.72	0.87	Koc=401.08-45.43pH
	10.0 to 29.9					*ns*
	≥30.0					*ns*
k	0 to 9.9					*ns*
	10.0 to 29.9	11	0.14	32.19	0.57	k=0.18+0.58 FDA
	≥30.0	12	0.083	58.87	0.77	k=0.66- 0.010 % sand
Max	0 to 9.9					*ns*
	10.0 to 29.9					*ns*
	≥30.0					*ns*

*ns: not significant, RMSE:Root mean square error; EXP : explained variance

3.5 Discussion

Controls (silica sand and autoclaved silica sand) showed no mineralization suggesting that the degradation of 17β-estradiol is biologically mediated by soil organisms. Other authors concluded the same for soil samples from agricultural soils in eastern Canada (Colucci et al., 2001), Australia (Ying and Kookana, 2005) and the USA (Fan et al., 2007; Xuan et al., 2008). A wide variety of enzymes can participate to estrogen breakdown including members of the catechol-*o*-methyltransferase family (Lakhani et al., 2003). Hence, many bacteria are able to participate in the biodegradation of estrogens (Hanselman et al., 2003) including *Pseudomonas spp*. which is a common bacterium in agricultural soils (Stumm-Zollinger and Fair, 1965).

17β-estradiol was mineralized following first-order kinetics in Alberta soils. Previous researchers concluded that the degradation of 17β-estradiol follows first-order kinetics in agricultural soils (Colucci and Topp, 2001; Colucci et al., 2001; Ying and Kookana, 2005; Fan et al., 2007; Xuan et al., 2008), but zero-order and second-order kinetics have been also reported (Stumpe and Marschner, 2007). The observed first-order kinetics for 2,4-D mineralization in agricultural soils also agrees with the results of others (e.g., Parker and Doxtader, 1983; Guo et al., 2000; Gaultier and Farenhorst, 2007).

Neither 17β-estradiol or 2,4-D demonstrated a lag-phase before the onset of mineralization. Mineralization rates of 17β-estradiol were slower in the Alberta soils than in the four agricultural soils from Israel and Germany used in Stumpe and Marschner

(2007), but faster than those observed by Xuan et al. (2008) in an American grassland soil (Table 3.7). Lucas and Jones (2006) observed a lag phase in the mineralization of 17β-estradiol in three soil samples obtained from agricultural soils amended with sheep urine and sheep manure in North Wales. They attributed this lag phase to the time taken for the community of bacteria to re-establish itself in response to the adverse changes in soil conditions caused by urine.

Table 3.7: Results from studies on 17ß-estradiol mineralization.

Location	**Characteristics**	**Organic content (gC kg^{-1})**	**Mineralization parameters**	**Soils**	**Reference**
Alberta, Canada	Chernozems and Luvisols	7 - 138	k 0.034 - 0.44 day^{-1} Max 5.82 % - 19.18%	36	Current study
Ontario, Canada	Loam, Sandy Loam and Silt Loam	5 - 18	k dissipation 1.45-3.12 Max 11.5 % - 17.1%	3	Colucci et al., 2001
Australia	Sand to Clay	9 - 30	k NA Max >90% aerobic soils	4	Ying and Kookana, 2005
North Wales	Sandy Clay, Sand, Sandy Loam grassland	12 - 27	k NA Max 15*-90%**	3	Lucas and Jones, 2006
U.S.A.	Sandy Loam	13	k 0.0006 h^{-1} aerobic soils Max 6% aerobic soils	1	Fan et al., 2007
Israel and Germany	Silty Loam, Clay	8-14	k 0.0023-0.0031 day^{-1} Max 5.1% -7.4%	4	Stumpe and Marschner, 2007
U.S.A.	Silt Loam	14.4	k 4.55 day^{-1} Max NA	1	Xuan et al., 2008

*hormone applied in distilled water, **hormone applied in natural sheep urine

NA:not available

The mineralized fraction was significantly smaller for 17β-estradiol than for 2,4-D (11.32 % for 17β-estradiol and 53.27 for 2,4-D). In a previous study conducted in the Alberta soils (Gaultier et al., 2008b), carboxyl-labelled 2,4-D showed higher mineralization than U-ring-labelled 2,4-D (13% higher in soil with less than 1% SOC to 21% higher in soils with a SOC higher than 4 %). The mineralized fraction of 17ß-estradiol was always less than 20% and hence residual estrogenic activity may remain through residual concentrations of 17β-estradiol or through some of the metabolites such as estrone or estriol that are known to be estrogenic. However, the identity or the estrogenicity (the ability to activate the estrogen receptor) of other metabolites further down the mineralization pathway is unknown. In other mineralization experiments with Canadian soils, the maximum mineralization of 17β-estradiol was also below 20% (Colucci et al., 2001) (Table 3.7). Maximum 17β-estradiol mineralization in other studies ranged from as low as 5.1% in soils from northwest Israel irrigated with freshwater to 90% in soils from North Wales amended with sheep urine (Table 3.7).

Soil properties that had the greatest influence on 17ß-estradiol mineralization were SOC and CEC. 2,4-D mineralization was additionally influenced by pH, % sand and % clay. Soil pH has an effect because 2,4-D is ionizable at pKa 2.8 (Wauchope et al., 1992) and the pH of the Alberta soils ranged from 4.6 to 7.5. In contrast, 17ß-estradiol is mainly in nonionic form because its pKa value is near 10.6 (Hurwitz and Liu, 1977, Lewis and Archer, 1979).

There was no correlation between SOC and bacterial activity, which disagrees with several previous publications (Willems et al. 1996; Bolan and Baskaran 1997; Nkedi-Kizza and Brown 1998; Smalling and Aelion, 2004). FDA is generally assumed to be a good indicator of bacterial activity (Schnürer and Rosswall, 1982). However, SOC might not be the only factor influencing microbial communities. Green et al. (2007) found no correlation between SOC and FDA in tropical agricultural soils and reasoned that N and P were limiting factors to microbial growth. Consequently, N and P measurements in Alberta soils may further be important in understanding the relation between microbial activity and the mineralization of pesticides and estrogens.

The significant negative correlation between Kd and Max for 2,4-D agrees with previous observations that bioavailability of 2,4-D decreases as sorption of 2,4-D to soil increases (Ogram et al., 1985; Greer and Sheldon, 1992; Gaultier et al., 2008b). In contrast, there was a significant positive correlation between Kd and Max for 17ß-estradiol. A possible explanation is that some of the steps in the 17ß-estradiol mineralization process occur in the sorbed phase, as observed for agricultural soils in North-Dakota (Casey et al., 2003) and for biosolids (Layton et al., 2000). Therefore, models aiming to predict the fate of 17ß-estradiol in soil should take into account such possibility. Certain pesticide fate models, such as Pesticide Root Zone Model (Carousel et al., 1997), offer the possibility to include transformations in the sorbed phase.

Given the relatively poor results for predicting either 17ß-estradiol or 2,4-D mineralization parameters based on regression, the use of probability density functions in

conjunction with Monte Carlo analysis will be more appropriate to assign such chemical input parameters when using pesticide fate models in agri-environmental policy assessments at the regional scale. The extreme values and the triangular distributions were frequently the best-fitted probability density functions to describe the variability of sorption and mineralization parameters and soil properties across the range of Alberta soils and, in 16 cases out of 19, the log-normal showed a better fit than the normal probability density function. Hence, the use of a normal probability density function for chemical and soil properties input parameters instead of the extreme values, triangular or log-normal functions would have resulted in an overestimation of input parameter values.

3.6 Conclusion

The mineralization of 2,4-D was limited by sorption, while 17β-estradiol sorption and mineralization were positively correlated suggesting that some of the steps in the mineralization of this compound occur in the sorbed phase. SOC was the main factor influencing the fate of 17β-estradiol and soils relatively low in SOC showed both a lesser sorption and extent of mineralization, which could potentially increase the risk of estrogen off-site movement. Regardless of the compound, the power of regressions models relating soil properties to 17β-estradiol and 2,4-D fate was stronger for sorption parameters than for mineralization parameters. Using sorption parameters of the same compound to predict mineralization parameters, or using the mineralization parameters of one compound to predict the mineralization parameters of the other compound, also resulted in weak predictive models for 17β-estradiol and 2,4-D mineralization

parameters. Consequently, 17β-estradiol and 2,4-D mineralization parameters are difficult to estimate using regression analysis and the use of probability density functions is more appropriate when using pesticide fate models in agri-environmental policy assessments at regional-scales.

3.7 Acknowledgments

The authors would like to acknowledge the financial support of the Natural Sciences and Engineering Research Council of Canada for this research under its Discovery program. We thank the University of Manitoba Graduate Fellowship for financial support to E. Caron. The authors thank the employees of Alberta Agriculture and Rural Development for sampling the soils and Mr. Paul Panich, Mr. Michael Mendenhall and Mr. Dan Inaba at AAFC Lethbridge for their assistance with laboratory experiments.

3.8 References

Adam, G. and Duncan, H. 2001. Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. Soil Biol. Biochem., 33:943-951.

Ahmad, R., Kookana, R.S., Alston, A.M. and Skjemstad, J.O. 2001. The nature of soil organic matter affects sorption of pesticides. 1. Relationships with carbon chemistry as determined by 13C CPMAS spectroscopy. Environ. Sci. Technol., 35:878-884.

Alberta Agriculture and Food, Economics and Competitiveness Division, Statistics and Data Development Unit. 2006. Census of Agriculture for Alberta, Provincial Highlights. AGDEX 852-5.

Bank, T.L., Kukkadapu, R.K., Madden, A.S., Ginder-Vogel, M.A., Baldwin, M.E. and Jardine, P.M. 2008. Effects of gamma-sterilization on the physico-chemical properties of natural sediments, Chemical Geology 251(1-4):1-7.

Benoit, P., Barriuso, E., Houot, S. and Calvet, R. 1996. Influence of the nature of soil organic matter on the sorption desorption of 2,4-dichlorophenol and the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), Eur. J. Soil Sci., 47:567-578.

Boesten, J.J.T.I., van der Linden, A.M.A. **1991**. Modelling the influence of sorption and transformation on pesticide leaching and persistence. J. Environ. Qual., 21(2):425-435.

Bolan, N.S. and Baskaran, S. 1997. Sorption and degradation of phorate as influenced by soil depth. Aust. J. Soil Res., 35(4):763-775.

Boivin, A., Amellal, S., Schiavon, M. and van Genntchen, M.Th. 2005. 2,4-Dichlorophenoxiacetic (2,4-D) sorption and degradation dynamics in three agricultural soils. Environ. Poll., 138:92-99.

Burns, J.M., Hall, S. and Ferry, J.L. 2009. The adsorption of Saxitoxin to clays and sediments in fresh and saline waters, Water Res., 43(7):1899-1904

Caron, E., Farenhorst, A., Gaultier, J., Rank, N., Goddard, T. and Sheedy, C. 2010. Sorption of four estrogens by surface soils from 41 cultivated fields in Alberta, Canada. Geoderma, 155:19-30.

Carousel., R.F., Imhoff, J.C., Hummel, P.R., Cheplick, J.M., Donigian, A.S. Jr. and Suarez, L.A. 1997. PRZM-3, A model for predicting pesticide and nitrogen fate in the crop root and unsaturated zones:User's manual for release 3.12.2, 420 pages. National Exposure Research Laboratory, U.S. Environmental Protection Agency, Athens, GA.

Casey, F.X.M., Larsen, G.L., Hakk, H. and Šimůnek, J. 2003. Fate and transport of 17β-estradiol in soil-water systems. Environ. Sci. Technol., 37:2400-2409.

Casey, F.X.M., Šimůnek, J., Lee, J., Larsen, G.L. and Hakk, H. 2005. Sorption, mobility, and transformation of estrogenic hormones in natural soils. J. Environ. Qual., 34:1372-179.

Cathcart, J., Cannon, K., and Heinz, J. 2008. Selection and establishment of Alberta agricultural soil quality benchmark sites. Can. J. Soil Sci., 88(3):399-408.

Chamignon, C., Haroune, N., Forano, C., Delort, A.M., Besse-Hogan, P. and Combourieu, B. 2008. Mobility of organic pollutants in soil components. What role can magic angle spinning NMR play? Eur. J. Soil Sci., 59(3):572-583.

Cullimore, D.R. 1981. The enumeration of 2,4-D degraders in saskatchewan soils. Weed Sci., 29(4):440-443.

Colucci, M.S. and Topp, E. 2001. Persistence of estrogenic hormones in agricultural soils: II. 17α-Ethynylestradiol. J. Environ. Qual., 30:2077-2080.

Colucci, M.S., Bork, H. and Topp, E. 2001. Persistence of estrogenic hormones in agricultural soils: I. 17β-estradiol and estrone. J. Environ. Qual., 30:2070-2076.

Dann, R.L., Close, M.E., Lee, R. and Pang, L. 2006. Impact of data quality and model complexity on prediction of pesticide leaching. J. Environ. Qual., 35:628-640.

Das, B.S., Wraith, J.M., Kluitenberg, G.J, Langner, H.M., Shouse, P.J. and Inskeep, W.P. 2005. Evaluation of mass recovery impacts on transport parameters using least-squares optimization and moment analysis. Soil Sci. Soc. Am. J., 69 (4):1209-1216.

Dubus, I.G. and Brown, C.B. 2002. Sensitivity and first-step uncertainty analyses for the preferential flow model MACRO. J. Environ. Qual., 31:227-240.

Dubus, I.G., Brown, C.D. and Beulke, S. 2003. Sources of uncertainty in pesticide fate modelling. Sci. Total Environ., 317: 53-72.

Dubus, I.G. and Janssen, P.H.M. 2003. Issues of replicability in Monte Carlo modeling: a case study with a pesticide leaching model. Environ. Toxicol. Chem., 22(12):3081-3087.

Ecological Stratification Working Group. 1996. A National Ecological Framework for Canada. Agriculture and Agri-Food Canada, Research Branch, Centre for Land and Biological Resources Research and Environment Canada, State of Environment Directorate, Ottawa/Hull. 125pp.

Estrella, M.R., Brusseau, M.L., Maier, R.S., Pepper, I.L., Wierenga, P.J. and Miller, R.M. 1993. Biodegradation, sorption, and transport of 2,4-Dichlorophenoxiacetic acid in saturated and unsaturated soils. Appl. Environ. Microbiol., 59(12):4266-4273.

Fan, Z., Casey, F.X.M., Hakk, H. and Larsen, G.L. 2007. Persistence and fate of 17ß-estradiol and testosterone in agricultural soils. Chemosphere, 67:886-895.

Farenhorst, A. Papiernik, S.K., Saiyed, I., Messing, P., Stephens, K.D., Schumacher, J.A., Lobb, D.A., Li, S., Lindstrom, M.J., Schumacher, T.E. 2008. Herbicide sorption coefficients in relations to soil properties and terrain attributes on a cultivated prairie. J. Environ. Qual., 37(3):1201-1208.

Gaultier, J. and Farenhorst, A. 2007. 2,4-D mineralization in soil profiles from a cultivated hummocky landscape in Manitoba, Canada. **J. Environ. Sci. Health, Part B: Pestic., Food Contam., Agric. Wastes,** 42(3):255-264.

Gaultier, J., Farenhorst, A., Cathcart, J. and Goddard, T. 2008a. Regional assessment of herbicide sorption and degradation in two sampling years. J. Environ. Qual., 37(5):1825-1836.

Gaultier, J., Farenhorst, A., Cathcart, J. and Goddard, T. 2008b. Degradation of [carboxyl-^{14}C] 2,4-D and [ring-U-^{14}C] 2,4-D in 114 agricultural soils as affected by organic carbon content. Soil Biol. Biochem., 40(1):217-227.

Gee, G.W. and Bauder, J.W. 1986. Particle size analysis. Pages 383-412. In A. Klute, ed. *Methods of soil Analysis*. Part 1. Physical and Mineralogical Methods. ASA/SSSA, Madison, WI.

Green, V.S., Stott, D.E., Cruz, J.C. and Curi, N. 2007. Tillage impacts on soil biological activity and aggregation in a Brazilian Cerrado Oxisol. Soil tillage Res., 92:114-121

Greer, L.E. and Sheldon, D.R. 1992. Effect of inoculant strain and organic matter content on kinetics of 2,4-Dichlorophenoxacetic acid degradation in soils. App. Environ. Microbiol., 58:1459-1465.

Guo, L., Jury, W.A., Wagenet, R.J. and Flury, M. 2000. Dependence of pesticide degradation on sorption: nonequilibrium model and application to soil reactors. J. Contam. Hydrol., 43:45-62.

Hanselman, T.A., Graetz, D.A. and Wilkie, A.C. 2003. Manure-borne estrogens as potential environmental contaminants: A review. Environ. Sci. Technol., 37(4):5471-5478.

Hurwitz, A.R., and Liu, S.T. 1977. Determination of aqueous solubility and pka values of estrogens. J. Pharm. Sci., 66(5):624-627.

Kozarek, J.L., Wolfe, M.L., Love, N.G. and Knowlton, K.F. 2008. Sorption of estrogen to three agricultural soils from Virginia, USA. Trans ASABE, 51(5):1591-1597.

Laegdsman, M., Andersen, H., Jacobsen, O.H. and Halling-Sorensen, B. 2009. Transport and fate of estrogenic hormones in slurry-treated soil monoliths. J. Environ. Qual. 38(3):955-964.

Lakhani, N.J., Venitz, J., Figg, W.D. and Sparreboom, A. 2003. Pharmacogenetics of estrogen metabolism and transport in relation to cancer. Curr. Drug Metab., 4:505-513.

Layton, A.C., Gregory, B.W., Seward, J.R., Schultz, T.W. and Sayler, G.S. 2000. Mineralization of steroidal hormones by biosolids in wastewater treatment systems in Tennessee U.S.A. Environ. Sci. Technol., 34:3925-3931.

Lee, L.S., Strock, T.J., Sarmah, A.J. and Rao, P.S.C. 2003. Sorption and dissipation of testosterone, estrogens, and their primary transformation products in soil and sediment. Environ. Sci. Technol., 37:4098-4105.

Lee, S., Pardue, J.H., Moe, W.M. and Kim, D.J. 2009. Effect of sorption and desorption-resistance on biodegradation of chlorobenzene in two wetland soils. J. Hazard. Mater., 161:492-498.

Legendre, P. and Legendre, L. 1998. *Numerical Ecology.* 2nd Ed. Elsevier B.V., Amsterdam, The Netherlands.

Leskiw, L.A., Yarmuch, M.S., Waterman, L.P., Sansom, J.J. 2000. Baseline soil physical and chemical properties of forty-three soil quality benchmark sites in Alberta. File #99-042, Can-Ag Enterprises Ltd., Edmonton, AB., 175pp.

Lewis, K.M. and Archer, R.D. 1979. pKa values of estrone, 17 beta-estradiol and 2-methoxyestrone. Steroids, 34(5):485-499.

Loeppert, R.H. and Suarez, D.L. 1996. Carbonate and Gypsum. Pages 437-475 In M. Bartels, ed. *Methods of Soil Analysis*. Part 3. Chemical methods. ASA/SSSA, Madison, WI.

Loffredo, E. and Senesi, N. 2006. Fate of organic pollutants in soils with emphasis on adsorption/desorption processes of endocrine disruptor compounds. Pure Appl. Chem., 78(5):947-961.

Lotrario, J.B., Stuart, J.B., Lam, T., Arands, R.R., O`Connor, O.A. and Kosson, D.S. 1995. Effects of Sterilization Methods on the Physical Characteristics of soil: Implications for sorption isotherm analyses, Bull. Environ. Contam. Toxicol., 54:668-675.

Lucas, S.D. and Jones, D.L. 2006. Biodegradation of estrone and 17β-estradiol in grassland soils amended with animal wastes. Soil Biol. Biochem., 38:2803-2815.

Martens, M. and Martens, H. 1986. Partial least square regression. In J.R. Piggott, *Statistical Procedures in Food Research* pp.292-360, London, Elsevier Applied Sciences.

Martens, H. and Martens, M. 2000. Modified Jack-Knife estimation of parameter uncertainty in bilinear modelling by Partial Least Squares regression (PLSR), Food Qual. Prefer., 11:5-16.

McKeague, J.A. (ed) 1978. *Manual on Soil Sampling and Methods of Analysis*. Canadian Society of Soil Science, Ottawa, Canada, 212 pp.

Moreale, A and Van Bladel, R. 1980. Behavior of 2,4-D in Belgian soils. J. Environ. Qual., 9:627-633.

Mortensen, S.K. and Jacobsen, C.S. 2004. Influence of frozen storage on herbicide degradation capacity in surface and subsurface sandy soils. Environ. Sci. Technol., 38(24):6625-6632.

Nelson, D.E. and Sommers, L.E. 1982. Total carbon, organic carbon, and organic matter. Pages 539-577 In A.L. Page, ed. *Methods of Soil Analysis*. Part 2. Chemical and microbial properties. ASA/SSSA, Madison, WI.

Nkedi-Kizza, P. and Brown, K.D. 1998. Sorption, degradation, and mineralization of carbaryl in soils, for single-pesticide and multiple-pesticide systems. J. Environ. Qual., 27(6):1318-1324.

Nofziger, D. L., Chen, J.-S. and Haan, C. T. 1994. Evaluating the chemical movement in layered soil model as a tool for assessing risk of pesticide leaching to groundwater J. Environ. Sci. Health. Part A Environ. Sci. Eng., 29(6):1133-1155.

Novak, J.M., Moorman, T.B. and Cambardella, C.A. 1997. Atrazine sorption at the field scale in relation to soils and landscape position. J. Environ. Qual., 26:1271-1277.

Ogram, A.V., Jessup, R.E., Ou, L.-T. and Rao, P.S.C. 1985. Effect of sorption on biological degradation rates of 2,4-dichlorophenoxyacetic acid in soils. App. Environ. Microbiol., 49:582-587.

Parker, L.W. and K.G. Doxtader. 1983. Kinetics of the microbial degradation of 2,4-D in soil: effects of temperature and moisture. J. Environ. Qual., 12:553-558 .

Ren, W.-X., Li, P.-J., Geng, Y. and Li, X.J. 2009. Biological leaching of heavy metals from a contaminated soil by Aspergillus Niger. J. Hazard. Mater., 167(1-3):164-169.

Rivera, F. and Hawari. J. 2008. Experimental Evidence of insitu natural attenuation of 2,4- and 2,6-dinitrotoluene in marine sediments. Chemosphere, 70:791-799.

Robertson, B.K. and Alexander, M. 1994. Growth-linked and cometabolic biodegradation: possible reason for occurrence or absence of accelerated pesticide biodegradation. Pest. Sci., 41:311-318.

Routledge, E.J., Sheahan, D., Desbrow, C., Brighty, G.C., Waldock, M., and Sumpter, J.P. 1998. Identification of estrogens chemicals in STW effluent. 2. In vivo responses in trout and roach. Environ. Sci. Technol., 32:1559-156

Schnürer, J. and Rosswall, T. 1982. Fluorescein diacetate hydrolysis as a measure of total microbial activity in soils and litter. Appl. Environ. Microbiol., 6:1256-1261.

Shaw, L.J. and Burns, R.G. 1998. Biodegradation of 2,4-D in a non-contaminated grassland profile. J. Environ. Qual., 27:1464-1471.

Shaw, L.J. and Hooker, J.E. 2008. The fate and toxicity of the flavonoids Naringenin and Formononetin in soil. Soil Biol. Biochem.,40:528-536.

Smalling, K.L. and Aelion, C.M. 2004. Distribution of atrazine into three chemical fractions: impact of sediment depth and organic carbon content. Environ. Toxicol. Chem., 23(5):1164-1171.

Smith, A.E. and Aubin, A.J. 1991. Effects of long-term 2,4-D and MCPA field applications on the soil breakdown of 2,4-D, MCPA, mecocrop and 2,4,5-T. J. Environ. Qual., 20:436-438.

Stumm-Zollinger, E. and Fair, G.M. 1965. Biodegradation of steroids hormones. Steroid Hormones, 37(11):1506-1511.

Stumpe, B. and Marschner, B. 2007. Long-term sewage sludge application and wastewater irrigation on the mineralization and sorption of 17β-estradiol and testosterone in soils. Sci. Total Environ., 374:282-291.

Stephens, K., Farenhorst, A., and Fuller, L.G. 2002. Effect of soil sterilization by mercuric chloride on the sorption of herbicides by soil. J. Environ. Sci. Health, Part B, Part B, 37(6):559-569.

Veeh, R.H., Inskeep, W.P. and Camper, A.K. 1996. Soil depth and temperature effects on microbial degradation of 2,4-D. J. Environ. Qual. 25(1):5-12.

Vergili, I. and Barlas, H. 2009. Removal of 2,4-D, MCPA and Metalaxyl from water using Lewatit VP OC 1163 as sorbent. Desalination, 249:1107-1114.

Wauchope, R.D., Buttler, T.M., Hornsby, A.G., Augusti, J.N., Beckers, P.W.M. and Burt, J.P. 1992. The SCS ARS CES pesticides properties database for environmental decision-making. Rev. Environ. Contamin. Toxicol., 123:1-155.

Wauchope, R.D., Yeh, S., Linders, J.B.H.J., Kloskowski, R., Tanaka, K., Rubin, B., Katayama, A., Kordel., W., Gerstl, Z., Lane, M . and Unsworth, J.B. 2002. Review:

Pesticide soil sorption parameters: theory, measurements, uses, limitations and reliability. Pest Manag. Sci., 58:419-445.

Willems, H.P.L., Lewis, K.J., Dyson, J.S. and Lewis, F.J. 1996. Mineralization of 2,4-D and atrazine in the unsaturated zone of a sandy loam soil. Soil Biol. Biochem., 28(8):986-996.

Wolf, D.C., Hao, T.H., Scott, H.D. and Lavy, T.L.1989.Influence of sterilization methods on selected soil microbiological, physical, and chemical properties. J. Environ. Qual., 18:39-44.

Wolt, J.D., Nelson, H.P., Cleveland, C.B. and van Wesenbeeck, I.J. 2001. Biodegradation kinetics for pesticide exposure assessment. Rev. Environ. Contam. Toxicol., 169:123-164.

Xuan, R., Blassengale, A.A. and Qiquan, W. 2008. Degradation of estrogenic hormones in a silt loam soil. J. Agric. Food Chem., 56:9152-9158.

Ying, G.-G. and Kookana, R.S. 2005. Sorption and degradation of estrogen-like – endocrine disrupting chemicals in soil. Environ. Toxicol. Chem., 24(10):2640-2645.

Yu, C.-P., Roh, H., Chu, K.-H. 2007. 17 beta-estradiol-degrading bacteria isolated from activated sludge. Environ. Sci. Technol., 41(2):486-492.

Zablotowicz R.M., Accinelli C., Krutz L.J. and Reddy, K.N. 2009. Soil depth and tillage effects on glyphosate degradation. J. Agric. Food.Chem., 57(11):4867-487.

4. MANURE APPLICATION EFFECTS ON 17ß-ESTRADIOL SORPTION AND MINERALIZATION PARAMETERS IN SOIL

4.1 Abstract

Naturally produced estrogens, of which 17ß-estradiol is the most potent, can be released in the environment when manure is applied onto agricultural land. There are limited data available on the fate of estrogens in agricultural soils particularly in those soils amended with manure. The objective of this study is to assess the impact of fresh and long-term (since 1973) manure applications on soil properties and the fate of 17ß-estradiol in a clay loam Dark Brown Chernozem in Alberta, Canada. The long-term manure applications to this soil consisted of annual solid beef manure applications to triplicated field plots (15.24 m by 7.62 m) at rates of 0, 60, 120 and 180 Mg ha^{-1} in case of an irrigated (I) agricultural system and 0, 30, 60 and 90 Mg ha^{-1} in case of a rainfed water regime (R). Fifteen soil samples (0-15 cm) were collected from each plot and composited for laboratory experiments on 17ß-estradiol sorption by batch-equilibrium and 17ß-estradiol mineralization by soil microcosm. To examine the effect of a fresh application of manure, solid beef manure was added (60 Mg ha^{-1}) in the laboratory to soil samples from I and R plots from the 0 and 60 Mg ha^{-1} treatment. Regardless of historical manure treatment, 17ß-estradiol sorption by soil ranged from 93% to 100% of that applied and generally increased with increasing manure rate applied to field soils with the 17ß-estradiol sorbed concentration being positively correlated to Total C, SOC (soil organic carbon content),

Total N, NO_3^- and NH_4^+. Mineralization followed the first-order degradation kinetics, with maximum mineralization (Max) ranging from 12.03 to 27.81% and mineralization half-lives ranging from 1.20 to 9.82 days. For the I plots, half-lives were significantly shorter in the 60 Mg ha^{-1} manure rate treatment, while in the R plots, half-lives were significantly shorter in the 90 Mg ha^{-1} manure rate compared to control plots (no manure) or plots amended with other manure rates but Max was not significantly influenced by historical manure rates in I and R. Regardless of the rotation time used in the batch-equilibrium experiments (ranging from 1 hour to 40 hours), half-lives were negatively correlated to concentrations sorbed and Max was positively correlated to the amount of 17ß-estradiol sorbed per unit of soil. For the samples receiving a fresh application of manure in the laboratory, a significant reduction was observed for Max and a significant increase was observed for half-lives. We conclude that land management practices such as manure management have an impact on soil properties affecting the sorption and mineralization of 17ß-estradiol in soil.

4.2 Introduction

Estrogens are feminizing compounds that are naturally produced and excreted by vertebrates such as humans and livestock. Estrogens produced by livestock can be released into soils when their urine and/or feces (manure) are spread onto agricultural land. Runoff from manure-applied plots have shown concentrations of 17ß-estradiol ranging from 0.3 to 1.3 μg L^{-1} (Nichols et al., 1997; Busheé et al., 1998; Finlay-Moore et al., 2000). Natural estrogens such as 17β-estradiol are known to have adverse effects on

fish reproduction when present in surface water at part per trillion concentrations (Lai et al., 2002).

The province of Alberta is the most important cattle-producing province in Canada, with more than 6 million head. In commercial cattle feedlots, it is estimated that 23% of N and 57% of P inputs are harvested in manure (Kissinger et al., 2007). Consequently, this manure is applied to soil to increase the fertility of Alberta's crop land which accounts for around 26% of the total area in crop land in Canada (Alberta Agriculture and Food, 2006). Most of Alberta's crop land is under semi-arid climatic conditions and hence irrigation is part of some cropping systems.

The fate of 17β-estradiol in soils has been studied predominantly in non-amended soils (Casey et al., 2003; Lee et al., 2003; Van Emmerick et al., 2003; Das et al., 2004; Casey et al., 2005; Ying and Kookana, 2005; Hildebrand et al., 2006; Loffredo and Senesi, 2006; Sangsupan et al., 2006; Caron et al., 2010a). These studies indicate that 17β-estradiol sorption by soil is strongly controlled by SOC (Yu et al., 2004; Hildebrand et al., 2006; Loffredo and Senesi, 2006), but also by specific surface area and texture (Yu et al., 2004; Loffredo and Senesi, 2006). Mineralization of organic chemicals sometimes decreases with increasing sorption because of the reduced chemical availability to microorganisms (Ogram et al., 1985; Greer and Sheldon, 1992; Gaultier et al., 2008). Other studies have observed a positive relation between 17β-estradiol sorption and mineralization in soils (Casey et al., 2003, Caron et al., 2010b) and biosolids (Layton et al., 2000) presumably because some steps in the mineralization process occur while 17β-estradiol is sorbed. Recent studies have demonstrated that a one-time addition of sheep,

swine, poultry or cattle manure on grassland and arable soils increased mineralization of 17β-estradiol (Lucas and Jones 2006, Stumpe and Marschner 2010). Stumpe and Marschner (2010) found that long-term organic waste (manure, wastewater, sewage sludge) applications increased SOC and consequently the sorption of 17β-estradiol to 15 different soils with SOC ranging from 70 to 350 g C kg^{-1} while both increases and reductions were observed for mineralization.

The objective of this study is to determine the impact of fresh and long-term cattle manure applications on soil properties and fate of 17β-estradiol in an irrigated and non-irrigated Dark Brown Chernozem soil in southern-Alberta.

4.3 Materials and Methods

4.3.1 Field Sampling and Soil Property Analysis

This study utilized samples collected from field plots associated with Agriculture and Agri-Food Canada's Long-term (since 1973) Manure Study (49°42'N, 112°48'W, elevation 915 m, Hao et al., 2003) in Lethbridge, Alberta. Each plot is 15.5 m long by 7.62 m wide. The 24 studied plots are divided equally in two types of water regimes: 12 plots are irrigated (I) (annual average of 159.9 mm of water applied from 1974 to 2008, ranging from 0 to 431.8 mm) and 12 plots are rainfed (R) (non-irrigated). The water used for irrigation is from an irrigation dugout less than 20 m from the plots. It is filled around

May 15 each year with water from the St Mary's Irrigation District. Within each water regime, the experimental design was completely randomized with four manure application rates in triplicates. For the I plots, the manure rates are: 0, 60, 120 and 180 Mg ha^{-1}. In the case of the R plots, manure application rates are: 0, 30, 60 and 90 Mg ha^{-1}. These rates were respectively once, twice and three times those recommended (N based) for irrigated and rainfed farmland respectively, when the plots were established in 1973 (Alberta Agriculture and Environment, 1973). Plots were cropped in barley at the time the experiment was initiated. Based on the Alberta soil test laboratory database summary (Kryzanowski, 1993) the recommended N-based rates ranged from 45 to 146 kg N ha^{-1} for irrigated stubble farmland; between 6 and 22 kg N ha^{-1} for barley on summer fallow and from 39 to 73 kg N ha^{-1} for barley on stubble in rainfed farm land in the 1970's in Alberta (personal communication Kryzanowski, 2010). The current recommended rates for feed barley based on N range from 45 to 146 kg N ha^{-1} for irrigated farm land and from 6 to 17 kg N ha^{-1} for barley on fallow and from 39 to 73 kg N ha^{-1} for barley on stubble in rainfed farm land. Every year since 1973, solid beef cattle manure has been applied following crop harvest in either September or October and in each year the manure was incorporated after application. The characteristics of the cattle manure varied over the 35 years of the on-going long-term experiment but were on average 2,459.4 ± 957.6 mg kg^{-1} of PO_4, 159.1 ± 237.9 mg kg^{-1} of NO_3-N, 1,626.7 ± 1,405.0 mg kg^{-1} of NH_3-N. 9,794.0 ± 3,540.2 mg kg^{-1} of Cl, 2,612.9 ± 1,279.6 mg kg^{-1} of sulphate, 2.37 ± 0.71 S m^{-1} for electrical conductivity and pH was 7.0 ± 0.4 from 1973 to 2007. Additional details on the site and previous studies have been reported in 11 other publications (Sommerfelt and Chang, 1985; Sommerfelt et al., 1988; Chang et al., 1991; Chang and

Entz, 1996; Chang and Janzen, 1996; Gao and Chang, 1996; Hao and Chang 2002 and 2003; Hao et al., 2003 and 2004; Chang et al., 2005).

On October 8, 2008, 15 soil cores (0-15 cm) were collected in a grid pattern in each plot after crop harvest but before manure application. Samples were collected using an auger (2.54 cm diameter) disinfected with ethanol (95 %) between each plots. The 15 cores for each plot were composited to achieve one bulk sample per plot for a total of 12 bulk samples in the I and R plots respectively. Soil was kept at 4^{o}C for three weeks prior to the determination of soil properties and 17β-estradiol mineralization, while a portion of soil was frozen for a period of eight months and then thawed before being used in the sorption experiments.

Soil texture was determined using the hydrometer method (Gee and Bauder, 1986). Soil pH was determined using 5 g of soil and 10 mL of 0.01M $CaCl_2$ (McKeague, 1978). SOC was determined after acidification using 6M HCl (Nelson and Sommers, 1982). Total C, SOC and Total N were determined on a C/N analyser (2100 Soil, Carlo Erba instruments, Milan, Italy). Available P was determined using the Olsen extraction with 2.5 g of soil (Schoenau and O'Halloran, 2008). Available N in 5 g of soil was extracted using 25 mL of 2 M KCl (Maynard et al., 2008). Available N and P were determined using a continuous flow analyzer (Bran Luebbe autoanalyzer 3, Mequon, WI, USA). Bacterial activity was determined by the fluorescein diacetate hydrolysis assay (FDA) (Adam and Duncan, 2001) on soil samples (2 g dry weight) wetted to 80% field capacity and incubated in sterile Falcon tubes at 20^{o}C for 7 days.

4.3.2 17β-estradiol Sorption

17β-estradiol sorption by soil was determined by batch-equilibrium experiments. Analytical grade 17β-estradiol (purity of 99%, from Sigma-Aldrich Chemical Company, St. Louis, MO) and 17β-estradiol [4-^{14}C] (99% radiochemical purity, specific activity 1.665x10^{12}-2.257x10^{12} Becquerels mmol^{-1}, from American Radiolabeled Chemicals, St-Louis, MO) (Figure 4.1) were mixed in autoclaved water (30 minutes at 121^{o}C) to prepare the stock solution. The stock solution was kept at 4^{o}C in glass amber bottles in the dark and was used on days one and two after preparation. In order to limit the potential impact of ethanol on 17β-estradiol sorption by soil, the percentage of ethanol contained in the stock solution was less than 0.005 %.

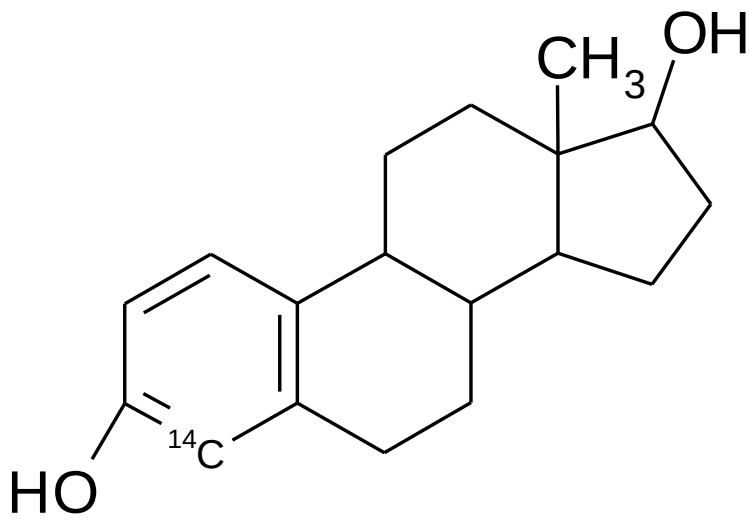


Figure 4.1: Structure of 17β-estradiol [4-^{14}C]

Air-dried soil (5 g) was weighted in glass tubes (in duplicate). Tubes were capped with aluminum foil and autoclaved (30 minutes at 121^{o}C and 21 psi). Sterilization of soils by autoclaving was necessary since estrogen biodegradation during the sorption experiment would have lead to an underestimation of Kd values (Wolf et al., 1989). Previous studies have showed that autoclaving soil will not impact its properties such as SOC, pH, cation exchange capacity and soil surface area (Wolf et al., 1989; Lotrario et al., 1995), nor the Kd values of some chlorinated hydrocarbons (Lotrario et al., 1995) and pesticides (Benoit et al., 1996).

Stock solution (10 mL with a specific activity of 16.67 Becquerels mL^{-1}) was added to soil and then the tubes were closed with the aluminum foil between the cap and the tube. The estrogen concentration in soil was equivalent to 50 µg kg^{-1} of soil, assuming 100 % sorption. The 50 µg kg^{-1} of soil is the same concentration used by Caron et al. (2010a and b) and is within the mid-range of the concentrations used by Casey et al. (2003). The concentration is also within the linear range of the 17ß-estradiol sorption isotherm for 17β-estradiol as determined by Casey et al. (2005) and Kozarek et al. (2008). Soil slurries were rotated at 5^{o}C in the dark for 1h, 2h, 16h, 24h and 40h and then centrifuged for 30 min at 7,000 rpm (6000g). Supernatant (1 mL) in duplicates was added to 7 mL scintillation vials containing 5 mL of scintillation cocktail (Fisher Scientific, Fairlawn, NJ). Scintillation Counting (LSC) with automated quench correction (#H method) (LS 7500 Beckman Instruments, Fullerton, CA) and a maximum counting time of 10 minutes was used to determine the amount of radioactivity in the stock solutions and supernatant samples. Concentration sorbed (Cs) was used in the statistical analysis and is obtained by

subtracting the concentration in the supernatant solution from that initially applied. Controls consisting of glass tubes with solution only were also included and demonstrated no loss of radioactivity during the experiment, suggesting that there was no loss of 17ß-estradiol due to sorption to glass. For the sorption data expressed in Cs, the coefficient of variation (CV) was on average 0.3 % between laboratory replicates as well as between field replicates.

4.3.3 17β-estradiol Mineralization

Mineralization in soil was determined by microcosm experiments consisting of a sealed 1 L glass Mason jar in triplicates. Each soil microcosms contained a glass jar with 17β-estradiol mixed in 25 g of soil (oven-dry weight basis), a glass test tube with 3 mL of acidified water (pH~3) to preserve humidity and a scintillation vial with 5 mL of 0.5 M NaOH to trap evolved $^{14}CO_2$. Additional microcosms were set-up as controls containing 17β-estradiol mixed in 25 g of autoclaved silica sand (triplicates) and 25 g of non-autoclaved silica sand (triplicates).

A second mineralization experiment was run simultaneously. This second experiment consisted of adding 0.66 g (dry weight) of solid cattle manure (moisture content of 34.35%) to 25 g of soil from the 0 and 60 Mg ha^{-1} field treatments of both I and R plots. This one-time manure application was equivalent to a wet weight manure application rate of 60 Mg ha^{-1} in the field, assuming that this manure is incorporated into the soil to 15 cm depth with a bulk density of 1,000 kg m^{-3} for both the 0 and 60 Mg ha^{-1} soils. The bulk

density used was based on field measurements (in the same plots) using the ring method (Blake and Hartge, 1986) and was the same for each plot. The solid cattle manure contained 250,100 mg C kg^{-1} (dry), 21,590 mg N kg^{-1}, 30 mg of NO_3^- kg^{-1} (dry), 520 mg of NH_4^+ kg^{-1} (dry), and 11,280 mg PO_4 kg^{-1} (dry). For the manure-spiked soils, Total N, NO_3^-, NH_4^+, FDA, PO_4, Total C, Inorganic C, SOC were also determined immediately after manure application to an additional set of soil samples.

Prior to applications of 17β-estradiol, soil moisture content was determined gravimetrically. Soils were then brought to 80% of their field capacity minus the 1 mL required for 17β-estradiol spiking. Field capacity was measured for each soil using the container method in which a mass of soil is saturated with water then drained under gravitational forces and oven-dried to determine the mass of water left in the soil.

Mason jars were incubated in the dark at 20^{o}C for a pre-incubation period of seven days. Soil or silica sand were then spiked with a 1 mL solution containing 1,667 Becquerels mL^{-1} 17β-estradiol [4-^{14}C] and analytical grade 17β-estradiol to yield a concentration of 50 μg kg^{-1} 17β-estradiol in soil. This solution was deposited drop wise in a grid pattern and then thoroughly mixed with the soil in order to ensure a homogeneous distribution. Mason jars were again incubated in the dark at 20^{o}C with 0.5 M NaOH traps being removed and replaced at 1, 4, 8, 10, 17, 35, 49, 65, 77 and 90 days. Microcosms were aerated weekly in order to avoid anaerobic conditions and moisture was maintained gravimetrically weekly throughout the experiment. The amount of radioactivity in the stock solution and traps was determined by adding 8 mL of scintillation cocktail (30%

Scintisafe scintillation cocktail; Fisher Scientific, Fairlawn, NJ) to vials and counting by LSC as previously described for sorption.

The cumulative amount of $^{14}CO_2$ was fitted to the first-order kinetics equation in Sigma plot, 2000 (SPSS Inc, Chicago, Il.) to obtain mineralization rate (k) and the % of applied 17β-estradiol mineralized at time infinity (Max). The first-order degradation equation is defined as M_t=Max ($1-e^{-kt}$), where M_t= % of applied 17β-estradiol mineralized at time t with t expressed in days. Half-lives were calculated by ln (2) divided by k. For the sample that did not receive manure in the laboratory, the average CV of half-lives was 17.2 and 16.1% and that of Max was 8.4 and 8.1 % for the laboratory and field replicates, respectively. For the samples that received manure in the laboratory, the average CV of half-lives was 10.7% and 12.5% and that of Max was 8.9% and 7.8 % for the laboratory and field replicates, respectively.

4.3.4 Statistical Analyses

Pearson correlation coefficients ($P < 0.05$) were determined (1989-2002, JMP version 5.0, SAS Institute) among the following parameters: Cs at each rotation time, half-lives, Max and the soil properties Total N, NO_3^-, NH_4^+, FDA, PO_4, Total C, SOC, Inorganic C, %sand, %clay and %silt. Correlations were conducted on untransformed data due to the robust nature of these analyses (Legendre and Legendre, 1998). All correlations were checked visually to confirm that the correlations were the result of general trends rather than extreme outliners.

For each of I and R, Cs was analyzed using a two-way ANOVA (SigmaStat for Windows version 3.1 Systat Software Inc.) with factors 1) manure rate and 2) rotation time followed by the Tukey-Kramer HSD multiple comparison test with $\alpha < 0.05$. Normality was tested by the Shapiro-Wilks test and data respected normality (Shapiro-Wilks statistic ≥ 0.90, Analyse-it for Microsoft Excel, version 2.20) and equality of variance (SigmaStat for Windows version 3.1 Systat Software Inc.).

For each of I and R, the impact of manure rate on mineralization parameters (half-lives and Max) and soil properties (Total N, NO_3^-, NH_4^+, FDA, PO_4, Total C, Inorganic C, SOC, %sand, %clay and %silt) was determined using a one-way ANOVA (SigmaStat for Windows version 3.1 Systat Software Inc.). The ANOVA was followed by the Tukey-Kramer HSD multiple comparison test with $\alpha < 0.05$. Normality and equality of variance was tested (SigmaStat for Windows version 3.1 Systat Software Inc.) and all data respected normality (Shapiro-Wilks statistic ≥ 0.90, Analyse-it for Microsoft Excel, version 2.20) except for the PO_4 and %sand data which were log-transformed. Normality testing was made on the entire dataset (I+R) in order to increase n. Equality of variance was respected except in case of log-transformed PO_4 data for I.

A paired t-test with $\alpha < 0.05$ (SigmaStat for Windows version 3.1 Systat Software Inc.) was used to determine the impact of a fresh addition of manure on mineralization parameters (half-lives and Max) and soil properties (Total N, NO_3^-, NH_4^+, FDA, PO_4, Total C, Inorganic C, SOC). Data for Max, half-life, FDA, Total N, Total C and SOC

respected normality (Shapiro-Wilks statistic ≥ 0.90, Analyse-it for Microsoft Excel, version 2.20) as was the data for PO_4 and Inorganic C after log-transformation. Many transformations were considered for the NO_3^- and NH_4^+ data with the optimum transformation being a square transformation (Shapiro-Wilks statistic of 0.87 for NO_3^- and 0.82 for NH_4^+) and used in the t-test. The t-tests were done on the entire data set, but also on data from either the I or R plots only, and either the 0 or 60 Mg ha^{-1} rate only.

4.4 Results

Concentration sorbed ranged from 46.6 to 50.1 μg kg^{-1}. For I, the long-term addition of manure significantly increased 17β-estradiol sorption in soil (Figure 4.2A). Concentration sorbed was significantly smaller at 1h rotation time but there were no significant differences across the other rotation times, suggesting that equilibrium conditions were met at 2h. For R, the interaction between manure rate and rotation time was significant ($P < 0.05$) (Figure 4.2B). Rotation time had no effect on Cs values in the 60 kg ha^{-1} manure plot, but at all other manure treatments, the 1h rotation time had significantly lesser Cs values than the 40h rotation time (Figure 4.2B). Within each rotation time, soils that had not received manure always demonstrated significantly lesser Cs values than soils that had received manure, with the exception of the 24h rotation time (Figure 4.2B). When all data were combined, Cs at every rotation time was significantly positively correlated to Total N, NO_3^- , NH_4^+, Total C and SOC (Table 4.1).

Figure 4.2 A

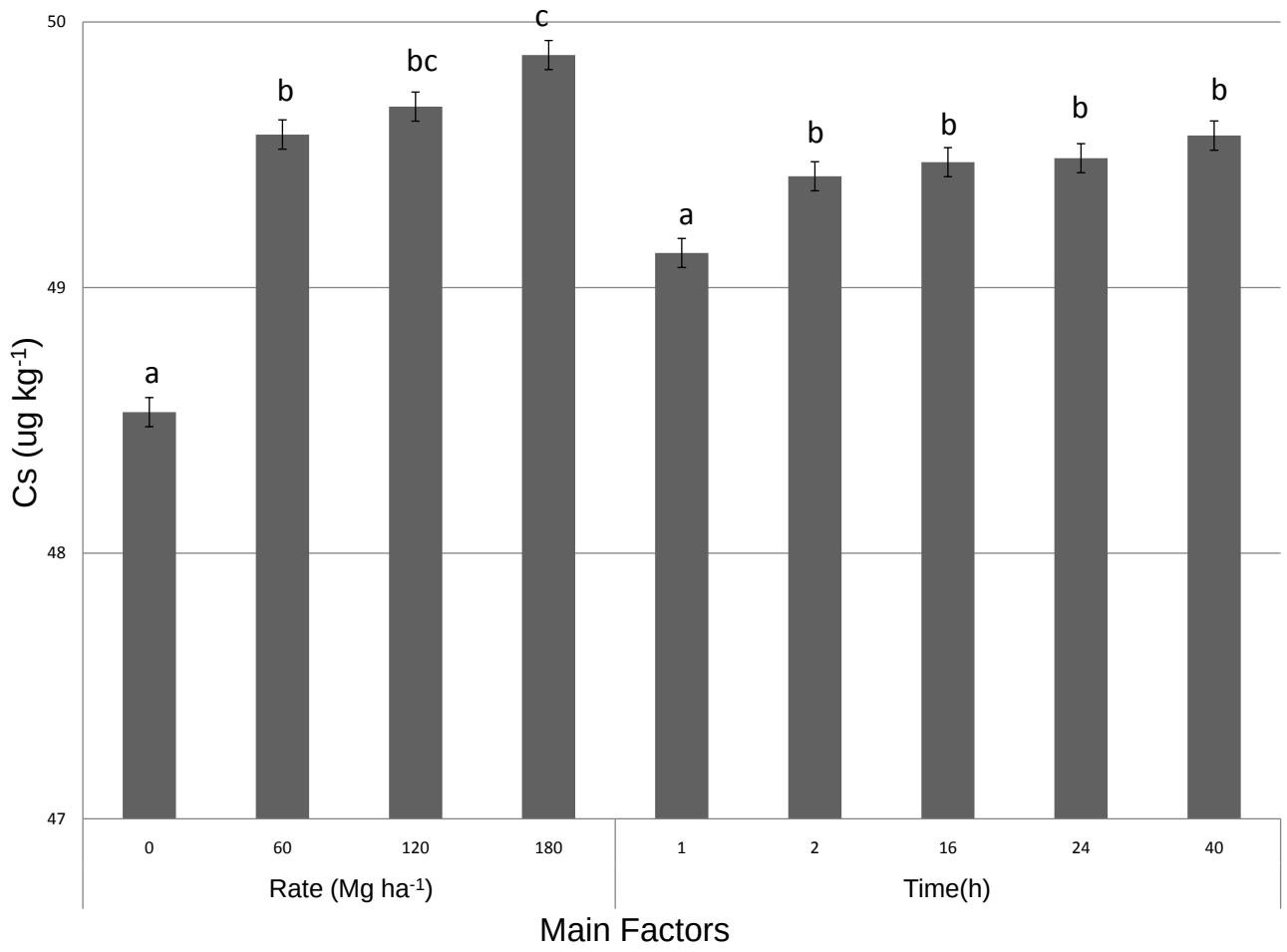

50
49
48
47
Cs (ug kg-1)
a
b
bc
c
a
b
b
b
b
0
60
120
180
1
2
16
24
40
Rate (Mg ha-1)
Time(h)
Main Factors

Figure 4.2 B

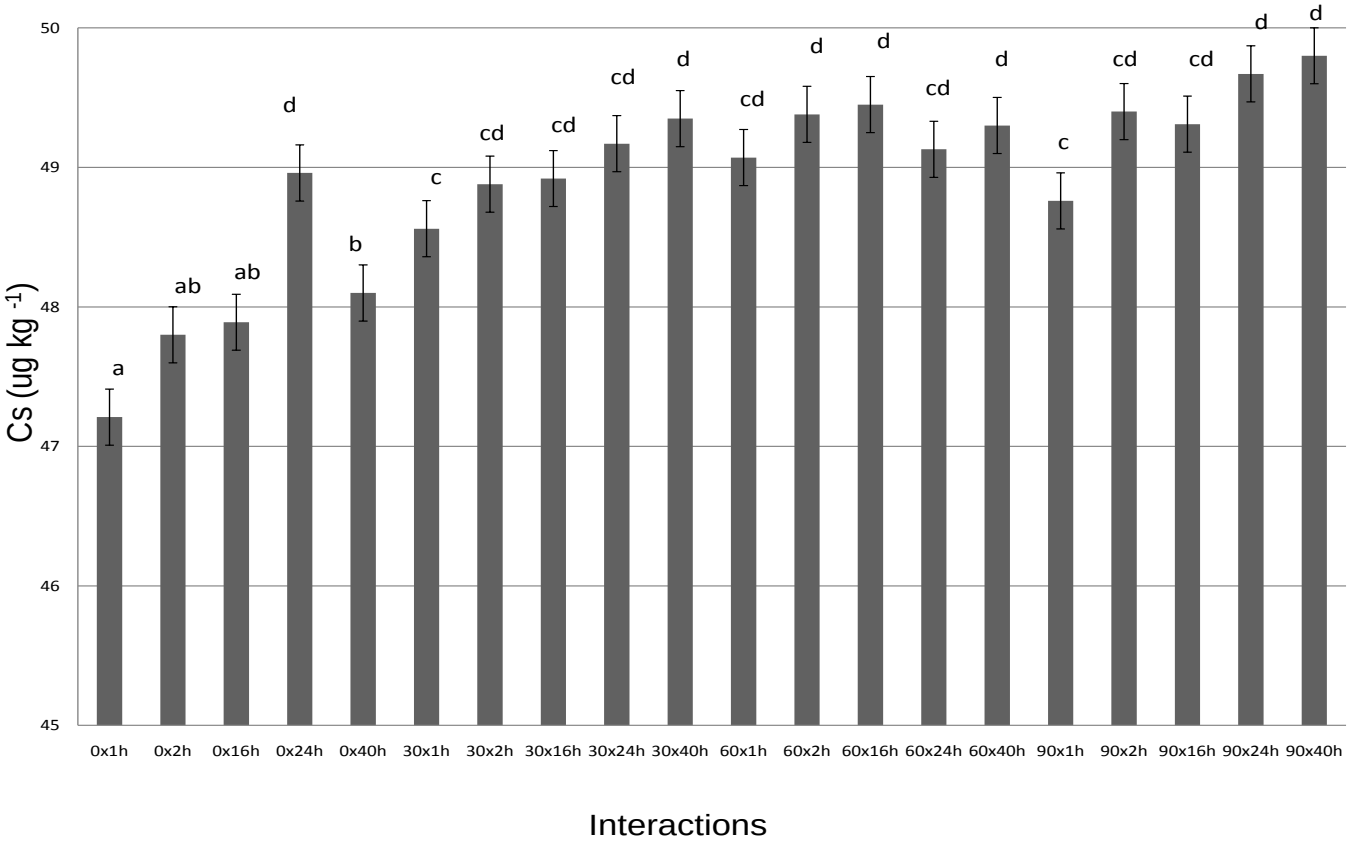


Figure 4.2: 2-Way ANOVA results for Cs in the A) I and B) R plots with factors rotation time and manure rate. Groups with the same letter are not statistically different and bars represent standard error.

Table 4.1: Correlation between Cs, mineralization parameters and soil properties.

	CS1	CS2	CS16	CS24	CS40	HL	Max	FDA	PO_4	Total N	NO_3^-	NH_4^+	Total C	SOC	IC	% sand	% clay	% silt
CS1	1																	
CS2	0.89***	1																
CS16	0.98***	0.88***	1															
CS24	0.59**	0.51*	0.62**	1														
CS40	0.88***	0.88***	0.88***	0.70***	1													
HL	-0.68***	-0.77***	-0.69***	-0.47*	-0.66***	1												
Max	0.51*	0.59**	0.51*	0.40*	0.45*	-0.77***	1											
FDA	0.49*	0.48*	0.53**	-	0.48*	-0.54***	0.41*	1										
PO_4	0.44*	-	0.41*	-	0.55**	-	-	-	1									
Total N	0.73***	0.65***	0.75***	0.66***	0.74***	-0.46*	0.50*	-	0.45*	1								
NO_3^-	0.84***	0.75***	0.86***	0.71***	0.84***	-0.52**	0.48*	-	-	0.90***	1							
NH_4^+	0.80***	0.68***	0.79***	0.56***	0.77***	-0.50*	0.48*	-	0.46*	0.83***	0.91***	1						
Total C	0.74***	0.66***	0.75***	0.64***	0.74***	-0.46*	0.50*	-	0.44*	1***	0.91***	0.84***	1					
OC	0.74***	0.65***	0.71***	0.66***	0.75***	-0.47*	0.50*	-	0.47*	1***	0.91***	0.84***	1***	1				
IC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1			
%sand	-	-	-	-0.42*	-	-	-	-	-	-	-0.48*					1		
%clay	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.80***	1	

%silt	-	-	-	-	-	-	-	0.45 *	-	-	-	-	-	-	-	-	- 0.56 **	1

[a] * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). *** Correlation is significant at the 0.001 level (2-tailed). - Correlation is not significant at the 0.05 level.

Mineralization of 17β-estradiol followed first order kinetics (r^2 ranging from 0.78 to 0.99). Half-lives ranged from 1.2 to 4.3 days in I but from 3.2 to 8.8 days in R. Max ranged from 18.7 to 27.8% in I and from 14.1 to 24.5% in R. For both I and R, half-lives were numerically shorter in soils with a history of manure applications, relative to plots free of manure (Table 4.2). Half-lives were significantly shortest in the 60t ha^{-1} manure plot for I and in the 90 Mg ha^{-1} manure plot for R. In contrast, Max was not significantly influenced by the history or rate of manure application in either I or R. Half-lives were significantly negatively correlated with Cs at all rotation times while Max was significantly positively correlated to Cs (Table 4.1). Max and half-lives were significantly negatively correlated.

Total N, NO_3^-, NH_4^+, Total C and SOC significantly differed across manure treatments in I (Table 4.2). For NO_3^- and NH_4^+, concentrations significantly increased in the order of 0<60<120=180 while values for Total N, Total C and SOC significantly increased in the order of 0=60<120=180 (Table 4.2). For R, NO_3^-, significantly increased in the order of 0<30<60=90, and FDA in the order of 0≤30=60<90, but no other soil property was influenced by the rate of long-term manure application (Table 4.2).

Additions of raw manure to soil in the laboratory resulted in 17β-estradiol half-lives ranging from 2.7 to 9.8 days and 17β-estradiol Max ranging from 13.1 to 22.3%. The corresponding samples that did not received the additional application of manure in the laboratory demonstrated half-lives ranging from 1.2 to 8.8 days and Max from 15.5 to 25.1%.

Table 4.2: Mean (coefficient of variation) of mineralization parameters (Half-life and Max) and soil properties. Group means in columns with the same letter are not significantly different from each other (One-Way ANOVA, $P < 0.05$ with factor: rate). Multiple comparisons were done using the Tukey-Kramer HSD test with $\alpha < 0.05$.

Irrigated													
Rate (Mg ha^{-1})	Hl (day)	Max (%)	FDA (µg g^{-1})	PO_4 (mg kg^{-1}) **	Total N (gN kg^{-1})	NO_3^- (mg kg^{-1})	NH_4^+ (mg kg^{-1})	Total C (gC kg^{-1})	SOC (gC kg^{-1})	Inorg C (gC kg^{-1})	Sand (%)	Clay (%)	Silt (%)
0	3.8 (17.5) B	22.1 (13.5) A	3.0 (83.7) A	129.7 (43.2) A	2.4 (12.5) A	11.4 (36.8) A	3.1 (16.1) A	30.2 (6.3) A	21.8 (17.9) A	8.4 (25.0) A	24.1 (15.8) A	46.7 (9.0) A	29.3 (5.1) A
60	1.6 (20.8) A	23.8 (6.4) A	2.6 (3.8) A	480.1 (76.7) A	5.6 (21.4) A	58.1 (32.0) B	4.6 (10.9) B	58.0 (20.9) A	51.2 (19.9) A	6.8 (4.2) A	24.0 (17.9) A	46.9 (10.2) A	29.2 (5.5) A
120	2.5 (25.4) AB	22.32 (1.2) A	3.5 (48.6) A	509.3 (49.7) A	10.0 (23.0) B	113.9 (16.5) C	6.3 (6.3) C	97.7 (23.5) B	91.8 (19.8) B	5.9 (81.4) A	20.6 (3.4) A	48.6 (7.0) A	30.8 (9.0) A
180	3.5 (19.6) B	25.4 (8.1) A	3.2 (18.8) A	425.0 (7.6) A	11.1 (85.6) B	131.0 (5.4) C	6.7 (7.5) C	110.6 (8.6) B	100.6 (9.4) B	10.0 (15.0) A	20.2 (5.0) A	50.6 (9.5) A	29.3 (13.0) A
Rainfed													
Rate (Mg ha^{-1})	Hl (day)	Max (%) *	FDA (µg g^{-1})	PO_4 (mg kg^{-1})	Total N (gN kg^{-1})	NO_3^- (mg kg^{-1})	NH_4^+ (mg kg^{-1}) *	Total C (gC kg^{-1})	SOC (gC kg^{-1})	Inorg C (gC kg^{-1})	Sand (%)	Clay (%)	Silt (%) *
0	8.1 (7.3) B	16.6 (6.3) A	1.0 (10.0) A	67.3 (28.2) A	2.7 (40.7) A	9.6 (14.6) A	3.2 (28.1) A	31.1 (29.3) A	24.1 (43.6) A	0.7 (42.9) A	22.3 (8.1) A	49.0 (0.8) A	28.8 (5.2) A
30	6.4 (32.1) B	16.1 (11.4) A	2.0 (25.0) AB	554.8 (112.0) A	4.9 (36.7) A	35.5 (11.0) B	4.4 (11.4) A	51.2 (33.8) A	44.8 (37.5) A	0.7 (28.6) A	25.6 (11.7) A	45.6 (6.1) A	28.8 (3.5) A
60	5.6	16.8	2.8	358.3	5.6	64.4	4.1	58.9	50.9	0.8	22.1	49.4	49.4

	(9.8)	(6.0)	(10.7)	(50.6)	(8.9)	(12.3)	(14.6)	(9.0)	(9.2)	(25.0)	(10.4)	(9.1)	(4.3)
	B	A	B	A	A	C	A	A	A	A	A	A	A
90	3.6	10.6	3.8	324.0	5.1	78.1	4.6	52.5	46.1	0.6	22.1	47.2	47.2
	(10.1)	(16.8)	(15.8)	(47.0)	(48.1)	(14.3)	(10.9)	(40.2)	(49.7)	(50.0)	(5.0)	(1.5)	(0.8)
	A	A	C	A	A	C	A	A	A	A	A	A	A

* Denotes a test where power was below 0.8 (due to the low number of replicates) but with a relatively low p value, suggesting that no conclusion can be made on significant differences.

** P value was 0.842 for the ANOVA condition of equality of variance not respected) and 0.789 for the ANOVA on Ranks. In both cases, there was non-significant difference.

An addition of fresh manure in the laboratory significantly ($P < 0.05$) decreased Max (on average from an initial value of 19.8% down to 15.6%) and increased ($P < 0.05$) half-lives (on average from initial half-lives of 4.8 up to 5.5 days), as well as NH_4^+ ($P < 0.05$) (on average from an initial content of 3.5 up to 52.9 mg kg^{-1}), NO_3^- ($P < 0.05$) (on average from an initial content of 33.1 up to 53.6 mg kg^{-1}) and Total C ($P < 0.05$) (on average from an initial content of 44.6 up to 49.9 g C kg^{-1}), but did not influence other soil properties. When only the I plots were considered, a significant ($P < 0.05$) decrease was observed for Max (from an initial value of 23.0% down to 17.3%) and a significant ($P < 0.05$) increase was observed for HL (from an initial value of 2.7 up to 3.6 days), Total C ($P < 0.05$) (from an intial content of 44.1 up to 53.3 g C kg^{-1}), SOC ($P < 0.05$) (from an intial content of 36.5 up to 43.2 g OC kg^{-1}), Total N ($P < 0.05$) (from an initial content of 4.0 up to 4.8 g N kg^{-1}) and NH_4^+ ($P < 0.05$) (from an intial content of 3.9 up to 53.5 mg kg^{-1}). When considering only the R plots, a significant ($P < 0.05$) decrease was observed for Max (on average from an initial value of 16.7% down to 14.3%) and a significant ($P < 0.05$) increase only for NH_4^+ (on average from an initial content of 3.6 up to 52.3 mg kg^{-1}). When considering only the 0 Mg ha^{-1} treatment, Max was significantly ($P < 0.05$) reduced with the addition of fresh manure (on average from an initial value of 19.3% down to 13.9%) while NH_4^+ was significantly ($P < 0.05$) increased from the initial value of 3.1 up to 56.4 mg kg^{-1}. When considering only the 60 Mg ha^{-1} treatment, Total N, NO_3, NH_4^+, Total C and SOC were significantly ($P < 0.05$) increased by a fresh addition of manure in the laboratory, but there was no affect ($P > 0.05$) on Max or half-lives.

4.5 Discussion

Mineralization of 17β-estradiol in soil was biologically mediated since the autoclaved silica sand and silica controls showed only 0.75% and 2.11% total mineralization. Max (ranging from 12.03 to 27.81%) was generally greater in soils from the present study than soils used in previous studies for which Max ranged from 5 to 19.2% (Colucci and Topp, 2001; Stumpe and Marschner, 2007; Fan et al. 2007; Caron et al., 2010b). Our soils generally had greater SOC and finer textures than the soils used by the previous authors. Max was never higher than 30%, suggesting that some estrogenic activity might remain due to the presence of 17β-estradiol or its metabolites, estrone or estriol.

Previous research also concluded that the degradation of 17β-estradiol occurred according to first-order kinetics in agricultural soils (Colucci and Topp, 2001; Ying and Kookana, 2005; Fan et al., 2007; Xuan et al., 2008; Caron et al., 2010b; Stumpe and Marschner, 2010), but zero-order and second-order kinetics have also been reported (Stumpe and Marschner, 2007).

Plots that had never received manure (0 Mg ha^{-1} treatment) possessed the intrinsic capacity to mineralize estrogens. Bacteria that participate to 17ß-estradiol degradation have been isolated from a variety of matrices (Yoshimoto et al., 2004, Yu et al., 2007) including *Pseudomonas* commonly-found in soils (Stumm-Zollinger and Fair, 1965). 17ß-estradiol breakdown in soil is thought to occur through co-metabolic means (Stumpe and Marschner, 2007).

Relative to control plots, after 35 years, the SOC concentrations in the Dark Brown Chernozem soil were statistically greater in plots receiving an annual manure application of 120 Mg ha^{-1} or more. This agrees with other studies demonstrating that long-term manure applications to soil can increase SOC (Gami et al., 2009; Huang et al., 2009; Mugwe et al., 2009; Huang et al., 2010). The sorption of 17β-estradiol was positively correlated to SOC, as shown by others (Hildebrand et al., 2006; Loffredo and Senesi, 2006; Caron et al., 2010a), and increased sorption was observed for plots with increasing annual manure rates. Sorption was correlated not only to SOC but also with NO_3^- and NH_4^+ because the concentration of N also increased due to the application of manure. In the current study, Total N, NO_3^-, NH_4^+, Total C and SOC significantly increased with manure rates in the I plots and NO_3^- increased with manure rates in the R plots. When comparing our results to those of previous research on the same experimental plots, other studies also concluded that NO_3^- and SOC are significantly influenced by manure rates under both I and R (Table 4. 3).

17β-estradiol half-lives were negatively significantly correlated with SOC and Cs. A possible explanation for this observation is that some of the steps in the 17ß-estradiol mineralization process occurred in the sorbed phase, as suggested for agricultural soils in Alberta (Caron et al. 2010b) and North-Dakota (Casey et al., 2003) as well as for biosolids (Layton et al., 2000). Half-lives were also negatively significantly correlated with Total N, NO_3^-, and NH_4^+, possibly because of the autocorrelation between SOC and N as a result of manure applications. However, also in other studies, when 17β-estradiol

was applied to soils using urine as the solvent, 17ß-estradiol mineralization in soils was greater than when 17β-estradiol was applied to soils using distilled water as the solvent (Lucas and Jones, 2006), suggesting that N containing constituents in manure could have contributed to decreased 17ß-estradiol half-lives. In addition to altering SOC and N contents in soil, it is also possible that organic waste, such as manure, contains bacteria able to biodegrade estrogens (Yoshimoto et al., 2004, Yu et al., 2007), hence increasing 17ß-estradiol mineralization rates. Additionally, an exposure to estrogens due to annual applications of manure could precondition microorganisms, leading to accelerated degradation, as has been observed for pesticides (Cullimore, 1981; Smith and Aubin, 1991; Robertson and Alexander, 1994; Shaw and Burns, 1998).

Max was significantly reduced for soils that received a fresh application of manure in the laboratory. Our study results are in contrast with those of Stumpe and Marschner (2010) who measured a 10 to 160 fold increase in mineralization when manure, biosolids or wastewater amendments were freshly applied to sand and 15 different soils from Israel and Germany. However, there is also evidence in the literature that amendments (in that case grape skins and winery wastewaters) can induce physicochemical changes in soils and thereby influence the structure and functions of soil microbial communities for some time after which the state of microbiological system returns (Saison et al., 2006). Perhaps the physicochemical changes that occurred upon the application of manure (e.g., increases in NH_4^+, NO_3^- and Total C were observed) caused changes to the soil microbial communities and Max was reduced because of corresponding changes in the structure and function of soil microbial communities. Hemmings and Hartel (2006) found less than

6% of 17β-estradiol mineralization in breeder and broiler litter at different temperatures and water potentials, also suggesting that manure or other organic wastes can impede maximum 17β-estradiol mineralization. Even if Max was reduced by a fresh application of manure in the laboratory, the long-term history of an annual application of manure in the field had no affect on Max, suggesting that the reduction of Max is short-lived and associated with the immediate manure application.

4.6 Conclusion

In surface soil samples collected from field plots with a history of 35 years of solid beef manure applications, 17β-estradiol sorption by soil significantly increased with the annual amount of manure applied but for all soil samples the sorption of 17β-estradiol was 93 to 100% of that initially applied. Concentrations sorbed and Max were positively correlated, suggesting that some steps in mineralization are occurring in the sorbed phase, but differences in Max among manure treatments (ranging from 0 to 180 Mg ha^{-1} in I and from 0 to 90 Mg ha^{-1} in R) were not consistent enough to result in statistically significant differences. Max was always less than 30% after 90 days, suggesting that residual estrogenic activity could remain for more than a season in soils exposed to estrogens. In the plots that received an addition of manure in the laboratory, Max was decreased and half-lives increased compared to plots that did not receive manure in the laboratory; however, we suggest that this impact is temporary since it was not reflected in plots with a history of manure application. Future studies should focus not only on basic soil properties but also on the influence that soil and manure properties have on the

metabolism of bacteria (soil and waste-borne) as well as the ecological interactions between bacteria themselves in order to better understand the impact of manure on the fate of estrogens.

Table 4.3: Comparison of the impact of manure rates on measured soil properties (0-15 cm) in this study with previous studies on the same experimental plots.

Sampling year	Significant	Not significant	Reference
	Irrigated		
2008	Total N, NO_3^-, NH_4^+, Total C and SOC	FDA, PO_4, Inorganic C, texture	This study
1973 to1983	SOC, bulk density	-	Sommerfelt and Chang, 1985
1973 to 1983	SOC, Total N	-	Sommerfelt et al., 1988
1984	SOC, pH, electrical conductivity (EC), sodium adsorption ratio (SAR), Total N, NO_3^-,Total P, PO_4, Cl, HCO_3, Na, Ca+Mg, Zn	NH_4^+, SO_4, Cu	Chang et al., 1991
1973 to 1991	NO_3^-	-	Chang and Entz, 1996
1973 to 1992	NO_3^-, organic N	-	Chang and Janzen, 1996
1973 to 1991	% sand, SOC, Total N, CEC	Inorganic C	Gao and Chang, 1996
1973 to 1998	SOC, Total N, NO_3^-	NH_4^+	Hao et al., 2003
1973 to 1998	Total P, soil test P, water-soluble P	Aggregate size distribution	Hao et al., 2004
1973 to 1998	-	Total P and soil test P **	Chang et al., 2005
	Rainfed		
2008(this study)	NO_3^-,FDA	PO_4, Total N, NH_4^+, Total C SOC, Inorganic C, texture	-
1973 to1983	organic matter, bulk density	-	Sommerfelt and Chang,1985
1973 to 1983	SOC, Total N	-	Sommerfelt et al., 1988
1984	SOC, pH, EC, SAR, Total N, NO_3^-, Total P, PO_4, Cl, SO_4, HCO_3, Na, Ca+Mg, Zn	NH_4^+, Cu	Chang et al., 1991
1973 to 1991	NO_3^-	-	Chang and Entz, 1996
1973 to 1992	NO3-, organic N		Chang and Janzen, 1996

1973 to 1991	% sand, SOC, Total N, CEC	Inorganic C	Gao and Chang, 1996
1973 to 1998	SOC, Total N, NO_3-	NH_4^+	Hao et al., 2003
1973 to 1998	Total P, soil test P, water-soluble P	Aggregate size distribution	Hao et al., 2004
1973 to 1998	-	Total P and soil test P**	Chang et al., 2005

*NO_3^- and NH_4^+ were also measured but no statistics were made.

** increased with the amount of manure applied but regardless of annual application rates.

4.7 Acknowledgments

The authors acknowledge the financial support of the Natural Sciences and Engineering Research Council of Canada for research funding. We also thank the University of Manitoba Graduate Fellowship for financial support to E. Caron. The authors thank Mr. Paul Panich, Mr. Brett Hill, Mrs. Pamela Caffin, Mr. Clarence Gilbertson and Mr. Dan Inaba at AAFC Lethbridge for their assistance to the laboratory experiments.

4.8 References

Adam, G. and Duncan, H. 2001. Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. Soil Biol. Biochem., 33:943-951.

Alberta Environment and Alberta Agriculture. 1973. Confinement livestock facilities waste management code of practice. Agdex 538/710. Alberta Environment and Alberta Agriculture, Alberta.

Alberta Agriculture, Food and Rural development. 2004. Alberta fertilizer guide. Agdex 541-1, Alberta Agriculture, Food and Rural development, Alberta

Alberta Agriculture and Food, Economics and Competitiveness Division, Statistics and Data Development Unit. 2006. Census of Agriculture for Alberta, Provincial Highlights. AGDEX 852-5.

Benoit, P., Barriuso, E., Houot, S. and Calvet, R. 1996. Influence of the nature of soil organic matter on the sorption desorption of 2,4-dichlorophenol and the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), Eur. J. Soil Sci., 47:567-578.

Blake, G.R. and Hartge, K.H. 1986. Bulk Density. Pages. 363-67 in A. Klute, ed. Methods of Soil Analysis. Part I. Physical and Mineralogical Methods. Agronomy No 9, 2nd ed. American Society of Agronomy, Madison, WI.

Busheé, E.L., Edwards, D.R. and Moore, P.A. Jr. 1998. Quality of runoff from plots treated with municipal sludge and horse bedding. Trans. ASAE, 41(4):1035-1041.

Caron, E., Farenhorst, A., Gaultier, J., Rank, N., Goddard, T. and Sheedy, C. 2010a. Sorption of four estrogens by surface soils from 41 cultivated fields in Alberta, Canada. Geoderma 155:19-30.

Caron, E., Farenhorst, A., Mc Queen, R., Sheedy, C., Goddard, T., Gaultier, J. 2010b. Mineralization of 17ß-estradiol in 36 surface Soils from Alberta, Canada.In PRESS, Agric. Ecosyst. Environ.

Casey, F.X.M., Larsen, G.L., Hakk, H. and Simunek, J. 2003. Fate and transport of 17β-estradiol in soil-water systems. Environ. Sci. Technol., 37:2400-2409.

Casey, F.X.M., Šimůnek, J., Lee, J., Larsen, G.L. and Hakk, H. 2005. Sorption, mobility, and transformation of estrogenic hormones in natural soils. J. Environ. Qual., 34:1372-179.

Chang, C., Sommerfelt, T.G. and Entz, T. 1991. Soil chemistry after 11 annual applications of cattle feedlot manure. J. Environ. Qual., 20(2):475-480.

Chang, C. and Entz, T. 1996. Nitrate leaching losses under repeated cattle feedlot manure applications in southern Alberta. J. Environ. Qual. 25:145-153.

Chang, C. and Janzen, H.H. 1996. Long-term fate of nitrogen from annual feedlot manure applications. J. Environ. Qual. 25:785-790.

Chang, C., Whalen, J.K. and Hao, X. 2005. Increase in phosphorus concentration of a clay loam surface soil receiving repeated annual feedlot cattle manure applications in southern Alberta. Can. J. Soil Sci. 85:589-597.

Colucci, M.S., Bork, H. and Topp, E. 2001. Persistence of estrogenic hormones in agricultural soils: I. 17β-estradiol and estrone. J. Environ. Qual., 30:2070-2076.

Cullimore, D.R. 1981. The enumeration of 2,4-D degraders in Saskatchewan soils. Weed Sci., 29(4):440-443.

Das, B.S., Lee, L.S., Rao, P.S. and Hultgren, R.P. 2004. Sorption and degradation of steroid hormones in soils during transport: Column studies and model evaluation. Environ. Sci. Technol., 38:1460-1470.

Fan, Z., Casey, F.X.M., Hakk, H. and Larsen, G.L. 2007. Persistence and fate of 17ß-estradiol and testosterone in agricultural soils. Chemosphere, 67:886-895.

Finlay-Moore, O., Hartel P.G. and Cabrera, M.L. 2000. 17β-Estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. J. Environ. Qual., 29:1604-1611.

Fujino, C., Wada, S., Konoike, T., Toyota, K., Suga, Y. and Ikeda, J. 2008. Effect of different organic amendments on the resistance and resilience of the organic matter decomposing ability of soil and the role of aggregated soil structure. Soil Sci. Plant Nutr., 54(4):534-542.

Gao, G. and Chang, C. 1996. Changes in CEC and particle size distribution of soils associated with long-term annual applications of cattle feedlot manure. Soil Sci., 161(2):115-120.

Gami, S.K., Lauren, J.G. and Duxbury, J.M. 2009. Soil organic carbon and nitrogen stocks in Nepal long-term soil fertility experiments, Soil Tillage Res., 106(1):95-103.

Gaultier, J., Farenhorst, A., Cathcart, J. and Goddard, T. 2008b. Degradation of [carboxyl-^{14}C] 2,4-D and [ring-U-^{14}C] 2,4-D in 114 agricultural soils as affected by organic carbon content. Soil Biol. Biochem., 40(1):217-227.

Gee, G.W. and Bauder, J.W. 1986. Particle size analysis. Pages 383-412. In A. Klute, ed. *Methods of soil Analysis*. Part 1. Physical and Mineralogical Methods. ASA/SSSA, Madison, WI.

Greer, L.E. and Shelton, D.R. 1992. Effect of inoculant strain and organic matter content on kinetics of 2,4-Dichlorophenoxiacetic acid degradation in soils. App. Environ. Microbiol., 58:1459-1465.

Hati, K.A., Swarup, A., Dwivedi, A.K., Misra, A.K., Bandyopadyay, K.K. 2007. Changes in soil physical properties and organic carbon status at the topsoil horizon of a vertisol of central India after 28 years of continuous cropping, fertiization and manuring. Agr. Ecosyst. Environ., 119(1-2):127-134.

Hao, X. and Chang, C. 2002. Effect of 25 annual cattle manure applications on soluble and exchangeable cations in soil. Soil Sci. 167(2):126-134.

Hao, X. and Chang, C. 2003. Does long-term heavy cattle manure application increase salinity of a clay loam soil in semi-arid southern Alberta? Agric. Ecosys. Environ. 94:89-103.

Hao, X., Chang, C., Travis, G.R. and Zhang, F. 2003. Soil carbon and nitrogen response to 25 annual cattle manure applications. J. Plant Nutr. Soil Sci. 166(2):239-245.

Hao, X., Chang, C. and Li, X. 2004. Long-term and residual effects of cattle manure application on distribution of P in soil aggregates. Soil Sci., 169(10):715-728.

He, Z., Tazisong, I.A., Senwo, Z.N. and Zhang, D. 2008. Soil properties and macro cations status impacted by long-term applied poultry litter. Commun. Soil Sci. Plant Anal., 39(5-6):858-872.

Hemmings, S.J.N. and Hartel, P.G. 2006. Mineralization of hormones in breeder and broiler litters at different water potentials and temperature. J. Environ. Qual., 35:701-706

Hildebrand, C., Londry, K.L. and Farenhorst, A. 2006. Sorption and desorption of three endocrine disrupters in soils. J. Environ. Sci. Health B, 41:907-921.

Huang, Q.R., Hu, F., Huang, S., Li, H.-X., Yuan, Y.-H., Pan, G.X. and Zhand, W.J. 2009. Effect of long-term fertilization on organic carbon and nitrogen in a subtropical paddy soil. Pedosphere, 19(6):727-734.

Huang, S., Peng, X, Huang, Q and Zhang, W. 2010. Soil aggregation and organic carbon fractions affected by long-term fertilization in a red soil of subtropical China. Geoderma, 154(3-4):364-369.

Kissinger, W.F., Koelsch, R.K., Erickson, G.E. and Klopfenstein, T.J. 2007. Characters of manure harvested from beef cattle feedlot. Appl. Eng. Agric.,23(3):357:365.

Kozarek, J.L., Wolfe, M.L., Love, N.G. and Knowlton, K.F. 2008. Sorption of estrogen to three agricultural soils from Virginia, USA. Trans ASABE, 51(5):1591-1597.

Kryzanowski, L. 1993. Agricultural Soils and Animal Nutrition Laboratory, Alberta Agriculture. In Rennie, D.A. 1993. Chapter 8: Trends in available soil N,P,K and S. In A Review of the impact of macronutrients on crop responses and environmental sustainability on the Canadian Prairies. D.A.Rennie, C.A.Campbell and T.L.Roberts (eds.). Canadian Society of Soil Science.

Lai, K.M., Scrimshaw, M.D. and Lester, J.N. 2002. The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit. Rev. Toxicol., 32 (2):113-132.

Layton, A.C., Gregory, B.W., Seward, J.R., Schultz, T.W. and Sayler, G.S. **2000**. Mineralization of steroidal hormones by biosolids in wastewater treatment systems in Tennessee U.S.A. Environ. Sci. Technol., 34:3925-3931.

Lee, L.S., Strock, T.J. Sarmah, A.K. and Rao, P.S.C. 2003. Sorption and dissipation of testosterone, estrogens and their transformation products in soil and sediment. Environ. Sci. Technol. 37:4098-4105.

Legendre, P. and L. Legendre. 1998. Numerical Ecology. 2nd Ed. Elsevier B.V., Amsterdam, The Netherlands.

Lithourgidis, A.S., Matsi, T., Barbayiannis, N. and Dordas, C.A. 2007. Effect of liquid cattle manure on corn yield, composition, and soil properties. Agron. J., 99(4):1041-1047.

Loffredo, E and Senesi, N. 2006. Fate of organic pollutants in soils with emphasis on adsorption/desorption processes of endocrine disruptor compounds. Pure Appl. Chem., 78(5):947-961.

Lotrario, J.B., Stuart, J.B., Lam, T., Arands, R.R., O`Connor, O.A. and Kosson, D.S. **1995**. Effects of Sterilization Methods on the Physical Characteristics of soil: Implications for sorption isotherm analyses, Bull. Environ. Contam. Toxicol., 54:668-675.

Lovitskii, P.S. 2005. Changes in the properties of dark chesnut soil soils under the impact of long-term irrigation (the Kakhovka irrigation system). Eurasian Soil Sci., 38(5):551-562.

Lucas, S.D. and Jones, D.L. 2006. Biodegradation of estrone and 17β-estradiol in grassland soils amended with animal wastes. Soil Biol. Biochem.,38:2803-2815.

Maynard, D.G., Kalra, Y.P. and Crumbaugh, J.A. 2008. Extraction of NO3-N and NH4-N with 2.0M KCl. In M.R. Carter and E.G. Gregorich, Canadian Society of Soil Science**,** CRC Press, *Soil Sampling and Methods of Analysis* 2nd Ed. chapter 6, pp 72, Taylor & Francis Group, LLC.

McKeague, J.A. (ed) 1978. Manual on soil sampling and methods of analysis. Canadian Society of Soil Science, Ottawa, Canada, 212 pp.

Mugwe, J., Mugendi, D., Mucheru-Muna, M., Odee, D. and Mairura, F. 2009. Effect of selected organic materials and inorganic fertilizer on the soil fertility of a Humic Nitisol in the central highlands of Kenya. Soil Use Manage., 25(4):434-440.

Nelson, D.E. and Sommers, L.E. 1982. Total carbon, organic carbon, and organic matter. In A.L. Page, ed. Methods of Soil Analysis. Part 2. Chemical and microbial properties. ASA/SSSA, Madison, WI. pp. 539-577.

Nichols, D.J., Daniel, T.C., Moore, P.A. Jr, Edwards, D.R. and Pote, D.H. 1997. Runoff of estrogen hormone 17β-Estradiol from poultry litter applied to pasture. J. Environ. Qual., 26:1002-1006.

Nunes, J.M., Lopez-Pineiro, A., Albarran, A., Munoz, A., and Coelho, J. 2007. Changes in selected soil properties caused by 30 years of continous irrigation under Mediterranean conditions. Geoderma, 139(3-4):321-328.

Ogram, A.V., Jessup, R.E., Ou, L.-T. and Rao, P.S.C. 1985. Effect of sorption on biological degradation rates of 2,4-dichlorophenoxyacetic acid in soils. App. Environ. Microbiol., 49:582-587.

Robertson, B.K. and Alexander, M. 1994. Growth-linked and cometabolic biodegradation: possible reason for occurrence or absence of accelerated pesticide biodegradation. Pest. Sci., 41:311-318.

Saison, C., Degrange, V., Oliver, R., Millard, P., Commeaux, C., Montange, D. and Le Roux, X. 2006. Alteration and resilience of the soil microbial community following compost amendment: effects of compost level and compost-borne microbial community. Environ. Microbiol., 8(2):247-257.

Sangsupan, H.A., Radcliffe, D.E., Hartel, P.G., Jenkins, M. B., Vencil, W.K. and Cabrera, M.L.2006. Sorption and transport of 17β-estradiol and testosterone in undisturbed soil columns. J. Environ. Qual. 35:2261-2272.

Schoenau, J.J. and O'Halloran, I.P. 2008. Sodium Bicarbonate-Extractable Inorganic P (Olsen et al. 1954), In M.R. Carter and E.G. Gregorich, Canadian Society of Soil Science CRC Press , Soil Sampling and Methods of Analysis 2nd Ed., chapter 8, pp 90, Taylor & Francis Group, LLC.

Shaw, L.J. and Burns, R.G. 1998. Biodegradation of 2,4-D in a non-contaminated grassland profile. J. Environ. Qual., 27:1464-1471.

Smith, A.E. and Aubin, A.J. 1991. Effects of long-term 2,4-D and MCPA field applications on the soil breakdown of 2,4-D, MCPA, mecocrop and 2,4,5-T. J. Environ. Qual., 20:436-438.

Sommerfelt, T.G. and Chang, C. 1985. Changes in soil properties under annual application of feedlot manure and different tillage practices. Soil Sci. Soc. Am. J., 49:983-987.

Sommerfelt, T.G., Chang, C. and Entz, T. 1988. Long-term annual manure applications increase soil organic matter and nitrogen and decrease carbon to nitrogen ratio. Soil Sci. Soc. Am. J. 52:1668-1672.

Stumm-Zollinger, E. and Fair, G.M. 1965. Biodegradation of steroids hormones. Steroid Horm., 37(11):1506-1511.

Stumpe, B. and Marschner, B. 2007. Long-term sewage sludge application and wastewater irrigation on the mineralization and sorption of 17β-estradiol and testosterone in soils. Sci. Total Environ. 374:282-291.

Stumpe, B. and Marschner, B. 2010. Organic waste effects on the behavior of 17β-estradiol, estrone and 17α-ethinylestradiol in agricultural soils in long-and short-term setups. J. Environ. Qual., 39:907-916.

Van Emmerik, T., Angove, M.J., Johnson, B.B., Wells, J.D. and Fernandes, M.B. 2003. Sorption of 17*β*-estradiol onto selected soil minerals. J. Colloid Interface Sci., 266:33-39.

Vogeler, I. 2009. Effect of long-term wastewater application on physical soil properties. Water Air Soil Poll., 196(1-4):385-392.

Watanabe, A., Kawasaki, S., Kitamura, S and Yoshida, S. 2007. Temporal changes in humis acids in cultivated soils with continuous manure application. Soil Sci. Plant Nutr., 53(5):535-544.

Wolf, D.C., Hao, T.H., Scott, H.D. and Lavy, T.L. 1989. Influence of sterilization methods on selected soil microbial, physical and chemical properties. J. Environ.Qual.,18:39-44.

Xuan, R., Blassengales, A.A. and Wang, Q. 2008. Degradation of estrogenic hormones in a silt loam soil. J. Agric. Food Chem., 56:9152-9158.

Ying, G.-G. and Kookana, R.S. 2005. Sorption and degradation of estrogen-like – endocrine disrupting chemicals in soil. Environ. Toxicol. Chem., 24(10):2640-2645.

Yoshimoto, T., Nagai, F., Fujimoto, J., Watanabe, K., Mizukoshi, H., Makino, T., Kimura, K., Saino, H., Sawada, H. and Omura, H. 2004. Degradation of estrogens by *Rhodococcus zopfii* and *Rhodococcus equi* isolates from activated sludge in wastewater treatment plants. Appl. Environ. Microbiol.,70(9):5283-5289.

Yu, C.-P., Roh, H. and Chu, K.-H. 2007. 17 beta-estradiol-degrading bacteria isolated from activated sludge. Environ. Sci. Technol., 41(2):486-492.

5. DEVELOPMENT OF COMPETITIVE ELISAS FOR 17B-ESTRADIOL AND 17B-ESTRADIOL+ESTRONE+ESTRIOL USING RABBIT POLYCLONAL ANTIBODIES

Caron E., Sheedy, C. and Farenhorst, A. 2010. Development of Competitive ELISAs for 17β-Estradiol and 17β-Estradiol+Estrone+Estriol using rabbit polyclonal antibodies. Journal of Environmental Science and Health Part B: Pesticides, food contaminants and agricultural wastes 45(2):145-151. Reproduced with permission from the editor.

5.1 Abstract

Estrogens are a family of feminizing hormones that are excreted by vertebrates. It has been documented that their presence in surface waters, even in the ng/L range, can have detrimental impacts on fish reproduction. Two competitive enzyme-linked immunosorbent assays using rabbit polyclonal antibodies were developed: one for 17β-estradiol and a second one for 17β-estradiol (E2)+estrone (E1)+estriol (E3). Two different conjugates were synthesized using the Mixed-anhydride (for the 17β-estradiol ELISA) and the Mannich (for the E1+E2+E3 ELISA) reactions. The 17β-estradiol ELISA was highly specific with an IC_{50} of 243 ng mL^{-1} for 17β-estradiol. The E1+E2+E3 ELISA exhibited cross-reactivity with estrone (85%) and estriol (62%) with an IC_{50} of 18 ng mL^{-1} for 17β-estradiol. Cross-reactivity was tested against 13 chemically related compounds and both immunoassays showed significant cross-reactivity with two estradiol conjugates: βestradiol-17-valerate and βestradiol-3-benzoate (from 57 to 84 %)

for which, to our knowledge, there are currently no commercially available ELISA. Characteristics (sensitivity, inter and intra assay variation, and cross-reactivity) of the E1+E2+E3 ELISA were further compared to those from a commercial estriol ELISA. The commercial ELISA was more specific, sensitive and its inter-assay variation was less (9.5% compared to 10% for the E1+E2+E3 ELISA) but the E1+E2+E3 ELISA had less intra-assay variation (4% compared to 5% for the commercial ELISA). Finally, a solid-phase extraction method compatible with the E1+E2+E3 immunoassay demonstrated that this combined approach of extraction and immunoassay had good potential for determining estrogen concentrations in environmental samples such as surface water in urban and agricultural ecosystems.

5.2 Introduction

Estrogens are compounds known to induce production of vitellogenin in fish when present in surface waters at concentrations as low as one ng/L (Tyler and Routledge, 1998). They are naturally produced and excreted by vertebrates, including humans and livestock. The concentrations and relative proportions of the excreted estrogens vary among species (Palme et al., 1996). Estrogens are sometimes conjugated to sulphates and glucuronides in order to facilitate their excretion and the conjugated forms can be found in both feces and urine (Ivie et al., 1986; Palme et al., 1996). Estrogens can be introduced in the environment by the release of wastewater from sewage treatment plants or the application of manure to agricultural land, for example.

Enzyme-linked immunosorbent assays (ELISAs) have gained popularity for the determination of estrogens in a wide variety of environmental samples because ELISAs exhibit low limits of detection, are user-friendly and cost effective, and samples can be processed more rapidly than analyses by conventional detection methods such as gas chromatography (GC) and high pressure liquid chromatography (HPLC).

Since the first development of antisera specific against estrogens (Chung-Hsiu and Lundy, 1971; Lindner et al., 1971), several ELISAs for the determination of estrogens have been developed commercially. Commercial ELISAs are now commonly used to detect estrogens in matrices of urban origin such as wastewaters from sewage treatment plants (Dorabawila and Gupta, 2001; Drewes et al., 2005; Hintemann et al., 2006; Lee et al., 2006; Suzuki and Maruyama, 2006). ELISAs are also commonly used in studies involving livestock manures and their application to agricultural land including poultry litter (Jenkins et al., 2006), broiler litter (Finlay-Moore et al., 2000), dairy manure wastewater (Hanselman et al., 2004) and swine and dairy waste (Raj Raman et al., 2004). They were also successfully used to study the impact of best management practices on exportation of estrogens from the farm to the environment (Nichols et al., 1997).

ELISAs have been compared with conventional chemistry techniques in a few publications. For 17ß-estradiol, the method detection limits for the commercial direct ELISA varied between 0.05 ng L^{-1} in surface waters to 0.1 ng L^{-1} for wastewater while for gas chromatography tandem mass spectrometry (GC-MS/MS), detection limits ranged from 0.2 to 0.4 ng L^{-1} (Huang and Sedlack, 2001). In the case of GC-MS/MS those limits

were influenced not only by the type of matrix but they also varied with instrument performance. In addition, contrary to traditional analysis by GC, ELISAs do not require any prior derivatization (Gray and Sedlack, 2005) and are therefore not subject to laboratory errors and cost associated with this extra step. ELISAs are also faster and less expensive than conventional chemistry techniques (Farré et al., 2006). However, ELISAs are not generally able to discriminate concentrations specific for the conjugated and free forms of estrogens (Suzuki and Maruyama, 2006).

In the present work, two ELISAs were developed: the first one as a broad scan for estrogens (17β-estradiol, estrone and estriol more specifically because they are the predominant natural estrogens found in the environment) and a second ELISA specific for the most potent of these estrogenic compounds, 17β-estradiol.

5.3 Material and Methods

5.3.1 Conjugation reactions

5.3.1.1 Mannich Reaction. All chemicals used for conjugation reactions and Phosphate Buffered Saline (PBS) were from Sigma-Aldrich Chemical Company (St. Louis, MO) and exhibited a purity of at least 95% (with analytical grade 17ß-estradiol, estrone and estriol being at least 98% pure, Figure 5.1). The Mannich reaction was used for the synthesis of estrone:OVA (ovalbumin) and estriol:OVA conjugates. 20 mg of OVA were dissolved in two mL of morpholinoethanesulfonic acid (MES) (pH 4.7). An amount

measuring 10 mg of hapten were dissolved in one mL ethanol, 500 μL of this hapten solution were taken and added to 500 μL MES buffer. A volume of 800 μL of this final hapten solution was added to 800 μL of the OVA solution and 200 μL of formaldehyde 37% were added. This mixture measuring 1.8 mL was left to stir three hours at 20^{o}C and was dialyzed overnight in PBS.

5.3.1.2 Mixed Anhydride Reaction. A mixed anhydride estradiol:OVA conjugate was synthesized following the protocol described in Yau et al (1998). Briefly, 100 μmol (35. 9 mg) of the hapten β-estradiol-6-one-6-(O-carboxymethyloxime) was dissolved in 250 μL of ethanol. Volumes measuring 250 μL of 1,4 dioxane, 7.5 μL of triethylamine and 7.5 μL of isobutyl chloroformate were added to the hapten solution. This mixture was vortexed, left to react for 30 minutes at 20^{o}C and filtered on glass wool. The filtrate was then added dropwise to 1.5 mL of 0.2 M $NaHC0_3$ (pH 9.5) in which 22 mg of OVA had previously been dissolved. This mixture was stirred for six hours at 20^{o}C and then dialyzed for 24 hours with four changes of ultra-pure water.

The compound β-estradiol 6-(O-carboxy-methyl)oxime:BSA (Bovine serum albumin) was purchased from Sigma-Aldrich Chemical Company, St. Louis, MO. In addition, estrone:BSA and estriol:BSA immunogens were synthesized using the Mannich reaction according to the same protocol described for the estrone:OVA and estriol:OVA conjugates. An estradiol:BSA immunogen was synthesized using the mixed anhydride reaction protocol described for the estradiol:OVA conjugate synthesis.

Figure 5.1A

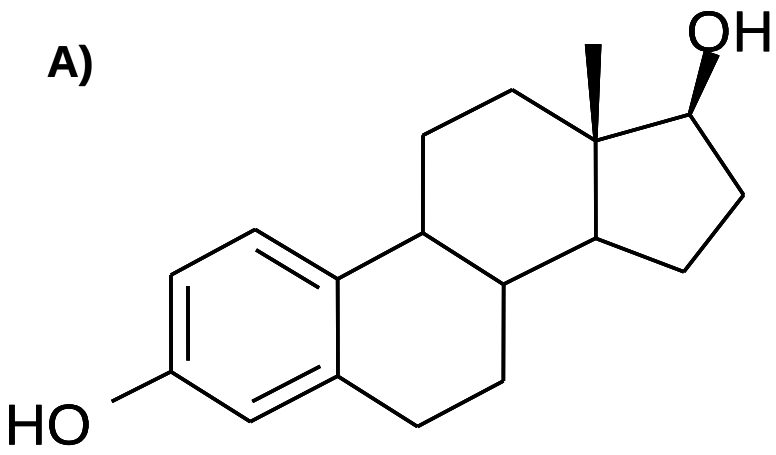


Figure 5.1B

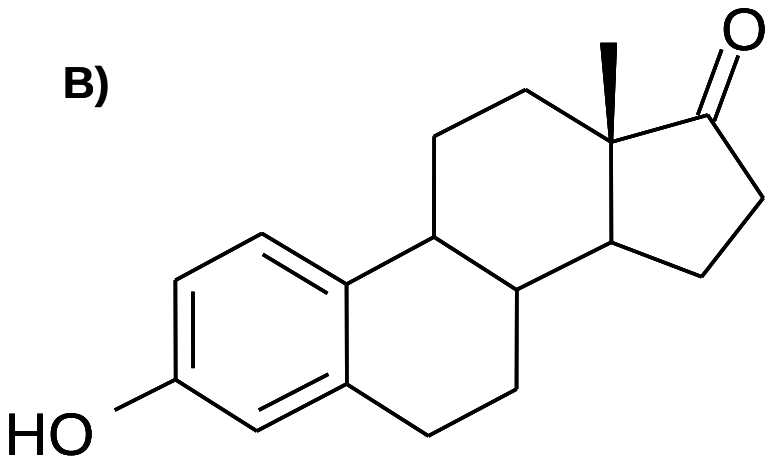


Figure 5.1C

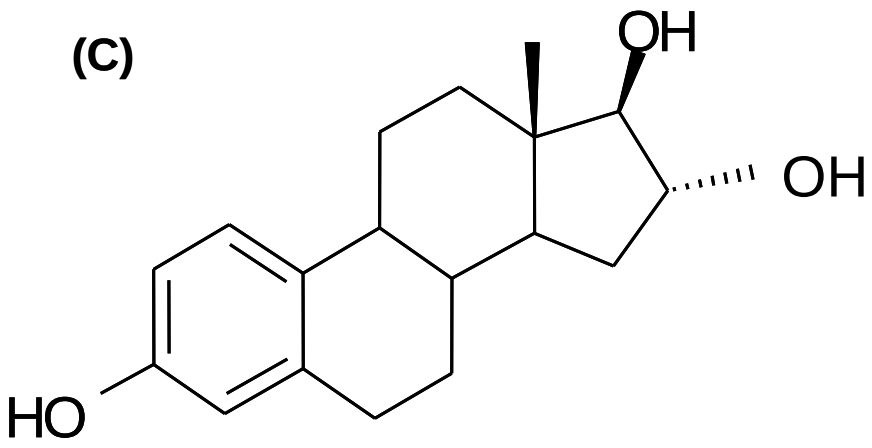


Figure 5.1: Structure of the three haptens: A 17β-estradiol, B) estrone and C) estriol

5.3.2 Immunizations

For the E1+E2+E3 ELISA, 18 New-Zealand white female rabbits were immunized (six with β-estradiol 6-(O-carboxy-methyl)oxime:BSA, six with estrone:BSA and six with estriol:BSA). The first injection was prepared in Freund's complete adjuvant (Sigma-Aldrich Chemical Company, St-Louis, MO) at a concentration of 0.5 mg conjugate mL^{-1}. The following booster shots were administered every three weeks over a period of six months in Freund's incomplete adjuvant (Sigma-Aldrich Chemical Company, St-Louis, MO), at 0.5 mg conjugate mL^{-1}.

For the 17β-estradiol ELISA, two New-Zealand white male rabbits (Rabbit 1 and 2) were immunized as follows with the mixed anhydride estradiol:BSA immunogen: first

injection at a concentration of 0.5 mg conjugate mL^{-1}, then booster shots once per week during three weeks at a concentration of 0.2 mg conjugate mL^{-1} and finally once per month for two months at the same concentration (Rao and Moore, 1977). Immunogens were prepared in Titer Max Gold (Sigma-Aldrich Chemical Company, St-Louis, MO).

5.3.3 Selection of Final Sera

Checkerboard ELISA was used to screen the final sera. Each serum was tested against both types of conjugates (Mannich and Mixed Anhydride) for its corresponding hapten. Respective working concentrations for serum and conjugate were obtained when B/Bo (where B=optical density of the standard and Bo=optical density of the 0 pg mL^{-1} standard) was near one. Inhibition curves with hapten concentrations ranging from 0.01 to 10 000 ng mL^{-1} were obtained using the optimized dilutions of sera and conjugate for each ELISA as determined by the checkerboard ELISA.

5.3.4 ELISA Protocols

Only the final serum from one immunized rabbit from each experiment was chosen: “Serum Green” immunized with β-estradiol 6-(O-carboxy-methyl)oxime:BSA for the E1+E2+E3 ELISA and “Serum 2” for the 17β-estradiol ELISA. During prior testing for the development of optimized protocols, it was also noticed that incubations for four hours or longer could lead to the partial degradation of the conjugate. For both ELISAs,

microtiter plate wells were coated with 100 µL per well of mixed anhydride estradiol:OVA conjugate (dilution 1/6400 in PBS for the E1+E2+E3 ELISA and dilution 1/3200 in PBS for the 17ß-estradiol ELISA) and incubated for two hours at 37°C. Wells were emptied, blocked with 200 µL per well of 3% milk in PBS, incubated one hour at room temperature and washed three times with PBS-Tween. During that time, samples were incubated for one hour at 20°C with an equal volume of a serum dilution (1/800 dilution of rabbit Green Serum in PBS for the E1+E2+E3 ELISA and 1/800 dilution of rabbit 2 Serum in PBS for the 17ß-estradiol ELISA). Amounts measuring 100 µL per well of that mix were added to each well and let to stand at 20°C for one hour. Wells were washed three times with PBS-Tween. 100 µL per well of HRPO goat anti-rabbit antibody (dilution according to manufacturer's instructions) were added to each well, let to stand for one hour at 20°C and washed three times with PBS-Tween. A volume of 100 µL per well of ABTS peroxidase substrate was added to each well and let to stand for 30 minutes at 20°C before reading at 415nm on a Bio-Rad Model 680 (Mississauga, ON) microtiter plate reader. Total time of analysis was 5.5 hours. Horseradish Peroxidase (HRPO) goat anti-rabbit antibody was obtained from Cedarlane Laboratories Limited, Hornby, ON, and ABTS (2,2'azino-di(3-ethylbenzthiazoline-6-sulphate) peroxidase substrate system was from Kirkegaard and Perry Laboratories (KPL), Gaithersburg, MD.

5.3.5 Characterization

Cross-reactivity was measured by comparing the IC_{50} (the concentration of estrogens necessary to inhibit signal development by 50%) for 17β-estradiol with those of

chemically related compounds obtained from calibration curves made on the same day, on the same plate with three wells for each concentration on the calibration curve. Cross-reactivity was tested against 13 compounds including three other natural estrogenic compounds (estrone, estriol and 17α-estradiol), one gestagen (progesterone), one androgen (testosterone), two corticosteroids (aldosterone, hydrocortisone), two naturally conjugated estrogens ([estradiol-17 (B-D glucuronide) sodium salt], and [estradiol-3 (B-D glucuronide) sodium salt]), three synthetic estrogens (ethinyl estradiol, βestradiol-17-valerate and βestradiol-3-benzoate) and one herbicide (atrazine) with known estrogenic activity (Nishihara et al., 2000). For cross-reactivity determination, all chemicals were of analytical grade and purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) and exhibited a purity of at least 95% except for atrazine (99% purity, analytical-grade; Riedel de Häen, Seelze, Germany).

5.3.6 Commercial ELISA

Using estriol as a free hapten, the characteristics (sensitivity, inter-assay variation, intra-assay variation and cross-reactivities) of the E1+E2+E3 ELISA were contrasted to the commercially available estriol EIA kit (strip-plate format) from Cayman Chemical Company (Ann Arbor, MI) in order to determine if the E1+E2+E3 ELISA presented some potential for the reliable measurement of estriol concentrations in environmental samples. Estriol was chosen for this test due to the linearity of the calibration curve of the E1+E2+E3 ELISA for this hapten. We decided not to do the same study using the 17β-estradiol and a commercially available 17β-estradiol EIA kit due to the fact that the 17β-

estradiol had a higher IC_{50} than the E1+E2+E3 ELISA and did not show a typical sigmoid calibration curve. Since the ranges of the estriol EIA kit and the E1+E2+E3 ELISA were different, each assay was tested for the following characteristics (inter-assay variation, intra-assay variation, sensitivity and cross-reactivity) within its own working range. For the E1+E2+E3 ELISA standards were 0, 0.01, 0.1, 1, 10, 100, 1000 and 10 000 ng mL^{-1} and for the Estriol ELISA, standards were 7.8, 15.6, 31.3, 62.5, 125, 250, 500, and a 1000 pg mL^{-1}.

In addition, the estriol antiserum provided in the Estriol kit was tested as a replacement for the E1+E2+E3 antiserum in the E1+E2+E3 ELISA using the dilution recommended for the commercial kit but with the same protocol as described above for the E1+E2+E3 ELISA, in order to see if the commercially available antibody was compatible with the present E1+E2+E3 ELISA format.

5.3.6.1 Data Analysis for the Comparison of the Two Immunoassays. Parameters for the two assays were calculated according to Hanselman et al. (2004). Briefly, the sensitivity was calculated as the mean of seven replicates of a 0 pg mL^{-1} standard minus twice the standard deviation. Intra-assay variation was calculated on the entire calibration curve by averaging the coefficients of variation of all the standards. Inter-assay variation was obtained by calculating the difference between the B/Bo for each standard and averaging this difference for the entire calibration curve. The inter-assay variation was repeated on two different days using fresh standards each time for both ELISAs.

5.3.7 Extraction Method of Estrogens in Water and Validation

We used the following extraction method of estrogens from water as previously described in Thompson et al. (2009) and Waters Corp. (2008). The HLB (Hydrophilic-lipophilic Balance) Oasis 3cc cartridge (Waters, Milford, MA) was conditioned using 3 mL of diethyl ether (BDH, Toronto, ON), 3 mL of Methanol (EMD Chemicals Inc, Gibbstown, NJ), and 3 mL of Milli-Q water. The 200 mL spiked water sample was then loaded onto the cartridge. The cartridge was rinsed sequentially with 1 mL 40% methanol, 1mL Milli-Q water and 1 mL of 10% methanol: 2% NH_4OH (Fisher Scientific, Ottawa, ON) in Milli-Q water. Elution was made using 2 mL of methanol, the extract was then taken to dryness under 0 grade nitrogen and reconstituted in 1:1 methanol, Milli-Q water.

In order to test the compatibility between the extraction method and the E1+E2+E3 ELISA, three spikes (1, 10 and 100 ng mL^{-1}, each in triplicate) of estriol and a blank (0 ng mL^{-1}, in triplicate) were prepared in water collected from an edge-of-field experiment in June 2008 and extracted. Spikes were prepared using a fresh solution of estriol in ethanol (EMD Chemicals Inc, Gibbstown, NJ) and extracted the same day. Extracts were kept frozen overnight at $-20^{o}C$ and analyzed by the E1+E2+E3 ELISA on the day following the extraction. A 50 μL sample of the extract were analyzed by ELISA following a dilution in 200 μL PBS. The results of the ELISA readings for these extracts were then compared to a calibration curve made in PBS and to another calibration curve containing 10 % of methanol in PBS. This concentration of 10% was chosen to be in

accordance with the 10 % methanol present in the spikes after they went through the extraction method and the dilution prior to the ELISA.

5.4 Results and Discussion

5.4.1 Assay Characterization

Typical calibration curves obtained for both ELISAs with 17β-estradiol as the free hapten or inhibitor are shown in Figure 5.2. The calibration curves for the E1+E2+E3 ELISA with estrone and estriol as the inhibitor are also shown. The 17β-estradiol ELISA had an IC_{50} of 243 ng mL^{-1} and a working range between 0.1 and 10 000 ng mL^{-1}. The intra-assay variation was 5.2% and the inter-assay variation was 5%. For the E1+E2+E3 ELISA, the working range is between 1 and 100 ng mL^{-1} with an IC_{50} of 18 ng mL^{-1} for 17β-estradiol. The intra-assay variation was 5% and the inter-assay variation was 9.5%.

The E1+E2+E3 ELISA showed high cross-reactivity to natural estrogens, estrone (85%) and estriol (62%), and synthetic estrogens, βestradiol-3-benzoate (77%) and βestradiol-17-valerate (84%), which makes it a valuable screening tool for the presence of those estrogens in water samples (Table 5.1). Screening might be suitable when the analysis of a large number of samples is required with a general objective to monitor for natural and synthetic estrogens while costs are limited for confirmatory analysis by GC or HPLC. The 17β-estradiol ELISA did not exhibit cross-reactivity with estrone nor estriol, and showed lesser cross-reactivity to the synthetic estrogens, βestradiol-3-benzoate (72%) and βestradiol-17-valerate (57 %) than the E1+E2+E3 ELISA (Table 5.1). Neither the

17β- estradiol ELISA nor the E1+E2+E3 ELISA demonstrated other cross-reactivities. Zhao et al. (2007) observed that antibodies generated against estrogens conjugated at C-3, C-4 or C-6 are generally more specific than when conjugated at C-16 or C-17. This is likely due to a better exposition of functional groups unique to this moiety (den Hollander et al., 1974; Zhao et al., 2007). The compounds βestradiol-3-benzoate and βestradiol-17-valerate are synthetic estrogenic compounds that are sometimes used in the cattle industry. Therefore, they could potentially be present in surface waters in dense agricultural areas and the present assays would be able to detect them. To our knowledge, there are no commercially available ELISA made to detect those compounds.

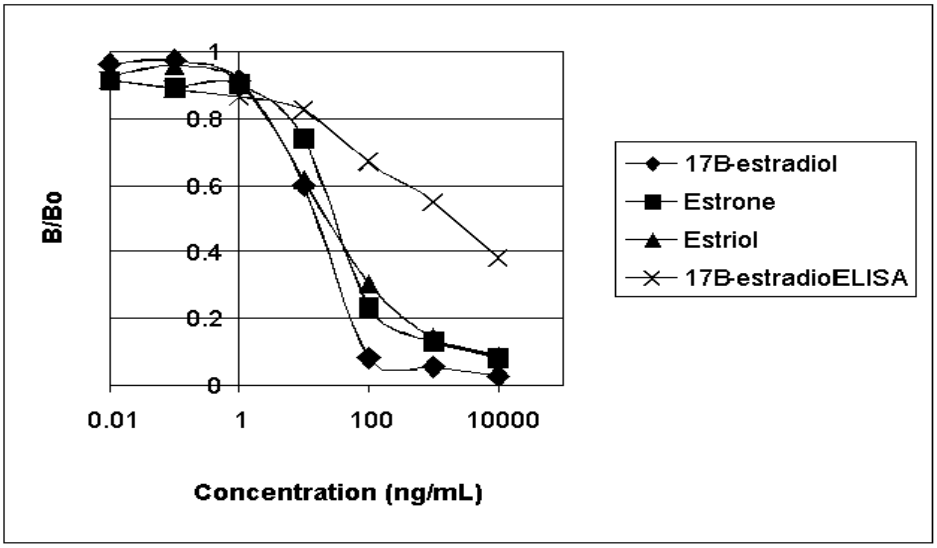


Figure 5.2: Calibration curves for the E1+E2+E3 ELISA (17β-estradiol, estrone and estriol as inhibitor) and for the 17β-estradiol ELISA.

Note: concentration is on logarithmic scale.

None of the ELISAs developed in the present study showed cross-reactivity to the conjugated estrogens estradiol-17 (B-D glucuronide) sodium salt and estradiol-3 (B-D glucuronide) sodium salt. It has previously been documented that common fecal bacteria, such as *Escherichia coli*, could hydrolyze conjugated estrogens (Belfroid et al., 1999) and that 90% of estrone glucuronide was deconjugated within 30 minutes in sow feces (Vos, 1996). Once unconjugated, these free estrogens could be detected by the ELISAs.

Other conjugation techniques have also been used for the 1,3,5(10)estratrien-3,17-diol-6-one-6-carboxymethyloxime (hapten conjugated to BSA in the present study). In Hintemann et al. (2006), 1,3,5(10)estratrien-3,17-diol-6-one-6-carboxymethyloxime was activated with N-hydroxysuccinimide and N,N-dicyclohexylcarbodiimide in order to be conjugated to peroxidase. The competitive ELISA from Hintemann et al. (2006) had a limit of detection of 0.05 ng 17ß-estradiol L^{-1} (taking into account a 50 x concentration factor) and a working range of 0.28-590 ng L^{-1}. The conjugation method used by Hintemann et al. (2006) had previously been used for the conjugation of triazines (Schneider and Hammock, 1992). The 17ß-estradiol-ELISA developed by Hintemann et al. (2006) was highly specific and showed significant cross-reactivity only for sulfated and glucuronide estrogens conjugated at the C-3 position (9 and 25% respectively) (Hintemann et al., 2006). The ELISA developed by Hintemann et al. (2006) showed a lower limit of detection and a wider working range than the ELISAs developed in the present study. However, the E1+E2+E3 ELISA from the present study was capable of detecting a wider variety of estrogenic compounds that could potentially be present in the environment.

5.4.2 Commercial ELISA

The Estriol ELISA was less labour intensive than the E1+E2+E3 ELISA; it required only two steps (adding all reagents to the microtiter plate wells and color development), while for the E1+E2+E3 ELISA, all steps are made sequentially (with a triple PBS rinse between each step of coating, blocking, inhibition, addition of the secondary antibody and color development). However, the total analysis time is longer for the Estriol ELISA when using the 18 hours incubation (as recommended by the manufacturer) compared with 5.5 hours for the E1+E2+E3 ELISA. The Estriol ELISA was more specific for estriol, had a lower working range and showed less intra-assay variation than the E1+E2+E3 ELISA. However, the E1+E2+E3 ELISA had a lesser inter-assay variation than the Estriol ELISA (Table 5.2). The competitive 17ß-estradiol and Estriol EIA kits from Cayman Chemical Company have been used in soil studies. Using soil columns and batch equilibrium experiments, Mansell et al. (2004) studied the removal of estrogens by biodegradation and sorption. Limits of detections of the method were 0.4 ng L^{-1} for 17ß-estradiol and 0.6 ng L^{-1} for estriol in aqueous samples collected during those experiments, extracted with a C18 disc and analyzed by ELISA.

Table 5.1: Cross-reactivity for the E1+E2+E3 and 17β-estradiol ELISAs.

Compound	E1+E2+E3 ELISA	17β-estradiol ELISA
17β-estradiol	100%	100%
Estrone	85%	0%
Estriol	62%	0%
Testosterone	0%	0%
Progesterone	0%	0%
βestradiol-17-valerate	84%	57%
βestradiol-3-benzoate	77%	72%
Aldosterone	0%	0%
Hydrocortisone	0%	0%
17-α-estradiol	1%	0%
Estradiol-17 (B-D glucuronide) sodium salt	0%	0%
Estradiol-3 (B-D glucuronide) sodium salt	0.2%	0.2%
Ethinyl estradiol	1%	0%
Atrazine	0%	0%

5.4.3 Extraction Method of Estrogens in Water and Validation

Extracts from spikes prepared in edge of field water samples resulted in lower absorbance values than those prepared in PBS or PBS + water + 10% methanol. However, the slopes of the curves prepared in all matrices were parallel (Figure 5.3). It could therefore be

possible to make a correction on the spiked extract absorbance values which could enable the use of the extraction method combined with the E1+E2+E3 ELISA to detect the presence of estrogens in edge of field water and other environmental samples. The equation of the calibration curve for the edge of field spiked sample was y=-0.1072Ln(x)+0.9128 (R^2=0.9986), while the equation for the PBS + water + 10% methanol calibration curve was y=-0.0895Ln(x)+1.0291 (R^2=0.9753).

Table 5.2: Characteristics of the Estriol Cayman kit and the E1+E2+E3 ELISA.

	Estriol Cayman	**E1+E2+E3 ELISA**
Sensitivity	0.4 pg mL^{-1}	0.04 ng mL^{-1}
Working range	15.6 to 250 pg mL^{-1}	1 to 100 ng mL^{-1}
Inter-assay	10 %	9.5%
Intra-assay	4 %	5 %
Cross-reactivity		
17β-estradiol	0.69%	100%
Estrone	0.02%	85%
Testosterone	<0.01%	0%
Progesterone	<0.01%	0%
Ethinyl estradiol	<0.01%	1%

Note: Cross-reactivity data for the Estriol kit is from manufacturer

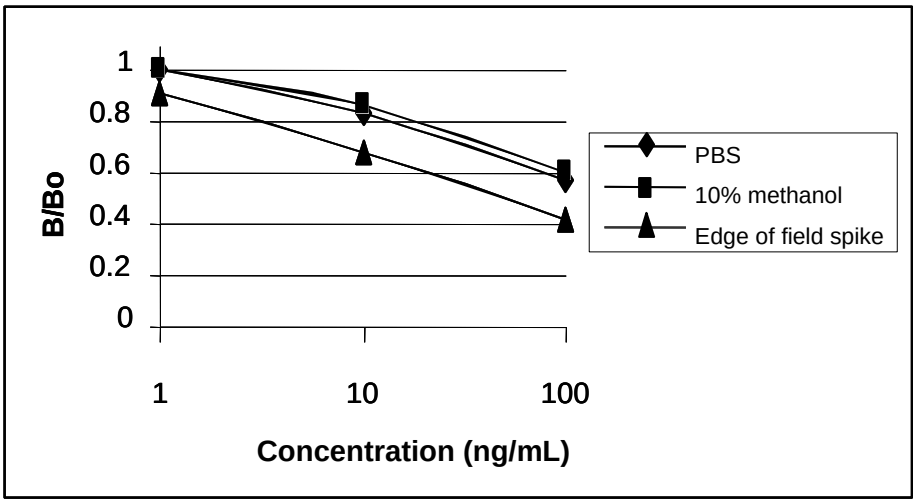


Figure 5.3: Calibration curves for the E1+E2+E3 ELISA in PBS, 10% methanol and for the extracts of spikes in edge of field water.

Note: concentration is on logarithmic scale.

5.5 Conclusion

Two competitive assays using rabbit polyclonal antibodies were developed. The 17β-estradiol ELISA is highly specific for 17β-estradiol (with cross-reactivities of 57% for βestradiol-17-valerate and 72% for βestradiol-3-benzoate) with an IC_{50} of 243 ng mL^{-1} while the E1+E2+E3 ELISA could be used as a wide screen assay for a variety of estrogens with an IC_{50} of 18 ng mL^{-1} for 17β-estradiol. The E1+E2+E3 ELISA exhibited cross-reactivity with estrone (85%), estriol (62%), βestradiol-17-valerate (84%) and βestradiol-3-benzoate (77%) but not with nine other chemicals. Both ELISAs presented good intra (about 5%) and inter-assay variability (5% for the 17β-estradiol ELISA and 9.5% for the E1+E2+E3 ELISA). The E1+E2+E3 ELISA had a lesser inter-assay variation than the commercially available Estriol ELISA. The E1+E2+E3 ELISA could

be used in conjunction with a solid-phase extraction method to process environmental samples if a correction is made to the calibration curve in 10% methanol+water+PBS.

5.6 Acknowledgments

The authors acknowledge the financial support of the Natural Sciences and Engineering Research Council of Canada. We also thank the University of Manitoba Graduate Fellowship for funding to E. Caron. The authors thank Mr. Dan Inaba, Mr. Paul Dawson, Mrs. Courtney Lamb and Ms. Brenda Pink at AAFC Lethbridge with their assistance with the laboratory experiments. We also thank Alberta Agriculture, Food and Rural Development for providing water samples.

5.7 References

Belfroid, A.C., Van der Horst, A., Vethaak, A.D., Schafer, A.J. Ris G.B.J., Wegener, J. *et al*. 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in the Netherlands. Sci. Tot. Environ., 225:101-108.

Chung-Hsiu, W and Lundy, L.E. 1971. Radioimmunoassay of plasma estrogens. Steroids 18 (1):91-111.

den Hollander, F.C., van Weemen, B.K. and Woods, G.F. 1974. Specificities of antisera against estrogens linked to albumin at different positions (c6, c11, c16, c17). Steroids 23(4):549-560.

Dorabawila, N., and Gupta, G. **2001**. Endocrine disrupter -estradiol- in Chesapeake Bay tributaries. J. Hazard. Mater., A120:67-71.

Drewes, J.E., Hemming, J., Ladenburger, S.J., Schauer, J., and Sonzogni, W. 2005. An assessment of endocrine disrupting activity changes during wastewater treatment through the use of bioassays and chemical measurements. Water Environ. Res., 77(1):12-23.

Farré, M., Brix, R., Kuster, M., Rubio, F., Goda, Y., López de Alda, M.J. and Barceló, D. 2006. Evaluation of commercial immunoassays for the detection of estrogens in water by comparison with high-performance liquid chromatography tandem mass spectrometry HPLC-MS/MS (QqQ). Anal. Bioanal. Chem., 385:1001-1011.

Finlay-Moore, O., Hartel, P.G. and Cabrera, M.L. 2000. 17β-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. J. Environ. Qual., 29:1604-1611 .

Gray, J.L. and Sedlak, D.L. 2005. The fate of estrogenic hormones in an engineered treatment wetland with dense macrophytes. Water Environ. Res., 77(1):24-31.

Hanselman, T.A., Graetz, D.A. and Wilkie, A.C. 2004. Comparison of three enzyme immunoassays for measuring 17β-estradiol in flushed dairy manure wastewater. J. Environ. Qual., 33:1919-1923.

Hintemann, T., Schneider, C., Schöler, H.F. and Schneider, R.J. 2006. Field study using two immunoassays for the determination of estradiol and ethinilestradiol in the aquatic environment. Water Res., 40:2287-2294.

Huang, C.-H. and Sedlak, D.L. 2001. Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. Environ. Toxicol. Chem., 20(1):133-139.

Ivie, G.W., Christopher, R.J., Munger, C.E. and Coppock, C.E.J. 1986. Fate and residues of [4-C-14] Estradiol-17-beta after intramuscular injection into Holstein steer calves. J. Anim. Sci., 62:681-690.

Jenkins, M.B., Endale, D.M., Schomberg, H.H. and Sharpe, R.R. 2006. Fecal bacteria and sex hormones in soil and runoff from cropped watersheds amended with poultry litter. Sci. Tot. Environ., 358:164-177.

Lee, Y.C., Wang, L.M., Xue, Y.H., Ge, N.C., Xang, X.M. and Chen, G.H. 2006. Natural estrogens in the surface water of Shenzhen at the sewage discharge of Hong Kong. Hum. Ecol. Risk Assess., 12:301-312.

Lindner, H.R., Perel, E., Friedlander, A. and Zeitlin, A. 1971. Specificity of antibodies to ovarian hormones in relation to the site of attachment of the steroid hapten to the peptide carrier. Steroids, 19(3):357-375.

Mansell, J., Drewes, J.E. and Rauch, T. 2004. Removal mechanisms of endocrine disrupting compounds (steroids) during soil aquifer treatment. Water Sci. Technol., 50 (2):229-237.

Nichols. D.J., Daniel, T.C., Moore, P.A.Jr., Edwards, D.R. and Pote, D.H. 1997. Runoff of estrogen hormone 17β-estradiol from poultry litter applied to pasture. J. Environ. Qual., 26:1002-1006.

Nishihara, T., Nishikawa, J.I., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S. and Utsimi, H. 2000. Estrogenic activities of 517 chemicals by yeast two-hybrid assay. J. Health Sci. 46(4):282-298.

Palme, R., Fischer, P., Schildorfer, H., and Ismail, M.N. 1996. Excretion of infused C-14-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci., 43:43-63.

Rao, P.N. and Moore, P.H.Jr. 1977. Synthesis of new steroidal haptens for radioimmunoassay. Part IV. 3-O-carboxymethyl ether derivatives of estrogens specific antisera for radioimmunoassay of estrone, estradiol 17β, and estriol. Steroids, 29(4):461-496.

Raj Raman, D., Williams, E.L., Layton, A.C., Burns, R.T., Easter, J.P., Daugherty, A.S., Mullen, M.D. and Syler, G.S. 2004. Estrogen content of dairy and swine wastes. Environ. Sci. Technol., 38(13):3567-3573.

Schneider, P. and Hammock, B.D. **1992**. Influence of the ELISA format and the hapten-enzyme conjugate on the sensitivity of an immunoassay for s-triazine herbicides using monoclonal antibodies. J. Agric. Food Chem., 40:525-530.

Suzuki, Y. and Maruyama, T. 2006. Fate of natural estrogens in batch mixing experiments using municipal sewage and activated sludge. Water Res., 40:1061-1069.

Thompson, M.L., Casey, F.X.M., Khan, E., Hakk, H., Larsen, G.L. and DeSutter, T. 2009. Occurrence and pathways of manure-borne 17β-estradiol in vadose zone. Chemosphere 76(4):472-479.

Tyler, C.R. and Routledge, E.J. 1998. Endocrine disruption in wildlife: A critical review of the evidence. Crit. Rev. Toxicol., 28:319-361.

Vos, E.A. **1996**. Direct ELISA for estrone measurements in the feces of sows: Prospects for rapid, sow-side pregnancy diagnosis. Theriogenology, 46:211-231.

Waters Corporation, 2008. Oasis HLB cartridges and 96-well plates care and user manual, support number USRM10076779, 6 pages, Milford, MA.

Yau, K.Y.F., Tout, N.L., Trevors, J.T., Lee, H. and Hall, J.C. 1998. Bacterial expression and characterization of a picloram-specific recombinant Fab for residue analysis. J. Agric. Food Chem., 46:4457-4463.

Zhao, M., Zhou, S. Yan, J. and Li, L. 2007. Immunochemical analysis of endogenous and exogenous estrogens. Curr. Pharm. Anal., 3:25-38.

6. CONCLUDING CHAPTER

6.1 Summary of Research Findings

The hypotheses are: (1) Sorption of estrogens is positively related to soil organic content (SOC), therefore, soils with a greater SOC have greater sorption. (2) Sorption and mineralization are negatively correlated because sorption reduces bioavailability, therefore, soils with a greater SOC have greater 17β-estradiol sorption but lesser 17β-estradiol mineralization. (3) Manure application to soil increases SOC, therefore increasing sorption and reducing mineralization of 17β-estradiol in soil.

A positive correlation between SOC and estrogen sorption by soil was previously found for 17β-estradiol by Yu et al. (2004), Hildebrand et al. (2006) and Loffredo and Senesi (2006). In agreement with the previous studies conducted on a smaller set of samples, SOC was the single most influential soil constituent determining the sorption of 17β-estradiol, but also the sorption of estrone, estriol and equol by soil. The ecoregion and the landscape position that had less SOC also showed lower estrogen sorption values. This corresponded to the Mixed Grassland ecoregion, in which soils were mainly classified as Brown Chernozems, as well as upper-slopes across ecoregions.

The sorption of estrogens by soil increased in the order 17β-estradiol=estriol <estrone<equol, suggesting that 17β-estradiol and estriol can be more mobile than

estrone and equol with water in the environment. However, sorption was relatively strong for all compounds (ranging from 89 to 94% of that applied). 17β-estradiol is an estrogen of particular interest because it is the most potent of natural steroidal estrogens and the sorption of this estrogen was further studied in an Albertan soil that had received long-term manure applications at rates of 0, 60, 120 and 180 Mg ha^{-1} in an irrigated system and at rates of 0, 30, 60 and 90 Mg ha^{-1} in a naturally rainfed system. 17ß-estradiol sorption by soil in that study was also high (from 93% to 100% of that applied) and significantly increased with manure rate because of the associated increases in SOC.

In all soils included in this research, mineralization of 17β-estradiol was biologically-mediated and followed first-order kinetics without a lag phase. Other researchers also used the first order kinetics to describe 17β-estradiol mineralization in agricultural soils (Colucci and Topp, 2001; Ying and Kookana, 2005; Fan et al., 2007; Xuan et al., 2008; Stumpe and Marschner, 2010). There was a positive association between 17β-estradiol sorption and maximum 17β-estradiol mineralization, possibly because some of the steps in the 17ß-estradiol mineralization process occur in the sorbed phase as observed for agricultural soils in North-Dakota (Casey et al., 2003) and for biosolids (Layton et al., 2000). None of the soils included in this research showed maximum 17β-estradiol mineralization exceeding 30% at 90 days, which suggest that even under optimum environmental conditions for mineralization, 17β-estradiol or its metabolites estrone and/or estriol appear to have a relatively long persistence and/or are soil-bound or fixed into microbial cells in Alberta soils

Prediction models for sorption and mineralization parameters of 17β-estradiol in Alberta soils at the regional scale were established by Partial Least Squares regression using soil properties. These models gave good regressions for 17β-estradiol sorption but not for 17β-estradiol mineralization, suggesting that probability density functions should be used when estimating mineralization at the regional scale. The log-logistic probability density function was the best-fitted model for the prediction of 17β-estradiol mineralization rates while maximum 17β-estradiol mineralization was best-predicted by the extreme values probability density function. 17β-estradiol and estriol sorption could mutually be predicted through ordinary linear regression but not estrone or equol.

Based on the results of the sorption and mineralization experiments, it is possible to pinpoint some areas that could potentially be at greater risk of contamination by estrogens. These areas would consist of areas that have a low SOC (less than 1% particularly) because sorption and mineralization would be particularly low. In addition, since mineralization requires oxygenated conditions, areas where soils are not well oxygenated would be at higher risk of estrogen accumulation. Agricultural practices that increase soil aeration as well as contact between soils and manure (in order to increase sorption) could potentially reduce risk of estrogen contamination of surface waters. However, a complete risk analysis needs to take into account other parameters such as for example: storage impact on estrogens concentrations in manure, applied manure quantities, climate as well as possibilities for preferential flow.

The experiments on the sorption and mineralization of estrogens were conducted using radioisotopes. Radioisotope techniques are relatively cheap and allow the study of lower concentrations of estrogens compared to GC or HPLC techniques. However, it is difficult to conduct field experiments using radioisotopes because of environmental concerns. ELISA is an alternate detection method to GC or HPLC techniques, also known to be cheaper than these conventional techniques. ELISA techniques should thus be developed and optimized in order to help conduct experiments in the field.

Two ELISAs were developed using rabbit polyclonal antibodies: one was specific for 17β-estradiol while the other could detect 17β-estradiol+estrone+estriol. Both immunoassays had cross-reactivity with two estradiol conjugates: βestradiol-17-valerate and βestradiol-3-benzoate, which are sometimes used for oestrus synchronization in the livestock industry (Cavestani et al., 2010). The 17β-estradiol+estrone+estriol ELISA demonstrated greater promise for further development than the ELISA specific for 17β-estradiol because of its more linear working range and lower IC_{50}. A solid-phase extraction method was thus utilized to determine the suitability of the 17β-estradiol+estrone+estriol ELISA for quantifying estrogen concentrations in environmental samples. These samples consisted of runoff water from an edge-of-field experiment and the ELISA could detect estriol concentrations as low as 1 ng mL^{-1}. When the characteristics of the 17β-estradiol+estrone+estriol ELISA were compared against those of a commercial ELISA for estriol (since the 17β-estradiol+estrone+estriol ELISA was more linear for estriol than for 17β-estradiol or estrone), the 17β-

estradiol+estrone+estriol ELISA showed less intra-assay variation but the commercial ELISA was more specific, more sensitive and showed lower inter-assay variation.

6.2 Methodological notes

During sorption experiments numerous precautions had to be taken in order to avoid degradation. This included that all material, including soil, was autoclaved and sorption experiments were done in the dark and in a cold environment (5^{o}C) in order to reduce biodegradation. In addition, solutions were prepared in autoclaved water, kept in the dark at 4^{o}C when not in use, and used within one or two days. The experiment included the use of glass tubes because preliminary experiments indicated that estrogens sorbed onto the walls of Teflon tubes.

6.3 Contribution to Advancement of Science

Overall, this research has provided a better understanding of the fate (sorption and mineralization) of estrogens in soils, an area where knowledge is limited. The current research included a large number of samples while previous studies in this area had been limited to less than 10 samples (Table 1.2). Hence, the results of this study on the fate of estrogens were the first to provide information on the fate of estrogens at the field and regional scales. This allowed for the identification of zones with greater or lesser estrogen persistence and/or sorption. Another innovative aspect is that this was the first study to quantify the sorption of the phytoestrogen equol in soils. This is particularly important

since equol can be a dominant source of estrogenic activity in manure (Lorenzen et al., 2006).

The larger number of samples included in this study contributed to the strength of the statistical analysis to establish correlations among soil properties, sorption and mineralization parameters. In addition, this also allowed, for the first time, the development of equations for estimating the fate of estrogens at a regional scale. Such equations could be useful for risk assessments at the provincial scale in the province of Alberta.

In addition to this Ph.D. research, there have been only one other study (Stumpe and Marschner, 2010) on the impact of a long-term history of manure application on 17β-estradiol sorption and mineralization. Studies on the impact of long-term and fresh manure applications on soil properties and estrogen fate will become increasingly important in the design of good manure management that reduces contaminant transport to water resources in a world where good quality water is increasingly limited (Chenini, 2010).

6.4 Future Research Needs

These research findings provide some information on the fate of estrogens in fresh and long-term manure-amended soils, but soils were spiked with fresh ^{14}C-estrogen solutions and hence the research did not consider (more aged) estrogens excreted by livestock and

entering the soil through manure applications after storage. Additional information also needs to be collected on the characterisation of the estrogen content in different types of manure and the impact that storage can have on these concentrations. This information would open the door for designing beneficial management practices to reduce the potential for contamination of surface waters by estrogens.

Additional research needs to be conducted in field in order to validate the results from laboratory experiments. Laboratory experiments can provide reliable information on basic processes such as the relationship between soil properties and fate processes; however, the experiments are often conducted at constant and optimized temperature and moisture conditions. Under field conditions, temperature and moisture will vary. It is further recommended that field research should focus on areas for which the laboratory experiments identified the estrogens to be more persistent. Such field studies would assist in our understanding of whether these zones are indeed higher risk zones for estrogens off-site movement, and could lead to define possible ways to limit or mitigate this risk.

Good methods of detection will be necessary in order to conduct extensive environmental monitoring. Although ELISAs have been developed for the determination of estrogens, it is necessary to improve on their compatibility with conventional detection techniques such that ELISAs will become less sensitive to the presence of solvent residues from the extraction procedure and other matrix effects. This is important because the use of ELISAs as a screening method could reduce analysis time, sample manipulation and cost of analysis. For example, ELISAs could be used as a good screening method to identify

the subset of samples that requires further confirmatory analysis through gas chromatography or liquid chromatography.

6.5 References

Casey, F.X.M., Larsen, G.L., Hakk, H. and Šimůnek, J. 2003. Fate and transport of 17β-estradiol in soil-water systems. Environ. Sci. Technol., 37:2400-2409.

Cavestany, D., Crespi, D. and Fernandez, A. 2010. Oestrus synchronisation and fixed time artificial insemination in beef heifers. Anim. Prod. Sci., 51(7):670-674.

Chenini, F. 2010. Implications of water quality on irrigation practices under water scarcity. *In* Water scarcity in the Mediterranean: Perspectives under global change. (Eds) Sabater, S. and Barcelo, D. Springer-Verlag, Berlin, Germany, pp. 161-172

Colucci, M.S., Bork, H.and Topp, E. 2001. Persistence of estrogenic hormones in agricultural soils: I. 17β-estradiol and estrone. J. Environ. Qual., 30:2070-2076.

Fan, Z., Casey, F.X.M., Hakk, H. and Larsen, G.L. 2007. Persistence and fate of 17ß-estradiol and testosterone in agricultural soils. Chemosphere, 67:886-895.

Hildebrand, C., Londry, K.L. and Farenhorst, A. 2006. Sorption and desorption of three endocrine disrupters in soils. J. Environ. Sci. Heal. B, 41: 907-921.

Layton, A.C., Gregory, B.W., Seward, J.R., Schultz, T.W. and Sayler, G.S. 2000. Mineralization of steroidal hormones by biosolids in wastewater treatment systems in Tennessee U.S.A. Environ. Sci. Technol., 34:3925-3931.

Loffredo, E and Senesi, N. 2006. Fate of organic pollutants in soils with emphasis on adsorption/desorption processes of endocrine disruptor compounds. Pure Appl. Chem., 78(5):947-961.

Lorenzen, A., Burnison, K., Servos, M. and Topp, E. 2006. Persistence of endocrine-disrupting chemicals in agricultural soils. J. Environ. Eng. Sci., 5:211-219.

Stumpe, B. and Marschner, B. 2010. Organic waste effects on the behavior of 17β-estradiol, estrone and 17α-ethinylestradiol in agricultural soils in long-and short-term setups. J. Environ. Qual., 39:907-916.

Xuan, R., Blassengales, A.A. and Wang, Q. 2008. Degradation of estrogenic hormones in a silt loam soil. J. Agric. Food Chem., 56:9152-9158.

Ying, G.-G. and Kookana, R.S. 2005. Sorption and degradation of estrogen-like – endocrine disrupting chemicals in soil. Environ. Toxicol. Chem., 24(10):2640-2645.

Yu, Z., Xiao, B., Huang, W. and Peng, P. 2004. Sorption of steroid estrogens to soils and sediments. Environ. Toxicol. Chem., 23(3):531-539.

APPENDIX

Figure mineralization from Chapter 3: example with 703U