Sorption, Degradation and Transport of Estrogens and Estrogen Sulphates in Agricultural Soils

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by

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Lincoln University
Te Whare Wānaka o Aoraki

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PUBLICATIONS/CONFERENCE PAPERS FROM THIS THESIS

Following papers have been published or are currently under review:

- Scherr, F.F., Sarmah, A.K., Di, H.J., Cameron, K.C (2008). Modelling degradation and metabolite formation kinetics of estrone-3-sulphate in agricultural soils. *Environmental Science & Technology 42* (22), 8388–8394. doi:10.1021/es801850a
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- Scherr, F.F., Sarmah, A.K., Di, H.J., Cameron, K.C. Sorption of estrone and estrone-3-sulphate from artificial urine and $CaCl_2$ solutions in agricultural soils. *Environmental Chemistry and Toxicology* (submitted 10/2008, in review).

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- Scherr, F**.**, Sarmah, A., Di, H., Cameron, K.: Sorption of estrogens and their sulphate conjugates from artificial urine. *Latsis Symposium 2007 – Research Frontiers in Environment and Sustainability*, 17.-19. September 2007,

Competence Center Environment and Sustainability, ETH Zurich, Switzerland. Invited poster presentation.

- Scherr, F., Sarmah, A., Di, H., Cameron, K.: Sorption of estrogens and their sulphate conjugates from artificial urine. *RAISEBIO – AXIOM – HIGRADE - Summerschool: "Chemicals in soil: interactions, availability and residue formation"*, 23.-27. September 2007, UFZ Leipzig, Germany. Invited poster presentation.
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ABSTRACT

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Ph.D.

Sorption, Degradation and Transport of Estrogens and Estrogen Sulphates in Agricultural Soils

by

Frank Scherr

The fate and behaviour of estrogens in the environment are of concern due to the compounds' endocrine disruption potential. Estrogens, namely 17ß-estradiol (E2), estrone (E1), and estrogen sulphates, i.e. 17ß-estradiol-3-sulphate (E2-3S) and estrone-3-sulphate (E1-3S) excreted by livestock constitute a potential source for estrogen contamination in the environment.

A method was developed to separate and quantify the hormones by high-performance-liquid-chromatography (HPLC) and ultraviolet detection (UV). A combination of dichloromethane (DCM) and dicyclohexylamine hydrochloride (DCH·HCl) gave recoveries from 97.3 to 107% for E1-3S extraction from aqueous solutions. The recoveries from soil samples ranged from 80.9 to 95.2% (E2-3S), and from 86.3 to 91.7% (E1-3S), respectively.

Results of batch sorption studies showed that Freundlich isotherms were nonlinear ($N \neq 1$) with K_f values ranging from 34.2 to 57.2, and from 3.42 to 4.18 $mg^{1-N}\ L^{N}\ kg^{-1}$ for E1, and E1-3S, respectively, indicating the sorption affinity of E1-3S was about an order of magnitude lower than that of E1. The hydrophilic sulphate group of E1-3S possibly shielded the compound from hydrophobic interactions with the soil organic matter and allophanic clay minerals that were proposed as sorbents for E1. Contraction of clay minerals, "salting out" and competitive sorption of artificial urine constituents were likely to have been responsible for observed changes in Freundlich parameters

when artificial urine was used as mediator matrix. Plotting the effective distribution coefficient as a function of hypothetical exposure concentrations facilitated the comparison of the sorption behaviour of both compounds as influenced by the mediator solution. The results emphasized that using the $CaCl_2$ matrix might result in false inferences for the sorption behaviour of these compounds in a dairying environment.

The four hormones rapidly degraded in the agricultural soils under aerobic conditions, and the majority of the compounds degraded > 50% within the first 24 hrs. Soil arylsulphatase activities were directly correlated with degradation rate constants of the estrogen sulphates. Estrone was identified as a metabolite of E2 and E1-3S, and these three compounds were observed as metabolites of E2-3S. Single-first order (SFO) and double first-order in parallel (DFOP) kinetics were used to model the degradation and metabolite formation data. The results showed that the DFOP model was in most cases better able to predict the parent compound degradation than the SFO model, and also enabled to estimate accurate degradation endpoints. ER-CALUX® analysis revealed the formation of estrogenicity during E2-3S degradation, which could partly be explained by the formation of the metabolites E2 and E1.

Transport studies with E1-3S and E1 showed that the transport and retention of both compounds were significantly influenced by the mediator matrix. While no breakthrough curves (BTCs) were recorded during hormone application in $CaCl_2$ (10 mM) both hormones were detected in the leachate when applied in artificial urine. Rate-limited sorption processes were proposed for the delayed arrival of the hormone BTCs compared with a conservative bromide tracer. Intense colouration of the leachate during the artificial urine experiments suggested the hormones were likely to be moved by colloid-facilitated transport. Furthermore, the detection of residue hormone and metabolite concentrations implied that degradation of E1-3S and E1 was hampered by urine constituents such as glycine and urea.

Keywords: 17ß-estradiol, estrone, 17ß-estradiol-3-sulphate, estrone-3-sulphate, sorption, degradation, transport, ER-CALUX®, arylsulphatase activity, kinetic modelling, metabolite formation, artificial urine

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

αE2 = 17α-estradiol

Ab = antibody

Ag = antigen

AIC = Akaike information criterion

AU = artificial urine

BTC = breakthrough curve

CDE = convection-dispersion equation

CLIA = chemiluminescent immunoassay

DAD = diode array detector

DCH = dicyclohexylamine

DCM = dichloromethane

DDT = dichlorodiphenyltrichloroethane

DFOP = double first-order in parallel

DNA = deoxyribonucleic acid

DT = dissipation time

E1 = estrone

E1-3S = estrone-3-sulphate

E2 = 17β-estradiol

E2-3S = 17β-estradiol-3-sulphate

E2-3,17S = 17β-estradiol-3,17-disulphate

E3 = estriol

EEQ = 17β-estradiol equivalent

ELISA = enzyme-linked immosorbent assay

ER = estrogen receptor

ER-CALUX® = estrogen-receptor-mediated, chemical-activated, luciferase gene expression

ERE = estrogen response element

FD = fluorescence detection

FIA = fluoroimmunoassay

GC = gas chromatography

HOC = hydrophobic organic contaminant

HPLC = high-performance-liquid-chromatography

K_{ow} = octanol-water distribution coefficient

LC = liquid chromatography

LOD = limit of detection

MBC = microbial biomass carbon

MDL = method detection limit

mRNA = messenger ribonucleic acid

MS = mass spectrometer(s)

4-NP = 4-nonylphenol, 4-nitrophenol

OC = organic carbon

PFIA = polarization fluorescent immunoassay

RE = relative estrogenicity

SPE = solid phase extraction

SSA = specific surface area

TRFIA = time-resolved fluoroimmunoassay

UV = ultraviolet

WWTP = wastewater treatment plant

YES = yeast estrogen screen

CHAPTER I
INTRODUCTION

1 BACKGROUND

The presence of anthropogenic manufactured organic chemicals that have the potential to interact with the normal functioning of human and wildlife biological systems was proposed as early as the 1920s and became a prominent issue following the publication of the book *Silent Spring* (Carson, 1962). The causal relationships between organo-chlorine pesticide exposure and detrimental health effects on wildlife and human beings that were presented to a wide audience (Carson, 1962), resulted in huge scientific interest to understand the relationship between anthropogenic manufactured chemicals and their adverse effects on human and wildlife health. A number of studies in the 1990s have established the link between sexually altered fish populations and effluents from wastewater treatment facilities (e.g., Purdom *et al.*, 1994; Jobling *et al.*, 1998), and the endocrine disruption hypothesis was publicly raised with the release of Colborn *et al.*'s book *Our Stolen Future* (1997). Elevated levels of the egg yolk protein precursor vitellogenin in male fish have uncovered the estrogen-like biological activity of many water contaminants and vitellogenin has become a biomarker for fish exposure to compounds that exhibit estrogen-like action (Sumpter and Jobling, 1995). While natural and synthetic hormones as well as degradation products of surfactants and detergents were among the first compounds to be identified as sources for the observed abnormalities in fish (Desbrow *et al.*, 1998), today hundreds of synthetic and natural compounds are suspected of having the potential to interfere with the normal functioning of human and wildlife hormonal systems (Fent, 2003).

Global population growth is putting increasing pressure on food supplies, and the increasing demand for meat and diary products has led to the intensification of livestock operations and the use of synthetic steroid hormones as growth promoters (Khan *et al.*, 2008a). Although the majority of steroidal growth promoters are now banned in many of the industrialized countries, the naturally produced steroid hormones excreted by livestock may still pose a risk to the aquatic environments. Despite the fact that some recent review papers have proposed a negligible risk associated with estrogen

excretions of livestock (Hanselman *et al.*, 2003; Johnson *et al.*, 2006), estrogens are continuously detected all around the globe in waterways (e.g., D'Ascenzo *et al.*, 2003; Isobe *et al.*, 2003; Petrovic *et al.*, 2004). Apart from wastewater treatment plant discharges, agricultural practices, such as land application of wastes, waste lagoons or feedlot effluent discharges, have been proposed as main sources for estrogens in water (Nichols *et al.*, 1997; Peterson *et al.*, 2000; Arnon *et al.*, 2008).

The natural mammalian excretion mechanism for estrogens involves an endogenous conjugation with hydrophilic functional groups such as sulphates and glucuronides, resulting in the ability to excrete the original hydrophobic compounds via the urinary route (Fent, 2003). These conjugates may constitute up to 92% of the total estrogen excretions of female cattle during late stages of pregnancy (Hanselman *et al.*, 2003) and have the potential to be back-converted to their free counterparts in the environment, believed to be mediated by enzymes. This back conversion has been shown in waste water from sewer systems in Italy (D'Ascenzo *et al.*, 2003). However, when this Ph.D. project began no information was available about the behaviour of estrogen conjugates in agricultural soils. This constituted a considerable gap in the scientific knowledge. Improved knowledge in this area is required urgently because information on the environmental fate of these compounds is essential for conducting sound risk assessments of estrogen emissions from livestock operations.

2 THE NEED FOR THE RESEARCH

New Zealand's agricultural sector is a major driver of the country's economy and in 2005/2006 contributed 47% to the total exports (agricultural economics of Australia & New Zealand, 2008). Furthermore, the majority of > 9.5 million head of cattle continuously graze pastures throughout the year (Statistics New Zealand Tatauranga Aotearoa, 2008) and are expected to produce 40 times more waste than does the human population, constituting a considerable potential source for estrogen exposure to the environment (Sarmah *et al.* 2006).

However, only a small number of studies have addressed the issue of estrogen fate in the New Zealand environment and the studies have focused on the endocrine disruption potential of waste water effluents (Leusch *et al.* 2006), estrogens and estrogenic activity in animal waste treatment pond effluents (Sarmah *et al.*, 2006) and the degradation of endocrine disrupting compounds in river sediments and groundwater aquifer material (Sarmah and Northcott, 2008). Sarmah *et al.* (2008) investigated sorption behaviour of estrogens in some agricultural soils from New Zealand and concluded that the unique mineral properties of New Zealand soils yielded very distinct sorption characteristics for these compounds in some of the investigated soils.

While there is a growing body of literature available on the sorption, degradation and transport of free estrogens (e.g., Ying *et al.*, 2002; Khanal *et al.*, 2006), recent research has shown that neglecting impacts of the exposure matrix on degradation and transport (Stumpe and Marschner, 2007; Lucas and Jones, 2009) as well as assuming equilibrated sorption behaviour (Casey *et al.*, 2005; Fan *et al.*, 2008) may often lead to false assumptions about the fate of these compounds in the environment. Moreover, the fate and behaviour of estrogen conjugates, such as estrogen sulphates, has never been studied; they might, however, play an important role in the distribution of their often unexpectedly detected parent compounds in the environment.

3 SCOPE AND STRUCTURE OF THE THESIS

Following this general introduction (Chapter I), a literature review (Chapter II) presents an introduction to estrogen-related endocrine disruption and an overview of the fate and behaviour of estrogens and estrogen sulphates in the soil/water environment. General gaps in the current scientific knowledge about the fate and behaviour of estrogens, and especially estrogen sulphates, have been identified in Chapter II, and while considering New Zealand's unique dairying practices and related activities, a number of objectives for this thesis were developed that conclude Chapter II. Chapter III describes the analytical methods that have been developed to simultaneously extract estrogens and estrogen sulphates from soil and liquid

matrices. A method to separate and quantify estrogens and estrogen sulphates by means of high-performance-liquid-chromatography coupled to UV-spectrometry was designed and is also described in Chapter III. To investigate the influence of the exposure matrix on the sorption parameters of estrone and estrone-3-sulpahte, a series of batch sorption experiments involving two mediator solutions (5 mM $CaCl_2$ and artificial cow urine) were conducted and the results are presented in Chapter IV. The degradation behaviour of 17ß-estradiol, estrone and their 3-substituted sulphate conjugates at three incubation temperatures was examined in detailed laboratory experiments, and the results are presented in Chapter V, along with some insights into the role of arylsulphatase enzyme activity in the degradation of estrogen sulphates. The observed degradation and metabolite formation kinetics of the compounds were subject to kinetic modelling in order to obtain degradation endpoints, and the possibility of estrogenicity formation during the degradation of 17ß-estradiol-3-sulphate was investigated (Chapter V). To clarify the impact of the exposure matrix on the transport and retention of estrone and estrone-3-sulphate, miscible displacement experiments with undisturbed soil lysimeters were conducted in the laboratory with two mediator solutions (10 mM $CaCl_2$ and artificial cow urine) and the breakthrough curves of the hormones and a bromide tracer were detected. The results are described in Chapter VI. Finally, Chapter VII presents a brief synopsis along with a general discussion, a general conclusion and recommendations for future research.

While Chapters IV–VI all refer to Chapter III for the analytical methodology, Chapters III-VI are written as stand-alone research papers, hence some repetition, particularly in the introduction to the respective chapters, was unavoidable.

CHAPTER II
LITERATURE REVIEW

1 ESTROGENS: NATURALLY OCCURRING STEROID HORMONES

Estrogens belong to the chemical class of hormones and are naturally secreted by the adrenal cortex, the testis, the ovary and the placenta of humans and mammals in general (Fent, 2003). With respect to livestock, the major sources of estrogens are the granulosa cells of the ovarian follicles and the placenta in females, and the testis in males, respectively (Lange *et al.*, 2002). The most important estrogens are 17ß-estradiol (E2) and estrone (E1), which are *inter alia* responsible for maintaining the health of reproductive tissues, breasts, skin and brain (Fent, 2003). The major functions of estrogens are in sex determination, sexual differentiation, and sexual development (Tyler *et al.*, 1998). After exhibiting their intended action in the mammalian body these hormones undergo a wide range of phase I reactions, including hydroxylation, reduction and oxidation, before a conjugation takes place in phase II reactions, predominantly with sulphate, glucuronide or glutathione. Ultimately, they are excreted as conjugates mainly in urine or, after minor metabolism, as free hormones in faeces (Lange *et al.*, 2002). The amount and ratio of estrogens and their conjugates in either urine or faeces depends to a large extent on species, gender, age, and, for females, on the state of cycling or the state of pregnancy (Hoffmann *et al.*, 1997; D'Ascenzo *et al.*, 2003). However, the transformation of estrogens in the mammalian body is much more complex than described above and as yet is not fully understood. This has led to the assumption that a variety of estrogen conjugates might be released into our environment. Giese (2003) describes some of the possible pathways in the metabolism of estrogens.

The large majority of estrogen conjugates excreted by humans is comprised of sulphates and glucuronides (D'Ascenzo *et al.*, 2003). Likewise, it is reported by Hoffmann *et al.* (1997) that female cattle also excrete mainly sulphate and glucuronide estrogen conjugates. Research investigating the fate and behaviour of these hormone conjugates in the environment is still in its infancy even though they are regarded as precursors to their free

counterparts and have the potential to contribute to environmental estrogen distribution and endocrine disruption in wildlife (Isobe and Shimada, 2003).

2 ENDOCRINE DISRUPTION

The International Programme on Chemical Safety (2002) reports that since the publication of Rachel Carson's book *Silent Spring* (1962), there has been increasing awareness that chemicals in the environment can exert profound and deleterious effects on wildlife populations and that human health is inextricably linked to the health of the environment. The last two decades, in particular, have witnessed growing scientific concerns and public debate over the potential adverse effects that may result from exposure to a group of chemicals that have the potential to alter the normal functioning of the endocrine system in wildlife and humans. For instance, the release of *Our Stolen Future* (Colborn *et al.*, 1997) highlighted the scientific concerns of potentially widespread endocrine disruption and aimed to arouse public interest beyond the realm of scientific expert and policy panels. According to the IPCS report (2002) concerns regarding exposure to endocrine disrupting chemicals (EDCs) are primarily due to 1) the adverse effects observed in certain wildlife, fish, and ecosystems; 2) the increased incidence of certain endocrine-related human diseases; and 3) endocrine disruption resulting from exposure to certain environmental chemicals in animals in laboratory trials.

Despite the surge of interest in the endocrine disruption field, there is still no globally accepted definition of the term (Fisher, 2004). According to Kavlock *et al.* (1996) an 'endocrine disruptor' can be described as:

> *An exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental process.*

Whereas the International Programme on Chemical Safety (IPCS) suggests:

> *An endocrine disruptor is an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse*

*health effects in an intact organism, or its progeny, or(sub)populations (*IPCS, 2002*)*.

The major point of contention over the efforts to define an endocrine disruptor is in the use of the phrase 'adverse'. Similarly, the language used to describe compounds that may alter the endocrine system presents a challenge in linguistic research as intriguing as much of the laboratory research in this area (McLachlan, 2001). For example, estrogenicity *per se* is not an adverse effect; it is a natural mechanism of hormone action controlled via homeostatic mechanisms (McLachlan, 2001). However, a chemical with estrogenic properties acting out of context within the endocrine system, or at a vulnerable developmental stage, may have the potential to induce an adverse effect (Fisher, 2004). Regarding steroid hormones, this fact has to be kept in mind when referring to the endocrine disrupting effects associated with them (*section 2.2.2*). Furthermore, estrogenicity on its own may not be adequate to describe the endocrine disrupting potential of these compounds (*section 2.2.1*). Nevertheless, from the various groups of substances with reported endocrine-disrupting properties estrogens and synthetic steroids are the most potent compounds (Rodriguez-Mozaz *et al.*, 2004a). In the context of this thesis, to simplify matters, the term estrogens is used to refer to the female sex hormones 17α-estradiol (αE2), 17ß-estradiol (E2), estrone (E1) and estriol (E3), while the term xenoestrogens is used to describe compounds that have been shown to act like estrogens in terms of their interference with the endocrine system. Endocrine disruption, furthermore, refers to adverse effects associated with the abnormal interaction of estrogens and/or xenoestrogens with the endocrine system of an animal or human being.

2.1 Mechanisms of endocrine disruption

Most of the known environmental chemicals with hormonal activity derive that activity from interaction with one or more receptors of the steroid/thyroid/retinoid family of nuclear receptors (McLachlan, 2001). Hence, the classical understanding of endocrine disruption is based on the abnormal binding of a hormone-like compound with one of the nuclear receptors of the endocrine system (Figure 2.1.) and its subsequent adverse

effects. Research in endocrinology over the past decade, however, has shown that hormonal signalling may be distributed in networks that have not yet been considered (McLachlan, 2001) and that endocrine disruption occurs also at non-genomic level (Janer and Porte, 2007; Ropero *et al.*, 2006).

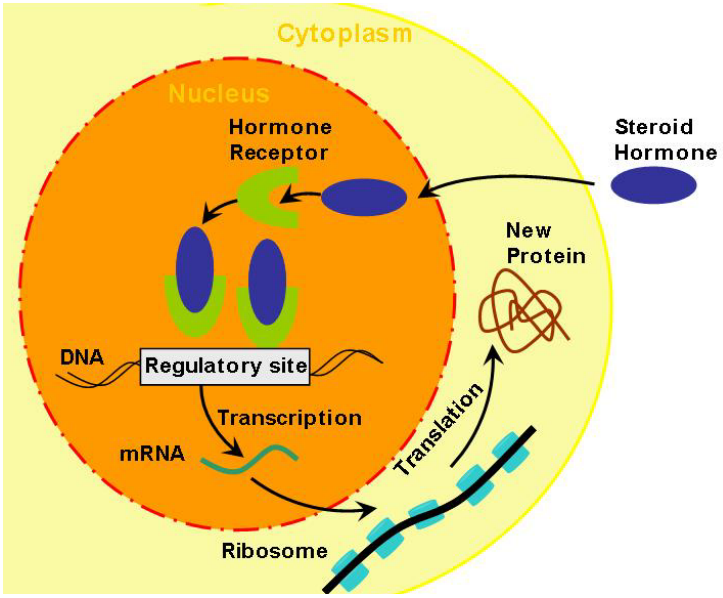


Figure 2.1. Steroid hormones and steroid hormone-like substances bind to the hormones' receptors in the nucleus of cells. Adopted from Fent (2003).

Figure 2.1. displays the classic principle of receptor-mediated hormone action. A hormone, e.g., E2, enters the target cell and travels to the nucleus where it connects to the hormone receptor, e.g., an estrogen receptor (ER). The hormone-receptor complex is then able to activate a regulatory site located on the DNA, i.e. the estrogen response element (ERE), which triggers the transcription of messenger RNA (mRNA), and in response the translation of new proteins at the cell's ribosome (Figure 2.1.). All vertebrates and many invertebrates have been reported to produce E2 and three iso-forms of the ER have been discovered (ERα, -ß, and -γ) also outside the nucleus (McLachlan, 2001; Ropero *et al.*, 2006), e.g., in the cell membrane (Ho and Liao, 2002). According to McLachlan (2001), the phylogenetic distribution of estradiol production in the animal kingdom suggests estrogenically active chemicals may be evolutionarily conserved signals, and further, that all animals may be sensitive to estrogens. Many structurally diverse chemicals have been

reported to bind to the various forms of the ER including pharmaceuticals (e.g., ethynylestradiol), pesticides (dichloro-diphenyl-trichloroethane, DDT), plasticisers (bisphenol A), surfactants (4-nonylphenol, 4-NP), and plant products (coumesterol) amongst others (IPCS, 2002; Watson *et al.*, 2005).

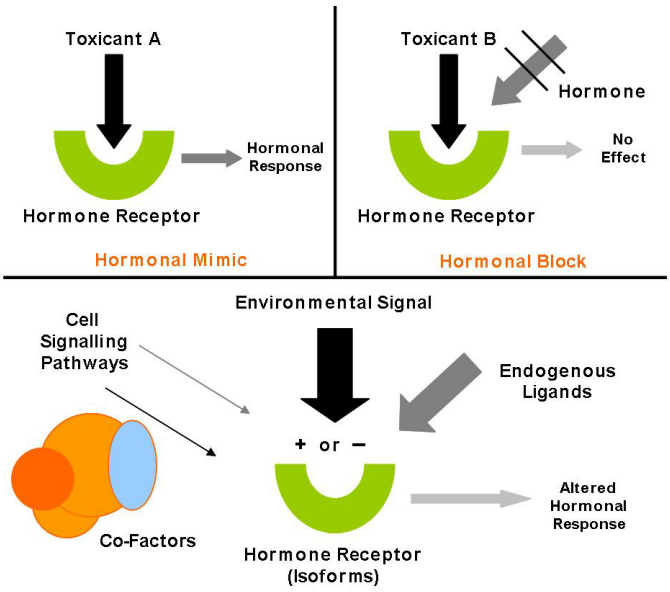


Figure 2.2. Functional mechanism of receptor based hormonal signalling (lower panel) and possible mechanisms of endocrine disruption. Source: McLachlan (2001).

The binding of E2 to the ER initiates a myriad of possible signal transduction pathways that, depending on the cellular context, elaborate responses as varied as survival, adhesion, and proliferation, and culminate in physiological processes as divergent as cardiovascular protection, bone preservation, organogenesis, and cancer (Ho and Liao, 2002). Consequently, endocrine disruptors interfere with the functioning of the endocrine system in at least three possible manners (Figure 2.2.): 1. by mimicking the action of naturally produced hormone, binding to their hormone receptors; 2. by blocking the receptors in target cells for these hormones and therefore preventing the action of natural hormones; or 3. by altering the synthesis and function of hormone receptors and modifying the synthesis, transport, metabolism and excretion of hormones (Ropero *et al.*, 2006).

Although E2 and its related estrogens E1 and E3 are naturally occurring steroid hormones they constitute a potential threat to wildlife because they

are constantly introduced into the environment as a result of human activity. Sanitation and agricultural practices are the major sources of estrogen pollution, and estrogens have been found in various environmental matrices (section 5).

2.2 (Estrogen-related) endocrine disruption in wildlife and laboratory studies

The effects of (xeno-)estrogens on living species have been predominantly constituted by altered sexual development such as intersex and feminization (Milnes *et al.*, 2006; Scott *et al.*, 2007). In fish, elevated levels of the egg yolk precursor protein vitellogenin in males and abnormal levels in females represent a widely accepted biomarker for exposure to endocrine disruptors (Sumpter and Jobling, 1995; Scott *et al.*, 2007). The pathways and actions of the endocrine systems are extremely complex, highly interdependent and not yet fully understood. Hence, the vast effects of endocrine disruption occur at different scales, ranging from cell to population, and emerge at various stages in the lifespan of an organism. Furthermore, it is difficult to extrapolate laboratory-based data to the routes of exposure and the consequent effects in the environment (Lai *et al.*, 2002). Thus, within the scope of this literature review only some of the reported effects that have been associated with estrogen induced endocrine disruption are briefly illustrated.

Table 2.1. displays a brief outline of such effects from laboratory and wildlife observations along with the respective exposure concentrations. Although estrogen conjugates are generally recognized as the biologically inactive form, based on the potential to bind to the ER family, the possibility of their contribution to endocrine disruption in wildlife may exist. The effects of E1-3S on the endocrine functions of Japanese quails were investigated by Isobe and Shimada (2003). The study reported a reduction in testis weights and apoptosis for E1-3S treated quails. However, the exposure concentration of 1 mg per day, via intramuscular injection over 4 days, is very unlikely to occur in the environment.

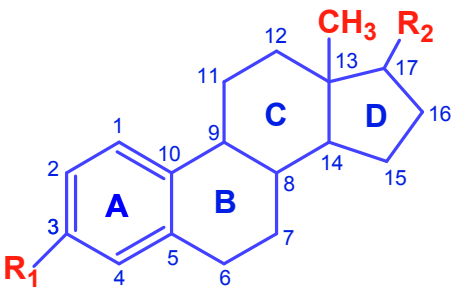
Table 2.1. Estrogen-related endocrine disruptive effects on wildlife species.

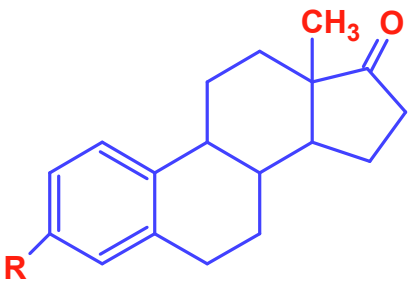
Exposure Pathway	Effects	Exposure Concentration	Reference
Male starling (*sturnus vulgaris*)			
Earthworms pretreated with E2	cell-mediated immune function humoral immune response	200 ng E2 per day, 5 days per week, 7 month	(Markman *et al.*, 2008)
Japanese quail (*coturnix japonica*)			
intramuscular injection	reduced testis weight reduced spermatozoon apoptosis	1 mg E1-3S in 1 mL ethanol, 4 days	(Isobe and Shimada, 2003)
Nematode (*caenorhabditis elegans*)			
via growth medium	increased number of germ cells	~3 µg E2 mL^{-1} in growth medium	(Hoshi *et al.*, 2003)
Zebrafish (*danio rerio*)			
via water	feminization of offspring increased vitellogenin	< 1–1 ng E2 L^{-1}, 21 days (adults), 41 days (offspring)	(Leo *et al.*, 2007)

3 MOLECULAR STRUCTURE AND PHYSICOCHEMICAL PROPERTIES OF ESTROGENS

The common feature in the structure of steroid hormones is a tetracyclic molecular framework composed of three phenol rings (rings A to C) and one cyclopentane ring (ring D), which is termed the cyclo-pentano-perhydro-phenanthrene structure (Figure 2.3.). Estrogens contain a condensed aromatic ring at position A, and key structural differences of the free hormones arise in the D-ring structure owing to the type and stereochemical arrangement of functional groups at the C-17 position (Hanselman *et al.*, 2003; Khanal *et al.*, 2006). The functional hydroxyl group at C-17 of E2 can either point downward from the molecule (α-configuration, αE2) or project upward from the molecule (ß-configuration, E2) (Hanselman *et al.*, 2003). The

isomeric nature of E2 has an important meaning for its endocrine action. The α-configuration has only about 11% and 56% of the binding affinity of the ß-configuration to the ERα and ERß, respectively (Kuiper *et al.*, 1997). Estrone, a degradation product of estradiol, shows a reduced carboxyl functional group at C-17 and its binding affinities to ERα and ERß account for 60% and 37% of E2, respectively.





R_1=OH, R_2=OH:	estradiol	R=OH:	estrone
R_1=SO_4, R_2=OH:	estradiol-3-sulphate	R=SO_4:	estrone-3-sulphate
R_1=OH,R_2= SO_4:	estradiol-17-sulphate		
R_1=SO_4,R_2= SO_4:	estradiol-3,17-sulphate		

Figure 2.3. Molecular structures of estrogens and their sulphate conjugates.

Possible positions for substituting functional groups to form estrogen conjugates are C-3 and C-17 for E2, and C-3 for E1, respectively. Hence, E2 has three sulphate conjugates and E1 has one sulphate conjugate, indicated by the form of the respective substituting groups (Figure 2.3.).

Table 2.2. displays some relevant physicochemical properties of the estrogens and sulphate conjugates under investigation. The low vapour pressure of the free hormones indicates that volatilisation can be excluded as major sources of environmental distribution. Therefore atmospheric dispersion processes may be restricted to particle co-transport. No literature data on the vapour pressure of the sulphate conjugates are available; however, their chemical nature and high molecular weight suggest volatilisation to be of low importance for their environmental behaviour. The octanol-water distribution coefficients (K_{ow}) indicate that both free estrogens (E1 and E2) have a very strong tendency to accumulate in organic matter and

the low values for their aqueous solubility suggest the same tendency (Table 2.2.).

Table 2.2. Physicochemical properties of natural estrogens and their sulphate conjugates.

Property	E1	E2	E1-3S	E2-3S	E2-3,17S
Molecular formula	$C_{18}H_{22}O_2$	$C_{18}H_{24}O_2$	$C_{18}H_{22}O_5S$	$C_{18}H_{24}O_5S$	$C_{18}H_{24}O_8S_2$
Molecular weight (g mol $^{-1}$)	270.4	272.4	350.5	352.4	432.5
Vapour pressure[a] (Pa)	3 x 10 $^{-8}$	3 x 10 $^{-8}$	n.a.	n.a.	n.a.
Log K_{ow}	3.43[c], 3.13[c],3.3 [b]	3.10[c], 3.94[b],4.0 [b]	0.95[d], 2.65[h]	1.46[d], 1.94[f]	0.52[f]
Solubility (mg L^{-1})	13, 2.1	13, 3.1	4.2–150[f]	10.2–170[f]	39.8-3×10^{3} [f]
pK_a	10.3–10.8 [a, e]	10.5–10.7 [a, e]	-3.0[f]	-3.0[f]	-3.0[f]
Melting point (°C)[c]	259	171	n.a.	n.a.	n.a.

[a]Yu *et al.* (2004); [b]cited by Lee *et al.* (2003); [c]cited by Lai *et al.* (2000); [d]calculated with KowWin Program (Meylan and Howard, 1995); [e]cited by Hanselman *et al.* (2003); [f]calculated with ALOGPS 2.1 (Tetko *et al.*, 2005); n.a. = not available

In contrast, the sulphate conjugates have lower values for log K_{ow} and higher aqueous solubility values indicating stronger hydrophilic features. The pK_a values in Table 2.2. suggest the free estrogens would only be charged at very high pH values and the sulphate conjugates will never be protonated under environmental pH values, leaving them ionic in nature at all times. These physicochemical properties have an important impact on the compounds' behaviour in environmental matrices and suggest distinct differences between free and conjugated estrogens.

4 ANALYSIS OF ESTROGENS AND THEIR SULPHATE CONJUGATES

Studying the fate and behaviour of estrogens requires analytical methods that are reliable, reproducible and meet the challenge of detecting trace concentrations in a variety of environmental samples. Furthermore, estrogens and their conjugates are labile compounds that can degrade in

between sampling and further processing of the sample before quantification (Kuster *et al.*, 2005). Therefore an appropriate sampling technique and preservation procedure needs to be applied that guarantees representative results. For laboratory-based studies this implies either demanding preservation techniques or the instantaneous treatment of samples.

4.1 Sample extraction and cleanup

Aqueous samples have been successfully treated with formaldehyde (Baronti *et al.*, 2000) or sulphuric acid (López de Alda and Barceló, 2000) to preserve steroid hormones without loss of concentrations. Controlling photo- and biodegradation through usage of amber glass bottles and storing samples in the cold (< 5ºC) seems to be sufficient for short storage times of up to 48 hrs (Kuster *et al.*, 2008). Subsequent sample clean-ups involving filtration and solid-phase-extraction (SPE) have been well demonstrated in the literature (Isobe *et al.*, 2003; Rodriguez-Mozaz *et al.*, 2004b; Kuster *et al.*, 2008), allowing compound pre-concentration and thus lowering the method and detection limits. In the context of laboratory studies liquid-liquid extraction employing dichloromethane has been proved to yield excellent estrogen recoveries from aqueous solutions reaching 100% (Lee *et al.*, 2003; Soto *et al.*, 2004; Sarmah *et al.*, 2008).

Since estrogen conjugates have a different chemical behaviour, their co-extraction from environmental matrices constitutes a complex task that has been addressed with sophisticated multiphase SPE schemes employing sequential elution schemes for separation of free and conjugated estrogens (D'Ascenzo *et al.*, 2003; Isobe *et al.*, 2003). However, there is some evidence in the early literature that liquid-liquid extraction may also be capable of yielding excellent recoveries for E1-3S when an ionization agent, namely dicyclohexylamine hydrochloride (DCH·HCl), is added to the organic solvent (Deans *et al.*, 1955). Compared with aqueous samples, the detection of estrogens and their conjugates in solid matrices, i.e. soils, sediments and sludge, is a bigger analytical challenge due to the more complex matrix in the solid media (Kuster *et al.*, 2004; Gabet *et al.*, 2007). In laboratory studies simple liquid extraction has been successfully employed to extract free

estrogens from soils and sediments using various solvents including dichloromethane (Lee *et al.*, 2003; Sarmah and Northcott, 2008; Sarmah *et al.*, 2008), acetone (Xuan *et al.*, 2008), and ethylactetate (Ying and Kookana, 2003). A subsequent solvent transfer step allows a pre-concentration. Studies investigating true environmental solid samples often involve further cleanup steps such as SPE and gel permeation chromatography (Sarmah *et al.*, 2006; Isobe *et al.*, 2006), and employ specialised technical machinery such as accelerated solvent extraction (Beck *et al.*, 2008) or microwave-assisted solvent extraction (Matějíček *et al.*, 2007).

4.2 Instrumental estrogen detection

The analytical technique of choice for quantifying estrogens during the past decade has been gas chromatography coupled with mass spectrometry (GC-MS), mainly because of the lower detection limits as opposed to liquid chromatography (LC). However, since analysis of estrogens with GC-MS involves derivatization prior to detection, the quantification of conjugated estrogens is difficult because typical derivatization takes place at the positions of the conjugated groups. Distinction of different conjugates is therefore very difficult. Better cleanup and pre-concentration techniques have recently overcome past constraints related to matrix interferences and led to the development of LC-MS and LC-MS/MS methods for quantification of estrogens and their conjugates. In the context of the present dissertation access to MS techniques was not available and therefore further discussion is not pursued. Recently, Gabet *et al.* (2007) provided a comprehensive review of available GC and LC methods including sample cleanup and pre-concentration steps.

In laboratory studies investigating the chemical behaviour of estrogens, high-performance-liquid-chromatography (HPLC) methods seem to be a conventional and reliable tool for compound quantification. Diode array detection (DAD), ultraviolet detection (UV), and fluorescence detection (FD) are the most commonly employed detection systems, apart from MS (Ying and Kookana, 2003; Jacobsen *et al.*, 2005; Bonin and Simpson, 2007; Sarmah *et al.*, 2008; Xuan *et al.*, 2008). However, these detection units are not able to

identify the analytes unambiguously, and hence complete separation within the chromatographic column is crucial for a successful analysis and quantification (Ingerslev and Halling-Sørensen, 2003). Analytical columns in use are mainly silica-based C-18 and ODS-2 columns, and the dominant mobile phase consists of acetonitrile and water, which is sometimes acidified. Separation of hormone conjugates employing HPLC photo spectrometric (PS) methods has received little attention, though a study by Blom *et al.* (2001) demonstrated the separation of eight estrogens, including five conjugates on a HPLC-UV system, suggesting the feasibility of HPLC-UV for detection of estrogens and their conjugates. Method detection limits for HPLC-UV methods considering sample extraction, cleanup and pre-concentration were in the lower $\mu g\ L^{-1}$ and $\mu g\ kg^{-1}$ range for aqueous and solid samples, respectively (Lee *et al.*, 2003). In general, method detection limits with MS detectors are an order of magnitude lower (e.g., Isobe *et al.*, 2006, Beck *et al.*, 2008).

4.3 Biological estrogen detection

There is an extensive body of literature covering biological methods to determine either the estrogenic potential of a compound or the concentration of the compound(s) in the respective matrices. The latter most often is based on a correlation of the former with a standardized colorimetric reaction of the biological system. In general, two different methods dominate the research interest, namely bioassays based on recombinant yeast or human cells and the enzyme-linked immunosorbent assays (ELISA).

The principle of the recombinant yeast screen was developed by Routledge and Sumpter (1996), who integrated the DNA sequence of the human estrogen receptor (ERα) into the main chromosome of the yeast. The cells also contained expression plasmids carrying the reporter gene *lac*-Z encoding the enzyme ß-galactosidase, which was used to measure the receptors' activity. In such a system the ERα is expressed in a form of binding to the ERE, which were situated within a strong promoter sequence on the expression plasmid. On binding an active ligand, the estrogen-occupied receptor interacts with transcription factors and other transcriptional components to modulate gene transcription. This causes expression of the

reporter gene *lac-Z*, and the enzyme produced (ß-galactosidase) is secreted into the medium, where it metabolizes the chromogenic substrate, chlorophenol red-ß-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorbance at 540 nm (Routledge and Sumpter, 1996). This principle of yeast estrogen screen (YES) has been further developed in recent years, and more sophisticated methods are now available. In order to be able to measure ß-galactosidase activity yeast cells had to be destroyed, which was carried out either mechanically or by means of chemical addition. A method performed by Jungbauer *et al.* (2002) used enzymatic-controlled hydrolyzation, which led to a decrease in sample preparation time and sample handling complexity. The method reached a detection limit of 0.1 ng L^{-1} for E2 provided the sample could be concentrated 1000-fold before analysis.

Almost a decade ago Legler *et al.* (1999) developed the estrogen receptor-mediated, chemical-activated, luciferase gene-expression (ER-CALUX®) assay using human T47D.Luc cells to measure substances with (anti-) estrogenic activity. Like the YES assay, the ER-CALUX® assay is based on a receptor gene construct, with activation of the endogenous estrogen receptors as the sole molecular mechanism leading to response (Houtman *et al.*, 2006). The response in the ER-CALUX assay is the induction of transcription and translation of luciferase protein as a response to ERE binding on the newly constructed estrogen-responsive luciferase reporter gene (pEREtata-Luc) (Legler *et al.*, 1999). This method has now been validated and can be applied to environmental samples (Houtman *et al.*, 2006).

The principle of immunochemical analytical detection such as ELISA is the capability of antibodies (Ab) to specifically recognise and form stable complexes with antigens (Ag) that can be subsequently analysed by means of detection of a label (Rodriguez-Mozaz *et al.*, 2004a). The assays can be run either in homogeneous or heterogeneous formats. In the former, all the immunoreagents are in solution, and there is no separation between the free (Ag and Ab) and the bound phase (Ag-Ab-labeled and nonlabeled) before the detection takes place. In the heterogeneous format, one of the immunoreagents is immobilized on a solid support, which facilitates the

isolation of the bound fraction (Ag–Ab). The most common markers are enzymes such as horseradish peroxidase and alkaline phosphatase (Estevez-Alberola and Marco, 2004). Apart from that, recent research has invented novel markers, and a brief overview is outlined in Estevez-Alberola and Marco (2004). Techniques applying novel markers are, for instance, the competitive binding chemiluminescent immunoassay (CLIA), competitive binding fluoroimmunoassays (FIA), time-resolved fluoroimmunoassay (TRFIA), and polarization fluorescent immunoassay (PFIA). According to Ingerslev and Halling-Sørensen (2003) the immunochemical techniques available for environmental detection of steroids are inferred from their clinical parents. The authors reported limits of detection (LOD) within those assays to range from 0.01 to 0.05 µg L^{-1} but also stressed that literature data often show higher LOD associated with prior difficulties in sample preparation. However, employing commercial available ELISA kits, Suzuki and Maruyama (2006) reported low LOD for E1 and E2 concentrations with 2.0 and 1.0 ng L^{-1}, respectively, obtained from municipal sewage and activated sludge, which constitutes a complex matrix. The technique is very sensitive, and fast improvements are promising a broad range of valid methods for detection of environmental steroid concentrations. At present, however, the development is still in an early stage and the immunoassays have several limitations (cited by Ingerslev and Halling-Sørensen, 2003), e.g., limitation to one analyte, vulnerability to cross-reactivity, and the difficulties in antibody syntheses.

5 SOURCES AND OCCURENCE OF ESTROGENS

As outlined in section 1, estrogens are naturally occurring female sex hormones that govern a plethora of biological functions. Not all the hormones are recycled internally and a major part is excreted in faeces or after conjugation in urine. Therefore the major sources for estrogens and their conjugates in the environment are on the one hand human excrement and on the other hand animal excrement originating from livestock breeding. These wastes are introduced to the environment either after extensive treatment in waste-water treatment plants (WWTPs, mainly urban domestic wastes), after

minor treatment, e.g., irrigation of effluents from oxidation ponds for livestock wastes, or without treatment through direct excretion by grazing livestock.

5.1 Human excrement

The amount of excreted estrogens by humans differs between the genders. The highest values of estrogens are excreted with the urine of pregnant women, which accounts for 259 µg d^{-1} (E2), 600 µg d^{-1} (E1), and 6,000 µg d^{-1} (E3) (cited by Johnson *et al.*, 2006). Based on a number of earlier published studies these authors assumed that males excrete 1.6 µg d^{-1} E2, 3.9 µg d^{-1} E1 and 1.5 µg $d^{-}1$ of E3 with their urine. The rate of urinary excretion by menstruating females ranges from 3.5 µg d^{-1} (E2), 6–8 µg d^{-1} (E1), to 4.8–6 µg d^{-1} (E3). In contrast, D'Ascenzo *et al.* (2003), who investigated hormone conjugates in Italian sewers, reported much higher values depending on the hormonal state of the females. These authors reported that on average (n=50) menstruating women excrete 20.9, 11.5, and 21.4 µg estrogens per day as E3, E2 and E1, respectively. Females in the menopause (n=22) still excrete 6.3, 7.0, and 12.7 µg estrogens per day as E3, E2 and E1. The study also investigated the urinary estrogen excretion of one pregnant female (6^{th} month); excretion values were 6,495 (E3), 258 (E2), and 940 µg (E1) per day. D'Ascenzo *et al.* (2003) noted that all these estrogens were mainly present in conjugated forms, with glucuronides being the dominant form while sulphates accounted for 23, 20 and 22% of the total estrogen derivatives excreted from menstruating, non-menstruating, and pregnant women, respectively.

The excreted free and conjugated steroids collected in sewer systems eventually end up in WWTPs. In general, these systems are capable of removing the majority of the estrogen loading (Khanal *et al.*, 2006). However, depending on geographical location and climate conditions as well as site-specific flow regimes, removal capacities vary and may be unsatisfactory at certain times (Ternes *et al.*, 1999; Schlüsener and Bester, 2008). Even though estrogens are not volatile, aerosol-bound emissions from WWTPs have been shown to occur (Beck and Radke, 2006). These losses contribute toward observed removal rates but might falsely be accounted to biological

degradation. Removal rates of WWTPs for estrogens range from as low as 34% to 100% (D'Ascenzo *et al.*, 2003; Kuster *et al.*, 2008; Schlüsener and Bester, 2008), and individual concentrations in the effluents are mainly in the lower ng L^{-1} range or below, potentially not bearing a risk to wildlife. However, there is evidence that estrogens are likely to act together and in an additive manner cause observed physiological effects such as the feminization of fish (Sumpter and Johnson, 2005; Thorpe *et al.*, 2003). Furthermore, it has been shown in Japan and Germany that concentrations of estrogen sulphates in WWTP effluents, as opposed to influents, can be elevated (Komori *et al.*, 2004; Schlüsener and Bester, 2008), leading to the assumption that re-conjugation processes had occurred within those engineered systems.

5.2 Animal excrement

The amount, type and proportions of excreted estrogens and conjugates depend on livestock species, and their reproductive stages and lactation periods. A comprehensive review by Hanselman *et al.* (2003) reveals that estrogen excretions by cattle seem to be evenly distributed between faeces and urine for non-pregnant cows, with values of 384 and 320 µg per day and per cow, respectively. However, for pregnant cattle more estrogens are excreted in urine and the ratio between urinary and faecal estrogens increases towards parturition. Similar trends are reported for sow wastes (Hanselman *et al.*, 2003; Johnson *et al.*, 2006). As for the prevalent form, free estrogens dominate in the faeces, while conjugated forms are mainly present in the urine. Estrone-3-sulphate appears to be the major sulphate conjugate in pregnant cattle urine, accounting for up to 92% of the total urinary estrogens at the end of gestation (Hoffmann *et al.*, 1997). Contributions of other livestock to environmental estrogen concentrations include sheep, poultry and horse breeding. One way of assigning environmental estrogen to livestock sources may be via the E2 epimers. Research has shown that the α-epimer is dominant in cattle waste, while the ß-epimer is dominant in sow wastes (Raman *et al.*, 2004; Sarmah *et al.*, 2006).

Typical concentrations of estrogens in waste lagoons range from > 1 to 5.9 µg L^{-1} (Raman *et al.*, 2004). The highest estrogen concentrations have been found in fresh dairy cattle manure, accounting for up to 640 and 1,230 µg kg^{-1} for E1 and E2, respectively (Shore and Shemesh, 2003).

5.3 Estrogens in the environment

Over the last decades, residues of the free hormones E1 and E2 have been reported mainly in river water and sediments due to the inevitable link to WWTP effluents discharging into the receiving waters. Recent improvements in the development of analytical methods for conjugates have allowed the measurement of trace concentration of these compounds in WWTP, receiving waters, and sediments. Typically, estrogen concentrations are elevated downstream of WWTP influents, with E1 being most frequently detected and at the highest concentrations (Labadie and Budzinski, 2005; Yamamoto *et al.*, 2006; Peng *et al.*, 2008). The reported concentrations range from > 1 to 65 ng L^{-1} (E1), > 1 to 2 ng L^{-1} (E2), and > 1 to 7 ng L^{-1} (E1-3S) (Kolpin *et al.*, 2002; Williams *et al.*, 2003; Rodriguez-Mozaz *et al.*, 2004b; Labadie and Budzinski, 2005; Yamamoto *et al.*, 2006; Kuster *et al.*, 2008; Peng *et al.*, 2008). Interestingly, both Kuster *et al.* (2008) and Rodriguez-Mozaz *et al.* (2004b) reported E1-3S concentrations in river water exceeding those of E1 in some cases. Kuster *et al.* (2008) even reported about 0.5 ng L^{-1} of E1-3S in a drinking water sample from Spain. Research from Japan and Germany reveals that WWTPs are not as efficient in eliminating estrogen conjugates as they are for their free counterparts (Komori *et al.*, 2004; Schlüsener and Bester, 2008), which could well explain the observed differences.

Barel-Cohen *et al.* (2006) investigated hormone concentrations over a 100-km stretch downstream of a sewage effluent discharge and found the E2 concentration to range from 2 to 4 ng L^{-1} over the entire distance during spring sampling, while in autumn E2 concentration were reduced from 4 to 2 ng L^{-1} within the first 30 km but dropped below the detection limit only at the end of the transect. Estrone has also been detected in estuary water that receives a high load of industrial and domestic waste waters over the course

of two years at concentrations from > 1 to 10 ng L^{-1}, and suspended matter was identified as a potential carrier for E1 (Noppe *et al.*, 2007). Estrogens are also abundant in the sediments of waters receiving wastes, with reported concentrations ranging from 0.05 to 3.6 ng g^{-1} for E1 (Reddy and Brownawell, 2005; Isobe *et al.*, 2006; Matějíček *et al.*, 2007) with a maximum of about 30 ng g^{-1} (Labadie *et al.*, 2007), and 0.08 to1.8 ng g^{-1} for E2. Estrone-3-sulphate has been detected in river sediments at about 0.4 ng g^{-1} (Matějíček *et al.*, 2007) and in Tokyo bay sediments at 0.05 ng g^{-1} (Isobe *et al.*, 2006).

Regarding environmental concentrations of estrogens, soil samples have largely been overlooked and data on this matrix is limited in the literature (Kuster *et al.*, 2004). However, there is some evidence for the occurrence of free estrogens in natural and agricultural soils. Very recently, Beck *et al.* (2008) reported estrogen concentrations both for a cropland soil that had been regularly amended with manure three times a year, and for an intensively grazed pasture soil. The concentrations of αE2, E2, and E1 were 7, 3, and 25 ng kg^{-1} in the cropland soil, and 5, 2, and 12 ng kg^{-1} in the pasture soil. Elsewhere in the U.S., estradiol at much higher concentrations was found in soil samples from a site being irrigated with recycled and pretreated brown water from a septic tank, with values of 10 and 6 µg kg^{-1} for the first and second 5 cm of the profile, respectively (Stanford and Weinberg, 2007).

As mentioned in section 3, the tendency for estrogens to evaporate is negligible, owing to their low vapour pressures. However, aerosol particles may act as estrogen carriers and could contribute to the dispersion of estrogens from WWTPs (Beck and Radke, 2006), an aspect that needs further investigations.

6 ENVIRONMENTAL FATE AND BEHAVIOUR OF ESTROGENS

Like other organic compounds, estrogens can undergo a variety of processes such as sorption, degradation, and leaching, after being released to the environment. Reduction in the concentration of the estrogens due to dilution could also play a role in their ultimate fate and distribution in the

environment. Figure 2.4. illustrates the possible distribution pathways and sinks of estrogens and their conjugates that are governed by the above-mentioned processes. Of all the processes, sorption is one of the most important factors, and the sorption characteristics of the hormones in a particular medium (e.g., soils, sludge and sediments) also affect other processes such as degradation and transport.

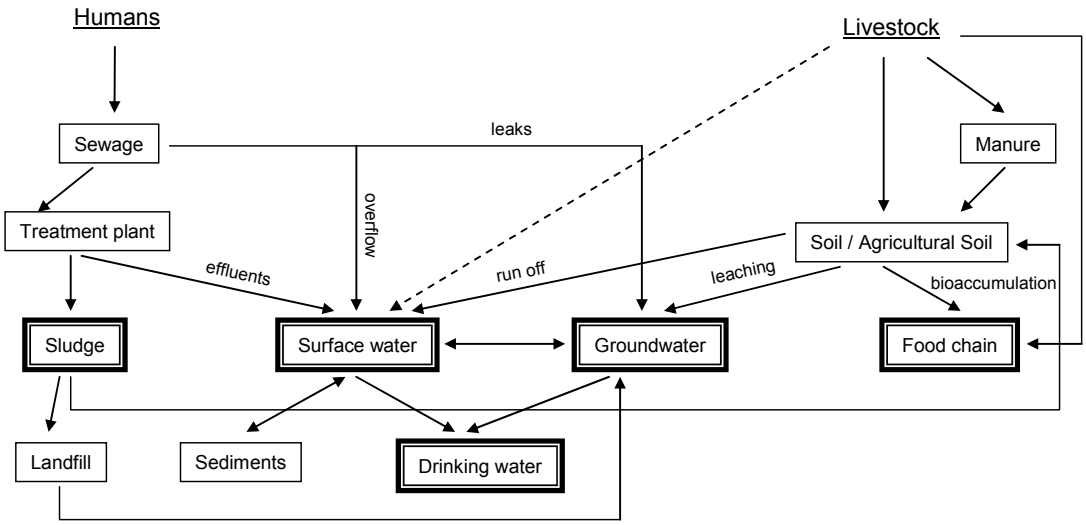


Figure 2.4. Pathways (arrows) and compartments (boxes) of environmental estrogen exposure and distribution. Adopted from Kuster *et al*. (2005).

Considering their high octanol-water partitioning coefficients (Table 2.2.), free estrogens are expected to exhibit substantial sorption to organic matter. Thus sorption behaviour has an important influence on the estrogen amounts that are available for biodegradation and transport processes in soils and sediments. Dilution factors were found not to be sufficient to explain decreasing estrogen concentrations downstream of waste water discharges (Williams *et al.*, 2003; Labadie *et al.*, 2007). The authors suggested biodegradation and sediment sorption are responsible for the observed concentration reductions, highlighting the importance of these processes for the environmental fate of estrogens. The following section therefore discusses the literature available on the sorption, degradation, and transport of estrogens in environmental matrices.

6.1 Sorption of estrogens

Sorption is the process in which chemicals become associated with solid-phases, and includes adsorption, if the compounds attach to a two-dimensional surface, and absorption, if the compounds penetrate into a three-dimensional matrix (Schwarzenbach *et al.*, 2003). Partitioning describes the process of distribution of a molecule between two phases (e.g., aqueous and solid) governed by equilibrium and can be considered as an umbrella term that comprises various processes that result in the distribution of organic contaminants between aqueous and solid phases. Sorption, therefore, can be considered as one type of a partitioning process since it involves the distribution of organic compounds between (pore) water and geo-sorbent matrices (Ehlers and Loibner, 2006). In the following context sorption refers to the reversible and irreversible uptake of a compound from an aqueous mediator solution by sorbents such as soil, sediment, minerals or colloids.

Several studies reported the sorption of estrogens to river-bed sediments and soils to be moderate to high. For instance, Holthaus *et al.* (2002) found sorption coefficients (K_d) ranging from 4 to 74 L kg^{-1} for E2 in river bed sediments while Ying and Kookana (2005) reported single point K_d values to range from 26 to 108 L kg^{-1} for E1, and from 30 to 123 for E2, respectively. However, sorption of hydrophobic organic chemicals (HOC) usually exhibits non-linear behaviour (Pignatello *et al.*, 2006) which was also observed for estrogens in sediments and soils (Lai *et al.*, 2000; Lee *et al.*, 2003; Yu *et al.*, 2004). The Freundlich equation, which has most frequently been used to describe non-linear sorption behaviour for many organic compounds, can be written as:

$$C_s = K_f \cdot C_w^N \tag{2.1}$$

where C_w is the solution concentration [mg L^{-1}], C_s is the sorbed phase concentration [mg kg^{-1}], K_f is the Freundlich capacity coefficient [mg^{1-N} kg^{-1} L^N], and N is the measure of isotherm linearity [dimensionless], an indicator of sorption site heterogenicity. Even though the model is considered as an empirical sorption equation, Freundlich-type isotherms can in fact result from the overlapping patterns of several Langmuir-type sorption phenomena

occurring at different sites on complex sorbents (Weber *et al.*, 1992) such as soil.

Most studies investigating the sorption of estrogens found *N* values < 1 indicating limited availability of specific sorption sites. Typical values for *N* range from as low as 0.57 to 1.0 (Lai *et al.*, 2000; Lee *et al.*, 2003, Yu *et al.*, 2004, Hildebrand *et al.*, 2006; Sarmah *et al.*, 2008). The organic matter domain of soils is considered to be the most important sorbent for HOC in soils and therefore literature often reports organic carbon normalized distribution coefficients (K_{oc}) for estrogens with log-transformed values ranging from < 3 to 4 (e.g. Lee *et al.*, 2003, Sarmah *et al.*, 2008), and in some cases even higher than 5 (Yu *et al.*, 2004). However, soils with a high specific surface area (SSA, [m^2 g^{-1}]) have especially shown to yield *N* values > 1 (Casey *et al.*, 2003; Hildebrand *et al.*, 2006; Sarmah *et al.*, 2008), suggesting that mineral surfaces, and in particular allophanic clay minerals, are important sorbents for estrogens (Sarmah *et al.*, 2008). Furthermore, montmorillonite was found to sorb E2 and to a lesser extend E1 (Van Emmerik *et al.*, 2003; Bonin and Simpson, 2007) probably by intercalation into expanding mineral layers. The observed pH dependence of E1 sorption onto montmorillonite with increased sorption capacity above pH 8 supports this assumption, and particle flocculation was also suggested to play an important role in the sorption of estrogens to montmorillonite at high pH values (Shareef *et al.*, 2006). Other typical soil mineral constituents such as goethite, illite, and kaolinite have been reported to exhibit either no or very low sorption capacities for E1 and E2 (Van Emmerik *et al.*, 2003; Shareef *et al.*, 2006; Bonin and Simpson, 2007).

In summary, the sorption behaviour of free estrogens appears to be very specific to the investigated sorbents and their compositions. While organic matter plays an important role, the impact of soil minerals can not be overlooked and they may contribute to the commonly observed sorption non-linearity of estrogens in soils. Partitioning into the organic matter domain can not be regarded as the sole driving process for sorption of estrogens, adsorption onto mineral surfaces has to be considered as well (Bonin and Simpson, 2007). Furthermore, non-linearity implies that the common approach of organic carbon normalization is a simplification that may not pass

for estrogens and thus lead to false inferences for risk assessments. There is no information about the sorption potential of estrogen conjugates such as sulphates even though these compounds are directly released to the soil environment by grazing livestock. Considering this exposure scenario, the aqueous matrix from where sorption takes place onto soil particles must also be taken into account. While there is evidence that salinity affects the sorption magnitude of free hormones (Bowman *et al.*, 2002) and that competition of several estrogens for specific sorption sites may occur (Yu *et al.*, 2004; Bonin and Simpson, 2007), little or no effort is being made to investigate the sorption behaviour of estrogens from environmental matrices such as cow urine for example. A point of contention may also be the differences in the experimental and analytical protocols used for parameter estimation that originate from sorption research focused on highly persistent HOC. Sterilizing agents are often used to avoid compound loss through biotic processes and to warrant a complete equilibration between sorbed and solution concentration. However, this seems to be a questionable approach for labile compounds such as estrogens that never reach a true equilibration under field situations (Hildebrand *et al.*, 2006*;* Sangsupan *et al.*, 2006). Thus the commonly used approach may produce an overestimation of the true sorption behaviour of estrogens and estrogen sulphates under field conditions.

6.2 Degradation of estrogens in soil

According to Klöpffer (1996), the term degradation refers to any chemical alteration a compound undergoes in the environment, and further, if the degradation results in the formation of simple inorganic compounds such as H_2O, CO_2, SO_4^{2-}, etc., the term mineralization is appropriate.

There is a historic, economic interest in the capability of microorganisms to convert and degraded steroids since steroidal compounds are an important part of modern medicine and have been used in various therapeutic applications, including anti-inflammatory, immunosuppressive, progestational, diuretic, anabolic, and contraceptive agents (Fernandes *et al.*, 2003). Furthermore, viable bacterial strains that are able to mineralize estrogens efficiently and actively would be of benefit for waste water

treatment processes and some advances in that respective field can be foreseen (Fujii *et al.*, 2002; Yoshimoto *et al.*, 2004).

The soil environment, however, has received little attention in terms of its estrogen degradation potential, and it is only over the last decade that researchers have started to investigate this matrix against the background of the compounds' potential adverse effects on human and wildlife health. In general, E2 degrades faster than E1 in soil, which seems logical, given the compounds' chemical structure (Figure 2.3., section 3). The oxidation of the hydroxyl group at position C-17 of the E2 molecule to yield the reduced carboxyl group of E1 can be catalysed by a broad range of dehydrogenase enzymes in the soil. Estrone degradation, in contrast, likely involves ring cleavage that is energetically less attractive and therefore could explain the different degradation patterns between E2 and E1. Results from laboratory experiments demonstrated that E2 rapidly degrades forming E1 as a major metabolite under aerobic conditions in soils, and degradation rates (k, [h^{-1}]) showed some dependence on soil type, temperature and moisture content (Colucci *et al.*, 2001, Xuan *et al.*, 2008). Furthermore, one study reported the degradation of E2 occurred in sterilized soils, which was attributed to abiotic oxidation probably facilitated by mineral surfaces, and the formation of non-extractable, slowly mineralizing soil residues was observed (Colucci *et al.*, 2001). Low to non-existent degradation in sterile soils and the increased degradation and mineralisation with increasing temperatures indicate that the degradation of estrogens is to a large extent governed by microbial processes in the soil environment. A study by Xuan *et al.* (2008) draws the same conclusion from an experiment where the degradation rate of E2 was found to be directly proportional to the amount of non-sterile soil. Soil enzymes such as ß-glucuronidases (E.C. 3.2.1.31) and arylsulphatases (3.1.6.1) are believed to be responsible for cleaving the conjugate structure and leading to the release of free estrogens (Lucas and Jones, 2006; Khanal *et al.*, 2006). However, this process has only recently been indirectly substantiated for waste water systems (D'Ascenzo *et al.*, 2003; Okayasu *et al.*, 2005), and a thorough investigation of the degradation of estrogen conjugates in the soil environment is not yet available.

While most studies involving directly spiked soil observed the immediate degradation of E2 (Colucci *et al.*, 2001; Lee *et al.*, 2003; Casey *et al.*, 2005; Jacobsen *et al.*, 2005), a recent study by Lucas and Jones (2006) revealed that natural exposure matrices such as sheep urine could temporarily inhibit the degradation of E2 and E1. The authors speculated that estrogens in soil would in general be metabolised in the co-metabolism of microorganisms, and that the microbial population would have to adapt to the estrogens before utilizing them, which could serve as an explanation for the delayed degradation pattern. Another possibility for the observed differences is the presence of veterinary antibiotics such as tylosin, sulphamethazine or tetracyclines in animal wastes. These have been shown to alter the dehydrogenase activity in soils that also received E2 (Chun *et al.*, 2005). 17ß-estradiol degradation rates were also found to be significantly slower under the presence of the antibiotic sulphadimethoxine (Xuan *et al.*, 2008) (Table 2.3.).

Dissipation times (DT) of estrogen in soil depend on the conditions mentioned above, and half-lives (DT_{50}) range from as low as a few hours to approximately 25 days. Table 2.3. lists a selection of estrogen degradation studies in soils where degradation and/or mineralisation rate constants and/or half-lives were reported, covering a variety of soil treatments and management histories. Half-lives are in general estimated assuming first-order degradation kinetics. However, research has shown that residues of the hormones can be expected to persist in soils for timeframes exceeding months (Casey *et al.*, 2005; Lucas and Jones, 2006; Stumpe and Marschner, 2007), and the simple single-first order kinetic approach may not be sufficient to describe their degradation patterns (Das *et al.*, 2004; Stumpe and Marschner, 2007).

In the reported literature there has been very little effort to model the formation of E1 during the degradation of E2, which is a considerable gap in the knowledge required for a sound risk assessment of estrogen applications onto agricultural lands. However, such models have successfully been employed to describe the degradation and metabolite formation of androgens (Khan *et al.*, 2008b), pesticides (Understrup *et al.*, 2005; Etzerodt *et al.*,

2008), and pharmaceuticals (Richter *et al.*, 2007). Even though these concepts are available they have rarely been applied to estrogens, and the degradation of estrogen sulphates in particular lacks experimental and subsequent modelling investigations to clarify the impact on free estrogen formation and the related estrogenic potential.

Table 2.3. Soil degradation studies and calculated DT_{50} values of estrogen degradation in soils for a variety of treatments reported in the literature.

Concentration/ Treatment	Process	Kinetic	DT_{50} [d]	Reference
1 mg kg^{-1} (three soils at 13% m.c.[a], 30°C)	mineralisation			
	estradiol	first-order	0.22—0.48[b]	Colucci *et al.* (2001)
	estrone		0.61—1.7[b]	
1 mg kg^{-1} (60% m.c., 20°C)	mineralisation			
waste water vs.	estradiol			Stumpe and Marschner (2007)
fresh water irrigated	estradiol	first order	288[b]	
			267[b]	
1 mg kg^{-1} (25°C)				
10, 15, 20% m.c. saturated	degradation of estradiol	first order	1.3, 0.92, 0.69, 1.2	Xuan *et al.* (2008)
1 mg kg^{-1} (25°C, 15 m.c.) with				
sulphadimethoxine	inhibition of estradiol degradation	first order		Xuan *et al.* (2008)
0 mg kg^{-1}			0.92	
12.4 mg kg^{-1}			1.4	
62.1 mg kg^{-1}			2.6	

[a]m.c.=moisture content. [b]DT_{50} values were calculated from the presented rate constants (k) ($DT_{50}=\ln(2)\ k^{-1}$).

6.3 Runoff and transport estrogens

Given their high octanol-water distribution coefficients (Table 2.2.) and the moderate to high sorption tendency (section 6.1), free estrogens are unlikely to exhibit significant mobility in the soil compartment (Sangsupan *et al.*, 2006). Similar conclusions could be drawn from the reported sorption and degradation studies that suggest moderate to high sorption and reasonably fast degradation for E2 and E1 in soils (see previous sections). However, field trials have revealed that realistic leaching risks can not easily be inferred

from laboratory scale experiments. For instance, E2 has been reported in karst springs in the U.S. (Peterson *et al.*, 2000) and positive correlations were found with faecal coliforms and *E.coli* in all instances, indicating that E2 originates from animal waste applications and all three compounds move similarly in the mantled karst. Furthermore, Nichols *et al.* (1997) have shown that E2 runoff occurred from poultry litter application to pasture with a maximum concentration of 1.28 μg L^{-1}, and E2 persisted for at least 7 days under their field conditions.

In order to better understand the realistic transport behaviour of estrogens in the field, a few researchers have studied the transport of estrogens in experimental soil columns. Casey *et al.* (2003) used packed micro-columns to monitor the break through curves of applied estradiol. Inverse modelling with the HYDRUS-1D transport model showed that although the inclusion of sorption and transformation processes resulted in good data fitting, the estimated sorption parameters did not match with the corresponding batch results. The estimated parameters had large confidence intervals, the metabolites could not be monitored, and hydrodynamic dispersivity values were high (3.5 to 62 cm) for their 15.2-cm-long homogenous soil columns. Unambiguous parameter estimation was therefore probably not warranted, and physical non-equilibrium conditions existed, which is in contrast to their reported chemical non-equilibrium (Das *et al.*, 2004). Inclusion of a first-order-rate limited sorption process was able to improve the description of column transport data in a subsequent study (Casey *et al.*, 2005) but degradation processes are still largely unknown. In a similar study, Das *et al.* (2004) used pulse and flow-interruption conditions with packed micro-columns to independently derive transport mechanisms and degradation parameters for E2 and its metabolite E1. While their forward modelling efficiency was satisfactory to describe the observed breakthrough curves, the authors pointed out that better models need to be developed to account for complex degradation processes.

Using undisturbed soil columns, Sangsupan *et al.* (2006) found lower sorption affinity for estrogens in subsoil as opposed to topsoil and revealed that hormone transport is affected by both chemical and physical non-

equilibrium conditions indicating that preferential flow patterns could lead to groundwater contamination. Pronounced macro-pore flow was also identified as one of the causes of estrogen leaching into the drainage of an agricultural site after manure treatment (Kjær *et al.*, 2007). The authors stressed that the area of agricultural land from which estrogens may potentially be transported to the aquatic environment is much larger than previously believed. More recently, Arnon *et al.* (2008) investigated the profile under a dairy farm waste lagoon for its estrogen distribution. The lagoon has been operated for > 40 years and trace estrogen concentrations in the range of 30–210 ng kg^{-1} were detected down to 32 m below surface, and in the groundwater at 47 m below surface. Forward modelling using HYDRUSS-1D was not fully able to explain the detected estrogen concentrations, and mechanisms including hormone-manure interactions and preferential flow paths were suggested to lead to enhanced transport rates. The authors also stressed that the common practice of clay lining to prevent leaching from such lagoons can not completely protect the groundwater environment from waste lagoon leachate under long-term exposure.

7 NEW ZEALAND CONDITIONS

To date, research involving estrogens and their environmental fate is very limited in New Zealand. An initial screening survey by Sarmah *et al.* (2006) involved analysis of estrogen concentrations in the effluents of livestock farms and sewage treatment plants around the Waikato region. While most of the farm effluent contained concentrations in the range of 46.2 to 4,416 ng L^{-1} of total estrogens (αE2, E2 and E1), only two out of three WWTP had estrogens in their effluent (Table 2.4.). The biologically determined estrogenicity exceeded the estrogenicity predicted by the chemical analysis, indicating other compounds contributed to the estrogenic activity in the samples. In another study (Leusch *et al.*, 2006), two New Zealand WWTPs were found to be very efficient in estrogen removal and no estrogenicity was reported in the plants' effluents.

Table 2.4. Concentration of estrogens in dairy (Farm 1–7), goat, piggery and sewage effluent (Pukete, Taupo and Temple View) samples. Source: Sarmah *et al*. (2006).

Compound	Concentration (ng/L)											
	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6	Farm 7	Piggery	Goat	Pukete	Taupo	Temple view
17α-estradiol	40.0	592	458	1028	ND	18.8	ND	10.9	172	ND	9.5	ND
17β-estradiol	28.8	289	147	331	ND	ND	ND	8.0	47.1	T	14.8	T
Estriol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Estrone	66.9	3123	696	3057	ND	40.9	ND	27.3	157	T	84.7	19.0
17α-ethynyl estradiol	ND	ND	ND	ND	ND	ND	ND	ND	ND	T	ND	ND
Total	135.7	4004	1301	4416	–	59.1	–	46.2	376.1	–	109	19.0

ND=not detected; T=trace level below MDL; Farm 7 sample was split and lost during derivatisation and therefore data was not available.

During the course of this thesis sorption potential of some New Zealand soils was found to be moderate to high and it was hypothesised that allophanic soils, typical of parts of the North Island of New Zealand, might have unique sorption capacities for estrogens and play an important role in the fate of estrogens in the New Zealand environment (Sarmah *et al.*, 2008).

In New Zealand, agricultural business makes a major contribution to the overall economy. As indicated in Figure 3.4., in 2005/2006, agricultural products contributed 47% to the country's total exports of $29.7 billion (agricultural economics of Australia & New Zealand, 2008). Given the fact that New Zealand's beef and dairy industry is mainly based on pasture grazing systems (Shorten and Pleasants, 2007) and a combined population of over 9.5 millions head of cattle (Statistics New Zealand Tatauranga Aotearoa, 2008) is estimated to currently graze New Zealand's paddocks, the potential exists for estrogen contamination of ground and surface waters.

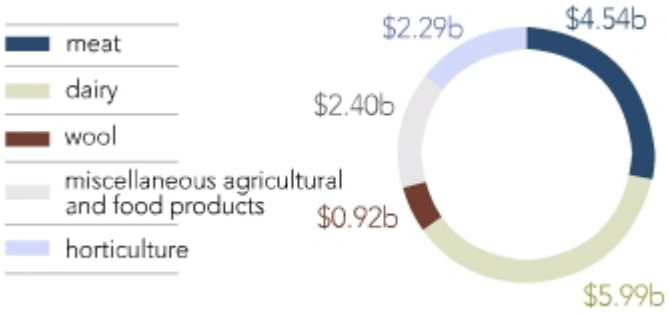


Figure 2.5. Agricultural export receipts, 2005/2006, New Zealand. Source: agricultural economics of Australia & New Zealand (2008).

According to one estimate, the combined livestock population in New Zealand excretes about 40 times the amount of the human population (Ministry for the Environment [MfE], 1997), and land application of dairy effluent has become increasingly popular. Research involving dairy grazing systems has furthermore shown that approximately 85% of defecations and urinations occur in the paddocks (White *et al.*, 2001), indicating that future research into the environmental fate of estrogens from animal wastes should not only focus on waste lagoons and their effluents but also address soil processes that govern estrogen distributions.

8 THESIS OBJECTIVES

The previous sections have highlighted the gaps in the scientific knowledge base about the fate of estrogens in the environment, and have stressed the fact that little is known in particular about the fate of their important sulphate conjugates in the soil environment. The aim of this research work was therefore to improve our knowledge and understanding of estrogen and estrogen sulphate behaviour in the soil environment where animal excretions are the main sources for these chemicals. The specific objectives were:

- to develop an extraction method capable of extracting estrogen sulphates from aqueous solutions and soil;
- to develop an analysis method capable of detecting sulphate hormone conjugates by means of HPLC-UV detection;
- to investigate the sorption behaviour of single free and sulphate-conjugated estrogens in a range of soils from the commonly used $CaCl_2$ solution and an artificial urine solution in the laboratory;
- to investigate and model the degradation behaviour and metabolite formation kinetics of single free and sulphate-conjugated estrogens in different soil substrates under a range of temperature regimes in the laboratory;

- to determine the transport behaviour of single free and sulphate-conjugated estrogens in undisturbed soil micro-columns as influenced by the mediator solution ($CaCl_2$ versus artificial urine).

CHAPTER III
ANALYSIS OF ESTROGENS AND THEIR SULPHATE CONJUGATES

1 INTRODUCTION

High-performance-liquid-chromatography (HPLC) offers a reliable analytical tool for separating organic contaminants from pre-treated (environmental) samples. Its application in combination with photo spectrometric (PS) detectors for the quantification of estrogens is commonly reported in the literature and constitutes a cheaper alternative to the more sensitive mass spectrometers (MS) (Ingerslev and Halling-Sørensen, 2003). The application of HPLC in laboratory-scale microcosm experiments that do not necessarily require the low detection limits demanded for environmental residue analysis has long been established. The most commonly employed PS detectors are diode array detection (DAD), ultraviolet detection (UV) and fluorescence detection (FD) (Ying and Kookana, 2003; Jacobsen *et al.*, 2005; Bonin and Simpson, 2007; Sarmah *et al.*, 2008; Xuan *et al.*, 2008). As these detectors, unlike MS, are not able to identify analytes unmistakably, the complete separation within the chromatographic column is crucial for a successful quantification of target compounds (Ingerslev and Halling-Sørensen, 2003). The most commonly used analytical columns are C-18 and ODS-2 silica columns and the dominant mobile phase consists of acetonitrile and water, which is sometimes acidified. The recent development of monolithic HPLC columns has led to a higher sample throughput capacity owing to the higher flow rates that can be employed on these columns (Cledera-Castro *et al.*, 2005); and the implementation for estrogen separation has also been reported (Mizuguchi *et al.*, 2005). Separation of hormone conjugates employing HPLC in combination with PS detection methods has received little attention, though a study by Blom *et al.* (2001) reported the separation of eight estrogens including five conjugates on a HPLC-UV system, suggesting the feasibility of HPLC-UV. The mobile phase in that study consisted of methanol and a 20 mM ammonium sulphate buffer, and separation was performed within about 40 minutes at a flow rate of 1 mL min^{-1}. Method detection limits for a HPLC-UV method, considering sample extraction, cleanup and pre-concentration, were in the lower µg L^{-1} and µg kg^{-1} range for aqueous and solid samples, respectively (Lee *et al.*, 2003).

There is an extensive body of literature available dealing with various methods for extraction of free conjugated estrogens from aqueous and water samples. The methods are sophisticated and often involve a series of cleanup and separation procedures comprising filtration, solid-phase-extraction, sonication, accelerated solvent extraction, and microwave assisted solvent extraction among others (López de Alda and Barceló, 2001; Díaz-Cruz *et al.*, 2003; Kuster *et al.*, 2004; Matějíček *et al.*, 2007; Beck *et al.*, 2008). The majority of these methods are tailored to the respective subsequent analytical technique, and even though methods are validated, the replication of procedures in a different laboratory environment often requires further modifications and adjustments. In laboratory-scale experiments the use of less extensive extraction methods has been proved suitable to investigate environmental fate processes such as sorption and degradation. In particular, the use of dichloromethane (DCM), comprising a subsequent solvent transfer process, achieved recoveries in the range of 77 to 105 % for free estrogens (Lai *et al.*, 2000; Lee *et al.*, 2003; Sarmah *et al.*, 2008). The feasibility of chlorinated solvents for the extraction of estrogen sulphates from urine samples was reported earlier and showed excellent recoveries when employed with dicyclohexylamine hydrochloride (Deans *et al.*, 1955). However, to date no studies have been conducted using these solvents and modifiers to extract estrogen sulphates from aqueous samples and no literature is available on the extraction of estrogen sulphates from soil samples.

This chapter illustrates the analytical methods that had been developed to extract and analyse estrogens and their sulphate conjugates in aqueous and soil samples. Most of the presented methods have been utilized throughout the entire Ph.D. project and constitute an integral part of the subsequent chapters investigating the fate and behavior of estrogens and estrogen sulphates in agricultural soils by means of laboratory-scale experiments.

2 MATERIALS AND METHODS

2.1 Chemicals

17β-estradiol (E2, > 98% purity), estrone (E1, > 99% purity), 17β-estradiol-3-sulphate (E2-3S), and estrone-3-sulphate (E1-3S) (all ≥ 95% purity) were purchased from Sigma-Aldrich Ltd, Australia. Acetonitrile (Mallinckrodt ChromAR, ≥ 99.8% purity), dichloromethane (DCM, Mallinckrodt UltimAR, ≥ 99.9% purity), methanol (Mallinckrodt ChromAR, ≥ 99.9% purity), and ammonium sulphate (BDH Laboratory Supplier AnalaR, > 99% purity), potassium chloride, potassium sulfate (BDH Laboratory Supplier AnalaR, all > 99% purity), urea (Labserv Analytical Grade, > 99% purity), potassium bicarbonate and glycine (Ajax Finechem Analytical) were obtained from Biolab Scientific Ltd, New Zealand. Dicyclohexylamine (Merck, > 99% purity) was obtained from the University of Waikato Chemical Store, New Zealand, and synthesized with concentrated hydrochloric acid (Ajax Finechem, 36%) to form solid dicyclohexylamine hydrochloride (DCH·HCl). HPLC grade deionised water was obtained from an onsite arium® 61316 high performance reverse osmosis system (Sartorius Stedim Biotech GmbH, Germany). Oxygen free nitrogen gas (gas code 152) was purchased from BOC gas supplier (BOC, New Zealand).

2.2 HPLC–UV system specifications

Analysis of the free and sulphate estrogens was performed on a Dionex Summit High Performance Liquid Chromatography (HPLC) system comprising a Dionex Solvent Rack SOR-100, a Dionex Thermo-stated Column Compartment TCC-100, a Dionex ASI-100 Automated Sampler, a Dionex P680 LP-Pump, and a Dionex UVD 170U detector. The UV lamp was a L2D2 deuterium lamp (Hamamatsu Photonic, K.K., Japan) and the injection syringe was a Gastight® from Hamilton Bondaduz (#1725, 0.25 mL, Switzerland).

The analytical columns used were a Luna 5u RP-C18 (150 × 4.6 mm, Phenomenex®, Australia) and a Chromolith Performance RP-18 (100 × 4.6 mm, Merck®, Germany). The monolithic column was later replaced by an equivalent Onyx® Monolithic C18 column (100 × 4.6 mm, Phenomenex®, Australia). All columns were operated with a Security Guard™ column

cartridge holder equipped with a C18 cartridge (4 mm, Phenomenex®, Australia).

2.3 HPLC–UV separation and detection

The elution scheme by Blom *et al.* (2001) was modified to separate the estrogens E2 and E1 and their sulphate conjugates E2-3S and E1-3S. The original organic solvent methanol was replaced by acetonitrile due to the higher auto-absorbance of methanol at lower detection wavelengths that were found to be more sensitive for estrogen detection (see below). The elution scheme was initially developed on the Luna 5u RP-C18 column and later implemented for the monolithic columns to achieve a reduction in runtime and consequently a higher sample throughput. Standard linearity was assessed in the concentration range of 0.1 to 1.0 µg mL^{-1} and 1.0 to 20 µg mL^{-1}. The limits of detection were calculated on a signal to noise ration of 3 to 1.

The optimal detection wavelength was determined by preparing a combined solution of E1, E2, E1-3S, and E2-3S each at 10 µg mL^{-1} in mobile phase. The UV absorbance of the solution was then measured on a Shimadzu UV 160A UV-Visible Recording Spectrophotometer (Shimadzu, Japan) from 200 -300 nm in a full-scan mode.

2.4 Extraction from aqueous matrices

An artificial urine solution was prepared in accordance with Early *et al.* (1998) and consisted of $KHCO_3$ (22.2 g L^{-1}), KCl (3.95 g L^{-1}), K_2SO_4 (6.7 g L^{-1}), $(NH_2)_2CO$ (23.5 g L^{-1}), and $C_2H_5NO_2$ (6.2 g L^{-1}). To extract the hormones from calcium chloride solution ($CaCl_2$, 5 mM) and artificial urine solutions, the method by Lee *et al.* (2003) was modified. Aqueous solutions of E1 and E1-3S were prepared at concentrations of 0.25, 0.5, 0.75, 1.5, 2.5 and 5.0 µg mL^{-1} by adding appropriate amounts of methanolic stock solutions (200, 400, and 600 µg mL^{-1}) to the respective mediator solution. An aliquot of 5 mL of the respective hormone solution was extracted by liquid-liquid extraction with DCM (4.9 mL). In order to extract E1-3S, an additional volume of 0.25 mL DCH·HCl (10 µg mL^{-1} in H_2O) was added to the aqueous matrix. The samples were extracted over night on an end-over-end shaker at 22 ± 1°C. After centrifugation at 2200 rpm, an aliquot of 2 mL of the DCM phase was carefully

removed with a glass pipette and transferred to an amber glass HPLC vial and evaporated under a gentle stream of N_2 at 22 ± 1°C. The dried sample was then reconstituted in 20% and 70% methanol for E1-3S and E1, respectively. Reconstituted samples were immediately analysed by means of HPLC-UV detection.

2.5 Extraction from soil samples

During the sorption study the extraction of the free estrogen E1 was performed in accordance with Lee *et al.* (2003); however, including a sonication step. In brief, 4.9 mL of DCM were added to 2-3 g of soil(slurry), sonicated for 10 minutes in a sonication bath (Lab Line, Elma, Germany), and placed on an end-over-end shaker over night at 22 ± 1°C. For extraction of the E1-3S, an additional volume of 0.25 mL DCH·HCl (10 µg mL^{-1} in H_2O) was added before sonication. After centrifugation at 2200 rpm, an aliquot of 2 mL of the DCM phase was then transferred to an amber glass HPLC vial and processed as described for the aqueous extraction (*section 2.4*)

During the degradation study extraction recovery was evaluated by means of the sterile controls. In brief, 150 g of soil, adjusted to 60% of the maximum water holding capacity (-33 kPa), was autoclaved three times in preserving jars (122.5°C, 1.13 Bar, Priorclave, Ltd, U.K.). After autoclaving, the water loss was determined gravimetrically and the lost volume was reapplied using sterilised deionised water containing appropriate amounts of E1-3, E2-3S, E1, and E2, respectively, to yield a nominal soil concentration of 5 mg kg^{-1} soil. To maintain sterility during the experiment, the entire procedure was conducted in a laminar flow cabinet and the aqueous spiking solution was carefully mixed with the soil with a sterilised spatula. Triplicate sub-samples of approximately 2–3 g were then removed and extracted by liquid-liquid extraction with 5 mL of DCM and 0.25 mL of DCH·HCl (10 µg mL^{-1}) on an end-over-end shaker at 22 ± 1°C over night. Further processing and analysis was conducted as described before (*section 2.4*).

3 RESULTS AND DISCUSION

3.1 HPLC–UV separation and detection

3.1.1 Optimum UV absorbance

Figure 3.1. displays the resulting UV absorbance spectrum of the combined estrogen standard for the wavelength range from 200 to 300 nm. The graph shows two absorbance optima at 201 and 279 nm. The relative absorbance at 201 nm was about 20 times higher than at 279 nm, indicating that both the free and the sulphate-conjugated estrogens have an optimum absorbance in the far UV range. A wavelength of 201 nm was selected for the detection of the hormones and their sulphate conjugates.

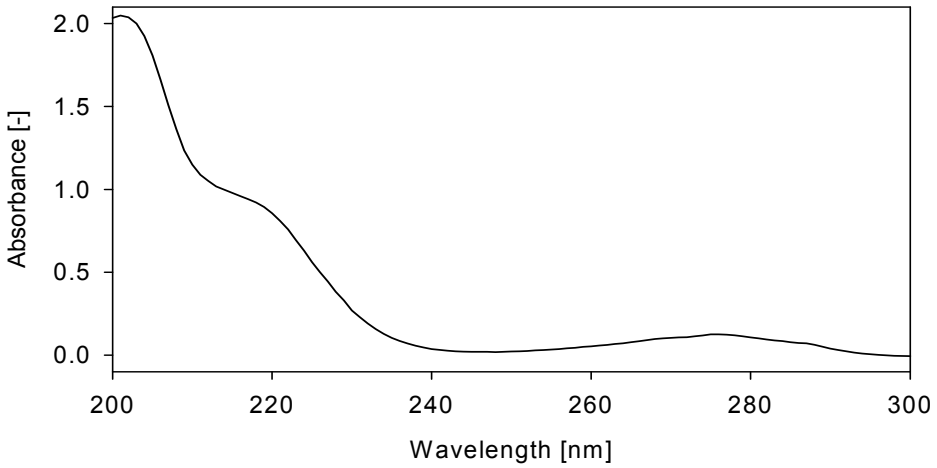


Figure 3.1. Absorbance spectrum of a combined estrogen standard in mobile phase.

The predominant wavelength for E1 and E2, as well as for estrogen conjugates detection via UV detectors reported in the literature, is 280 nm (Blom *et al.*, 2001; Van Emmerik *et al.*, 2003; Yu *et al.*, 2004; Bonin and Simpson, 2007), which is in close agreement with the second optimum found in the present study. Lee *et al.* (2003) and Sarmah *et al.* (2008) reported wavelengths of 205 and 225 nm, respectively, for the detection of E1 and E2, indicating that lower wavelengths may also be suitable for estrogen detection. The differences may be explained by the diverse UV detectors in use. Older detectors often lack the capability to run wavelengths in the lower

UV range and therefore the wavelength optimum found in this study may not have been available in some of the earlier studies.

3.1.2 Compound separation

The aqueous mobile phase for estrogen sulphate separation was derived from the study by Blom *et al.* (2001), where a number of six estrogen conjugates, sulphates and glucuronides, were separated in 40 minutes together with the parent compounds E1 and E2 on a C18 (4.6 × 250 mm) HPLC column employing a gradient system of ammonium sulphate (20 mM, pH 6.8) and methanol. Methanol has a high auto-absorbance at the previously determined optimum detection wavelength and was replaced by acetonitrile, which has been employed for the free estrogen separation on HPLC-UV systems (Lee *et al.*, 2001; Ying *et al.*, 2007). Furthermore, the ammonium sulphate concentration of the buffer solution was reduced to 5 mM and acidified with concentrated sulphuric acid (H_2SO_4) to pH 3 in order to prevent the sulphate conjugates being flushed through the HPLC column without significant retention. Additionally, high purity deionised water was employed as a third mobile phase to prevent salt precipitations during gradient operations. Implementation of the optimized gradient on a monolithic HPLC column reduced the runtime from approximately 22 minutes to 9.3 minutes due to the higher flow rate of 2 mL min^{-1} applied to the monolithic column. The optimized gradient system allowing for separation of E1-3S, E2-3S, E1 and E2 is illustrated in Figure 3.2., while Figure 3.3. displays a matching standard chromatogram (5.0 µg mL^{-1}) for an injection volume of 50 mL. The column temperature was maintained at 22 ± 1°C.

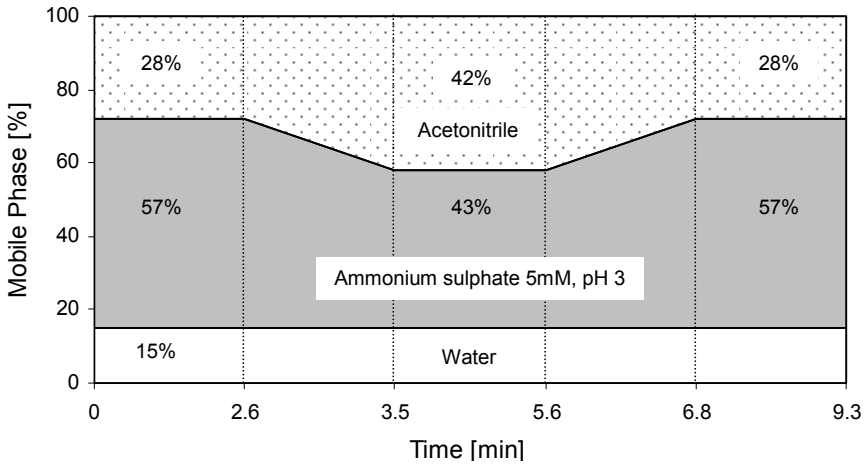


Figure 3.2. Mobile phase gradient system for the separation of E1-3S, E2-3S, E1 and E2 prior to UV detection. Dotted vertical lines indicate a change in mobile phase composition.

The standard chromatogram shown in Figure 3.3. indicates that the gradient system yielded a distinct separation of the four target compounds and that the matrix influence was negligible. However, blank matrix runs were always conducted both as a matter of quality assurance and to identify possible additional retention mechanisms that might occur on analytical columns as a result of high sample throughput and/or guard column failure.

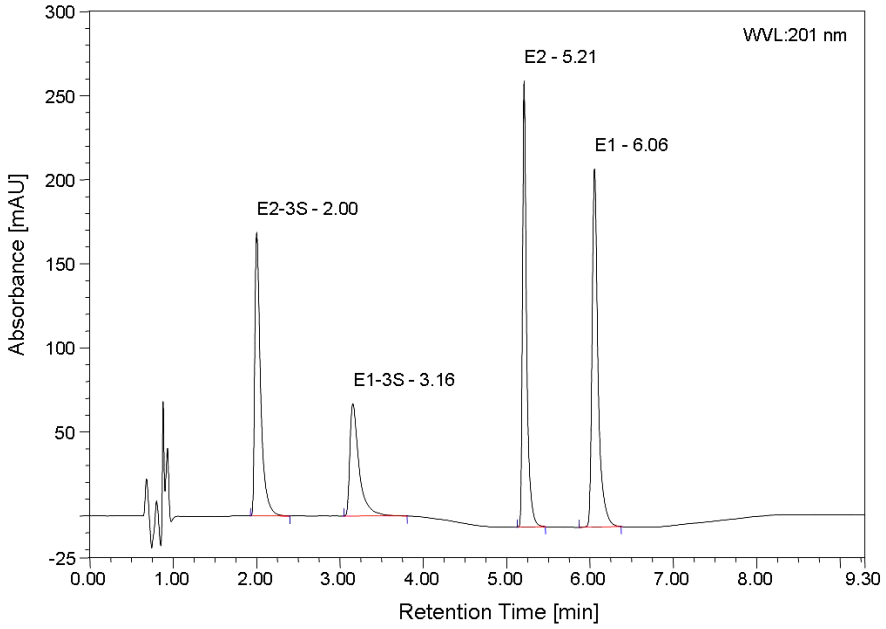


Figure 3.3. Standard chromatogram for the separation of E2-3S, E1-3S, E2 and E1 (5.0 µg mL^{-1}) on a monolithic C18 column (Phenomenex Onyx®, 4.6 × 100 mm).

The free estrogens E1 and E2 have been successfully separated on a commonly packed C18 analytical column using mixtures of acetonitrile and water as the mobile phase. Typical runtimes are in the range of 11–20 minutes to separate E1 and E2 at flow rates of 1.0–1.5 mL min^{-1} with varying acetonitrile/water ratios (Gatti *et al.*, 1998; Lee *et al.*, 2003; Choi *et al.*, 2006). In the present study, the implementation of a monolithic C18 analytical column resulted in a significant reduction of the sample runtime, and separation of E1 and E2 was achieved within 2.5 minutes with a mobile phase consisting of 47% acetonitrile and 53% water and a flow rate of 2.0 mL min^{-1}. Figure 3.4. displays a standard chromatogram for E1 (5.0 µg mL^{-1}) and E2 (5.2 µg mL^{-1}) at a column temperature of 22 ± 1°C. Similar reductions in retention times have been reported earlier, and the application of monolithic silica columns for the separation of E2 and E1 was found to be suitable (Mizuguchi *et al.*, 2005).

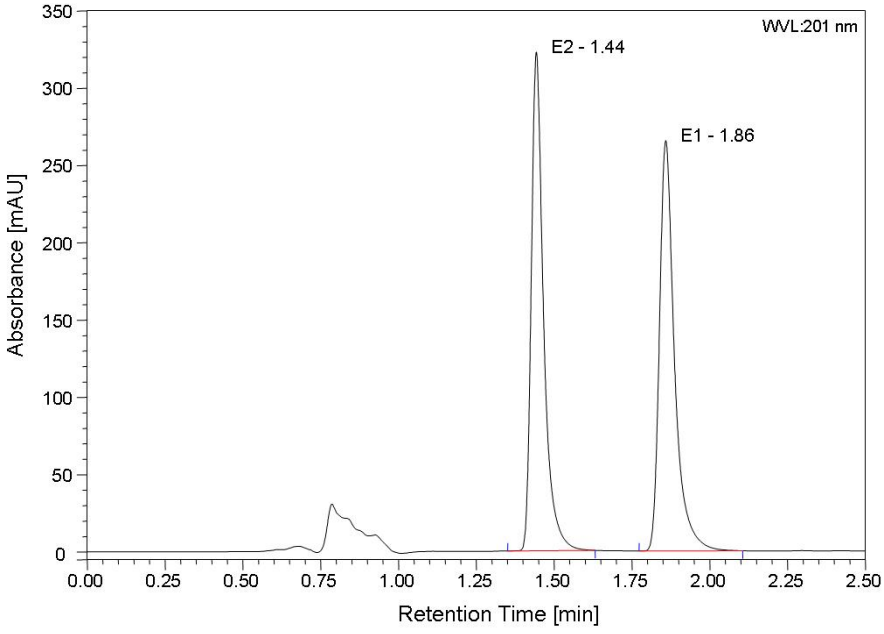


Figure 3.4. Standard chromatogram for the separation of E2 (5.2 µg mL^{-1}) and E1 (5.0 µg mL^{-1}) on a monolithic C18 column (Phenomenex Onyx®, 4.6 × 100 mm).

3.1.3 Standard linearity and detection limits

The standards for all four compounds were linear in the range of 0.01-1.0 µg mL^{-1} (n=6) and 1.0-20 µg mL^{-1} (n=6), respectively. The correlation

coefficients for the standards were > 0.999 over the course of the entire experimental studies, and deviations in the correlation coefficient constituted a reliable indicator of faulty standard preparation. If variations occurred, a new standard series was prepared and rerun. The on-column limits of detection at an injection volume of 50 µL and S/N (signal/noise) ratio of 3 were: 9.0 ng/mL, 10 ng/mL, 5.0 ng/mL, and 7.0 ng/mL for E2-3S, E1-3S, E2 and E1, respectively.

3.2 Extraction from aqueous matrices

The extraction recovery for E1 and E1-3S from the aqueous matrices $CaCl_2$ (5 mM) and artificial urine at six concentrations is displayed in Figure 3.7. In general, the recoveries were excellent and ranged from 97.3 to 107 % for both compounds across the six concentrations. A conclusive trend in relation to the aqueous matrix or the aqueous concentration was not, however, detected. Matrix effects that could have been expected from urea and glycine interference in the artificial urine extraction did not occur. The method detection limits accounted for 1.0 ng mL^{-1} for both compounds.

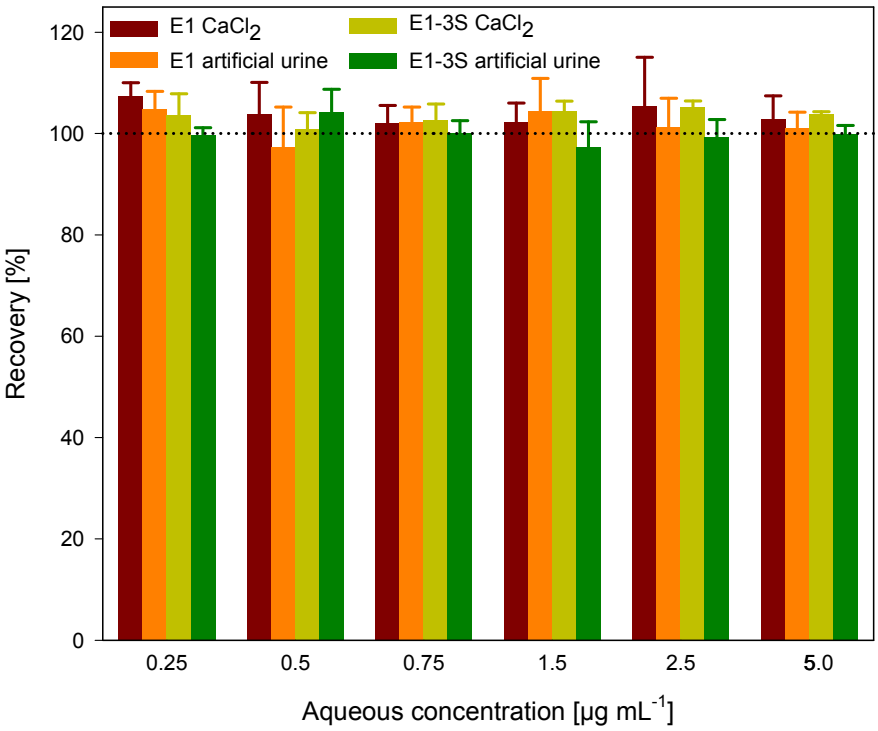


Figure 3.5. Recoveries for the extraction of E1 and E1-3S from the aqueous matrices $CaCl_2$ (5 mM) and artificial urine at six concentrations. Dotted line indicates 100% mark. Average values and relative standard deviations are shown.

Dichloromethane has been reported to be suitable for liquid-liquid extraction of estrogens from aqueous samples and the recoveries reached in the present study are in the range of previously reported values. For instance, Soto *et al.* (2004) achieved a recovery of 102% for E1 with DCM extraction. A lower recovery of 82.5% for E1 was reported by Lai *et al.* (2000); however, Lee *et al.* (2003) found DCM extractions to be excellent for their intended purpose to investigate sorption of estrogens in soils and river sediments. The lower recoveries found by Lai *et al.* (2000) may be also a result of their subsequent derivatization for GC-MS analysis during which compound loss could have occurred. The method detection limits of the present work are five times lower than reported by Lai *et al.* (2000) and two times higher than reported by Lee *et al.* (2003), which is attributed to the different DCM/aqueous matrix ratios employed amongst the three studies. Lai *et al.* (2000) used a similar soil to solution ratio but were not able to achieve a similar pre-concentration due to their lower absolute DCM volume of 1 mL. Lee *et al.* (2003) were able to achieve a lower method detection limit by using a higher volume of the aqueous matrix. Regarding the extraction of E1-3S from aqueous samples using DCM there is no information available. Dean *et al.* (1955) noted the successful recovery of E1-3S with ethylene dichloride and DCH·HCl from which the present extraction scheme was derived. Compared with the LC-MS method detection limits reported in the literature (D'Ascenzo *et al.*, 2003; Rodriguez-Mozaz *et al.*, 2004; Kuster *et al.*, 2008), the values reported here are about an order of magnitude higher and therefore probably not sufficient to detect estrogen residues in aged environmental field samples. However, for the purpose of laboratory scale investigations to study sorption, degradation and column transport of estrogens the method is suitable in order to obtain quality data and to infer environmental fate parameters. Furthermore, the presented methods do not include the usage of expensive SPE procedures and therefore also minimize costs and the risk of compound loss due to extensive solvent transfers. The latter may be overcome by online-SPE techniques (Kuster *et al.*, 2008) that are still in the early stages of development and available only at highly specialised laboratories.

3.3 Extraction from soil

The standardised extraction of estrogens from natural soils constitutes a challenge because the free hormones and in particular their sulphate conjugates are labile compounds that undergo fast degradation in natural soils. The study of recoveries is therefore always influenced by degradation processes that alter soil concentration. Sterilization techniques such as autoclaving or treatment with sodium azide change soil properties and may lead to false conclusions in relation to recovery studies (Lotrario *et al.*, 1995). Gamma radiation, which is usually implemented to avoid those limitations, was not available. The presented results therefore constitute a synopsis of recoveries found during the course of the various experimental stages of this thesis.

3.3.1 Recoveries during the sorption study

Figure 3.6. displays the combined recovery from soil and aqueous phase extractions during the sorption of E1 and E1-3S to three different soils. The values for E1-3S include the recovery for its parent compound E1, which occurred as a metabolite. Except of E1 in the Hamilton soils from $CaCl_2$ the recoveries were > 80%, indicating acceptable extraction with the employed method. It has been earlier reported that a second extraction step can increase recoveries (Lee *et al.*, 2003; Sarmah *et al.*, 2008), but not in the present study. Poor soil recoveries for E1 with DCM extraction, on the other hand, were reported by Beck *et al.* (2008) as accounting for only 46%. However, excellent recoveries for estrogen extraction from sediments have been reported using variety of solvents including acetone, acetonitrile/water, ethyl acetate and dichloromethane/water (Kuster *et al.*, 2004).

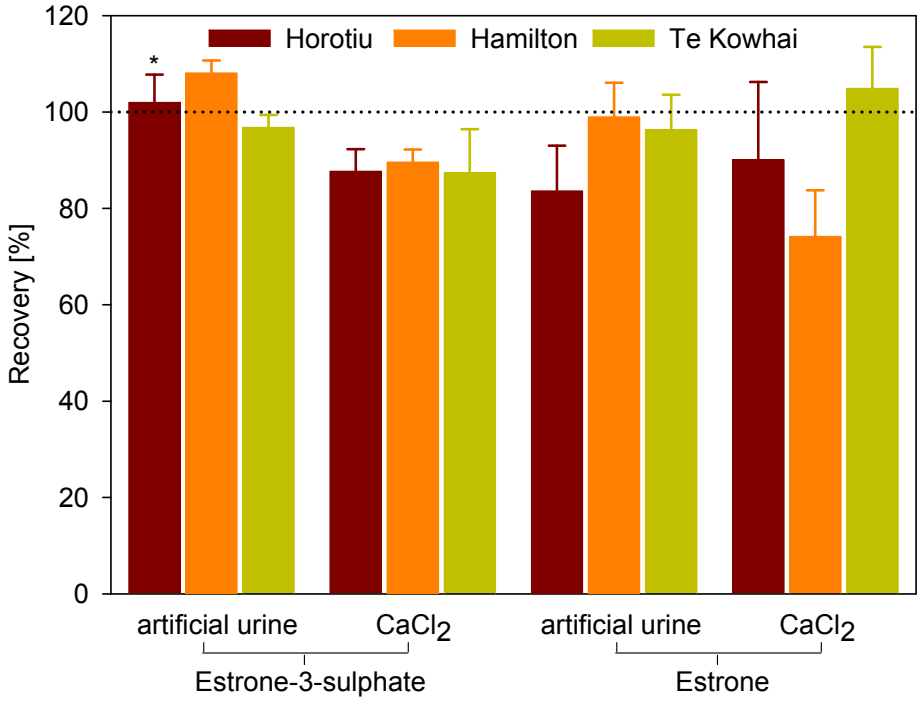


Figure 3.6. Combined recovery from soil and aqueous solutions during the sorption of E1 and E1-3S to three different soils. Estrone-sulphate recoveries include the metabolite E1. Average values and relative standard deviations for n = 12 samples. *indicates n = 10 samples. Dotted line represents 100% mark.

The extraction of estrone-sulphate is covered only by a minority of studies and a preferred solvent is not clearly identified. In general, it can be concluded from the literature that either multiple SPE schemes (Isobe *et al.*, 2006) or the implementation of microwave-assisted extraction at very high temperatures (Matějíček *et al.*, 2007) is necessary to yield sufficient extraction recoveries for E1-3S. Against this background the use of DCH·HCl in combination with DCM or other solvents has potential for further development. In a few instances, the target peaks were interfered with by matrix impurities originating from the soil and in such cases the sample was withdrawn from the subsequent data evaluation.

3.3.2 Recoveries during the degradation study

Figure 3.7. displays the recovery of E2-3S, E1-3S, E2 and E1 from the sterile controls in the three investigated soils. The solvent extraction with DCM and the modifier DCH·HCl yielded good recoveries in the three soils ranging from 80.9 to 95.2%, and from 86.3 to 91.7% for E2-3S, and E1-3S,

respectively. Recovery for the free hormone E2 was comparable in the Hamilton and Matawhero soils; however, it accounted for only 66.5% in the Gibsons soil. The lowest recoveries were obtained for E1; in particular in the Gibsons soil, where only 41.3% were recovered.

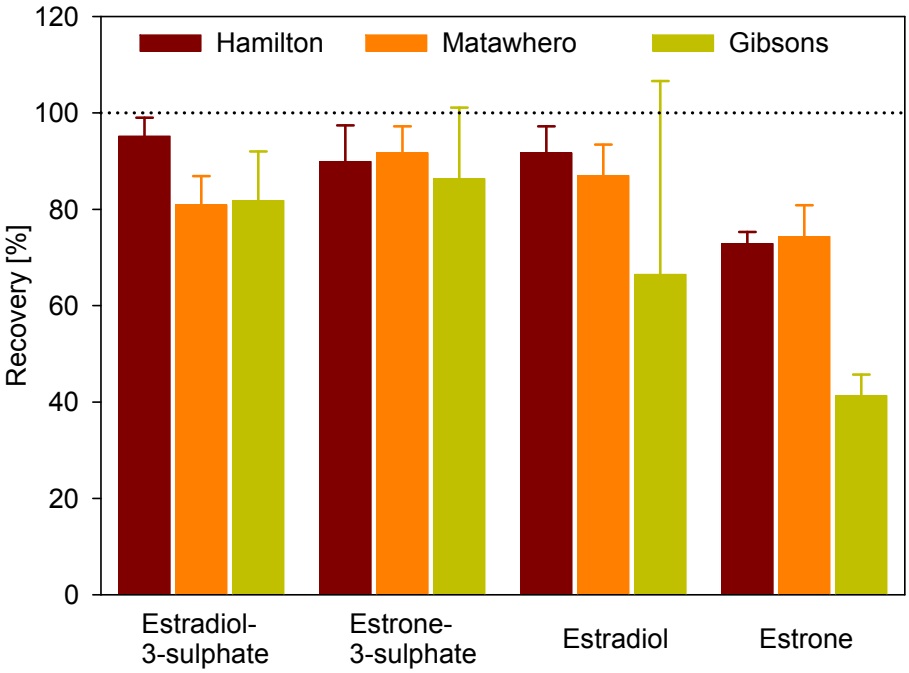


Figure 3.7. Recovery for Estradiol-3-sulphate, estrone-3-sulphate, estradiol and estrone extraction from three different sterilized soils fortified to 5.0 mg kg^{-1}. Average values and relative standard deviations for n = 3 samples are shown. Dotted line indicates 100% mark.

The values for estrogen sulphate extraction from soil samples are difficult to discuss, since there is no information available in the literature. However, a few studies investigated the occurrence of estrogen conjugates in river and bay sediments. An extensive investigation comparing different extraction methods including soxhlet extraction and microwave-assisted extraction and different SPE columns for cleanup was presented recently by Matějíček *et al.* (2007). Their results suggest that microwave-assisted extraction employing aqueous methanol (25:75, v/v) at 100°C, followed by a clean-up step using solid-phase extraction (SPE) on a ion-exchange sorbent Oasis WAX cartridge was most useful in extracting free and conjugate estrogens from spiked river sediments. Recoveries in this study were in the range of 92.5–100 % for a spiked concentration of 20 ng g^{-1} sediment. Similar

recoveries were also obtained by Isobe *et al.* (2006) employing aqueous acetonitrile (9:1, v/v) and sonication followed by an extensive cleanup procedure. The low recovery of E1 in particular in the Gibsons soil may be a result of the spiking procedure. Autoclaving can change the physical properties of soils (Lotrario *et al.*, 1995), and visible structural changes were most significant in the Gibsons soil where autoclaving led to a dense aggregation of the soil. During the fortification, the equal distribution of the spiking solution was therefore more difficult here than in the other soils. This assumption is supported by the high relative standard deviations in the Gibsons soil (Figure 3.9.). Furthermore, the soil aggregates in the Gibsons soil appeared especially repellent to the uptake of the spiking solution, which consequently led to some loss of the spiking solution to the glassware and uneven distribution. Dichloromethane was found to be inefficient for extraction of free estrogens from agricultural soils, yielding only 28 and 46% for E2 and E1, respectively (Beck *et al.*, 2008). However, Lai *et al.* (2000), and later Lee *et al.* (2003), found DCM to be suitable for free estrogen extraction from soil samples with excellent recoveries accounting for > 80% for E2 and E1.

It has to be noted that the high recoveries obtained by Matějíček *et al.* (2007) and Isobe *et al.* (2006) were based on small sample sizes of 0.5–1 g of sediment, which reduce the possibility of compound loss during the fortification procedure. The aim of the present research was to investigate the recovery yield in order to study degradation of estrogens and estrogen sulphates in microcosm experiments. The whole experimental process was therefore investigated involving sub-sampling from a fortified soil mass of 150 g.

The method detection limits (based on a signal to noise ratio of 3:1) for the free and sulphate-conjugated estrogens are summarized in Table 3.1. They show some variation with the soil type due to the varying matrix interferences from different soils. These method detection limits were only achieved, however, with an increase in the soil mass to 5 g and an increase of the DCM volume to 6 mL, as well as the volume of DCM that was evaporated (increased to 4 mL).

Table 3.1. Method detection limits [ng g^{-1}] for estrogen and estrogen sulphates extracted from three agricultural soils followed by HPLC-UV detection.

Soil	E2-3S	E1-3S	E2	E1
Hamilton	2.4	2.0	2.9	1.0
Matawhero	2.4	2.0	2.8	1.0
Gibsons	1.8	2.0	1.3	1.0

The presented method detection limits (Table 3.1.) are about 2–10 times higher than reported by Matějíček *et al.* (2007), and about 14–100 times higher than reported by Isobe *et al.* (2006) and Beck *et al.* (2008) for sediment and soil samples, respectively. In these studies, MS detectors have been used and they are in general more sensitive than UV detectors to analyse very low concentrations (Ingerslev and Halling-Sørensen, 2003). Furthermore, these studies used sophisticated technical equipment during the extraction process, i.e. microwave extractor, accelerated solvent extractor, or gel permeation chromatography devices that were not available for the present work. Moreover against the background of an expected total sample size of > 1300 samples for the degradation study, the application of SPE cartridges, which come at a cost of NZ$3–10,- per sample, was not affordable and the employment of simple solvent extraction was favoured. The achieved method detection limits were found to be suitable to study the laboratory degradation of the free and sulphate-conjugated estrogens.

4 CONCLUSIONS

The separation of the free estrogens E1 and E2 and their sulphate conjugates has been successfully developed using a mobile phase gradient system consisting of acetonitrile, ammonium sulphate (5 mM, pH 3), and water and UV detection at 201 nm. The implementation of this gradient system on a monolithic C18 column resulted in significant runtime reduction, which can be regarded as a crucial achievement for the standardized analysis of a high number of samples. A shorter runtime has a number of positive effects, including shorter sample retention time on the autosampler before analysis, less usage of mobile phase leading to reduced costs, and the

decrease of deuterium lamp dead times in between target peaks, which consequently increases the number of samples that can be analysed by one lamp.

Novel solvent methods were developed to extract E1-3S from aqueous matrices, and E1-3S and E2-3S from solid matrices. Dichloromethane in combination with DCH·HCl resulted in excellent recoveries for the extraction of estrogen sulphates from aqueous matrices and were also found to be suitable to extract these compounds together with their free counterparts from soil. However, extraction recovery for E1 was found to be poor in one soil, which was attributed to the effects originating in the sterilizing procedure that may have altered the physical properties of the soil and hindered the uniform fortification of the soil. The method detection limits in this study were about 2–100 times higher than comparable studies found in the literature involving the analysis of estrogen sulphates in solid samples, but deemed to be suitable for the intended purpose. Given the fact that highly specialised extraction equipment and MS detectors were not available in our laboratory, the developed extraction methods can be considered excellent for the study of the sorption and degradation behaviour of estrogens and estrogen sulphates in soils through laboratory investigations. Moreover, the soil extraction method can be employed in laboratory column transport experiments to extract estrogen soil residues.

CHAPTER IV
SORPTION OF ESTRONE AND ESTRONE-3-SULPHATE

1 INTRODUCTION

Adverse effects, such as the induction of intersex and alteration of sex, caused by estrogens at concentrations in the lower ng L^{-1} range have been reported from laboratory studies on fish (Metcalfe *et al.*, 2001). Martinović *et al.* (2007) reported that male fathead minnows were no longer able to compete successfully for reproduction when exposed to estrogenic sewage treatment effluent and estradiol (E2) at a concentration of about 50 ng L^{-1}. Similar and lower concentrations of estrogens have been detected in the environment. For instance, Isobe *et al* (2003) reported estrone (E1) concentration of 3.4 to 6.6 ng L^{-1} in river samples from Japan, and Noppe *et al.* (2007) found E1 concentrations to range from < 1 to 10 ng L^{-1} in a European estuary over the course of 2 years. The major sources for estrogens in the environment are animal (Hanselman *et al.*, 2003; Tashiro *et al.*, 2003; Khan *et al.*, 2008a) and medical and household wastes (Kolpin *et al.*, 2002).

While the free forms of estrogens dominate in faeces, estrogens are primarily present in a conjugated, more hydrophilic form in mammalian urine (D'Ascenzo *et al.*, 2003), and estrone-3-sulphate (E1-3S) appears to be the dominant estrogen conjugate in cattle urine (Hoffmann *et al.*, 1997). Estrogen concentrations in livestock urine depend on the state of pregnancy and increase towards parturition with the total excreted mass perhaps exceeds 100 mg per day and per cow (Hanselman *et al.*, 2003). Estrogen sulphates are considered biologically inactive in terms of their potential to bind to the estrogen receptor family and therefore are not expected to exhibit any endocrine disruption potential. However, a recent study (Isobe and Shimada, 2003) observed apoptosis in the testicular cells of Japanese quails, with reduction of testicular weights after exposure to E1-3S. The authors concluded E1-3S may be one of the risk factors for endocrine disruption in wildlife because it can be de-conjugated to E1. De-conjugation of estrogen sulphates consequently forming their free counterparts has already been reported in sewers (D'Ascenzo *et al.*, 2003) and waste water treatment plants (Komori *et al.*, 2004). The soil environment, however, has received little

attention even though it is a major sink for animal wastes potentially containing these hormone conjugates.

New Zealand's agricultural sector plays an important role in its overall economy and dairy products, accounting for nearly 50% of the annual exports in 2005/2006 (agricultural economics of Australia & New Zealand, 2008). The dairy cattle population of nearly 5,300,000 outnumbers the human population by over a million, and most of the livestock graze the pastures continuously throughout the year (Statistics New Zealand Tatauranga Aotearoa, 2007). According to one estimate, the combined livestock population in New Zealand excretes about 40 times the amount of waste produced by the human population (Ministry for the Environment [MfE], 1997), and land application of dairy effluent has become increasingly popular. Furthermore, it can be expected that about 80% of the defecations and urinations in a grazed dairy system occur on the paddocks (White *et al.*, 2001). The potential therefore exists for estrogens, and in particular E1 and E1-3S, to reach the receiving waters via surface runoff or leaching through the soils.

Initially, the fate of estrogens in the soil environment is determined by sorption to the soil constituents such as organic matter and soil minerals. Sorption of estrogens has been studied over the past decade, and most studies found the sorption potential of agricultural soils to be moderate to high (Lee *et al.*, 2003; Casey *et al.*, 2005; Hildebrand *et al.*, 2006; Sarmah *et al.*, 2008); and suggested soil organic carbon was the major sorption domain for estrogens (Lee *et al.*, 2003; Yu *et al.*, 2004; Sarmah *et al.*, 2008). Sorption non-linearity, typical for many organic contaminants (Pignatello and Xing, 1996; Hinz, 2001), has also been reported for E1 and E2. However, the results are to some extent controversial due to differences in the experimental protocols and the unique characteristics of the sorbents investigated (Sarmah *et al.*, 2008). Estrogen sorption is also dependent on the background electrolyte or mediator solution (Bowman *et al.*, 2002), but there is a dearth of information on the sorption potential from cow urine and essentially no information exists on the sorption behaviour of E1-3S to soils. Both types of information are, however, necessary to address risks associated with hormone exposure in grazed dairy systems.

Batch equilibration studies were therefore conducted to study the sorption of E1 and E1-3S to three agricultural soils from the Waikato, a major dairying region in New Zealand, using a solvent-extraction technique previously adopted by Lee *et al.* (2003) and Sarmah *et al.* (2008). The aim of the study was to (a) investigate the differences in sorption parameters between E1 and its sulphate conjugate E1-3S; and (b) to elucidate the influence of mediator solution by comparing the commonly used $CaCl_2$ solution (5 mM) with an artificial urine solution.

Apart from general isotherm parameters, the effective distribution coefficients were calculated as a function of the aqueous hormone concentration. Based on a simplistic estrogen exposure scenario, these functions illustrate the difference between both compounds and the effect of the different mediator solutions so that some implications can be drawn for environmental risk assessment.

2 MATERIALS AND METHODS

2.1 Soils and chemicals

Three soils from the Waikato region were selected for this study. The soils were chosen to represent three major soil series under dairy farming in the Waikato region (Figure 4.1., Molloy, 1998) varying in their clay mineralogy, organic matter content, and particle size distribution (Table 4.1.). The specific surface area (SSA, $m^2\ g^{-1}$) was estimated from the clay percentage, organic matter content, and clay mineral composition of the soils using SSA values for single mineral components and organic matter cited by Hedley *et al.* (2000). Detailed descriptions of soils and the methods used to determine the remaining properties can be found elsewhere (New Zealand Soil Bureau (NZSB), 1968; Hewitt, 1992).

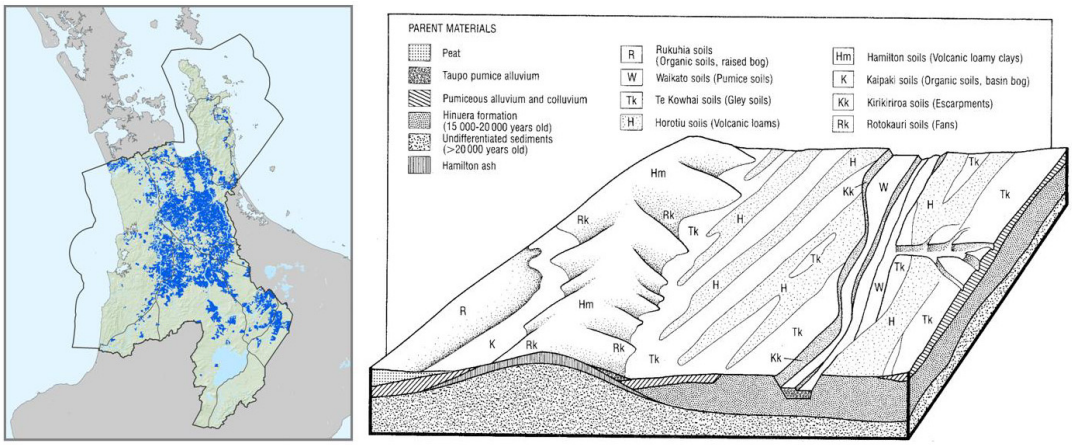


Figure 4.1. The Waikato district with dairy farming land use indicated in blue (left panel). Right panel shows the major soil types in the central Waikato basin near Hamilton (Source: Molloy, 1998).

Table 4.1. Selected properties of investigated soils.

pH[a]	OC [%]	Sand [%]	Silt [%]	Clay [%]	CEC[b] [$cmol_c$ kg^{-1}]	SSA [m^2 g^{-1}]	Clay mineralogy[c] [% of clay fraction]
Horotiu silt loam							
5.4	8.2	34	48	17	28.2	19.7	k[35], vga[30], im[30], cr[5]
Hamilton clay loam							
5.1	4.0	19	51	30	17.2	22.3	k[35], v[30],ha[25], q[3], fs[7]
Te Kowhai silt loam							
5.1	5.0	9	54	37	21.7	19.7	vga[50], ha[35], k[10], cr[4], fs[1]

[a]1:2.5 soil to solution ratio in $CaCl_2$. [b]cation exchange capacity. [c]clay minerals: cr=cristobalite, fs=feldspar, ha=halloysite, im=imogolite, k=kaolinite, q=quartz, v=vermiculite, vga=volcanic glass amorphous.

2.2 Apparent equilibrium and batch sorption isotherm determination

In order to determine a suitable contact time for the subsequent sorption experiments, a $CaCl_2$ solution was spiked with an appropriate volume of methanolic stock solution of E1 (600 mg L^{-1}) to yield an aqueous concentration of 2.58 mg L^{-1}. An aliquot of 30 mL of the stock solution was then added to 2 g of soil pre-weighed in glass centrifuge tube equipped with a Teflon® lined screw cap. Duplicate samples were prepared and the tubes were wrapped with aluminium foil to avoid photodegradation. Samples were placed

on a flatbed shaker in the dark at 22 ± 1°C. At increasing time intervals (0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 49, 72, 97, 111, 135 hrs) the tubes were centrifuged at 2200 rpm and a sub-sample of 0.5 mL was removed and transferred into an amber glass HPLC vial for HPLC-UV analysis (see Chapter III for details). The sorbed amount C_s [mg kg^{-1}] was estimated by mass balance as follows:

$$C_s = \frac{\left(C_w(t=0) - C_w(t)\right) * V_w(t-1)}{m_s} \quad (4.1)$$

where C_w is the aqueous phase concentration [mg L^{-1}], t is the time index, V_w is the aqueous phase volume [L], and m_s is the soil mass [kg].

A modified batch-equilibration method previously described by Li and Lee (1999) and Lee *et al.* (2003) was used to determine sorption of estrone and estrone-3-sulphates to soils from (I) a 5 mM $CaCl_2$ solution (pH 7.2, EC 1.4 dS m^{-2}), and (II) an artificial urine solution (AU, pH 8.3, EC 30.7 dS m^{-1}) consisting of $KHCO_3$ (22.2 g L^{-1}), KCl (3.95 g L^{-1}), K_2SO_4 (6.7 g L^{-1}), $(NH_2)_2CO$ (23.5 g L^{-1}), and $C_2H_5NO_2$ (6.2 g L^{-1}). Preparation of AU was according to Early *et al.* (1998); however, no KBr was used in the present experiment.

Stock solutions of the two hormones at concentrations of 200, 400, and 600 mg L^{-1} for E1 and E1-3S were prepared by dissolving appropriate amounts of the compounds in methanol. An appropriate amount of stock solution of each compound was added to the mediator solutions to yield 6 initial aqueous solution concentrations (0.25, 0.5, 0.75, 1.5, 2.5, and 5 mg L^{-1}). Air-dried soils (2 ± 0.1 g) were shaken with 30 mL of the two mediator solutions containing the single hormone at the above concentrations in 35-mL glass centrifuge tubes sealed with Teflon® lined screw-caps. To minimize photolysis, the tubes were covered with aluminium foil and placed on a flat bed shaker for 2 h at 22°C (± 2) in the dark.

After apparent equilibration the tubes were centrifuged for 20 minutes at 2200 rpm and an aliquot of 5 mL of the clear supernatant solution was removed for liquid-liquid extraction while the remaining supernatant solution was carefully decanted. The residual liquid phase in soil was determined gravimetrically and it was assumed that hormone concentration in the residual soil pore water was the same as that in the bulk supernatant solution.

To determine any losses to the glassware and to check for interfering peaks during analysis, blank controls were conducted with (I) mediator solutions containing the hormones but no soil, and (II) mediator solutions without hormones in contact with the soils.

The residual soil as well as the 5 mL of supernatant solution were extracted and analysed by means of HPLC-UV as described in Chapter III.

2.3 Isotherm modelling and statistics

The sorption isotherms were modelled with the Freundlich sorption model:

$$C_s = K_f C_w^N \tag{4.2}$$

where C_s [mg kg^{-1}], and C_w [mg L^{-1}] are the sorbed and solution phase concentrations, respectively, and K_f [mg^{1-N} L^N kg^{-1}] and N [unitless] are the Freundlich sorption coefficient and exponent signifying sorption magnitude and nonlinearity (N = 1 represents a linear isotherm). Isotherms were fitted utilizing the non-linear regression feature in SigmaPlot 2002 for Windows (Version 8.0, SPSS Inc.) with equal weighting across the data ranges. For isotherms of organic contaminants are often non-linear (Hinz, 2001) a corresponding linear partitioning coefficient is not useful for comparing sorption behaviour and was hence not computed. However, Freundlich coefficients are dependent on their Freundlich exponent and are therefore not suitable to compare sorption behaviour between different sorbents. A common simplification constitutes the normalization of sorption coefficients to the OC content (K_{oc}), which is concentration dependent in the case of nonlinear sorption: $K_{oc} = K_f C_w^{N-1} / f_{oc}$ (Pignatello *et al.*, 2006). However, the number of investigated soils in the present study was too small to significantly identify the OC domain as the primary sorbent, and E1 as well as E1-3S may exhibit sorption behaviour that is not essentially governed by the OC domain, thus from OC normalization was refrained. To be able to compare compounds and sorbents, the concentration-dependent effective distribution coefficient $K_d^{eff} = K_f C_w^{N-1}$ [L kg^{-1}] was calculated. A range of effective distribution coefficients were computed for each soil/compound/mediator-solution combination to account for minimum and maximum exposure concentrations

in a pasture environment. Minimum and maximum exposure scenarios were based on excretion data from the literature and calculated as described below.

Non-pregnant cows excrete an average of 320 µg estrogens per day in urine, predominantly as E1 (52%) and E1-3S (48%), while pregnant cows excrete up to 104,320 µg per day mainly as E1-3S (92%) (Hoffmann *et al.*, 1997; Hanselman *et al.*, 2003). Considering 8–12 urination events per cow per day each measuring 1.5 to 3.5 L (Shorten and Pleasants, 2007), a range of possible exposure concentrations would amount to 4–8,000 and 0.6–700 µg/L for E1-3S and E1, respectively.

The goodness of fit was assessed with the adjusted coefficient of determination R^2_{adj}, which corrects the normal coefficient of determination R^2 for the sample size and the number of fitted parameters:

$$R^2_{adj} = 1 - \left(1 - R^2\right)\frac{n-1}{n-p-1} \tag{4.3}$$

where n is the number of data points, and p the number of fitted parameters, which amount to two in the case of the Freundlich equation.

The statistically significant difference of the fitted model parameters K_f and N for different isotherms was tested with the Student's t statistic calculated as the difference between the tested variables divided by the standard error of the difference between the two tested variables:

$$t_{\exp} = \frac{|P_1 - P_2|}{SE_{P_1 - P_2}} \tag{4.4}$$

where t_{exp} is the experimental t value, P_1 and P_2 are the two variables to be compared, and SE is the corresponding standard error of the difference computed as:

$$SE_{P_1 - P_2} = \sqrt{SE_{P_1}{}^2 + SE_{P_2}{}^2} \tag{4.5}$$

where SE_P are the standard errors of variable one and two, respectively. The null hypothesis that both parameters are statistically not significantly different from each other was rejected for $t_{exp} > t_{tab}$ at $p < 0.01$.

3 RESULTS

3.1 Sorption kinetics of estrone

The results of the preliminary kinetic sorption study are illustrated in Figure 4.2. The displayed pattern may be explained by sorption and degradation processes. A rapid initial sorption was observed within the first hours of the experiment. Some differences amongst the soils are apparent. A first maximum of the theoretically sorbed amount was already attained after 1 h in the Te Kowhai soil while it took between 2 and 8 hours for the Horotiu and Hamilton soils.

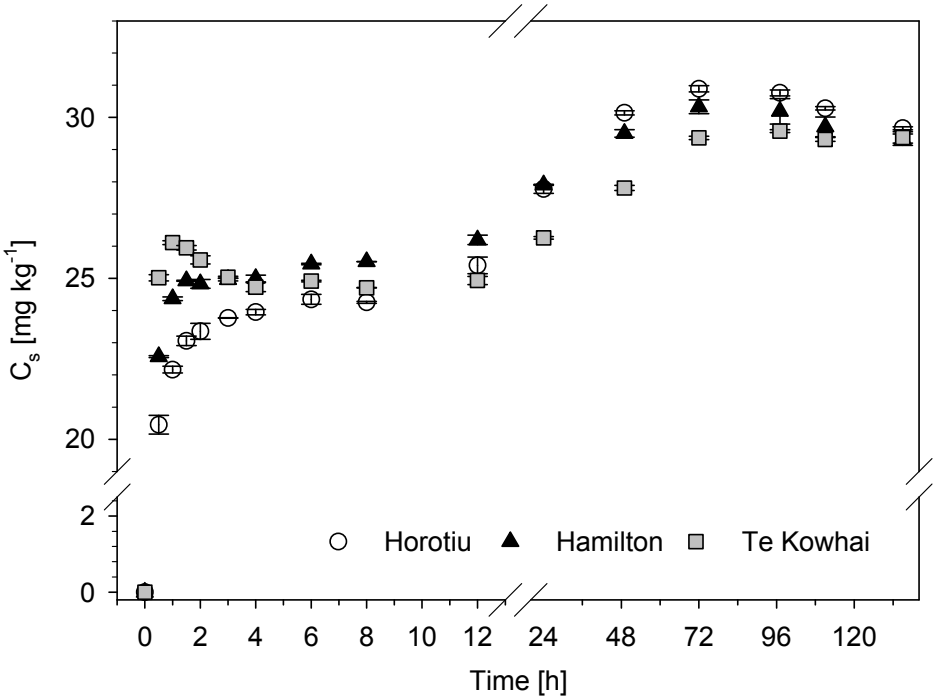


Figure 4.2. Theoretically sorbed amount (C_s) of E1 onto three different soils as a function of the contact time. Average of n = 2 samples is displayed. Standard deviations lay within symbols.

Since sterilization agents were deliberately excluded it can be assumed that degradation processes reduced the total mass of E1 in the system shortly after the initial rapid sorption phase and therefore also influenced the measured aqueous concentration. Due to the empirical nature of the mass balance, assuming the conservation of the initially applied mass of E1 and the reduction of the available aqueous volume by sub-sampling, the theoretically

sorbed mass decreases when the matching measured aqueous concentration does not decrease fast enough (see equation 4.2). However, it may also be a result of the degradation of the hormone. This occurred in the case of the Te Kowhai soil where the theoretical sorbed concentration decreased after the maximum at 1 h. Similar observations have been reported by Lai *et al.* (2000) for a number of estrogens sorbing to river sediments. Even though the authors explained the decrease in sorption with desorption and the possibility of partitioning onto dissolved organic matter it is likely that their observed effect could also be a phenomenon resulting from compound degradation since their study did not include sample sterilization either.

The influence of the degradation processes on the available mass may also explain the second (left) part of Figure 4.2. Continuing degradation reduces the total available mass of E1. This may result in a new apparent equilibration process and therefore the measured aqueous concentration was further reduced, which yields an increase in the theoretically sorbed amount (considering a false assumption of mass conservation). It is therefore unlikely that a true equilibrium would have been attained at any stage of the experiment, though it can be assumed that sorption is dominant in the first 2–10 hours of the experiment. It has to be noted that the aim of this study was not to determine the true equilibration time but rather assess when an initial fast sorption process is exhausted so that degradation process would further dominate the subsequent partitioning process.

It has been shown that partitioning coefficients (K_d) as well as Freundlich coefficients from non-sterilised batch experiments employing radiolabeled hormones (Sangsupan *et al.*, 2006) were lower than what was obtained with sterilized samples (e.g., Yu *et al.*, 2004; Hildebrand *et al.*, 2006), which is possibly due to degradation processes. Also, recent research has shown that assuming relatively high estrogen sorption in soils based on sterilized, fully equilibrated batch experiments infers a low risk of estrogen leaching and runoff that contradicts field studies. Estrogen leaching has been reported from agricultural sites and livestock waste lagoons (Kjær *et al.*, 2007; Arnon *et al.*, 2008) and Peterson *et al.* (2000) linked E2 concentrations

in wells from mantle karst to the sub-soil transport of the hormone through agricultural lands.

Chemical parameters obtained from batch sorption experiments cannot easily be extrapolated to interpret sorption behaviour of the compound in porous media. However, results are useful as a preliminary step (Limousin *et al.*, 2007), for instance to study the differences of the sorption behaviour between an estrogen and its sulphate conjugate or the influence of the background electrolyte on the sorption behaviour of estrogens and their sulphate conjugates. For the subsequent batch experiments a contact time of 2 hours was chosen for all soils, both compounds (E1 and E1-3S), and for both mediator solutions. Similar short contact times have been used by Lai *et al.* (2000) and Ying and Kookana (2005) to study estrogen sorption in soils and sediments.

The kinetic study suggests the first rapid sorption in the present systems would occur also within the first two hours of contact time (Figure 4.2.) and it is assumed that thereafter degradation processes significantly influence the partitioning of the compounds. Since both phases were extracted in the subsequent isotherm study, the amount of degradation occurring can be assessed. Hence, a comparison based on a standardised contact time was found to be more feasible for studying the initial sorption behaviour than finding a true equilibrium optimum for each sorbent/sorbate system that would never be reached under field conditions.

3.2 Sorption from $CaCl_2$ solution

Figure 4.3. displays the measured sorption isotherms of E1 and E1-3S from 5 mM $CaCl_2$ solution with the corresponding Freundlich fits for the three investigated soils. High values for R^2_{adj} (Table 4.2.) indicate the good fit of the Freundlich model to the measured isotherm data for both compounds. The K_f values for E1 were 44.0, 34.2, and 57.2 $mg^{1-N}\ L^N\ kg^{-1}$, for the Horotiu, Hamilton, and Te Kowhai soils, respectively. The matching N values also corresponded well with previously reported values for E1 (Lee *et al.*, 2003; Yu *et al.*, 2004; Hildebrand *et al.*, 2006; Sangsupan *et al.*, 2006) and imply linear sorption of E1 in the Hamilton soil ($N \sim 1$), limitless sorption potential in the Horotiu soil ($N > 1$) and limited sorption potential in the Te Kowhai soils.

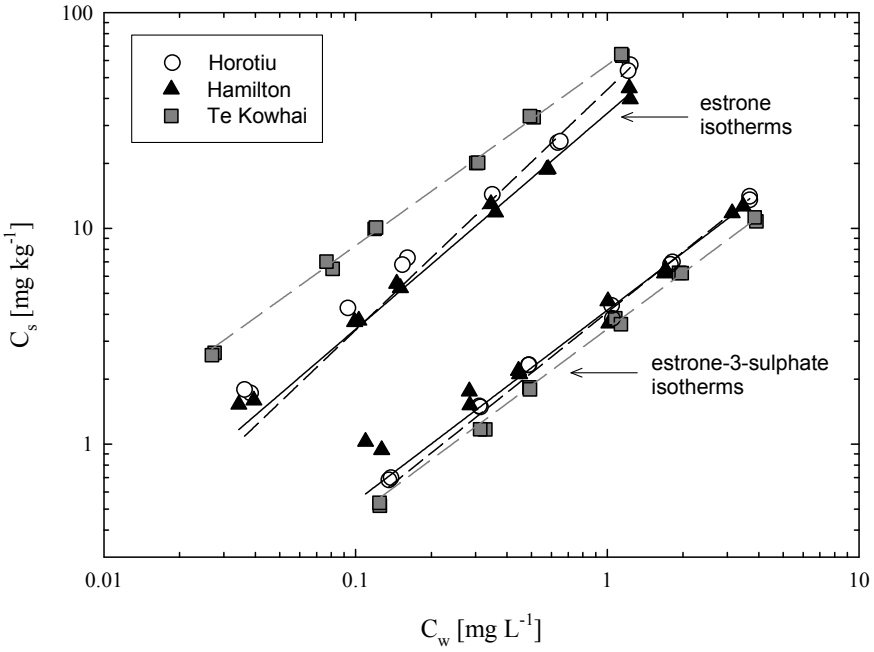


Figure 4.3. Sorption isotherms of E1 and E1-3S from $CaCl_2$ (5 mM) mediator solution. Lines indicate Freundlich fits.

In general, the sorption of estrogens in agricultural soils appears to be limited, i.e. only a limited number of specific sorption sites exist that are dominantly allocated within the organic matter domain of the soils (Lee *et al.*, 2003; Yu *et al.*, 2004). In contrast, soils with a high specific surface area (SSA) have been reported to exhibit limitless sorption potential for estrogens. For instance, Casey *et al.* (2003) found *N* values > 1 for 5 U.S. loam soils with a range of OC (3.3–9.2%) and SSA (106–175 m^2 g^{-1}). High organic matter (8.2%) and the high content of imogolite (30%, Table 1), an allophanic clay mineral with hydrophobic features, may explain the limitless sorption of E1 in the Horotiu soil. Recently, Sarmah *et al.* (2008) also observed limitless sorption of E2 in a similar soil from the Horotiu soil series and attributed it to the OC content and the presence of high allophane contents in the soil. In contrast to the Horotiu soil, the clay mineralogy of the Hamilton and Te Kowhai soils (Table 4.1.) is dominated by kaolinite and halloysite, with major fractions of amorphous volcanic glass (Te Kowhai) and vermiculite (Hamilton). Kaolinite has been found to bind E2 only weakly (Van Emmerik *et al.*, 2003; Casey *et al.*, 2003) and appears to have no sorption potential for E1 (Bonin and Simpson, 2007). Furthermore, Bonin and Simpson (2007) stressed that E1 has a

lower sorption affinity to montmorillonite than E2. Because it has a higher negative charge than the montmorillonite, the vermiculite fraction in the Hamilton soil is expected to contribute negligibly towards E1 sorption.

Table 4.2. Freundlich isotherm parameters for the sorption of E1 and E1-3S from $CaCl_2$ solution. Parameters are illustrated with on standard error (SE). Regressions were all significant at p < 0.001.

Soil	K_f ± SE [$mg^{1-N}\ L^N\ kg^{-1}$]	N ± SE	R^2_{adj}
	Estrone		
Horotiu	44.0 ± 0.7	1.115 ± 0.045	0.995
Hamilton	34.2 ± 0.7	1.001 ± 0.043	0.991
Te Kowhai	57.2 ± 0.4	0.837 ± 0.013	0.999
	Estrone-3-sulphate		
Horotiu	4.08 ± 0.09	0.932 ± 0.020	0.997
Hamilton	4.18 ± 0.15	0.887 ± 0.034	0.992
Te Kowhai	3.42 ± 0.07	0.886 ± 0.019	0.998

The sorption capacity for E1-3S was about one order of magnitude lower than for E1 in the investigated soils (Figure 4.3. and Table 4.3.). The K_f values accounted for 4.08, 4.18, and 3.42 $mg^{1-N}\ L^N\ kg^{-1}$, for the Horotiu, Hamilton, and Te Kowhai soils, respectively. The equivalent N values indicate more limited sorption of E1-3S as opposed to E1 in the Horotiu (0.932 vs. 1.115) and Hamilton (0.887 vs. 1.001) soils, and a slightly higher sorption capacity in the Te Kowhai (0.886 vs. 0.837) soil. Given the ionic and rather hydrophilic nature of E1-3S it is expected to exhibit lower sorption affinity to the organic matter domain of soils than its free counterpart. The log K_{ow} value for E1-3S calculated from the KOWIN software within the virtual computational chemistry laboratory (Tetko *et al.*, 2005) accounts for 0.95, while values for E1 range from 2.45 to 3.43 (cited by Lee *et al.*, 2003). At a pK_a of -3.0 (Tetko *et al.*, 2005) the E1-3S molecule is always negatively charged, and therefore electrostatic interaction, which governs anion sorption, depends on the net charge of the clay minerals and organic matter constituents in the soils. However, under the given conditions with a solution pH of 7.2, the eligible clay minerals present in the soils would not be charged positively

(Schachtschabel *et al.*, 1998), thereby excluding the possibility of significant anion retention by the clay minerals.

It is well established that the sorption of weakly acidic compounds is pH dependent and their sorption is favoured at pH values close to and below their pK_a values. Conformational changes in the soil organic matter have been proposed to contribute to sorption of such compounds at low pH values (Spadotto and Hornsby, 2003). The presence of the sulphate group in E1-3S reduces the possibility for hydrophobic interaction as opposed to E1. Unspecific interactions with organic matter and clay minerals, such as ligand binding, intercalation, and weak hydrogen bonding may be a plausible explanation for the observed weak sorption of E1-3S to the soils.

3.3 Sorption from artificial urine solution

Figure 4.4. displays the measured sorption isotherms along with the Freundlich fits for E1 and E1-3S for sorption from AU for the three investigated soils. The estimated Freundlich parameters are given in Table 4.3. The K_f values for E1 decreased significantly to 36.0 and 34.9 $mg^{1-N}\ L^N\ kg^{-1}$ in the Horotiu and Te Kowhai soils, while the value for the Hamilton soil increased to 39.8 $mg^{1-N}\ L^N\ kg^{-1}$ as opposed to sorption from the $CaCl_2$ solution. A significant change in the *N* value was only notable for the Horotiu soil (Table 4.3). Similarly, the Freundlich sorption parameters changed for E1-3S, with a significant increase in the K_f values being observed for the Hamilton soil. In contrast, the K_f value for the Te Kowhai soil decreased, while no change was observed in the Horotiu soil. The matching *N* values slightly increased, indicating a more linear sorption isotherm for the Hamilton and Te Kowhai soils.

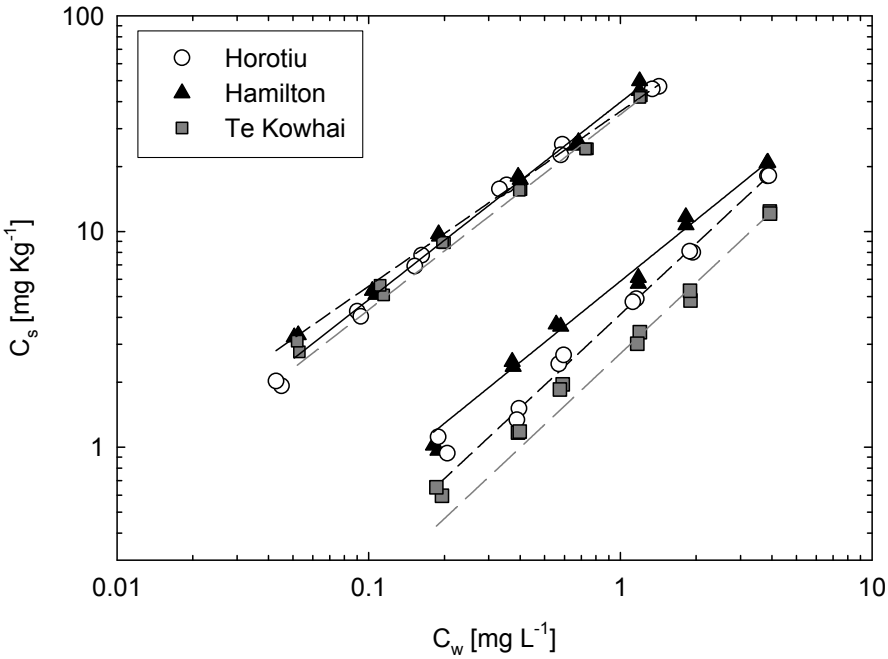


Figure 4.4. Sorption isotherms of E1 and E1-3S from artificial urine mediator solution. Lines indicate Freundlich fits.

There have been few investigations to determine the influence of the mediator solution on the sorption of estrogens and their sulphate conjugates in batch equilibration studies, hence it is difficult to discuss the finding in the context of similar studies. Bowman *et al*. (2002) reported a significant "salting out" effect for estrone sorption from estuarine water to river sediments. They observed an increase in the partition coefficient for E1 with increased salinity of the mediator solution that was attributed to the lowered aqueous solubility of the compound and the resultant higher hydrophobic interactions with the mediator solution. The AU had a higher conductivity than the $CaCl_2$ solution and the increased K_f value for E1 in the presence of AU in the Hamilton soil could therefore be associated with the "salting out" effect. Increased sorption in the presence of increasing soluble ions has also been reported for another hydrophobic organic chemical (PCB). The increased sorption was attributed to a combination of the salting-out effect and changes in the net charge of the organic matter toward neutral charge and therefore enhancing HOC sorption (Turner and Rawling, 2001).

Table 4.3. Freundlich isotherm parameters for the sorption of E1 and E1-3S from artificial urine solution. Parameters are illustrated with on standard error (SE). Regressions were all significant at p < 0.001.

Soil	K_f ± SE $[mg^{1-N} L^N kg^{-1}]$	N ± SE	R^2_{adj}
	Estrone		
Horotiu	36.0* ± 0.5	0.810* ± 0.023	0.995
Hamilton	39.8* ± 0.8	0.915 ± 0.041	0.990
Te Kowhai	34.9* ± 0.6	0.905 ± 0.034	0.993
	Estrone-3-sulphate		
Horotiu	4.13 ± 0.10	1.089* ± 0.021	0.998
Hamilton	5.89* ± 0.24	0.943 ± 0.035	0.993
Te Kowhai	2.72* ± 0.13	1.094* ± 0.038	0.994

* indicate significant difference ($p < 0.01$) between artificial urine and $CaCl_2$.

While the aqueous solubility of E1-3S was possibly decreased in the AU, the higher concentration of ions might have facilitated the sorption of the ionic sulphate part of E1-3S, for instance by alteration of clay minerals. The presence of potassium in the AU can cause the contraction of vermiculite to illite (Schachtschabel *et al.*, 1998), during which temporary stronger intercalation of E1-3S could have occurred resulting in slightly higher K_f values in the Hamilton clay loam. In contrast, the clay mineralogy of the Te Kowhai soil (Table 4.1.) indicates very low potential for sorption of E1-3S (as discussed above), which leads to the assumption that the observed decrease in K_f is a result of conformational changes in the organic matter domain due to the slightly higher pH value and the high concentrations of hydrated ions in the AU. This hypothesis, however, needs further investigation to elucidate the involved sorption mechanisms when sorption of estrogens takes place from complex matrices such as AU or real livestock urine.

It has been shown that the extent of E1 sorption decreases in multi-sorbate systems (Yu *et al.*, 2004; Bonin and Simpson, 2007) when other estrogens such as E2 or ethynylestradiol are present. The AU solution used in the present study contained glycine and urea, and both compounds could possibly also compete for discrete sorption sites with E1 and therefore lower the hormone's sorption magnitude as observed in the Horotiu and Te Kowhai

soils (Table 4.3). The lack of a similar observation for E1-3S indicates that sorption of the conjugate is likely controlled by other mechanisms than hydrophobic interactions. For example, its higher aqueous solubility indicates lower affinity to the hydrophobic sorption sites and therefore the competitive effect of glycine and urea no longer had an effect on the sorption parameters.

3.4 Sorption of E1 as a metabolite of E1−3S

During the equilibration of E1-3S, the formation of E1 as a metabolite was observed. In the AU treatment the formation of E1 accounted for < 0.1% of the initial mass in all three soils and the data obtained were too scattered to construct feasible isotherms. However, in the $CaCl_2$ treatment E1 was formed with values of 4.6, 4.8, and 6.7% of the initial mass of E1-3S in the Horotiu, Hamilton, and Te Kowhai soils, respectively. The construction of additional metabolite isotherms for E1 was therefore warranted. These isotherms and corresponding Freundlich fits are given in Figure 4.5., with the associated sorption parameters being summarized in Table 4.4. The K_f values for the Horotiu and Te Kowhai soils were 16.7 and 28.5 units lower for the metabolite isotherms; no change in the K_f value was observed for the Hamilton soil. The corresponding N values decreased for all soils, with a significant ($p < 0.01$) change being observed only for the Horotiu soil (Table 4.4.).

Table 4.4. Isotherm parameters for the sorption of E1 formed during E1-3S sorption from $CaCl_2$. Parameters are illustrated with on standard deviation (SE). Regressions were all significant at p < 0.001.

Soil	K_f ± SE [$mg^{1-N}\ L^N\ kg^{-1}$]	N ± SE	R^2_{adj}
Horotiu	27.3* ± 3.0	0.766* ± 0.031	0.991
Hamilton	37.8 ± 4.6	0.946 ± 0.041	0.993
Te Kowhai	28.7* ± 3.6	0.762 ± 0.040	0.987

* indicate significant difference ($p < 0.01$) to estrone sorption as parent compound.

Metabolite isotherms for E1 have been reported before (Sarmah *et al.*, 2008; Lee *et al.* 2003); however, for E1 as a metabolite of E2 and both studies

did not perform a separate batch experiment for E1 as a parent compound. Using a soil from the Horotiu series, Sarmah *et al.* (2008) obtained a *N* value of 0.75 for a E1 metabolite-isotherm, which is comparable to the value of 0.766 presented here for a similar concentration range. Their parent compound (E2) isotherm, however, had a *N* value > 1, which is in agreement with the *N* value for E1 sorption as a parent compound in the present study (Table 4.3), thus confirming limitless sorption capacity for both E1 and E2 in the Horotiu soils.

The differences in the *N* values are likely due to the different concentration ranges, since *N* is sensitive to the experimental concentration range (Pignatello *et al.*, 2006). It is common for Freundlich-type sorption isotherms with $N < 1$ to exhibit stronger sorbate affinity at lower aqueous concentrations (Pignatello and Xing, 1996; Huang *et al.*, 1997) and Yu *et al.* (2004) presented increasing organic carbon normalized partitioning coefficients for E1 and E2 with decreasing aqueous concentrations. To evaluate the concentration range dependence of the Freundlich parameters, both data sets (E1 as parent and metabolite) were combined and Freundlich parameters were derived for the combined isotherms for each soil. In all three cases the additional data points provided by the E1 metabolite isotherms did not significantly alter the overall Freundlich parameters (data not shown) as opposed to the parent isotherms. This indicates that it is absolutely necessary to investigate a broad range of aqueous concentrations in order to obtain representative and meaningful sorption parameters for these compounds. Nevertheless, it is *inter alia* necessary to obtain separate isotherms for estrogenic metabolites of estrogens such as E2 and E1-3S. It has been shown here and in previous studies (Sarmah *et al.*, 2008; Lee *et al.*, 2003) that E1, a major metabolite of estradiol and estrogen conjugates (Scherr *et al.*, *in press*), has different Freundlich parameters when sorption takes place as a metabolite.

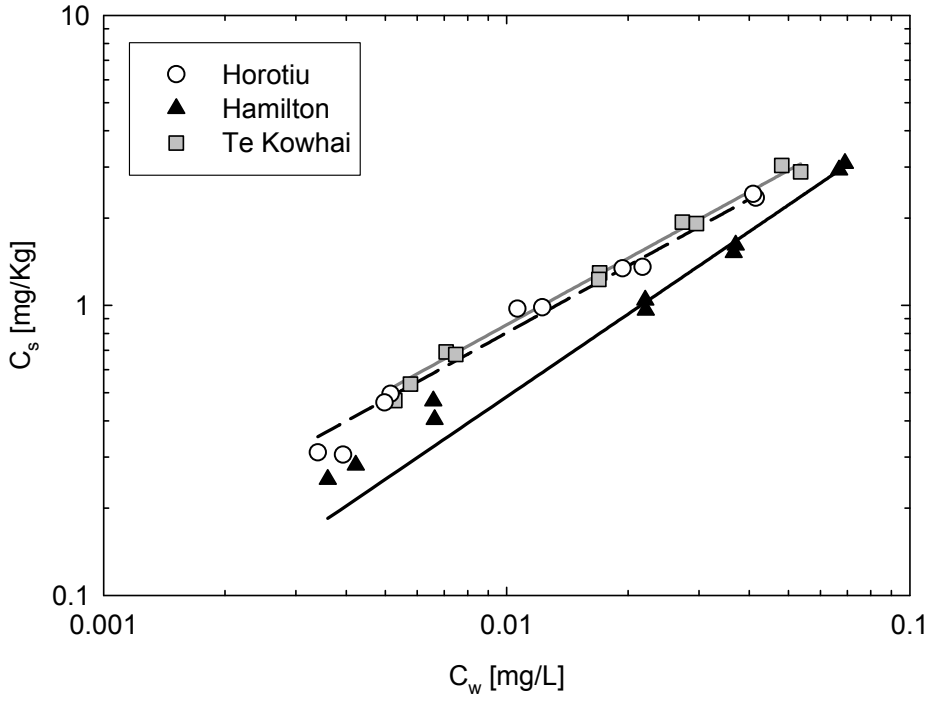


Figure 4.5. Sorption isotherms of E1 formed during sorption of E1-3S from $CaCl_2$ solution. Lines indicate Freundlich fits.

3.5 Environmental significance

The previous section has discussed the fact that sorption of E1 and E1-3S in the investigated soils cannot be related only to the organic matter constituents of the soils but depends also on their clay mineralogy and may be governed by processes that are yet to be investigated in more detail. Therefore, the common simplification of normalizing partition coefficients to the organic carbon content of soils is not useful for inferring environmental implications and risk assessment advice concerning estrogens and estrogen sulphates. However, many risk assessment and leaching models often require partitioning coefficients, and the concentration-dependent effective distribution coefficient (K_d^{eff}) may serve as an alternative.

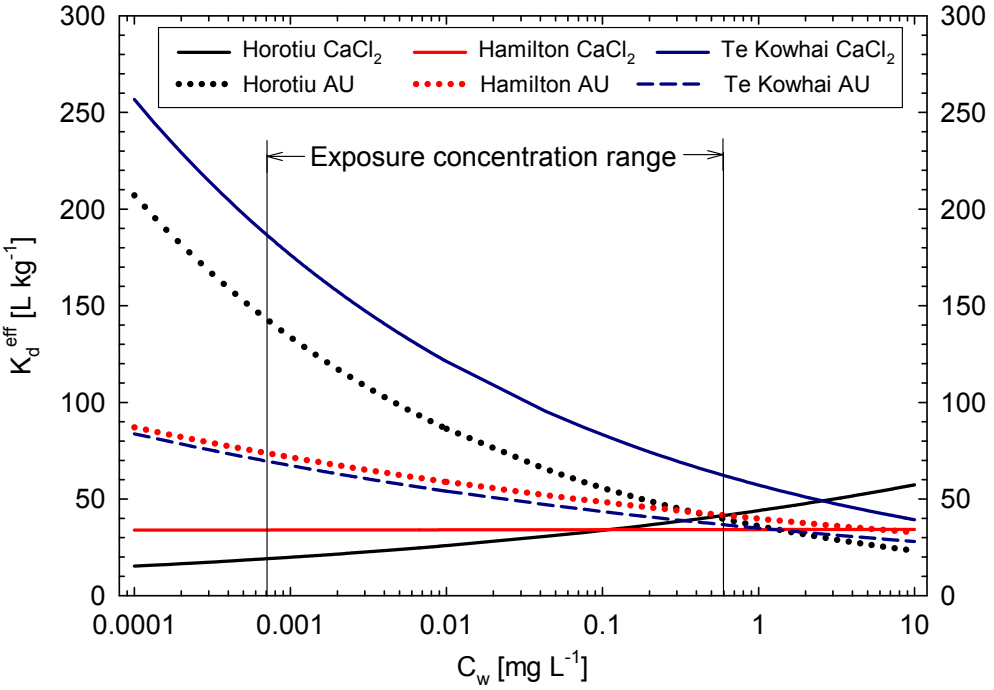


Figure 4.6. Effective distribution coefficients (K_d^{eff}) of E1 sorption from $CaCl_2$ and artificial urine solutions as a function of the aqueous concentration C_w. Vertical lines indicate the hypothetical range of exposure concentrations in a grazed pasture environment.

Based on the exposure scenarios illustrated in section 2, the effective distribution coefficient ($K_d^{eff} = K_f C_w^{N-1}$) was calculated for a concentration range of 0.0001–10 mg L^{-1} for both compounds and both treatments. Figures 4.6. and 4.7. illustrate the K_d^{eff} as a function of the aqueous hormone concentration for E1 and E1-3S, respectively. Figure 4.6. indicates that E1 sorption from AU would be considerably higher than from $CaCl_2$ at aqueous concentrations < 0.1 mg L^{-1} for the Hamilton and Horotiu soils, while the opposite applies to the Te Kowhai soil. The difference becomes more distinct at lower C_w and it consequently implies that, by using the common $CaCl_2$ isotherm, one would underestimate E1 sorption in particular at low exposure concentrations in a grazed pasture system on the Horotiu and Hamilton soils. For the Te Kowhai soil, an overestimation would occur using the common $CaCl_2$ isotherm (Figure 4.6.).

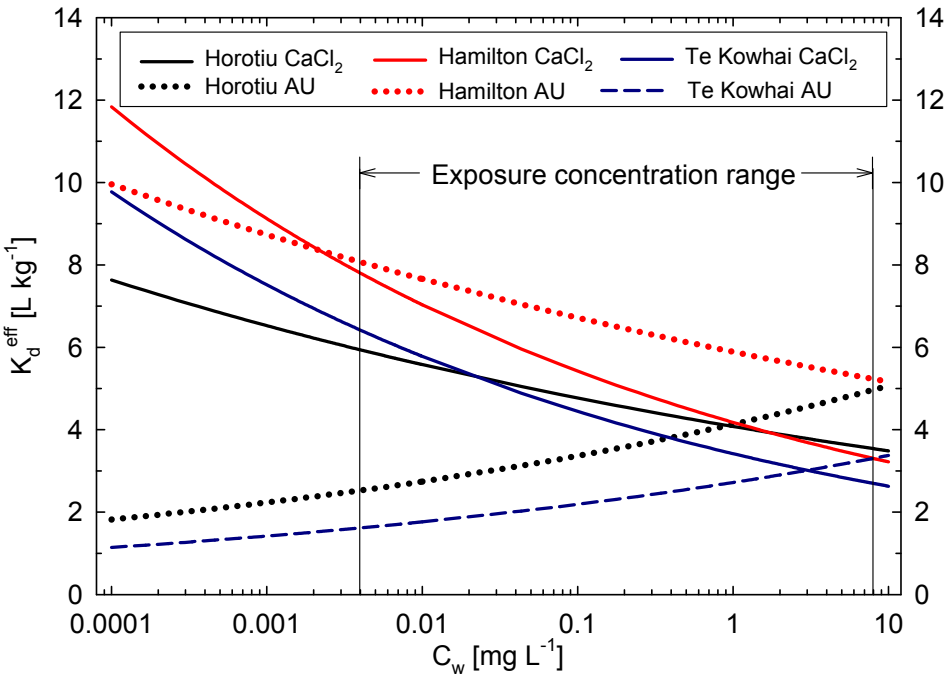


Figure 4.7. Effective distribution coefficients (K_d^{eff}) of E1-3S sorption from $CaCl_2$ and artificial urine solutions as a function of the aqueous concentration C_w. Vertical lines indicate the hypothetical range of exposure concentrations in a grazed pasture environment.

Likewise, Figure 4.7. presents the differences that arise for the K_d^{eff} of E1-3S at possible exposure concentration under grazed pasture employing the common $CaCl_2$ and the AU isotherm. The effect is again more pronounced at lower exposure concentrations and it becomes evident that the common $CaCl_2$ isotherm would overestimate sorption of E1-3S in the Horotiu and Te Kowhai soils. In contrast, sorption of E1-3S would be higher than expected from the common $CaCl_2$ isotherm in the Hamilton soil in the given exposure concentration range.

4 CONCLUSIONS

The sorption behaviour of estrogens and their sulphate conjugates play a significant role for all subsequent and concomitant environmental fate processes such as degradation and transport. In this study it was demonstrated that the sorption process of E1 is quickly influenced by degradation processes and it can be assumed that under field conditions in

agricultural soils a constant interaction between sorption and degradation determines the ultimate partitioning for these compounds. In order to compare the sorption behaviour of E1 and E1-3S under the influence of the mediator solution, it was found to be adequate to standardise batch experiments to a relative short contact time of 2 hours, a time frame that would also be relevant under field conditions.

The data from the batch experiments were successfully modelled with the Freundlich equation and the results highlighted that the hydrophilic E1-3S has much lesser sorption affinity with the investigated agricultural soils than its free counterpart E1. Moreover, it was demonstrated that the mediator solution has a significant effect on the sorption parameters. No clear trends of the sorption behaviour in relation to the soil properties were identified. However, some evidence exists to assume that sorption of E1 and E1-3S may also be influenced by the clay mineralogy and, in particular, that allophanic clay minerals such as imogolite may contribute and enhance E1 sorption in soils. Furthermore, it was illustrated that the common simplification of organic carbon normalization of partitioning coefficients may lead to false inferences in terms of the risk assessment of these compounds. Based on the batch isotherm parameters, the use of the effective distribution coefficient for a range of realistic exposure scenarios was proposed as an alternative way to assess hormone partitioning in soils. Utilizing the effective distribution coefficient as a function of the aqueous concentration it became evident that the impact of the mediator solution was quite pronounced in some of the soils. Therefore the common approach of using $CaCl_2$ in batch sorption experiments may lead to deceptive results for pasture environments where direct exposure of estrogens via grazing livestock is common.

Certainly more detailed work is needed to clarify the mechanisms involved in the sorption of estrogens and their conjugates in soils. In particular, although field experiments have been largely overlooked, they may actually reveal more realistic sorption behaviour for these compounds since varying temperature and climate conditions would have a direct effect on the compounds' fate and ultimately govern their endocrine disruptive potential in the environment.

CHAPTER V
DEGRADATION OF ESTROGENS AND THEIR SULPHATE CONJUGATES

1 INTRODUCTION

The estrogen sulphates 17β-estradiol-3-sulphate (E2-3S) and estrone-3-sulphate (E1-3S) are naturally occurring conjugates of the female steroid hormones 17β-estradiol (E2) and estrone (E1). Conjugates play a major role in the maternal circulation of pregnant livestock, and sulphate conjugates appear to be the major estrogen compounds in cattle urine during pregnancy (Hoffmann *et al.*, 1997). Sulphate-conjugated estrogens might be de-conjugated by arylsulphatase enzymes present in the environment to release their free counterparts which are of public and scientific concern because of their potential to interfere adversely with the normal hormonal functioning of wildlife (Jobling *et al.*, 1998). Moreover, a recent study by Isobe and Shimada (2003) showed that exposure to E1-3S can induce apoptosis in the testicular cells of Japanese quails and it was concluded that E1-3S could be one of the risk factors for endocrine disruption in wildlife.

Residues of E2, E1, and E1-3S have been detected across the globe in various environmental media such as sewers (D'Ascenzo *et al.*, 2003), wastewater (Gomes *et al.*, 2005), wastewater in treatment plants (Schlüsener and Bester, 2008) and wastewater in treatment plant effluents (Isobe *et al.*, 2003), river water (Rodriguez-Mozaz *et al.*, 2004b), Tokyo bay sediments (Isobe *et al.*, 2006), and river sediments (Matějíček *et al.*, 2007). Furthermore, ground and spring water affected by agricultural activities were found to be contaminated with free estrogens (Peterson *et al.*, 2000; Arnon *et al.*, 2008) despite the fact that a number of laboratory experiments suggested fast degradation and high sorption of free estrogens in agricultural soils (Casey *et al.*, 2003; Lee *et al.*, 2003; Ying and Kookana, 2005). These studies show that the degradation of estrogens and estrogen sulphates might be incomplete, and that estrogen sulphates may contribute toward elevated environmental concentrations of the free estrogens, especially when catchments are under the influence of agricultural activities. Direct excretal input by grazing animals is considered one of the primary routes of exposure of estrogens and estrogen sulphates in the New Zealand environment due to the dominance of continuously pasture grazing livestock all year round. In

addition, farmers are allowed to apply effluents onto the land as long as they follow the prescribed conditions set out by their respective regional council (Sarmah *et al.*, 2006). The potential therefore exists for hormones and hormone conjugates to reach receiving waters and impact the aquatic wildlife. Thus, knowledge about the degradation behaviour of these compounds is crucial for proper risk assessments of hormone exposure in the environment.

The degradation of the free estrogens E1 and E2 has been studied in a variety of environmental media including agricultural soils (Colucci *et al.*, 2001; Das *et al.*, 2004; Ying and Kookana, 2005; Lucas and Jones, 2006; Stumpe and Marschner, 2007; Xuan *et al.*, 2008), sewage sludge (Shi *et al.*, 2004; Weber *et al.*, 2004), river water and sediments (Jürgens *et al.*, 2002; Ying and Kookana, 2003), groundwater and aquifer sediments (Sarmah and Northcott, 2008), and pure culture media (Yoshimoto *et al.*, 2004; Yu *et al.*, 2007). Compared with the biodegradation by sewage microbes, which is fast and often complete, degradation of free estrogens by soil microbes is rather slow and incomplete (Khanal *et al.*, 2006). 17ß-estradiol is rapidly degraded in non-sterile soils forming E1 as a metabolite (Colucci *et al.*, 2001; Lee *et al.*, 2003; Ying and Kookana, 2005), which appears to be more persistent in the soil environment than the parent compound (Colucci *et al.*, 2001). The mineralization of estrogens appears to be dependent on the exposure matrix (Lucas and Jones, 2006), soil physical properties such as moisture content, and the incubation temperature (Colucci *et al.*, 2001).

However, to date no information is available on the degradation of estrogen-sulphates in soils. It has been speculated that the underlying mechanism in the degradation of estrogen-sulphates is the hydrolysis of these compounds and release of free estrogens in the presence of arylsulphatase enzymes (Lucas and Jones, 2006; Khanal *et al.*, 2006). D'Ascenzo *et al.* (2003) gave some evidence for this assumption through a laboratory incubation study of estrogen-sulphates using waste water collected from a septic tank. They found that the bacteria in the waste water needed an acclimatisation period of about 10 hrs before the hydrolysis started, and reported approximate half lives of 2.5 d for E1-3S and E2-3S at an initial concentration of 25 µg L^{-1},

without detecting any metabolites. The conditions in the soil environment are different from an aqueous matrix, and the abundance and activity of arylsulphatase often depend on soil mineralogy and organic matter and vary with the temperature and vegetation (Quiquampoix *et al.*, 2002; Speir and Ross, 2002). Agricultural practices and pollution history (Gianfreda *et al.*, 2005) are also factors that could affect the activity of soil hydrolases such as the arylsulphatase. Therefore, the basic assumption that estrogen-sulphates undergo an immediate degradation process in agricultural soils has yet to be demonstrated experimentally.

Degradation patterns are often described with first-order kinetics to derive degradation endpoints for risk assessment purpose (Colucci *et al.*, 2001; Fan *et al.*, 2007). When degradation does not follow a first-order kinetic other kinetic models, such as double first-order in parallel have been applied to describe observed degradation patterns of organic chemicals (e.g., FOCUS, 2006; Stumpe and Marschner, 2007). The mathematical description of metabolite formation and degradation of estrogens has received little attention. Many kinetic models, however, are available (FOCUS, 2006) and have already been applied to model androgen (Khan *et al.*, 2008b) and natural pesticide (Etzerodt *et al.*, 2008) degradation and metabolite formation in soils. In order to assess the goodness of fit of the models, the common approach involves the calculation of coefficient of determination (R^2). More complex models, however, often comprise more parameters to be fitted, consequently reducing the statistical degrees of freedom which is not accounted for in the R^2 measure. Alternatives that consider reduced degrees of freedom to facilitate model selection are the adjusted coefficient of determination, and the scaled root mean square error (FOCUS, 2006). Furthermore, information theory approaches such as the Akaike information criterion additionally consider sample bias (Burnham and Anderson, 2002). These statistical measures have rarely been employed in estrogen degradation studies. An accurate description of degradation and metabolite formation data might, however, facilitate risk assessment of environmental hormone exposure.

Given that the conversion and degradation of estrogen sulphates (e.g., E2-3S and E1-3S) to free estrogens can play a major role in the persistence of bioactive hormones (E2 and E1) in soil, and given there is a dearth of information on the degradation of estrogens in New Zealand soil and estrogen sulphates in soils in general, the aerobic dissipation and metabolite formation dynamics of E2, E1, E2-3S, and E1-3S in three pasture soils of New Zealand were investigated. The hypothesis that arylsulphatase is the main driver initiating the degradation of estrogen-sulphates was examined by relating the degradation kinetics to the arylsulphatase activity of the soils and further by comparing the degradation dynamics in a normal assay versus an assay where the enzyme was inhibited by an irreversible competitive inhibitor. The degradation dynamics were modelled with first-order or double-first order in parallel kinetic models and model selection was based on an array of statistical measures. Furthermore, the degradation of E2-3S at one temperature was monitored for the formation of estrogenicity by means of the ER-CALUX® assay (Legler *et al.*, 1999).

2 MATERIALS AND METHODS

2.1 Soils

The degradation of E1, E2, E1-3S and E2-3S was investigated in three top-soils (0–5 cm) collected from three different geographical locations in New Zealand. The Hamilton clay loam and Matawhero silt loam soils are from the Waikato and Hawke's Bay regions in the North Island, while the Gibsons fine sandy loam soil is from the Marlborough region in the South Island. The soils were selected to represent a range in organic carbon and other physicochemical properties such as particle size distribution (Table 5.1.). After sampling the field-fresh soils were sieved (ø 2mm) and stored in the cold (4°C) until used.

Microbial biomass carbon (MBC) was determined by a fumigation-extraction method using a K_C (fraction of biomass C mineralized to CO_2) factor of 0.41 (Wu *et al.*, 1990). In brief, 25 g of chloroform ($CHCl_3$) fumigated and non-fumigated soils (adjusted to 60% of maximum water holding capacity

(MWHC) at -33 kPa) were extracted with 0.5 M K_2SO_4 for 30 min. Soluble organic C in fumigated and non-fumigated soil samples was determined using a Lachat IL550 TOC-TN analyzer. In-depth descriptions of the soils and methods used to determine the particle size, OC content and cation exchange capacity can be found elsewhere (New Zealand Soil Bureau (NZSB), 1968; Hewitt, 1992).

Table 5.1. Physico-chemical properties of selected soils.

Soil	pH[a]	OC [%]	Sand [%]	Silt [%]	Clay [%]	CEC[b] [$cmol_c\ kg^{-1}$]	MBC[c] [$\mu gC\ g^{-1}$]
Hamilton clay loam	5.1	4.0	13.7	51.0	30.4	17.2	1724
Matawhero silt loam	4.3	2.1	15.6	55.2	25.0	15.4	481.7
Gibsons fine sandy loam	6.4	1.1	38.5	41.0	16.0	8.5	255.5

[a]At 1:2.5 soil to solution ration in $CaCl_2$. [b]Cation exchange capacity. [c]Microbial biomass C.

2.2 Arylsulphatase activity

Arylsulphatase enzyme (AryS, EC 3.1.6.1) activity was determined based on a method of Tabatabai and Bremmer (1970) as modified by Speir *et al.* (1984). In brief, 0.5 g of soil was adjusted to 60% MWHC in 2.0 mL acetate buffer (pH 5.8), and was incubated for 4 hrs at 7.5,15 and 25°C in duplicates with 0.5 mL 50 mM nitrophenyl-sulphate (dissolved in acetate buffer). Controls were prepared without substrate. After incubation, 0.5 mL of 0.5 M $CaCl_2$, and 2.0 mL of 0.5 M NaOH were added; 0.5 mL substrate solution was added to the controls. The soil suspension was then mixed and centrifuged at 2200 rpm. The 4-nitrophenol (4-NP) content of the supernatant solution was estimated spectrophotometrically at 400 nm and the enzyme activity is expressed as µg 4-NP formed $g^{-1}\ h^{-1}$.

2.3 Soil microcosm incubation

After adjusting the soils to 60% of their MWHC (-33 kPa), 150 g of each soil was pre-incubated in 250-mL preservation jars at 7.5, 15, and 25 (±1)°C in the dark for 5 days. The headspace in the jars was aerated regularly

throughout the experiment to maintain aerobic conditions (CO_2 < 2%), and a glass beaker containing 5 mL of water was placed in the jars to prevent the soil from drying. One jar per soil was prepared for each hormone, i.e. E1, E2, E1-3S, and E2-3S, respectively. Before fortification, a sub-sample of 50 g each was dried at 30°C overnight, and the water content was determined gravimetrically thereafter. The lost water was reapplied with an aliquot of 1.875 mL of hormone stock solution (400 µg mL^{-1} in methanol) to the designated jar, and the spiked soil was then thoroughly mixed with the remaining 100 g of the pre-incubated soil to obtain a nominal concentration of 5 mg kg^{-1}. One sterile control was prepared for each soil by autoclaving thrice (35 min at 122.5°C and 1.13 bar) and incubated at 15°C in the dark.

Given the fact that no data on the environmental soil concentrations of estrogen sulphates exist, a worst-case scenario was calculated for estrogen concentration that can be hypothetically expected under a dairying environment in New Zealand. Hanselman *et al.* (2003) reported maximum urinary excretion of estrogen to be 163,000 µg day^{-1} based on 1000-kg live animal mass. Assuming a typical dairy cow weighs 640 kg, a maximum total urinary mass of estrogen can be calculated as 104,320 µg day^{-1}. Estrogen sulfate contributes a maximum of 92% to the total estrogen in urine of dairy cows (Hoffmann *et al.*, 1997), and hence, the excreted mass in urine amounts to 95,974 µg day^{-1}. Considering cows urinate a minimum of eight times per day (Shorten and Pleasants, 2007), the actual estrogen sulphate mass would be 11,997 µg per urination event. Given the minimum surface area covered per urine deposit from cows is 0.2 m^2 (Shorten and Pleasants, 2007), and further assuming incorporation of urine in the top 2 cm of pasture soil, and an average soil bulk density of 1100 kg m^{-3}, a nominal soil concentration of estrogen sulphate is 2.7 mg kg^{-1}. However, urine patch overlap occurs sometimes in intensive pastoral grazing systems (Afzal and Adams, 1992) and patchy distribution of urine has been found to double the nitrogen application rate (Jarvis *et al.*, 1995). Based on this information and anecdotal evidence from farmers about the nature of urinary deposition by dairy cows a theoretical hormone concentration of approximately two-fold higher was used (5 mg kg^{-1}). Considering the detection limits presented in Chapter III (*section*

3.3.2) it was therefore possible to investigate the compounds' degradation within one order of magnitude.

Orthophosphate inhibits the AryS enzyme family as an irreversible competitive inhibitor (A. Tabatabai, personal communication, 1.-15. April 2008). In order to further assess the role of AryS activity on E2-3S degradation, a subset of assays was prepared with air dried Hamilton clay loam. In brief,150 g air-dried soil was adjusted to 60% MWHC with (I) water; and (II) with a solution of 0.5 M K_2HPO_4, yielding a soil concentration of 102 µM PO_4^{3-} g^{-1} soil. The jars were pre-incubated at 25°C for 5 days and spiked as described above.

The jars were sub-sampled in triplicate at increasing time intervals that were adjusted dynamically to the observed remaining hormone concentration in the respective microcosm. As discussed in Chapter III the sub-sampling had to be increased from initially 2 g per sample to a final mass of ca 5 g over the period of the incubation in order to achieve the desired method detection limits.

The sub samples were extracted and analysed for the parent compound degradation as well as for metabolite formation and dissipation dynamics as described in Chapter III (*section 2.5*). A subset of microcosms was also assessed by means of an ER-CALUX® assay (see below) to monitor the estrogenicity of the soil extract. ER-CALUX® analysis was conducted on the 15°C assays investigating E2-3S dissipation in the three soils. An aliquot of 0.2 mL of the DCM phase was then removed from the tubes at the completion of the extraction procedure and transferred into an amber glass campaign vial. The DCM was carefully evaporated under a gentle stream of N_2 and the dried sample was reconstituted in 25 µL of dimethyl sulfoxide (Anhydrous Grade) ready for ER-CALUX® analysis.

2.4 ER−CALUX®

Estrogenic activity of soil extracts were analysed via the estrogen receptor-mediated, chemical-activated luciferase reporter gene-expression (ER-CALUX®, BioDetection Systems, Netherlands) assay. All laboratory analyses for the ER-CALUX® assay were undertaken in an ISO9001:2000 quality certified

facility at the New South Wales Department of Primary Industries, Wollongbar, NSW, Australia.

In brief, T47-D human breast cancer cells were incubated in 96-well microtiter plates in a CO_2 incubator (5% CO_2 at 30°C) for 24 hrs. The plates were removed from the incubator and the assay medium was then removed and replaced to exclude any anti-estrogenic material present in the medium. A second incubation cycle of 24 hrs was conducted and at the end of the 48-hrs incubation period, the wells were examined under the microscope for signs of contamination or abnormal cell growth. Cells were then exposed to the soil extract samples and replaced in the CO_2 incubator for a further 24 hrs. After the medium was removed from the cells, 50 µL of lysis reagent were added and the plates were incubated at room temperature (< 20°C) for 15 min. After shaking the plates for 2 min at 300 rpm (2 mm orbit) one microtiter plate at a time was analysed by a BMG Lumistar luminometer. A glowmix reagent (luciferin; luciferase substrate) was automatically dispensed into each well prior to reading the sample, and the sample was quenched using a 1 M NaOH solution prior to the next well being read. Analysis of data was undertaken using Biodetection Systems Software (BiodetectionSystems, 2006) and data are reported in ng EEQ (17ß-estradiol equivalents) g^{-1} soil (Legler *et al.*, 1999).

2.5 Data analysis and modelling

2.5.1 Data handling

The data-handling activities were conducted in accordance with the recommendations illustrated in the FOCUS (FOrum for Coordination of pesticide fate models and their USe) guidance document (2006), i.e. any concentrations of the metabolite(s) detected at t = 0 were added to the parent compound concentration. The arithmetic average of those corrected triplicate sub-samples at t = 0 was used as the initial parent compound concentration (P_0), which was set to 100% and all subsequent parent compound (P_t) and metabolite (M_t) concentrations were expressed as percent forming/remaining of P_0. An initial screening showed negligible variation in parameter predictions when the fitting was performed with the normalized

data sets compared with the absolute values. Therefore, modelling was performed on the normalized data sets.

2.5.2 Parent compound degradation

The parent compound degradation was fitted with a single first-order exponential decay model (SFO) and a double first-order in parallel (DFOP) decay model, assuming no back conversion and no altering due to microbial growth. Both of these models can be described mathematically:

SFO: $$P_t = P_0 e^{-k_1 t} \quad (5.1)$$

DFOP: $$P_t = P_0[g e^{-k_1 t} + (1-g) e^{-k_2 t}] \quad (5.2)$$

where t is time (h), k_1 and k_2 are the degradation rate constants [h^{-1}], P_0 is the initial amount of hormone, P_t is the total amount of hormone at time t, and g is the fraction of P_0 applied to compartment 1 of the DFOP model [unitless]. According to eq. 5.2, degradation takes place in two compartments: in the first compartment rapid degradation is expected to occur within the soil-water phase, where microorganisms have easy access to the compound. In the second compartment, degradation is slow, and the compound is expected to be adsorbed to soil particles or to be located in micro-pores within the soil matrix, with the degradation rate being governed by the slow desorption-diffusion processes (Hamaker and Goring, 1976). The speed at which the compound is transformed in the two compartments is expressed by their respective rate constants k_1 (first compartment) and k_2 (second compartment), and usually $k_1 > k_2$. Two compartment models may also fit to degradation of compounds that have two isomers. However, the presence and the nature of the thio-ester bond in E1-3S and E2-3S relates to one distinctive form of the molecule. Therefore isomer specific degradation patterns can be excluded here.

2.5.3 Metabolite formation and degradation

The metabolite formation and dissipation dynamics were modelled using the best fit for the respective parent compound as input (eqs. 5.1 and 5.2), assuming metabolite degradation also follows a single first-order kinetic

(SFO) or a two-compartment first-order biexponential decay model (DFOP). For E2 and E1-3S both degrading to E1 this can be formulated mathematically:

SFO: $$M_t = ff_M (P_0 - P_t) e^{-k_{M1} t} \quad (5.3)$$

DFOP: $$M_t = ff_M (P_0 - P_t)[g_M e^{-k_{M1} t} + (1 - g_M) e^{-k_{M2} t}] \quad (5.4)$$

where, M_t is the total amount of the respective metabolite at time t, ff_M is the formation fraction of the metabolite [unitless, expressed in %], k_{M1} and k_{M2} are the metabolite degradation rate constants [h^{-1}], and g_M is the fraction of the metabolite applied to compartment 1 [unitless, expressed in %].

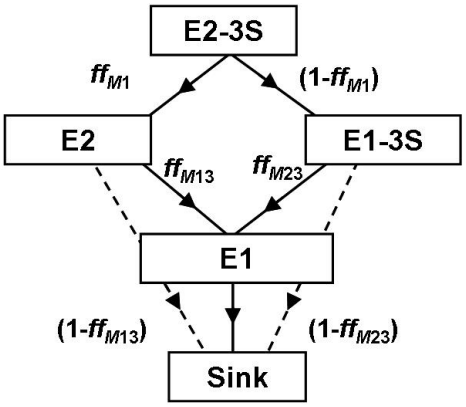


Figure 5.1. Illustration of the degradation and metabolite formation of E2-3S.

The degradation of E2-3S is more difficult to describe since the parent compound degradation is in theory equally likely to produce E1-3S or E2 as metabolites through oxidation at position C-17 or hydrolyzation of the thio-ester bond at position C-3, respectively. Basically, the same SFO and DFOP equations as described above can be applied to describe the system using the best fit for the parent compound. However, some constraints have to be applied to the metabolite formation fraction. The sum of the formation fractions of the two metabolites of E2-3S, E2 (M1) and E1-3S (M2), must theoretically not exceed 1, and therefore ff_{M2}=(1-ff_{M1}) (Figure 5.1.). Substituting the appropriate formation fraction in equations 5.3 and 5.4 the formation and degradation of M1 and M2 can be described mathematically. Both primary metabolites E2 and E1-3S have in theory a common metabolite E1 (M3), a secondary metabolite of E2-3S, and hence the degradation of both

primary metabolites results in the formation of M3 (Figure 5.1.). The sink term in Figure 5.1. was needed to compute the degradation of E1 with ModelMaker 4.0 (Modelkinetix.com) (*section 2.5.4*).

2.5.4 Modelling procedure and statistical measures

The statistical software R (version 2.6.1) with the nonlinear mixed effects (nlme) library employing nonlinear least-squares regression (nls) analysis with the Levenberg-Marquardt algorithm for parameter optimization was used to fit the measured degradation and metabolite formation data of E1, E2, and E1-3S, respectively (Pinheiro and Bates, 2000). It was also used to compute initial degradation parameters for the degradation of E2-3S. However, to compute the complex metabolite formation and degradation pathways (Figure 5.1.) the kinetic modelling software ModelMaker 4.0 was used, which employs the same optimization algorithm as the nlme library. The starting values and parameter constraints for the nonlinear regression are illustrated in Table 5.2.

Table 5.2. Parameter starting values and constraints for the nonlinear least squares regression optimization.

Model	Parameter starting values and constrains			
Parent	P_0 (min, max)	g (min, max)	k_1 (min, max)	k_2 (min, max)
SFO	100 (80, 120)		0.1 ($1x10^{-5}$, 2)	
DFOP	100 (80, 120)	0.6 (0.01, 0.99)	0.1 ($1x10^{-5}$, 2)	0.01 ($1x10^{-6}$, 0.5)
Metabolites	ff_M (min, max)	g_M (min, max)	k_{M1} (min, max)	k_{M2} (min, max)
SFO	0.8 (0.01, 0.99)		0.1 ($1x10^{-5}$, 2)	
DFOP	0.8 (0.01, 0.99)	0.6 (0.01, 0.99)	0.1 ($1x10^{-5}$, 2)	0.01 ($1x10^{-6}$, 0.5)

A number of statistical indices were computed for model comparison and goodness-of-fit evaluation at a given soil and temperature, which included one way analysis of variance (*ANOVA*), the adjusted coefficient of determination (R^2_{adj}), the Akaike Information Criterion for small sample size (AIC_c), a measurement error percentage to pass the chi-square (χ^2) statistic at 5% significance level [err (5%)], and the scaled root mean squared error (SRMSE). The model with the majority of the statistical measures in its favour was chosen to represent the data graphically and to serve as the input

function for the subsequent metabolite modelling. In the case of E2-3S the inbuilt functions for goodness of fit assessment in ModelMaker 4.0 were used to compare the two models and to choose the best fit.

The adjusted coefficient of determination (5.5) is a modification of the coefficient of determination (R^2) that accounts for the number of parameters (p) of the given regression model:

$$R^2_{adj} = 1 - (1 - R^2)\frac{n-1}{n-p-1} \tag{5.5}$$

where: $R^2 = 1 - \frac{SSE}{SST}$, is the coefficient of determination (5.6)

$SSE = \sum_i (O_i - P_i)^2$, is the residual sum of squares (5.7)

$SST = \sum_i (O_i - \overline{O_i})^2$, is the total sum of squares (5.8)

O_i = observed value; P_i = predicted value

$\overline{O_i}$ = mean of all observed values; n = sample size

p = number of optimized parameters.

Akaike's information criterion (*AIC*) uses estimates of goodness-of-fit (accuracy) and model variability (precision) to quantitatively rank different models in their abilities to describe a given data set. The *AIC* produces numerical values that reflect both accuracy and precision, such that the lowest *AIC* value identifies the model that is most likely justified by the data. *AIC* is only used to compare among (nested) model fits for a single data set and can not be used to rank or compare model performance across different datasets. The *AIC* is computed as the sum of two penalty terms, the first for bias (inaccuracy) and the second for variability (Burnham and Anderson, 2002; Saffron *et al.*, 2006):

$$AIC = n \ln\left(\frac{SSE}{n}\right) + 2K \tag{5.9}$$

where n is the sample size and $K = p+1$ is the number of estimated model parameters plus one for the model variance.

Unless the sample size exceeds 40, the use of AIC_c (Akaike information criterion for small sample size) is recommended (Saffron *et al.*, 2006), which includes a second bias correction term:

$$AIC_c = AIC + \frac{2K(K+1)}{n-K-1} \tag{5.10}$$

As stated above the model with the lower AIC_c score is more likely to represent the investigated dataset.

The χ^2-test is recommended by FOCUS (2006) to evaluate nested models and considers the deviations between observed and predicted values for a given model relative to the uncertainty of the measurements:

$$\chi^2 = \sum_i \frac{(P_i - O_i)^2}{\left(\frac{err}{100}\overline{O}\right)^2} \tag{5.11}$$

where: *err* = measurement error percentage.

The calculated value is then compared to a tabulated value $\chi^2_{df,\,\alpha}$ (with df = degrees of freedom, and α = probability that the obtained value is result by chance). The test is passed when $\chi^2 \leq \chi^2_{df,\,\alpha}$. An α of 0.05 is usually employed. The error term in equation 5.11 reflects the measurement uncertainty and is scaled with the mean of the observed data, thus, keeps constant throughout the whole measurement period (FOCUS, 2006). The minimum error-% of the error term in eq. 5.11 can be calculated as:

$$err = 100\sqrt{\frac{1}{\chi^2_{df,\alpha}}\sum_i \frac{(P_i - O_i)}{\overline{O}^2}} \tag{5.12}$$

Consequently, the model with the smallest error percentage is defined as most appropriate, because it describes the measured data in the most robust way (FOCUS, 2006).

The Scaled Root Mean Squared Error (SRMSE) gives an indication of the deviation from the ideal case where $P_i = O_i$. The error is scaled in relation to the mean of all observed values (FOCUS, 2006). The SRMSE is always larger than zero and a smaller SRMSE indicates a better fit.

$$SRMSE = \frac{1}{\overline{O}}\sqrt{\frac{\sum_i (P_i - O_i)^2}{n}} \tag{5.13}$$

The modelling procedure is illustrated schematically in Figure 5.2. The left panel describes the case for E2 and E1-3S, respectively, which both form E1 as a quantifiable metabolite. The right panel gives the proceeding for E2-3S where two quantifiable primary metabolites, E1-3S and E2, degrade then to

a common secondary metabolite E1. In the case of E1 degradation as a parent compound, the procedure stopped after the parent compound fitting.

The abbreviation DT_x in Figure 5.2. stands for the time it takes for the compound to dissipate to 50% (DT_{50}) and 90% (DT_{90}) of its initial amount, respectively. For a SFO model these values were calculated directly from the optimized first-order rate constants k ($DT_{50} = \ln(2)k^{-1}$ and $DT_{90} = \ln(10)k^{-1}$). For the DFOP model no analytical solution exists, and therefore an iterative procedure was employed using the solver tool in Excel (Microsoft Excel 2003 SP2) to derive dissipation times. It has to be noted that the iterative procedure to determine the values for DT_{50} and DT_{90} from the DFOP model requires a hypothetical value for the maximum amount of metabolite formed, which in theory is determined by the predicted formation fraction in equation 5.4 (calculated as $M_{max} = ff_M P_0$). However, a solution cannot be found unless the percentage of the metabolite remaining at the given dissipation time (i.e., $M_{t=DT50/90} = nM_{max}$ with, n= 0.5 for DT_{50} and n = 0.1 for DT_{90}) is covered by the fitted curve.

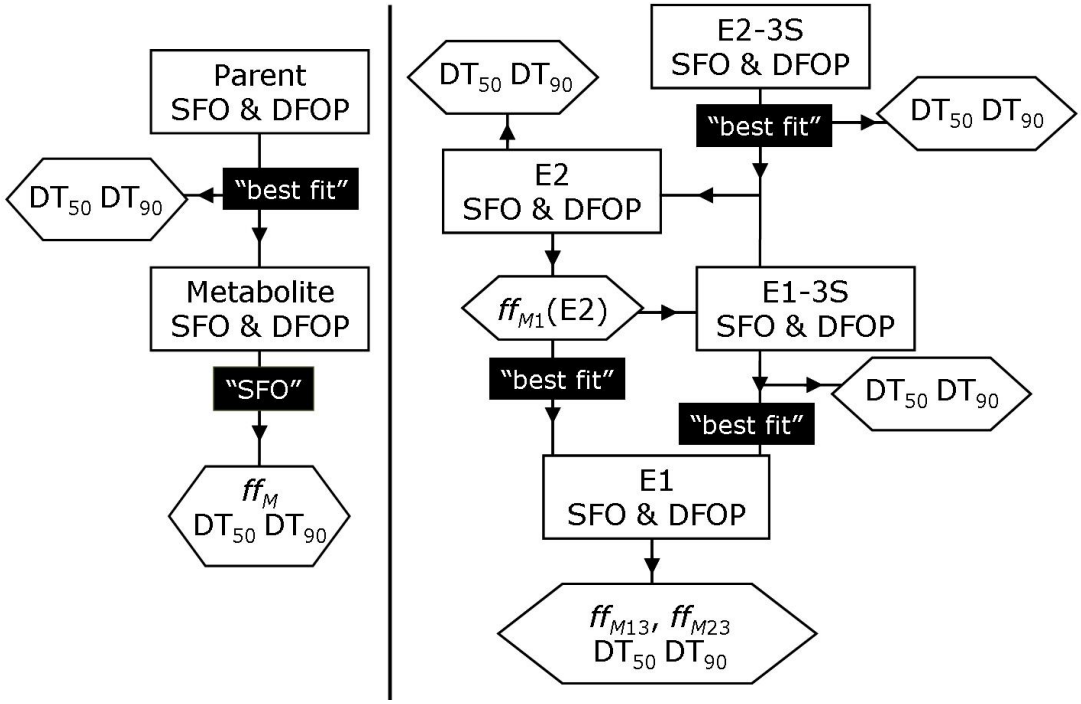


Figure 5.2. Illustration of the modelling procedure to model the degradation and metabolite formation kinetics of E1, E2, E1-3S (left panel) and E2-3S (right panel).

3 RESULTS

3.1 Degradation and metabolite formation of free estrogens

3.1.1 Estrone degradation

The degradation of E1 in the Hamilton clay loam soils is displayed in Figure 5.3. Table 5.3. gives the modelling results and the best fit is plotted as a line together with the data in Figure 5.3. Estrone degraded fast in the Hamilton soil with > 40% and > 60% of the initial concentration being removed within the first 2 hrs of the incubation at 7.5, 15, and 25°C, respectively.

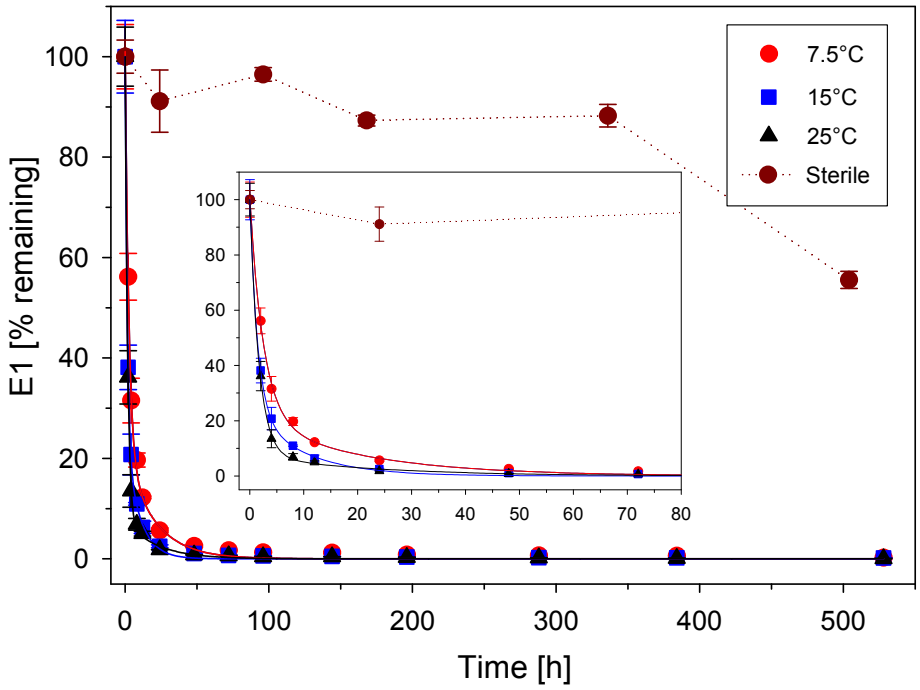


Figure 5.3. Estrone degradation in Hamilton clay loam at three incubation temperatures. Mean values of n = 3 samples are displayed with one relative standard deviation. Insert illustrates the first 80 hrs in detail. Solid lines represent the best fit.

A difference between the temperatures was most noticeable between 2 and 72 hrs of incubation where the initial fast degradation appeared to slow down. In the sterile control, E1 dropped to 88% of the initial mass within 336 hrs of incubation and a sharp decrease to 56% (505 hrs) was observed thereafter. Applying the DFOP model to the data improved the statistical measures as opposed to the SFO model for all three temperature datasets

(Table 5.3.) and the parameter estimates had small standard errors. The resulting DT values (Table 5.3.) decreased with increasing temperatures. In general E1 dissipated to < 10% of its initial within the first 24 hrs in the Hamilton soil.

Table 5.3. Optimized parameters with one standard error (SE), statistical measures, and dissipation times for the SFO and DFOP model fitted to the measured degradation data of E1 in three soils at three temperatures. Blue letters indicate best fit.

T [°C]	Model	Optimized parameters				Statistical measures				Dissipation times	
		P_0 (SE) [%]	g (SE) [%]	k_1 (SE) [h^{-1}]	k_2 (SE) [h^{-1}]	R^2_{adj}	AIC_c	err (5%)	SRMSE	DT_{50} [h]	DT_{90} [h]
Hamilton clay loam											
7.5	SFO	97.3 (3.59)		0.245 (0.020)		0.981	43.7	17.2	0.210	2.83	9.40
	DFOP***	100 (1.27)	79.0 (3.86)	0.397 (0.030)	0.050 (0.011)	0.998	20.5	5.81	0.066	2.33	16.1
15	SFO	98.6 (3.18)		0.416 (0.030)		0.985	39.0	18.7	0.229	1.67	5.54
	DFOP ***	100 (0.55)	75.9 (2.91)	0.701 (0.039)	0.104 (0.013)	0.999	-3.93	3.12	0.036	1.37	8.66
25	SFO	99.7 (2.09)		0.494 (0.024)		0.993	27.1	13.4	0.164	1.40	4.66
	DFOP ***	100 (0.71)	92.4 (1.49)	0.591 (0.020)	0.046 (0.013)	0.999	3.14	4.41	0.050	1.30	5.30
Matawhero silt loam											
7.5	SFO	81.6 (6.34)		0.016 (0.005)		0.840	61.8	18.4	0.229	43.3	144
	DFOP *	92.3 (5.95)	58.3 (12.5)	0.073 (0.034)	0.004 (0.002)	0.932	61.8	11.4	0.129	21.9	356
15	SFO	97.4 (4.44)		0.034 (0.005)		0.962	50.2	11.3	0.140	20.4	67.7
	DFOP *	102 (3.31)	72.1 (12.0)	0.063 (0.005)	0.007 (0.004)	0.993	49.3	6.70	0.076	16.7	150
25	SFO	92.5 (3.74)		0.044 (0.017)		0.975	45.0	10.5	0.130	15.8	52.3
	DFOP	92.9 (4.36)	96.5 (11.8)	0.047 (0.011)	0.003 (0.017)	0.968	57.1	11.3	0.127	15.4	55.5
Gibsons fine sandy loam											
7.5	SFO	90.8 (4.16)		0.026 (0.004)		0.960	53.6	12.4	0.154	26.7	88.6
	DFOP***	99.7 (3.36)	42.9 (11.3)	0.122 (0.051)	0.013 (0.003)	0.985	49.4	7.19	0.082	18.2	137
15	SFO	96.3 (3.07)		0.027 (0.003)		0.981	46.1	7.84	0.146	25.7	85.3
	DFOP**	102 (3.12)	33.0 (15.0)	0.115 (0.064)	0.017 (0.004)	0.991	45.8	5.28	0.068	20.7	111
25	SFO	96.3 (3.72)		0.065 (0.007)		0.979	45.6	11.2	0.138	10.7	35.4
	DFOP***	101 (1.97)	64.7 (8.86)	0.135 (0.024)	0.020 (0.005)	0.996	34.2	4.78	0.054	8.62	64.5

*, **, *** indicate statistical difference between SFO and DFOP at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Figure 5.4. shows the degradation of E1 in the Matawhero silt loam soil at the three investigated temperatures. The model fitting results are given in Table 5.3. and the best fit is plotted together with the data in Figure 5.4. Estrone degraded rapidly within the first 24 hrs of the incubation and the percent of E1 remaining was reduced to 38 (7.5°C), 34 (15°C) and 28% (25°C), respectively. Slower degradation was observed thereafter for all three temperatures and the percent remaining at the end of the incubation (504 hrs) accounted for 4.7, 0.71, and 0.24%, respectively, for increasing temperatures. In the sterile control, E1 did not vary within the first 168 hrs of incubation; however, > 20% were degraded at the end of the experiment. The statistical measures in Table 5.3. indicate that the DFOP model was slightly favoured over the SFO model to describe the degradation data at 7.5 and 15°C, but no improvements were achieved fitting the 25°C data with the

DFOP model. The calculated DT values decrease with increasing temperatures and show that 10% of the E1 persisted > 14 (7.5°C), 6 (15°C) and 2 days (25°C), respectively, in the Matawhero soil.

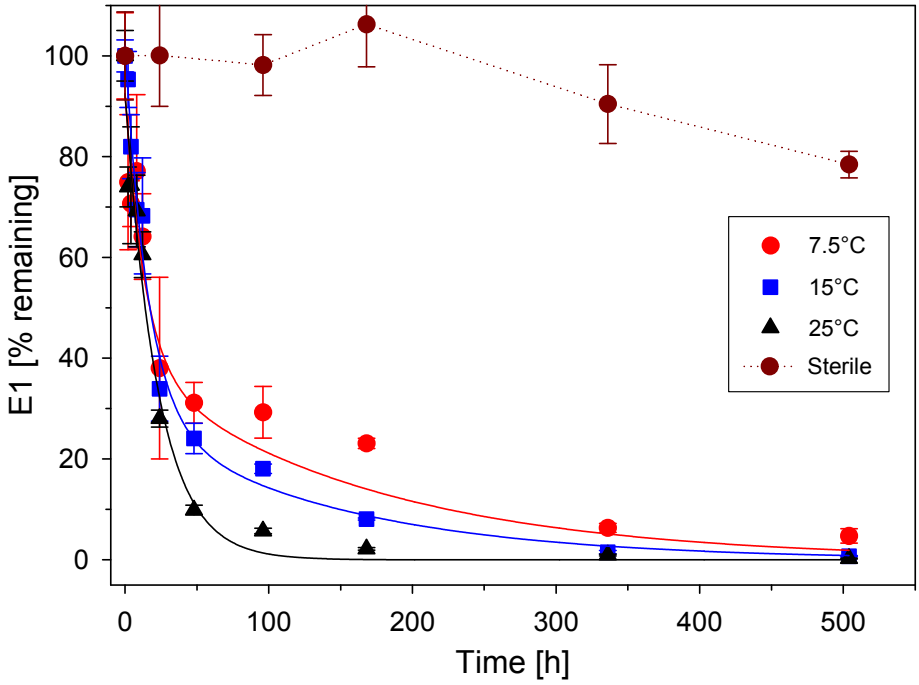


Figure 5.4. Estrone degradation in Matawhero silt loam at three incubation temperatures. Mean values of n = 3 samples are displayed with one relative standard deviation. Solid lines represent the best fit.

Figure 5.5. displays the degradation of E1 in the Gibsons fine sandy loam soil and the corresponding modelling results are summarized in Table 5.3. Rapid degradation of E1 was again observed in the first 96 hrs of incubation with 12, 9.3, and 4.6% remaining at 7.5, 15, and 25°C, respectively. Slower degradation occurred thereafter with 1.4% (7.5°C), 0.82% (15°C), and 0.24% (25°C) of E1 remained at the end of the experiment (672 hrs). Dissipation of E1 in the sterile control was noticed as well and the average measured values showed large standard deviations (Figure 5.5.). At the end of the incubation, 44% of the initial E1 was detected in the sterile control. The statistical measures in Table 5.3. indicate that the DFOP model performed better than the SFO model to fit the degradation data for all three temperatures, and the resulting DT_{90} values decrease with increasing temperatures.

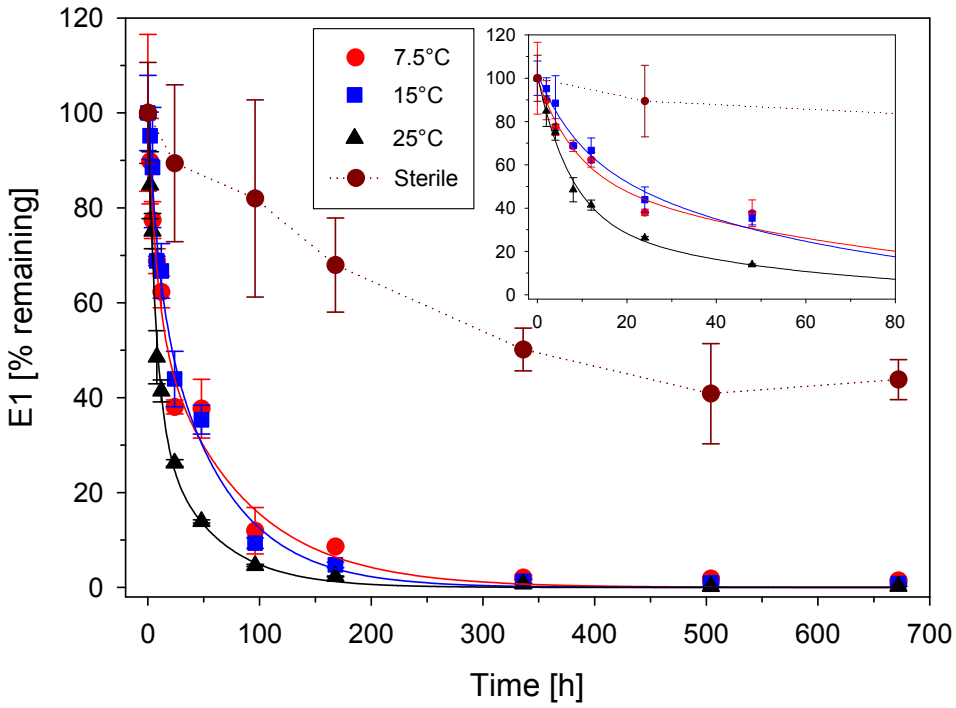


Figure 5.5. Estrone degradation in Gibsons fine sandy loam at three incubation temperatures. Mean values of n = 3 samples are displayed with one relative standard deviation. Insert illustrates the first 80 hrs in detail. Solid lines represent the best fit.

3.1.2 17β–Estradiol degradation and metabolite formation

17β-Estradiol degraded rapidly in the Hamilton soil with 53% (7.5°C), 66% (15°C) and 89% (25°C) being removed in the first 2 hrs of the incubation (Figure 5.6, left panel). The degradation decelerated after 4 hrs at 25°C and after 8 hrs at 15 and 7.5°C, respectively. 17β-estradiol was no longer detected after 120 hrs at 25°C, while residues < 1% were detectable until 384 hrs at 15 and 7.5°C. The sterile control sample showed some fluctuations at the start of the incubation and after 336 hrs 88% of the initially applied E2 was still detectable in the Hamilton soil. Table 5.4. shows that the DFOP model was better than the SFO model to describe the observed degradation pattern in the Hamilton soil; all statistical measures favour the DFOP model and the matching DT values indicate a temperature dependence of the E2 dissipation which was also supported by the plotted dataset (Figure 5.6.).

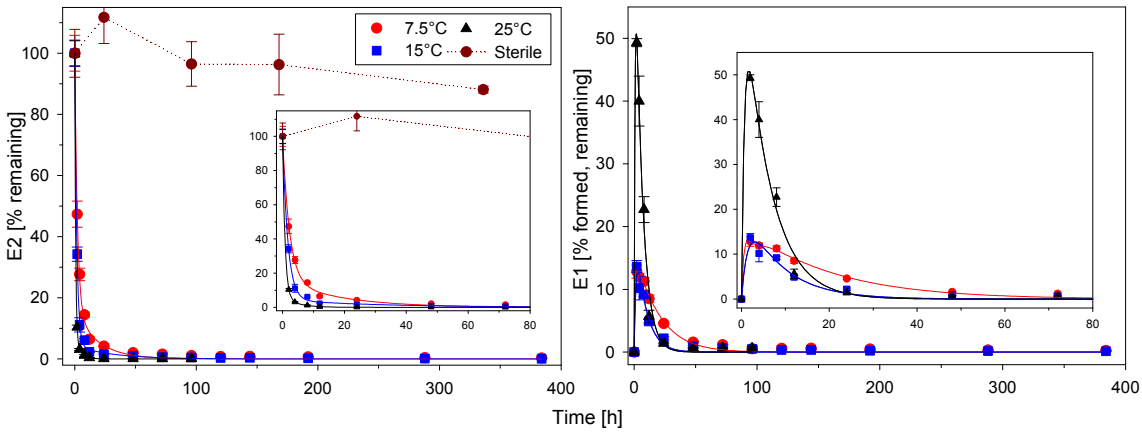


Figure 5.6. Degradation of E2 (left panel) and the concomitant formation and degradation of its metabolite E1 (right panel) at three incubation temperatures in the Hamilton clay loam soil. Mean values of n = 3 samples are displayed. Inserts illustrates the first 80 hrs in detail. Solid lines represent the best fit.

With E2 being degraded the appearance of a new peak was observed, which was identified as E1 based on the retention time in the HPLC-UV chromatograms. The concomitant formation and degradation of E1 is displayed in Figure 5.6. (right panel). The maximum formation of E1 in the Hamilton soil occurred after 2 hrs of incubation for all temperatures and accounted for 13, 14, and 49% of the initially applied E2 at 7.5, 15, and 25°C, respectively. Estrone degraded fast after that maximum at 15 and 25°C while at 7.5°C the degradation was comparably slower. Table 5.5. summarizes the modelling results for the metabolite E1. According to the statistical measures the DFOP was better able to fit the 7.5°C data but a solution for the matching DT values was not found (see section 2.5.4 for explanation). For 15 and 25°C the iteration process to fit the DFOP model did not converge and the SFO model was chosen to represent the data. The DT values in Table 5.5. suggest a temperature dependence of E1 degradation as a metabolite of E2. With the exception at 7.5°C, the DT_{90} values were < 24 hrs.

Table 5.4. Optimized parameters with one standard error (SE), statistical measures, and dissipation times for the SFO and DFOP model fitted to the measured degradation data of E2 in three soils at three temperatures. Blue letters indicate best fit.

T [°C]	Model	Optimized parameters				Statistical measures				Dissipation times	
		P_0 (SE) [%]	g (SE) [%]	k_1 (SE) [h^{-1}]	k_2 (SE) [h^{-1}]	R^2_{adj}	AIC_c	err (5%)	SRMSE	DT_{50} [h]	DT_{90} [h]
Hamilton clay loam											
7.5	SFO	98.0 (3.00)		0.314 (0.021)		0.986	37.9	15.8	0.193	2.21	7.33
	DFOP***	99.8 (1.24)	83.9 (4.06)	0.450 (0.033)	0.054 (0.017)	0.998	19.1	6.24	0.071	1.91	10.3
15	SFO	99.9 (1.61)		0.531 (0.020)		0.997	19.8	10.8	0.133	1.31	4.34
	DFOP **	100 (0.91)	95.6 (1.37)	0.591 (0.022)	0.033 (0.018)	0.999	10.3	5.98	0.068	1.24	4.62
25	SFO	100 (0.87)		1.11 (0.040)		0.999	6.09	4.81	0.060	0.624	2.07
	DFOP ***	100 (0.07)	93.3 (1.49)	1.31 (0.020)	0.226 (0.016)	1.00	-22.4	0.382	0.004	0.551	2.04
Matawhero silt loam											
7.5	SFO	88.8 (5.24)		0.099 (0.014)		0.955	51.7	18.4	0.227	7.00	23.3
	DFOP **	100 (2.07)	36.8 (4.64)	0.837 (0.264)	0.057 (0.006)	0.995	32.6	5.71	0.065	4.45	32.6
15	SFO	95.7 (2.71)		0.164 (0.010)		0.990	33.6	10.3	0.127	4.23	14.0
	DFOP [a]										
25	SFO	98.4 (3.35)		0.351 (0.026)		0.988	29.4	10.0	0.126	1.97	6.56
	DFOP*	100 (0.64)	51.5 (7.11)	0.795 (0.123)	0.184 (0.018)	0.999	27.2	1.75	0.019	1.63	8.64
Gibsons fine sandy loam											
7.5	SFO	97.5 (1.96)		0.112 (0.004)		0.996	27.5	6.65	0.082	6.15	20.5
	DFOP***	100 (1.24)	48.4 (16.9)	0.227 (0.059)	0.065 (0.014)	0.999	20.5	3.45	0.039	5.50	25.4
15	SFO	99.1 (1.94)		0.149 (0.007)		0.996	24.7	6.31	0.078	4.65	15.5
	DFOP	101 (1.91)	61.0 (53.1)	0.225 (0.107)	0.085 (0.059)	0.996	31.7	5.40	0.061	4.34	17.5
25	SFO	100 (1.41)		0.395 (0.012)		0.998	15.4	4.50	0.056	1.75	5.83
	DFOP	100 (1.40)	98.4 (1.76)	0.411 (0.019)	0.016 (0.039)	0.998	39.9	4.17	0.045	1.73	5.95

*, **, *** indicate statistical difference between SFO and DFOP at $p < 0.05$, $p < 0.01$, and $p < 0.001$. [a]did not converge.

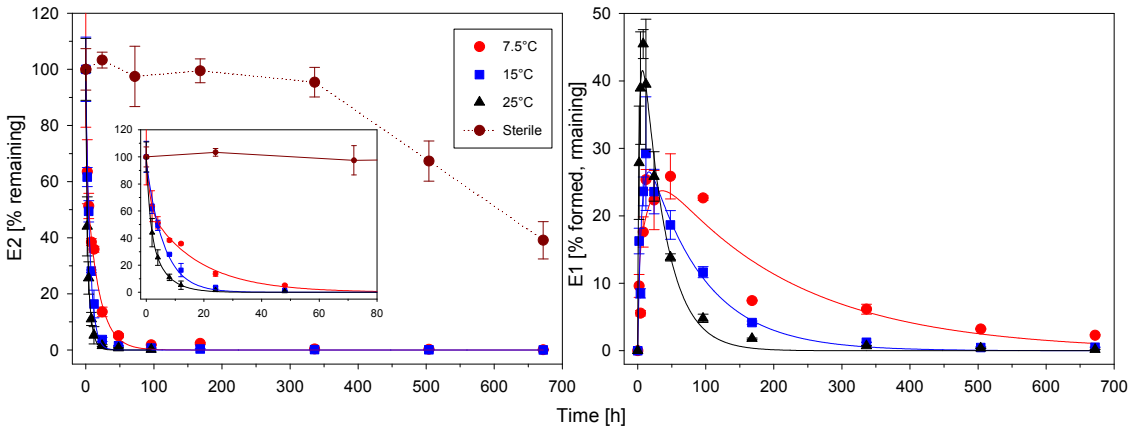


Figure 5.7. Degradation of E2 (left panel) and the concomitant formation and degradation of its metabolite E1 (right panel) at three incubation temperatures in the Matawhero silt loam soil. Mean values of n = 3 samples are displayed. Insert illustrates the first 80 hrs in detail. Solid lines represent the best fit.

Figure 5.7. displays the degradation of E2 (left panel) and the concomitant formation and degradation of E1 (right panel) in the Matawhero soil. 17β-Estradiol degradation was rapid in the first hours of incubation with 36 (7.5°C), 16 (15°C), and 5.3% (25°C) of the applied E2 remaining after 12 hrs. In the 25°C incubation, E2 was last detected after 96 hrs but remained

detectable throughout the incubation at the two lower temperatures, *albeit* only at trace levels (<1%). In the sterile control no significant reduction of E2 was observed within the first 336 hrs. However, a decline of E2 was detected thereafter and only 39% remained at the end of the incubation (672 hrs). The DFOP model was most suitable to fit the data at 7.5 and 25°C (Table 5.4.); however, no solution was obtained for the 15°C dataset and the SFO model was chosen to represent the measured data. While the model-derived DT_{90} values (Table 5.4.) suggest a faster degradation with higher temperatures, only a small difference was found between the DT_{50} values at 7.5 and 15°C. More than 90% of the E2 dissipated within the first day of incubation in the Matawhero soil.

The formation of E1 in the Matawhero soil rapidly reached a peak of 45% after 8 hrs at 25°C and E1 degraded fast thereafter (Figure 5.7., right panel). At 7.5 and 15°C the formation of a distinct peak was not pronounced. The maximum of formed E1 fluctuated around 20–25% (7.5°C) in the first 12–96 hrs, and around 24–29% (5°C) in the first 4–48 hrs, respectively. The subsequent E1 degradation was slower at 7.5°C compared with 15, and 25°C and E1 was detected until the end of the incubation (672 hrs) at all temperatures. The SFO model was found to be the best model to fit the formation and degradation data of E1 in the Matawhero soil (Table 5.5.). In fact, the DFOP model yielded a solution on only one occasion and only one statistical measure (err%) suggested a better performance compared to the SFO model. The resulting DT values (Table 5.5.) indicate once more the temperature dependence on the persistence of E1 and DT_{90} accounted for 460 hrs (>19 days) at 7.5°C.

In the Gibsons soil, the difference in the degradation of E2 between the three temperatures was most noticeable from 2 to 48 hrs of incubation (Figure 5.8., left panel). A fast degradation occurred within the first 24 hrs with > 90% being removed at 15 and 25°C. After 96 hrs the rate of degradation reduced and only trace levels of E2 were detected at 15 and 7.5°C. In the sterile control E2 varied between 100 and 90% within the first 336 hrs and decreased afterwards to 59% on the last sampling event (672 hrs). The DFOP model gave a solution for all three temperatures but the statistical measures

supported its choice only for the 7.5°C data (Table 5.4.). The resulting DT values decreased with increasing temperatures and suggest that > 90% of E2 was degraded within the first 25 hrs at all three temperatures.

Table 5.5. Optimized parameters with one standard error (SE), statistical measures, and dissipation times for the SFO and DFOP model fitted to the measured formation and degradation data of E1 as a metabolite of E2 degradation in three soils at three temperatures. Blue letters indicate best fit.

T [°C]	Model	Optimized parameters				Statistical measures				Dissipation times	
		ff_M (SE) [%]	g (SE) [%]	k_{M1} (SE) [h^{-1}]	k_{M2} (SE) [h^{-1}]	R^2_{adj}	AIC_c	err (5%)	SRMSE	DT_{50} [h]	DT_{90} [h]
Hamilton clay loam											
7.5	SFO	24.6 (1.67)		0.075 (0.009)		0.978	3.80	35.0	0.217	9.24	30.7
	DFOP***	82.0 (66.0)	76.3 (18.0)	1.05 (0.560)	0.055 (0.006)	0.993	-5.54	9.82	0.112	n.s.	n.s.
15	SFO	22.9 (1.93)		0.120 (0.015)		0.969	4.72	40.6	0.283	5.78	19.2
	DFOP [a]										
25	SFO	80.2 (4.61)		0.173 (0.014)		0.991	22.1	40.9	0.140	4.01	13.3
	DFOP [a]										
Matawhero silt loam											
7.5	SFO	30.9 (3.12)		0.005 (0.001)		0.922	42.2	91.8	0.323	139	460
	DFOP [a]										
15	SFO	36.3 (3.28)		0.012 (0.003)		0.951	36.5	75.0	0.273	57.8	192
	DFOP	36.6 (4.25)	96.0 (56.0)	0.013 (0.010)	0.002 (0.030)	0.937	47.4	24.0	0.273	55.3	200
25	SFO	59.3 (2.24)		0.030 (0.003)		0.993	23.8	36.8	0.111	23.1	76.8
	DFOP[a]										
Gibsons fine sandy loam											
7.5	SFO	42.1 (3.32)		0.010 (0.002)		0.962	36.8	70.6	0.239	69.3	230
	DFOP[a]										
15	SFO	72.9 (8.01)		0.028 (0.006)		0.943	48.5	103	0.317	24.8	82.2
	DFOP[a]										
25	SFO	61.9 (3.76)		0.032 (0.012)		0.981	37.6	64.0	0.190	21.7	72.0
	DFOP										

*, **, *** indicate statistical difference between SFO and DFOP at $p < 0.05$, $p < 0.01$, and $p < 0.001$. [a]did not converge. n.s.=no solution.

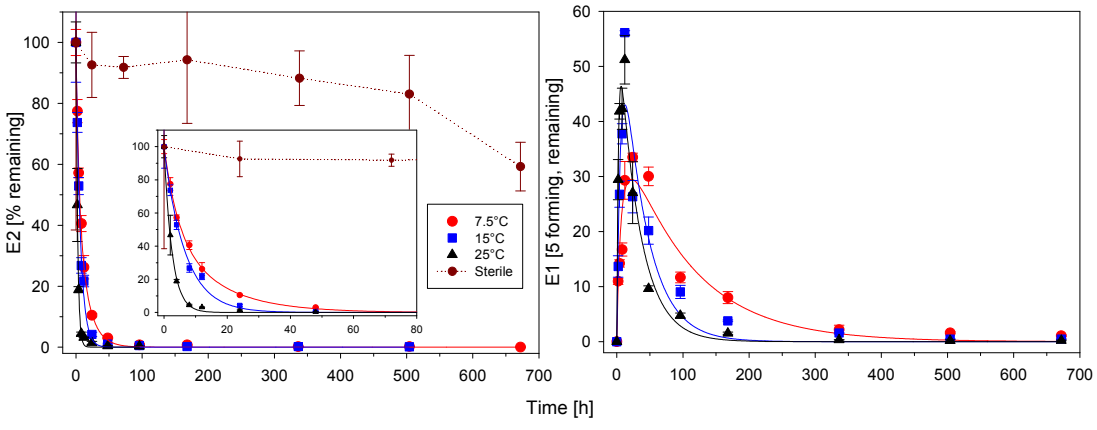


Figure 5.8. Degradation of E2 (left panel) and the concomitant formation and degradation of its metabolite E1 (right panel) at three incubation temperatures in the Gibsons fine sandy loam soil. Mean values of n = 3 samples are displayed. Insert illustrates the first 80 hrs in detail. Solid lines represent the best fit.

Estrone was rapidly formed in the Gibsons soil as a result of E2 degradation and showed distinct peaks accounting for 33% (7.5°C), 56% (15°C), and 51% (25°c) of the parent compound, respectively (Figure 5.8, right panel). While the peak occurred after 24 hrs at 15 and 25°C it took 48 hrs to reach a peak at 7.5°C. Estrone subsequently degraded in the Gibsons soil and the degradation was faster with increasing temperatures. At the end of the incubation (672 hrs) E1 was still detectable at ca 1% in the 7.5°C assay, while it was < 0.5% for the two higher temperatures. The modelling procedure resulted only in solutions for the SFO model to describe the formation and degradation pattern in the Gibsons soil at all temperatures and the corresponding DT values indicate shorter persistence of E1 with increasing temperatures (Table 5.5.).

3.2 Degradation and metabolite formation of sulphate-conjugated estrogens

3.2.1 Estrone-3-sulphate degradation and metabolite formation

The degradation of E1-3S in the Hamilton clay loam soil is displayed in Figure 5.9. (left panel) together with the observed formation and dissipation of its metabolite E1 (right panel). The dissipation of E1-3S was fast in the non-sterile incubations and in the first 24 hrs 91% (7.5°C), 94% (15°C), and 99% (25°C) were degraded, respectively. After 96 hrs the E1-3S concentration was below the MDL at 25°C, and no E1-3S was detected after 144 hrs at 15°C but it remained detectable at 7.5°C until 240 hrs of incubation. In the sterile control incubated at 15°C, no significant reduction in the E1-3S concentration was observed, although E1-3S varied between 90 and 113% throughout the experiment. Table 5.6. illustrates the modelling results and the statistical measures indicate that the DFOP model was superior to the SFO model to fit the data. The resulting DT values support the observed temperature dependence and indicate that > 90% of E1-3S was removed from the Hamilton soil within the first 24 hrs even at the lowest temperature of 7.5°C.

Estrone was rapidly formed during the degradation of E1-3S (Figure 5.9., right panel) and the formation reached a peak after 8 hrs at 7.5 and 15°C accounting for 10 and 11% of the parent compound, respectively. The maximum of 6.8% formed at 25°C was observed already after 2 hrs of

incubation. After the maximum, E1 degraded fast at all temperatures and ca 1% remained after 48 hrs. The rate of degradation declined thereafter and E1 was detectable only at trace amounts (< 1%) until the last sampling event (240 hrs). No E1 was detected in the sterile control throughout the experiment. The iterative process to fit the DFOP model to the formation and degradation of E1 did not converge for the 7.5 and 15°C data (Table 5.7.) and the SFO model was chosen to represent the data. All statistical measures, however, were in favour of the DFOP model to describe the 25°C data. The resulting DT values show no clear trend with the incubation temperatures but indicate that E1 as metabolite of E1-3S dissipated to ca 90% of its maximum formation with the first day in the Hamilton soil.

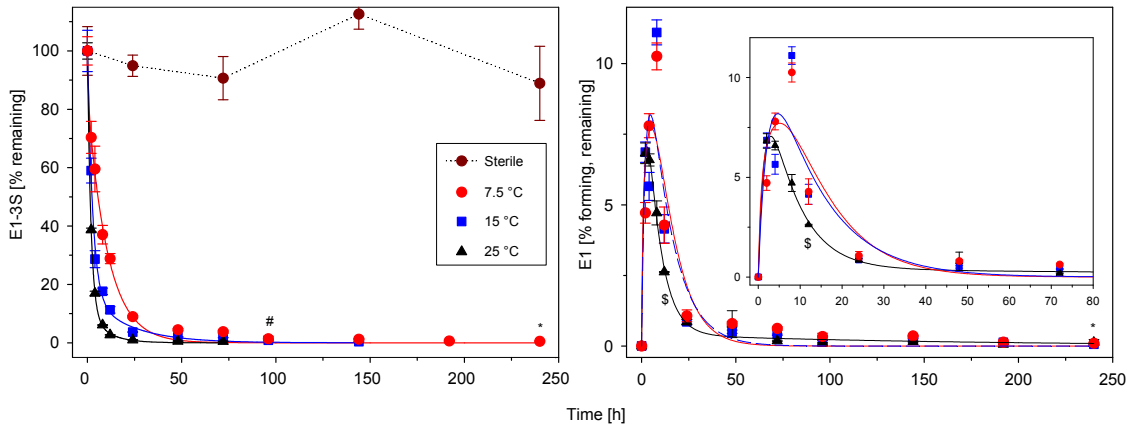


Figure 5.9. Degradation of E1-3S (left panel) and the concomitant formation and degradation of its metabolite E1 (right panel) at three incubation temperatures in the Hamilton clay loam soil. Mean values of n = 3 samples are displayed. $^{*,\#,\$}$ indicate n = 2 samples at 7.5,15, and 25 °C, respectively. Insert illustrates the firs 80 hrs in detail. Solid lines represent the best fit.

Table 5.6. Optimized parameters with one standard error (SE), statistical measures, and dissipation times for the SFO and DFOP model fitted to the measured degradation data of E1-3S in three soils at three temperatures. Blue letters indicate best fit.

T [°C]	Model	Optimized parameters				Statistical measures				Dissipation times	
		P_0 (SE) [%]	g (SE) [%]	k_{M1} (SE) [h^{-1}]	k_{M2} (SE) [h^{-1}]	R^2_{adj}	AIC_c	err (5%)	SRMSE	DT_{50} [h]	DT_{90} [h]
Hamilton clay loam											
7.5	SFO	95.2 (2.83)		0.112 (0.008)		0.989	36.3	9.58	0.118	6.17	20.5
	DFOP**	100 (2.30)	15.5 (5.89)	1.88 (5.85)	0.094 (0.009)	0.995	35.1	6.26	0.071	5.61	22.8
15	SFO	98.7 (3.93)		0.258 (0.022)		0.982	36.1	13.2	0.164	2.68	8.91
	DFOP **	101 (2.60)	84.0 (8.42)	0.363 (0.054)	0.046 (0.030)	0.992	39.3	8.06	0.090	2.36	12.3
25	SFO	99.6 (2.05)		0.453 (0.021)		0.996	21.8	5.86	0.074	1.53	5.09
	DFOP ***	100 (0.04)	89.1 (3.32)	0.547 (0.022)	0.107 (0.028)	0.999	52.3	1.13	0.012	1.43	5.65
Matawhero silt loam											
7.5	SFO	90.2 (5.06)		0.059 (0.009)		0.954	53.7	16.1	0.199	11.8	39.3
	DFOP ***	102 (3.13)	41.6 (6.89)	0.322 (0.098)	0.030 (0.005)	0.990	42.9	7.06	0.080	7.60	58.2
15	SFO	97.5 (2.91)		0.110 (0.008)		0.988	35.0	8.87	0.110	6.29	20.9
	DFOP *	101 (2.81)	35.9 (26.6)	0.296 (0.189)	0.073 (0.024)	0.993	40.1	6.94	0.078	5.51	25.6
25	SFO	103 (3.66)		0.214 (0.017)		0.987	31.7	9.04	0.113	3.24	10.8
	DFOP[a]										
Gibsons fine sandy loam											
7.5	SFO	108 (2.31)		0.025 (0.002)		0.991	40.3	6.00	0.074	27.4	91.2
	DFOP[a]										
15	SFO	108 (4.37)		0.038 (0.005)		0.974	52.9	11.8	0.146	18.3	61.0
	DFOP[a]										
25	SFO	96.9 (2.50)		0.171 (0.010)		0.992	31.4	9.53	0.118	4.05	13.5
	DFOP***	100 (1.30)	32.4 (12.6)	0.533 (0.204)	0.118 (0.017)	0.998	21.6	4.34	0.049	3.46	16.2

*, **, *** indicate statistical difference between SFO and DFOP at $p < 0.05$, $p < 0.01$, and $p < 0.001$. [a]did not converge.

Figure 5.10. shows the degradation of E1-3S in the Matawhero soil (left panel) and the concomitant formation and degradation of E1 (right panel). The rapid degradation of E1-3S appeared temperature dependent in the Matawhero soil which was most noticeable between 8 and 72 hrs. Estrone-3-sulphate fell below the MDL at 96 and 192 hrs for 25 and 15°C, respectively. Trace amounts (< 1%) of the parent compound were detectable at 7.5°C until the end of the experiment. Fitting the DFOP model to the degradation data improved the statistical measures for the 7.5 and 15°C data as opposed to the respective SFO fits (Table 5.6.). However, for the 25°C data no solution was found with the DFOP model. The corresponding DT values clearly show the observed temperature dependence and it became evident that especially at 7.5°C E1-3S persisted with values > 10% for almost 40 hrs (Table 5.6.).

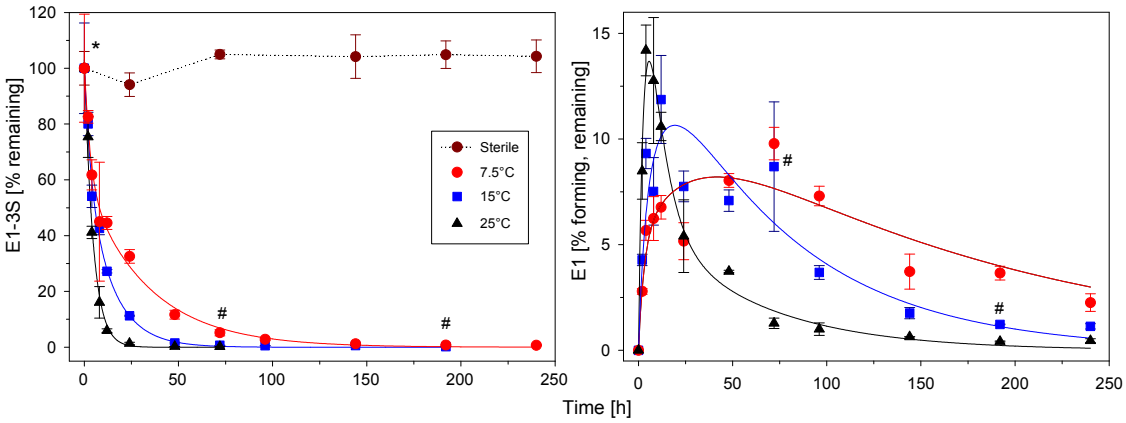


Figure 5.10. Degradation of E1-3S (left panel) and the concomitant formation and degradation of its metabolite E1 (right panel) at three incubation temperatures in the Matawhero silt loam soil. Mean values of n = 3 samples are displayed. *,# indicate n = 2 samples at 7.5 and 15°C, respectively. Solid lines represent the best fit.

Similar to the Hamilton soil, E1 was rapidly formed in the Matawhero soil during the degradation of E1-3S (Figure 5.10., right panel). However, in the Matawhero soil the influence of the temperature was more noticeable. A peak of 14% was rapidly reached at 25°C (4 hrs) and the subsequent degradation occurred rapidly as well. At 15 and 7.5°C the appearance of a peak was not distinct and the percentage of E1 formed fluctuated between 4 and 74 hrs of incubation, and after that a steady rate of degradation was observed. At the last sampling event (240 hrs) the remaining percentage of E1 accounted for 2.3% (7.5°C), 1.1% (15°C), and 0.5% (25°C), respectively. While the SFO model was found to be superior (statistical measures in Table 5.7.) to fit the 7.5 and 15°C data, fitting the DFOP model yielded an improvement in the measures for the 15°C data. The DT values reflect the observed temperature relation and suggest that E1 as a metabolite of E1-3S can persist with values of > 10% for at least 14 days (DT_{90}= 361 hrs) in the Matawhero soils at 7.5°C.

Table 5.7. Optimized parameters with one standard error (SE), statistical measures, and dissipation times for the SFO and DFOP model fitted to the measured formation and degradation data of E1 as a result of E1-3S degradation in three soils at three temperatures. Blue letters indicate best fit.

T [°C]	Model	Optimized parameters				Statistical measures				Dissipation times	
		ff_M (SE) [%]	g (SE) [%]	k_{M1} (SE) [h^{-1}]	k_{M2} (SE) [h^{-1}]	R^2_{adj}	AIC_c	err (5%)	SRMSE	DT_{50} [h]	DT_{90} [h]
Hamilton clay loam											
7.5	SFO	27.2 (4.79)		0.102 (0.022)		0.903	13.2	60.6	0.470	6.80	22.6
	DFOP[a]										
15	SFO	16.9 (3.52)		0.086 (0.027)		0.868	17.6	73.2	0.510	8.06	26.8
	DFOP[a]										
25	SFO	14.0 (0.72)		0.132 (0.009)		0.991	-22.3	15.8	0.141	5.24	17.4
	DFOP*	14.8 (0.62)	93.4 (3.57)	0.159 (0.015)	0.015 (0.010)	0.996	-24.3	7.23	0.082	n.s.	18.2
Matawhero silt loam											
7.5	SFO	12.4 (1.22)		0.006 (0.001)		0.954	12.3	41.2	0.225	108	361
	DFOP	16.1 (12.1)	26.5 (52.7)	0.215 (0.690)	0.006 (0.002)	0.943	22.8	19.3	0.220	n.s.	332
15	SFO	16.4 (1.94)		0.014 (0.003)		0.930	20.3	56.0	0.298	48.7	162
	DFOP	24.0 (9.35)	46.7 (21.7)	0.135 (0.171)	0.011 (0.004)	0.931	28.1	23.0	0.262	n.s.	154
25	SFO	27.7 (2.60)		0.068 (0.010)		0.971	11.3	40.2	0.224	10.2	33.7
	DFOP***	32.7 (2.70)	80.6 (8.80)	0.122 (0.028)	0.017 (0.008)	0.990	6.54	10.2	0.116	n.s.	41.1
Gibsons fine sandy loam											
7.5	SFO	17.2 (1.95)		0.015 (0.002)		0.956	1.11	32.7	0.227	47.5	158
	DFOP*	62.9 (31.2)	75.7 (11.6)	0.324 (0.165)	0.013 (0.002)	0.975	2.57	13.4	0.153	n.s.[b]	n.s.
15	SFO	27.7 (4.37)		0.023 (0.002)		0.980	0.39	27.4	0.164	30.0	100
	DFOP**	41.0 (8.09)	48.4 (9.77)	0.138 (0.072)	0.019 (0.002)	0.989	0.87	9.28	0.106	n.s.	84.5
25	SFO	8.84 (1.01)		0.023 (0.005)		0.933	3.6	37.3	0.338	30.0	100
	DFOP[a]										

*, **, *** indicate statistical difference between SFO and DFOP at $p < 0.05$, $p < 0.01$, and $p < 0.001$. [a]did not converge. n.s.=no solution.

The degradation of E1-3S in the Gibsons soil is illustrated in Figure 5.11. (left panel) together with the concomitant formation and degradation of E1 (right panel). Estrone-3-sulphate degraded slower in the Gibsons soil as compared to the remaining soils. However, after 24 hrs only 2.9% was left at 25°C, while at 15 and 7.5°C 37 and 59% remained. The rate of degradation for E1-3S slowed at all temperatures towards the end of the incubation (240 hrs) in the Gibsons soil, and 1.1 (7.5°C), 0.70 (15°C) and 0.21% (25°C) were still remaining, respectively. There was no substantial degradation noted in the sterile control and the values for E1-3S ranged between 90 and 100% throughout the incubation. The SFO model was sufficient to describe the 7.5 and 15°C data, while with the DFOP model no solution was found (Table 5.6.). However, for the 25°C data the DFOP model performed better as indicated by the statistical measures in Table 5.6. Similar to the two other soils the resulting DT values indicate a temperature dependence of E1-3S degradation in the Gibsons soil as well.

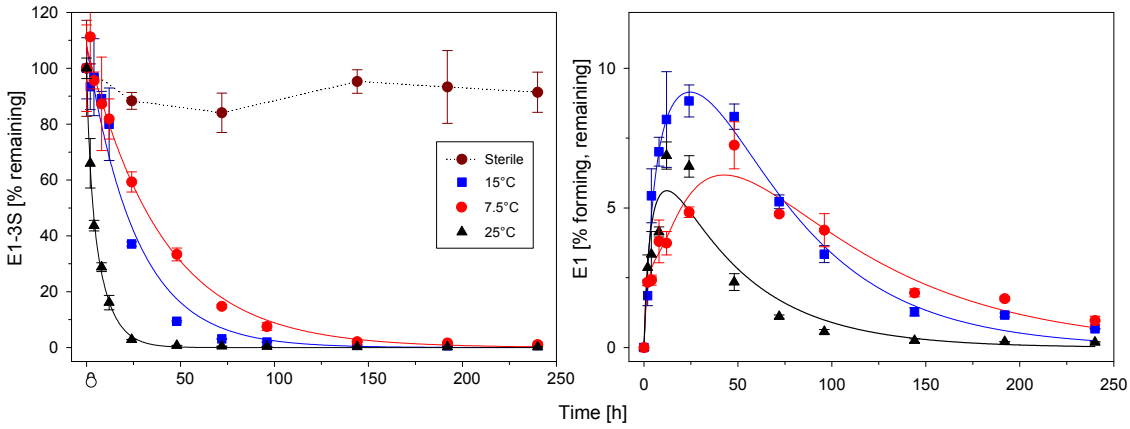


Figure 5.11. Degradation of E1-3S (left panel) and the concomitant formation and degradation of its metabolite E1 (right panel) at three incubation temperatures in the Gibsons fine sandy loam soil. Mean values of n = 3 samples are displayed. Solid lines represent the best fit.

The formation and degradation pattern of E1 in the Gibsons soil was similar to the Matawhero soil. However, the formation of a clear peak of E1 was pronounced at all three temperatures and the peak occurred at later times with decreasing temperatures (Figure 5.11., right panel). The maximum of E1 formed accounted for 7.2 (7.5°C), 8.8 (15°C), and 6.9% (25°C). The fitting procedure established the DFOP model as the favoured one to fit the 7.5 and 15°C data (Table 5.7.); however, the solution was such that a subsequent calculation of DT values was not warranted for all desired values. The SFO model was sufficient to fit the 25°C data, while fitting the DFOP model gave no converging solution. The matching DT values showed no clear trend towards temperature dependence for the persistence of E1 as a metabolite of E1-3S in the Gibsons soil.

3.2.2 17β-Estradiol-3-sulphate degradation and metabolite formation

17β-Estradiol-3-sulphate degraded fast in the Hamilton soil at the three investigated temperatures (Figure 5.12., top left panel). The % remaining decreased very fast within the first hours of incubation and E2-3S fell below the MDL after 8 (25°C), 12 (15°C), and 24 hrs (7.5°C), respectively. However, the sterile control did not show any significant reduction in E2-3S until the end of the incubation. The degradation data was fitted well with a SFO model and due to the small number of data points the DFOP model resulted in an

over-parameterisation and gave no useful results. The results of the SFO model fits are given in Table 5.8., and the matching DT values indicate the fast and temperature dependent dissipation of E2-3S in the Hamilton soil.

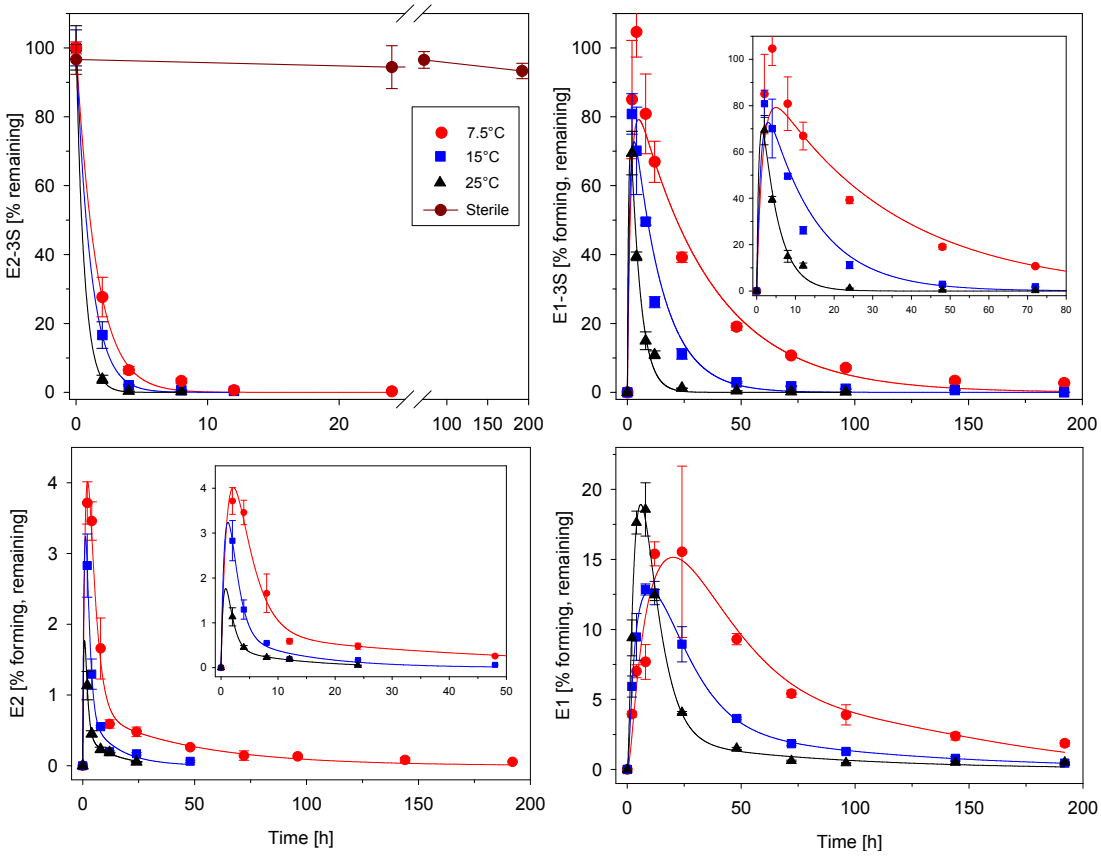


Figure 5.12. Degradation of E2-3S (top left panel) and the concomitant formation and degradation of its metabolites E1-3S (top right panel), E2 (bottom left panel), and E1 (bottom left panel) at three incubation temperatures in the Hamilton clay loam soil. Mean values of n = 3 samples are displayed. Inserts show first hrs in detail. Solid lines represent the best fit. Note the different scales for Y-axis in bottom plots.

Based on matching retention times, E2 was identified as a metabolite of E2-3S degradation and the simultaneous formation and degradation of E2 in the Hamilton soil is illustrated in Figure 5.12. (bottom left panel). The maximum of E2 formed was observed right after 2 hrs of incubation with values of 1.1% (25°C), 2.8% (15°C) and 3.7% (7.5°C). Thereafter, E2 degradation was rapid and E2 fell below the MDL at 48 and 96 hrs at 25 and 15°C, respectively. Traces (< 0.5%) were detected at 7.5°C until the end of the incubation (Figure 5.12.). Estradiol was not detected in the sterile control. Based on the ModelMaker inbuilt functions to asses the goodness of fit

it was found that for all temperatures the DFOP model fitted the data the best. The results are illustrated in Table 5.9., however, the matching DT values were calculated from the corresponding SFO fit as the DFOP model failed to provide a solution in most cases.

Table 5.8. Parameter estimates with their standard error and resulting DT_{50} and DT_{90} values for the single first-order fits to the measured degradation data of E2-3S in the Hamilton clay loam soil.

T [°C]	P_0 ± SE [%]	k ± SE [h^{-1}]	R^2_{adj}	DT_{50} [h]	DT_{90} [h]
7.5	100 ± 1.52	0.648 ± 0.025	0.998	1.07	3.55
15	100 ± 0.54	0.903 ± 0.016	0.999	0.768	2.55
25	100 ± 0.21	1.63 ± 0.028	1.00	0.424	1.41

A second metabolite of E2-3S degradation was identified as E1-3S on the basis of matching retention time in the HPLC analysis. The formation and degradation of E1-3S in the Hamilton soil is illustrated in Figure 5.12. (top right panel). While estrone-3-sulphate reached a peak, taking 4 hrs to reach a maximum of 104% at 7.5°C, a peak was attained only after 2 hrs at 15 (81%) and 25°C (69%). Following the maximum occurrence, the rate of E1-3S degradation indicated temperature dependence and fell below the MDL at 144 hrs (25°C). Trace amounts of 2.7 and 0.03% were still detectable at 192 hrs for the 7.5 and 15°C incubations. No E1-3S was detected in the sterile control. The SFO model was found to fit the data the best and the fits are also illustrated in Figure 5.12. The formation fraction was pre-determined by the equation: ff_M (E1-3S) = 1-ff_M (E2) (see Figure 5.1.), and the corresponding first-order rate constants resulted in DT_{50} values of 22.9, 8.77, and 2.89 h, for incubations at increasing temperatures.

A third metabolite was identified as E1, by means of comparing retention times against a reference standard. Its formation and degradation is displayed in Figure 5.12. (bottom right panel). Maximum formation of E1 was attained after 8 (15 and 25°C) and 24 hrs (7.5°C) and accounted for 13, 19, and 16% respectively. Estrone degraded thereafter, and the measured data in Figure 5.12. suggest the speed of degradation was temperature dependent. Using ModelMaker the DFOP model was identified as the best to fit the data for E1 at all temperatures. The resulting formation fractions and kinetic

degradation parameters are summarized in table 5.10. The DT values again originate from the corresponding SFO fits because it was not possible to calculate DT values from the DFOP model fits.

Table 5.9. Optimised parameters with their standard error for the formation and degradation of E2 as a result of E2-3S degradation in the Hamilton clay loam. DT values were calculated from the corresponding SFO fit.

T [°C]	ff_M (SE) [%]	g_M (SE) [%]	k_{M1} (SE) [h-1]	k_{M2} (SE) [h-1]	R^2_{adj}	DT_{50} [h]	DT_{90} [h]
7.5	8.04 (0.50)	94.7 (90.5)	0.352 (1.32)	0.022 (0.578)	0.986	3.66	12.2
15	8.14 (1.19)	97.0 (1.30)	0.807 (0.146)	0.079 (0.048)	0.999	1.43	4.76
25	3.70 (0.54)	94.0 (1.07)	1.00 (0.145)	0.081 (0.015)	0.999	1.92	6.37

Table 5.10. Optimised parameters with their standard error for the formation and degradation of E1 as a result of E2-3S degradation in the Hamilton clay loam. DT values were calculated from the corresponding SFO fit.

T [°C]	ff_{M13} (SE) [%]	ff_{M23} (SE) [%]	g_M(SE) [%]	k_{M1} (SE) [h^{-1}]	k_{M2} (SE) [h^{-1}]	R^2_{adj}	DT_{50} [h]	DT_{90} [h]
7.5	41.2 (82.2)	71.3 (40.1)	99.0 (2.78)	0.077 (0.043)	0.021 (0.027)	0.894	9.59	31.9
15	100 (126)	22.1 (50.9)	96.8 (15.9)	0.076 (0.133)	0.010 (0.037)	0.997	13.7	45.4
25	1.00 (104)	43.7 (8.30)	97.8 (1.57)	0.159 (0.029)	0.014 (0.013)	0.993	4.98	16.6

Figure 5.13. displays the degradation of E2-3S in the Matawhero soil (top left panel) with E2-3S being detected only up to 12 (25°C), 24 (15°C) and 48 hrs (7.5°C). However, no substantial decrease was observed in the sterile control and values were always ca 100%. The degradation kinetics were well described with the SFO model and the resulting parameter estimates are given in Table 5.11. The corresponding DT values decrease with increasing temperatures and suggest that ca 90% of E2-3S would be removed within the first day even at the lowest temperature of 7.5°C.

The degradation of E2-3S resulted in the formation of E2 as a metabolite (Figure 5.13., bottom left panel). The maximum formation was 7-8% for all three temperatures and occurred within the first 8 hrs. The metabolite (E2) degraded rapidly after that; however, continued to persist

until the end of the incubation at trace amounts (< 0.1%) at 7.5 and 15°C. In contrast, E2 fell below MDL after 96 hrs at 25°C. The fitting procedure with ModelMaker yielded particularly good fits for the 7.5 and 25°C datasets employing the DFOP (7.5°C) and SFO model (25°C) as indicated by the R^2_{adj} values (Table 5.12.). The DFOP model fits to the 15°C data gave no solution; however, the goodness of fit for the SFO model was comparably poor (R^2_{adj} < 0.900, Table 5.12.). The DT values indicate that E2 as a metabolite of E2-3S degraded to > 90% in the first 24 hrs in the Matawhero soil.

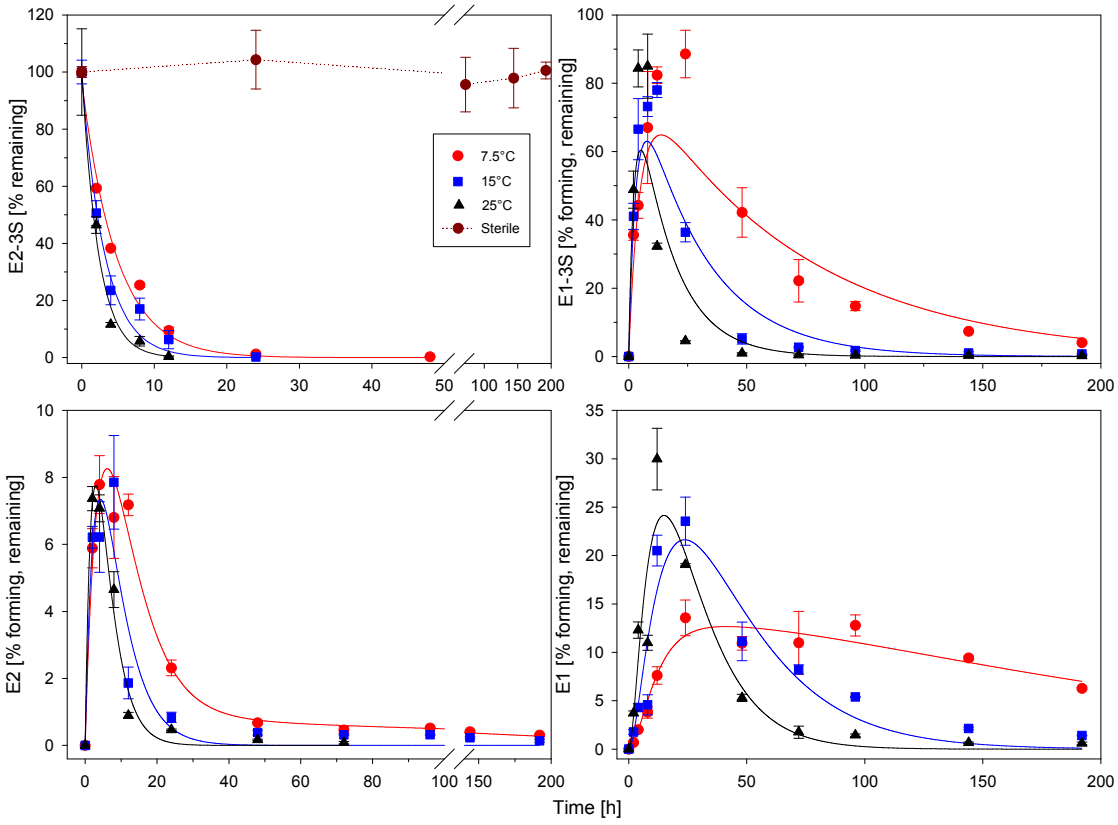


Figure 5.13. Degradation of E2-3S (top left panel) and the concomitant formation and degradation of its metabolites E1-3S (top right panel), E2 (bottom left panel), and E1 (bottom left panel) at three incubation temperatures in the Matawhero silt loam soil. Mean values of n = 3 samples are displayed. Inserts show first hrs in detail. Solid lines represent the best fit. Note the different scales for Y-axis in bottom plots.

Table 5.11 Parameter estimates with their standard error and resulting DT_{50} and DT_{90} values for the single first-order fits to the degradation data of E2-3S in the Matawhero silt soil.

T [°C]	P_0 ± SE [%]	k ± SE [h^{-1}]	R^2_{adj}	DT_{50} [h]	DT_{90} [h]
7.5	95.8 ± 4.09	0.209 ± 0.019	0.981	3.32	11.0
15	98.6 ± 5.41	0.315 ± 0.038	0.970	2.20	7.31
25	101 ± 4.50	0.436 ± 0.044	0.982	1.59	5.28

During the degradation of E2-3S, a major metabolite was identified as E1-3S and its formation and degradation pattern is shown in Figure 5.13. (top right panel). After an initial formation phase that reached a maximum of 89 (7.5°C), 78 (15°C), and 84% (25°C), respectively, E1-3S degraded and a clear influence of the temperature was noted with faster degradation at higher temperatures. The occurrence of the maximum of formed E1-3S showed also the influence of the incubation temperature and occurred after 4, 12, and 24 hrs with decreasing temperatures. The formation fraction constraint by the E2 fits (see above) was responsible for poor fitting results of the SFO model to the E1-3S data, and employing the DFOP model did not improve the results. The lines in Figure 5.13. (top right panel) present the SFO fits and the corresponding R^2_{adj} values were 0.819 (7.5°C), 0.907 (15°C) and 0.795 (25°C). From the first-order rate constants, DT_{50} values of 50.0 (7.5°C), 19.3 (15°C), and 10.7 h (25°C) were calculated for the dissipation of E1-3S as a metabolite of E2-3S.

Table 5.12. Parameter estimates with their standard error and resulting DT_{50} and DT_{90} values for the best fits to the formation and degradation data of E2 as a metabolite of E2-3S degradation in the Matawhero soil.

T [°C]	ff_{M1} (SE) [%]	g_M (SE) [%]	k_1 (SE) [h^{-1}]	k_2 (SE) [h^{-1}]	R^2_{adj}	DT_{50} [h]	DT_{90} [h]
7.5	18.4 (1.58)	97.8 (2.34)	0.133 (0.021)	0.006 (0.013)	0.971	5.98	19.8
15	15.3 (2.38)		0.173 (0.038)		0.877	4.01	13.3
25	16.3 (1.27)		0.260 (0.026)		0.975	2.67	8.9

The formation and degradation of E1 was also observed in the Matawhero soils as a result of the degradation of E2-3S. The displayed datasets (Figure 5.13., bottom right panel) indicate a temperature

dependence. The maximum formation of E1 was 13.5 (7.5°C), 23 (15°C) and 29% (25°C) of the parent compound and was this reached as a distinct peak after 24 and 12 hrs at 15 and 25°C, respectively. While E1 degraded thereafter at 15 and 25°C a plateau of ca 12% was attained at 7.5°C between 8 and 96 hrs. The SFO model was found to best describe the E1 data at all three temperatures and the fits are plotted in Figure 5.13. The corresponding parameter estimates are illustrated in Table 5.13. The estimates for the formation fractions from E2 (ff_{MI13}) and E1-3S (ff_{M23}) had high standard errors associated for the 15 and 25°C datasets for which ff_{M23} also reached its constraint (99%). The DT values calculated from the matching SFO fits indicate that E1 as a metabolite of E2-3S can persisted at values > 50% for at least 116 hrs at 7.5°C but would be degraded to > 90% in the first 24 hrs at 25°C.

Table 5.13. Optimised parameters with their standard error for the formation and degradation of E1 as a result of E2-3S degradation in the Matawhero silt loam soil. DT values were calculated from the corresponding SFO fit.

T [°C]	ff_{M13} (SE) [%]	ff_{M23} (SE) [%]	k_{M1} SE) [h^{-1}]	R^2_{adj}	DT_{50} [h]	DT_{90} [h]
7.5	71.1 (13.1)	9.71 (10.4)	0.006 (0.003)	0.976	116	384
15	1.00 (46.7)	99.0 (22.5)	0.066 (0.016)	0.808	10.5	34.9
25	3.45 (2133)	99.0 (1182)	0.100 (0.090)	0.808	6.93	23.0

The degradation of E2-3S in the Gibsons soil is illustrated in Figure 5.14. (top left panel). The degradation in the non-sterile incubations occurred fast and > 90% of the initial E2-3S were removed within the first 24 hrs. The rate of degradation was temperature dependent and E2-3S was only detected until the end of the incubation (240 hrs) at 7.5°C. While E2-3S varied between 90% and 113% in the sterile control during the first 144 hrs only 86% were recovered at 240 hrs. The SFO model resulted in good fits and was chosen to represent the data. Table 5.14. gives the corresponding parameter estimates and the matching DT values.

Table 5.14. Parameter estimates with their standard error and resulting DT_{50} and DT_{90} values for the single first-order fits to the degradation data of E2-3S in the Gibsons fine sandy loam soil.

T [°C]	P_0 ± SE [%]	k ± SE [h^{-1}]	R^2_{adj}	DT_{50} [h]	DT_{90} [h]
7.5	95.8 ± 2.24	0.090 ± 0.005	0.993	7.69	25.5
15	98.1 ± 1.69	0.213 ± 0.008	0.996	3.25	10.8
25	101 ± 1.74	0.376 ± 0.014	0.993	1.84	6.13

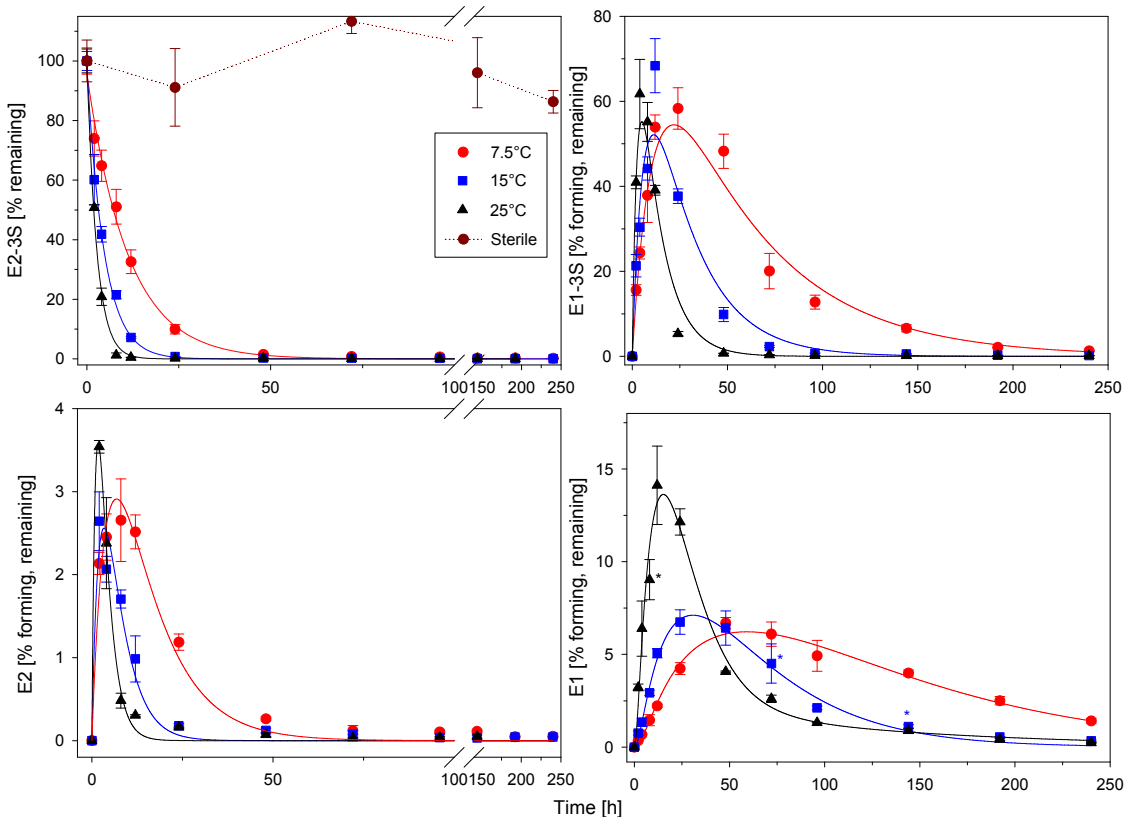


Figure 5.14. Degradation of E2-3S (top left panel) and the concomitant formation and degradation of its metabolites E1-3S (top right panel), E2 (bottom left panel), and E1 (bottom left panel) at three incubation temperatures in the Gibsons fine sandy loam soil. Mean values of n = 3 samples are displayed.*indicates n = 2 samples. Inserts show first hrs in detail. Solid lines represent the best fit. Note the different scales for Y-axis in bottom plots.

In the Gibsons soil, E2 was also identified as a metabolite and its maximum occurrence was in the first hours of the incubation and accounted for 2.6-3.6%. 17β-Estradiol degraded rapidly thereafter and was only detectable in trace amounts (< 1%) after 48 hrs. The modelling procedure resulted in excellent fits of the SFO model and the best combination of the formation fraction and the first-order rate constant is plotted in Figure 5.14. (bottom left panel) with the corresponding parameter estimates being

displayed in Table 5.15. The resulting DT values indicate a fast degradation of E2 as a metabolite of E2-3S in the Gibsons soil.

Figure 5.14. displays the formation and degradation of E1-3S, which was observed in the Gibsons soil. At higher temperatures the maximum formation of E1-3S was observed earlier, and accounted for 58% (7.5°C), 68% (15°C), and 61% (25°C). The subsequent degradation was also faster with increasing temperatures, and fitting the data with the SFO model resulted in a good representation of the data with R^2_{adj} values of > 0.9. The corresponding rate constants were 0.019, 0.043, and 0.088 h^{-1} for 7.5, 15, and 25°C, respectively.

Table 5.15. Parameter estimates with their standard error and resulting DT_{50} and DT_{90} values for the best fits to the formation and degradation data of E2 as a metabolite of E2-3S degradation in the Gibsons soil.

T [°C]	ff_{M1} (SE) [%]	k_1 (SE) [h^{-1}]	R^2_{adj}	DT_{50} [h]	DT_{90} [h]
7.5	13.8 (1.14)	0.221 (0.032)	0.973	3.14	10.4
15	10.0 (1.23)	0.399 (0.060)	0.947	1.74	5.77
25	15.0 (1.19)	0.826 (0.066)	0.992	0.84	2.79

A third metabolite was identified as E1 and its formation and degradation is shown in Figure 5.14 (bottom right panel). With increasing temperatures more E1 was formed and the subsequent degradation was faster at higher temperatures. The modelling exercise resulted in the SFO model being the best to describe datasets for 7.5, and 15°C while the 25°C dataset was predicted better by the DFOP model. Table 5.16. presents the parameter estimates together with the resulting DT values. The estimated formation fractions had lower standard errors compared to the results for the Matawhero soil and the DT values indicate the observed temperature dependence of the E1 persistence in the Gibsons soil.

Table 5.16. Optimised parameters with their standard error for the formation and degradation of E1 as a result of E2-3S degradation in the Gibsons fine sandy loam soil. DT values at 25°C were calculated from the corresponding SFO fit.

T [°C]	ff_{M13} (SE) [%]	ff_{M23} (SE) [%]	g (SE) [%]	k_{M1} (SE) [h^{-1}]	k_{M2} (SE) [h^{-1}]	R^2_{adj}	DT_{50} [h]	DT_{90} [h]
7.5	26.9 (4.61)	14.4 (2.12)		0.013 (0.002)		0.977	53.3	177
15	27.2 (5.19)	13.6 (2.20)		0.026 (0.003)		0.982	26.7	88.6
25	20.9 (19.0)	34.5 (10.5)	97.6 (3.40)	0.069 (0.024)	0.008 (0.014)	0.966	13.5	45.0

3.3 Arylsulphatase activity

Figure 5.15. displays the AryS activities in the three soils at 7.5, 15 and 25°C. A clear temperature dependence of the enzyme activity was observed and the activities at each incubated temperature followed an order: Hamilton > Matawhero > Gibsons. The maximum AryS activity was 58.0 µg 4-NP $g^{-1}h^{-1}$ in the Hamilton soil 25 °C, and the minimum value was 2.92 µg 4-NP$g^{-1}h^{-1}$in the Gibsons soil at 7.5 °C. Temperature dependence of AryS activity can be described by the Arrhenius equation (Elsgaard and Vinther, 2004), which can be expressed in a linear form as follows:

$$\ln k = \ln A \frac{E_a}{RT} \tag{5.14}$$

where k is the rate constant and in this case the activity, A is a regression constant, R is the gas constant (8.314472 J $K^{-1}mol^{-1}$), and E_a is the apparent activation energy of the reaction. Equation (5.14) described the temperature dependence of the AryS activity well with $R^2 \geq 0.989$ for all three soils. The activation energies were calculated from the slope of the regression lines and were 50.4, 49.2 and 46.1 kJ mol^{-1} for the Hamilton, Matawhero, and Gibsons soils, respectively.

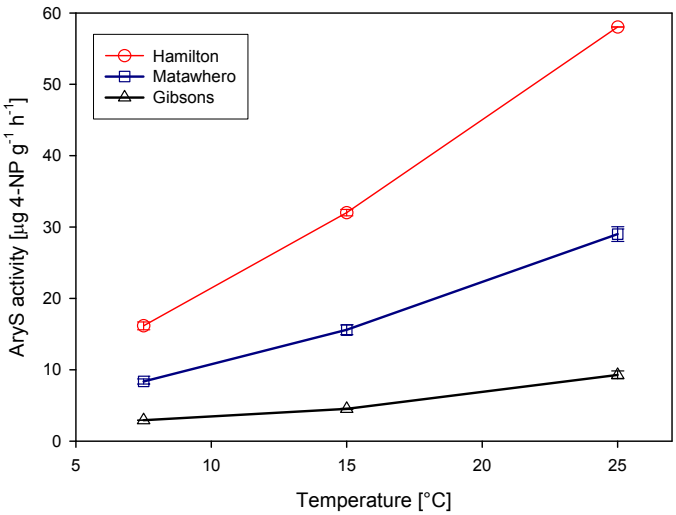


Figure 5.15. Arylsulphatase activity of the three soils at the three incubation temperatures. Standard deviations of duplicate samples lay within symbols.

3.4 Influence of orthophosphate on the arylsulphatase activity and the degradation of E2-3S in the Hamilton clay loam soil

The AryS activity in the inhibited soil accounted for only 54% of the non-inhibited soil. Accordingly, degradation of E2-3S in the inhibited soil was slower than in the non-inhibited soil and the matching first order rate constant differed by a factor of 3 (Figure 5.16. and Table 5.17.). The persistence of E2-3S was extended from 24 hrs in the non-inhibited soil to 72 hrs in the inhibited soil. Furthermore, the formation and persistence of the metabolites E1-3S and E1 were more pronounced in the inhibited assay.

Figure 5.16. illustrates the importance of the arylsulphatase in the initial degradation of the sulphate conjugates. The metabolite E1-3S reached its peak 2 hours after incubation started in the non-inhibited assay and the peak was reached after 4 hours under inhibition. The maximum percentage of E2-3S converted to E1-3S was 95% and 42% in the inhibited and non-inhibited assay, respectively. A similar pattern was observed for E1, the metabolite of E1-3S and E2. However, the maximum percentage of conversion was higher in the non-inhibited soil (52%) but depleted rapidly after 2 hrs (Figure 5.16., right panel). In the inhibited soil, E1 approached a plateau accounting for nearly 40% over the incubation period of 48–72 hrs (Figure 5.16, left panel); however, E1 continued to degrade with ca 20% loss accounted for by the last

sampling time (144 h). Estradiol (E2), the second metabolite of E2-3S, was detected only in minor proportions with values of 0.9% and 0.8% in inhibited and non-inhibited assays, respectively, but no residues were found after 24 hrs in both assays.

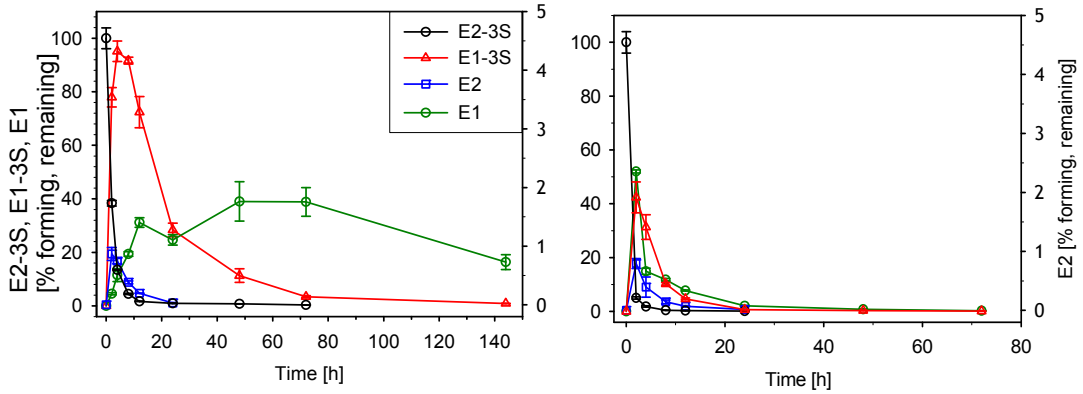


Figure 5.16. Degradation of E2-3S in Hamilton clay loam at 25°C with (left panel) and without (right panel) inhibition of arylsulphatase. Note scale differences in X-axis.

Table 5.17. Parameter estimates of single first-order fits and DT_{50} and DT_{90} values for the degradation of E2-3S in Hamilton clay loam at 25°C with and without inhibition of arylsulphatase.

Assay	P_0 ± SE [%]	k ± SE [h^{-1}]	R^2_{adj}	DT_{50} [h]	DT_{90} [h]
inhibited	100 ± 1.30	0.482 ± 0.014	0.998	1.44	4.78
non-inhibited	100 ± 0.82	1.48 ± 0.079	0.999	0.468	1.56

3.5 ER–CALUX® results

During the degradation experiment involving E2-3S at 15°C, samples were also extracted to measure the behaviour of the estrogen activity, or estrogenicity, at various sampling times using ER-CALUX® assay (Legler *et al.*,1999). The result of the assay was expressed as 17β-estradiol equivalent (EEQ) [µg g^{-1}]. An additional measure was introduced to assess the proportional estrogenicity of the total detected estrogens, i.e. the relative estrogenicity (*RE*) [%], and was calculated as per the following equation:

$$RE = \frac{EEQ}{\sum E2-3S, E2, E1-3S, E1} \cdot 100 \qquad (5.15)$$

Figure 5.17. illustrates the results for the Hamilton soil. The EEQ corresponded well with the occurrence of E2 and E1 over time and reached a

maximum of 0.8 µg g^{-1} after 8 hrs of incubation. Thereafter, EEQ decreased but estrogenicity remained detectable until the end of the incubation at concentrations of < 0.1 µg g^{-1}. The *RE* increased over time and at 192 hrs the *RE* measure suggested that about 90% of the remaining estrogens in the soil were estrogenically active.

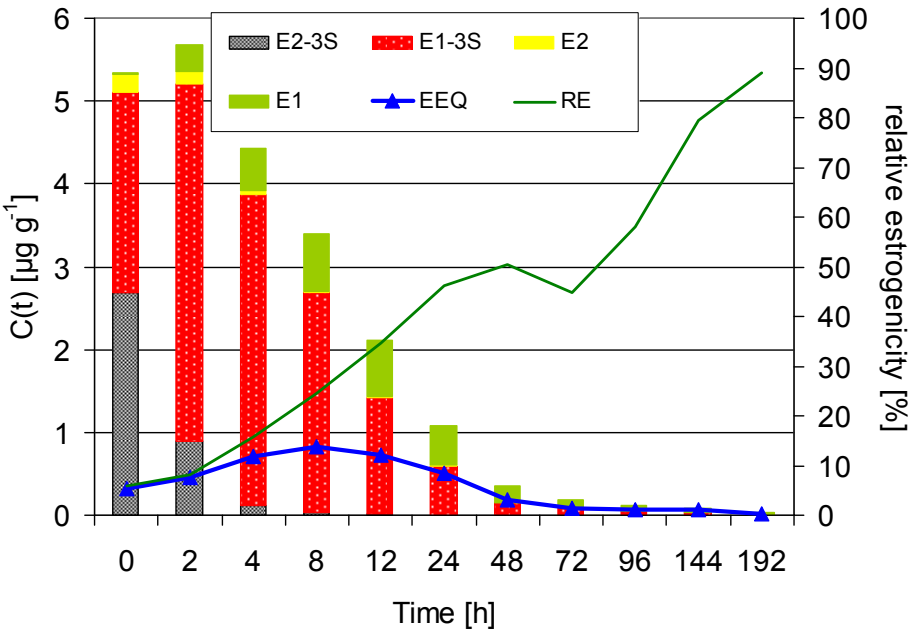


Figure 5.17. Absolute concentration of the estrogens, the corresponding 17ß-estradiol equivalents (EEQ), and the relative estrogenicity (RE) of the estrogens in the Hamilton clay loam soil.

The results for the Matawhero soil are given in Figure 5.18. The EEQ value rapidly reached 0.9 ng g^{-1} after 2 hrs of incubation and dropped to ca 0.5 ng g^{-1} at 4 and 8 hrs. A maximum of 1.0 ng g^{-1} was reached at 12 hrs followed by a subsequent slow decrease. At 192 hrs the EEQ accounted for 0.24 ng g^{-1}. The RE was < 50% in the first 24 hrs but rapidly increased thereafter and its final value exceeded 350%, implying that the measured estrogenicity could not be solely explained by the detected estrogens in the soil.

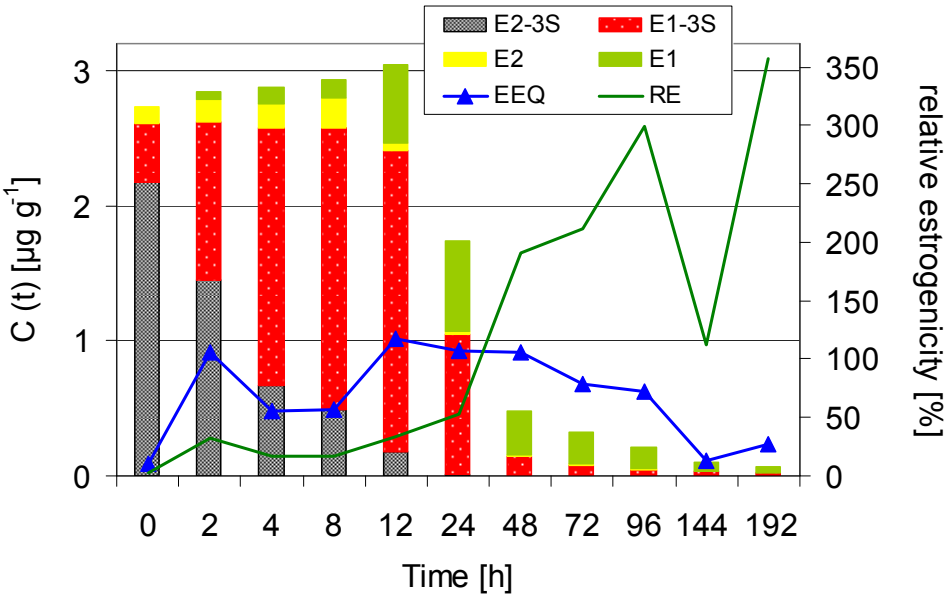


Figure 5.18. Absolute concentration of the estrogens, the corresponding 17β-estradiol equivalents (EEQ), and the relative estrogenicity (RE) of the estrogens in the Matawhero silt loam soil.

The results of the ER-CALUX® analysis for the Gibsons soil are illustrated in Figure 5.19. The EEQ values slowly increased over the first 12 hrs to a value of 0.8 µg g^{-1}. A second maximum was observed at 48 hrs (0.7 µg g^{-1}) after which the EEQ slowly decrease to a value of 0.02 µg g^{-1} at 192 hrs. The *RE* measure indicated that during the first 24 hrs < 20% of the detected estrogens contributed towards estrogenicity. A steep increase from 24 to 96 hrs reaching a maximum of 123%, indicated that the estrogenicity in this period was higher than what could be explained by the detected estrogens. The *RE* measure, however, dropped to 48% at 192 hrs.

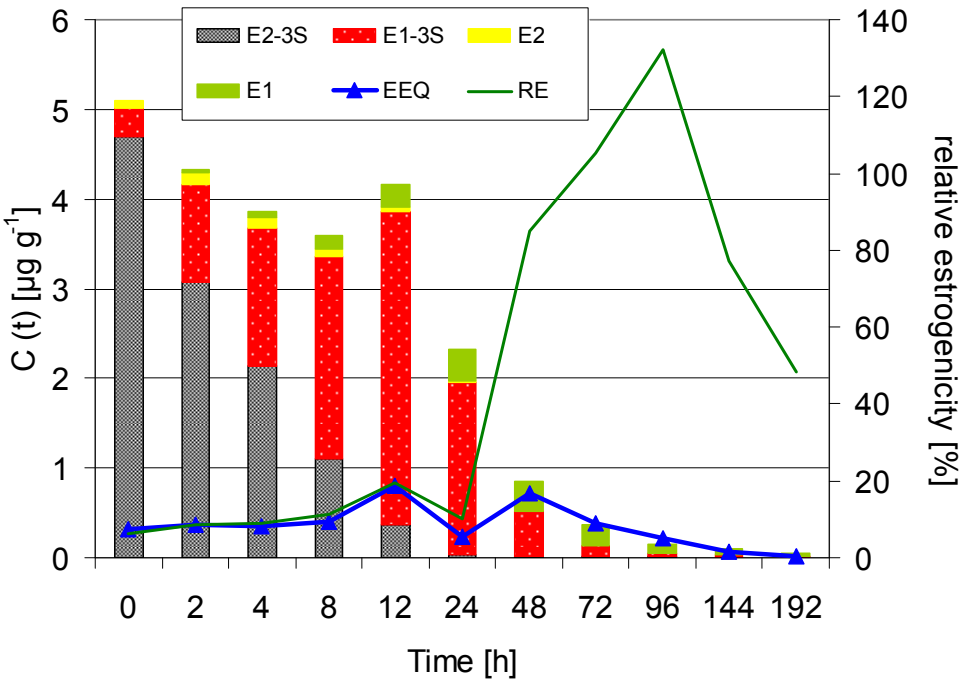


Figure 5.19. Absolute concentrations of the estrogens, the corresponding 17ß-estradiol equivalents (EEQ), and the relative estrogenicity (RE) of the estrogens in the Gibsons fine sandy loam soil.

3.6 Relationship between degradation rate constants and soil properties

In order to elucidate the driving factors for the estrogen degradation in the investigated soils the first-order rate constants (*k*) for each degradation data set were analysed for their correlation with the soil properties. Table 5.18. presents the correlation between the first-order degradation rate constants (*k*) of the free estrogens E1 and E2 with the clay and organic carbon (OC) content and with the microbial biomass carbon (MBC). High correlations of *k* with OC and MBC were found; however, the correlation with OC was significant only for E2 degradation at 15°C, while the correlation with MBC was significant for the degradation of both compounds at all three temperatures.

The correlation of the first-order degradation rate constants for the estrogen sulphates E1-3S and E2-3S with the soil properties is displayed in Table 5.19. High correlations were obtained for OC, MBC, clay and the Arylsulphatase activity (AryS). The correlations of *k* with MBC and OC were significant in the three soils for all three temperatures. In a similar way, *k*

was significantly correlated to AryS for all soil-temperature conditions except one (E2-3S, 25°C). Though the correlation of *k* with the clay content was high, a significance was only recorded for E1-3S at 7.5°C.

Table 5.18. Correlation matrix for single first-order degradation rate constants of E1 and E2 degradation with soil properties.

	Estrone (E1)			Estradiol (E2)		
	7.5°C	15°C	25°C	7.5°C	15°C	25°C
clay	0.766	0.795	0.759	0.751	0.807	0.753
OC	0.929	0.946	0.926	0.921	0.952*	0.922
MBC	0.985*	0.992**	0.983*	0.981*	0.994**	0.981*

*, **indicate significance at $p < 0.1$ and $p < 0.05$ (n = 3).

Table 5.19. Correlation matrix for single first-order degradation rate constants of E1-3S and E2-3S degradation with soil properties.

	Estrone-3-sulphate (E1-3S)			Estradiol-3-sulphate (E2-3S)		
	7.5°C	15°C	25°C	7.5°C	15°C	25°C
AryS	1.00***	0.996**	0.963*	0.977*	0.963*	0.932
clay	0.964*	0.943	0.865	0.895	0.863	0.811
OC	0.999**	1.00***	0.979*	0.990**	0.978*	0.954*
MBC	0.968*	0.983*	1.00***	0.998**	1.00**	0.995**

*, **,***indicate significance at $p < 0.1$, $p < 0.05$, and $p < 0.01$ (n = 3).

The AryS activity, measured at the three temperatures, was significantly correlated to the MBC and OC content of the three soils and the soil properties MBC and OC content were also significantly correlated (Table 5.20.).

Table 5.20. Correlation of AryS activity with soil properties.

	AryS				
	7.5°C	15°C	25°C	clay	OC
clay	0.970*	0.968*	0.968*		
OC	0.997**	0.998**	0.998**	0.949	
MBC	0.962*	0.964*	0.964*	0.866	0.980*

*, **indicate significance at $p < 0.1$ and $p < 0.05$ (n=3).

4 DISCUSSION

4.1 Persistence of estrogens and estrogen sulphates

The free estrogens E1 and E2 as well as their sulphate conjugates E1-3S and E2-3S were rapidly removed without a lag phase in the three investigated agricultural top-soils under aerobic incubation. In fact, with the exception of E1-3S in the Gibsons soil at 7.5°C, > 50% of the initial amount of all the compounds degraded within the first 24 hrs of incubation. In general, E2 degraded faster than E1, and E2-3S degraded faster than E1-3S irrespective of the soil and the compounds degraded faster with increasing temperatures.

Elevated degradation of E2 as opposed to E1 was reported by Colucci *et al.* (2001) for a microcosm study involving three agricultural soils with a similar range of physicochemical properties to the soils investigated in the present study. The temperature used by Colucci *et al.* (2001) was 30°C, and despite lower temperature, the resulting DT_{50} values in the present study were similar (Table 5.3. and 5.4.) accounting for < 24 hrs. The higher value obtained for E1 in one soil by Colucci *et al.* (2001) (40.8 h in the sandy loam, calculated from the presented rate constant) might be a result of the different moisture contents used in the two studies. The soils in the present study were adjusted to 60% MWHC, while the moisture content in Collucci *et al.* (2001) was 13%. The authors also showed that E2 degradation was faster with increasing moisture content in that particular soil; a relationship that would likely apply to E1 degradation as well. Increased degradation of E2 with increases in moisture content in a silt loam soil was also shown by Xuan *et al.* (2008). The corresponding DT_{50} value of 4.08 h for the degradation of E2 at 25°C (15% moisture content) is in good agreement with the values found in the present study, considering the different moisture contents between the studies. Fast degradation of E2 was also reported by Ying and Kookana (2005) in an agricultural loam soil, and even though the authors stated a DT_{50} value of 3 days, examination of their data revealed that E2 degraded > 50% within the first day of the incubation, similar to the rapid degradation of E2 observed in the present study.

In contrast, Lucas and Jones (2006) found E2 and E1 to possess similar degradation behaviour when studying their mineralization by measuring $^{14}CO_2$ release form soil fortified with radiolabeled estrogens. Moreover, DT_{50} values reported by Lucas and Jones (2006) were considerably higher than in the present study with values ranging from 1.5 to 46 days, and from 1.3 to 34 days for E1 and E2, respectively. These higher DT_{50} values can be explained by the way the hormones were added to soil, i.e. in combination with the amendment of manure or urine solutions. These matrices possibly comprise compounds that could slow estrogen degradation. For instance, readily available carbon sources for microorganisms (e.g., lipids or sugars) that could be a plausible explanation for the observed lag phase in some of the incubation studies conducted by Lucas and Jones (2006). Furthermore, the presence of veterinary antibiotics in livestock wastes (Kahn *et al.*, 2008a) is also likely to increase the persistence of estrogen in agricultural soils (Chun *et al.*, 2005; Xuan *et al.*, 2008). Large DT_{50} values for E2 mineralization were suggested by Stumpe and Marschner (2007). Investigating the mineralization of E2 in a soil receiving long-term waste water irrigation, a first-order rate constant of 0.0024 d^{-1} was reported by Stumpe and Marschner (2007), which corresponds to a DT_{50} value of 298 days. However, literature data on the effects of manure amendment on estrogen degradation are not consistent. For instance, Jacobsen *et al.* (2005) reported faster E2 mineralization in manure-amended soils than in soils without addition of manure. Although type and age of manure and urine may play a key role in hormone mineralization and may either hinder or facilitate hormone degradation (Lucas and Jones, 2006), information is still scarce and this needs to be investigated further.

To date, the available literature almost exclusively reports half-life (DT_{50}) values for estrogens. However, given that estrogens exhibit their potentially adverse biological activity also at trace concentrations (ppt), the calculation and presentation of DT_{90} in combination with DT_{50} values might give a more comprehensive representation of the compounds' persistence in soils, especially when their degradation does not follow a first-order process. From the present study it can be ascertained, for instance, that E1 persisted for longer than 14 days at 7.5°C in the Matawhero soil (DT_{90} = 356 h, Table

5.3.) in contrast to the corresponding DT_{50} value suggesting a fast degradation.

In summary, it appears that free estrogens rapidly degrade in soils under aerobic conditions when they are added as a single compound. However, the estrogens E2 and E1 seem to persist longer when applied to soils in a particular exposure matrix such as manure or urine (including urine surrogates) (Lucas and Jones, 2006) or when they are applied to soils with a pollution history such as waste water irrigation (Stumpe and Marschner, 2007). Moreover, research has shown that anaerobic conditions can considerably prolong the persistence of estrogens in soils and sediments (Ying and Kookana, 2005; Fan *et al.*, 2006; Sarmah and Northcott, 2008). The microcosms in the present study were regularly aerated and oxygen depletion can be excluded.

The degradation of estrogen sulphates in agricultural soils has so far received only theoretical consideration in the literature and generally it has been assumed that estrogen conjugates would be easily and readily degraded under natural conditions in soils (e.g., Hanselman *et al.*, 2003; Khanal *et al.*, 2006). The present study substantiated these assumptions and the calculated DT_{50} values imply that both estrogen sulphates E2-3S and E1-3S degraded to > 50% within the first 12 hrs after fortification. An exception was E1-3S degradation in the Gibsons soil where DT_{50} values accounted for 27.4 h (7.5°C) and 18.3 h (15°C). In comparison, DT_{50} values < 24 h can be calculated from the data of Okayasu *et al.* (2005) for estrogen sulphate degradation in an activated sludge microcosm. Higher DT_{50} values (2.5 days) were reported by D'Ascenzo *et al.* (2003) investigating the degradation of E2-3S and E1-3S in a waste water microcosm, where a lag-phase was observed at the start of the incubation. Similar to the argumentation above, the lag-phase might have occurred as a result of easier available substrates that were available in the waste water for the degrading microorganisms, even though the authors argue that the scarcity of arylsulphatases has caused the lag-phase. In the present study, the estrogen sulphates were added to the soils with water, and it can be expected that the primary exposure matrix animal urine would contain a

plethora of other chemicals, e.g., antibiotics and organic salts, that would slower the degradation of the compounds under field conditions.

4.2 Metabolite formation and persistence

The degradation of E2 consistently resulted in the formation of E1 across the three investigated soils and temperatures. Furthermore, E1 was also consistently identified as a metabolite of E1-3S degradation, and all three compounds E2, E1-3S and E1 were detected as metabolites in the experiments investigating E2-3S degradation.

It is well established that E1 is a major metabolite of E2 (e.g., Lee *et al.*, 2001; Chun *et al.*, 2005). Nevertheless, most recent studies studying E2 degradation in soil microcosms used radiolabeled compounds and inferred compound degradation from captured $^{14}CO_2$ without identifying degradation metabolites (Casey *et al.*, 2003; Lucas and Jones, 2006; Stumpe and Marschner, 2007). Colucci *et al.* (2001) quantified the formation of E1 as a metabolite of E2 and reported a transient accumulation of E1 with a maximum 6 hrs after incubation in one soil. While E1 was not detected during the subsequent sampling events in the respective soil, in two other soils previously spiked with E2, 100% of the measured radioactivity was attributed to E1 after 3 days of incubation (Colucci *et al*, 2001). Likewise, Chun *et al.* (2005) and Jacobsen *et al.* (2005) observed the formation, accumulation and subsequent degradation of E1 as a result of E2 degradation. The formation of E1 in the present study was consistent for all soils and temperatures, and while the maximum occurrence appeared to be temperature dependent, the results were not conclusive. However, the highest percentage of E1 formed was detected at 25°C in the Hamilton and Matawhero soils. In general, 13-49% (Hamilton soil), 26-45% (Matawhero soil), and 33-56% (Gibsons soil) of the parent compound E2 were identified as E1, which is consistent with other recent reports (Chun *et al.*, 2005, Jacobsen *et al.*, 2005; Ying and Kookana, 2005, Xuan *et al.*, 2008) where the metabolite E1 did not account for 100% of the parent compound. The fact that E1 was detectable in the investigated soils may also substantiate a faster degradation process of E2 (Chun *et al.*, 2005; Xuan *et al.*, 2008). Estrone was also investigated in this study as a parent compound and therefore a comparison of DT values is warranted. It is

apparent from Table 5.5., that the DT values for E1 as a metabolite were prolonged as opposed to the DT values for its dissipation as a parent compound (Table 5.3.). The differences was most noticeable at 7.5°C and the corresponding DT_{50} values were 3.9, 6.3, and 3.8 times higher in the Hamilton, Matawhero, and Gibsons soils, respectively. The prolonged persistence might be a result of a Michaelis-Menten enzyme-mediated degradation kinetic which usually decreases with decreasing substrate concentration (Khan *et al.*, 2008b), and the concentration of E1 in the soils was lower as a metabolite than as a parent compound.

Likewise, the persistence of E1 was prolonged when it is degraded as a metabolite of E1-3S (Table 5.7.); however, it was detected at lower percentages as opposed to the E2 metabolite with maximum values ranging from 6.8-11%, 6.7-14%, and 6.9-8.8%, in the Hamilton, Matawhero, and Gibsons soils, respectively. Apart from the Matawhero soil at 7.5°C, where E1 was degraded faster as a metabolite, the DT_{50} values were 1.4-5.9 times higher than the corresponding values for E1 degradation as a parent compound. Unlike above, no clear trend with respect to the investigated soils or temperatures was observed. This is the first time E1-3S was investigated in the soil environment, and comparison is therefore difficult. However, research in engineered systems has shown that E1 concentrations in wastewater treatment plants were higher than expected from degradation rate constants, and a number of authors have attributed these elevated concentrations of E1 to the degradation of estrogen sulphates subsequently releasing free estrogens (e.g., Ternes *et al.*, 1999, D'Ascenzo *et al.*, 2005; Schlüsener and Bester, 2008).

The persistence of E1 as a metabolite of E2-3S was not conclusively prolonged across all the soils and temperatures, however, E1 had 1.3-2.9, and 3.8-10 times higher DT_{50} values in the Gibsons and Hamilton soil. A considerable extension of DT_{50} values was observed for the metabolite E1-3S as opposed to its persistence as a parent compound. The corresponding DT_{50} values were 4.4-5.0, 6.7-14.8, and 6.8-21.3 times higher in the Gibsons, Matawhero, and Hamilton soils, respectively. The persistence of the metabolite E2 was consistently prolonged in the Hamilton soils (1.2-3.5

times), but was shorter in the Gibsons soil (ca 0.5 times) while no steady trend was observed in the Matawhero soil.

These results show that the degradation behaviour of estrogens and estrogen sulphates constitutes a complex system. In particular, the results imply that the persistence of the free estrogens in agricultural soils receiving animal wastes can not solely be assessed by studying their degradation as parent compounds, but one must also consider their formation from estrogen sulphates, which play a crucial role in the excretion pathways of livestock. Possible biological reasons for these findings are discussed in the following section.

4.3 Mechanisms of (bio)-degradation

The degradation of E2 and E1 in the soil environment is mainly associated with biological activity and in most cases limited or zero degradation was observed in sterile controls (e.g. Jacobsen *et al.*, 2005, Ying and Kookana, 2005; Xuan *et al.*, 2008). There are several lines of evidence that support biological mediated degradation being the cause of E2 and E1 degradation in the present study as well. First, the sterile controls for E1 and E2 showed no substantial loss during the first ca 300 hrs of incubation. An exception was the sterile control for E1 in the Gibsons soil. The constant depletion in that particular microcosm, however, can be attributed to the problems encountered during the spiking procedure (see Chapter III for details). The sterile controls were all sampled together and the fact that estrogen concentrations started to decrease after one particular sample event (336 h) suggest that a contamination had occurred that triggered a biological degradation process. Second, the first-order rate constants for E2 and E1 degradation were all significantly positively correlated to the soils microbial biomass carbon (MBC) (Table 5.18.). No other soil property showed consistent significant correlations. These findings strongly support the microbial biomass as primary degradation domain for E2 and E1 in the investigated soil which is in agreement with Xuan *et al.* (2008) who reported higher degradation rates with increasing proportion of non sterile soil in their microcosms. Third, the DT_{90} values exclusively decreased with increasing temperatures indicating that biologically mediated degradation had occurred. These observations are

consistent with literature reports. For instance, Colucci *et al.* (2001) and Xuan *et al.* (2008) observed increased estrogen degradation with increases in the incubation temperatures and both studies suggested the biological activity in the soils as the major driver for estrogen degradation.

In general, it is believed that the degradation of aromatic compounds such as estrogens occurs in the co-metabolism of microbes providing there are easier degradable carbon sources available (Stumpe and Marschner, 2007). However, not all biological degradation reactions are intra-cellular and exo-enzymes might contribute towards estrogen degradation. Therefore, two major degradation mechanisms for free estrogens are plausible. First, high log K_{ow} values (Table 2.2.) allow passive diffusion of free estrogen into microbial cells (e.g., Casey *et al.*, 2005; Stumpe and Marschner, 2007) where they are possibly eliminated by internal enzymes. Internal cytochrome P-450 oxigenases (Lucas and Jones, 2006) would be responsible for the oxidation of E2 to E1 or could hydroxylate E2 to estriol (E3) which was observed as a minor metabolite of E2 in soils (Casey *et al.*, 2005; Xuan *et al.*, 2008), though not in the present study. Furthermore, internal transferase enzymes can be expected to deactivate estrogens through addition of alkyl groups which would contribute to compound dissipation. Second, soils comprise a vast group of dehydrogenase enzymes that are, to a large extent, present as exo-enzymes and associated with organic matter and clay particles (Killham and Staddon, 2002). These enzymes are expected to be responsible for E2 oxidation to E1 and a direct correlation of E1 concentrations with dehydrogenase enzyme activity has been reported (Chun *et al.*, 2005).

The results from the present study suggest that a combination of both pathways is responsible for estrogen degradation. Because E1 never constituted 100% of its parent compound E2, oxidation likely occurred but a proportion of E2 was likely converted intra-cellular to other (non-)steroidal compounds. Increasing non-extractable radioactivity was reported for radiolabeled E2 degradation in soils (e.g., Colucci *et al.*, 2001; Lucas and Jones, 2006), which supports the assumption that a proportion of E2 was incorporated into the microbial biomass without conversion to E1. On the other hand, with a temperature increase from 15 to 25°C, more E1 was

detected as a metabolite of E2 in the Hamilton and Matawhero soils. This may be explained by higher extra-cellular dehydrogenase activity at the higher temperature, which consequently results in less E2 available for intra-cellular pathways that do not involve the formation of E1. While there is little evidence in the literature to support this hypothesis, the soils MBC could serve to indirectly substantiate it. The soils MBC was the lowest in the Gibsons soil, and even though it was not investigated, it can be assumed that the dehydrogenase activity is proportional to the soils OC (Killham and Staddon, 2002) and possibly also to MBC. Therefore, as observed, the effect of an increased enzyme activity would be most noticeable in the Hamilton soil followed by the Matawhero and Gibsons soils.

The degradation of estrogens sulphates has not been investigated before in the soil environment, and the present study was unambiguously able to substantiate the often proposed assumption that arylsulphatase (AryS) enzymes are responsible for the cleavage of the conjugates and the release of free estrogens. Three lines of evidence support the necessity of AryS for estrogen sulphate degradation: (I) The sterile controls for both compounds, E2-3S and E1-3S, in the three soils did not show any substantial loss of the compounds and metabolites were never detected (Figures 5.12.-5.14.). Typical soil AryS enzymes start to denature at ca 60°C and their activity extinguishes beyond 75°C (Elsgaard and Vinther, 2004). Therefore, autoclaving the soils three times at 121°C had denatured AryS enzymes, and the soils consequently lost their ability to de-conjugate estrogen sulphates which implies that AryS activity is the major driver of estrogen sulphate degradation. (II) The first-order degradation rate constants of estrogen sulphates were all but one (E2-3S, 25°C) significantly correlated to the AryS activity at the respective temperature (Table 5.19.), highlighting the importance of AryS activity for the conversion of estrogen sulphates. Moreover, the consistent significant correlation of AryS activity at the three investigated temperatures with the soil properties OC, clay content and MBC (Table 5.20.), indicates AryS enzymes were of microbial origin and associated with the organic matter and clay domain of the soils which was also reported before (Speir and Ross, 2002). (III) The incubation with orthophosphate, an

irreversible inhibitor of AryS, decreased the AryS activity of the Hamilton soil by 47% and consequently prolonged the presence of E2-3S and its major metabolite E1-3S in the inhibited soil (Figure 5.16.) underlining the importance of AryS activity for estrogen sulphate degradation.

Lucas and Jones (2006) proposed the existence of estrogen sulphate membrane transporters that facilitate the uptake of estrogen sulphates into microbial cells. Results from the present study may indirectly substantiate that hypothesis. The relatively high formation of E1-3S (60-100%) as a metabolite of E2-3S suggests that the oxidation of E2-3S to E1-3S likely occurred extra-cellular. However, the fact that comparably more E1 was detected during E2-3S degradation than during E1-3S degradation suggests that a proportion of E1-3S was transferred into cells, maybe by membrane transporters, and transformed via a pathway excluding the formation of E1. In contrast, fast external deconjugation of E2-3S by AryS enzymes produced E2 as a metabolite, which subsequently contributed to the extra-cellular formation of E1 explaining the higher detected percentage of E1.

4.4 Model performance

4.4.1 Parent compound degradation

All but one (Matawhero soil, 7.5°C) datasets of the E1 degradation experiment were fitted well by the SFO model with R^2_{adj} values ≥ 0.960 (Table 5.3.). Both parameter estimates of the SFO model, P_0 and k_1, had low standard errors and the estimated rate constants reflected the observed temperature dependence, i.e. faster degradation at higher temperatures. However, the estimates for P_0 deviated from 100%, which were pronounced for the Matawhero and Gibsons datasets in particular. Applying the DFOP model gave a solution for all datasets, and in all but one (Matawhero soil, 25°C) case the DFOP model improved the goodness of fit parameters (Table 5.3.). For instance, the err-% of the χ^2 test was reduced as opposed to the SFO model fits and accounted for values < 15%, a threshold value recommended in FOCUS (2006). Likewise, R^2_{adj} values, SRMSE scores and the ANOVA all favoured the DFOP model for the respective datasets, indicating its better performance. As stated in section 2, the model with the lower AIC_c score is

more likely to represent the investigated dataset. However, one might be interested in the associated probability to justify the use of a more complex model (DFOP, model B) over a simpler model (SFO, model A). It is therefore useful to calculate the difference between the two AIC_c scores (Burnham and Anderson, 2002):

$$\Delta AIC_c = AIC_c^B - AIC_c^A \tag{5.16}$$

The associated percentage chance that model B is more likely than model A [P(B>A)] can then be computed as:

$$P(B > A) = \frac{e^{-0.5\Delta AIC_c}}{1 + e^{-0.5\Delta AIC_c}} \times 100 \tag{5.17}$$

Figure 5.20. depicts such a relationship for $-10 \leq \Delta AIC_c \geq 10$.

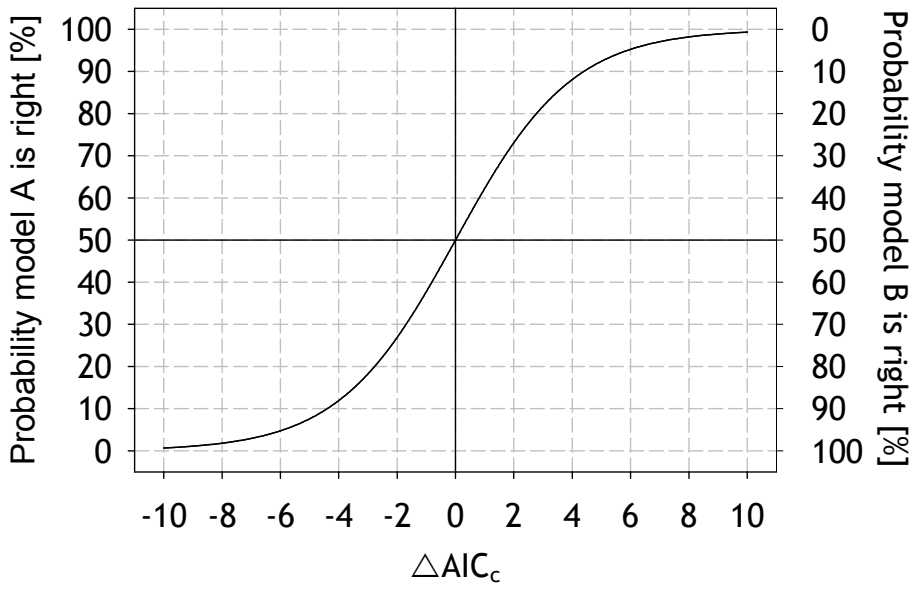


Figure 5.20. Probability distribution for model comparison of a simpler model A and a more complex model B as a function of the Akaike difference ΔAIC_c

An investigation of the AIC_c scores in Table 5.3. and calculating the respective ΔAIC_c, it becomes apparent that using the information theory approach the choice of the more complex model for the two Matawhero datasets (7.5 and 15°C) would not necessarily be justified. The models were found to be equally likely to represent the dataset for 7.5°C ($\Delta AIC_c = 0$), and for 15°C, the DFOP model was only about 22% more likely than the SFO model ($\Delta AIC_c = -0.9$).

Nevertheless, given the fact that the remaining statistical indices confirmed the better performance of the DFOP model, it was chosen to describe the data. The matching estimates for P_0 (Table 5.3.) were improved as indicated by their lower standard errors. However, the estimates for the split factor *g* had partially high standard errors that occurred for datasets where the matching data was more scattered (e.g. Matawhero and Gibsons soils, 7.5°C and 15°C).

The degradation of E2 was in general well described by the SFO model and the corresponding R^2_{adj} values in Table 5.4. (> 0.95) suggested a good fit for all datasets. The DFOP model gave a solution for 8 of the 9 datasets and improved the fit for 6 datasets and all the statistical indices, including ΔAIC_c, favoured the DFOP model. The corresponding standard errors of the parameter estimates for P_0 were in general < 5%; however, the estimates for the split factor *g* showed high standard errors for a few datasets (e.g., Matawhero, 25°C) and that uncertainty was also expressed in the associated rate constant k_1.

Similar results were obtained for the E1-3S datasets. While the SFO model described the data in general well (R^2_{adj} values > 0.95 in Table 5.6.), the fit to 6 out of the 9 datasets was improved using the DFOP model. It is noteworthy, that the DFOP model did not converge for the remaining three datasets. As observed above, improved statistical indices support the choice of the DFOP model. However, based on Figure 5.20., the ΔAIC_c scores would suggest the DFOP model to be a less likely choice than the SFO model in three cases (Hamilton 15 and 25°C, Matawhero 15°C). This circumstance can be regarded as a result of the sample size. The penalty term in equation 5.10 is sensitive to small sample size (Pinheiro and Bates, 2000), and this effect was most pronounced for the Hamilton 25°C dataset, where, at n = 8 data points, the AIC_c score was more than doubled with the introduction of two additional parameters in the DFOP model, a result of the reduced degrees of freedom.

The degradation of E2-3S was chosen to be represented solely by the SFO model because the majority of the datasets had a small sample size that resulted in high standard errors for DFOP parameter estimates. Furthermore, repeated model fits yielded different parameter solution which was not

observed for E2, E1, and E1-3S. The corresponding R^2_{adj} values in Tables 5.8., 5.11., and 5.14. indicate good fitting of the SFO model and the parameter estimates had low standard errors.

In general, the DFOP model was superior to describe the degradation data for E2, E1, and E1-3S, providing its choice was supported by the statistical indices. The investigation of more complex kinetic models than SFO has been recommended to describe laboratory degradation data, in order to avoid an underestimation of degradation rates at later sampling times and to obtain appropriate degradation endpoints (DT values) (e.g., Beulke and Brown, 2001; FOCUS, 2006; Herman and Scherer, 2006). The DFOP model fails to provide a solution when data are well described with the SFO model (Herman and Scherer, 2006), which also observed in a few instances in the present study. Herman and Scherer (2006) also proposed the preparation of composite box-whisker plots for visual residual assessment. Their method allows to assess whether a given model correctly predicts the observed degradation for a pre-determined time interval (e.g., around DT_{50}, DT_{90}). Figure 5.21. displays an example of such a residual comparison between the SFO and DFOP model fitted to the E1-3S degradation data.

The box-whisker plots in Figure 5.21. indicate that the DFOP model was superior in predicting the observed DT_{50} and DT_{90} values to the SFO model. The medians of the DFOP residuals are in excellent agreement with the zero line for all but the last time intervals. Especially in the important intervals 60-40% and 15-5%, relevant for DT_{50} and DT_{90}, the SFO model over- and under-predicted degradation, which consequently resulted in false DT calculation.

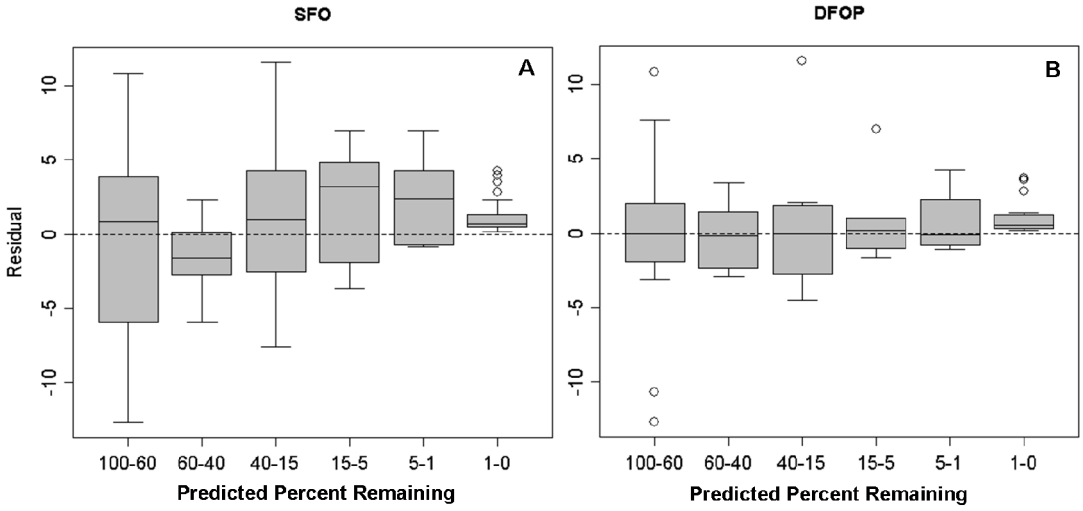


Figure 5.21. Residuals (observed – predicted) for single first-order (SFO, A) and bi-exponential (DFOP, B) degradation models fitted to the degradation of E1-3S (adjusted for the predicted intercept), plotted against the predicted percent remaining. The boxes cover the medial 50% of the data with the median dividing the box. Whiskers range to the largest and smallest points within 1.5 times the interquartile distance, and outliers are depicted as circles beyond the whiskers.

A DFOP model was used to describe some of the estrogen degradation data in Lucas and Jones (2006). However, due to their experimental setup the DFOP model was required to explain the $^{14}CO_2$ release from a primary compound mineralization (first compartment) and a secondary mineralization of the microbial community both contributing $^{14}CO_2$. In contrast, Stumpe and Marschner (2007) reported the usage of the DFOP model to describe some of their observed estrogen degradation data, explicitly highlighting the division between a fast and a slow compartment in agreement with the present data. As an alternative to explain slower degradation with increasing incubation, Xuan *et al.* (2008) presented an adjusted first-order kinetic model. They proposed that E2 degradation occurs only for the non-adsorbed proportion in soil. With increasing degradation the molar ratio between adsorbed and non-adsorbed E2 changes due to a slow desorption process and therefore the degradation kinetic deviates from a SFO kinetic. While this concept seems plausible and yielded good description of some of the data in Xuan *et al.* (2008), the application to the present data was not scrutinized.

4.4.2 Metabolite formation and degradation

The concept to model metabolite formation and degradation from parent compound kinetics presented in equation 5.3 was recently reported in a similar expression for the description of androgen degradation and metabolite formation data (Khan *et al.*, 2008b). However, no formation fraction was considered and the parent compound degradation was assumed to follow a SFO kinetic. Both these factors are likely causes for generally low R^2 values obtained for their fits to the metabolite data (Khan *et al.*, 2008b). In the present study the best fit from the parent compound degradation was used as an input function for the metabolite fitting, and both models, SFO and DFOP (eqs 5.3 and 5.4), were fitted to the datasets.

The SFO model performed well describing the formation and degradation of E1 as a metabolite of E2 in terms of the resulting R^2_{adj} values (all > 0.92 in Table 5.5.). In fact, the DFOP model only converged for two of the nine datasets. While the statistical measures favoured its choice for the Hamilton soil at 7.5°C, the standard errors were high for all parameter estimates and DT values could not be calculated (see section 2 for explanation). However, DT values were obtained from the SFO fits. Similar patterns were obtained from the fitting of the two models to the formation and degradation data of E1 as a metabolite of E1-3S (Table 5.7.). While the DFOP model improved the fit for four of the 9 datasets, the corresponding parameter estimates had high standard errors and in most cases the DFOP model was subsequently not able to provide DT values. The SFO model results were, however, appropriate to calculate DT values and in general the statistical measures indicated a good fit.

In general, inclusion of the formation fraction can be seen as a crucial step for the success of the model application and it might reflect possible degradation differences that can occur in estrogen degradation processes, i.e. not the entire amount of the parent compound is converted to the detectable metabolite (see previous section).

In order to model the more complex system of E2-3S degradation forming two metabolites, E2 and E1-3S, which subsequently have a common metabolite E1 (Figure 5.1.), the kinetic modelling tool ModelMaker 4.0 was

applied to solve the kinetic equations following the schematic illustration in Figure 5.2. This is the first time the degradation and metabolite formation of E2-3S has been attempted to be described kinetically to derive DT values for the metabolite. Therefore, a comparison with literature investigating this compound was not possible. However, ModelMaker has been successfully applied to model human pharmaceutical mineralization in soil (Richter *et al.*, 2007) and was used to describe similar complex systems of natural pesticides degradation in soil (Understrup *et al.*, 2005, Etzerodt *et al.*, 2008).

FOCUS (2006) recommends a stepwise approach to model metabolite formation and degradation, i.e. use the "best fit" for parent compound degradation as the input function for metabolite modelling, and subsequently find a solution for the metabolite(s). When using ModelMaker 4.0 that approach was found necessary since the modelling results from that particular software are highly dependent on the starting values (Erzgräber *et al.*, 2002). In the present study, starting values and parameter constraints (Table 5.2.) were kept constant for the entire range of datasets. However, on a few occasions the starting value for modelling the formation fraction of E2 as a metabolite of E2-3S had to be lowered from 0.8 to 0.5. In general, E2 formation and degradation was well described with the DFOP model for the Hamilton soil (Table 5.9.) and the SFO model for the Gibsons soil (Table 5.12.), while for the Matawhero soil (Table 5.15.) one dataset was best represented with the DFOP and two with the SFO model. The model estimated formation fractions were 3.7-8.1%, 15.3-18.4%, and 10.0-15.0 for the Hamilton, Matawhero, and Gibsons soils, respectively, and had in general low standard errors. The corresponding DT values were exclusively calculated from the SFO model fits.

The subsequent modelling procedure to fit the E1-3S metabolite data was dominated by the formation fraction constraint, i.e. ff_M(E1-3S) ≤ 1-ff_M(E2), which was violated on most occasions. Only the SFO model converged to a solution for E1-3S degradation which was not able to accurately describe the observed data for all soils (Figures 5.12.-5.14.). Especially, the initial fast formation and accumulation followed by a sharp decrease failed to be described, which leads to the assumption that the SFO model was not

sufficient to describe the biological processes. It is very likely that the necessary AryS enzymes were saturated with E2-3S at the start of the incubation, and therefore the degradation of formed E1-3S would be inhibited by substrate competition, which would explain the initial accumulation of E1-3S. With the depletion of E2-3S more AryS became available for E1-3S degradation explaining the sharp decrease of E1-3S. While such behaviour could theoretically be described with Michaelis-Menten kinetics, the implementation in ModelMaker was not pursued, *inter alia* because the calculation of DT values from such kinetics is not straightforward and depends on the initial substrate concentration (FOCUS, 2006). For practical reason the SFO model was used as an input function for the subsequent E1 modelling.

While the fits for the E1 metabolite datasets yielded in general high values for R^2_{adj}, the estimated formation fractions from E2 and E1-3S had high standard errors in particular for the Hamilton (Table 5.10.) and Matawhero (Table 5.13.) datasets. This uncertainty was also expressed in the corresponding rate constant estimates and consequently the derived DT values for E1 degradation as a metabolite of E2-3S have to be treated with caution. High standard errors of parameter estimates were also reported in earlier studies (Understrup *et al.*, 2005; Etzerodt *et al.*, 2008) implementing SFO kinetics to describe metabolite formation and degradation of natural pesticides. As a consequence, the application of the SFO or DFOP models can be regarded as insufficient to accurately describe the complex formation and degradation pathways for the metabolite of organic chemicals in soils. Analysis of mineralization data for human pharmaceutical degradation in soil has shown that model consideration of reversibly sorbed metabolite fractions as well as recognition of microbial activity and growth yield excellent fitting results (Richter *et al.*, 2007). However, these approaches ideally require a combination of analytical metabolite quantification and radioactive residue tracking. Estrogen sulphates with a ^{14}C label were not available and hence the application of these concepts could not be scrutinised.

4.5 Estrogenicity during the degradation of 17β-estradiol-3-sulphate

The formation of estrogenicity during the degradation of E2-3S at 15°C coincides with the formation of the estrogenic free hormones E2 and E1. In the Hamilton soil, estrogenicity accounted for a maximum of ca 20% of initially applied hormones and the occurrence and dissipation of estrogenicity corresponded well with the formation of E2 and E1 (Figure 5.17.). The relative estrogenicity measure indicates that the majority of the remaining hormones at 192 hrs had estrogenic activity which is agreement with the dissipation of estrogen sulphates. In the Matawhero soil (Figure 5.18.) a comparable amount of estrogenicity (ca 1 μg EEQ g^{-1}) was formed, more rapidly, however, and it persisted longer than in the Hamilton soil, which is in accordance with the longer persistence of the free hormones in that soil. The relative estrogenicity measure beyond 24 hrs was > 100%, and since estrogen sulphates have a negligibly low binding affinity to the used receptor (Kuiper *et al.*, 1997) the measure implies that E2-3S was transformed to one or more metabolite(s) that were not detected with the analytical method but still had estrogenic activity. In the Gibsons soil (Figure 5.19.), although estrogenicity was formed in a similar way to the Hamilton soil, it peaked 4 h later, in good agreement with the observed pattern of free estrogen formation and degradation. In contrast, the relative estrogenicity measure was > 100% for two sampling times (72 and 96 h) in the Gibsons soil, which might refer to a temporal formation of undetected estrogenic metabolites. Eligible undetected metabolites with estrogenic activity are estriol or 17α-estradiol, which were confirmed as E2 metabolite in a recent study (Xuan *et al.*, 2008).

In summary, the ER-CALUX® results showed that the degradation of E2-3S involves a temporal formation of estrogenicity in the investigated soils. While a majority of the observed estrogenicity can be explained by the release and subsequent degradation of estrogenic E2 and E1, results also indicated that degradation of E2-3S likely followed different pathways in the different soils, which may have resulted in the formation of unknown metabolites that possess estrogenic activity in some of the soils. This is also in agreement with the above illustrated hypothesis that a variety of intra- and

extra-cellular degradation processes were responsible for the dissipation of E2-3S.

5 CONCLUSIONS

This study has demonstrated that the estrogens E1 and E2, as well as their 3-sulphates, E1-3S and E2-3S, rapidly degrade in agricultural soils incubated in controlled aerobic microcosms at temperatures relevant for field conditions. Furthermore, the formation and degradation of estrogenic metabolite were monitored and revealed that both E2 and E1-3S formed E1 as a major metabolite, while all three compounds were formed as a result of E2-3S degradation.

The lack of considerable compound dissipation in sterile controls in combination with high and significant correlations to the soils microbial biomass carbon strongly suggests microbial mediated degradation in the investigated soils. The *hitherto* only speculated assumption that arylsulphatase enzymes are responsible for estrogen sulphate de-conjugation has been substantiated in the present study. The arylsulphatase activity was significantly correlated with the majority of the first-order degradation rate constants of estrogen sulphates across the three investigated soils and temperatures.

The patterns of metabolite formation and degradation in combination with estrogenicity measures suggest that the degradation of estrogens and estrogen sulphates occurred as a combination of intra- and extra-cellular enzyme-mediated degradation processes, which were to some extent influenced by soil type and temperature, and may have produced intermediate metabolites that were not identified but still possessed some estrogenic activity. Moreover, in one of the investigated soils the estrogenicity measures imply that these undetected metabolites may have temporarily accumulated. In general, however, the occurrence of estrogenic active substances in the degradation of E2-3S at 15°C amounted to a maximum of 20% of the parent compound and estrogenicity did not persist in the investigated soil.

The degradation of E1 as well as the degradation and metabolite formation of E2 and E1-3S were successfully modelled with either a single-first order (SFO) or a double-first order in parallel (DFOP) degradation model. The use of an array of statistical measures was found to be useful to make an informed decision of model choice and to consequently derive useful DT_{50} and DT_{90} values as degradation endpoints. Although the DFOP model was statistically superior to the SFO model to describe some of the metabolite datasets, the calculation of DT values was not always warranted and is a shortfall of the mathematical concept inherent to the DFOP model. However, selection of the DFOP model in the parent compound degradation can be regarded as a crucial step to successfully model metabolite formation and degradation. Applying both these models to the metabolite formation data of E2-3S degradation, it became apparent that despite delivering good fits, the majority of the model estimated parameters had high standard errors indicating high uncertainties. Therefore, better models need to be applied that, in combination with radioactive residue tracking, could reveal some of the complex biological processes involved in the degradation and metabolite formation of E2-3S in soils. This can be especially true in cases where the free hormones were degraded as metabolites with elevated persistence as opposed to their degradation as parent compounds.

In the present study the hormones were applied to the soils with an aqueous solution and the soil microcosms were mixed thoroughly and regularly aerated, and the soil water content was maintained at the same level throughout the duration of the incubation. Under field conditions, the deviation from these ideal conditions could possibly prolong the persistence of estrogens and estrogen conjugates. Also, the typical exposure matrices, i.e. faeces, urine and effluents usually contain compounds that could possibly influence the degradation pathways and further extend the persistence of estrogens and estrogen sulphates in the agricultural soil environment.

CHAPTER VI
TRANSPORT AND RETENTION OF ESTRONE AND ESTRONE-3-SULPHATE IN MICROLYSIMETERS

1 INTRODUCTION

The female steroid hormone estrone (E1) and its sulphate conjugate estrone-3-sulphate (E1-3S) are major components of the estrogenic steroids excreted in the urine and faeces of pregnant cattle (Hoffmann *et al.*, 1997). Of these, the total urinary excretions of estrogens per cow amount to a maximum of 104,000 µg day^{-1} during the late stages of pregnancy and comprise about 92% of E1-3S (Hanselman *et al.*, 2003). Despite the facts that E1 is known for its potential to cause endocrine disruption in aquatic wildlife and that E1-3S has been shown to cause apoptosis in quails (Isobe and Shimada, 2003), no effort has been made to date to understand the contribution of E1-3S originating from cattle urine to overall concentrations of estrogens in the environment. However, de-conjugation of E1-3S by naturally occurring arylsulphatase enzymes can lead to the formation of E1 which has a higher endocrine disrupting potential than E1-3S (Kuiper *et al.*, 1997) and livestock operations are suspected to contribute to environmental concentrations of pharmaceuticals and hormones (Khan *et al.*, 2008a; Kolpin *et al.*, 2002). Literature reports indicating the presence of E1-3S in river sediments (Matějíček *et al.*, 2007), river water (Rodriguez-Mozaz *et al.*, 2004b), and one drinking water sample from Spain (Kuster *et al.*, 2008) highlight the potentially important role of E1-3S in the distribution of its parent compound E1 in the environment.

In New Zealand, animal wastes containing estrogens are applied to agricultural land (Sarmah *et al.*, 2006) and continuously grazing livestock also constitute a direct source of steroid hormones. These hormones can be leached from soil in drainage water and knowledge about the transport behaviour of estrogens and estrogen sulphates through soil is therefore necessary to conduct a thorough risk assessment of animal waste-related estrogen exposure. Studies investigating the transport behaviour of the free estrogens 17ß-estradiol (E2) and E1 in pre-packed micro columns (Das *et al.*, 2004; Casey *et al.*, 2005) and in intact soil cores (Sangsupan *et al.*, 2006; Fan *et al.*, 2008) have shown that both physical and chemical non-equilibrium process contribute to enhanced estrogen transport, which is in contrast to

batch sorption studies that suggest high to moderate sorption of these compounds in topsoil (Yu *et al.*, 2004). Furthermore, as these hormones are labile compounds, degradation kinetics appear to play an important role in estrogen transport (Fan *et al.*, 2008). It has recently been reported that the degradation of E2 and E1 was significantly influenced by the exposure matrix (Lucas and Jones, 2006) and that estrogen transport in pre-packed soil columns was facilitated when the compounds were applied in artificial sheep urine (Lucas and Jones, 2009).

However, to date no information is available about the transport of estrogen sulphates in porous media, or the influence of the exposure matrix on the transport behaviour of these compounds. This study therefore investigated the transport of E1-3S and its parent compound E1 in undisturbed soil columns (7.55 cm internal diameter by 18 cm length) under saturated conditions. Two different mediator solutions were used to apply the hormones at a concentration of 0.5 mg L^{-1} to the microlysimeters: a weak ionic solution of $CaCl_2$ (10 mM), and an artificial cow urine solution. The leachate from the lysimeters was collected and the concentration of a bromide tracer and the hormone measured. A physical non-equilibrium transport model was subsequently used to describe the transport of the solutes. After elution of the solute breakthrough curve the soil cores were sectioned into 6 equal parts and analysed to determine the hormone concentration in each depth.

2 MATERIALS AND METHODS

2.1 Soil lysimeters

The lysimeter casings were made of polyvinylchloride (PVC) with a length of 20-22 cm and an internal diameter of 7.55 cm. Undisturbed soil cores of the Hamilton clay loam soil were obtained in situ by hand carving from the ground surface. The internal walls of the lysimeter casings were smeared with petroleum jelly and each casing was progressively pressed down to encase the exposed column of soil. After the desired length of 18 cm was reached, the lysimeter cores were carefully detached from the soil with a blade. The bottom was sealed with a PVC cap containing 5 sampling ports

secured with a fine mesh (ø 1 mm) to allow leachate to drain through for collection. In the laboratory the lysimeters were saturated from bottom up with water, and liquid petroleum jelly was injected into any voids at the soil-casing interface, thus preventing preferential flowing between the soil and the PVC casing. By assuming a porosity of 0.51 (Pang *et al.*, 2008) the pore volume of the lysimeters was calculated as 1644 cm^3.

2.2 Transport experiment

The transport and retention behaviour of E1 and E1-3S from an artificial urine solution and a conventionally used $CaCl_2$ solution (0.01M) was investigated. One lysimeter was assigned to each hormone/solution combination, and the saturated lysimeter was placed on a wooden rack, equipped with an irrigation head comprising of a reservoir and 41 needles to uniformly irrigate the lysimeter (Figure 6.1.). The irrigation head was supplied with $CaCl_2$ (10 mM) through a peristaltic pump (Cole-Parmer Instrument Company) from a reservoir and the lysimeter leachate was collected in a beaker from which leachate fractions were sampled with an ISCO (ISCO 6700, ISCO Nebraska, U.S.A) automated water sampler (Figure 6.1.). Tubes and containers were covered in aluminium foil to prevent photodegradation.

Each lysimeter was irrigated with at least 4 pore volumes of $CaCl_2$ (10 mM) before the respective hormone solution was applied. Hormone solutions of 0.5 mg L^{-1} were prepared by adding an appropriate amount of methanolic stock solution (600 mg L^{-1}) to either $CaCl_2$ (10 mM) solution or artificial urine. The artificial urine solution was prepared as described in Early *et al.* (1998) and consisted of $KHCO_3$ (22.2 g L^{-1}), KCl (3.95 g L^{-1}), K_2SO_4 (6.7 g L^{-1}), $(NH_2)_2CO$ (23.5 g L^{-1}), and $C_2H_5NO_2$ (6.2 g L^{-1}). Both solutions contained KBr (6.35 g L^{-1}) as a conservative tracer.

Four transport experiments were investigated: HC_01 = Estrone-3-sulphate with artificial urine; HC_02 = Estrone-3-sulphate with $CaCl_2$ (10 mM); HC_03 = Estrone with artificial urine; HC_04 = Estrone with $CaCl_2$. One litre of the respective hormone solution was applied at approximately steady state flow, followed by $CaCl_2$ (10 mM) solution without hormones or KBr tracer until the bromide concentration in the leachate was close to zero. The leachate was collected in fractions on a time average basis, and each fraction was

analyzed for the bromide concentration using an ion-selective electrode (Metrohm 6.0502.100, Herisau, Switzerland). Additionally, to determine the hormone concentration in the leachate fractions, 25 mL of each fraction were extracted with 5 mL of dichloromethane and 0.25 mL of dicyclohexylamine hydrochloride (10 mg L^{-1} in H_2O) over night. Analysis was performed on an HPLC-UV system as described in Chapter III.

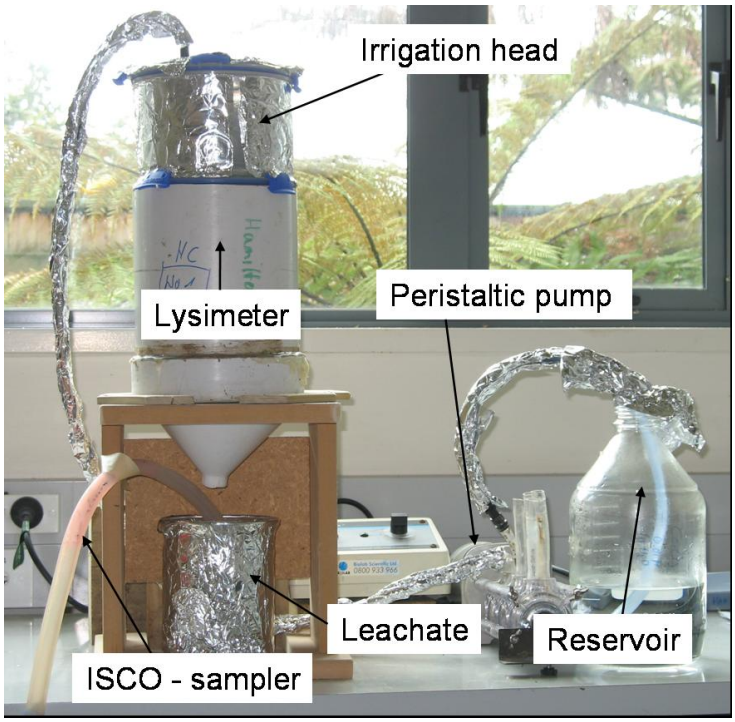


Figure 6.1. Setup for the miscible displacement experiments. Each lysimeter was irrigated through an irrigation head supplied with solution from a peristaltic pump. Leachate was collected in a beaker and sampled with an automated water sampler.

At the end of the transport experiment, the lysimeters were carefully cut open and sectioned into 6 equal parts, each of 3 cm. The soil from each section was thoroughly mixed and analysed to determine its volumetric water content. To determine the residual hormone concentrations of E1-3S and E1, respectively, triplicate samples of 5 to 6 g of each section were extracted with 6 mL of dichloromethane and 0.25 mL of dicyclohexylamine hydrochloride (10 mg L^{-1} in H_2O) over night and analysed by means of HPLC-UV (Chapter III).

2.3 Transport model

The convection-dispersion equation (CDE) at equilibrium was used to model the breakthrough curves (BTCs) of the tracer and the hormones. In a dimensionless form the equilibrium CDE can be written as (Toride *et al.*, 1995):

$$R\frac{\partial C}{\partial T} = \frac{1}{P}\frac{\partial^2 C}{\partial Z^2} - \frac{\partial C}{\partial Z} - \mu C \tag{6.1}$$

Where R [= $1+\rho K_d\theta^{-1}$] is the retardation factor; P [= vLD^{-1}] is the column Peclet number, an index of combined effect of mass flow and dispersion; and μ [= $L\mu_s(R\text{-}1)v^{-1}$]is a dimensionless rate coefficient for the degradation of the solute in the solid phase (μ_s) assuming no degradation occurs in the liquid phase. For the conservative tracer bromide $\mu = 0$. C [= CC_0^{-1}], T [= vtL^{-1}] and Z [= xL^{-1}] are the dimensionless aqueous concentration, time and length, respectively.

Breakthrough curves of bromide are often asymmetrical and exhibit significant tailing, which is regarded as an indicator of physical nonequilibrium processes in the experimental system resulting from heterogeneous porous media. In such cases a two-region mobile-immobile transport model can be used (Toride *et al.*, 1995; Šimůnek *et al.*, 2008). Assuming degradation of the reactive solute occurs in the immobile and mobile region, the model can be formulated by two partial differential equations:

$$\beta R\frac{\partial C_m}{\partial T} = \frac{1}{P}\frac{\partial^2 C_m}{\partial Z^2} - \frac{\partial C_m}{\partial Z} - \omega(C_m - C_{im}) - \mu_m C_m \tag{6.2}$$

$$(1-\beta)R\frac{\partial C_{im}}{\partial T} = \omega(C_m - C_{im}) - \mu_{im} C_{im} \tag{6.3}$$

where the subscripts *m* and *im* refer to the mobile and immobile region, respectively; β [= $(\theta_m+f\rho K_d)(\theta+\rho K_d)^{-1}$] is the dimensionless parameter related to the fraction of adsorption sites that equilibrate with the mobile liquid phase (θ_m); for a conservative tracer [$K_d = 0$], β [= θ_m/θ] is a direct measure of the mobile water available for solute transport; ω [= $(\alpha L)(\theta v)$] is the Damköhler number related to the mass transfer coefficient between the two regions; μ_m and μ_{im} are degradation rate constants in the mobile and immobile region, respectively. The constants used to calculate the dimensionless

parameters were: the input solution concentration (C_0); the volumetric water content (θ), which was obtained from the average of the six sections; soil bulk density (ρ) (from Pang *et al.*, 2008, 1.16 g cm^{-1}); and the soil column length (L).

The CXTFIT code within the software STANMOD (Studio for Analytical MODels, Šimůnek *et al.*, 1999) was used to find analytical solution for the CDEs by means of inverse fitting of the obtained BTCs. Initially, parameter estimates were obtained for the bromide BTCs. Estimated parameters were *v* (pore water velocity), *D* (dispersion coefficient), and for the nonequilibrium CDE, additionally, *β* and *ω* were estimated. These parameters were used in the hormone BTCs, and parameters estimated for the hormone BTCs were *R* (retardation factor) and μ_m and μ_{im}.

3 RESULTS AND DISCUSSIONS

3.1 Solute transport analysis

The peak concentration of the conservative bromide tracer in the column effluents occurred within 0.9-1.1 pore volumes for HC_01, HC_02, and HC_04. However, the HC_03 lysimeter showed significant preferential flow and the bromide concentration in the effluent peaked early after 0.3 pore volumes (Figure 6.2., left panel). The significant tailing in the bromide BTCs can be regarded as an indication for a two region process where the soil water is divided in a high permeability and a low permeability zone (Torride *et al.*, 1995; Pang *et al.*, 2008). A portion of the solute is being transported within the high permeability zone, while the remaining portion diffuses into the low permeability zone. When the maximum concentration of solute has moved through the high permeability zone, the portion from the low permeability zones slowly diffuses back into the high permeability zone following the concentration gradient (Pang *et al.*, 2008), which caused the observed tailing of the bromide BTCs.

Detectable hormone concentrations in the column effluents were only obtained for the lysimeters HC_01 and HC_03 (Figure 6.2., right panel), where artificial urine was applied to investigate the transport of E1-3S and E1,

respectively. The maximum amount of recovered hormone in the effluent was < 6% (E1-3S, HC_01) and about 18% (E1, HC_03), and both maxima occurred later than the corresponding bromide peaks. The later arrival of the hormone maxima indicates that despite the assumption of preferential flow domains, the retardation of the hormones due to sorption prevailed. The pronounced tailing of the hormone BTC further indicates the possibilities of slow desorption and/or reverse diffusion back into the high permeability zone. In the past, peak hormone concentration break through has been observed occurring simultaneous to chloride break through (Sangsupan *et al.*, 2006) and preferential flow patterns were suggested to contribute to fast hormone movement through intact soil cores. In the present study, however, even under pronounced preferential flow conditions (HC_03, Figure 6.2.) the hormone BTC still showed retardation compared with the conservative tracer indicating the influence of sorption processes.

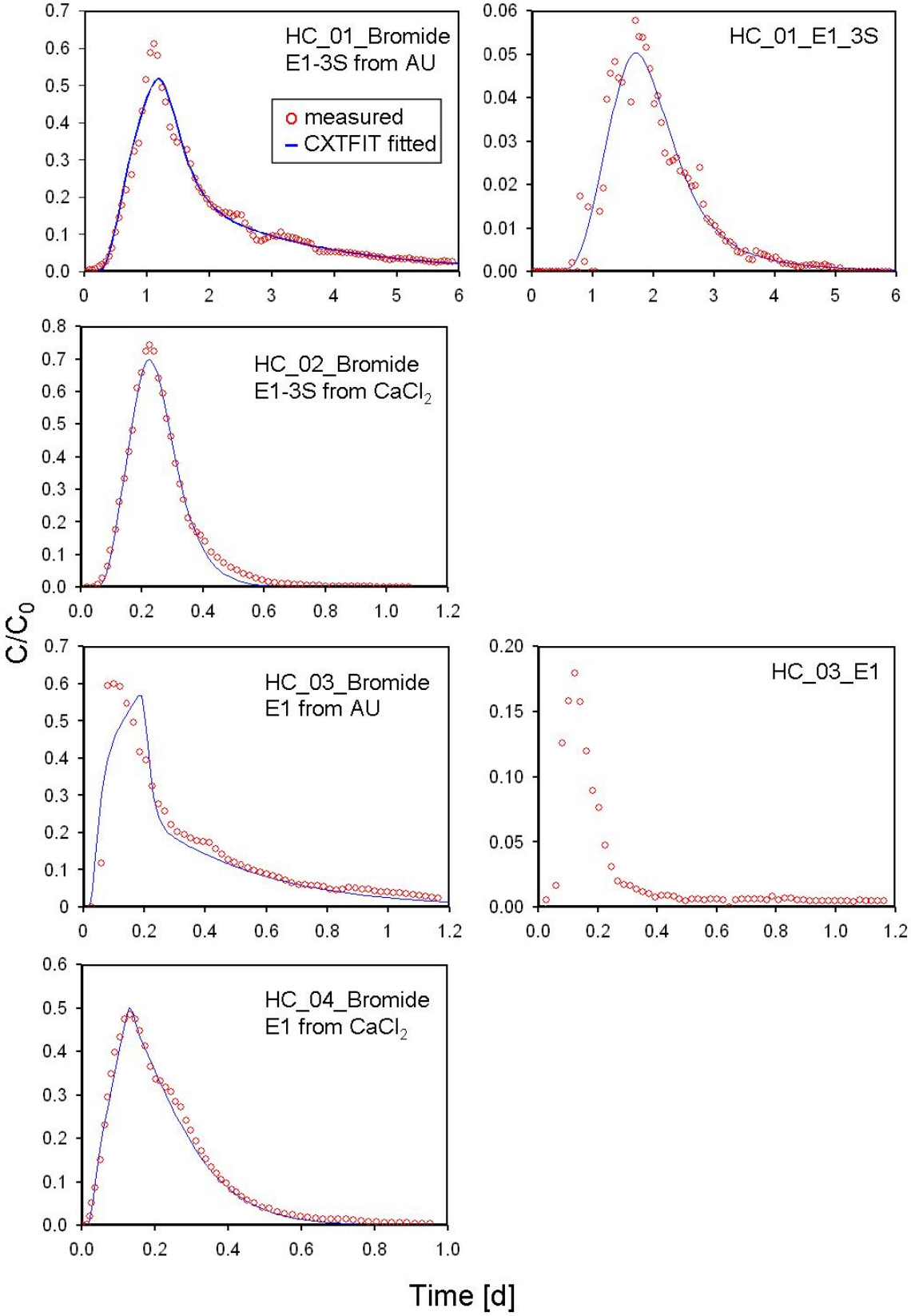


Figure 6.2. Breakthrough curves of bromide (left panel), E1-3S (top right panel), and E1 (centre right panel), applied with a conventional solution of $CaCl_2$ (10 mM) (HC_02, HC_04) or an artificial cow urine solution (HC_01, HC_03). Blue solid lines indicate the CXTFIT fitted model.

The leachate fractions of the columns HC_01 and HC_02 where E1-3S and E1, respectively, were applied with artificial urine, showed a distinct yellow colour development (Figure 6.3.). The intensity of the coloured leachate reached its maximum in good agreement with the peaks of E1-3S and E1 in the leachate. These results indicate that the hormones might travel slower than a conservative tracer; however, when applied with artificial urine, E1 and E1-3S might be transported at a similar speed to their transport matrix in undisturbed microlysimeters. The yellow colour is considered to be an indication of organic matter dissolution and dispersion which could have resulted in colloid enhanced transport of the hormones (Dizer *et al.*, 2002). The dissolution of soil organic matter is likely to be due to the high salt concentrations in the artificial urine and this may have resulted in an increased amount of dissolved organic matter which became available for estrogen co-transport in the soil lysimeter. An increase in soil dissolved organic carbon as a response to artificial urine application has been also reported in the past (Shand *et al.*, 2002).

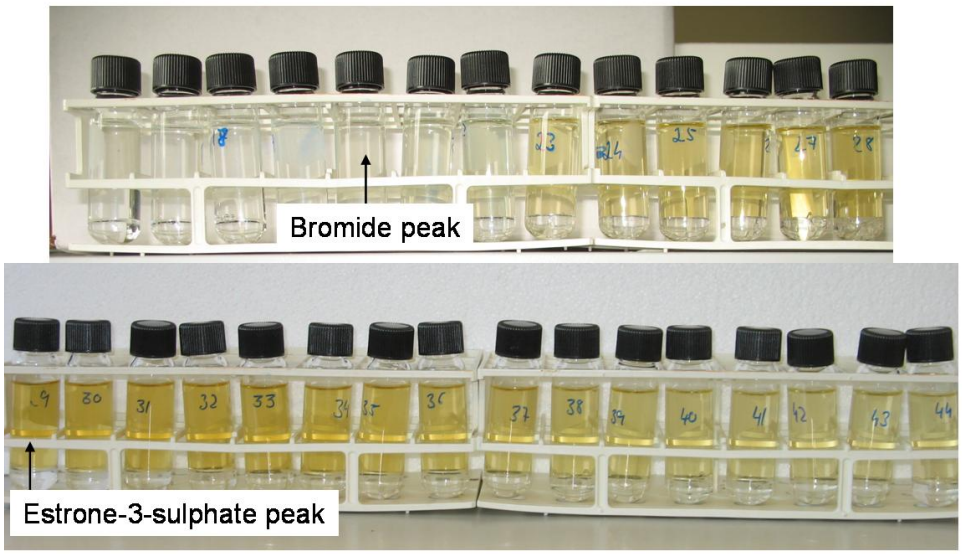


Figure 6.3. Colour of a subset of leachate fractions from lysimeter HC_01, where E1-3S was applied in artificial urine (1 L of 0.5 mg L^{-1}).

Furthermore, the formation of volatile ammonia was noticed during the application of the artificial urine, which is typical due to microbial breakdown of the urea and glycine in the urine (Liang *et al.*, 2007). Since organic

contaminants such as hormones are expected to be metabolised by co-metabolism (Lucas and Jones, 2006; Stumpe and Marschner, 2007) the presence of readily available carbon sources like urea and glycine possibly suppressed the rapid degradation of E1 and E1-3S when both the hormones were applied in artificial urine. Suppression of microbial degradation was recently suggested as a reason for enhanced transport of E2 and E1 with artificial sheep urine as compared with transport with rain water in packed micro-soil columns (Lucas and Jones, 2009).

3.2 Hormone retardation

The results of the soil extraction from the sectioned lysimeters are presented in Figure 6.4. In the lysimeter HC_01, E1 was detected in the soil extracts of the first 12 cm; however, no resident concentration of E1 was detected deeper in the profile and the parent compound E1-3S was not observed throughout the profile. Estrone is a metabolite of E1-3S resulting from enzyme mediated microbial degradation processes (see Chapter V). The fact that neither the metabolite E1 nor its parent compound E1-3S was extracted from the sections of the lysimeter HC_02, supports the earlier stated assumption that the exposure matrix likely had an important influence on the biodegradation processes of the hormones in natural soil. The artificial urine matrix likely suppressed the biodegradation of E1-3S and its metabolite E1 in the lysimeter HC_01, while fast degradation below method detection limit without suppressions was observed in the lysimeter HC_02. The effect of the artificial urine becomes even more pronounced when the timeframes of both miscible displacement experiments are compared. Despite the fact that it took ca 6-fold longer for the solution to percolate through HC_01 than through HC_02 (Figure 6.2.), trace amounts of the metabolite E1 were still detectable in the profile of HC_01 (Figure 6.4.), highlighting the impact of the artificial urine on the microbial community responsible for hormone degradation.

The occurrence of a pronounced preferential flow pattern in the lysimeter HC_03 (Figure 6.2.), in combination with the impacts of the artificial urine on the microbial degrader community, may likely have been responsible for the observed E1 resident concentration in the soil extracts

(Figure 6.4.). In comparison with HC_04, where E1 was only detected in the 0-3 and 6-9 cm sections, a clear vertical allocation of E1 resident concentrations was observed in HC_03. Apart from the 3-6 cm section, E1 was detected throughout the profile of HC_03 in concentrations well above the method detection limit, with a maximum of > 12 ng g^{-1} in the 12-15 cm section. Changes in hormone resident concentrations resulting from different mediator solutions have been reported by Lucas and Jones (2009); however, their results were not conclusive and did not show a clear trend in relation to the respective mediator solution. These inconclusive results in Lucas and Jones (2009) are likely a result of the short column length of only 7 cm, which probably did not provide a spatial range long enough to detect differences in hormone resident concentrations. However, from the present study it is clear that preferential flow patterns and effects of the exposure matrix on the microbial degrader community may contribute to enhanced vertical transport of E1 and E1-3S in agricultural soils.

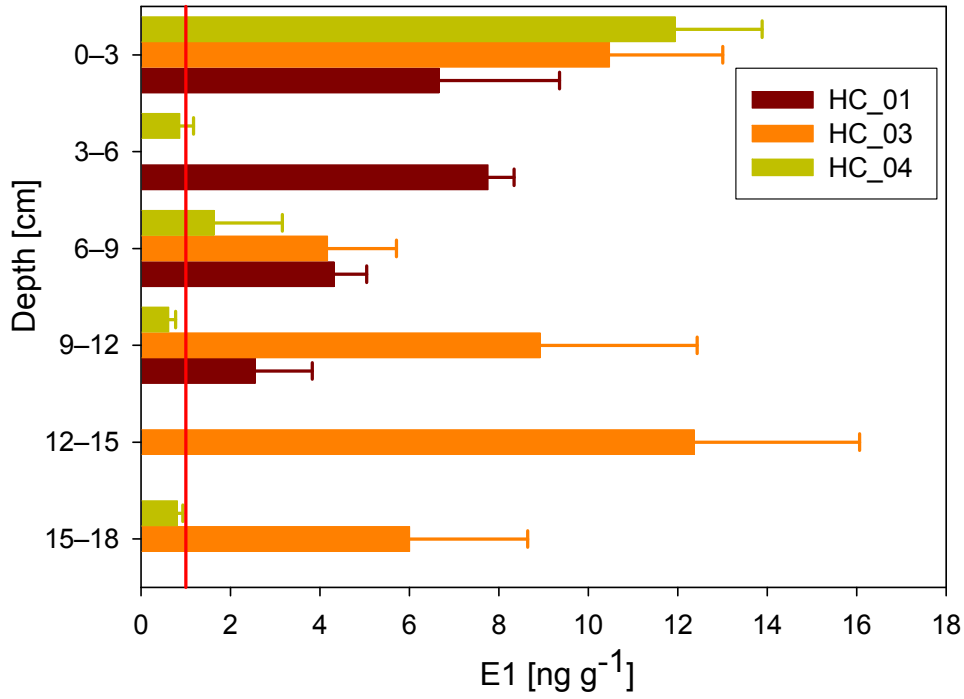


Figure 6.4. Estrone resident concentration in the sectioned cores HC_01, HC_03, and HC_04, respectively. Average values of n = 3 samples are displayed with one standard deviation. The red line indicates the method detection limit of 1 ng g^{-1}.

3.3 Transport modelling

The two-region physical non-equilibrium model available within CXTFIT (Toride *et al.*, 1995) was used to inversely fit the bromide tracer breakthrough data and to obtain transport parameter estimates for v, D, β, and ω, respectively. The model yielded a good description of the datasets for the lysimeters HC_01, HC_02, and HC_04 as supported by the R^2 values (Table 6.1.); however, the observed preferential flow pattern in the HC_03 core was not well described (Figure 6.2.). Furthermore, the results for the HC_04 data were highly dependent on initial parameter starting values and independent parameter estimates were only obtained by setting the dispersion coefficient to a value similar to that of HC_02. The estimated dispersion coefficient of HC_03 data was associated with a large standard error indicating the poor fitting capability of the two region model for pronounced preferential flow. The estimated pore water velocities were in good agreement with experimentally determined values and small differences can be attributed to the heterogeneous nature of the soil with spatially varying porosity.

Table 6.1. The CXTFIT model parameter estimates (including one estimated standard error) for the two-region physical nonequilibrium model fitted to the bromide breakthrough data.

Lysimeter	v ± SE [cm d^{-1}]	D ± SE [cm^2 d^{-1}]	β ± SE [-]	ω ±SE [-]	R^2
HC_01	10.2 ± 3.59	18.4 ± 3.46	0.46 ± 0.03	0.78 ± 0.07	0.971
HC_02	95.8 ± 1.22	102 ± 22.9	0.87 ± 0.06	0.37 ± 0.29	0.995
HC_03	68.2 ± 6.77	117 ± 102	0.23 ± 0.05	1.01 ± 0.10	0.891
HC_04	113 ± 1.63	n.e.	0.31 ± 0.02	2.3 ± 0.13	0.991

n.e.= not estimated

A solution for the hormone BTC was only obtained for HC_01 using the two region physical nonequilibrium model. Furthermore, the parameter estimates were only independent when v, D, β, and ω from the bromide BTC were used, while only R and μ_{im} and μ_m were being optimised. The estimated R value of 2.69 (± 0.04) corresponds to a linear partition coefficient (K_d) of 0.87 L kg^{-1}, which is lower than expected from the sorption experiment in Chapter IV. The model estimated dimensionless degradation rate constants were 2.52 (± 0.04) and 1.67 (± 0.64) for the mobile and immobile region,

respectively. Since the metabolite E1 was not detected in the column leachate, it can be assumed that degradation occurred only in the instantaneously sorbed fraction of E1-3S, and, therefore, dimensional degradation rate constants for the mobile and immobile region were calculated as 15.2 d^{-1} and 10.1 d^{-1} (Toride *et al.*, 1995). The latter is in good agreement with the first-order rate constant obtained from the degradation study of E1-3S in Hamilton clay loam at 25°C (Chapter V).

Modeling hormone transport in intact soil cores appears to be a major challenge. The use of either physical or chemical nonequilibrium models alone has been reported to be insufficient (Das *et al.*, 2004, Sangsupan *et al.*, 2006). Accurate and satisfactory description of data from miscible displacement experiments has only been achieved with consideration of both physical and chemical nonequilibrium processes (Sangsupan *et al.*, 2006). A crucial step in obtaining appropriate model results lies in the independent determination of sorption and degradation kinetics through separate batch studies. For instance, Fan *et al.* (2008) independently determined sorption and degradation kinetics of E2, its metabolite E1, and another polar metabolite, in batch experiments and used the obtained values later in a HYDRUS 1D model to fit mass transfer rates between solid and liquid phase to data from miscible displacement experiments. The authors were also able to describe the resident concentration of the compounds in the sectioned soil cores with their model, a feature that was not available with the limited capacities of the CXTFIT model implementation.

4 CONCLUSIONS

This study has demonstrated the effect of the exposure matrix on the transport and retention of E1-3S and E1 in undisturbed microlysimeters. On the one hand, artificial cow urine facilitated the transport, but then also hampered the degradation of E1-3S and E1. The dissolution and dispersion of soil organic matter as a result of artificial urine application, led to an increase in dissolved organic carbon thus allowing colloid facilitated transport of the hormones. Furthermore, the presence of readily available carbon sources in

the artificial urine possibly suppressed the fast co-metabolic degradation of E1-3S and E1 in the soil cores.

The two-region physical nonequilibrium model was appropriate to describe the BTCs of the conservative tracer bromide; however, it did not deliver information about potential chemical nonequilibrium processes involved in hormone transport. The recently developed concepts for hormone transport in undisturbed soil columns (Fan *et al.*, 2008) may hold better explanations of miscible displacement data. Future studies, however, should include considerations of the exposure matrix. The present study has shown that the exposure matrix significantly impacts on hormone behaviour in saturated porous media, and implies that a comprehensive risk assessment for transport of steroid hormones in agricultural environments cannot be obtained by using only weak ionic mediator solutions in lysimeter studies. Further research is certainly warranted to study the coupled sorption, degradation and transport processes of these hormones using large undisturbed soil monoliths.

CHAPTER VII
GENERAL DISCUSSION, CONCLUSIONS AND FUTURE RESEARCH

1 GENERAL DISCUSSION

Chapter III presented the development of a method to extract estrone (E1) and estrone-3-sulphate (E1-3S) from aqueous matrices by liquid/liquid extraction, a method to extract 17ß-estradiol (E2), E1 and their 3-substituted sulphate conjugates from soil samples by solvent extraction, and an improved HPLC-UV method to simultaneously detect and quantify the 4 hormones, respectively. These analytical methods were used to analyse the samples on the following detailed experiments conducted in the laboratory:

1. the sorption behaviour of E1 and E1-3S in agricultural soils as influenced by the mediator matrix (Chapter IV);
2. the degradation and metabolite formation behaviour of E2, E1 and their 3-substituted sulphate conjugates in agricultural soils at three incubation temperatures (Chapter V);
3. and the transport and retention behaviour of E1 and E1-3S in undisturbed soil lysimeters as influenced by the mediator matrix (Chapter VI).

While extraction of estrogens from aqueous and soil samples has been successfully conducted using dichloromethane (DCM) in a number of previous studies (e.g., Lai *et al.*, 2000; Lee *et al.*, 2003; Soto *et al.*, 2004), the developed methods to extract the estrogen sulphates from aqueous and soil samples using solvent extraction constitute a novelty in this thesis. The addition of an organic modifier, namely dicyclohexylamine hydrochloride (DCH·HCl) as recommended by Dean *et al.* (1955) to extract E1-3S from urine, was crucial to obtain acceptable extraction recoveries for the estrogen sulphates with values ranging from 97.3 to 107 % for aqueous samples, and 80.9 to 95.2% for soil samples, respectively. Similar recoveries for the free estrogens have been reported by Lee *et al.* (2003) and Soto *et al.* (2004) and the values in this study are also comparable with recoveries obtained with more sophisticated extraction techniques, e.g., solid phase extraction or microwave assisted solvent extraction (D'Ascenzo *et al.*, 2003; Matějíček *et al.*, 2007). However, the free hormones showed somewhat lower recoveries

from soil samples and especially in one soil, E1 recovery was poor (41.3%) with the present method. Recently Beck *et al.* (2008) recently reported poor recoveries for E1 and E2 extraction from soil samples using DCM. However, earlier studies have successfully demonstrated the use of DCM to extract estrogens from soils and sediments (e.g., Lee *et al.*, 2003; Sarmah *et al.*, 2008). The poor recoveries of E1 in the present study could be attributed to the specific physico-chemical properties of that particular soil which likely hampered the fortification process and thus may have led to low recoveries. In general, the developed extraction methods constituted a cost-effective and reliable tool to study the fate and behaviour of estrogens and estrogen sulphates in laboratory scale experiments.

The implementation of a monolithic high-performance-liquid-chromatography column to separate the four hormones resulted in a significant reduction in runtime which warranted the throughput of a high number of samples that was needed to conduct detailed laboratory experiments that involved a high temporal resolution. UV absorption of the four hormones was found to be at maximum at 201 and 279 nm which is in good agreement with previously reported detection wavelengths (Lee *et al.*, 2003; van Emmerik *et al.*, 2003; Yu *et al.*, 2004). The developed HPLC method allowed for excellent separation of E1-3S, 17ß-estradiol-3-sulphate (E2-3S), E1 and E2 in < 10 minutes by employing a gradient system comprising of an acidified ammonium sulphate buffer (5 mM), acetonitrile and water. Similar reductions in retention times have been reported earlier, and the application of monolithic silica columns for the separation of E2 and E1 was found to be suitable (Mizuguchi *et al.*, 2005). The use of ammonium sulphate buffer for separation of estrogen conjugates was reported by Blom *et al.* (2001), *albeit* with a higher buffer concentration (20 mM) and without pH adjustment. The overall method detection limits in the present study were 1.0 ng mL^{-1} for estrone and estrone-sulphate extraction from aqueous matrices, and ranged from 1.0 to 2.9 ng g^{-1} for extraction from soil samples. Compared with the LC-MS method detection limits reported in the literature (D'Ascenzo *et al.*, 2003; Rodriguez-Mozaz *et al.*, 2004; Kuster *et al.*, 2008), the detection limit for aqueous extraction in the present study was about an order of magnitude

higher, however, comparable to other studies that employed HPLC-UV detection (Lai *et al.*, 2000; Lee *et al.*, 2003). The present detection limits for soil extraction were about 2–10 times higher than reported by Matějíček *et al.* (2007), and about 14–100 times higher than reported by Isobe *et al.* (2006) and Beck *et al.* (2008) for sediment and soil samples, respectively. In these studies however, MS detectors have been used that are in general more sensitive than UV detectors and therefore better suited to detect very small concentrations (Ingerslev and Halling-Sorensen, 2003). In general, for the purpose of laboratory scale investigations to study sorption, degradation and lysimeter transport of estrogens and estrogen sulphates, the developed extraction methods were found to be very suitable in order to obtain quality data and to infer environmental fate parameters for these compounds.

Although E1-3S is a major constituent of the total estrogen excretions of cattle during late stages of pregnancy (Hoffmann *et al.*, 1997) and trace concentrations are continuously being reported in various environmental media (e.g., Isobe *et al.*, 2006; Matějíček *et al.*, 2007; Kuster *et al.*, 2008) the sorption behaviour of this compound has never been studied. Also, the possibility of an influence of the mediator solution on the sorption behaviour of estrogens has not been investigated, despite the fact that grazing livestock constitute a direct exposure source for estrogens in the environment, which is of particular interest for New Zealand conditions. Therefore, this thesis examined the sorption behaviour of E1 and E1-3S in three agricultural soils from New Zealand. Batch sorption experiments were conducted to study the influence of an artificial cow urine solution in comparison with the common approach of using a weak ionic solution of $CaCl_2$ (5 mM).

Typically, the sorption behaviour of organic pollutants is determined at equilibrated conditions between solid and liquid phases (Pignatello and Xing, 1996) and a study by Yu *et al.* (2004) reported equilibration times for 17β-estradiol up to 160 h. However, more recent studies found that relevant sorption kinetics occur in shorter time periods ranging from a few hours to 2 days in non-sterile soils (Casey *et al.*, 2005; Fan *et al.*, 2008). In the present study an apparent equilibrium for E1 sorption was reached in the first 1 to 8 hours before degradation processes significantly impacted the sorption in the

three investigated soils. Therefore, a contact time of 2 hours was chosen for the subsequent batch experiments. Similar contact times have been used earlier to study estrogen sorption in river sediments (Lai *et al.*, 2000).

The sorption isotherms of E1 and E1-3S in the three soils were well described by the Freundlich equation and the resulting Freundlich parameters for E1 sorption were in good agreement with values previously reported (Lee *et al.*, 2003; Yu *et al.*, 2004; Hildebrand *et al.*, 2006; Sangsupan *et al.*, 2006; Sarmah *et al.*, 2008). Apparent limitless sorption of E2 in a Horotiu soil ($N > 1$) was reported by Sarmah *et al.* (2008) and the present study confirmed a similar behaviour for sorption of E1. In the present study, the apparent limitless sorption capacity in the Horotiu soil was attributed to the specific clay mineralogy with a high presence of allophane and the high OC content of the soil. In comparison with E1, the sorption capacity for E1-3S was about one order of magnitude lower in the investigated soils. Given its ionic, hydrophilic nature, E1-3S is expected to exhibit lower sorption affinity to the organic matter domain of soils than its free counterpart. Anion retention by allophone as a possible retention mechanism was excluded because the solution pH was above the isoelectric point of the mineral (Schachtschabel *et al.*, 1997). Unspecific interactions with organic matter and clay minerals, such as ligand binding, intercalation, and weak hydrogen bonding were proposed as a plausible explanation for the observed weak sorption of E1-3S to the soils.

The Freundlich parameters for E1 and E1-3S showed small changes when sorption occurred from the artificial urine solution as compared to the $CaCl_2$ solution. A clear mechanism responsible for these differences could not be identified due to the limited dataset. However, it is likely that the higher conductivity of the artificial urine caused a “salting out” effect (Bowman *et al.*, 2002) leading to increased sorption of E1 in the Hamilton soil. In contrast, competitive behaviour of artificial urine constituents for specific sorption sites may have been the cause for reduced sorption of E1 in the Horotiu and Te Kowhai soils which is in good agreement with previous studies where decreased hormone sorption was observed in multi-sorbate systems (Yu *et al.*, 2004; Bonin and Simpson, 2007). The lack of a similar observation for E1-3S

indicates that sorption of the conjugate is likely controlled by other mechanisms than hydrophobic interactions.

During the sorption of E1-3S from $CaCl_2$, E1 was detected as metabolite in both the soil and aqueous phase and hence the calculation of a metabolite isotherm was warranted. The corresponding Freundlich exponent *N* was comparable to a previously reported estrone metabolite isotherm in the Horotiu soil (Sarmah *et al.*, 2008). The lower *N* values of the metabolite isotherms confirmed general isotherm non-linearity and increasing sorption affinity at lower aqueous concentrations which was reported for E1 before (Yu *et al.*, 2004) and is a common phenomenon for Freundlich-type sorption isotherms (Pignatello and Xing, 1996).

Due to a lack of correlation between isotherm parameters to soil organic carbon and the general nonlinearity of the calculated isotherms, the commonly used organic carbon normalization was not favoured. In order to compare the differences between the two mediator solutions a hypothetical exposure concentration range for E1-3S and E1 in cattle urine was calculated ranging from 4–8,000 and 0.6–700 µg/L, respectively. The effective distribution coefficient $K_d^{eff} = K_f C_w^{N-1}$ was then plotted against the aqueous concentration. The results showed that at low aqueous concentrations the K_d^{eff} for both compounds was subject to significant changes between the two mediator solutions. Depending on the soil type and compound, either an over- or an underestimation of sorption would be inferred from the common $CaCl_2$ isotherms as compared to the artificial urine solution.

The degradation of the estrogen sulphates E2-3S and E1-3S in the soil environment was studied for the first time in this thesis. In general, the compounds were rapidly degraded in the three investigated soils with DT_{50} values < 24 hrs for the three incubation temperatures. Compound degradation occurred faster with increasing temperatures, and no significant degradation was observed in the sterile control samples supporting the assumption that estrogen sulphate degradation was biologically mediated. The strong correlation of first-order degradation rate constants to measured arylsulphatase activities together with the suppressed degradation in an inhibited assay substantiated that assumption and confirmed the *hitherto* only

speculated theory of thio-ester bond cleavage of the estrogen sulphates by arylsulphatase enzymes. Correlations with soil properties revealed that the arylsulphatase activity was mainly of microbial origin and located in the soil's organic matter and clay domains. This is in agreement with previously reported findings (Chen *et al.*, 2001; Speir and Ross, 2002). Compared to waste water treatment plants where estrogen sulphates have been detected in the effluents due to incomplete degradation (D'Ascenzo *et al.*, 2003; Schlüsener and Bester, 2008) the degradation of estrogen sulphates was faster and more complete in the agricultural soils.

The detection of the metabolites E1-3S and E2 as a result of the degradation of E2-3S suggested that two degradation reactions occurred simultaneously. Hydrolysis of E2-3S led to the formation of E2 while oxidation at position 3 resulted in the release of E1-3S. Both metabolites then formed E1 as a common metabolite. From the detected amounts of E2 and E1-3S it was clear that the oxidation prevails over the hydrolyzation. This is the first time estrogen sulphates have been investigated in the soil environment, and therefore comparison is difficult. However, research in engineered systems has shown that E1 concentrations in wastewater treatment plants were higher than expected from degradation rate constants, and a number of authors have attributed these elevated concentrations of E1 to the degradation of estrogen sulphates subsequently releasing free estrogens (e.g., Ternes *et al.*, 1999, D'Ascenzo *et al.*, 2005; Schlüsener and Bester, 2008). In comparison to their free counterparts, estrogen sulphates were degraded faster in the investigated soils. However, when the free compounds were degraded as metabolites of the sulphate conjugates, they persisted in general longer than when they were degraded as single parent compounds. Overall, E1 persisted the longest in the investigated soils with DT_{50} of 6 days at maximum which is in good agreement with previously published studies showing E1 degraded slower than its parent compound (Colucci *et al.*, 2001; Casey *et al.*, 2003; Sarmah and Northcott, 2008).

In terms of the mechanisms of biodegradation the results in this thesis suggested that a combination of intra and extra cellular degradation processes mediated by relevant enzymes, such as dehydrogenases, cytochrmone P-450

oxigenases, and/or transferases. These enzymes were likely to have been responsible for the observed degradation kinetics of free and sulphate-conjugated estrogens. A combination of extra and intra cellular co-metabolic processes responsible for the degradation of E2 and E1 was also recently proposed by Stumpe and Marschner (2007).

The use of kinetic models to determine degradation endpoints is recommended for pesticide dissipation in soils (Beulke and Brown, 2001; Focus, 2006) and have also been applied to estrogen dissipation data (Colucci *et al.*, 2001, Xuang *et al.*, 2008). While a pseudo-first order model (SFO) was appropriate to fit the degradation data of E2-3S, some of the datasets for the degradation of E2, E1-3S and E1 were better described with a double-first-order in parallel model (DFOP) which delivered more accurate estimations for degradation endpoints than the SFO model. The model choice was facilitated by the use of an array of statistical measures including analysis of variance, the adjusted coefficient of determination, the Akaike information criterion, the scaled root mean squared error, and an error percentage to pass a χ^2 test. Herman and Scherer (2006) found that the DFOP model in general provided the best estimates of degradation endpoints for a number of 64 datasets and the use of other models than SFO to describe dissipation kinetics is also recommended by the FOCUS guidance document (FOCUS, 2006). A recently presented concept to model metabolite formation of androgens (Khan *et al.*, 2008b) with SFO kinetics was extended by incorporating the DFOP kinetics and including metabolite formation fractions, and was subsequently used to model the observed metabolite formation kinetics. While this improved modelling concept provided excellent description of the metabolite data of E2 and E1-3S degradation, especially due to the consideration of DFOP kinetics for the parent compound data, it failed to deliver degradation endpoints in some instances caused by iterative procedures that were necessary to estimate these values in the case of the DFOP model. Furthermore, despite acceptable fitting results the simple SFO and DFOP model did not provide enough information about the complex degradation mechanisms involved in the degradation and metabolite formation of E2-3S discussed above.

The possibility of estrogenicity formation during the degradation of E2-3S at 15°C was investigated by means of ER-CALUX® measurements. The results showed that the degradation of E2-3S involved a temporal formation of estrogenicity in the investigated soils. While a majority of the observed estrogenicity was explained by the release and subsequent degradation of the free hormones E2 and E1, results also indicated that the degradation of E2-3S was likely to have followed different pathways in the different soils which may have resulted in the formation of unknown metabolites that possessed estrogenic activity in some of the soils. This is in agreement with the above illustrated hypothesis that a variety of intra- and extra-cellular degradation processes were responsible for the dissipation of E2-3S.

Knowledge about the transport and retention behaviour of estrogens is a prerequisite for a thorough risk assessment and several studies have paid attention to the topic in the past (Das *et al.*, 2004; Casey *et al.*, 2005; Sangsupan *et al.*, 2006; Fan *et al.*, 2008). However, to date there is no published information available about the transport of E1-3S, and it is just recently that the impact of the exposure matrix on free estrogen transport has been highlighted (Lucas and Jones, 2009). In this thesis the transport behaviour of E1 and E1-3S were investigated in undisturbed soil lysimeters as influenced by the exposure matrix. Results showed that the hormones were rapidly degraded during the transport in the 18 cm long columns and no hormones were detected in the leachate when they were applied with $CaCl_2$ (10 mM). However, application with artificial urine resulted in hormone breakthrough curves and about 6 and 18% of the initially applied hormone were recovered at the maximum in the leachate for E1-3S and E1, respectively. Facilitated hormone transport and hampered hormone degradation processes were proposed by Lucas and Jones (2009) as reasons for observed differences in breakthrough curves of E2 and E2 from rain water and artificial urine applications. Intense yellow/brown colouration of leachate during the transport experiments involving artificial urine lent support to the assumption that colloid-facilitated estrogen transport might have occurred. High salt concentrations in the artificial urine likely caused the dissolution and dispersion of soil organic matter which subsequently became available for

estrogen co-transport. Furthermore, the readily available carbon sources glycine and urea may have suppressed the rapid co-metabolic transformation of E1-3S and E1. Results from soil resident concentration analysis in the sectioned soil cores supported that assumption. Only the lysimeters that received the hormones in artificial urine showed significant soil resident concentrations throughout the profile.

In contrast to previously reported results (Sangsupan *et al.*, 2006, Fan *et al.*, 2008) the estrogen maxima in the leachate occurred later than that of a conservative bromide tracer indicating the existence of a retention mechanism, which was possibly rate-limited sorption to soil constituents. Even under pronounced preferential flow conditions, the maxima of E1 occurred delayed highlighting the important role of sorption related retention processes under saturated flow conditions.

A physical non-equilibrium model available in CXTFIT (Toride *et al.*, 1995) was found to be suitable to describe the bromide breakthrough data and explained the observed tailing of the break through curves well, except of the preferential flow pattern observed in one lysimeter. However, the CXTFIT capacities were too limited to deliver information about possible physical and chemical non-equilibrium processes involved in estrogen transport. More recently developed concepts incorporating both non-equilibrium processes appear to be better suited in order describe estrogen transport in undisturbed soil media (Sangsupan *et al.*, 2006; Fan *et al.*, 2008) and may also likely to be appropriate to model estrogen sulphate transport behaviour, although this is yet to be scrutinised.

2 GENERAL CONCLUSIONS

In conclusion, the fate and behavior of E2, E1 and their respective 3-substituted sulphate conjugates in agricultural soils of New Zealand is strongly dependent on the exposure matrix and the specific soil properties. Although degradation of the hormones occurred rapidly in the laboratory incubations, some of the metabolites could persist for prolonged periods and the biological degradation of E2-3S has led to the formation of estrogenicity that could not

be entirely explained by the detected metabolites E2 and E1. Furthermore, sorption and transport behaviour of E1 and E1-3S was strongly impacted by the exposure matrix and the consideration of artificial urine as an exposure matrix has revealed novel insights into estrogen transport and sorption processes. With the knowledge gained from this thesis agricultural grazing practices may be managed in an improved way in the future in order to protect receiving waterways from possible estrogen contamination.

With respect to the research objectives set in this thesis following key conclusions were drawn:

- Dicyclohexylamine hydrochloride as an organic modifier in dichloromethane was found to be suitable for the extraction of estrogen sulphates from aqueous and soil samples.
- A gradient system comprising of ammonium sulphate (5 mM, pH 3), water and acetonitrile is appropriate to separate and detect estrogens and estrogen sulphates with a HPLC-UC system. The implementation of monolithic analytical columns can lead to significant runtime reductions allowing for higher sample throughput.
- Sorption of estrone is moderate in the investigated soils and was about an order of magnitude higher than sorption of estrone-3-sulphate. The presence of the sulphate group changes the governing sorption mechanisms for the compounds and the Freundlich-type sorption isotherms were significantly changed by the use of artificial urine as mediator solution.
- Estrogens and estrogen sulphates are degraded rapidly via biological processes in New Zealand agricultural soils. The activity of arylsulphatase enzymes is necessary for the cleavage of the thio-ester bond in estrogen sulphates which leads to the formation of free estrogens and corresponds to the formation of estrogenicity.
- Single-first order and double-first-order in parallel kinetic models provide a good description of degradation and metabolite formation datasets and are suitable for obtaining degradation endpoints, even though they lack conceptual explanation of the complex interplay of intra and extra cellular degradation processes.

- Artificial urine significantly facilitates the transport of estrone and estrone-3-sulpahte in undisturbed soil lysimeters as compared to $CaCl_2$, most likely through colloid-facilitated transport phenomenon and suppression of the co-metabolic degradation of the compounds caused by artificial urine constituents.

3 FUTURE RESEARCH

Over the course of this three year Ph.D. project, research progress related to the environmental fate of estrogens and estrogen sulphates has advanced substantially and this thesis has contributed two peer-reviewed publications and a third manuscript is currently under revision. The continuous release of novel information about specific processes involved in the fate and behaviour of estrogens and estrogen conjugates in the environment highlights the scientific need to better understand the compounds fate. A better understanding of estrogen fate is also is also necessary from a regulatory and agricultural management perspective. Despite the recent progress, there are still many gaps in the scientific knowledge about the fate and behaviour of single free and conjugated hormones under dairying environment, and the present thesis provides some recommendations for future research:

- More steroid conjugates need to be investigated in respect to their environmental fate and behaviour and their potential to contribute to the concentrations of free steroids detected in the environment.
- The influence of alternative exposure matrices such as artificial urine solutions on the sorption kinetics of estrogens and estrogen conjugates constitutes another research area requiring further investigations.
- While the impact of antibiotic presence has been shown to change degradation kinetics of free estrogens, no information is yet available about the impact on estrogen conjugate degradation. This is a significant gap in the current knowledge given the global presence of antibiotics in livestock operations.

- Recently-developed transport models for the free estrogens that incorporate both physical and chemical non-equilibrium processes, also need to be adapted for estrogen conjugates in order to better predict their transport behaviour in saturated porous media.
- The leaching potential of both free and conjugated estrogens under unsaturated soil water conditions needs to be investigated, since such information would be very useful to manage dairying operations in a manner that protects receiving waters from estrogen contamination.

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