STEROID ESTROGENS AND ESTROGENIC ACTIVITY IN FARM DAIRY SHED EFFLUENTS

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Abstract

Estrogenic contamination of waterways is of world-wide concern due to the adverse effects observed in aquatic biota. Recently, wastes from agricultural activities have been identified as likely sources of steroid estrogens released into the environment. Wastes from dairying activities are of particular concern in New Zealand. This project included development of analytical methods to measure free and conjugated estrogens, measurement of estrogens from the source to receiving environments and an investigation of effluent treatment technologies.

The analytical method developed in this study was based on GC-MS measurement of free estrogens (17α-estradiol (17α-E2), 17β-estradiol (17β-E2) and estrone (E1)) and LC-IT-MS measurement of their sulfate-conjugates (17α-E2-3S, 17β-3S, E1-3S) in raw and treated farm dairy shed effluents (DSE). Effluents from farms in the Canterbury and Waikato Regions, two regions where dairy farming is the dominant land-use, were collected and analysed. All effluents demonstrated high concentrations of steroid estrogens, particularly 17α-E2 (median 760 ng/L). Estrogenic activity was also elevated, at up to 500 ng/L 17β-E2 equivalents using the E-Screen, an *in vitro* cell proliferation bioassay. Comparison to the chemical data indicated that for most samples, the highest proportion of estrogenic activity was derived from steroid estrogens naturally excreted by dairy cows. Conjugated estrogens were measured in several raw effluent samples, at similar concentrations to those of free estrogens, particularly E1.

Dairy effluent treatment systems reduced free estrogen concentrations by 63-99% and reduced estrogenic activity by up to 89%. In spite of high removal efficiencies, estrogens remained elevated in the treated effluents that are discharged into waterways. Steroid estrogens and estrogenic activity were detected in streams and groundwater in areas impacted by dairy farming. Although concentrations were generally low, in two streams the concentrations were above levels regarded as safe for aquatic biota (<1 ng/L). The results demonstrate that dairy effluents are indeed a major source of estrogens to the environment and to waterways.

Abbreviations

CALUX	Chemically activated luciferase gene expression
DCM	Dichloromethane
DES	Diethylstilbestrol
DMSO	Dimethylsulfoxide
DSE	Dairy shed effluent
DVB	Divinylbenzene
E1	Estrone (IUPAC name: Estra-1,3,5(10)-triene-17-one, 3-hydroxy-)
E1-d_4	[2,4,16,16-$^2H_4$]-estrone
E1-3S	Estrone-3-sulfate
E1-3S-d_4	[2,4,16,16-$^2H_4$]-Estrone-3-sulfate-d_4
17β-E2	17β-estradiol (IUPAC name: Estra-1,3,5(10)-triene-3,17-diol-(17β)-)
17β-E2- d_4	[2,4,16,16-$^2H_4$]-17β-estradiol
E2-3,17S	17β-Estradiol-3,17-disulfate
E2-3G	17β-Estradiol-3-glucuronide
E2-3G,17S	17β-Estradiol-3-glucuronide, 17-sulfate
E2-3S	17β-Estradiol-3-sulfate
E2-3S-d_4	[2,4,16,16-$^2H_4$]-17β-Estradiol-17-sulfate-d_4
E2-3S,17G	17β-Estradiol-3-sulfate, 17-glucuronide
E2-17Ac	17β-Estradiol-17-acetate
E2-17G	17β-Estradiol-17-glucuronide
E2-17S	17β-Estradiol-17-sulfate
17α-E2	17α-estradiol (IUPAC name: Estra-1,3,5(10)-triene-3,17-diol-(17α)-)
17α-E2-3S	17α-estradiol-3-sulfate
17α-E2-17G	17α-estradiol-17-glucuronide
E3	Estriol (IUPAC name: Estra-1,3,5(10)-triene-3,16,17-triol-(16α,17β)-)
E3-3G	Estriol-3-glucuronide
E3-3S	Estriol-3-sulfate
EDC	Endocrine disrupting compound
EE2	17α-ethynyl estradiol
EE2-d_4	[2,4,16,16-$^2H_4$]-17α-ethynyl estradiol
EEQ	17β-estradiol equivalents
EIA	Enzyme immunoassay
EPA	Environmental Protection Agency
ER	Estrogen receptor
ERE	Estrogen response element
ESI	Electrospray ionisation
FWHM	Full width at half maximum height (measure of resolution of MS instrument)

GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
GC-MS-MS	Gas Chromatography Mass Spectrometry Mass Spectrometry (tandem mass spectrometry)
GPC	Gel Permeation Chromatography
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
hER	Human estrogen receptor
ISTD	Internal standard
IT	Ion trap (mass spectrometry)
LC	Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS-MS	Liquid Chromatography Mass Spectrometry Mass Spectrometry (tandem mass spectrometry)
MCF-7	Michigan Cancer Foundation -7 (cell line)
medER	Estrogen receptor from Japanese medaka (*Oryzias latipes*)
MeOH	Methanol
MQ	Milli-Q water
MTBE	Methyl tert-butyl ether
NP	Nonylphenol
OP	Octylphenol
PCB	Polychlorinated biphenyl
PNEC	Proposed No Effect Concentration
RIA	Radioimmunoassay
SIM	Selected ion monitoring
SRM	Selected reaction monitoring
SPE	Solid Phase Extraction
STP	Sewage treatment plant
TEF	Toxic equivalency factor
THF	Tetrahydrofuran
TMACl	Tetramethyl ammonium chloride
TOF	Time of flight (mass spectrometry)
TFA	Trifluoroacetic acid
TFAA	Trifluoroacetic anhydride
YES	Yeast estrogen screen, an *in vitro* assay developed to measure estrogenicity of compounds and environmental samples

CHAPTER ONE

INTRODUCTION

Chapter 1: Introduction

1.1 BACKGROUND

Estrogenic compounds have been linked to a wide range of effects in wildlife, particularly in aquatic environments. As early as 1978, a high prevalence of intersex fish (having both male and female gonadal characteristics) was discovered in a sewage effluent lagoon and downstream of a sewage treatment plant (STP) discharge in the River Lea, United Kingdom (Thames Water Authority, unpublished report cited in (1)). Further investigations showed that male rainbow trout and carp produced the egg-yolk protein vitellogenin when exposed to sewage treatment plant effluents, whereas fish in control waters did not (2). Although male fish have the gene for this protein, they do not usually produce it as the female sex hormone estrogen is required to express the gene (3). These 'estrogenic effects' persisted in receiving waters downstream of effluent discharge locations, as demonstrated by elevated vitellogenin concentrations and lower gonad weight in wild and caged male fish compared to control fish (4,5); and by a high incidence of intersex in wild fish (6).

These observations led to investigations into the cause of this 'feminizing' effect of the effluent. Initially the synthetic estrogen ethynylestradiol (in the contraceptive pill) and nonylphenols (degradation products of nonylphenol ethoxylate detergents) were postulated as potential causes of this feminising effect of effluent (7). Nonylphenols had been shown to act in a similar way to estrogen in biological assays (8). However, toxicity identification evaluation procedures suggested that the natural estrogen hormones 17β-estradiol and estrone excreted in human urine and faeces were equally likely to cause the observed estrogenic effects from sewage effluents (9,10). Subsequently, numerous studies have examined natural and synthetic estrogens in STP influents, effluents and downstream receiving environments.

1.2 ENDOCRINE SYSTEM AND ENDOCRINE DISRUPTION

1.2.1 Endocrine System

All multi-cellular organisms require intercellular communication mechanisms which allow them to respond to their environment and maintain homeostasis (11). The endocrine (or hormone) system is a cell signalling system that plays a critical role in the key processes of development, growth, reproduction, behaviour, and metabolism (11,12). This system consists of several glands (e.g., adrenal, pituitary, gonads) that synthesise and secrete hormones which are then transported via the circulatory system to target cells. On reaching a target cell, the hormone binds to a specific hormone receptor, and then the receptor/hormone complex attaches to a specific segment of DNA called the response element to activate or inhibit gene expression, ultimately leading to protein synthesis. Figure 1.1 shows that the hormone 17β-estradiol binds to the estrogen receptor (ER), then the ligand-ER activated complex binds to the estrogen response element (ERE) as a dimer to up or down regulate genes that are modulated by estrogens.

1.2.2 Steroid Metabolism and Excretion

The most active form of the endogenous estrogen hormone is the steroid 17β-estradiol (17β-E2) which is found in the circulatory system of all vertebrates. Less active forms of estrogen are also present in vertebrates, including estrone (E1), estriol (E3) and 17α-estradiol (17α-E2) (Figure 1.2), though each varies in importance between species. Hormones that do not reach target cells or have completed their task of receptor binding and protein synthesis are inactivated, primarily via the liver and kidneys, before excretion (11). Estrogens are inactivated by conversion to weaker forms; or by conversion to more water soluble compounds that can be readily excreted by the addition of sulfate or glucuronide groups. These groups can be attached at either the 3 or 17 position, or both and are referred to as conjugated estrogens (Figure 1.3).

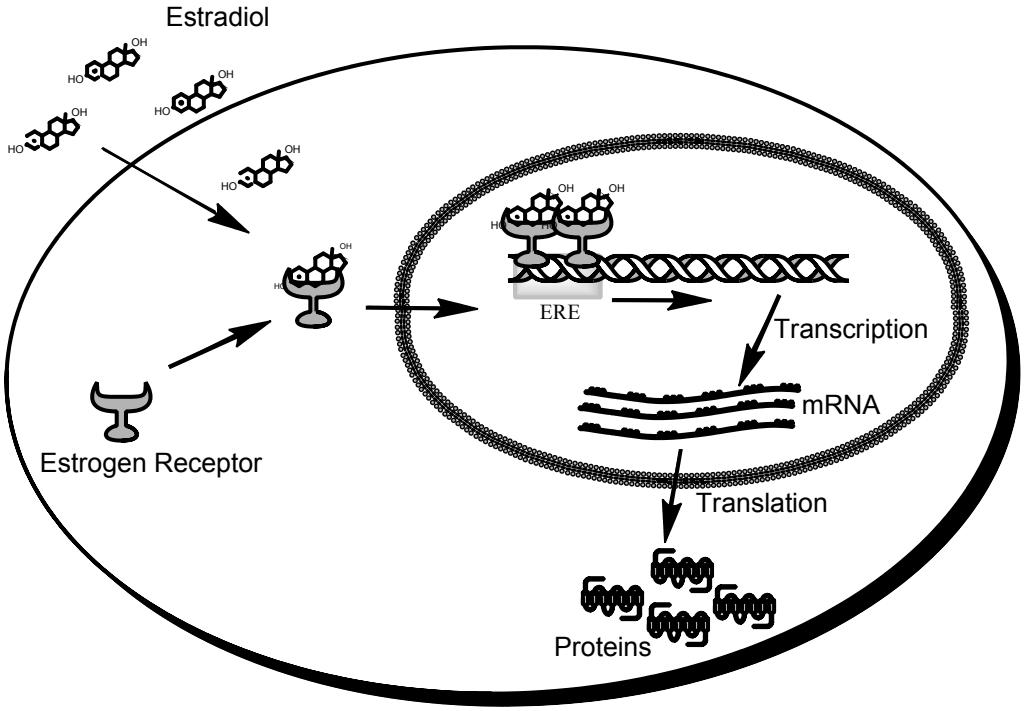


Figure 1.1: Simplified process of estrogen receptor binding and protein synthesis. The steroid hormone 17β-estradiol enters the cell and binds to the receptor. The ligand-ER complex enters the nucleus and forms a homodimer that binds to the estrogen response element (ERE) on the DNA chain. This initiates gene transcription and subsequently production of new proteins.

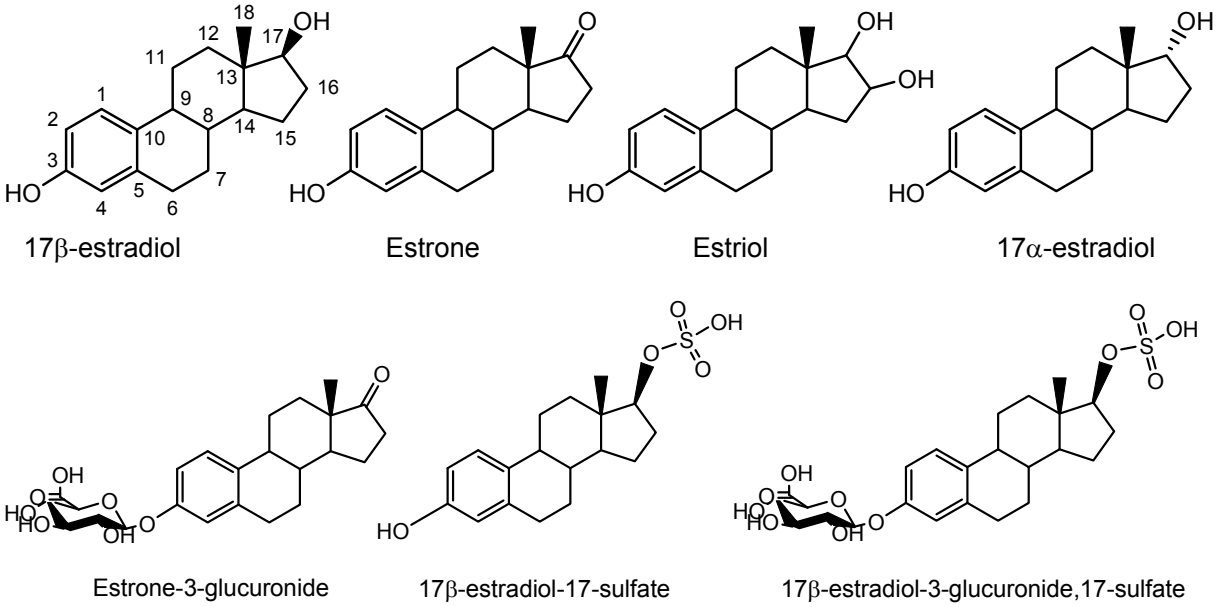


Figure 1.2: Structures of steroid estrogens and conjugated steroid estrogens. Carbon atom numbers indicated on 17β-estradiol.

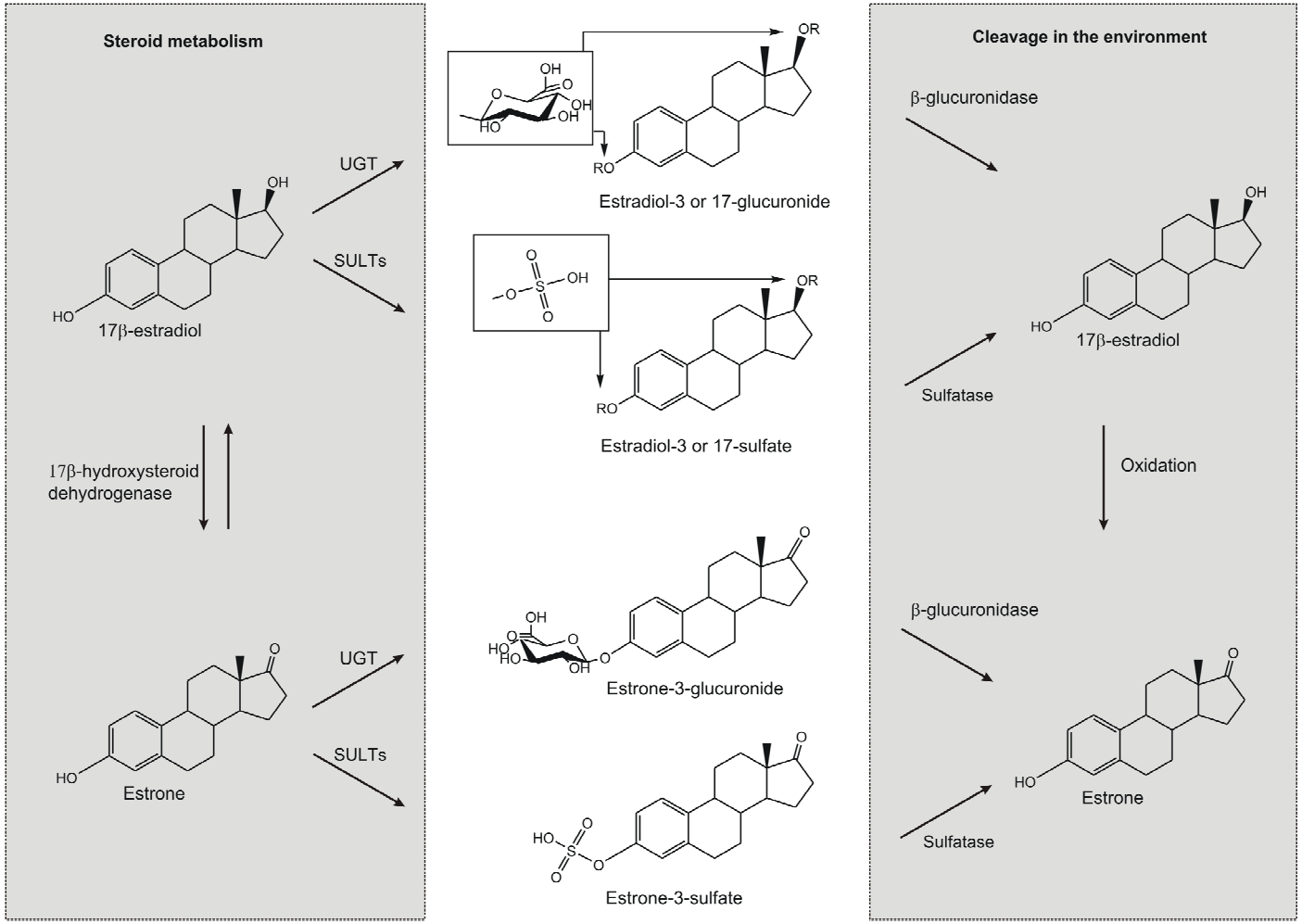


Figure 1.3: Inactivation of 17β-estradiol through oxidation to estrone and conjugation to glucuronide and sulfate groups. Glucuronidation and sulfation reactions catalysed by UDP-glucuronosyltransferase enzyme (UGT) and sulfotransferases (SULTs) respectively. Deglucuronidation and desulfation reactions catalysed by β-glucuronidase and sulfatase enzymes respectively.

Daily excretion rates of estrogens depend on gender and reproductive state. Pregnant women can excrete more than 30 mg of E3 per day as conjugated forms in urine (13), whereas usual cycling (menstruating) levels of E3 in urine are 5-14 μg/day with similar levels of E1 (8-21 μg/day) and 17β-E2 (3-11 μg/day) (14). Males excrete lower levels at 1.5 μg/day for E1, 1.5 μg/day for 17β-E2 and 4 μg/day for E3 (14). Although predominantly excreted as conjugates, the sulfate and glucuronide groups can be removed through hydrolysis reactions to reform the 'free' estrogen. This process has been demonstrated in sewage (15,16) and results in the presence of the free estrogens in sewage effluents.

Likewise, other mammals excrete estrogen in urine and faeces and rates may be much higher: for example, cows excrete ~200 μg/day of total estrogens in urine when cycling (17), and 43-100 mg/day in urine at the end of pregnancy (18,19). These rates do not include faecal excretion, which is an equally or more important excretion route for cattle (20). Estrogen excretion from some species is lower, such as chickens, where layer hens excrete ~3.3 μg/day of E1 and ~3.0 μg/day of 17β-E2 while non-laying hens excrete 0.93 μg/day of E1 and 2.3 μg/day of 17β-E2 (21). Livestock excretion results in their presence in agricultural wastes (e.g., manures) and potentially increases estrogenic contamination of aquatic receiving environments through discharges of agricultural wastes.

1.2.3 Endocrine Disruption

Endocrine disrupters interfere with the normal process of hormone/receptor binding and protein synthesis, predominantly via interaction with the hormone receptors (11). Endocrine disrupting compounds (EDCs) can bind to receptors and activate gene expression analogous to the endogenous hormone (an agonist). Alternatively, a compound can bind to the receptor without activating gene expression and in doing so, prevent binding and gene expression by endogenous hormones (antagonists). Non-receptor mediated mechanisms are also possible, including alterations in the synthesis, metabolism or transport of endogenous hormones (22); alterations in the synthesis or binding affinity of hormone receptors; alteration of post-receptor activation (23) and by disrupting 'cross-talk' between different hormone receptors (11). Although endocrine

disruption has been most clearly demonstrated for reproductive hormones, particularly estrogen and testosterone, and thyroid hormones (11,12), it is likely that other hormones will be similarly susceptible (24).

A wide range of adverse effects in wildlife and humans were linked to an endocrine disruption mechanism by Colburn et al. (25). Wildlife effects include imposex in marine gastropods caused by the anti-foulant tributyltin; masculinisation of fish downstream of bleached Kraft pulp and paper mills; intersex and vitellogenin induction in fish downstream of sewage effluents (as described earlier); and abnormalities in steroid hormone levels, decreased penis size and poor reproductive success in alligators in a pesticide contaminated lake (25,26).

The form of endocrine disruption most studied to date is mediated through the estrogen receptor, with a wide range of compounds identified as estrogen agonists (also known as estrogenic compounds). Estrogenic compounds are defined not by a common chemical structure, but by their common action. In most cases, this is through interaction with the estrogen receptor, however there is also evidence of cross-talk and non-ER mediated mechanisms (12). The definition proposed by Hertz (27), that 'estrogens are substances which elicit the proliferative activity of the organs of the female genital tract' has been used in this thesis as it relates most appropriately to the bioassay method predominantly used (the E-Screen, Chapters 4-7).

These structurally diverse compounds include natural and synthetic hormones, compounds produced by plants and fungi (phyto- and myco-estrogens respectively) and the so-called xenoestrogens. Xenoestrogens are anthropogenic estrogenic compounds such as nonylphenols, bisphenol A and some phthalates and pesticides (Table 1.1). Xenoestrogens tend to bind only weakly to the estrogen receptor, reflected in their low binding affinities compared to the synthetic and natural hormones, and their low potency in a prolilferation assay (Table 1.1). The wide range in binding affinities and estrogenic potencies are coupled with a wide range in environmental concentrations of estrogenic compounds. Industrial compounds, such as nonylphenol, can be present at sub µg/L to low µg/L in riverine waters while the more potent hormones are present only at sub to low ng/L concentrations (28).

Table 1.1: Known estrogenic compounds and their relative potencies in ER binding assays and a cell proliferation assay.

Compound	Chemical Class / Use	Relative ER binding affinity (29-31) [a]	Relative potency in cell proliferation assay (E-Screen) (29,31,32) [b]
17β-estradiol	Endogenous sex hormone	1	1
Estrone	Endogenous sex hormone	0.007-0.60	0.01-0.012
Estriol	Endogenous sex hormone	0.03-0.75	0.071-0.1
17α-ethynyl estradiol	Synthetic hormone	1.2-8.7	1-1.25
DES	Synthetic hormone	1.3-4.7	2.5-10
Zearalenone	Mycoestrogen	0.05	0.01
Coumestrol	Phytoestrogen	0.0012-0.93	0.00001-0.00011
Genistein	Phytoestrogen	0.0001-0.14	0.000013
p-nonylphenol	Industrial chemical	0.00007-0.00018	0.000013-0.00008
4-octylphenol	Industrial chemical	0.0003-0.0019	0.0001-0.0002
Bisphenol A (BPA)	Plasticiser	0.00023-0.0023	0.00001-0.000025
Benzylbutylphthalates	Plasticiser	0.00003	0.000003
2′,4′,6′-trichloro-4-hydroxybiphenol	PCB	0.054	0.0001
Kepone	Pesticide	0.0019	0.000001
p,p′-DDT	Pesticide	0.0000054	0.000001
o,p′-DDT	Pesticide	0.00003-0.00089	0.000001

Note: [a] ER binding affinity relative to 17β-estradiol, unitless. [b] Potency in E-Screen assay relative to 17β-estradiol, unitless.

1.3 AGRICULTURE AS A POTENTIAL SOURCE OF ESTROGENS

1.3.1 Potential Loads Estimated from Excretion

Urinary and faecal excretion rates have been used by several authors to estimate and evaluate the potential load of estrogens from different human and livestock sources. Estimates for human and livestock populations in the UK (33), the Netherlands (34), the EU and the US (35) indicate the importance of livestock as a source of steroid estrogens (Table 1.2). For the UK, the estimated total estrogen (E1 plus 17β-E2) excretion from the human population was 365 kg/year, while excretion from farm animals was 1520 kg/year, with the majority of this from dairy cattle (1058 kg/year, 70% of livestock load) (33). This is despite substantially fewer dairy cattle (2.2. million) than people (59 million) and reflects the higher daily excretion rates from cattle compared to that from humans

(33). In the Netherlands, the relative livestock contribution is even higher at 94% of the total estimated estrogen excretion, and dairy cattle are again the dominant source (63% of total livestock emissions) (34). Dairy cattle were also estimated to be the largest contributors of estrogens in the EU and USA compared to pigs, sheep and chickens (35). These loading calculations are at best an estimate of the potential load that may enter the environment and must be supported by actual data from analyses of agricultural wastes.

Table 1.2: Estimated loads of estrogens from livestock from various countries/ regions (kg/year).

	UK (33) [a]	**Netherlands** (34)	**EU** (35)	**US** (35)
Humans	365	1,170	NC	NC
Dairy cattle	1,058	11,700	24,600	45,000
Laying hens	NC [b]	440	2,700	2,400
Broiler chickens	49	NC	93	280
Pigs	386	3,900	3,000	830
Sheep	27	NC	1,300	92
Total Livestock	1,520	16,900	31,700	48,600
Total	1,885	18,000	31,700	48,600

Notes: [a] Sum of 17β-E2 and E1. [b] NC: Not calculated.

1.3.2 Measured Concentrations in Agricultural Wastes

Steroids were in fact identified in agricultural wastes as early as 1978, when estrogenic activity was measured in poultry litter processed for livestock feed (36). Despite this, and another early study highlighting potential issues with estrogens administered to animals subsequently entering the environment (37), there was little research in this field. In the late 1990s, widespread publicity regarding endocrine disrupting chemicals and their effects on wildlife (38) rekindled research into the presence of estrogens in agricultural wastes as potential sources of environmental estrogens. Most of the research reviewed has been published only in the last 5 years, highlighting the novelty of this research area.

These recent studies demonstrate high concentrations of steroid estrogens in agricultural wastes, including dairy wastewaters and manures, swine wastewaters, slurries and manures and poultry litter (Table 1.3). These concentrations are well above those found in sewage treatment plant effluents, indicating the potential magnitude of dairy wastes as a source of estrogens. Nevertheless, most studies are limited to a small number of samples from a small number of agriculture operations, with the exception of a comprehensive

study by Raman et al. (39), who analysed triplicate samples collected from multiple locations (up to eight) within a range of animal waste facilities.

Table 1.3: Estrogen concentrations in agricultural wastewaters (ng/L, mean reported where n>1).

Agricultural operation	Country	E1	17α-E2	17β-E2	E3	N [a]	Ref.
Piggery/Swine Facilities							
Farm	NZ	27.3	8.0	10.9	BDL	1	(40)
Farm WWTP	Japan	5300	665	1250	2600	2	(41)
Nursery	US	392	NM	48	208	4	(42)
Nursery	US	731	63	37	351	3	(43)
Finisher	US	74,700	NM	125	302	1	(42)
Finishing lagoon [b,c]	US	10,000	3000	3000	NM	24	(39)
Finishing hoop structure [b,c]	US	54,000	3000	40,000	NM	9	(39)
Finisher	US	1547	179	123	1543	3	(43)
Farrowing sows	US	14,124	NM	1971	7831	5	(42)
Farrowing facility [d]	US	6100	NM	1300	700	2	(44)
Farrowing lagoon [b,c]	US	6000	3000	4000	NM	8	(39)
Farrowing pit [b,c]	US	56,000	6000	18,000	NM	16	(39)
Farrowing sows	US	9940	1197	194	6288	3	(43)
Dairy Facilities							
Shed effluents	NZ	1164	356	159	ND	6	(40)
Drain with shed effluent	Australia	38	NM	8.6	NM	1	(45)
Dry stack manure sludge [b,c]	US	48,000	84,000	22,000	NM	18	(39)
Dry stack manure solids [b,c]	US	30,000	28,000	12,000	NM	12	(39)
Holding ponds [b,c]	US	8000	3000	2000	NM	16	(39)
Wastewater	US	70	224	148	<8	3	(43)
Flushed manure wastewater [c]	US	672	NM	344	NM	2	(46)
Flushed manure wastewater [c]	US	551	2114	672	BDL	5	(47)
Wastewater ponds	US	~300	~2200	~200	NM	6	(48)
Other Livestock Facilities							
Beef feedlot	US	17	6	<20	<8	3	(43)
Poultry	US	2246	265	40	340	3	(43)
Goat	NZ	157	47.1	172	BDL	1	(40)

Notes: [a] N = Number of samples. [b] Estimated from chart to nearest whole number. [c] Data for whole samples, not pre-filtered. [d] Estimated from chart to nearest 100 ng/L.

Wastes from different species have different steroid profiles, with 17β-E2 and E1 dominating in swine and poultry wastes while 17α-E2 and E1 dominate in dairy wastes. The mean results of Raman et al. (39) for 17β-E2, 17α-E2 and E1 are substantially higher than results reported by many other authors (Table 1.3). Their data were confirmed by comparing results obtained by GC-MS, ELISA and an estrogenic assay (YES). Fine et al. (42) similarly reported very high concentrations of E1 in swine wastewaters, with a mean concentration of 14.1 μg/L for wastewaters from farrowing sows and a concentration of 74.7 μg/L in a wastewater sample from a piggery finishing facility. Both authors (39,42)

reported higher steroid estrogen concentrations in samples containing comparatively higher solids. Heterogeneity between samples, sites and facilities is a likely cause of the observed variation in reported concentrations by different authors (Table 1.3). Piggery wastes typically contain higher estrogen concentrations than dairy wastes and may be due to lower dilution of piggery wastes compared to dairy wastes, due to different on-farm waste management practices.

The estrogenic activity of the agricultural wastes has also been analysed using biological assays to provide an integrated estimate of potential effects. This is limited to just four studies, again with typically a small number of samples (Table 1.4). These preliminary data with 17β-estradiol equivalents (EEQ) of 33-3100 ng/L further demonstrate the potential risk from agricultural wastes in the environment.

Table 1.4: Estrogenic activity in agricultural wastewaters (ng/L) and solid wastes (ng/g, mean reported where n>1).

Agricultural Operations	Country	EEQ	N	Ref.
Wastewaters				
Dairy farms	NZ	341	6	(40)
Goat farm	NZ	61	1	(40)
Piggery	NZ	33	1	(40)
Piggery operations	Japan	650 & 3100	2	(41)
Piggery – farrowing facility	US	843 & 858	2	(44)
Solid wastes				
Piggery operations	Canada	~4000	25	(49)
Dairy & veal manure	Canada	~3000	7	(49)
Dairy cow manure	Canada	~850	6	(49)
Beef cattle faecal pats	Canada	~6	6	(49)
Chicken litter	Canada	~150	24	(49)

In spite of conjugated estrogens being the dominant forms of estrogens excreted in urine, only one study has measured these compounds in agricultural wastes, possibly due to a lack of available analytical methods. Hutchins et al. (43) analysed conjugated estrogens in wastes from a range of concentrated animal feeding operations in the United States. For most animal facilities, concentrations of estrogen sulfate conjugates were at low ng/L in wastes and glucuronide conjugates were not detected (Table 1.5). In contrast, wastes from dairy facilities contained sulfate conjugates at concentrations of 39-174 ng/L, within the range of concentrations measured for free estrogens in the same samples. This illustrates

the potential importance of conjugated estrogens, if they can hydrolyse to free bioactive forms under environmental conditions or within effluent treatment systems.

Table 1.5: Range in conjugated estrogens concentrations in agricultural wastes (ng/L, n=3, from ref (43)).

Source	E1-3S	17α-E2-3S	17β-E2-3S	17β-E2-17S
Swine sow	1.7-2.0	<1.0	<1.0	72.0-84.3
Swine farrowing	<1.0	<1.0	<1.0	<1.0
Swine nursery	<1.0	<1.0	<1.0	<1.0
Poultry operation 1	2.0-3.8	<1.0	8.0-13.9	<1.0
Poultry operation 2	0.0-2.2	<1.0	<1.0	<1.0
Dairy farm	85.0-91.0	141-174	39-44	<1.0
Beef feedlot	<1.0	<1.0	<1.0	<1.0

1.3.3 Potential Loads from New Zealand Agricultural Sources

Livestock are likely to be an important source of estrogens in New Zealand, due to the predominance of agriculture and in particular, dairy farming. Although agriculture is widespread throughout New Zealand, the Waikato Region is the largest dairying region, with 1,669,000 dairy cattle, 32% of the national dairy herd. The Canterbury region of the South Island has fewer dairy cattle, but numbers have increased substantially, almost tripling in less than 10 years, from 275,000 in 1999 to 755,000 in 2007.

The estimated loads from different livestock sources have been calculated for New Zealand from excretion rates used for previously published estimates of estrogen loads (33-35). As each author used a different method and different daily excretion rates in their estimated loads, each method has been used to estimate the New Zealand loads (Table 1.6). The total load of estrogens calculated based on the Blok and Wosten (34) method is substantially higher than estimated based on the Lange et al. (35) and Johnson et al. (33) methods; however both indicate that dairy cattle are by far the most important contributors of estrogens to the environment. The estimated load from the human population is minor by comparison.

On New Zealand dairy farms, the majority of animal excreta is deposited onto paddocks during free range grazing and is not subject to any treatment. However, 10-20% of excreta is deposited within the farm dairy shed or yard area during milking (50), where it is captured as farm dairy shed effluent (DSE), which also contains wash-down water and chemical products used to clean the milking plant (50). This effluent is then either applied

to land, or treated and discharged into waterways. Discharge of treated effluents to waterways represents a potential risk for aquatic biota downstream.

Table 1.6: Estimated loads of estrogen excreted from New Zealand livestock and human populations based on different emission rates.

		Estimated total estrogen excretion (kg/yr) based on:		
Animal	**Population (000s)**	**Lange et al. (35)**	**Johnson et al. (33)**	**Blok & Wosten (34)**
Pig	356 [a]	16	27	72
Laying hens	3,325 [b]	24	11	8,561
Dairy cattle [c]	5,110 [d]	4,202	2,281	43,995
Broiler chicken	12,513 [b]	2.6	5.5	937
Sheep	35,897 [d]	619	124	NC
Total livestock	**60,514**	**4,918**	**3,928**	**54,896**
Humans	4,135 [e]	NC	26	391
Total humans & livestock	**64,649**	**4,918**	**3,953**	**55,286**

Note: [a] Data from 2003 agricultural production census (51). [b] Data from 2002 agricultural production census (52). [c] Dairy cattle estimate does not include contribution from calves as this was not included by Johnson et al. (33). [d] Data from 2007 agricultural production census (53). [e] Data from 2006 census (54).

1.3.4 Treatment and Disposal of Agricultural Wastes

Removal of steroid estrogens in sewage treatment plants (STPs) has been well-characterised over the past ten years, with the primary removal processes identified as sorption to solids and aerobic degradation (55). Conjugated estrogens are removed by initial hydrolysis to free forms within the sewerage system and in sewage treatment plants (55), and are then removed as free estrogens. Removal in agricultural waste treatment systems is not as well-characterised, particularly for liquid wastes. Furthermore, the treatment of agricultural wastes is typically more rudimentary than treatment of sewage. Initial studies indicate 44-99% of steroid estrogens in swine or dairy wastewaters are removed following anaerobic sludge treatment or passage through lagoons or wetlands (41,44,48). Initial studies in New Zealand (40) demonstrated the presence of steroid estrogens in DSE treated through pond systems, suggesting removal is not as good in these systems.

Approximately one-third of farms in the Waikato Region, the largest dairy farming region in New Zealand, continue to discharge treated DSE to waterways. If estrogens are not

adequately removed during treatment, as suggested from preliminary data, DSE discharged into waterways potentially represent the major source of estrogens to New Zealand's aquatic environments and a high risk to aquatic biota downstream.

1.4 THESIS RATIONALE AND OBJECTIVES

As described in the preceding sections, endocrine disruption and the presence of estrogenic compounds in aquatic environments is of world-wide concern. Much of the research to date has investigated sewage treatment plants as major contributors of steroid estrogens and xenoestrogens. Recently agricultural effluents have been identified as likely sources of steroid estrogens into the environment and this is of particular concern in New Zealand, a country whose economy largely depends on agriculture. Initial investigations in New Zealand (40,56) indicate that significantly higher concentrations of steroid estrogens are likely be found in dairy effluents when compared to concentrations in STP effluents.

Based on this, the overall aim of this thesis was to assess whether dairy shed effluents contribute significant amounts of steroid estrogens to New Zealand's aquatic environment.

Specific objectives were as follows:

1. To develop a method to enable the analysis of free and conjugated estrogens in dairy shed effluents and environmental samples.

2. To determine levels of free and conjugated estrogens in dairy effluents and compare results with those generated using biological assays.

3. To assess whether estrogens are removed in dairy effluent treatment systems used prior to discharge of effluents to aquatic receiving environments.

4. To investigate the presence of steroid estrogens and estrogenic activity in aquatic receiving environments in catchments with a high proportion of dairy land use.

1.5 THESIS STRUCTURE

This thesis is presented in six chapters following this introduction. Because each chapter, with the exceptions of this introduction and the final chapter, is presented as a stand-alone paper, there is some repetition between chapters particularly with respect to experimental methods.

Chapter 2 reviews analytical methods for analysis of free and conjugated estrogens in wastewaters.

Chapter 3 describes the development of analytical methods to detect and quantify steroid estrogens and conjugated estrogens in dairy shed effluents and environmental samples.

Chapter 4 presents the results of a survey of estrogens and estrogenic activity in dairy shed effluents from farms in the Canterbury and Waikato regions of New Zealand.

Chapter 5 presents an investigation of estrogen removal comparing a traditional two-pond based system with an advanced pond system used to treat dairy effluents in the Waikato Region of New Zealand.

Chapter 6 compares the estrogenic activity of DSE samples measured in the E-Screen assay with that in receptor-reporter gene yeast based assays.

Chapter 7 describes preliminary investigations into the presence of steroid estrogens and estrogenic activity in aquatic receiving environments in catchments with predominantly dairy land use.

Chapter 8 combines the findings of this thesis, discusses overall conclusions with reference to the objectives of the research and presents recommendations for future research.

CHAPTER TWO

REVIEW OF METHODS TO ANALYSE ESTROGENS IN WASTEWATERS

Chapter 2: Review of Methods to Analyse Estrogens in Wastewaters

2.1 INTRODUCTION

The steroid estrogens 17β-E2, E1, E3 and 17α-E2 can be measured either biologically utilising their common mechanism of action, or by targeted chemical analysis. The classic biological method for measuring estrogen induction of cell proliferation is an increase in rat uterine wet weight (23). Due to the length of time and ethical considerations with this *in vivo* test, a range of *in vitro* assays have been developed and have since been used to screen compounds and environmental samples for estrogenic activity. The mechanisms, advantages and disadvantages of *in vitro* bioassays are briefly discussed in this chapter. Chemical methods for the analysis of free estrogens in sewage influents and effluents, and in some agricultural wastes have been published. These methods are typically based on GC-MS or LC-MS and these are briefly reviewed.

The analysis of conjugated estrogens is very challenging, and is particularly demanding in wastewaters due to their high complexity. There are far fewer published studies of these compounds. Nonetheless, there are a growing number of studies analysing conjugated estrogens in addition to free estrogens in sewage (16,57-64), surface waters and river sediments (65,66). Additionally, a recent paper has assessed conjugated estrogens in agricultural wastewaters (43).

Historically, analysis of conjugated estrogens (e.g., in urine or plasma) used methods based on the hydrolysis of conjugated estrogens into the free form followed by analysis as free estrogens. These hydrolysis methods have been used to measure conjugated estrogens in STP influents and effluents. However, these polar compounds are well-suited to analysis by LC-MS and these instruments are now widely available. The methods reviewed in this chapter focus on those utilising LC-MS(MS) analysis. In addition to analysis, sample preservation and preparation procedures are reviewed.

2.2 *IN VITRO* BIOASSAYS

In general, *in vitro* assays tend to be very sensitive, repeatable and low cost, and can provide information on mechanisms of estrogen action (67). *In vitro* bioassays measure all estrogenic compounds present in environmental samples based on their biological activity, and do not require prior knowledge of the chemical constituents. Measurements include the contribution from any steroid estrogens, phytoestrogens, mycoestrogens and xenoestrogens present in a sample. In this way they provide an integrated measurement of estrogenic activity that organisms may be exposed to. In environmental samples, results are typically expressed as 17β-estradiol-equivalents (EEQ, usually in ng/L) by comparison to the response of pure 17β-estradiol in that assay.

The *in vitro* assays can be classified into three groups: receptor binding assays, receptor-reporter gene assays and cell proliferation assays. The mechanisms, advantages and disadvantages of these groups are summarised briefly below. These aspects, specific examples of assays, and their protocols have been previously reviewed in detail (67-70).

Receptor binding assays are based on the primary mechanism of action of estrogenic compounds, which is binding to the estrogen receptor (ER). Typically, samples are added to a preparation of estrogen receptors along with radiolabelled 17β-E2 and the amount of 17β-E2 competitively displaced is determined. Estrogen receptors for these assays have been isolated from mammalian tissues, such as rat (71,72) or sheep (31) uteri, and fish tissues such as rainbow trout livers (73,74). A more novel method is to extract receptors produced by recombinant *E. coli* (75) or human liver cancer cells (76). A disadvantage of receptor binding assays for environmental samples is that they merely indicate the presence of compounds able to bind to the estrogen receptor. This does not imply that they can elicit an ER-mediated response, as the compounds may be either estrogen agonists or antagonists. Furthermore, as these assays are extra-cellular, they cannot account for differences in cell uptake or metabolism of chemicals.

Receptor-reporter gene assays require not only that a compound bind to the estrogen receptor, but that they induce receptor-mediated transcription and therefore measure agonists only. Reporter gene assays are usually based on yeast, fish or mammalian cells, genetically modified by adding a reporter gene sequence that encodes for transcription of

a protein such as galactosidase or luciferase, which is then measured directly or indirectly. In yeast cell assays, an estrogen receptor (typically hERα) must also be inserted into the cell along with the reporter-gene sequence, while the mammalian and fish cell lines contain endogenous receptors. These assays have been used the most frequently for assessing estrogenicity of environmental samples, particularly the YES (Yeast Estrogen Screen) assay.

Cell proliferation assays use estrogen responsive cancer cell lines that proliferate in the presence of estrogenic compounds. The most commonly used assay is the E-Screen, which uses MCF-7 (human breast cancer) cells. Cells are exposed to estrogenic samples, incubated for 4-6 days, and the increase in number of cells is directly or indirectly measured (e.g., measuring protein or metabolised dyes) (70). The long incubation time, variation between cell lines, and poor reproducibility between laboratories may decrease the utility of the E-Screen assay, however it remains one of the most sensitive *in vitro* assays for assessing estrogenic activity in environmental samples (67).

2.3 CHEMICAL ANALYSIS OF FREE ESTROGENS

While bioassays provide information on the potential effects of effluents or other environmental samples, when used in isolation they provide no information on the causative compounds, which is essential for managing sources of estrogenic contamination. Chemical analyses typically target particular compounds or groups, although broad screen approaches have also been used. The targeted approach is most applicable when measuring trace levels of analytes in complex samples, as procedures are specifically adapted for the chemical properties of target analytes while interfering compounds in the samples are discarded or eliminated as far as possible.

Specific analytical methods for quantifying free estrogens in environmental samples have been reviewed for a range of matrices including surface waters (77-79), municipal wastewaters (80), sludges, sediments and soils (81). The analyses of a broader range of estrogenic compounds and other endocrine disrupting compounds are reviewed in references (28,82,83) and those cited therein.

Because free estrogens are present at low concentrations in wastewaters (ng/L), large volume samples must be extracted and concentrated to allow their measurement. Solid phase extraction is a convenient way to do this and has been most widely used in this field, whereas liquid-liquid extraction is rarely used. Reverse-phase sorbents are typically used, such as C18 or carbograph, though more recently there has been a move towards newer phases such as divinylbenzenes (DVBs) and mixed-mode sorbents. After extraction of typically 0.5 L (sewage influents), 1 L (sewage effluents) or 2 L (waters), free estrogens retained on the cartridges can be eluted in a small volume of solvent, thus concentrating samples by a factor of 50-200.

Subsequent cleanup of free estrogens depends on the sensitivity and selectivity of the quantitation method, but is generally required for sewage influents, effluents and agricultural wastes. The most commonly used clean-up strategies for free estrogens are based on adsorption/partition chromatography on SPE columns, using silica (84,85), florisil (58,63,86), C18 (57), aminopropyl (87) or a combination (88). GPC has been used to remove high molecular weight interferences before GC-MS analysis (65,89). Novel methods reported include strong anion exchange (65) and immunoaffinity adsorption chromatography (90).

Most methods for quantitation of natural estrogens in waters and wastewaters use selective and sensitive techniques such as LC-MS(MS) or GC-MS(MS), though derivatisation to more volatile compounds is required for the latter. While LC-MS is more appropriate for semi-polar analytes such as estrogens without the need for derivatisation, detection limits have generally been too high for environmental samples, particularly for complex matrices such as sewage wastes, where ionisation suppression further increases detection limits. LC-MS-MS is becoming more common for analysis of environmental estrogens as these instruments become more widely available and they are able to achieve detection limits comparable to, or better than, GC-MS. Aspects of mass spectrometry have been previously reviewed for the environmental analysis of free estrogens (91) and for analysis of a broader range of EDCs (92).

2.4 CHEMICAL ANALYSIS OF CONJUGATED ESTROGENS

Analysis of conjugated estrogens can be important as they are the primary form excreted in urine by mammals and there is potential for them to be hydrolysed in the environment to the more potent free estrogens. There are a large number of conjugated estrogens that may be excreted by mammals and some of these are listed in Table 2.1 along with their physico-chemical properties. These compounds demonstrate a wide range in aqueous solubility and pKa, factors which have relevance to their extraction and subsequent analysis.

Table 2.1: Physico-chemical properties of conjugated estrogens [a].

Name	Abbrev.	CAS. No.	Mol. wt.	Water solubility (g/L)	logP	Est. pKa
Estrone-3-glucuronide	E1-3G	2479-90-5	446.5	549	1.375	2.80
Estrone-3-sulfate	E1-3S	481-97-0	350.4	7.4	3.508	-3.84
17β-Estradiol-3-glucuronide	E2-3G	15270-30-1	448.5	410	1.818	2.80
17β-Estradiol-17-glucuronide	E2-17G	1806-98-0	448.5	440	2.109	2.82
17β-Estradiol-3-sulfate	E2-3S	481-96-9	352.4	4.2	3.951	-3.82
17β-Estradiol-17-sulfate	E2-17S	3233-69-0	352.4	2.0	4.399	-3.50
17β-Estradiol-3,17-disulfate	E2-3,17S	3233-70-3	432.4	6.9	4.219	-3.99
17β-Estradiol-3-glucuronide, 17-sulfate	E2-3G,17S	84123-28-4	528.5	914	2.087	-3.49
17β-Estradiol-3-sulfate, 17-glucuronide	E2-3S,17G	26923-03-5	528.5	999	1.929	-3.82
17α-estradiol-3-sulfate	17α-E2-3S	22139-70-4	352.4	4.2	3.951	-3.82
17α-estradiol-17-glucuronide	17α-E2-17G	33602-53-8	448.5	440	2.109	2.82
Estriol-3-glucuronide	E3-3G	2479-91-6	464.5	999	0.631	2.80
Estriol-3-sulfate	E3-3S	481-95-8	368.4	20	2.764	-3.82

Notes: [a] Data sourced from SciFinder Scholar (American Chemical Society), calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris.

2.4.1 Sample Handling and Preservation

Appropriate sample preservation is required to prevent the hydrolysis of conjugated estrogens to their free forms in the presence of bacteria, particularly in sewage influents. However, few studies have preserved samples and have relied on rapid extraction of the samples and storage at < 4 °C. Reddy et al. (93) compared sample preservation for conjugated estrogens and found that the addition of mercury chloride (50 mg/L) or

formaldehyde (0.4%) did not prevent hydrolysis of 17β-estradiol-3-glucuronide in sewage effluents and influents, even within the short period between sample collection, filtration and extraction, while acidification with sulfuric acid to pH 2 did prevent hydrolysis. Similarly, a Danish Environmental Protection Agency study (64) demonstrated the stability of conjugated estrogens spiked into STP effluents that were preserved by acidifying to pH 3 with sulfuric acid and storing at 4 °C. This study also demonstrated no appreciable loss of conjugated estrogens to glass sampling bottles, and no difference between untreated and silanised glassware (64).

2.4.2 Sample Extraction

All methods reviewed (Table 2.2) have utilised SPE, generally in cartridge form, to extract conjugated estrogens from aqueous samples after filtering to remove gross particulate material (usually through glass fibre filters, in particular GF/C). Internal standards are usually added at this stage and in some cases samples have been acidified prior to SPE procedures (58,60).

Solid phases used include C18 silica-bonded phases that are frequently used for extracting free steroids, but divinylbenzene phases or graphitised carbon sorbents are now more commonly used (Table 2.2). Divinylbenzene sorbents (EDS-1, Oasis HLB) have hydrophilic properties in addition to the hydrophobic properties of C18 sorbents, which enhance the adsorption of the more polar conjugated estrogens, in addition to extracting free estrogns. Graphitised carbon sorbents (ENVI-Carb and Carbograph) are increasingly used as they also have good affinity for polar compounds within aqueous matrices. Matejicek et al. (66) demonstrated that Oasis WAX could also be used to extract conjugated and free steroid estrogens from a water:methanol extract of sediment, with recovery slightly better than Oasis HLB; however these cartridges have not yet been applied to the extraction of wastewater or surface water samples.

Conjugated and free estrogens may be eluted in a single step (88,94-96) or can be selectively eluted from the SPE phases and recovered as separate fractions allowing for separate and optimised clean-up methods and/or instrumental analysis. The solvent used varies but for conjugated estrogens is typically methanol, or methanol mixtures, with

Table 2.2: Analytical methods for the analysis of conjugated estrogens in wastewaters and other aqueous environmental samples.

Analytes	Sample type & vol.	SPE	Elution solvent	Clean-up	Analysis	Detection limits	Year	Ref
E1-3G, E1-3S, E2-3G, E2-3S, E2-17G, E3-3G, E3-16G, E3-3S	100 mL STP influent 250 mL STP effluent 2 L river water	Carbograph	10 mL DCM:MeOH (80:20), with 5mM TMACl	None	LC-ESI(-)-MS-MS	0.005-0.63 ng/L river water 0.04-6.0 ng/L STP effluent 0.2-15.0 ng/L STP influent	2002	(62)
E1-3G, E1-3S, E2-3G, E2-3S, E2-17G, E3-3G, E3-16G, E3-3S	5mL urine 50 mL septic tank 100 mL STP influent 250 mL STP effluent	Carbograph	20 mL DCM:MeOH (60:40), with 10 mM sodium acetate	None	LC-ESI(-)-MS-MS	0.3-12 ng/L effluent 0.8-30 ng/L influent	2003	(16)
E1-3G, E1-3S, E2-3G, E2-3S, E2-17G, E2-3G,17S, E2-3S,17G, E2-3,17diS, E3-3G, E3-3S	1 L effluent, river water	EDS-1	10 mL MeOH with 5 mM TEA	None	LC-ESI(-)-MS-MS	0.1-3.1 ng/L	2003	(58)
E1-3S, E1-3G, E2-S, E2-G, E2-S,G, E2-diS, E3-G, E3-S[b]	500 mL STP effluent	Oasis HLB	6 mL MeOH (incl. free estrogens)	Florisil and NH_2-SPE	LC-ESI(-)-MS-MS	0.1 – 1.3 ng/L	2004	(88)
E1-3G, E1-3S, E2-3G, E2-3S, E2-17G, E2-17S	0.5 L STP influent 1 L STP effluent	Oasis HLB	8mL MeOH:MQ (75:25) with 2% NH_4OH,	Fractionation using WAX HPLC column with phosphate saline buffer	LC-ESI(-)-MS-MS	0.04-0.28 ng/L MQ 0.05-0.16 ng/L influent	2005	(93)
E1-3S, E1-3G, E3-16G, EE2-3S, EE2-3G	500 mL STP influent	tC18 (Sep-Pak)	2mL ACN/MQ (70:30) (incl. free estrogens)	None	LC-ESI(-)-MS	0.8 – 4.5 ng/L DW; 1.9 – 7.1 ng/L influent	2005	(94)
E1-3S and E2-3S	1 L STP effluent	DVB-Phobic Speedisk (Baker)	15 mL MTBE then 15 mL MeOH (incl. free estrogens)	GPC on Phenogel columns with THF:acetone (70:30)	LC-ESI-MS	0.6-1.8 ng/L tap water [a] 4-28 ng/L influent [a]	2005	(96)
E1-3S, E1-3G, E2-3S, E2-3G, E3-3S, E3-3G	2 L river, estuarine waters	ENVI-CARB	DCM:MeOH (80:20), with 5 mM TMACl	None	LC-ESI(-)-MS-MS	0.4-0.9 ng/L, matrices not stated, 12 ng/L for E3-3G	2006	(63)
E1-3G, E1-3S, E2-3G, E2-3S, E2-17G, E2-17S, αE2-3S, E2-3G,17S, E2-3S,17G, E2-3,17diS, E2-3,17diG, E3-3G, E3-3S	100 mL agricultural effluents	Carbopack	10 mL DCM:MeOH (80:20), with 5mM TMACl	None	LC-ESI(-)-MS-MS	Not reported	2007	(43)
E1-3S	1 L sewage	C18	10 mL MeOH then 10 mL DCM (incl. free estrogens)	GPC on PLgel columns with DCM:MeOH (90:10) then NH_2-SPE	LC-ESI(-)-MS-MS	0.1 ng/L, influent and effluent (E1-3S only)	2007	(95)

Notes: [a] Limit of quantitation reported only. [b] Positions of glucuronide and sulfate groups not specified.

the addition of an ion pair reagent such as tetramethyl-ammonium chloride (16,43,94), triethylamine (58,60) or ammonium hydroxide (93) to improve the recovery of the acidic conjugates. Free estrogens may be eluted before or after elution of conjugated estrogens, usually with MeOH (60) MeOH:DCM mixtures (16,43,62,63) or ethylacetate (58,65,93). To elute conjugated estrogens along with free estrogens from C18 sorbents, an acetonitrile:water mixture (70:30) (94) and MeOH followed by DCM (95) have been successfully used. MeOH has been used for elution from Oasis HLB sorbent (88) and MTBE followed by methanol for DVB sorbents (96).

2.4.3 Purification of Extracts

In many cases, conjugated estrogen analysis proceeded with no further purification of sample extracts (Table 2.2); however, to reduce matrix suppression in LC-MS-MS analysis, sample clean-up is generally required for complex samples such as sewage influents. Sample cleanup has been based on selective removal of high molecular weight interferences using GPC (95,96), or utilising the anionic nature of conjugated estrogens on anion exchange columns in either SPE (65,88,95) or HPLC format (93).

2.4.4 LC-MS Analysis

Separation of conjugated estrogens (usually along with free estrogens) has invariably been performed on C18 columns or modified C18 columns with methanol:water or acetonitrile:water mobile phases. There are contradictory reports for the most appropriate solvent, with some authors reporting better sensitivity with acetonitrile (93) while others reported improved sensitivity with methanol (66). Acetonitrile has been used for the most part, possibly due to better separation achieved with this solvent (66).

Mobile phase modifiers are generally required for adequate retention of the most polar conjugates. These are usually basic modifiers, such as ammonium hydroxide (63,66,93), methylamine (43) or triethylamine (58,65), to prevent tailing of the acidic conjugates. Conversely Gentili et al. (62) developed a separation method using formic acid in the mobile phase at pH 2.88, which provided sharper peaks than at higher pHs (adjusted with

ammonia). Other authors have reported adequate retention and separation without modifiers (94,96).

Due to the anionic nature of conjugated estrogens, all studies reviewed (Table 2.2) have employed ESI interfaces in the negative mode to ionise the conjugates. Analysis has been by tandem mass spectrometry using triple quadrupole instruments, with only two exceptions. One study demonstrated a method using an ion trap mass spectrometer to measure conjugates in sediments (66), and a single quadrupole instrument was used by Gomes et al. (94) to analyse STP influents.

Limits of detection (LODs) vary according to analyte and matrix, with detection limits for river water samples typically in the sub ng/L range (0.1-1.0 ng/L) when using tandem mass spectrometry. Lower detection limits were usually achieved for 17β-E2- and E1-sulfates, with higher limits for glucuronides, especially E3-glucuronides, generally due to poorer recovery of these more polar conjugates (e.g., ref (62)) and higher instrument detection limits (e.g., ref (63)).

LODs are often 10-fold higher for sewage effluents, and 20-fold higher for influents, with most studies reporting detection limits in the low ng/L range (Table 2.2), depending on the compound. Reddy et al. (93) achieved the lowest detection limits reported for sewage influents at 0.05-0.16 ng/L, analysing only 17β-E2 and E1 sulfates or glucuronides.

2.4.5 Hydrolysis Methods

As stated, several of the reviewed papers which analysed conjugated estrogens in environmental samples first subjected samples to a hydrolysis step to cleave conjugated estrogens. The liberated free estrogens were then measured using GC-MS or LC-MS methods, and concentrations of conjugates calculated from the difference in concentration when compared to the results obtained for untreated samples. The hydrolysis step has been undertaken both on raw samples (43,59,61), so that subsequent extraction procedures follow those for free estrogens; and on extracted samples (57,60,64), requiring that sample storage, extraction and elution methods are optimised for conjugated estrogens.

Hydrolysis of conjugated estrogens is typically carried out using the β-glucuronidase enzyme, extracted from either the bacteria *Escherichia coli* or the terrestrial snail *Helix pomatia*, both of which are commercially available. The source of the enzyme used may be important as β-glucuronidase from *H. pomatia* also has some sulfatase activity and therefore will also cleave both glucuronide and sulfate conjugates, while the enzyme from *E. coli* reportedly has no sulfatase activity (16,97,98). This difference can be utilised to provide information on the forms of conjugates present in a sample if enzymes from both sources are used, as demonstrated by Hoffman et al. (99) in their analysis of conjugated estrogens in urine from pregnant cows.

Solvolysis can also be used to cleave sulfate-conjugates and may be required for disulfates and sulfo-glucuronides, which are reportedly resistant to enzyme treatment (59,64). Labadie and Budzinski (60) used acidic solvolysis (TFA in THF:MeOH 9:1), after an enzyme hydrolysis step using β-glucuronidase from *H. pomatia,* to further cleave conjugated estrogens in STP effluents.

Although the cleavage of conjugated estrogens is reported to proceed with high efficiency when standard solutions are tested, conflicting results have been reported for analysis of environmental samples. Hutchins et al. (43) reported loss of 17β-E2 after enzyme hydrolysis and suggested that the extended reaction time at 37 °C allowed degradation of free estrogens in the unpreserved samples. Finlay-Moore et al. (100) similarly reported losses of free steroid hormones after a methanolysis step prior to measurement of free estrogens using enzyme immunoassay (EIA). The reaction using anhydrous methanolic HCl was substantially faster (5 min, at 60 °C), suggesting the free steroids may also degrade through other (non-biologically mediated) mechanisms.

2.4.6 Alternative Methods

Immunochemical methods of analysis such as enzyme immunoassays (EIA) and radioimmunoassays (RIA) are available for E1-3S and have been used to measure steroid concentrations in cattle urine (101) and faeces (102), though they have not yet been applied to environmental samples. Similar assays for the free estrogens have been used to measure estrogens in sewage effluents (103) and agricultural effluents (39,46), but suffer from problems with cross-reactivity, low reproducibility and false positives (79). It is

likely that the increasing availability of LC-MS instruments with high sensitivity and selectivity for multiple compounds will result in less frequent use of immunochemical methods in the future.

2.4.7 Limitations in Analytical Methods

There is no consistency between the methods published for analysis of conjugated estrogens, with respect to sample preservation, extraction sorbents, clean-up methods and analysis. Most studies did not preserve samples, despite two studies published in 2005 indicating the importance of appropriate sample preservation to avoid losses. LC methods differ between studies, with conflicting results for sensitivity in the presence of different solvents or modifiers. For the many studies that used triple quadrupole instruments to generate tandem mass spectra, sample clean-up was unnecessary, however, this may not be the case when analysing samples with more difficult matrices than sewage effluents or river water. There is a need for analytical methods that are applicable to a range of matrices including agricultural wastes and use widely available equipment and instrumentation.

2.5 SUMMARY

Biological methods for analysis of steroid estrogens are well-established, with numerous sensitive assays available which integrate the activity of mixtures. Chemical methods for free estrogens are typically based on GC-MS or LC-MS after sample extraction and concentration using SPE.

Analysis of conjugated estrogens is more difficult due to their higher polarity and acidity. As for free estrogens, sample extraction is typically based on SPE methods, however, common reversed phase sorbents like C18 appear to be less suitable. Conjugated estrogens have most frequently been analysed using LC-MS-MS; when using triple quadrupole instruments, sample clean-up is often unnecessary and low detection limits (< 1 ng/L) can be achieved. Detection limits for sewage influents and effluents are typically higher than for surface or ground water samples. Samples with complex matrices are more likely to require clean-up prior to LC-MS analysis.

CHAPTER THREE

DEVELOPMENT OF AN ANALYTICAL PROCEDURE FOR DETERMINATION OF ESTROGENS AND CONJUGATED ESTROGENS IN DAIRY SHED EFFLUENT AND ENVIRONMENTAL WATER SAMPLES

Chapter 3: Development of an analytical procedure for determination of estrogens and conjugated estrogens in dairy shed effluent and environmental water samples

3.1 INTRODUCTION

The measurement of free and conjugated estrogens in DSE samples requires a sensitive and selective method. Chemical analysis of these compounds is challenging due to their low concentrations and is particularly demanding in wastewaters due to their high complexity. Consequently, methods based on mass spectrometry are the most applicable and have been used in many published studies (reviewed in Chapter 2).

Triple quadrupole LC-MS-MS instruments have been the most frequently used for analysis of conjugated estrogens, due to their superior selectivity and sensitivity, however alternative instruments such as time-of-flight and ion trap are lower cost and being increasingly used for environmental applications (104). Time-of-flight MS (TOF-MS) instruments provide high mass resolution (typically >5000 FWHM), allowing the separation of analytes from isobaric interferences and measurement of accurate mass aids in unequivocal identification (105). Ion trap MS (IT-MS) instruments can perform tandem MS, making them highly selective, and can perform MS^n experiments, aiding the identification of unknowns.

LC-MS methods based on ESI can experience ionisation suppression due to matrix components, which results in loss of sensitivity (106). Sample preparation is therefore an important step in enriching trace analytes while reducing matrix interferences to improve method detection limits. This is extremely important for analyses of free and conjugated estrogens in treated and untreated animal wastes and effluents. These represent highly complex mixtures, containing extremely high concentrations of a multitude of organic compounds, with a wide range of chemical structures and functionality.

Though sample clean-up is essential, each additional step in an analysis method can lead to reduced recovery. The overall recovery can be assessed by spiking blanks and samples,

and using the measured recovery to adjust the measured concentration of analytes in the samples. However this approach relies on the assumption that recovery will be constant for each sample. In addition, efficacy in derivatising analytes, variation in injection volumes, and matrix suppression or enhancement in the instrumental analyses each contribute to inaccuracies in the final concentration (107). These errors can be overcome by the use of surrogate internal standards (107,108). Isotopically labelled versions of the analytes make ideal surrogates, being as chemically similar to the analyte as possible, thus ensuring they act similarly through sample extraction, preparation and chromatography (107,108). Isotope dilution analysis has been widely used in trace environmental analysis, including that of emerging contaminants (104).

This study aimed to develop a method to analyse both free and conjugated estrogens in DSE samples. The applicability of LC-TOF-MS and LC-IT-MS instruments for analysis of conjugated estrogens was assessed. A previously developed method to analyse free estrogen steroids based on derivatisation and GC-MS analysis (40) was adapted for analysis of DSE samples. Sample clean-up methods were trialled for their suitability for analysis of DSE samples, and isotope dilution was used for target analyte quantitation. Whilst many of the procedures used in this study were adapted from previously published steps, considerable changes were made to develop a method to allow for the analysis of both free and conjugated estrogens in DSE samples, a matrix in which they had not both been examined previously. The developed method was also assessed for its applicability to analysing free and conjugated estrogens in stream water samples.

3.2 MATERIALS AND METHODS

3.2.1 Materials

17β-estradiol (E2), 17α-estradiol (17α-E2), estrone (E1), estriol (E3), ethynylestradiol (EE2) and 17β-estradiol-17-acetate (E2-17Ac) were purchased from Sigma-Aldrich (Auckland, New Zealand). The following conjugated estrogens were also purchased from Sigma-Aldrich as sodium or potassium salts: estrone-3-glucuronide (E1-3G), estrone-3-sulfate (E1-3S), 17β-estradiol-17-glucuronide (E2-17G), 17β-estradiol-3-glucuronide (E2-3G), 17β-estradiol-3-sulfate (E2-3S), estriol-3-sulfate (E3-3S), estriol-3-glucuronide (E3-3G), 17β-

estradiol-3,17-disulfate (E2-3,17diS) and estradiol-3-sulfate-17-glucuronide (E2-3S,17G). 17α-estradiol-3-sulfate sodium salt (17α-E2-3S) and 17β-estradiol-17-sulfate sodium salt (E2-17S) were purchased from Steraloids (Rhode Island, United States). 17β-estradiol-2,4,16,16-d_4 (E2-d_4, min. 98% labelled), estrone-2,4,16,16-d_4 (E1-d_4, min. 98% labelled), ethinylestradiol-2,4,16,16-d_4 (EE2-d_4, min. 98% labelled), sodium 16β-estradiol-2,4,16,16-d_4-3-sulfate (E2-3S-d_4, min. 98% labelled) and sodium estrone-2,4,16,16-d_4-3-sulfate (E1-3S-d_4, min. 98% labelled) were purchased from C/D/N Isotopes (Quebec, Canada). Stock standards were prepared from the solids to concentrations of approximately 1 mg/mL in either acetone (free estrogens) or MeOH (conjugated estrogens). Working standards of the isotopically-labelled standards were prepared at 10 µg/mL in acetone (free estrogens) or MeOH (conjugated estrogens) for spiking samples. This ensured that the volume of solvent used in spiking did not affect sample extraction or recovery.

ENVI-18 cartridges (1 g) were purchased from Supelco (Auckland, New Zealand), Oasis HLB cartridges (500 mg) were purchased from Waters (Auckland, New Zealand), florisil columns (1 g) and bulk aminopropyl packing were purchased from IST (Auckland, New Zealand). JT Baker aminopropyl, silica, florisil and diol SPE cartridges (each 500 mg) and cyclohexyl, cyano and C8 SPE cartridges (each 100 mg) were purchased from Biolab Group (Auckland, New Zealand). Phenyl SPE cartridges (100 mg) were purchased from Alltech (Auckland, New Zealand). Sephadex ™ LH-20 was purchased from Pharmacia Biotech AB (Uppsala, Sweden). Solvents used were of HPLC or pesticide residue grade (Malinkrodt, Biolab Group NZ) or double distilled from analytical grade. Other reagents were analytical grade. Purified water (MQ) was obtained from in-house water purification systems (Millipore).

3.2.2 SPE Procedures

The SPE systems tested for the extraction and recovery of free estrogens are listed in Table 3.1. Mixed standards in MeOH at concentrations of 1 and 10 µg/ml were used to add 25 and 250 ng of each free and conjugated estrogens. Approximately 5 mL of water was added to the column reservoir of each cartridge and then a 250 µL aliquot of each mixed standard was added. Cartridges were eluted with the solvents listed (Table 3.1).

Table 3.1: SPE systems used for extraction and elution of free estrogens

SPE cartridge	Pre-conditioning	Elution
ENVI-18 (1 g)	10 mL acetone, 10 mL water	2 x 10 mL acetone
Oasis HLB (500 mg)	10 mL MeOH, 10 mL water	8 mL MeOH
Oasis HLB (500 mg)	10 mL MeOH, 10 mL water	8 mL ethyl acetate
Oasis HLB (500 mg)	10 mL MeOH, 10 mL water	30 mL DCM:diethylether:MeOH (40:10:1)

To assess the extraction of conjugated estrogens, standards were loaded onto Oasis HLB columns. The cartridges were washed after loading using the procedure described by Reddy and Brownawell (109), with 30% MeOH:water containing 2% acetic acid (8 mL), followed by 60% MeOH:water containing 2% acetic acid (8 mL) and finally 30% MeOH:water containing 2% ammonium hydroxide (4 mL). The conjugated estrogens were then eluted with 75% MeOH:water containing 2% ammonium hydroxide (8 mL, (109)). The SPE columns were then dried under vacuum to remove residual water before elution of free estrogens using the DCM:diethylether:MeOH mixture.

3.2.3 Sample Clean-Up Procedures for Free Estrogens

Initially clean-up procedures for free estrogens used GPC followed by silica gel adsorption chromatography based on Sarmah et al. (40). Standards and sample extracts from SPE elution were dried under nitrogen and reconstituted in 0.5 mL of DCM for injection onto GPC. The GPC system consisted of a Shimadzu LC-10AT VP pump, SIL-10AF auto-injector, SPD-10A UV-Vis detector and FRC-10A fraction collector. Biobeads SX-8 was packed into two 440 mm by 10 mm ID glass columns connected in series, with DCM as mobile phase, pumped at a flow rate of 1.4 mL/min. The fraction where free estrogens eluted (from 24-42.5 min) was collected for analysis.

The analyte fraction from GPC was next dried down and reconstituted in 1 mL of hexane:acetone (65:35) for silica gel chromatography, based on the method reported by Ternes et al. (2002). One gram of silica gel (Merck Si 60, activated at 150 °C for 8 h, deactivated with 1.5% water) was made into a slurry with hexane:acetone and packed into a small glass column (ID 8 mm). The extract collected from GPC was quantitatively added to the column with 3 x 1 mL washes of hexane:acetone, and eluted with 5 mL hexane:acetone.

The second method for purification of the free estrogens combined elution from the Oasis SPE cartridges and clean-up in a single step. The dried Oasis HLB cartridge was connected in series to aminopropyl sorbent (500 mg) added to the top of a florisil cartridge (1 g), previously washed with acetone (10 mL). Free estrogens were eluted from the Oasis SPE and through the clean-up cartridges with 30 ml of DCM:diethyl ether:MeOH (40:10:1).

An alternative GPC procedure followed this step, using the same set-up as described above, but with two Phenogel SEC columns (5 μm particle size, 7.8 mm I.D., 600 mm and 300 mm, Phenomenex, Auckland, NZ) connected in series. Again, DCM was used as mobile phase, pumped at a flow rate of 0.5 mL/min. Standards (for recovery testing) and sample extracts from the SPE were dried under nitrogen and reconstituted in 0.25 mL of DCM for injection. The fraction from 42 to 62 min, containing the estrogens, was collected. This fraction was dried under nitrogen in preparation for derivatisation and GC-MS analysis.

3.2.4 Sample Clean-Up Procedures for Conjugated Estrogens

Several methods were tested for their suitability to clean-up samples for conjugated estrogen analysis, including GPC, HPLC fractionation and various SPE adsorbents (Table 3.2). Mixed standards of conjugated estrogens were used to assess recovery through each method. Quantitation of analyte recovery was undertaken using LC-TOF-MS as described below. The final method selected for sample clean-up is described in more detail below.

For the finalised clean-up procedure, the conjugated estrogen fraction eluted from the Oasis HLB was dried under nitrogen and resuspended in MeOH (250 μL) for clean up through two SPE phases. Pre-packed silica columns (500 mg) were preconditioned with 5 mL of chloroform, then samples were transferred with three rinses of chloroform that were also transferred to the column reservoir. A further 1.55 mL of chloroform was added to the column reservoir give a maximum of 11% MeOH in chloroform. This mixed solvent solution was passed through the silica columns. The retained conjugate steroids were eluted from the silica columns with 20 mL of chloroform:MeOH (1:1) and dried under nitrogen. The conjugate steroids were reconstituted in MeOH in preparation for cleanup on aminopropyl columns (500 mg). The cartridges were conditioned with a sequence of

Table 3.2: Methods tested for clean-up of conjugated estrogens

Method	Column / stationary phase details	Mobile Phase / Solvent	Instrumentation
GPC	Phenogel SEC columns (7.8 mm x 200 mm and 300 mm, 5 µm particle size, Phenomenex)	THF / acetone (70:30)	Shimadzu system incorporating LC-4A pump attached to Shimadzu SPD-2AS UV spectrophotometer and a Hewlett-Packard 3390A integrator
GPC	Phenogel SEC columns (as above)	DCM / MeOH (50:50)	As above
GPC	Sephadex ™ LH-20 packed in glass column 9 mm x 450 mm)	MeOH	None required
GPC/Partition	Sephadex ™ LH-20 packed in glass column 9 mm x 450 mm)	Chloroform / MeOH (50:50)	None required
HPLC (reversed phase)	Luna C18 (10.0 mm x 250 mm, 5µm, Phenomenex)	Water with 0.1% TFA, acetonitrile (variable concentrations)	Dionex system incorporating P680 HPLC pump, ASI-100 autosampler and TCC-100 column oven attached to a UVD340U diode array detector, controlled by HP computer
SPE (normal phase)	Florisil, silica and diol (500 mg)	DCM / MeOH mixtures and chloroform / MeOH mixtures	None required
SPE (reversed phase)	Cyclohexyl, cyano, C8, phenyl (100 mg)	MeOH / water mixtures	None required
SPE (anion exchange)	Aminopropyl (500 mg)	MeOH containing 5% ammonia	None required

MeOH, water and MeOH again. The MeOH extracts were loaded onto the aminopropyl columns, washed with 1 ml MeOH and eluted with 2 x 3mL MeOH containing 5% ammonia. Samples were dried under nitrogen and reconstituted in ammonium acetate buffer (0.01 mM) with 12% acetonitrile for LC-MS determination.

3.2.5 LC-MS Determination of Conjugated Estrogens

LC-TOF-MS analysis

Conjugated steroid estrogens were quantified using a Waters 2690 LC (Waters, USA) liquid chromatograph attached to a Micromass LCT TOF-MS operating in the negative ion mode. Separation was performed on an X-Bridge C18 column (150 mm x 1.0 mm I.D., 3.5 µm particle size, Waters, USA) at 28 °C. The mobile phases were (A) MQ containing ammonium acetate buffer (0.01 mM), and (B) 60% acetonitrile with ammonium acetate buffer (0.01 mM); the flow rate was 50 µL/min. Initial conditions of 10%B were held for 1 min, then increased linearly to 80% B at 24 min, held for 4 min then reset to the original conditions.

The mass spectrometer was operated in negative mode electrospray ionization, with conditions optimized as follows: capillary voltage of -2600 V, sample cone voltage of -38 V, extraction cone voltage 4V, source temperature of 100 °C, desolvation temperature 200 °C, and MCP detector at 2600 V. Spectra were acquired over the 70-800 m/z range. The instrument was calibrated externally with sodium formate and the resolution was at least 4500 FWHM.

Retention times and m/z ratios used for detection of the individual compounds are listed in Table 3.3. Data acquisition was carried out using MassLynx 4.0 software and the All File Accurate Mass Measure (AFAMM) software was used to calibrate the mass of analytes according to the reference mass of the isotopically labelled internal standards (E2-3S-d_4 and E1-3S-d_4). Linear nine-point calibration curves were made for each of the analytes (5-1000 ng/ml, r^2>0.99). Analytes were quantified by isotopically labelled internal standardization against E2-3S-d_4 and E1-3S -d_4.

Table 3.3: Retention times and TOF-MS detection parameters for the conjugated estrogens

Compound	Rt (min)	Selected Ion	*m/z*
E3-3G	3.47	$[M-H]^-$	463.1968
E2-3S,17G	4.19	$[M-H]^-$	527.1507
E3-3S	6.77	$[M-H]^-$	367.1215
E2-3,17-diS	9.94	$[M-SO_3H]^-$	350.1188
E2-3G	12.02	$[M-H]^-$	447.2019
E2-17G	13.89	$[M-H]^-$	447.2019
E1-3G	16.74	$[M-H]^-$	445.1862
E2-3S	21.41	$[M-H]^-$	351.1266
E2-3S-d_4	21.41	$[M-H]^-$	355.1517
17α-E2-3S	22.61	$[M-H]^-$	351.1266
E1-3S	23.68	$[M-H]^-$	349.1110
E1-3S-d_4	23.68	$[M-H]^-$	353.1361

LC-IT-MS analysis

Conjugated estrogens were analysed using an LTQ linear ion trap mass spectrometer fitted with an ESI interface (ThermoQuest, Finnigan, San Jose, CA) operating in negative ion mode, coupled to an Ettan Multi-Dimensional Liquid Chromatography system (GE Healthcare BioSciences). Separation was performed on an Extend C18 column (150 mm x 1.0 mm I.D., 3.5 µm particle size, Agilent, USA) maintained at 30°C. The mobile phases used on the LC-IT-MS differed slightly to those used on the LC-TOF-MS due to the different columns used on each system. Mobile phases were (A) ammonium acetate buffer (0.01 mM) in 12% acetonitrile, and (B) ammonium acetate buffer (0.01 mM) in 89% acetonitrile; the flow rate was 50 µL/min. Initial conditions of 0%B were held for 10 min, increased linearly to 12% B at 12 min, held for 5 min, increased linearly to 30% B at 25 min, held for 10 min, then increased linearly to 100% B at 40 min and held for 3 min to flush unwanted contaminants from the column before re-equilibrating to the original conditions.

Mass spectral data were acquired in the negative mode by using a selective reaction monitoring (SRM) method that monitored the distinctive daughter ions formed by fragmenting the precursor ion $[M-H]^-$ for each of the conjugated estrogens (Table 3.4). The electrospray ionization voltage, capillary temperature, sheath gas pressure, sweep gas, and auxiliary gas were set at -10 V, 350 °C, 25 psi, 3 psi, and 3 psi respectively. Collision energy was 35 V. Ionisation parameters were previously optimised for each of the

compounds by directly injecting a constant concentration of a mixed standard, delivered by syringe pump.

Table 3.4: Retention times and IT-MS detection parameters for the conjugated estrogens.

Compound	Rt (min)	Precursor Ion	Precursor Ion m/z	Daughter Ions m/z
E3-3G	6.67	$[M-H]^-$	463.2	287.2, 445.2
E2-3S,17G	8.23	$[M-H]^{2-}$	263.2	254.2, 350.2, 379.2,405.2, 451.2
E2-3,17-diS	12.34	$[M-H]^{2-}$	215.2	175.2, 215.2, 333.2, 350.2
E3-3S	14.23	$[M-H]^-$	367.2	287.2
E2-3G	16.55	$[M-H]^-$	447.2	175.2, 271.2, 429.2
E2-17G	16.76	$[M-H]^-$	447.2	175.2, 271.2, 429.2
E1-3G	20.61	$[M-H]^-$	445.2	175.2, 269.2, 427.2
E2-3S	30.01	$[M-H]^-$	351.2	271.2
E2-3S-d_4	29.86	$[M-H]^-$	355.2	275.2
17α-E2-3S	33.23	$[M-H]^-$	351.2	271.2
E1-3S	35.30	$[M-H]^-$	349.2	269.2
E1-3S-d_4	35.02	$[M-H]^-$	353.2	273.2

Data acquisition was carried out using XCalibur 2.0 software. Linear eight-point calibration curves were made for each of the analytes (5-1000 ng/ml, r^2>0.99). Analytes were quantified by internal standardisation against isotopically labelled 17β-E2-d_4-3-sulfate and E1-d_4-3-sulfate.

3.2.6 GC-MS Determination of Free Estrogens

Free steroid estrogens were derivatised to their respective trifluoroacetates using a minor modification of the procedure described by Lerch and Zinn (110). Samples were dried completely under nitrogen and redissolved in toluene (50 µl). Trifluoroacetic anhydride (TFAA, 10 µl) was added, and the reaction proceeded at room temperature for 5 min. Iso-octane (200 µl) containing E2-17Ac (500 ng/ml) was added as a volumetric internal standard, and the mixture washed with aqueous potassium carbonate (1%, 2.5 ml) to remove acidic reaction by-products. The separated organic layer was transferred to GC vials and stored under refrigeration before analysis.

Steroid estrogens were quantified by GC-MS using an Agilent 6890 gas chromatograph fitted with an Agilent split/splitless injector, PAL autosampler and an Agilent 5975 quadrupole mass selective detector (MSD). Individual compounds were separated using a HP-5MS column (length 30 m, film thickness 0.25 µm, inner diameter 0.25 mm, Agilent) with an initial oven temperature of 90 °C for 1.5 min, then increasing at 20 °C per min to

130 °C, followed by 4 °C per min to 236 °C and 8 °C per min to 320 °C with a hold for 5.5 min (total run time 46 min). Helium was used as carrier gas. The sample was injected (2 µL) into a split/splitless injector held at 270 °C. Transfer of volatile analytes onto the column was enhanced using pressure pulsed injection at 35 psi for 1.1 min. Following this pressure pulse, the column pressure was increased using a constant pressure ramp that optimised separation of the target analytes. Pressure was ramped throughout the analysis from an initial 17.50 psi for 1.5 min, increased at 1.5 psi/min to 20.5 psi, followed by an increase of 0.3 psi/min to 28.5 psi and finally 5.0 psi/min to 50.0 psi. The GC–MS interface and the quadrupole temperatures were set at 230 and 150 °C, respectively. Electron Impact Spectra (EIS) were obtained at 70 eV. The MSD was calibrated against PTFBA using the autotune function. The resulting electron multiplier voltage (EMV) was increased by 300 EMV for increased sensitivity. Retention times and m/z ratios used for detection of the individual compounds are listed in Table 3.5. Mass spectral data was acquired using the synchronous scan / single ion monitoring (SIM) mode.

Table 3.5: Retention times and GC-MS detection parameters for the trifluoroacetylated estrogens

Analyte	Rt (min)	Quantitation Ion	Qualifier Ions
E2-d_4	27.621	468	311, 258
17α-E2	27.020	309	351, 309
17β-E2	27.676	464	351, 309
E3	27.950	576	463, 349
E1-d_4	29.262	370	324, 311
E1	29.328	366	322, 309
EE2-d_4	30.749	311	258, 396
EE2	30.793	392	256, 309
E2-17Ac (ISTD) [a]	35.407	314	315, 356

Note: [a] Not a TFAA derivative.

Data analysis was undertaken using the Chemstation software using data acquired in the SIM mode. Quantitation of estrogens in samples and spiked samples was by isotope dilution, based upon the relative response factors for the following pairings: 17α-E2/E2-d_4, E2/E2-d_4, E3/E2-d_4, E1/E1-d_4, EE2/EE2-d_4. Six-point calibration curves from 0-250 ng/mL were prepared for quantitation at lower concentrations, and eleven-point calibration curves were prepared from 0-10,000 ng/mL for quantitation of spikes at 2500 ng/L (see below). Although a linear regression fitted the data adequately, a quadratic curve provided higher r^2 values (full range $r^2 > 0.99$; lower range: $r^2 > 0.99$ except E3 at 0.96).

During initial method development, recovery of each target analyte was quantified against the relative response of E2-17Ac (as ISTD). This was also used to calculate absolute recovery of spiked samples. Eight-point calibration curves from 0-1000 ng/mL were used for method development and eleven-point calibration curves were made from 0-2,500 ng/mL for quantitation of spikes at 2500 ng/L (see below). Quadratic curves were fitted to the data and weighted to the inverse of concentration, resulting in slightly poorer r^2 values (r^2 0.92 – 0.97, E3 0.88).

3.2.7 Sample Preservation Experiments

Fresh samples of DSE were collected and split into nine replicates in 2.5 L solvent-rinsed glass bottles. Three samples were immediately preserved with H_2SO_4 to pH < 2, following the method of Reddy et al. (93); three samples were preserved with formaldehyde at 1% v/v (16) and three samples were not preserved. All samples were stored at < 4 °C in the dark throughout the test period. Sub-samples were extracted immediately after collection, then at 24 hours, 48 hours, 96 hours and 168 hours (7 days) after collection.

At each time period, ~500 mL of sample was removed from each bottle, centrifuged at 3000 rpm at 4 °C for 10 min to remove bulk solids, then filtered through glass fibre filters (GF/C, pore size 1.2 µm Whatman) with the addition of filter aid. Samples were spiked with surrogate standards (250 ng each E2-d_4, E1-d_4, E1-3S-d_4 and E2-3S-d_4). Extraction, SPE elution, extract clean-up and analysis were as described above, with some modifications.

During clean-up of the conjugated estrogen extract, aminopropyl columns were rinsed with acetonitrile (3 mL), acetonitrile with 10 mM ammonia (3 mL), and MeOH with 10 mM ammonia (3 mL), before elution with two aliquots of MeOH containing 2% ammonia (3 mL). Eluted samples were dried under nitrogen and made up in the acetate buffer (0.01 mM ammonium acetate in water:acetonitrile, 88:12) for LC-MS analysis.

For GC-MS analysis, six-point calibration curves from 0-250 ng/mL were used for quantitation of 17β-E2 and E1; eleven-point calibration curves from 0-2500 ng/mL were used for quantitation of 17α-E2 as this was present at higher concentrations. Although a

linear regression fitted the data adequately, a quadratic curve provided higher r^2 values ($r^2 > 0.997$).

3.2.8 Matrix Spike Experiments

Recovery of target analytes from various matrices was obtained by spiking samples of MQ water, stream water and DSE samples with mixed standards of unlabelled and isotopically-labelled analytes.

MQ water

This test was conducted in triplicate by spiking 25 ng and 250 ng of conjugated and free estrogens and labelled surrogate internal standards (250 ng each E2-d_4, E1-d_4, EE2-d_4, E2-3S-d_4 and E1-3S-d_4) into 1 L of MQ water. Samples were extracted through 500 mg Oasis HLB cartridges at 5-10 ml/min, previously conditioned with MeOH (2 x 5 mL) and MQ water (2 x 5 mL). Sample bottles were rinsed three times with 10 mL MQ water, which was also loaded onto the Oasis HLB cartridges.

Cartridges were then washed using the procedure described by Reddy and Brownawell (109), with 30% MeOH/water containing 2% acetic acid (8 mL), followed by 60% MeOH/water containing 2% acetic acid (8 mL) and finally 30% MeOH/water containing 2% ammonium hydroxide (4 mL). The fraction containing conjugates was eluted with 8ml of 2% ammonium hydroxide in MeOH:water (75:25). The Oasis SPE cartridges were dried under vacuum, connected to aminopropyl/florisil cartridges, and the fraction containing free estrogens eluted with a mixture of DCM (DCM):diethyl ether:MeOH (40:10:1) (30 ml) as previously described. The collected extract was dried under nitrogen and subjected to GPC clean-up (on Phenogel columns) before derivatisation and GC-MS analysis.

The fraction containing conjugated estrogens was dried under nitrogen and resuspended in 0.25 ml of MeOH. This crude extract was further cleaned up using silica gel (500 mg) and aminopropyl (500 mg) SPE columns to remove polar interferences. The purified extract was dried under nitrogen and reconstituted in 250 µL of acetate buffer (0.01 mM ammonium acetate in water:acetonitrile, 88:12) for LC-IT-MS analysis.

Stream water

A large volume (20 L) grab sample was collected from a local stream and immediately acidified to pH<2 with conc. H_2SO_4. On return to the laboratory, the sample was filtered through GF/C to retain particulate material. Due to low turbidity, the stream sample did not require centrifuging. The bulk sample was divided into 10 x 2 L replicates, then spiked with either 0 or 25 ng of conjugated and free estrogens, corresponding to samples containing 0 and 12.5 ng/L of analytes. Each sample was also spiked with 250 ng labelled surrogate internal standards. Sample treatment was as described for MQ water, with the exception that samples were not subjected to further clean-up using GPC (free fraction) or silica and aminopropyl (conjugate fraction) as the extracts were expected to be sufficiently clean for final analysis.

Dairy Shed Effluent

DSE was collected from a dairy farm on the Ruakura Research Station operated by AgResearch. Grab samples of DSE were collected from the collection sump and drains of the milking shed yard. Samples were immediately preserved by the addition of conc. H_2SO_4 (to pH ≤ 2). Sample processing began immediately on return to the laboratory and was completed within 36 hours. Samples were stored at <4 °C during this period where necessary. Due to the high suspended solids loading of DSE, samples were centrifuged at up to 1780 *g* for 10-20 min then the supernatant filtered through GF/C filters topped with Hi-flo supercel filter aid (BDH). The filtered samples were combined, then divided into 20 x 500 mL aliquots for the spike recovery experiments. Five replicates were used for each spike concentration of 0 ng, 12.5 ng, 125 ng and 1250 ng, corresponding to 0 ng/L, 25 ng/L, 250 ng/L and 2500 ng/L respectively, to cover the range of concentrations expected to be encountered in field samples. Each sample was also spiked with labelled surrogate internal standards, extracted and prepared as outlined above for MQ water.

3.3 RESULTS AND DISCUSSION

3.3.1 Analysis of Free and Conjugated Estrogens by LC-MS

The initial objective of method development was to develop an LC-MS method to analyse both free estrogens and conjugated estrogens, following extraction and clean-up. Initial attempts to analyse 17β-E2 using APCI (negative mode) and ESI on both LC-TOF-MS and LC-IT-MS indicated high instrument detection limits (IDLs) for pure standards (>10 ng injected), substantially higher than that reported by others (59,111,112). With such a relatively high IDL, the resulting method detection limits (MDLs) would not be within the required range for environmental samples owing to the effect of ion suppression associated with their complex matrices. Poor detection limits for estrogens by LC-MS have been previously reported by Hájková et al. (113). A decision was made to analyse the free estrogens by GC-MS after derivatisation, owing to the substantially lower detection limits that could be achieved.

Conjugated estrogens cannot be derivatised and analysed by GC-MS but are amenable to LC-MS analysis. Therefore, LC-TOF-MS and LC-IT-MS systems were compared for their suitability for quantitative measurement of conjugated estrogens in DSE samples. Ammonium acetate buffer was selected as it is commonly used for analysis of weakly acidic analytes (114) and has also been used for analysis of conjugated estrogens in pharmaceutical formulations (115,116). The gradient conditions were optimised for separation of mixed standards and were specific to each column and LC system.

Chromatograms of standard solutions analysed by each system are shown in Figures 3.1 and 3.2. E2-3G and E2-17G were not completely resolved on either column. IDLs were determined from the lowest calibration standards injected that provided a signal to noise (*S/N*) ratio > 3 (Table 3.6). This indicated better sensitivity with the LC-IT-MS versus LC-TOF-MS for the more polar conjugates, while a similar level of sensitivity was obtained for the estrogen sulfate conjugates with both LC-MS instruments.

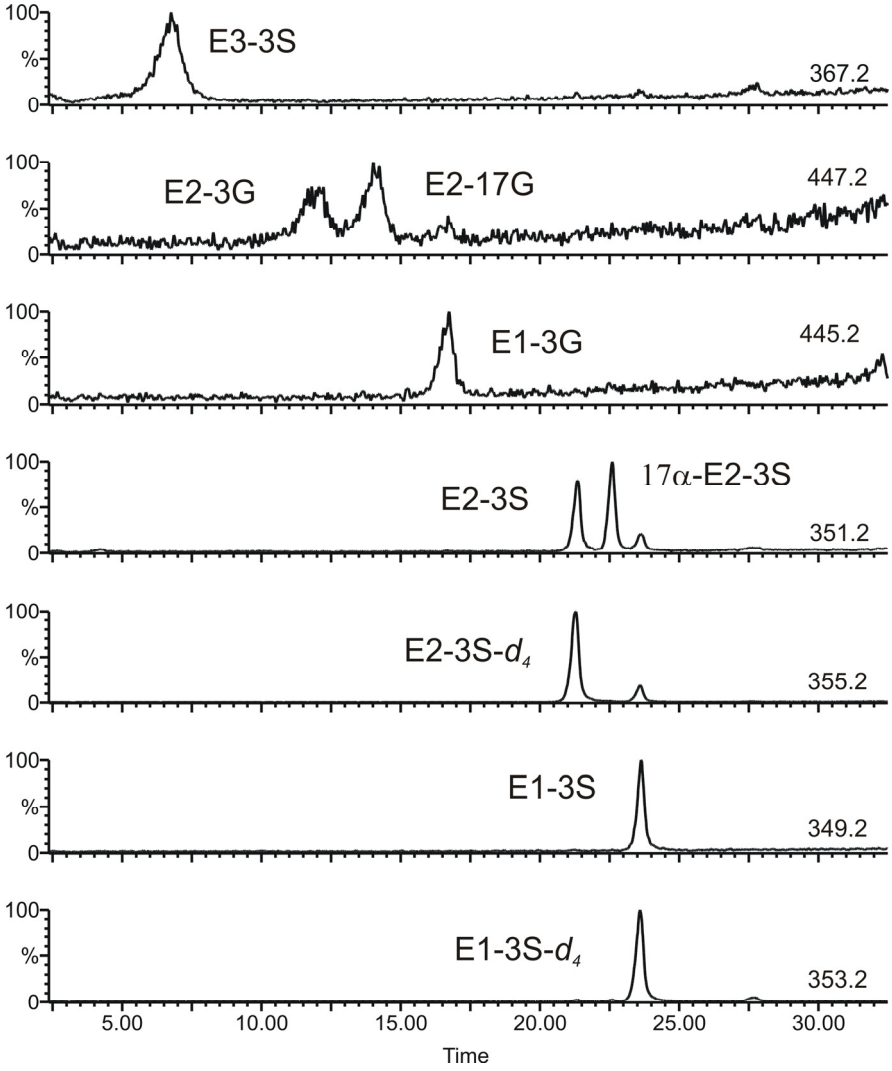


Figure 3.1: Selected m/z chromatograms of 0.6 ng of each of the conjugated estrogens separated on X-bridge C18 column and analysed by LC-TOF-MS. E3-3G, E2-17G,3S and E2-3,17diS were not adequately retained on the column.

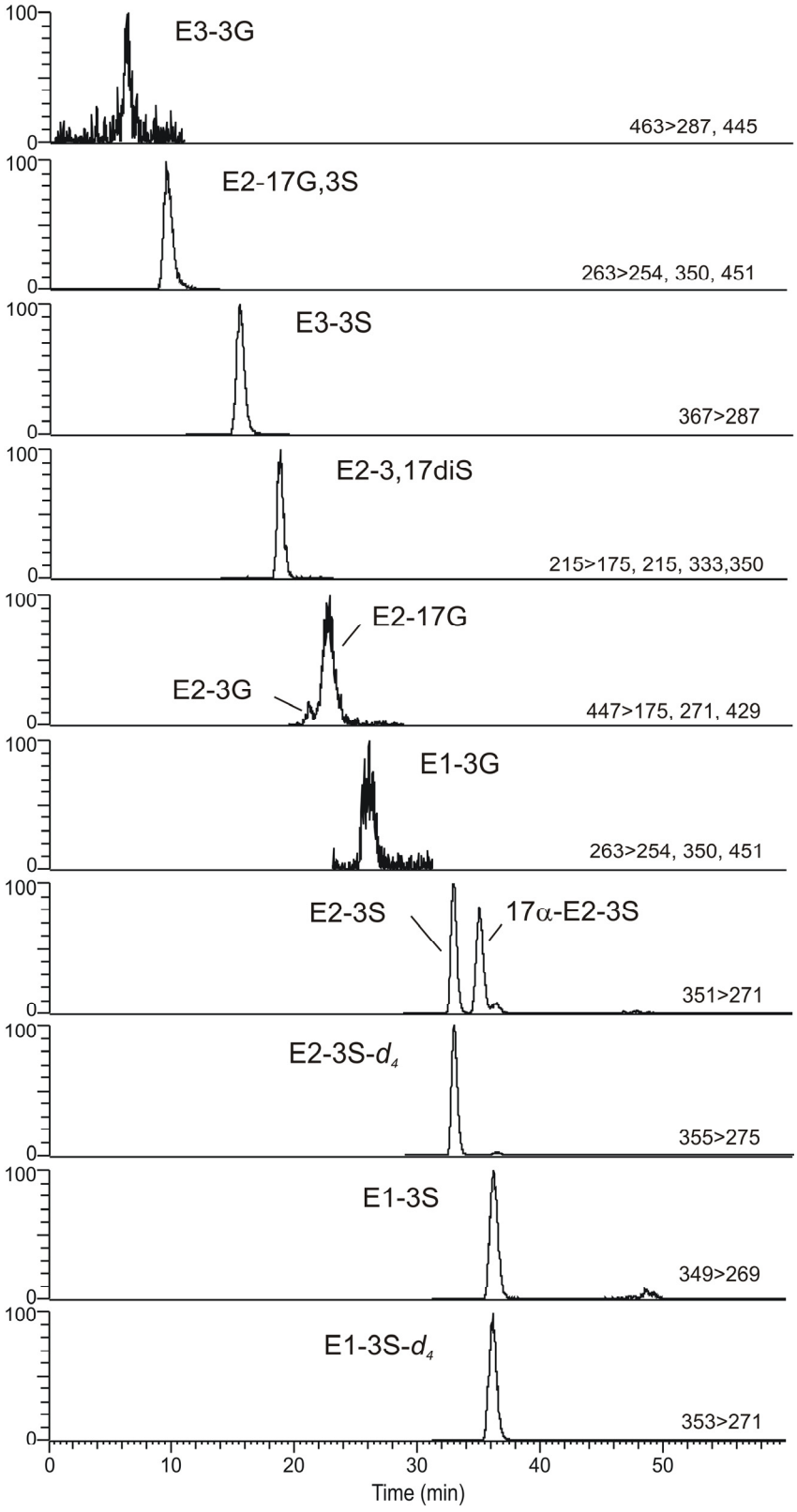


Figure 3.2: SRM chromatograms of 1 ng of each of the conjugated estrogens separated on Extend-C18 column and analysed by LC-IT-MS.

Table 3.6: Comparison of instrument detection limits (IDLs) obtained by LC-TOF-MS and LC-IT-MS for the conjugated estrogens

	Instrument detection limit (ng/mL)	
Compound	LC-TOF-MS	LC-IT-MS
E3-3G	500	10
E3-3S	40	5
E2-3S,17G	500	5
E2-3,17-diS	500	5
E2-3G	40	10
E2-17G	40	10
E1-3G	40	25
E2-3S	5-10	5
17α-E2-3S	5-10	5
E1-3S	5-10	5
E2-3S-d4	5-10	5
E1-3S-d4	5-10	5

However, in the presence of the sample matrix from DSE samples, the resulting background contribution obtained by LC-TOF-MS was excessively high, resulting in decreased sensitivity. Improved signal to noise was obtained utilising the high resolution of TOF-MS by decreasing the mass window from 0.5 Da to 0.1 Da (Figure 3.3). Narrowing the mass window further reduced signal to noise as the overall ion count decreased. Despite the improvement using a mass window of 0.1 Da, the level of sensitivity achieved in the presence of sample matrix remained inadequate using LC-TOF-MS. By comparison, a low level of background noise was obtained by LC-IT-MS operating in SRM mode (Figure 3.4).

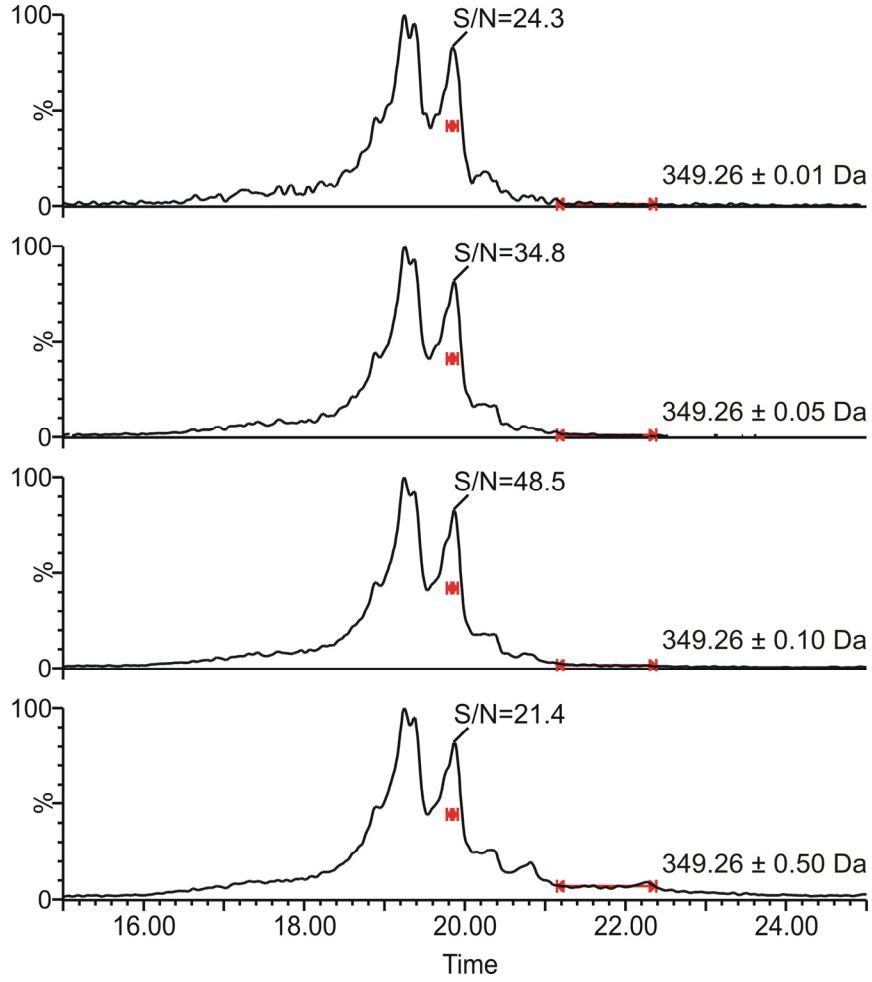


Figure 3.3: Increasing signal to noise (S/N) ratio calculated by peak-to-peak method for 17a-E2-3S peak with narrower mass window using LC-TOF-MS.

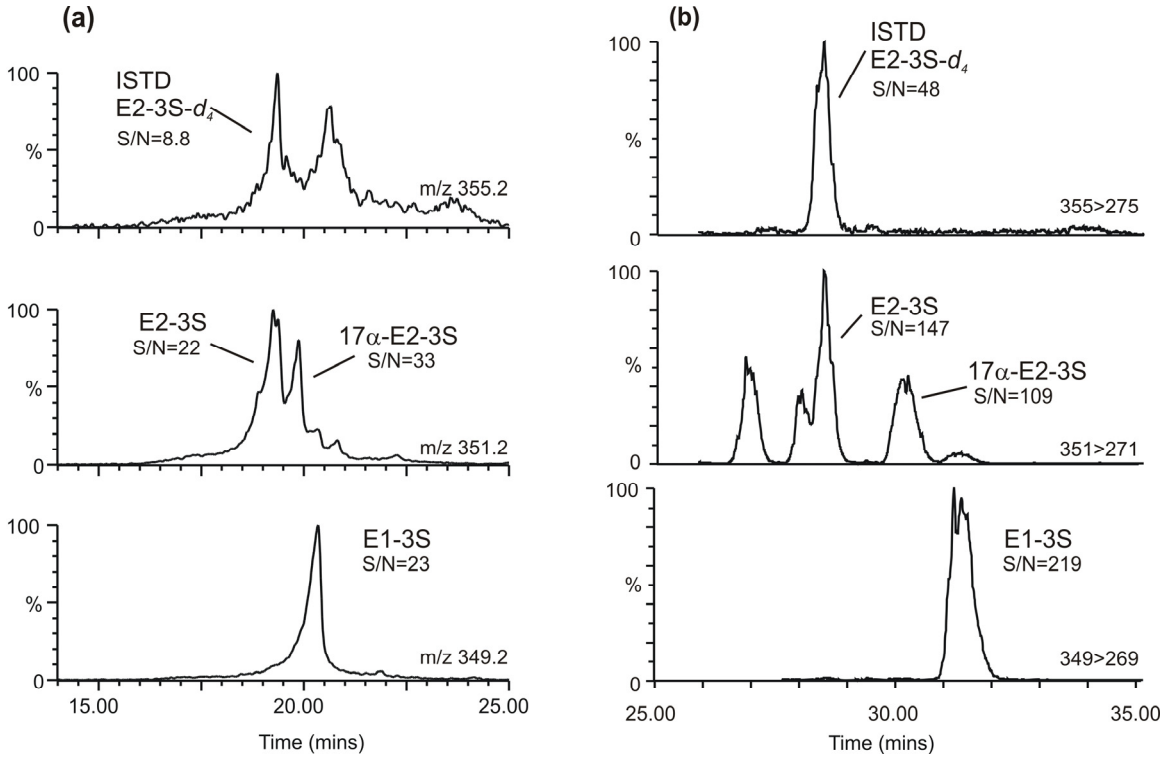


Figure 3.4: Comparison of LC-TOF-MS (a) and LC-IT-MS (b) traces for a DSE sample spiked with conjugated estrogens.

3.3.2 Comparison of SPE Methods

Initially Supelco ENVI-18 (1 g, Supelco) cartridges were tested for extraction of free steroid estrogens (Table 3.7). To extend the method to enable the simultaneous extraction of conjugated estrogens, a range of commercially available cartridges were evaluated, including ENVI-18 (1 g, Supelco), Oasis HLB (500 mg, Waters), Strata-X (500 mg, Phenomenex), Strata SDB-L (500 mg, Phenomenex), Strata C18-E (500 mg, Phenomenex), Isolute C18/Env+ (300 mg, Isolute), Isolute C18(EC) (500 mg, Isolute). The C18 sorbents provided poor recovery of conjugated estrogens (data not shown), while Oasis HLB cartridges showed promising recoveries of both conjugated and free estrogens compared to the other SPE types, and were selected for further evaluation. The use of Oasis HLB cartridges has been reported by several researchers for analysing both conjugated and free estrogens (58,87,109), owing to the advantages provided by their combination of lipophilic and hydrophilic properties. In addition, recovery of organic compounds is reportedly not affected by drying of the sorbent (117), a useful characteristic when extracting numerous samples simultaneously.

Table 3.7: Comparison of percentage recovery of free estrogens from MQ water spiked at 25 ng/L and 250 ng/L, extracted through either ENVI-18 or Oasis HLB and eluted with various solvents [a].

Sorbent and solvent	Spike conc.	17α-E2	17β-E2	17β-E2-d_4	E3	E1	EE2
ENVI-18	25	115 ± 12	98 ± 12	116 ± 13	118 ± 27	117 ± 12	114 ± 12
Acetone	250	119 ± 10	115 ± 6	121 ± 11	144 ± 3	127 ± 12	125 ± 13
Oasis HLB							
MeOH	25	58 ± 35	59 ± 33	59 ± 33	101 ± 88	68 ± 13	74 ± 10
	250	80 ± 24	77 ± 24	80 ± 25	105 ± 38	63 ± 27	53 ± 26
Ethyl Acetate	25	141 ± 26	135 ± 26	151 ± 28	188 ± 27	147 ± 23	144 ± 24
	250	120 ± 5	112 ± 5	121 ± 4	113 ± 7	194 ± 15	NM
DCM mix	25	329 ± 13	374 ± 10	NM	118 ± 10	257 ± 8	311 ± 16
	250	107 ± 3	89 ± 1	109 ± 4	49 ± 5	107 ± 1	109 ± 2

Notes: [a] Mean ± standard deviation, n = 3 except for DCM mix, n = 5. NM = Not measured.

Elution of free estrogens from Oasis HLB cartridges has been reported using ethyl acetate (6-8 ml) (58,109), MeOH (8 ml, (87)), or a mixture of DCM:diethyl ether:MeOH (40:10:1) (30 ml, M. Burkhardt, pers. comm). The recovery of free estrogens was assessed for each of these solvents (Table 3.7). Recovery regularly exceeded 100% due to signal enhancement when measured by GC-MS and quantified by external standards.

Several solvents have been reported for elution of conjugated estrogens from Oasis HLB (58,87,93). Isobe et al. (58) and Labadie and Budzinski (87) used triethylamine as an ion pair reagent to elute the conjugated estrogens. However, as triethylamine has been shown to result in severe contamination of mass spectrometers (118), an alternative ion pair was sought to avoid this possibility. Ammonium hydroxide had been successfully used by Reddy et al. (93). The dairy wastes are a highly complex matrix containing significant quantities of polar acidic and basic compounds that are co-extracted with free and conjugated estrogens by the combined lipophilic and hydrophilic phases of the Oasis HLB cartridges. These interfering compounds were preferentially removed from the Oasis HLB cartridges, with the solvent elution scheme of Reddy et al. (93), followed by elution of the retained conjugated estrogens.

Recovery was acceptable for the less polar conjugates, but was <80% for E2-17G, E2-3,17diS and E3-3S (Table 3.8). Increasing the concentration of MeOH in the final wash step to 35% or 40% resulted in a slightly cleaner extract, but further reduced the recovery of the more polar conjugates (data not shown). E3 was also eluted along with the conjugated estrogens using this solvent (75% MeOH:25% MQ water with 2% ammonium hydroxide),

which reduces the application of this particular method when analysing STP influents and effluents where E3 is of interest.

Table 3.8: Percentage recovery of conjugated estrogens from MQ water extracted through Oasis HLB [a].

	Percentage recovery (mean ± standard deviation)	
Conjugate	**Spike conc. of 25 ng/L**	**Spike conc. of 250 ng/L**
E3-3G	NM [b]	NM
E2-3S,17G	NM	NM
E2-3,17-diS	76 ± 12	44 ± 5
E3-3S	65 ± 6	40 ± 3
E2-17G	84 ± 1	73 ± 2
E1-3G	94 ± 6	84 ± 4
E2-3S	90 ± 6	92 ± 5
17α-E2-3S	NM	NM
E1-3S	90 ± 7	91 ± 5

Notes: [a] n = 3. [b] NM = Not measured.

3.3.3 Clean-up of Free Estrogens

Initially the protocol for free estrogen clean-up followed the scheme reported by Sarmah et al. (40) using a coarse GPC step on Biobeads SX-3 to remove high molecular weight interferences, followed by clean-up with silica gel chromatography. Initial tests indicated that though there was complete recovery through the GPC, recovery of E3 was poor with silica gel, and recovery of other analytes was variable when tested at a concentration equivalent to 25 ng/L in samples (Table 3.9). Clean-up on aminopropyl (NH_2) cartridges as described by Labadie and Budzinski (60) was assessed as a substitute for silica gel and provided improved recovery of E3 (Table 3.9).

Table 3.9: Comparison of percentage recovery of free estrogens from various cleanup steps (results expressed as mean values ± standard deviation; n = 3).

Clean-up method	Spike conc.	Percentage recovery					
		17α-E2	**E2-d_4**	**17β-E2**	**E3**	**E1**	**EE2**
Silica [a]	25	76 ± 23	83 ± 33	72 ± 27	0 ± 0	76 ± 22	77 ± 24
	250	102 ± 7	108 ± 2	98 ± 5	0 ± 0	109 ± 12	108 ± 12
NH_2 [a]	25	118 ± 11	132 ± 12	123 ± 15	66 ± 53	167 ± 5	154 ± 3
	250	119 ± 36	122 ± 37	113 ± 34	135 ± 48	116 ± 48	61 ± 30
NH_2/Fl [b]	25	99 ± 14	NM [c]	81 ± 6	55 ± 7	107 ± 7	110 ± 4
	250	183 ± 6	174 ± 7	152 ± 4	79 ± 3	118 ± 4	111 ± 4
GPC (Phenogel) [b]	25	79 ± 11	72 ± 13	70 ± 9	35 ± 9	71 ± 18	141 ± 107
	250	75 ± 7	85 ± 7	84 ± 8	60 ± 4	89 ± 10	77 ± 12
GPC (Biobeads) [a]	25	106 ± 33	119 ± 34	114 ± 37	120 ± 59	110 ± 55	101 ± 45
	250	103 ± 17	106 ± 11	102 ± 18	119 ± 15	112 ± 17	108 ± 17

Notes: [a] Quantitated by external quantitation. [b] Quantitated against E2-17Ac. [c] Not measured, tested at 250 ng only.

Florisil has also been used instead of (58,63,86) or in combination with aminopropyl sorbents to purify environmental extracts of estrogenic steroids (88). A method based on these sorbents has been used by the USGS (M. Burkhardt, pers. comm) and incorporates elution from the SPE extraction and cleanup in a single step (119). This reduces sample handling, the potential for cross-contamination, and reduces potential losses of more volatile components during nitrogen blow-down of the sample solutions. There was less variability in recovery rates with this method compared to that obtained by silica or off-line aminopropyl columns (Table 3.9). Although the recovery exceeded 100% at a spike level equivalent to 250 ng/L in samples, re-quantitation against the surrogate standards indicated mean recoveries of 91 to 106%, with the exception of E3, at 12 and 8% for the spike levels of 25 and 250 ng/L respectively.

After the NH_2/Fl clean-up steps, substantial interference from the matrix of the DSE samples remained in the extracts (Figure 3.5a and 3.5c). An additional GPC step was included in the method to reduce this (Figure 3.5b and 3.5d). With the reduction in sample matrix following NH_2/Fl cleanup, high resolution commercially packed Phenogel columns could be used, which provided better separation and used lower volumes of DCM compared to the Biobeads SX-8 laboratory packed columns.

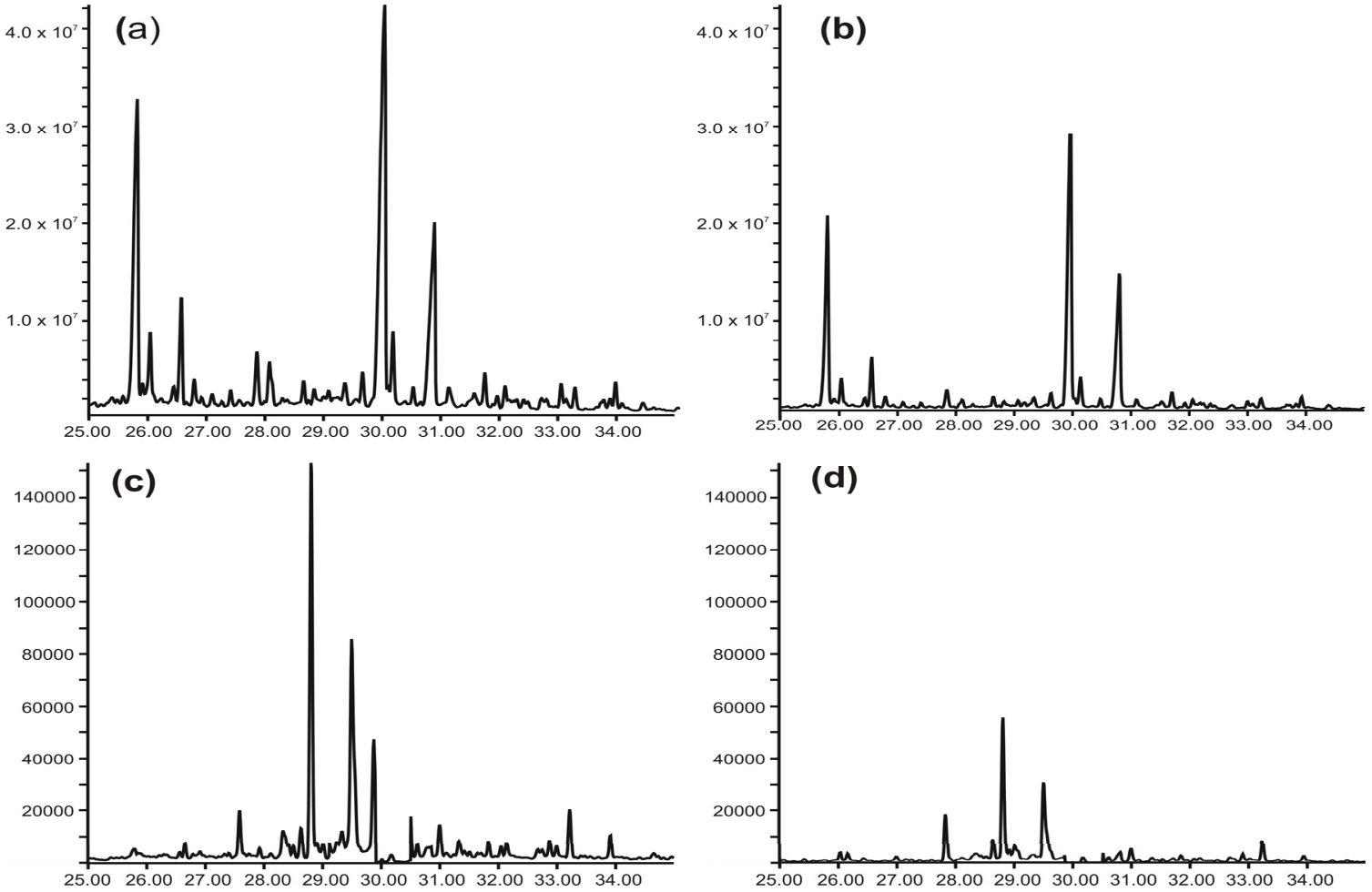


Figure 3.5: TIC chromatograms of DSE sample by before (a) and after (b) GPC polish and SIM chromatograms before (c) and after (d) GPC polish.

There was no statistical difference (95% confidence level) between the purification steps at a low spike level for 17α-E2, 17β-E2, E3 or EE2. There was a significant difference in the amount of E1 recovered ($p=0.013$), with a statistical difference between NH_2 and both Phenogel and silica purification. At the high spike level, there was a significant difference for 17α-E2, 17β-E2, 17β-E2-d_4 and E3. This was due to higher recovery from NH_2/Fl for 17α-E2, 17β-E2, 17β-E2-d_4 which was above that for all other cleanup methods. For E3, recovery from silica was significantly lower than with other tested methods.

Recovery of E3 was also low from NH_2/Fl and Phenogel column cleanups. In addition, much of the E3 was eluted with the conjugated estrogens, meaning a minimal amount of E3 was recovered from the full method. Many published studies for STP influents and effluent do not include E3, despite its likely occurrence in those samples, suggesting other researchers have also observed reduced recovery. This was not of concern for the study of dairy wastes, as E3 has not been identified as a metabolite in dairy cattle and was therefore not expected to be present in the samples. Because E3 and EE2 are not components expected to be present in DSE, they were not included in the final method validation. However, this may reduce the applicability of the method to analysis of other agricultural wastes where E3 is expected to be present.

3.3.4 Clean-up of Conjugated Estrogens

As matrix interferences greatly reduced detection of conjugated estrogens by LC-MS, several methods were tested for their suitability to clean-up sample extracts containing conjugated estrogens, including HPLC fractionation, GPC and various SPE adsorbents.

HPLC fractionation using a semi-preparative C18 column gave good recovery; however, the wide variation in polarity between the estrogen conjugates meant that a long fraction needed to be collected, resulting in ineffective cleanup. Reverse phase (phenyl, cyclohexyl, cyano and C8) SPE cartridges were also trialled; however, conjugated estrogens were not adequately retained on C8 or cyano columns, and phenyl or cyclohexyl columns did not provide any clean-up. Ideally a clean-up procedure should use a different mode of adsorption to those used for the sample extraction and for separation during analysis, so alternative methods were sought.

Gel permeation chromatography of E2-3S and E1-3S using Phenogel SEC columns has recently been reported by Schlusener (96), using THF/acetone (70:30) as mobile phase, and this was also trialled for its application to DSE. Though glucuronide conjugates were eluted within the expected elution volume, sulfate conjugates were irreversibly bound to the column with this solvent mixture. Though this was not reported by Schlusener (96), adsorption effects have been previously reported with polystyrene/ divinylbenzene columns (120). These effects are thought to result from active sites associated with residual components of the monomer material used to the make the column packing, and can be extremely variable from column to column (121). The columns used by Schlusener et al. (96) differed from those used in this study in internal diameter (21.2 mm versus 7.8 mm I.D.), length (300 mm versus 2 x 300 mm), particle size (5 µm versus 3 µm), column batch, and previously used solvents (not stated versus DCM). These differences could all contribute to subtle differences in the activity of binding sites for the acidic sulfate conjugates. A mixture of DCM:MeOH (1:1) was also trialled but conjugated estrogens eluted earlier than expected, co-eluting with other material in the sample matrix. This may be due to ionic exclusion, which has been reported for compounds with acidic functional groups (120).

Gel permeation chromatography using Sephadex LH-20 has been extensively used for the preparative extraction and/or separation of conjugated estrogens from urine (122,123), bovine liver (124) and, more recently, used to remove impurities in the analysis of steroid estrogens from sediments (65). Its potential to remove matrix interferences from DSE samples was assessed in this study. Sephadex LH-20 was swelled in MeOH and packed into a glass column (9 mm ID x 450 mm). Standards or samples were diluted in MeOH, applied to the GPC column and eluted under gravity using MeOH as the mobile phase, and fractions collected according to time or elution volume. Recovery for standards spiked into the mobile phase was complete; however, when spiked samples were tested, changes in viscosity resulted in changes in the flow rate and the recovery of spiked samples was poor. In addition, some compounds in the matrix were irreversibly bound to the LH-20 material, potentially affecting its longer term performance.

Sephadex LH-20 has also been used to separate conjugated estrogens with chloroform:MeOH containing low concentrations of salts (125,126). Under this solvent

system, partition is the major separation mechanism, rather than gel permeation (125). This solvent system was also trialled for purification of DSE samples; however, the problems observed with MeOH as solvent recurred, such as changes in viscosity and irreversible binding of matrix components to the Sephadex LH20 stationary phase.

In comparison, partition chromatography on SPE cartridges offered several advantages: disposable sorbents eliminate potential issues with irreversible binding of matrix components; substantially smaller columns decrease solvent use and elution time; and many samples can be purified at once using a vacuum manifold system. Florisil, silica and diol cartridges were trialled, with mixtures of chloroform:MeOH based on the solvents used for LH-20 in partition mode (125), and also with DCM:MeOH mixtures.

Initial trials indicated that silica could be suitable, as more polar interferences were retained on the columns. Silica SPE columns (500 mg) using a 1:1 mixture of chloroform: MeOH mixtures provided adequate recovery of the more polar glucuronides (Table 3.10). A higher proportion of MeOH was required using florisil, possibly due to the basic nature of florisil. An improvement on the silica clean-up can be achieved for sulfates with a mixture of 5:1 chloroform: MeOH. Under these conditions, recovery of E2-3S was 94 ± 6% at a spike concentration of 25 ng and 87 ± 3% at a concentration of 250 ng. Recovery of E1-3S was 93 ± 5% and 93 ± 1% respectively. However, the recovery of glucuronides was unacceptably low and therefore this solvent mixture could not be used in their analysis.

*Table 3.10: Comparison of percentage recovery of conjugated estrogens from various cleanup steps **

	Recovery on silica (1:1 CHCl3:MeOH)		**Recovery on aminopropyl**	
Compound	**Spike 25 ng**	**Spike 250 ng**	**Spike 25 ng**	**Spike 250 ng**
E3-3G	41	43	NM	NM
E3-3S	102	123	NM	NM
E2-3S,17G	83	40	97	80 ± 13
E2-3,17-diS	88	43	111	90 ± 5
E2-3G	43	52	NM	NM
E2-17G	102 ± 17	96 ± 12	NM	86 ± 3
E1-3G	100 ± 15	83 ± 10	92	85 ± 14
E2-3S	115 ± 25	87 ± 16	NM	102 ± 2
17α-E2-3S	114	145	NM	100
E1-3S	80 ± 20	60 ± 13	6	76 ± 7
E2-3S-d_4	NM	96 ± 29	NM	NM
E1-3S-d_4	73%	85 ± 23	NM	NM

Note: * Results expressed as mean values ± standard deviation where n = 3; single value reported where n=1.

Better cleanup was expected to be achieved when following this step with a sorbent orthogonal in selectivity to the reverse phase used in the LC-MS separation or the normal phase silica step. Aminopropyl cartridges were therefore assessed, using a mixture of 5% ammonia in MeOH, amended from Komori et al. (88) and Koh et al. (95), and provided acceptable recovery (Table 3.10).

The final method included cleanup on both silica and aminopropyl cartridges. The use of both phases effected removal of different interferences, allowed for simultaneous cleanup of samples in batches of 12-20, and was substantially quicker than methods using GPC or preparative HPLC. Recovery of the polar conjugates was undesirably low, however sulfates were expected to be the most important conjugates in the dairy wastes, as these are found at highest concentrations in dairy cattle urine (99) and tend to be more recalcitrant in wastewaters (16). For these reasons, final method validation included E2 and E1 sulfates only.

3.3.5 Comparison of Sample Preservation Methods

Sample preservation is required to prevent microbially-mediated degradation of conjugated estrogens into free estrogens and further degradation products. Methods were compared by measuring steroids in triplicate samples over the period up to 7 days after sample collection. E1-3S was the only conjugate that was detected in these DSE samples and was quantified by external calibration. Therefore some of the variation in E1-3S concentration may be due to differences in recovery between samples. However, the stability of E1-3S and other conjugated estrogens can be inferred from changes in free steroid estrogen concentrations.

Higher concentrations of E1 and 17α-E2 were measured in unpreserved samples compared to the acid and formaldehyde preserved samples (Table 3.11). One explanation for this is hydrolysis of conjugates in the short period between sample collection and the end of extraction (approximately 8 hours), as previously reported by Reddy et al. (93). In the absence of preservative, 17β-E2 concentrations increased over the storage period, while 17α-E2 increased between 1 and 2 days after collection, then decreased slightly, and E1 was relatively stable. E1-3S was detected in unpreserved samples only during the first three days of storage.

Table 3.11: Percentage of initial estrogen concentration (day 0) determined in the effluent after storage for 1, 2, 4 and 7 days.

	Concentration measured (ng/L)				Conc. relative to initial conc. (Day 0)			
	E1	**17α-E2**	**17β-E2**	**E1-3S**	**E1**	**17α-E2**	**17β-E2**	**E1-3S**
Unpreserved								
Day 0	49 ± 3	607 ± 2	6 ± 1	61 ± 24	100 ± 6	100 ± 0	100 ± 24	100 ± 51
Day 1	42 ± 1	650 ± 10	25 ± 6	11 ± 0	87 ± 2	107 ± 2	456 ± 24	26 ± 60
Day 2	44 ± 5	698 ± 55	30 ± 8	20 ± 5	89 ± 10	115 ± 8	548 ± 27	19 ± 0
Day 4	42 ± 1	667 ± 25	45 ± 2	ND	86 ± 2	110 ± 4	806 ± 4	0 ± 0
Day 7	50 ± 0	648 ± 33	55 ± 4	ND	103 ± 0	107 ± 5	991 ± 8	0 ± 0
Formaldehyde								
Day 0	46 ± 1	429 ± 11	11 ± 1	63 ± 23	100 ± 3	100 ± 3	100 ± 6	100 ± 44
Day 1	44 ± 2	503 ± 16	8 ± 0	22 ± 1	96 ± 5	117 ± 3	69 ± 5	75 ± 10
Day 2	46 ± 0	536 ± 37	6 ± 0	35 ± 8	101 ± 0	125 ± 7	52 ± 7	24 ± 12
Day 4	46 ± 3	537 ± 27	5 ± 1	25 ± 2	99 ± 7	125 ± 5	41 ± 12	35 ± 2
Day 7	48 ± 2	534 ± 31	4 ± 1	ND	105 ± 5	125 ± 6	39 ± 25	0 ± 0
Acid								
Day 0	38 ± 2	468 ± 17	18 ± 0	44 ± 27	100 ± 6	100 ± 4	100 ± 2	100 ± 80
Day 1	34 ± 3	449 ± 16	19 ± 1	21 ± 0	90 ± 9	96 ± 4	102 ± 3	28 ± 0
Day 2	36 ± 4	455 ± 17	20 ± 1	27 ± 6	93 ± 11	97 ± 4	107 ± 6	15 ± 22
Day 4	35 ± 3	422 ± 10	19 ± 0	29 ± 1	86 ± 2	92 ± 1	101 ± 3	82 ± 10
Day 7	35 ± 3	422 ± 10	19 ± 0	29 ± 1	90 ± 8	90 ± 2	102 ± 2	0 ± 0

Prior studies of unpreserved samples of sewage influent and effluent have not reported increases in free estrogen concentrations over time. A possible explanation for this discrepancy could be higher concentrations of conjugated estrogens in the DSE samples. These contained freshly excreted urine and faeces, whereas human excreta can take some time to travel to sewage treatment plants. Faecal bacteria, including *E. coli*, are also expected to be present at high concentrations in unpreserved dairy and sewage effluents and they produce the β-glucuronidase enzyme which can hydrolyse conjugated estrogens, increasing free estrogen concentrations.

In formaldehyde-preserved samples, 17β-E2 decreased slightly over the 7 day storage period and 17α-E2 concentrations demonstrated an initial increase then stabilised. E1 concentrations were similar throughout the period and E1-3S was detected up to 5 days of storage. In acid-preserved samples, 17β-E2 and E1 were measured at constant concentrations throughout the 7 days of storage while 17α-E2 decreased slightly over the period. E1-3S was detected after up to 7 days of storage in the acid preserved samples; however, the concentrations were extremely variable over this time.

The results suggest that acidification is the best preservation method, based on the stable concentrations of free estrogens in those samples compared to those measured in

unpreserved and formaldehyde preserved samples. However, given the decrease in concentrations of E1-3S measured on day 2 compared to day 1, it may be prudent to extract samples immediately, even if preserved by acidification.

3.3.6 Method Detection Limits

Method detection and quantitation limits were based on concentrations in actual samples corresponding to *S/N* ratio of 3 and 10 respectively. The detection limits (Table 3.12) for free estrogens for surface waters are comparable to those previously published based on GC-MS with large volume samples (127-129), and are a slight improvement on Lee et al. (130), Kolpin et al. (131) and methods using LC-MS-MS (e.g., (63)). Most importantly, the limit of detection for these compounds is within the range of environmental concern, with no effect concentrations suggested to be 1 ng/L (132).

Table 3.12: Method detection and quantitation limits in stream water and DSE samples.

	Stream water 2 L sample>250 µl		**DSE** 0.5 L sample>250 µl	
Compound	**MDL**	**MQL**	**MDL**	**MQL**
E1	0.2	0.4	0.8	2.5
17α-E2	0.1	0.4	0.8	2.5
17β-E2	0.1	0.4	0.8	2.5
E1-3S	3	9	15	45
17α-E2-3S	12	26	15	45
17β-E2-3S	3	9	15	45

Detection limits for the free estrogens in DSE are higher, reflecting the effect of sample matrix on instrument performance. The detection limits are an improvement on early studies by Raman et al. (39), who reported a detection limit of 10 µg/L and on Hanselman et al. (125 ng/L for 17α-E2, 17β-E2 and E1, (47)), and Hutchins et al. (43) (4 ng/L, 20 ng/L and 12 ng/L for 17α-E2, 17β-E2 and E1 respectively). As the latter two studies used substantially lower sample volumes (40 mL and 25 mL respectively) compared to 500 mL in this study, it is likely that their detection limits could have improved with a higher concentration factor. The current method could also be adapted to use a lower sample volume if conjugated estrogens are not of interest. A further study (44) did not report the detection limits achieved using LC-MS-MS to analyse estrogens in swine wastewater lagoons, though the lowest concentration of 17β-E2 reported was 12 pM (3.3 ng/L), suggesting similar detection limits to the present study.

The detection limits obtained for the conjugated estrogens in surface waters are higher than those reported using triple quadrupole LC-MS-MS systems (16,58,62,63,93,94), despite the high concentration factor used for this study. The MQLs obtained for this study are above likely concentrations in most environmental waters, indicating that LC-IT-MS instruments may not be appropriate for analysis of environmental samples.

MDLs were not required to be as low when analysing effluent samples as much higher concentrations are expected in those samples. The MDLs for DSE samples were approximately 5-fold higher compared to cleaner stream water, due to sample matrix effects (ionisation suppression and higher background). This is consistent with the findings of previous studies; for example, detection limits for E2-3S and E1-3S were up to 40-fold higher in sewage influent and 8-fold higher for sewage effluent compared to river waters (62). This indicates sample matrix can adversely affect detection limits even when using more sensitive triple quadrupole instruments. The only other published report on the analyses of conjugated estrogens in agricultural wastes reported a limit of detection of 1 ng/L (43). However this LOD was based on the lowest quantitation standard and does not account for ionisation suppression and increased noise experienced when analysing samples, due to the presence of sample matrix. The corresponding MDL would most likely be considerably higher.

3.3.7 Variation in Replicate Samples

Replicate samples were analysed to provide an indication of the precision of the overall method. Samples were collected from the same location in separate sample bottles then extracted and analysed following the procedures developed. The results (Table 3.13) indicated good precision for the free estrogens, but variable concentrations of E1-3S.

Table 3.13: Concentration of estrogens in replicate samples of DSE (ng/L).

	E1	17α-E2	17β-E2	E1-3S
Replicate 1	41	472	18.6	34
Replicate 2	37	482	18.2	74
Replicate 3	37	449	17.9	24
Average	38	467	18.2	44
Standard Deviation	2	17	0.3	27
CV	6%	4%	2%	61%

3.3.8 Recovery in Different Matrices

The performance of the entire method was assessed by spiking MQ water, stream water, and DSE samples with free and conjugated estrogens at a range of environmentally relevant concentrations. Recovery of free estrogens ranged from 65–144% at concentrations of 25 ng/L, with the exception of 17α-E2 in the DSE (Table 3.14), where interference in some of these particular samples resulted in apparently higher recovery (91-222%). Recovery was 91-129% at concentrations of 250-2500 ng/L. For the conjugated estrogen sulfates, recovery ranged from 71-176%, with the exceptions of E2-3,17diS and E2-17G,3S which were not recovered in the spiked samples, probably due to low recovery through SPE extraction and clean-up stages.

Table 3.14: Mean recovery of estrogens in MQ, stream water and DSE samples (percentage recovery ± standard deviation, n=5).

	MQ water		Stream water	DSE		
Compound	**25**	**250**	**12.5**	**25**	**250**	**2500**
17α-E2	144 ± 47	103 ± 9	123 ± 2	172 ± 50	125 ± 16	129 ± 3
17β-E2	99 ± 10	91 ± 14	92 ± 2	88 ± 2	90 ± 3	91 ± 2
E1	104 ± 10	93 ± 8	92 ± 2	92 ± 4	91 ± 4	83 ± 1
E2-3S	107 ± 8	92 ± 7	106 ± 8	158	98 ± 10	103 ± 2
17α-E2-3S	124 ± 28	71 ± 48 98 ± 15 [a]	176 ± 32	236	100 ± 37	101 ± 21
E1-3S	97 ± 9	75 ± 15	111 ± 8	228	121 ± 10	111 ± 9

Notes: [a] Excluding outlier.

During method development considerable signal enhancement was observed following the SPE steps, demonstrating external calibration was not a reliable method for quantitating the free steroid estrogens. Therefore, in order to assess the absolute recovery of free estrogens in the spiked samples, quantitation was based on the response relative to E2-17Ac, added to sample extracts as an internal standard. The resultant calculated recoveries (Table 3.15) suggest substantial loss of analytes at high concentrations

(2500 ng/L) in DSE, with recoveries averaging 31-43%. However the response of target analytes at 2500 ng/L was approximately 7.5-12-fold higher than that for samples spiked at 250 ng/L, which is close to the 10-fold increase in response expected from the linear calibration curves.

Table 3.15: Absolute recovery of free estrogens spiked into MQ, stream water and DSE samples.

	MQ water		Stream water	DSE		
Compound	**25**	**250**	**12.5**	**25**	**250**	**2500**
17α-E2	143 ± 82	71 ± 22	166 ± 10	186 ± 24	73 ± 17	31 ± 6
17β-E2	99 ± 41	67 ± 23	130 ± 11	67 ± 3	60 ± 13	31 ± 6
E1	118 ± 41	73 ± 20	163 ± 9	111 ± 14	83 ± 7	43 ± 6

It appears E2-17Ac is not a good choice of internal standard for the target analytes in these samples, as its response was enhanced in the presence of sample matrix, eluting at a similar time in the GC-MS chromatogram. Conversely, the response of the isotopically labelled surrogate internal standards and analytes was reduced in the presence of sample matrix, due to signal suppression in this earlier region of the chromatogram. Ratios of the deuterated standard response compared to E2-17Ac (ISTD) response are compared for the solvent standards, stream matrix and DSE (Table 3.16), and demonstrate the effect of residual sample matrix on signal response.

Table 3.16: Comparison of ISTD response ratios between different matrices.

	Solvent standards	**Stream samples**	**DSE samples**
Response in matrix relative to solvent standard response			
ISTD E2-17Ac	1.0	1.3	1.4
17β-E2-d_4	1.0	0.54	0.42
E1-d_4	1.0	0.68	0.51
Ratio of response within each matrix			
Ratio E2-d_4 : E2-17Ac	1.0	0.41	0.31
Ratio E1-d_4 : E2-17Ac	1.1	0.58	0.43

Preparation of calibration standards in matrix extracts can reduce this effect (107,133), but requires that the matrix of the sample is the same as the matrix used for the standards (106). This may be difficult to ensure when environmental samples are collected from different sources. On the basis of the recovery data, it is considered that the isotope dilution method is more accurate for quantification of estrogens in environmental samples compared to the internal standard method using E2-17Ac or other structural analogues of steroid estrogens. This is consistent with a previous study which demonstrated that the relative response of steroid estrogens was variable and when E2-

17Ac was used as internal standard, their concentrations were more likely to be under- or over-estimated, when compared to concentrations calculated using the isotope dilution method (129).

3.3.9 Comparison to Standard Addition Method

The standard addition method can also be used for quantification in the presence of sample matrix. Concentrations of 17α-E2 and E1 in the non-spiked DSE samples were calculated based on the standard addition method from the samples spiked at 25, 250 and 2500 ng/L. The response was first corrected against the response of E2-17Ac to allow for differences in final sample volume or injected volume. As the matrix was the same for all samples, this was considered acceptable. The concentration of 17α-E2 by standard addition was calculated to be 24 ng/L in the original sample, compared to 39 ng/L when calculated by isotope dilution. E1 was calculated at 3 ng/L and 7 ng/L for standard addition and isotope dilution methods respectively. For both analytes, the isotope dilution method gave a slightly higher concentration; however, given the complexity of the samples and the range in concentration observed in environmental samples, the difference in results obtained by isotope dilution and standard addition was considered acceptable for this study. The standard addition method requires the analysis of up to four injections for each sample, increasing the time (and cost) of analysis, and importantly for DSE samples and other complex waste matrices, increasing the negative impact on injector, column and detector performance resulting from accumulated sample matrix.

3.4 CONCLUSIONS

A sensitive and selective method was developed for analysis of the free estrogens 17β-E2, 17α-E2 and E1 at ng/L levels in environmental and DSE samples. The developed method used SPE extraction with Oasis HLB cartridges, followed by elution and clean-up on aminopropyl and florisil sorbents. The extract was further purified through high resolution GPC, before derivatisation and analysis by GC-MS in SIM mode. Isotopically-labelled standards were used for quantitation and the overall method detection limit was 0.8 ng/L for free estrogens in DSE samples. The method developed was reliable and

robust for multiple matrices, and over a concentration range of several orders of magnitude.

DSE samples required additional purification for the analysis of conjugated estrogens when compared to methods published for water samples. Substantial difficulties were experienced in determining a clean-up step that was suitable for all conjugated estrogens, due to the wide range in polarities and acidities of this group of compounds. LC-TOF-MS was shown to be unsuitable for DSE samples due to high background noise, despite the inclusion of rigorous purification steps prior to analysis. While LC-IT-MS could achieve acceptable method detection limits for the sulfate conjugates, detection limits for the glucuronide conjugates were not satisfactory. Neither LC-TOF-MS nor LC-IT-MS could achieve the lower detection limits that were required for environmental water samples.

Despite these difficulties, a method was developed to measure sulfate conjugates in DSE samples. The developed method was based on SPE extraction and purification, and gave acceptable recovery for most conjugated estrogens. LC-IT-MS in SRM was used for analysis, and deuterated E2-3S was used for quantitation of conjugated estrogens, using istope dilution. The overall method provided detection limits of ~15 ng/L for sulfate conjugates in DSE samples.

CHAPTER FOUR

SURVEY OF STEROID ESTROGENS AND ESTROGENIC ACTIVITY IN NEW ZEALAND FARM DAIRY SHED EFFLUENTS

Chapter 4: Survey of steroid estrogens and estrogenic activity in New Zealand farm dairy effluents

4.1 INTRODUCTION

Estrogenic contamination of surface waters is of considerable concern world-wide, with accounts of feminisation of male fish, reproductive abnormalities and skewed sex ratios attributed to the presence of steroid estrogens and xeno-estrogens (7). Since initial studies demonstrated intersex fish downstream of sewage treatment plants (STPs) outfalls (2), a significant body of research has focussed on municipal effluents as sources of estrogenic chemicals, particularly natural estrogens including 17β-E2, E1, E3 and the synthetic steroid estrogen EE2 (9,16,85). In the past five years agricultural wastewaters have become widely recognised as potential sources of environmental estrogens (33-35,134). In fact, estimates of the loads of steroid estrogens excreted from humans and livestock in the United Kingdom (33), the United States (35) the Netherlands (34) and New Zealand (this study) suggest that, rather than humans, dairy cattle are the most significant contributor of steroid estrogens into the environment.

Dairy farming is a major and growing industry in New Zealand, particularly in the Canterbury and Waikato Regions. There are 820 dairy farms in Canterbury, with a total of 755,000 dairy cows (53). The Waikato Region has 5100 farms and a total of 1,669,000 dairy cows, over 30% of New Zealand's total herd (53). On NZ dairy farms, the majority of animal excreta is deposited onto paddocks during grazing and is not subject to any treatment. However, 10-20% of excreta is deposited within the farm dairy shed or yard area during milking (50) where it is captured as farm DSE that also contains wash-down water and chemical products used to clean the milking plant (50). This effluent is then either applied to land, or treated and discharged into waterways.

Recent studies of dairy manures and wastewaters confirm that steroid estrogen concentrations are high in these wastes, but there is limited data to properly assess the risk of dairy wastewaters to the receiving environment, especially aquatic environments. With the exception of the extensive study undertaken by Raman et al. (39), studies

published to date have focussed on characterising steroid content in a small number of dairy operations (typically one to three). Data varies between each study, with median concentrations of 17β-E2 ranging from 87 ng/L to 1700 ng/L (39,40,43,47).

Furthermore, the majority of these studies have not considered the presence of conjugated estrogens. Steroid estrogens are excreted by mammals as conjugated metabolites, where they are attached to either a sulfate or glucuronide group (99). While these metabolites have lower estrogenic activity than the free forms, studies show that glucuronide forms can be readily deconjugated by bacteria found in sewage systems (15). As this process can take up to several days (16,97,98,135), the measurement of free estrogens alone may underestimate the total load of estrogens entering the environment from freshly excreted manures or wastewaters. A recent study, examining three replicate samples, provided preliminary data indicating their presence in dairy wastes with 17α-E2-3S present at 141-182 ng/L, E1-3S at 85-91 ng/L and 17β-E2-3S at 39-44 ng/L, similar concentrations to those of free steroid estrogens in the samples (43).

Because steroid estrogens and estrogen mimics have an additive effect *in vivo* (136,137), potential effects of complex effluents are best evaluated using bioassays. These integrate the estrogenic activity of all compounds present within a sample and thereby provide an estimate of the overall potential estrogenic effect. *In vitro* bioassays have been used in New Zealand (56) and internationally (reviewed in (28,68)) to assess potential estrogenic activities of sewage effluents, which can contain a mixture of steroid estrogens and estrogen mimics, such as alkylphenols. When used in combination with chemical analysis of target compounds, bioassays can indicate potential causation of observed estrogenic activity in environmental samples (68). This approach has been widely used in assessing estrogenicity of STP influents and effluents (e.g., (138-140)), rivers (141), and marine waters (142).

This study has used chemical analysis and bioassay to investigate steroid estrogens in DSE, potentially a major source of estrogens to the New Zealand environment. Conjugated estrogens have been measured to provide an assessment of their significance in these wastes and of how much they contribute to the total load of estrogens released to the New Zealand environment from this agricultural waste source.

4.2 MATERIALS AND METHODS

4.2.1 Sampling Locations

DSE samples were collected from 18 privately owned farms operating with herd sizes of 140 to 1000 cows. Because access on to private property was essential and information about the farms was required, farms were not selected at random throughout the two regions, but were located where farmers had existing relationships with scientists and regulators. Farms were located within three stream catchments, the Toenepi Stream (Waikato), Waikuku Stream and Pahau River (Canterbury), where dairy farming was a major land use.

Samples were collected from the farms during the milking season, which begins around July to August and ends in April to May, with some variation by location. Samples were collected from farms in Toenepi and Waikuku catchments at the start and end of the 2006/2007 milking season. The Pahau catchment, where farm sizes are generally larger, was only sampled at the end of the season. Three farms in the Toenepi catchment (T2, T4 and T5) were sampled five times over two seasons to give some indication of variation in steroid levels and estrogenic activity with time. On these farms, sampling was undertaken in August 2006 and in May, August, November and December 2007.

4.2.2 Sampling and Extraction

In this study, samples were either collected directly from the milking shed collection sump or at the outlet from the final treatment pond depending on the effluent system. Grab samples were collected in solvent- and MQ water- rinsed amber glass bottles with Teflon-lined lids. Samples were immediately preserved by the addition of sulfuric acid (to pH ≤ 2) and transported to the laboratory on ice. Sample processing began immediately on return to the laboratory and was completed within 36 hours. Samples were stored at < 4 °C during this period where necessary. In addition, subsamples were collected from the Waikuku catchment, and at the three farms repeatedly sampled, for analysis of total nitrogen, total Kjedahl nitrogen, total phosphorus and total organic carbon. These were analysed using standard methods at a commercial laboratory.

Samples were centrifuged at up to 1750 *g* for 20 mins at 4 °C, and then filtered through glass fibre filters (GF/C, pore size 1.2 μm Whatman) with the addition of filter aid. Samples were extracted in duplicate (500 mL each) for i) chemical analysis of steroid estrogens and conjugated estrogens, and ii) estrogenic activity using the E-screen assay. In the August 2006 survey, samples for chemical analysis were spiked with 250 ng 17β-E2-d_4 as a surrogate standard. In later surveys, samples for chemical analysis were spiked with a range of surrogate standards (250 ng each E1-d_4, 17β-E2-d_4, E1-3S-d_4 and 17β-E2-3S-d_4). All centrifuged and filtered samples were loaded onto 500 mg Oasis HLB cartridges (preconditioned with 10 mL of MeOH followed by 10 mL of MQ water) at a flow rate of 5-10 mL/min. As samples collected in the Pahau catchment appeared to have a higher solids and organic content, 1 g cartridges were used to ensure that sorbents were not overloaded or blocked by fine particulates and colloidal components not removed by filtering.

4.2.3 Chemical Analysis

Samples for chemical analysis were eluted from the Oasis HLB cartridges in two fractions to separate conjugated estrogens and free estrogens, as described in detail in Chapter 3 and summarized briefly here. Cartridges were washed with MeOH water mixtures, then conjugated estrogens were eluted with 75% MeOH/water containing 2% ammonium hydroxide (8 mL). Free estrogens were then eluted with 30 ml of DCM:DEE:MeOH (40:10:1) through aminopropyl and florisil sorbents, to provide preliminary clean-up before the extract was further purified by gel permeation chromatography (GPC). TFAA was added to the extract collected from GPC to derivatise estrogens prior to GC-MS analysis in SIM/Scan mode (as fully described in Chapter 3).

In the first round of sampling, free estrogens were quantified against 17β-E2-17-acetate, added to the purified sample extracts as an internal standard, and their recovery corrected against the concentration of the surrogate 17β-E2-d_4 measured in the samples. For the second sampling round, estrogens were quantified by isotope dilution, based upon the relative response factors for the following pairings: 17α-E2/E2-d_4, E2/E2-d_4, E3/E2-d_4, E1/E1-d_4. Only data collected in SIM mode was used for quantitation.

Samples collected in the second round (at the end of the milking season) were also analysed by LC-IT-MS to assess the presence of conjugated estrogens. The conjugated estrogen fractions from the Oasis columns were dried under nitrogen and reconstituted in MeOH (250 µL), then cleaned up through silica gel (10% deactivated, 1 g) and aminopropyl columns (500 mg, JT Baker) connected in series. The silica columns were eluted with 20 mL of chloroform:MeOH (1:1) onto the aminopropyl columns under a low vacuum. The aminopropyl columns were rinsed with acetonitrile (3 mL), acetonitrile with 10 mM ammonia (3 mL), and MeOH with 10 mM ammonia (3 mL), then conjugated estrogens were eluted by applying 2 x 3 mL aliquots of MeOH containing 2% ammonia. Eluted extracts were dried under nitrogen and made up in acetate buffer (0.01 mM ammonium acetate in water:acetonitrile (88:12)), then analysed for conjugated estrogens using LC-IT-MS in negative ESI mode as described in Chapter 3. MS data was acquired by selected reaction monitoring (SRM) and conjugates were quantified by isotope dilution against 17β-E2-3S-d_4.

4.2.4 E-Screen Analysis

Samples for E-screen assay were simply eluted from the Oasis HLB cartridges with 30 ml of DCM:DEE:MeOH (40:10:1), dried down to approximately 1 mL, and solvent exchanged into DMSO (500 µL). Samples were stored at <20 °C in the dark until analysed by the E-screen assay.

The E-Screen assay method followed that previously described by Soto et al. (32), Korner et al. (143) and Leusch et al. (144) with minor modifications. Estrogen receptor-positive human MCF-7 BOS breast cancer cells, obtained as a gift from A. Soto (Tuft University), were cultivated in an atmosphere with 5% CO_2 at 37 °C in 25-cm² flasks (Sarstedt) in growth media consisting of phenol-red-free Dulbecco's modification of Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 0.025 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 4 mM L-glutamine and 0.1 mM non-essential amino acids. The steroid-free experimental medium consisted of phenol-red-free DMEM supplemented with 10% charcoal-stripped FBS, 0.025 mM HEPES, 4 mM L-glutamine and 0.1 mM non-essential amino acids. The MCF-7 cells were reconstituted in steroid-free experimental medium, seeded at a concentration of 25,000

cells/mL in sterile 96-well flat-bottom tissue culture plates (Nunclon), and incubated overnight at 37 °C in 5% CO_2/air.

Serial dilutions of samples or 17β-E2 (standard) were prepared in DMSO and added to the 96-well plate in triplicate. DMSO was used as the negative control. Outside wells were not used, due to the effects of evaporation (145). All plates were incubated for a further 5 days after cell exposure. Cell proliferation was assessed at the end of this period by analyzing formazan production after addition of 20 µL CellTiter 96 AQueous non-radioactive cell proliferation assay (Promega) to each assay well. After incubation (3 h), absorbance at 492 nm was measured for each well using a plate reader (FLUOStar model 403, BMG Lab Tech).

Absorbance in each set of wells was plotted against dilution and a curve was fitted by least squares regression based on the symmetric logistic model shown in *Equation 4.1*.

Equation 4.1: $$y = Min + \frac{Max - Min}{[1 + 10^{(\log EC_{50} - \log x) * slope}]}$$

The slope was fixed at 1.0 for all standards and samples and the maximum response was constrained to that of the positive control (17β-E2) for that experiment, in order to fit realistic curves. In addition, where reductions in cell response were observed at high concentration due to cytotoxicity, the affected data were excluded from the regression analysis. The EC50 for each sample was determined from the modelled curve. If the calculated EC50 did not fall within the range of concentrations measured for that sample it was considered unreliable and that sample considered below the detection limit. Estrogen equivalent concentrations for each sample (EEQ) were calculated from EC50 [17β-E2, ng/L] / EC50 [sample].

The relative proliferative effect (RPE) was calculated for samples where a plateau of maximum estrogenic activity was reached. For consistency, maximal cell yield was set at EC95 and calculated from the modelled curve, and RPE calculated as the ratio of the maximum proliferation induced by a sample to that induced by the positive control, 17β-E2. Samples were considered below detection limits if the maximum proliferation was less than 20% of the maximum proliferation of the standard curve.

Method quality control procedures included analysis of a standard curve for each 10 samples; at least two independent assays were performed for each sample with the average value reported. The quantitation limit varied with the cytotoxicity of the samples in the assay; it was typically 5 ng/L, but ranged from 0.3-19 ng/L.

4.2.5 Model Compounds and Calculation of Predicted Estrogens

Steroid estrogens and their conjugates were tested using the E-Screen to determine the relative potency of compounds likely to be present in DSE samples. Free estrogens were prepared at a concentration of 10 μM in DMSO, while conjugated estrogens were prepared at a higher concentration of 1-2 mM due to their expected lower potency. All compounds were tested in the E-Screen following the procedure described above. The final concentrations in the microtitre plate wells were ~0.8-50,000 pM for the free estrogens and from 0.08-10,000 nM for the conjugated estrogens.

To calculate EC50s, absorbance in each set of wells was plotted against concentration, the logistic curve was fitted and EC50 calculated as described above. The relative potency (RP) for tested compounds was calculated from the ratio EC50 [17β-E2, nM] to EC50 [test compound, nM]. The relative proliferative effect (RPE) was also calculated for pure compounds as described above. Full agonistic activity can be distinguished as RPE from 80–100%, while values less than 80% represent partial agonist activity (138).

Estrogen equivalents were predicted for the DSE samples from the measured concentration of each steroid (by GC-MS) multiplied by the relative potency (RP) of that steroid obtained by the E-Screen. The EEQs for each compound measured in a sample were summed to obtain the total predicted EEQ for that sample. This approach, based on the toxic equivalency factor (TEF) approach, has been shown to be appropriate for mixtures where each component acts through the same pathway and the dose-response curves are parallel for individual compounds (137).

4.2.6 Nutrient analysis

Samples of effluent were analysed by a commercial laboratory for total organic carbon (TOC), total phosphorus (TP), total nitrogen (TN), total Kjeldahl nitrogen (TKN) and oxidised forms of nitrogen (NOxN: nitrate-N and nitrite-N) using standard methods (146).

4.3 RESULTS AND DISCUSSION

4.3.1 Response of Free and Conjugated Estrogens in the E-Screen

All conjugated and free estrogens expected to be found in the DSE samples were analysed by bioassay to establish their potencies relative to 17β-E2. Although there are published potencies for many estrogenic compounds in E-Screen (29,31,32,138), there can be considerable variation due to inter-laboratory variation in the protocol and cell-lines used in the E-Screen (147). Potencies were therefore derived in this assay following the same procedures used to test environmental samples.

The response of free estrogens in the E-Screen assay is shown in Figure 4.1 and demonstrates the characteristic S-shaped dose-response curve expected for this assay. Dose response curves were parallel, demonstrating that the TEF approach is suitable for combining the effect of mixtures of estrogenic compounds. Of the free estrogens, estriol (E3) was the most potent compared to 17β-estradiol (E2), followed by estrone (E1) and 17α-estradiol (17α-E2, Figure 4.1). All the free estrogens tested demonstrated full proliferation, with RPE close to 100% of 17β-E2.

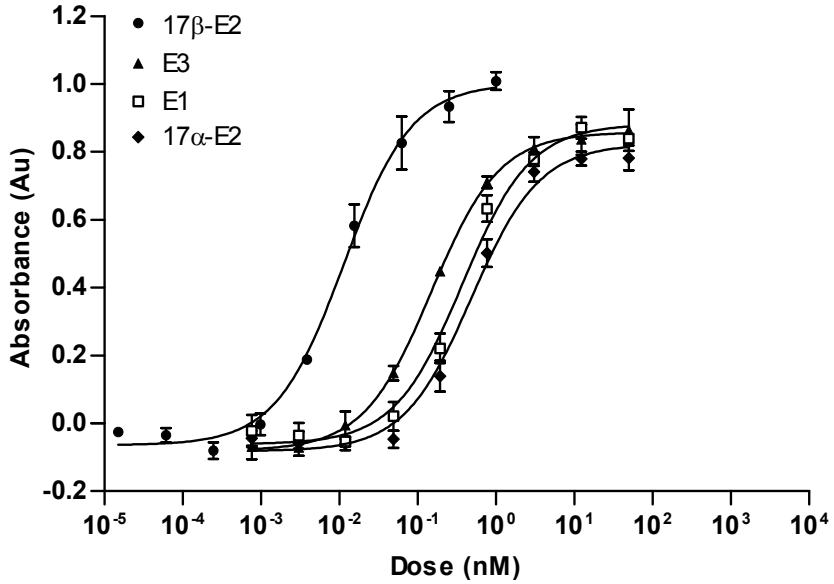


Figure 4.1: Estrogenic response of free steroid estrogens in the E-Screen assay. Results are expressed as the mean (± standard error) absorbance of the triplicate exposed wells with background absorbance subtracted. Absorbance is directly proportional to cell number.

The response of conjugated estrogens in the E-Screen assay similarly displayed the characteristic S-shaped dose-response curve. Many conjugated estrogens also demonstrated full proliferation at the highest concentrations tested (Figure 4.2), although their potency was several orders of magnitude lower than the free steroids, evidenced by the higher concentrations required to cause a response in the assay. Estradiol-disulfate (E2-diS) and estradiol-17-glucuronide-3-sulfate (E2-3S,17G) did not reach an asymptopic range in cell proliferation at the concentrations tested, and the RPE is therefore a minimum. The dose response curves were parallel only for those compounds that demonstrated full proliferation, suggesting that the TEF approach may not be accurate for combining the effect of mixtures of these compounds.

The potency of E1 and 17α-E2 was within the range of reported values for estrogenic potencies measured previously using the E-Screen (Table 4.1), and the potency of E3 was slightly lower. The RPE values for E1 and 17α-E2 were also very similar to literature values, whereas E3 was slightly higher (91% in this study compared with 82% (30)).

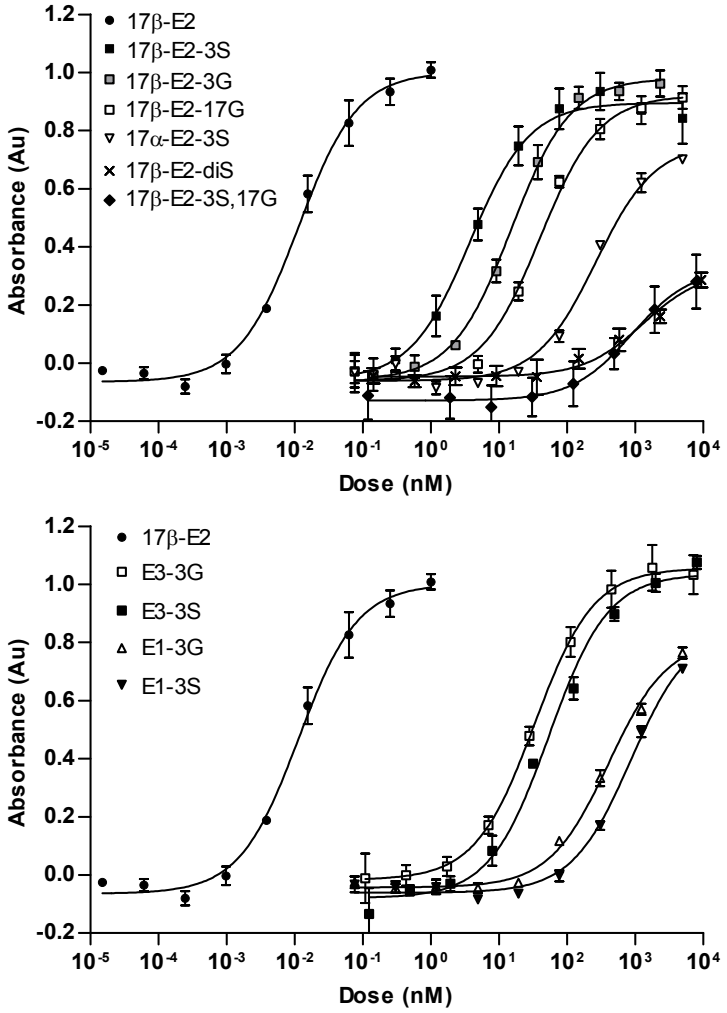


Figure 4.2: Estrogenic response of conjugated estrogens compared to 17β-E2. Results are expressed as the absorbance of the exposed wells with background absorbance subtracted. Absorbance is directly proportional to cell number.

No comparative data was available for the estrogenicity of conjugated estrogens obtained by E-Screen, but data obtained using other assays was available (Table 4.2). 17β-E2-3S elicited estrogenic responses in two receptor-reporter gene yeast assays (148,149) with relative potencies 3-4 orders of magnitudes lower than 17β-E2, slightly lower than was measured in this study. 17β-E2-3G was estrogenic in one receptor-reporter gene yeast assay when tested at a concentration up to 10 μM (148), but not in the YES (149) or ER-CALUX (150) assays, possibly due to the lower concentrations used in these assessments (maximums of 50 and 10 nM respectively). 17β-E2-3G,17S did not demonstrate estrogenic activity in a recombinant yeast assay (maximum concentration 10 μM, (148)), and less than 1% of E1-3S bound to rat ERα and ERβ at a maximum concentration of 100 μM (71).

Table 4.1: Potency of free estrogens relative to 17β-estradiol as measured in this study, and compared to literature values using the E-Screen assay.

	17β-E2	17α-E2	E1	E3
Relative Potency				
This study [a]	1	0.02	0.024	0.054
Gutendorf & Westendorf (29)	1	NM [b]	0.01	0.071
Soto et al. (32)	1	0.1	0.01	0.1
Soto, unpubl., cited in Fang et al. (30)		0.0079	0.044	0.25
Korner et al. (138)	1	NM	0.096	NM
Leusch et al. (31)	1	NM	0.012	NM
Relative Proliferative Effect				
This study [a]	100	91	94	91
Soto, unpubl., cited in Fang et al. (30)	100	90	95	82
Korner et al. (138)	100	NM	112	NM
Leusch et al. (31)	100	NM	77	NM

Note: [a] Values are average of at least two independent assays conducted in triplicate. [b] NM = Not measured.

Table 4.2: Potency of conjugated estrogens relative to 17β-estradiol as measured in this study, and compared to literature values using a range of assays.

	Relative Potency					RPE	RIE
	E-Screen [a] This study	ER-CALUX [b]	Yeast Assay [c]	Yeast Assay [d]	Receptor binding [e]	E-Screen [a] This study	Yeast Assay [c]
17β-E2	1	1	1	1	1	100	100
E1-3G	0.000029	-	-	-	-	93	-
E1-3S	0.000012	-	-	-	<0.01	94	-
17β-E2-3G	0.0013	NR [f]	0.0032	NR [f]	-	95	90
17β-E2-17G	0.00037	-	-	-	-	101	-
17β-E2-3S,17G	0.000017	-	-	-	-	41 [g]	-
17β-E2-3S	0.0026	-	0.0001	0.001	-	93	20
17β-E2-diS	0.000012	-	-	-	-	33 [g]	-
17α-E2-3S	0.000036	-	-	-	-	87	-
E3-3G	0.00055	-	-	-	-	98	-
E3-3S	0.00055	-	-	-	-	101	-

Note: [a] Values are average of at least two independent assays conducted in triplicate. [b] (150) [c] (148). [d] (149) [e] (71). [f] No response in assay, potency not calculated. [g] Minimum RPE, asymptope not reached.

Overall the results in this study confirm conjugated estrogens are weakly estrogenic when tested at μM concentrations, with potencies comparable to weak estrogen mimics such as phytoestrogens and nonylphenols (reviewed in (28) and (31)). Because the environmental concentrations of conjugated estrogens (ng/L) are expected to be much lower than industrial compounds (μg/L, (28)), they are considered to be inactive or have extremely low estrogenic potency (9,16,98,151). However, they remain a significant estrogenic risk as they can readily be deconjugated to their highly active free forms.

4.3.2 Steroid Estrogen Concentrations

Steroid estrogens were detected in all samples of DSE, with 17α-E2 present at the highest concentration in all samples (Table 4.3), followed by E1 and then 17β-E2 (with one exception). Steroid concentrations in effluents from milking farms ranged from 110 to 11,000 ng/L for 17α-E2 (median of 730 ng/L), 1 to 310 ng/L for 17β-E2 (median 24 ng/L), and 10 to 580 ng/L for E1 (median 100 ng/L). The concentration of free estrogens for samples collected in August was corrected by recovery of the surrogate standard 17β-E2-d_4. Differences in 17α-E2 and E1 recovery may mean that these were over- or under-estimated; however, results obtained from later sampling provided similar concentrations.

Steroid estrogen concentrations appeared to be higher in effluents from farms with treatment ponds for samples collected at the start of the season. It is hypothesised that hydrolysis of conjugated estrogens within the effluent system produced these higher concentrations. The retention time of a typical dairy farm pond system is 60-90 days (152), exceeding the half-lives for hydrolysis of 17β-E2-3S and E1-3S, which are estimated at 2.5 days (16). By comparison, irrigated effluent is discharged almost immediately after collection (RT < 3 hours). Measurement of conjugated estrogens in samples at the end of season could neither support nor contest this hypothesis, as unfortunately three of the five treatment pond systems sampled had stopped milking prior to sampling (which meant that effluent from within those ponds was not representative of that obtained during milking).

Conjugated estrogens were detected in most samples obtained in the end of season sampling. E1-3S was the most prevalent, with concentrations from 12 to 177 ng/L (median 21 ng/L) (Table 4.3). 17α-E2-3S was detected in only 4 of the 18 samples, at concentrations below the quantitation limit to 225 ng/L. 17β-E2-diS was also measured in four samples at 17-320 ng/L, although these results should be treated with caution due to the low recovery obtained in spiked samples. Similarly, quantitation of 17β-E2-3S was hampered by the presence of a large coeluting peak, and glucuronide conjugates were not quantified due to poor detection limits.

Table 4.3: Concentrations of estrogens and conjugated estrogens in dairy effluent samples (ng/L) [a].

				Start of milking season [b]				End of milking season [c]						
Farm No.	Treat-ment	Dis-charge [d]	No. stock	E1	17α-E2	17β-E2	Total free estrogens	E1	17α-E2	17β-E2	E1-3S	E2-diS	17α-E2-3S	Total free estrogens
Toenepi														
T1 [e]	2-pond	Water	160	200	880	55	1100	16	ND [f]	2	ND	ND	ND	18
T2	None	Land	250	35	160	9	190	190	1800	17	180	ND	130	2300
T3	2-pond	Land	144	240	500	45	780	450	1300	160	ND	ND	ND	1900
T4 [e]	APS [g]	Water	350	580	760	310	1700	7	ND	1	ND	ND	ND	8
T5 [e]	2-pond	Water	152	270	570	78	920	14	ND	2	ND	ND	ND	16
T6	Pond	Land	264	370	1400	170	1900	260	1600	92	56	ND	29	2000
Waikuku														
W1	None	Land	420	26	230	44	300	NM [h]	NM	NM	43	ND	ND	
W2	None	Land	220	100	720	85	900	21	230	5	12	ND	ND	260
W3	None	Land	380	45	130	24	170	26	290	3	29	ND	ND	350
W4	None	Land	300	100	110	33	250	19	310	9	50	ND	BQL [i]	390
W5	None	Land	260	120	380	62	560	20	210	3	58	ND	ND	290
W6	None	Land	200	50	250	18	300	10	250	9	45	ND	ND	320
Pahau														
P1	None	Land	1000	NM	NM	NM	NM	210	1700	44	ND	320	ND	2200
P2	None	Land	823	NM	NM	NM	NM	76	640	12	13	17	ND	760
P3	None	Land	490	NM	NM	NM	NM	42	830	22	25	ND	230	1100
P4	None	Land	600	NM	NM	NM	NM	49	1200	27	16	250	ND	1500
P5	None	Land	350	NM	NM	NM	NM	40	750	8	ND	ND	ND	800
P6	None	Land	720	NM	NM	NM	NM	480	11,000	64	110	60	ND	11,000

Notes: [a] All data rounded to no more than two significant figures. [b] Quantitated against 1717β-E2-acetate as internal standard, recovery corrected. [c] Quantitated by isotope dilution. [d] Effluents discharged either to receiving waterways ("Water"), or spray-irrigated onto land ("Land"). [e] Samples collected after end of milking season on these farms. [f] ND = Not detected. [g] APS = Advanced Pond System. [h] Not measured as this sample destroyed during work-up. [i] BQL = Below quantitation limit, trace concentration detected.

The steroid estrogen concentrations in dairy effluent are expected to depend on the reproductive state of the cattle, as excretion varies to a great extent with reproductive state. Estrogen excretion increases significantly during pregnancy, particularly within the last month (99,153,154), followed by a marked decrease within two days post-partum (20,99). Faecal excretion is reportedly the major excretion route, as free steroids, while conjugated estrogens are primarily excreted in urine (20,153). Although there are many studies of reproductive steroids in cattle, most of these were undertaken in the 1960s and 1970s using colourimetric and fluorometric methods that lack sensitivity and specificity (20) compared to modern methods of analysis. As such the data provided by these studies should be treated with caution. Recent studies have used radio- or enzyme-immunoassays to measure estrogens in faeces and urine.

The concentration of 17α-E2 was reported at ~10 ng/g in faeces from non-pregnant cows, and more than 22 ng/g in faeces from pregnant cows (155). Estrone and 17β-E2 concentrations were lower at 1.0 ng/g and 1.6 ng/g respectively in non-pregnant cows and 3.3 and 5.7 ng/g in pregnant cows (155). If it is assumed that DSE contains approximately 10% excreta (156), concentrations in effluent from cycling cattle could be estimated at 1000 ng/L for 17α-E2, 100 ng/L for E1, and 160 ng/L for 17β-E2. In effluent from pregnant cows, the concentrations would be higher at 2200 ng/L for 17α-E2, 330 ng/L for E1, and 570 ng/L for 17β-E2. In cycling cattle, urinary excretion of E1-3S has been measured at 0.4-4 μg/L; however, levels increase in pregnant cattle, and range between 10 and 40 μg/L during mid-pregnancy (101). Again, assuming that DSE contains ~10% excreta (156), E1-3S would be measured in the DSE at 40-400 ng/L in the early milking season and 1000-4000 ng/L at the end of the season. No recent data is available for other conjugated estrogens in urine, though E1-3S is reportedly the dominant conjugate in cattle urine (99).

In this study, samples were collected at the start of the milking season (August), reflecting excretions from stock just a few days post-partum. Cattle return to estrus and are typically bred in November. Samples collected near to the end of the season (March to May) were derived from pregnant stock approximately 90-150 days pre-partum (157). The concentrations of steroid estrogens measured in the DSE were within the expected range, given the variation in excretion levels and effluent dilution. E1-3S concentrations were

typically lower than these estimated values, and the measurement of other conjugated estrogens at higher concentrations (Table 4.3) suggests that there may have been some loss of E1-3S, possibly during sample work-up, and that these concentrations may be an under-estimate.

Despite differences in cattle estrogen excretion with reproductive state, total estrogen concentrations in the effluents collected near the end of the milking season were not consistently higher than at the start of the milking season, and there was no statistical difference in a t-test (two-tailed, p-value = 0.67). Repeated sampling at three farms demonstrated considerable variation over time but did not indicate a clear trend in the total or individual concentration of steroid estrogens over the sampling period (Figure 4.3). Variation in the amount of excreta within these samples, due to differences in dilution from wash-down water or differences in the depth and timing of sampling, may mask any seasonal differences due to the reproductive cycle. Excreta (both faeces and urine) collected directly from the cattle may provide a clearer picture of seasonal differences, but was not examined in this study. Since milking stops at least 8 weeks prior to calving in August, DSE is not produced during the period when estrogen excretion reportedly peak (101,155).

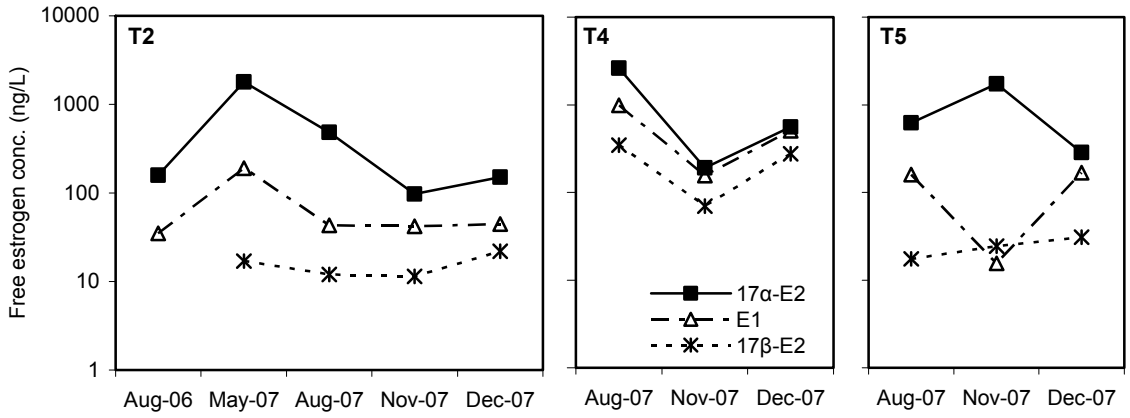


Figure 4.3: Comparison of steroid estrogen concentrations for three sites within the Toenepi catchment sampled three to five times during milking season.

The profile of individual steroids within urine and faeces also varies with reproductive stage. During pregnancy, 17α-E2 is the dominant estrogen excreted by cattle, predominantly in the form of a glucuronide conjugate in urine and a free steroid in faeces

(20,99,153). The amount of E1 and 17β-E2 excreted is lower, though the ratio of E1 to 17β-E2 differs in urine (where E1, predominantly excreted as E1-3S, dominates) to that in faeces (where the ratio appears to change during pregnancy (99)). However, during the estrus cycle, the relative concentrations of 17α-E2, 17β-E2 and E1 fluctuate (17). 17α-E2 was the most abundant steroid in all but three samples collected from ponds on farms after the milking season had ended where E1 dominated. This may represent degradation of 17α-E2 and 17β-E2 to E1, which is more stable (158,159). E1 was the next most abundant steroid in the samples, where it was present at concentrations around 4-times higher than 17β-E2. This may reflect the relative concentrations excreted from stock, and the transformation of 17β-E2 to E1 following excretion.

It has been suggested that larger herd sizes may increase the concentration of steroid estrogens in DSE (40). In this study, there was no relationship between the number of stock and the total concentration of steroids in all analysed samples (R^2 = 0.15), or when samples collected from pond systems were excluded (R^2=0.25). The volume of wash-down water used in the milking shed is estimated at 25 L per cow (152), suggesting that any increase in the amount of urine or faecal matter deposited with larger herds would be diluted by a larger volume of water. This would result in similar concentrations of steroid estrogens in effluents from all farms regardless of herd size, as observed in this study.

It has been reported that as herd sizes increase, farms may use progressively lower volumes of wash-down water per cow (156), suggesting there may be some difference in steroid concentrations in effluent from the larger farms. One of the large farms visited in this study reported they minimised water use (P6). The sample from this farm contained the highest steroid concentrations, with 17α-E2 present at a concentration more than ten-fold higher than the median across all analysed samples. As the majority of the effluents were obtained from farms with less than 350 cows (consistent with 66% of New Zealand farms (157)), further samples need to be collected from large farms and analysed to conclusively determine differences.

4.3.3 Estrogenic Activity

All effluent samples demonstrated an estrogenic response using the E-Screen assay, though most samples also demonstrated cytotoxic effects at high concentrations,

evidenced by a decrease in measured absorbance and by microscopic examination of the cell plates. Toxic effects were also noted in two-hybrid yeast and ERBA assays (data not presented). Despite the cytotoxicity, it was possible to calculate an accurate EC50 and therefore EEQ for each sample using the E-Screen. The EEQs obtained ranged from 1.4 to 670 ng/L (Table 4.4). Calculation of RPE was possible for only four samples due to cytotoxicity at high concentrations, and each demonstrated full agonistic activity.

Table 4.4: Estrogenic activity of DSE samples measured by bioassays (EEQ, ng/L) [a].

	Start of milking season			End of milking season		
Farm No.	**Measured**	**Predicted [b]**	**Ratio Meas:Pred**	**Measured**	**Predicted [b]**	**Ratio Meas:Pred**
Toenepi Catchment						
T1	110	77	1.4	1.4	2.0	0.7
T2	27	13	2.1	69	58	1.2
T3	71	60	1.2	140	200	0.7
T4	670	337	2.0	1.3	1.0	1.3
T5	120	96	1.2	3.6	2.0	1.8
T6	300	209	1.4	97	130	0.7
Waikuku Catchment						
W1	NM [c]	49	NC [d]	32	NC [e]	NC
W2	59	101	0.6	18	9.8	1.8
W3	20	28	0.7	18	9.7	1.9
W4	NM	38	NC	18	16	1.1
W5	NM	73	NC	26	7.9	3.3
W6	30	24	1.2	11	15	0.7
Pahau Catchment						
P1	NM	NM	NM	55	82	0.7
P2	NM	NM	NM	32	27	1.2
P3	NM	NM	NM	36	39	0.9
P4	NM	NM	NM	65	52	1.3
P5	NM	NM	NM	27	24	1.1
P6	NM	NM	NM	570	290	2.0

Notes: [a] All data rounded to two significant figures. [b] Predicted from potency of steroids in E-Screen (17β-E2=1, 17α-E2=0.020, E1=0.024). [c] Not measured. [d] Not calculated. [e] Sample lost during work-up and chemical analysis not completed.

EEQs predicted from chemical analysis and estrogenic potencies in the E-screen were similar to those measured by the E-Screen assay, with ratios typically within the range 0.6-1.8 (Table 4.4). Comparisons within the same order of magnitude are considered to be within the precision of the assay and chemical analyses (138,160). Four samples had a ratio ≥ 2.0, which was outside the variation expected from the E-Screen assay; potential reasons for this are discussed later.

A comparison of the estrogenic activity predicted from the concentrations of the measured steroid estrogens in the DSE samples to that obtained by bioassay is shown in Figure 4.4. The steroid estrogens contributed 90 ± 38% of the EEQ measured in the E-Screen (range 30-171%), indicating that most of the observed activity in the E-Screen was attributable to these compounds. This percentage is much higher than reported for STP influents and effluents, where steroids contribute up to 80% of measured activity in influents but regularly less than 50% in STP effluents (28,68). This is presumably because of a broader range of domestic and industrial chemicals that exhibit estrogenic activity entering these systems. Of the individual steroids, the largest mean contribution was from 17β-E2 at 60 ± 35% (range 11–143%), followed by 17α-E2 at 25 ± 18% (range 0-60%), then E1 at 6 ± 5% (range 2-28%). The calculated contribution from conjugated estrogens to total estrogenic activity was negligible (0.000–0.035%), due to the significantly lower potency of these compounds.

Despite the lower potency of 17α-E2 (~2% of 17β-E2), the concentrations in effluent samples are substantially higher than those of its isomer 17β-E2. This highlights the importance of measuring 17α-E2 in agricultural wastes, and contrasts with a previous suggestion that the contribution of 17α-E2 is negligible due to its low activity (33). Even at a potency of 1%, upon which that statement was based, the contribution of 17α-E2 remains significant, at up to 30% (12 ± 9%) of the total EEQ. In contrast, estrone is commonly measured by authors assessing the estrogenicity of livestock wastes, but its contribution to the EEQ (6 ± 5%) was relatively minor for most samples analysed in this study.

If conjugated estrogens are hydrolysed to free forms in the environment they could contribute additional estrogenic activity, particularly the 17β-E2-conjugates. For samples containing conjugated estrogens, the additional activity they could contribute, assuming complete hydrolysis of conjugates and no change to the parent steroid, would be relatively low for most samples, with a median of 1.5 ng/L. However, for those samples containing residues of 17β-E2–diS, the potential contribution would be significantly higher, up to 200 ng/L EEQ. The potential risk from conjugated estrogens depends on their likelihood to hydrolyse to free estrogens. It is therefore important to understand the

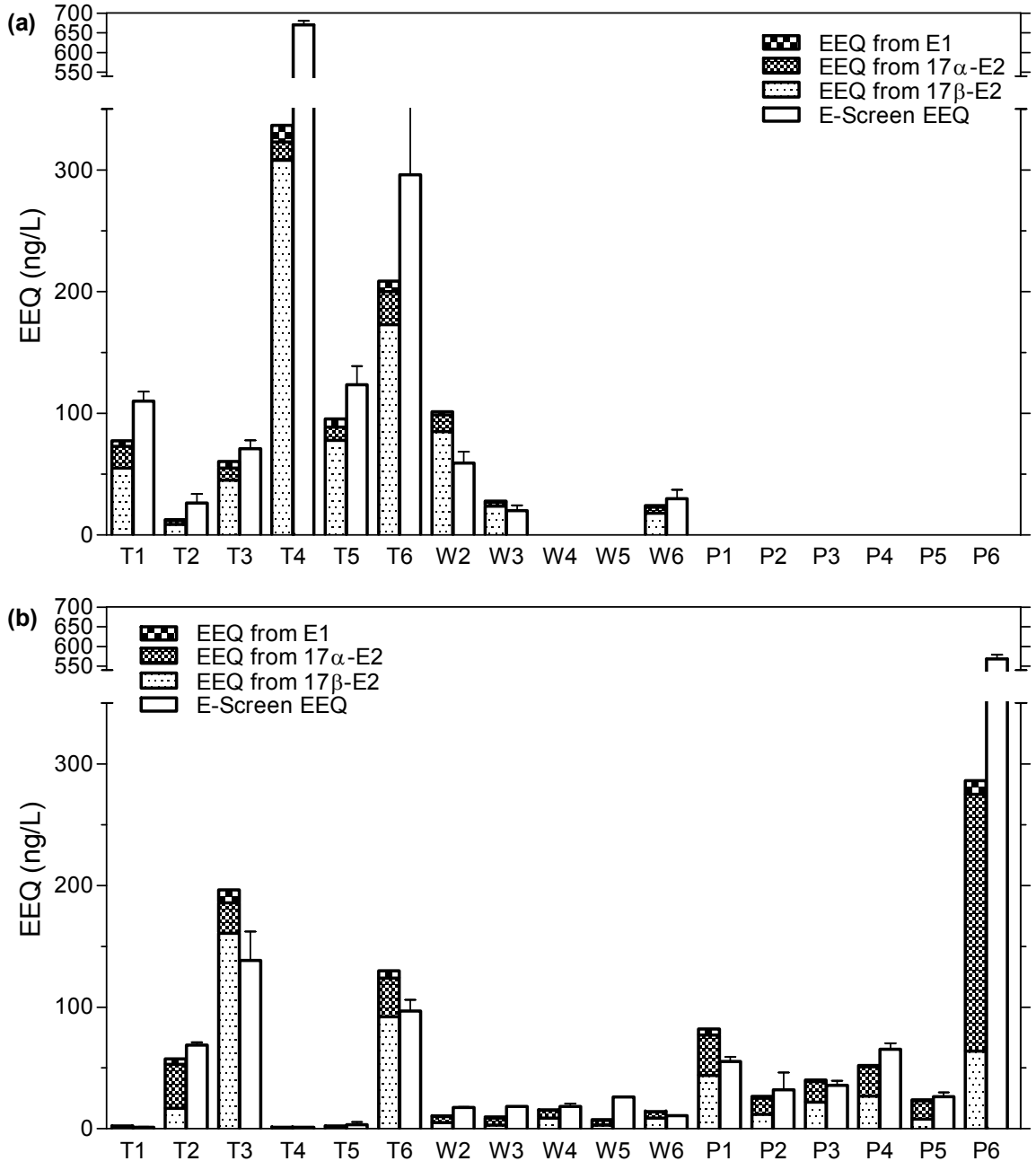


Figure 4.4: Comparison of EEQ predicted from potencies and concentrations of steroid estrogens with the EEQ measured by E-Screen for each sample (error bars represent standard error of duplicate measurements). Predicted EEQs based on the following potencies: 17β-E2 = 1, E1 = 0.024 and 17a-E2 = 0.02.

fate of conjugated estrogens in receiving environments to develop realistic assessments of the sources and risks of estrogens in the environment.

As mentioned, four of the analysed samples (T2 and T4 at the start of season and W5 and P6 at the end of season) demonstrated substantial differences between measured and

predicted EEQs, with ratios from 2.0 to 3.3. For sample T2 and W5, this may be due to the lower precision associated with the measurement of 17β-E2 at low concentrations (3 and 9 ng/L in these samples). As this is the most potent estrogen, it can have a large influence on the predicted total EEQ. For the other two samples, the differences were outside the expected variation, and may be due to the presence of estrogenic compounds not quantified by chemical analysis. Bisphenol A and 4-nonylphenol, two weakly estrogenic compounds, were detected using GC-MS at ~20 ng/L in these samples (not recovery corrected). Due to their low potency (2.5-6.0 x 10^{-5} and 1.3-10 x 10^{-5} respectively in the E-Screen assay (28)), their contribution to overall estrogenic activity of the sample would be negligible (<0.001 ng/L). The estrogenic potency of other nonylphenols present in the sample (identifiable only as part of the nonylphenol technical mix, at up to 60 ng/L) is about a tenth lower than the recognised xenoestrogen 4-nonylphenol (32), and therefore their contribution to measured activity is again negligible.

Cleaning products used in milking sheds are predominantly based on inorganic acids such as sulfuric acid, or bases such as sodium hydroxide. Previously, products authorised for use in New Zealand included nonylphenol ethoxylate (NPE) surfactants (161). In addition, NPEs are found in several veterinary medicines, such as teat dips to reduce mastitis and bloat control remedies (161). The use of these products is under review (161), but it is likely they will continue to be used until effective alternatives are available.

Phytoestrogens and mycoestrogens have been detected in manures (74,162) but were not measured in this study. Equol has previously been identified as a contributor to estrogenic activity in hog manure, at a concentration of 6.9-16.6 mg/L (74). The mycoestrogen zearalenone is slightly more potent than equol (0.01 cf. 0.005, (32)), and could contribute to estrogenic activity if present at the maximum concentration previously measured in cattle manure (up to 197 ng/g dry weight (163)). Zearalenone and its analogue zearalenol are produced by the fungus *Fusarium*, which is common in New Zealand grass. Zearalenone and zearalenol have also been measured in New Zealand maizes (164,165), and have have been shown to persist for 12 weeks in silage (163). It is highly probable these estrogen mimics, and/or their metabolites are present in DSE from grass-fed cattle. Their presence in DSE is likely to be site-specific, and be related to the

type and condition of pasture, and the use of additional stock feeds such as grain or silage.

4.3.4 Comparison to Literature Data

Free estrogens

The free steroids measured in DSE in this study are comparable with previously published data for dairy wastes (Table 4.5), but 17β-E2 concentrations measured in this study (median 24 ng/L) were generally lower, especially when compared to E1 and 17α-E2 concentrations (medians from 88-1700 ng/L, Table 4.5).

Table 4.5: Median concentrations of free estrogens in dairy wastes (ng/L, median (range)).

Sample description	E1	17α-E2	17β-E2	Total free estrogens	N [a]	Ref.
DSE [b]	100 (10-580)	730 (110-6800)	24 (3-310)	728 (8-11,500)	27	This study
DSE after pond treatment	382 (ND[c]-3123)	249 (ND-1028)	88 (ND-331)	719 (ND-4416)	6	(40)
Dairy lagoons	75 (57-80)	212 (177-283)	167 (110-168)	436 (362-531)	3	(43)
Drain with dairy effluent	38	NM [d]	8.6	46.6	1	(45)
Flushed dairy wastewater [c]	672	NM	344	2		(46)
Flushed dairy wastewater	551 (370-2356)	2114 (1750-3270)	672 (351-957)	3462 (2742-5103)	5	(47)
Fresh dairy wastewater [e]	~300	~2200	~200	~2700	6	(48)
Dairy holding ponds	5500 (2500-5600)	~10,000 [e]	1700 (800-1900)	NC [f]	48	(39)
Manure (solids) [g]	50 (28-72)	155 (120-190)	48 (46-50)	NC [f]	3	(166)

Notes: [a] Number of samples analysed. [b] Data for three samples collected after milking season not included. [c] ND = Not detected. [d] NM = Not measured. [e] Data estimated from graph. [f] Could not be calculated from data reported. [g] Data for solid samples (units ng/g).

This was not due to degradation of 17β-E2 in the effluent systems, as untreated samples had particularly low levels of 17β-E2 compared to E1 and 17α-E2. A possible explanation for this observation is hydrolysis of 17β-E2 conjugates in inadequately preserved samples during storage and extraction. In this study and that of Raman et al. (39), all samples were preserved with acid to pH ≤ 2 and extraction was completed within 36 hours of collection. Preservation methods used by other researchers (formaldehyde, < 4 °C) do not prevent the hydrolysis of glucuronides in effluent samples (93).

Conjugated estrogens

There is only one previous study reporting conjugated estrogens in agricultural wastes to compare the data to. Hutchins et al. (43) measured E1-3S, 17α-E2-3S and 17β-E2-3S in three dairy effluent samples at median concentrations of 87, 170 and 42 ng/L respectively, similar to concentrations found in this study (Table 4.3). 17β-E2-17S and glucuronides were not detected in the study of Hutchins et al., possibly due to low and variable recovery (43). For example, recovery of 17β-E2-17S was 23 ± 20% and 91% in blank spikes and a matrix spike (swine lagoon sample), and glucuronide recovery was 23-125% and 71-150% in blank and matrix spikes (43). The variable recoveries demonstrated the difficulties involved in analysing these compounds in complex matrices.

Hutchins et al. also examined conjugated estrogens by subjecting raw effluent samples to enzyme treatment, followed by extraction and analysis of free estrogens by GC-MS (43). The results were compared with those obtained for untreated samples and the difference used to calculate the contribution from conjugated estrogens in the samples. Their results suggested the presence of significant amounts of E1 and 17α-E2 conjugates, with increases of 420-570 ng/L of E1 and 52-270 ng/L of 17α-E2 (43). The observed increase in 17α-E2 was similar to the concentrations of 17α-E2-3S measured by LC-MS-MS. In contrast, the increase in E1 was higher than the concentration of E1-3S measured by LC-MS-MS, though some of that increase results from the conversion of 17β-E2 and its conjugates. 17β-E2 was lower in two out of three samples after enzyme treatment compared with samples not subjected to enzyme treatment. Hutchins et al. (43) hypothesised that free estrogens in the sample degraded to E1 during the enzyme treatment process, as samples were maintained at 37 °C for 16 hours. Rapid degradation of 17β-E2 to E1 has previously been demonstrated in unpreserved dairy manure samples stored at 30 °C (167).

The present study indicates conjugated estrogens are present in DSE at significant concentrations when compared with the concentration of free estrogens. Specifically, the free and sulfate-conjugated forms of E1 were at similar concentrations in effluents analysed in this study, confirming the previous results of Hutchins et al. (43). Conversely, the free form of 17α-E2 was more prevalent than the sulfate-conjugate in analysed samples. This contrasts with the previous results for dairy wastes which suggested sulfate-conjugates comprised 40-45% of total identified 17α-E2 (43), but agrees with

studies of bovine excretion that suggest the glucuronide-conjugate is the principal conjugate of 17α-E2 rather than the sulfate form (99,153,154). Further improvements to the analytical methods used to analyse conjugated estrogens are required to provide additional information on the significance of conjugated forms of 17β-E2.

Estrogenic Activity

EEQs measured using the E-Screen assay in this study (Table 4.6) are comparable to those previously measured in DSE in New Zealand by ERBA assay (40), and in dairy manures in the Netherlands by ER-CALUX (166). The EEQs measured for an Australian DSE sample using the YES and ER-CALUX assays (45) were at the minimum end of the range measured in this study using E-Screen. To date, there are no comparative data for the estrogenic activity of dairy effluent or manure derived from the E-Screen assay, which was used most extensively in this study.

Table 4.6: Comparative data (median (range)).

Sample description	EEQ (ng/L unless stated)	Assay	N	Ref.
Dairy shed effluents [a]	46 (18-670)	E-Screen	24	This study
Dairy shed effluents after pond treatment	143 (ND-521)	ERBA	6	(40)
Dairy shed effluent	14 ± 0	YES	1	(45)
Dairy shed effluent	11 ± 6	ER-CALUX	1	(45)
Dairy manure	190 (16-370)	ER-CALUX	2	(166)
Dairy manure (solids, ng/g)	~280 (22 – 2900)	Yeast assay	7	(49)

Notes: [a] Data for three samples collected after milking season not included.

Lorenzen et al. (49) measured the estrogenic activity of solid dairy manure samples using the YES assay, and established a median of ~280 ng/g on a dry weight basis. Assuming 0.9% solids in the dairy effluent (156), this would be equivalent to 2520 ng/L EEQ in a liquid sample, higher than the median and maximum EEQs determined in this study using the E-Screen, but similar to the maximum EEQ determined by the ERBA assay in this study (results not presented).

Differences in estrogenic activity between the current study and those reported in literature may be due to differences in the *in vitro* assays used. The ERBA provides information on chemicals that can displace 17β-E2 and includes chemicals that can bind to

the ER without causing an estrogenic response. The E-Screen assay gives an indication of the potential estrogenicity on a cellular basis. Order of magnitude differences have been demonstrated when comparing receptor binding assays with the E-Screen (144) or receptor-reporter gene assays (72). Nelson et al. (168) reported a four-fold difference in the EEQ of wastewater samples measured using the ER-CALUX and receptor-reporter gene yeast assays. In the current study, the estrogenic activity of dairy effluent samples was considerably higher when measured with a two-hybrid yeast assay (Chapter 6) and by ERBA (data not shown) than with the E-Screen assay.

4.4 CONCLUSIONS

Steroid estrogens were detected in all DSE samples collected. 17α-E2 was measured at the highest concentrations, at a median of 730 ng/L. Conjugated estrogens were also present in some samples at significant concentrations; in particular E1-3S (13-180 ng/L) was present at similar concentrations to free E1 in those samples (10-480 ng/L). These values may be an under-estimate of the total free estrogens present in the samples, as free estrogens are known to sorb to particulates, which were not measured in this chapter.

All effluent samples had measurable estrogenic activity in the E-Screen, with a median of 46 ng/L by the E-Screen assay. Most of the observed estrogenic activity appears to be due to the presence of steroid estrogens. The majority of activity was attributable to 17β-E2 and 17α-E2, despite the lower potency of 17α-E2. Although conjugated estrogens demonstrated weak estrogenic activity when measured in the E-Screen, they did not contribute to estrogenic activity, due to their low relative potencies.

The presence of conjugated estrogens in the DSE is important as they can degrade to active steroids; however, they are rarely determined in agricultural wastes. Analytical methods need to be refined to reliably measure all forms of conjugated estrogens (including glucuronides) in complex waste matrices. The data from this study demonstrates that conjugated estrogens must be measured in future studies so that they can be included in risk assessments for these wastes. Because of the contribution of estrogens derived from conjugates, the total potential impact of steroid estrogens in regions of intensive dairy farming is significantly more than is immediately apparent from previous literature.

CHAPTER FIVE

ASSESSING THE EFFICACY OF TWO-POND AND ADVANCED POND TREATMENT SYSTEMS TO REMOVE ESTROGENS FROM DAIRY SHED EFFLUENT

Chapter 5: Assessing the Efficacy of Two-Pond and Advanced Pond Treatment Systems to Remove Estrogens from Dairy Shed Effluent

5.1 INTRODUCTION

The presence of steroid estrogens in waterways can result in feminising effects on aquatic biota including skewed hormone ratios, the induction of the precursor yolk protein vitellogenin in male fish, and in extreme cases, intersex fish (7). In New Zealand, dairy farms are the major source of steroid estrogens to the environment, and potentially to aquatic ecosystems. While most of the animal wastes on New Zealand dairy farms are dispersed over the land during free-range grazing, wastes are also produced during dairy shed milking operations. The animal wastes (faeces and urine) excreted in the milking shed and yard are combined with cleaning products used in the cleaning and rinsing of milking equipment and storage tanks, and diluted with wash-down water from cleaning of the shed and yards (50).

In many regions of New Zealand treated DSE was historically discharged to waterways after treatment, with two-pond systems (2PS) being widely used from the 1970s to reduce loads of total suspended solids (TSS) and biological oxygen demand (BOD) in discharged effluent. As this system is not effective at removing nutrients and pathogens (169), improvements have been sought, and the advanced pond system (APS) has recently been promoted. This reportedly removes dissolved nutrients (nitrogen and phosphorus) and faecal indicator bacteria considerably better than 2PS, and is more consistent at removing BOD and TSS (170,171). Neither of these systems was designed to remove micro-contaminants such as steroid hormones from DSE, and their removal efficiency for these contaminants has not been characterised.

Internationally, agricultural waste treatment systems have recently been examined for their ability to remove steroid estrogens, including systems for dairy wastewaters (48) and

swine manures (41,44). Lagoons, constructed wetlands and trickling filter systems have demonstrated the removal of steroid estrogens through loss mechanisms that include aerobic biodegradation, sorption and photolysis (41,44,48). However these systems are different to those used in New Zealand for the treatment of DSE and typically treat waste from much larger farms. For example, the dairy wastewater system described by Zheng et al. (48) includes three large lagoons, each approximately 100 m x 50 m and treats dairy manure and wastewater from a farm of 2000 cows. The majority of farms in New Zealand (63%) have between 100 and 350 cows, and only 2.3% milk over 1000 cows (157). Effluent ponds are accordingly much smaller, with design values of 19 x 35 m for the anaerobic and 22 x 53 m for the aerobic pond for a typical farm with 250 cows in the upper North Island. Despite being different to treatment systems used internationally, the New Zealand pond systems have potential to remove steroid estrogens as they incorporate aerobic degradation and photodegradation processes that are known to remove estrogens from wastewaters (159).

Removal of steroid estrogens from sewage has been thoroughly investigated using laboratory batch reactors, pilot-scale plants and numerous sewage treatment plants (reviewed in refs (159,172)). The findings, which likely apply to dairy effluents, indicate that the most important removal processes are sorption to sludge and aerobic degradation. Under non-sterile aerobic conditions, 17β-E2 is converted to E1, which is somewhat more stable (159,172). It has been suggested that 17α-E2, the primary metabolite found in dairy effluent, also produces E1 during aerobic degradation (43,48). This suggestion was based on the prevalence of E1 measured in treated dairy effluents in the field; however, this has not been confirmed by laboratory tests. Degradation to E1 would not decrease the potential for adverse effects, as E1 has similar estrogenic potency to 17α-E2, at least in *in vitro* assays (71,148,150).

This chapter reports on an investigation into the removal of estrogens in DSE through two-pond and advanced pond systems. The objectives of this study were to identify and quantify steroid estrogens within each stage of the treatment systems and assess their contribution to measured estrogenic activity; investigate conversion of conjugates and free estrogens within the systems and a laboratory batch experiment; and to assess and

compare the overall removal of steroid estrogens and estrogenic activity by each treatment system.

5.2 METHODS

5.2.1 Study Sites

The effluent systems investigated were both located in the same geographic area to minimise any differences in treatment efficiencies due to climatic effects. Both farms milked Fresian cattle, twice daily at approximately 6-7 am and 3-4 pm. Each milking shed and yard was hosed directly after milking and effluent was drained by gravity into the treatment system. Samples were collected during or directly after morning milking (7-8 am). Sampling from each system was undertaken three times during the milking season to provide replication. The milking season begins in late July/August and ends around April, when cows dry off before calving in July/August. Samples were collected August, November and December 2007, being at the start and peak of the milking season. These months correspond to late winter, spring and early summer.

Two-pond system

Traditional two-pond DSE treatment systems are a low-cost, low-maintenance system consisting of an anaerobic pond (AP) followed by a facultative pond (FP) to treat effluent before discharge to receiving waters. Farm DSE is piped to the anaerobic pond where solids settle out of solution and organic matter is digested by anaerobic bacteria. Sludge is periodically removed (every 2-4 years) to maintain effective water depth and thus hydraulic retention time. The partially treated effluent drains by gravity into the facultative pond, which is usually shallower and has a larger surface area. The facultative pond provides further anaerobic treatment in the lower layer and aerobic treatment at the pond surface. The facultative pond typically discharges into a farm drain which flows into a natural stream.

The system investigated consisted of two ponds approximately 20 m by 20 m, and received effluent from a farm milking 150 cows. Grab samples were collected from the

drain carrying wastes from the dairy shed to the anaerobic pond (Raw); at the point of exit from the anaerobic pond or point of entry into the facultative pond (AP); and at the discharge point from the facultative pond (FP).

Advanced Pond System

The advanced pond system is designed to optimise natural wastewater treatment processes, thereby minimising maintenance and operating costs (170). The initial anaerobic pond (AP) provides the same functions as that in a two-pond system. Effluent from the AP is discharged into a high rate pond (HRP), a long shallow pond continually mixed by a paddle-wheel to prevent thermal stratification and to maintain aerobic conditions (170). This promotes algal growth, with a corresponding uptake of nutrients, and further degrades organic matter. This is followed by two algal settling ponds in series (ASP) which are deep to promote the algae to settle, thereby removing nutrients from the effluent. The final stage is the maturation pond (MP), which promotes further removal of micro-organisms through a combination of solar UV-radiation, sedimentation and protozoan grazing (171).

The APS assessed within this study contained all of the described elements; the treated effluent was then discharged to a tile drain before release to a nearby farm drain and subsequent receiving water (Figure 5.1). A full description of this system and its treatment efficiency is provided by Craggs et al. (171). During the sampling period, approximately 350 cows were milked twice daily in the dairy shed. Raw effluent samples were collected from the input to the pond, from within the drain at the dairy shed prior to entering the AP (November and December). In August there was no effluent in the drain at the dairy shed, due to the time of sampling, and the sample had to be collected from within the AP at the point of entry. Effluent samples were also collected from between each component of the pond system (AP, HRP, ASP, MP) and after the tile drain at the final discharge point to the receiving environment (TD).

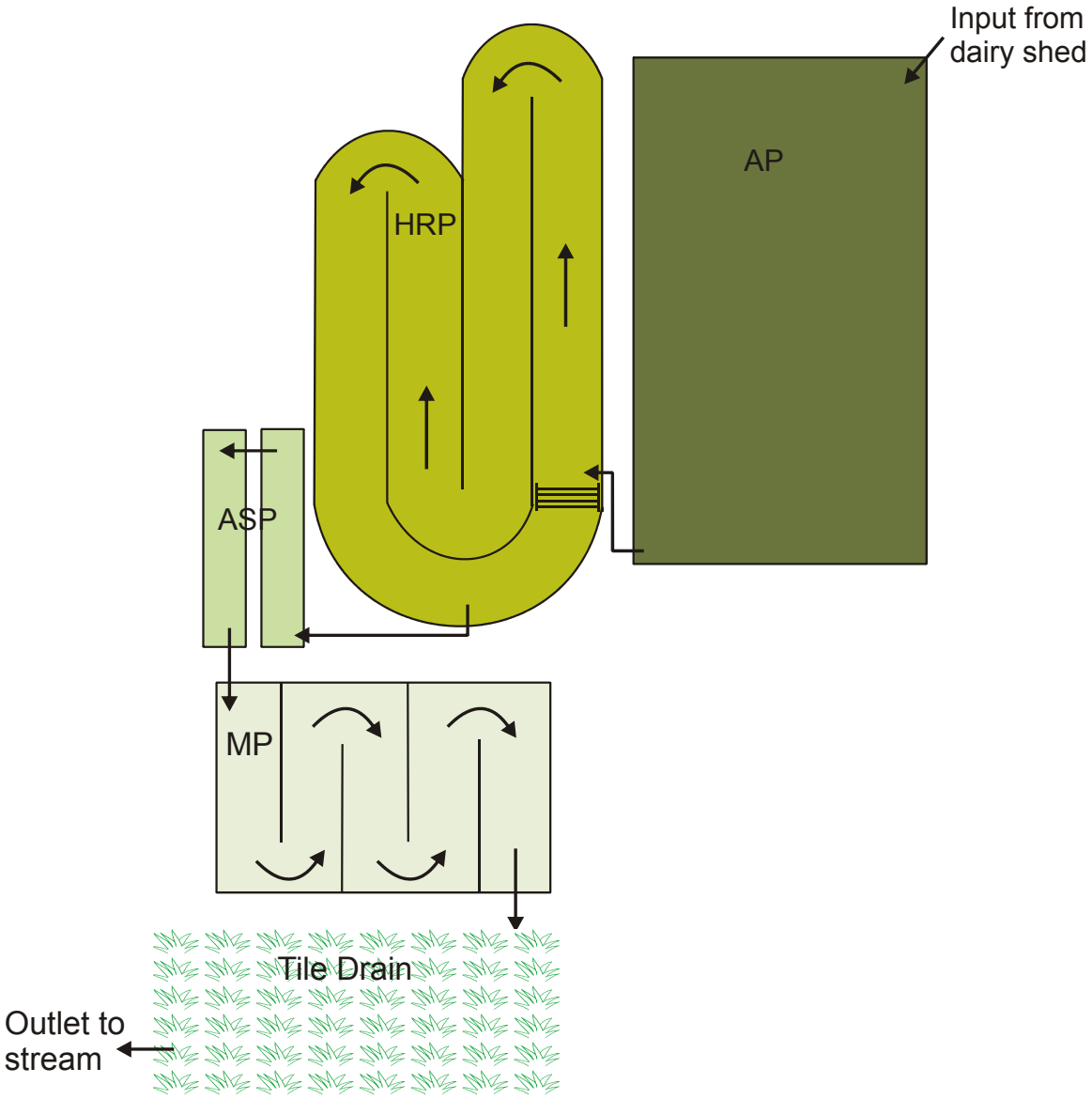


Figure 5.1: Schematic diagram of the Advanced Pond System (adapted from Craggs et al. (171)). AP = Anaerobic pond, HRP = High rate pond, ASP = Algal settling ponds, MP = Maturation Pond and TD = Tile Drain.

5.2.2 Sampling and Extraction

Grab samples were collected during morning milking in solvent-rinsed amber glass bottles with Teflon-lined lids. Samples were immediately preserved by the addition of sulfuric acid (to pH ≤ 2) and stored at <4 °C until extraction (within 24 hours).

Samples were extracted in duplicate for chemical analysis of free and conjugated estrogens and for biological analysis by E-Screen assay. Samples were also collected for

nutrient analysis in laboratory-supplied bottles containing preservative and were immediately delivered to a commercial laboratory for analysis.

Samples for estrogen analysis were centrifuged at up to 1780 *g* for 20 mins at 4 °C, then approximately 2 L was filtered through glass fibre filters (GF/C, 47mm, pore size 1.2 µm) with the addition of filter aid. Samples were divided into two aliquots of 1 L each, and samples for chemical analysis were spiked with surrogate standards (250 ng each E2-d_4, E1-d_4, E2-3S-d_4 and E1-3S-d_4). Both aliquots were loaded at a flow rate of 5-10 mL/min onto separate preconditioned 1 g Oasis HLB cartridges (20 mL of MeOH followed by 20 mL of MQ water). As part of quality control procedures, a method blank and two spike recovery samples were extracted with each batch of samples. Spike recovery samples (1 L MQ) were spiked with either 25 ng or 250 ng of the following free and conjugated estrogens: 17α-E2, 17β-E2, E1, 17β-E2-3G, 17β-E2-17G, 17β-E2-3,17S, 17β-E2-3S,17G, 17β-E2-3S, 17α-E2-3S, E1-3G and E1-3S.

The particulate fraction was extracted to assess the particulate associated concentration and the total concentration of free estrogens in each sample. The particulates recovered during centrifugation were stored at -20 °C prior to extraction. After defrosting at room temperature, approximately 6 g of wet solids was transferred to a centrifuge tube and spiked with 250 ng of E2-d_4 and E1-d_4 (25 µL of a 10 µg/mL solution). A mixture of isopropanol and MQ water (80:20, 10 mL, (173)) was added and the sample was extracted using sonication (10 min) followed by shaking on a reciprocal shaker at 235 rpm (30 min). The samples were centrifuged at 1000 *g* for 10 min to settle solids so that the supernatant could be removed. The extraction procedure was repeated with a 50:50 mixture of isopropanol and MQ water (173). The supernatants from each extraction were combined and diluted with 170 mL phosphate buffer (pH 7.0, 0.1M). The aqueous mixture was extracted through Oasis HLB cartridges (500 mg) conditioned as previously described. The extracted samples were eluted, purified and analysed for free estrogens as described below. Quality control procedures for the particulate analysis included blanks, solvent spikes, matrix spikes and duplicate extractions for each batch of samples. Spikes were prepared by addition of 250 ng of the free estrogens 17α-E2, 17β-E2, E1 to either solvent (isopropanol and MQ water) or solid samples (matrix spike).

5.2.3 Laboratory Biodegradation Experiment

A laboratory batch degradation test was conducted to assess the degradation of 17α-E2 in DSE and its potential to produce E1. Fresh samples of DSE were collected in 4 L solvent-rinsed glass bottles. These were combined into a single bulk sample, mixed and split into two samples of 5 L each in 10 L Schott bottles. One of the duplicate samples served as a control, while the other sample was spiked with 17α-E2 at a concentration of 25,000 ng/L. This concentration was selected to be in excess of the concentration of 17α-E2 or E1 that could potentially be found in the effluent sample. Both experimental bottles were stored in the dark at room temperature, and were frequently opened to the air and shaken to promote exchange of air and ensure that aerobic conditions were maintained within the effluent bottles.

Subsamples were removed from the control and treatment bottles at the following times: immediately (0 hours), 1 hour, 3 hours, 24 hours, 48 hours, 120 hours (5 days) and 192 hours (8 days). At each time period, 400-500 mL of sample was removed from each bottle, centrifuged at 1000 *g* at 4 °C for 10 mins to remove bulk solids, then filtered through glass fibre filters and filter aid as previously described. Samples were split into three replicates (100 mL each), spiked with surrogate standards (500 ng each 17β-E2-d_4, E1-d_4), and loaded onto 500 mg Oasis HLB cartridges (preconditioned with 10 mL of MeOH followed by 10 mL of MQ water) at a flow rate of 5-10 mL/min. Sample elution, clean-up and analysis of free estrogens is described below.

5.2.4 Chemical Analysis

The extracted samples for chemical analysis were eluted from the Oasis cartridges in two fractions to separate conjugated estrogens and free estrogens, as described in detail in Chapter 3 and summarized briefly here. The Oasis HLB cartridges were washed with a series of MeOH/MQ water mixtures to remove polar interferences (93) then conjugated estrogens were eluted with 75% MeOH/MQ water containing 2% ammonium hydroxide (8 mL, see Chapter 3 for details). The Oasis HLB cartridges were dried under vacuum to remove residual water and attached to the top of a Florisil cartridge (1 g cartridges, IST) onto which aminopropyl sorbent (500 mg, IST) was packed. Free estrogens were eluted

from the Oasis HLB cartridge and purified through the combined adsorbent column with 30 ml of DCM:DEE: MeOH (40:10:1), which was collected for further purification.

The free estrogen fraction was dried under nitrogen and further cleaned up using gel permeation chromatography (GPC) on Phenogel columns as detailed in Chapter 3. TFAA was added to the GPC extract to derivatise steroid estrogens prior to GC-MS analysis in SIM/Scan mode (see Chapter 3 for details). The steroid estrogens were quantified by isotope dilution against their deuterated surrogates, 17β-E2-d_4 and E1-d_4.

The conjugated estrogen fraction eluted from the Oasis HLB cartridge was dried under nitrogen and reconstituted in MeOH for clean-up. Half of each extract was purified through silica gel (500 mg, JT Baker) and aminopropyl (500 mg, JT Baker) columns to remove matrix interferences (described further in Chapter 3). The purified extracts were reconstituted in ammonium acetate buffer for analysis of conjugated estrogens using LC-IT-MS in negative ESI mode, as described in Chapter 3. MS data was acquired by selected reaction monitoring (SRM), and conjugates quantified by isotope dilution against 17β-E2-3S-d_4.

5.2.5 Enzymolysis and solvolysis procedure to assess conjugated estrogens

For samples collected from the 2PS and APS in December, the remaining half of the conjugated estrogen fraction of each sample was subjected to sequential enzymatic hydrolysis and solvolysis, to investigate whether conjugated estrogens not measurable by LC-MS were present in the samples. The sample extracts were dried under nitrogen blow-down to remove MeOH and reconstituted in sodium acetate buffer (0.2 N acetate buffer with 10 mg/ml ascorbic acid, 5 mL). β-glucuronidase from *Helix pomatia* (Sigma HP-2) was used, as this preparation has glucuronidase activity (131,100 Fishman Units/mL) and some sulfatase activity (≤ 7500 units Sulfatase/mL). After adding 50 µL of the enzyme (corresponding to 1,311 FU/mL), extracts were incubated overnight at 37 °C. The reaction extracts were passed through Oasis HLB cartridges to retain any liberated free estrogens. The cartridges were washed with MeOH/water mixtures as described above for raw sample extracts, residual conjugate estrogens were then eluted, and free estrogens that had been liberated by the enzymatic hydrolysis reaction were eluted and purified using

aminopropyl and florisil adsorbents as described above. GPC, derivatisation and analysis of this fraction was undertaken as described above.

The conjugated estrogen fraction recovered from the Oasis HLB cartridge was subjected to solvolysis to check for the presence of conjugated estrogens not readily cleaved by enzymatic hydrolysis such as sulfo-glucuronides and disulfates. A solvolysis procedure, modified from al Alousi et al. (174), was applied. The conjugate extract was completely dried under nitrogen and reconstituted in freshly distilled THF (5 mL). Acid was added (5 mL of 4M H_2SO_4) and extracts incubated at 53 °C for 2 hours. Extracts were diluted with 7 mL of potassium hydroxide (4M) and passed through Oasis HLB cartridges as above. Cartridges were washed as described above to remove polar interferences, and the free estrogen fraction was eluted with 30 ml of DCM: DEE: MeOH (40:10:1). These sample extracts contained little co-extracted material and did not require further purification. Samples were derivatised and analysed for free estrogens as described above. Free estrogens in the enzymolysis and solvolysis extracts were quantified against internal standards to account for differences in detector response due to signal enhancement or suppression. Because samples had been spiked with deuterated conjugates (17β-E2-3S-d_4 and E1-3S-d_4) prior to extraction, and these may also be cleaved to their respective free forms, 17β-E2-d_4, E1- d_4 could not be used for quantitation and 17β-E2-17-acetate was used as an internal standard instead.

The efficiency of the enzymolysis and solvolysis reactions was investigated by subjecting standards of conjugated estrogens to these reactions. For the enzymolysis reaction, mixed standards were prepared containing deuterated sulfates (17β-E2-3S-d_4 and E1-3S-d_4), glucuronides (17β-E2-3G, 17β-E2-17G, 17β-E2-3S,17G, E1-3G and E3-3G), sulfates (17β-E2-3,17S, 17β-E2-3S,17G, 17β-E2-3S, 17α-E2-3S, E1-3S and E3-3G) and a mixture of all non-deuterated forms. For the solvolysis reaction, the conjugates listed above were assessed in individual reactions. Enzymolysis and solvolysis reactions and Oasis extraction were undertaken as described above but samples were not subjected to clean-up prior to derivatisation and GC-MS analysis.

5.2.6 E-Screen Analysis

Samples for E-Screen assay were simply eluted from the Oasis HLB cartridges with 30 ml of DCM:DEE:MeOH (40:10:1), dried down to approximately 1 mL and transferred to brown glass vials. Solvent was evaporated under nitrogen, redissolved in DMSO (500 µL) and extracts stored at <20 °C in the dark until tested in the E-Screen assay. The E-Screen assay was conducted as described in Chapter 4. Method quality control procedures included analysis of a standard and a duplicate sample for each 10 samples; the assay was performed at least twice for each sample and the average value was reported.

5.2.7 Fractionation of Samples for E-Screen Analysis

Samples from November sampling were fractionated through florisil to investigate alternative causes of estrogenic activity. The sample extract eluted from the Oasis HLB cartridge was split into two, with one half subjected to further fractionation on florisil cartridges following a method previously reported (175). Extracts were dried under nitrogen then reconstituted in 1 mL hexane:DCM (3:1). Florisil cartridges (500 mg, Varian) were conditioned with 3 mL hexane. Samples were applied quantitatively using an additional 1.5 mL hexane:DCM then a further 2.5 mL hexane:DCM (3:1) was added to wash analytes through the cartridge. All eluent from these steps was collected (fraction NP). The second fraction (MP) was eluted with 2x 2.5 mL of acetone:DCM (1:9), and the third fraction (P) was eluted with 2x 2.5 mL of MeOH. Each fraction was completely dried under nitrogen and resuspended in 100 µl DMSO for analysis by E-Screen as described above. The unfractionated half of each sample was also analysed by E-Screen.

5.2.8 Nutrient analysis

Samples of effluent were analysed by a commercial laboratory for total organic carbon (TOC), total phosphorus (TP), total nitrogen (TN), total Kjeldahl nitrogen (TKN) and oxidised forms of nitrogen (nitrate-N and nitrite-N, NOxN) by standard methods (146). Total suspended solids (TSS) were measured using an in-house gravimetric method based on standard methods (146).

5.3 RESULTS AND DISCUSSION

5.3.1 Two-pond System

DSE Composition and Treatment System Performance

Common indicators of effluent quality were measured at each stage of the two-pond system (2PS) investigated in this study. This demonstrated that, although there was substantial variation in the initial effluent quality on each monitoring occasion, there was efficient removal of TOC, phosphorus and nitrogen through the treatment system (Figure 5.2).

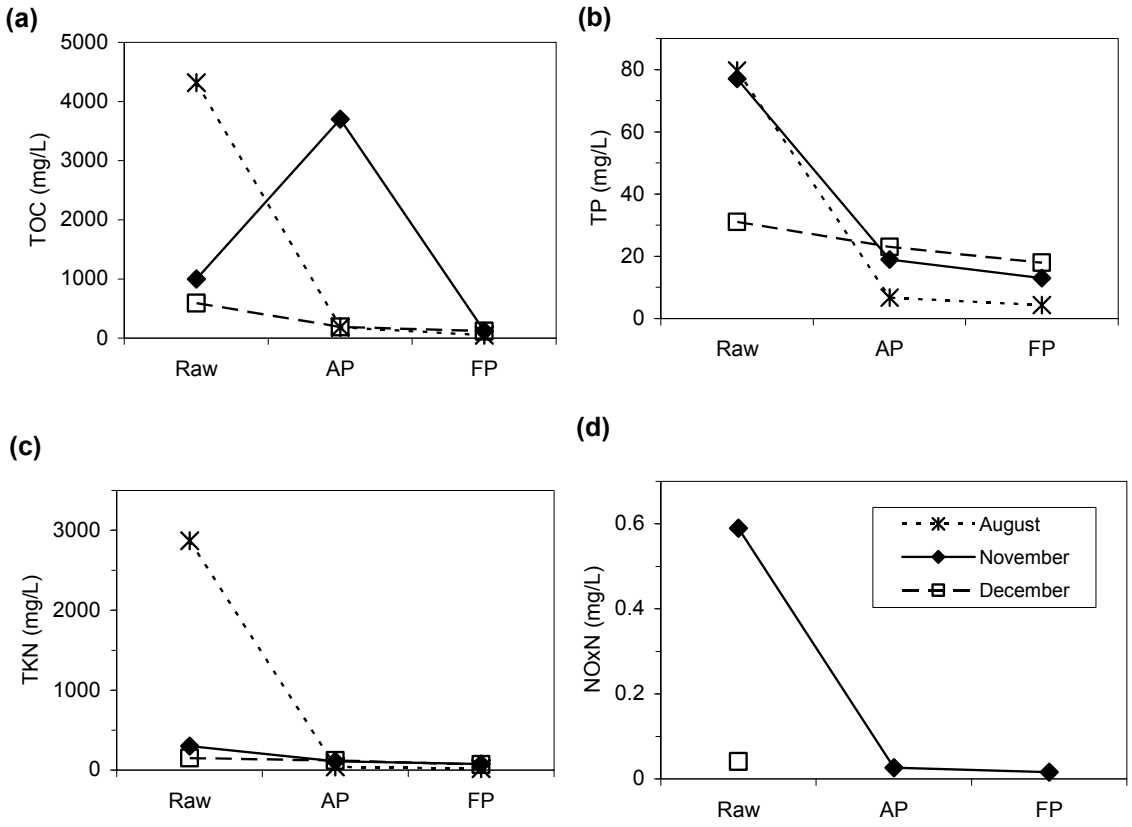


Figure 5.2: TOC (a), TP (b), TKN (c) and NOxN (d) concentrations at each stage in each effluent treatment system for each time sampled. Note: Most measurements for NOxN were below detection limits and are not plotted on this chart.

A comparison of effluent quality from the 2PS with literature values indicates it was within the range expected for such a system (Table 5.1). Final concentrations of nitrogen and phosphorus were at the lower end of literature values. Pond effluent quality is typically poorest in spring and summer (176), and higher concentrations of TP, TKN and TOC were observed in November and December compared to August. Overall the data suggest this particular pond is working well to treat the DSE. This may be due to the ponds being approximately the correct size based on guidelines for dairy effluent ponds (177).

Table 5.1: Performance and final effluent quality for nutrients in the 2PS.

	Final effluent quality (mg/L)				% Reduction
Parameter	This study	Lit. [a]	Lit. [b]	Lit. [c]	This study
TSS	200 [d]	198	105-307	220	84 [d]
TOC	97 ± 40	98 [e]	NM [f]	87	89 ± 10
TP	12 ± 7	27	NM	20	73 ± 28
TN	55 ± 31	NM	46-134	91	76 ± 23
TKN	55 ± 31	>75 [g]	46-134	91	76 ± 23
NOxN	0.016 (n=1)	0.065	0.066-0.36	0.44	NC [h]

Notes: [a] Literature values, median for 11 ponds in Manawatu and Southland (169). [b] Range in median of 6 ponds in Waikato (178). [c] Median for 12 ponds in Waikato (176). [d] n=1. [e] BOD data. [f] NM = Not measured. [g] Ammonia data, TKN not reported. [h] Not calculated due to large number of not detects.

Steroid Estrogens in Effluent through the Two-Pond System

E1, 17α-E2 and 17β-E2 were consistently measured in the untreated DSE (Raw, Figure 5.3), after the anaerobic pond (AP), and in the final effluent (FP). In both the aqueous and particulate fractions 17α-E2 and E1 were the predominant steroids, with 17β-E2 as a minor component (0.3 – 35 ng/L in aqueous fraction and 2.6-39.7 ng/L in particulate fraction). There were insufficient solids retained in the final effluent samples (FP) to enable measurement of estrogens associated with particulate material at this sampling point. The samples measured indicated that a high proportion (56 ± 27%) of the total steroids was associated with the particulate fraction. The total concentration of steroids in the particulate fraction was highest in the untreated effluent, which had the highest corresponding concentration of solids. This highlights the importance of measuring the 'whole' sample when assessing steroids in treatment systems, particularly for complex effluents like DSE with very high solids content.

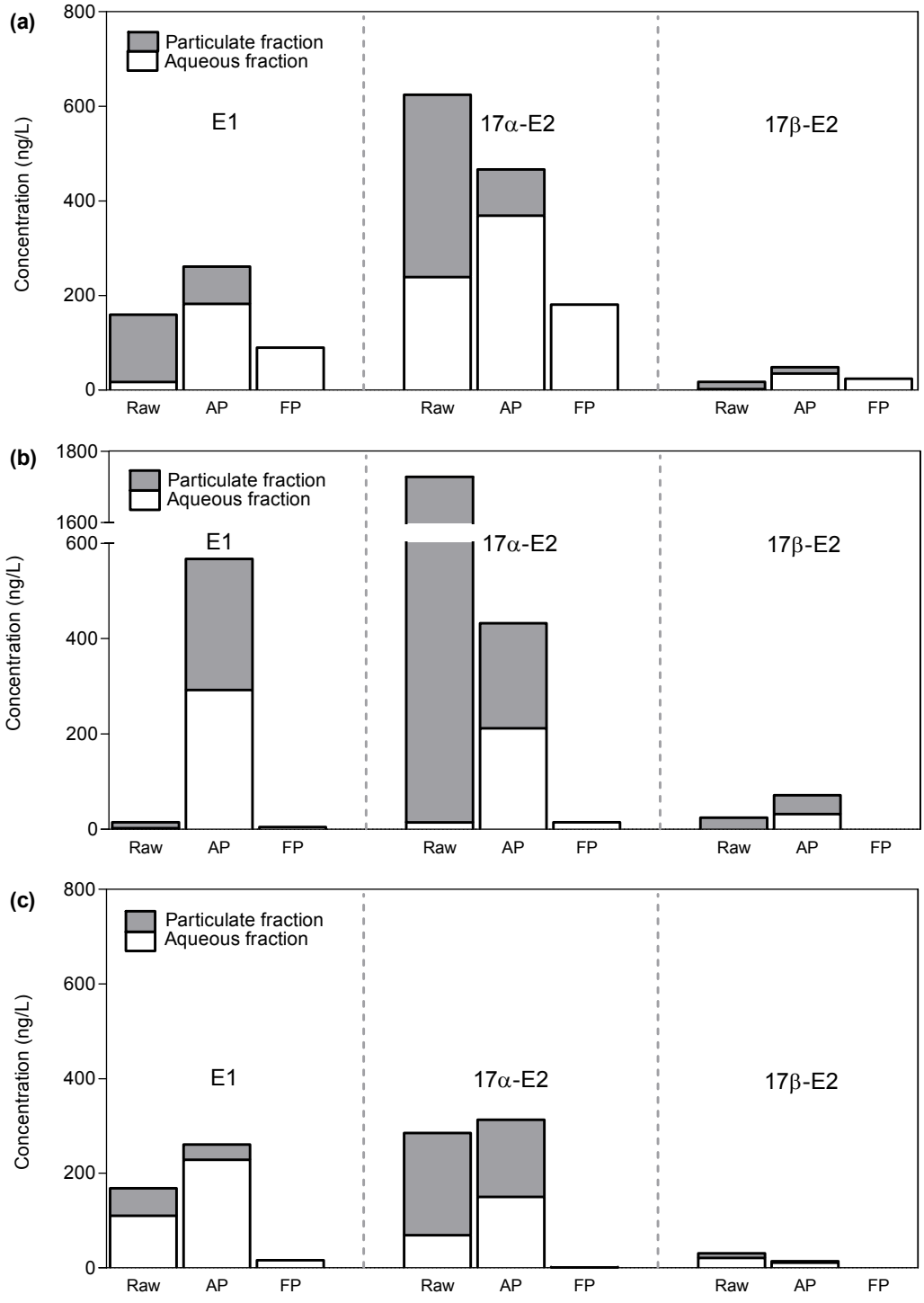


Figure 5.3: Steroid estrogen concentrations in aqueous and particulate fractions at each location in the 2PS for the three times sampled ((a) August; (b) November; (c) December).

The presence of steroids within the particulate fraction may be due to their excretion in faeces, immediate sorption of free steroids in urine to particulates, or from adsorption

during sample work-up. Acidification of the samples, necessary for preservation and sample treatment, is expected to increase adsorption of free steroids to the high organic matter content solid phase and to dissolved organic carbon. Schafer et al. (179) showed that adsorption of steroids to sludge at pH 2 was substantially higher than at pH 3 or more, and also that adsorption increased in the presence of higher concentrations of particulate matter. This resulted in increased adsorption of steroids to the particulate phase in raw effluents compared to treated effluents from the 2P system, which had much lower particulate content.

Conjugated Estrogens in Effluent through the Two-Pond System

Conjugated estrogens were investigated using LC-MS for all samples collected in August, November and December. An estradiol-glucuronide was detected in the input to the two-pond system in August at an estimated concentration of 90 ng/L. This may have been either 17β-E2-3G or 17β-E2-17G, as these could not be differentiated by the LC-MS method. 17β-E2-3S,17G was measured once in the outlet at an estimated concentration of 15 ng/L (December). As glucuronides detection limits were high and recovery sometimes low with this LC-MS method, the concentrations should be considered semi-quantitative only. No conjugates of either E1 or 17α-E2 were detected. 17β-E2-3S could not be quantified in these samples using LC-MS due to co-elution of an interfering compound.

The presence of conjugated estrogens in samples collected in December was also assessed by enzymatic hydrolysis and solvolysis of samples, followed by GC-MS analysis of the liberated free estrogens. After enzymolysis, concentrations of 17α-E2 measured 0.2-1.2 ng/L, while 17β-E2 measured 1.1-2.8 ng/L and E1 measured 3.6-12.1 ng/L. This suggests that glucuronide conjugates were present at only low concentrations in the samples, compared to the concentration of free steroids measured by GC-MS before enzymolysis (up to 228 ng/L, Figure 5.3). On the other hand, 30 ng/L of E1 was liberated by solvolysis of the sample collected after the anaerobic pond. This suggests that the parent conjugate was E1-3S, as E1-3G should have been cleaved during the enzymolysis step.

This comparison of the two methods to analyse conjugated estrogens shows confounding results. The LC-MS results for samples collected in December indicated the presence of 17β-E2-3S,17G and no other conjugates, whereas the solvolysis results suggested the presence of E1-3S, which was not detected by LC-MS. The discrepancy in E1-3S measurement does not appear to be due to limitations in the LC-MS method, as the method detection limit of 15 ng/L for E1-3S should have been adequate to measure it if present in the sample at ~30 ng/L (as was suggested by the solvolysis results).

Hutchins et al. (43) similarly found inconsistent results when using both LC-MS and enzyme hydrolysis to measure conjugated estrogens in agricultural effluents, including dairy effluent. Although E1 was liberated following enzyme treatment of three replicate samples from a poultry lagoon, neither E1-3S or E1-3G were detected using LC-MS-MS despite recoveries above 80% in matrix spikes and a limit of detection of 1 ng/L. In a dairy lagoon sample, 17β-E2-3S was detected by LC-MS but 17β-E2 did not increase after enzyme treatment (43). Finlay-Moore et al. (100) noted that methanolysis was unreliable for analysis of runoff samples from grasslands amended with broiler litter. In some samples free estrogen and testosterone concentrations increased by up to 150% but in others concentrations decreased by 63%.

Matrix spike experiments performed by Hutchins et al. (43) indicated that the enzyme treatment of 17β-E2-conjugates (17β-E2-3G, 17β-E2-17G, 17β-E2-3S,17G and 17β-E2-3,17S) resulted in increased concentrations of free E1, indicating transformation either during the enzyme reaction or subsequent degradation of the released 17β-E2 to E1. The transformation of 17β-E2 to E1 did not occur in blank water spikes treated by enzyme hydrolysis, suggesting something in the sample matrix could mediate this reaction (43).

The production of free steroids differing from the form present in the parent conjugate has been reported previously during enzyme hydrolysis (180,181), and was investigated for the enzymolysis and solvolysis reactions used in this study. There was no evidence of production of E1 from 17α-E2, 17β-E2 or E3 conjugates during either enzymolysis or solvolysis; however, recovery from enzymolysis was less than 50%, much lower than reported previously (43,57,59,61). The enzymolysis reaction had similar efficiency for sulfates as for glucuronides, possibly due to the sulfatase activity of the enzyme. Recovery from solvolysis was 60-87% for the sulfates but less than 5% for glucuronides. Around

25% of 17β-E2-3S,17G was recovered, though >80% of that was transformed to E3 during the reaction. E3 was also produced from the cleavage of 17β-E2-3G, and 17α-E2 was produced from the cleavage of 17β-E2-3S.

These results, along with those previously published (43,180,181) demonstrate that the presence of a free estrogen following cleavage reactions does not confirm the presence of that particular estrogen in conjugated form prior to cleavage. Confounding results are likely to be exacerbated in effluent samples where microbially-mediated degradation of the released free estrogen can occur during sample treatment. Rapid enzymolysis as described by Al Alousi and Anderson (174) (53 °C for 3-4 hours) may reduce the degradation of released steroids, but when trialled in this study the method was unsuccessful as the enzyme denatured at the higher temperature.

Estrogenic activity in Effluent through the Two-Pond System

The aqueous fraction of all samples elicited an estrogenic response in the E-Screen assay, with 17β-E2 equivalents (EEQ) of 4–66 ng/L (Figure 5.4a). Samples also demonstrated full agonism (RPE > 90%) in those samples that did not show cytotoxicity at high concentrations. In August and November, estrogenic activity was low in the raw effluent at 11 and 4 ng/L respectively, while in December, estrogenic activity was higher at 32 ng/L. On all occasions, estrogenic activity was higher at the anaerobic pond outlet (AP) and lower at the facultative pond outlet (FP). Activity at the FP outlet ranged from 3 to 14 ng/L.

Predicted estrogen equivalents based on known potencies in the E-Screen for the identified and quantified steroid estrogens were within the error of the E-Screen assay for most samples (Figure 5.4b), though the estrogenic activity at the AP outlet in December was higher than predicted. The overall increase in estrogenic activity from the raw effluent to the anaerobic pond outlet was not specifically attributable to any particular steroid estrogen, as all hormones proportionally increased in concentration in the aqueous phase (Figure 5.3, discussed further below).

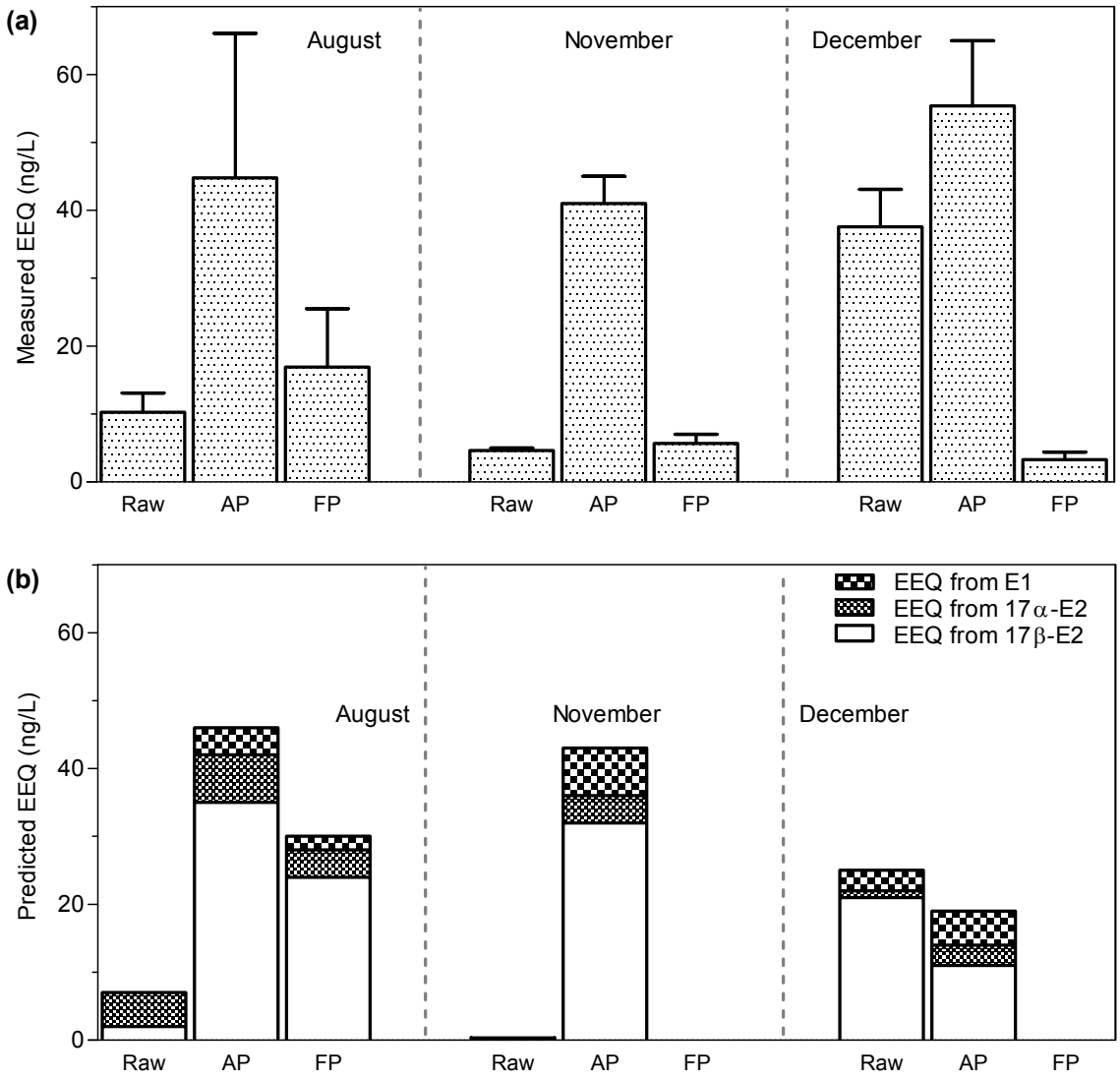


Figure 5.4: (a) EEQ measured by E-Screen (error bars represent standard errors) throughout the two-pond system. (b) EEQ predicted for each steroid hormone (E1, 17a-E2, 17β-E2) throughout the two-pond system. Raw effluent was collected from dairy shed drain, AP after anaerobic pond and FP after facultative pond.

Estrogenic activity was also measured in the conjugated estrogen fraction (eluted from Oasis HLB cartridges) for the August samples. This indicated 2.6 ng/L EEQ was present in the conjugated estrogen fraction of the untreated effluent, compared to 14.7 ng/L in the free estrogen fraction. Similar proportions were measured in the conjugated estrogen fraction of raw effluent samples from a different treatment system (18 & 31%, data not shown). This is a relatively high proportion (15%), considering the potency of conjugated estrogens is two to four orders of magnitude lower than free estrogens. The conjugated

estrogen fraction could contain other acidic polar compounds such as conjugated phytoestrogens and mycoestrogens. Lower activity was measured in the conjugate fraction after the anaerobic and facultative ponds, at 0.8 and 0.6 ng/L EEQ respectively.

Samples from November fractionated through florisil indicated 0.2 ng/L EEQ in the non-polar fraction and 0.8 ng/L in the polar fraction of the raw effluent, compared to 3.1 ng/L in the moderately-polar fraction. The final effluent also measured some activity in the non-polar and polar fractions at 0.6 ng/L and 0.2 ng/L respectively, compared to 3.8 ng/L in the moderately-polar fraction. The majority of the activity was associated within the moderately-polar fraction, which contains free steroid estrogens, nonylphenol, genistein and other similar polarity phytoestrogens (unpublished data, LH pers comm.). Anthropogenic compounds such as PCBs and PAHs are expected to be within the non-polar fraction, while the polar fraction is expected to contain more polar compounds, including conjugated metabolites of steroid estrogens and phytoestrogens.

Conversion and Removal of Estrogens in the Two-Pond System

The estrogenic activity and aqueous concentrations of steroid estrogens increased from the raw effluent to the anaerobic pond outlet in both August and November (Figures 5.3 and 5.4). This appears to be due to changes in distribution between dissolved and particulate phases of the steroid estrogens, conversion between conjugate and free forms, and transformation to E1.

Desorption from the particulate phase may explain the initial increase in aqueous 17α-E2, as there was a concurrent decrease in particulate concentration. However, desorption does not explain the increase in 17β-E2 or E1, as similar concentrations were measured in the particulate fractions both before and after the anaerobic pond. The total (aqueous + particulate) concentrations of E1 and 17β-E2 actually increased after the anaerobic pond (Table 5.2).

Table 5.2: Reduction in total steroid estrogen concentrations through the 2PS (% reduction [a]).

	August	November	December
Anaerobic pond			
E1	-64%	-3546%	-55%
17α-E2	25%	75%	-10%
17β-E2	-172%	-196%	56%
Sum	3%	39%	-22%
EEQ	-349%	-967%	-47%
Facultative pond			
E1	65%	99%	94%
17α-E2	61%	96%	100%
17β-E2	50%	100%	98%
Sum	62%	98%	97%
EEQ	62%	88%	94%
Total removal			
E1	43%	67%	90%
17α-E2	71%	99%	100%
17β-E2	-37%	100%	99%
Sum	63%	99%	96%
EEQ	-69%	-29%	91%

Note: [a] Reduction from aqueous and particulate fractions, does not include contribution from conjugated estrogens. [b] Negative value indicates increase in concentration.

Cleavage of conjugated estrogens has been suggested as the cause of increases observed in the initial stages of sewage treatment plants (85,144,151). It is likely that the observed increase in the concentration of E1 and 17β-E2 in the anaerobic pond is due to cleavage of their conjugated forms. While considerable effort was made to quantify the concentration of conjugated estrogens in the raw and treated effluents to investigate this hypothesis, the results were not conclusive. One of the major metabolites, 17β-E2-3S, could not be quantified by LC-MS due to interference, and the recovery of glucuronides was poor. The enzymolysis and solvolysis reactions demonstrated poor efficiencies for many of the conjugates and in some cases resulted in conversion to free estrogens that differed from those in the parent compound.

The conjugated estrogen 17β-E2-G was detected in the influent to the 2PS. This could account for the increase in both steroid concentrations and estrogenic activity on that date, if it was cleaved to 17β-E2 and subsequently partially degraded to E1 within the anaerobic pond. Although cleavage of conjugated estrogens is reported to be incomplete under anaerobic conditions (135,182), it may be that there is sufficient β-glucuronidase enzyme released by *E. coli* within the anaerobic pond to cleave at least a portion of the conjugated estrogens present.

Although the anaerobic pond did not decrease concentrations of estrogens, or descrease estrogenic activity, it is likely to indirectly aid in degradation of steroids. This pond removes a large proportion of the organic matter in dairy effluents (177) and higher organic loadings can lead to reduced degradation rates for 17β-E2 (183).

A considerable reduction in total estrogens was observed at the outlet from the facultative pond (Table 5.2), consistent with studies on estrogen degradation noting higher removal under aerobic than anaerobic conditions (184-186). The hydraulic retention time of more than 30 days in this pond is sufficient to degrade E1 to substantially lower concentrations. Aerobic degradation of E1 and 17β-E2 has been determined to be <3 to 14 days in the presence of activated sludge (98,186), and approximately 10 days in aquifer and river sediments (187). There are no similar studies for the degradation of 17α-E2, the major steroid detected in these effluents.

Removal efficiency in the facultative pond was higher in November and December than August, possibly due to warmer temperatures in these months or the presence of adapted microbial populations. Higher temperatures have been shown to increase aerobic degradation of estrogens in dairy wastes (167), municipal wastes (188) and soils (189). Adapted microbial populations may also increase the rate of aerobic biodegradation of steroid estrogens, as demonstrated by differences in 17β-estradiol and estrone degradation rates when using sludges from municipal wastewaters compared to industrial wastewaters (188), aquifer materials or river sediments (187,190). Sampling in August was undertaken near the start of the milking season, when the degrading microbial population required may not have been acclimated, compared to in November and December, when it had experienced considerable exposure to estrogens.

5.3.2 Advanced Pond System

DSE Composition and Treatment System Performance

The analysis of nutrients indicated a substantial difference in total nutrient concentrations in raw effluent samples obtained in August compared to those from November and December (Figure 5.5). This was due to a change in the sample collection point: in August this was adjacent to the inlet pipe of the anaerobic pond, while in November and

December the sample was collected from the farm dairy drain. These samples also differed in that they contained considerably higher amounts of solids compared to the pond inlet samples (data not shown).

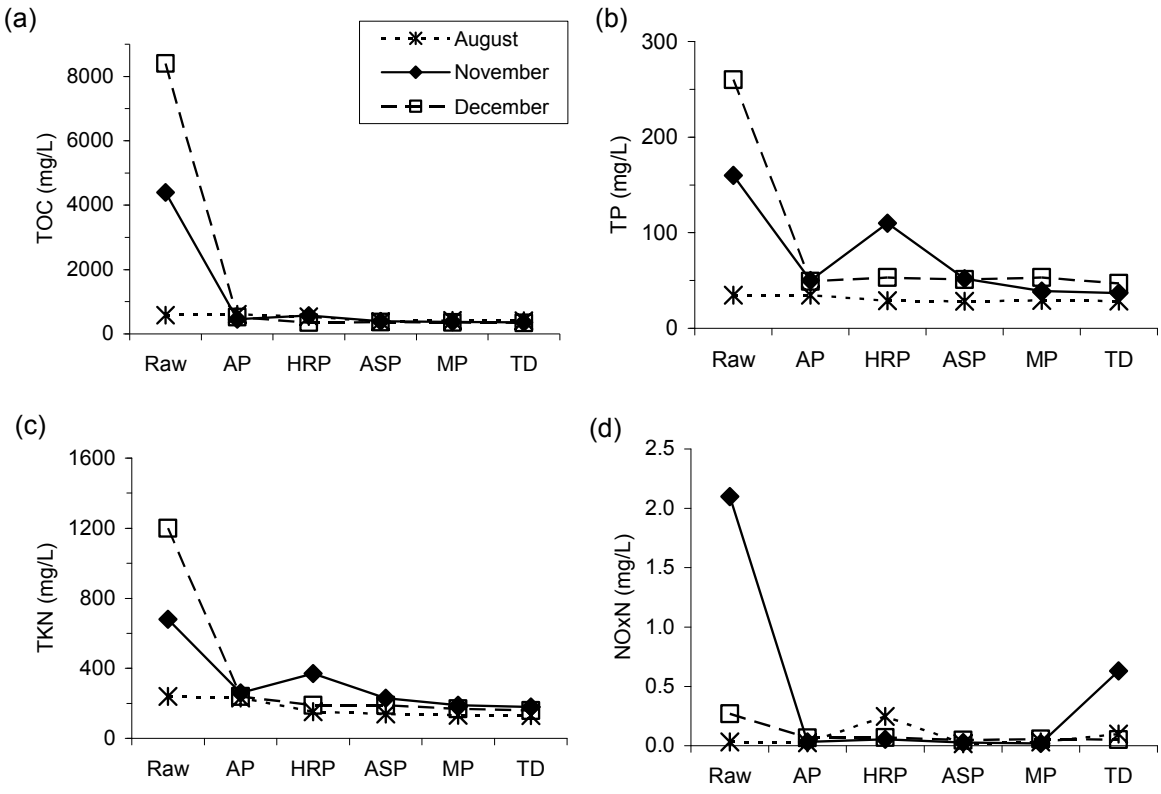


Figure 5.5: TOC (a), TP (b), TKN (c) and NOxN (d) concentrations at each stage in each effluent treatment system for each time sampled. Note several measurements for NOxN were below detection and are not plotted on this chart.

At the other sampling locations within the APS, nutrient concentrations at different times were less varied and the system provided efficient removal of solids and associated nutrients (phosphorus and nitrogen). Increases in TP and TKN in the HRP compared to the preceding anaerobic pond may result from the higher solids in the HRP effluent (data not shown). The increase in solids is due to both the growth of suspended algae and resuspension of bottom sediments in this shallow pond, which is designed for vertical mixing to facilitate algae growth.

The APS appears to be under-performing when compared to its first two years of operation (Table 5.3). This may be partly due to seasonal variation, poor pond operation or maintenance, or an overall decline in performance since pond commissioning. Seasonal variation in chemical and biological constituents of two-pond effluents has been reported with highest values reported for spring and summer (176). This was observed for TP and TKN in this treatment system, both being highest in the final effluents during November and December; in contrast TOC was higher in August.

Table 5.3: Performance and final effluent quality for nutrients in the APS.

	Final effluent quality (mg/L)		% Reduction
Parameter	**This study**	**Literature [a]**	**This study**
TSS	650 [b]	87	98 [b]
TOC	369 ± 35	43 (BOD)	73 ± 37
TP	38 ± 9	19	59 ± 36
TN	157 ± 25	NM	69 ± 21
TKN	157 ± 25	61	69 ± 21
NOxN	0.34 ± 0.41	NM	-21 ± 166

Notes: [a] Data from (171) [b] n=1

The paddle-wheel used in the high rate pond to maintain vertical mixing was not operating during November and December, which likely contributed to poor performance of the pond as the HRP is a critical component of the nutrient removal process (170,171). Appropriate pond maintenance is also important, as excess sludge build-up results in poor effluent quality (152).

Steroid Estrogens in Effluent through the Advanced Pond System

As with the two-pond system, E1, 17α-E2 and 17β-E2 were consistently found in the untreated DSE, and at most stages of the APS treatment system (Figure 5.6). Free steroid concentrations in the raw effluent were substantially higher in August compared to November and December. This is likely to be due to the change in sample collection point from near the inlet of the anaerobic pond to the dairy shed drain. The sample collected from the pond inlet would reflect not only the effluent discharged into the pond on the morning of sampling, but also effluent from previous days. The difference in steroid concentrations is likely due to hydrolysis of conjugated estrogens from previously discharged effluents.

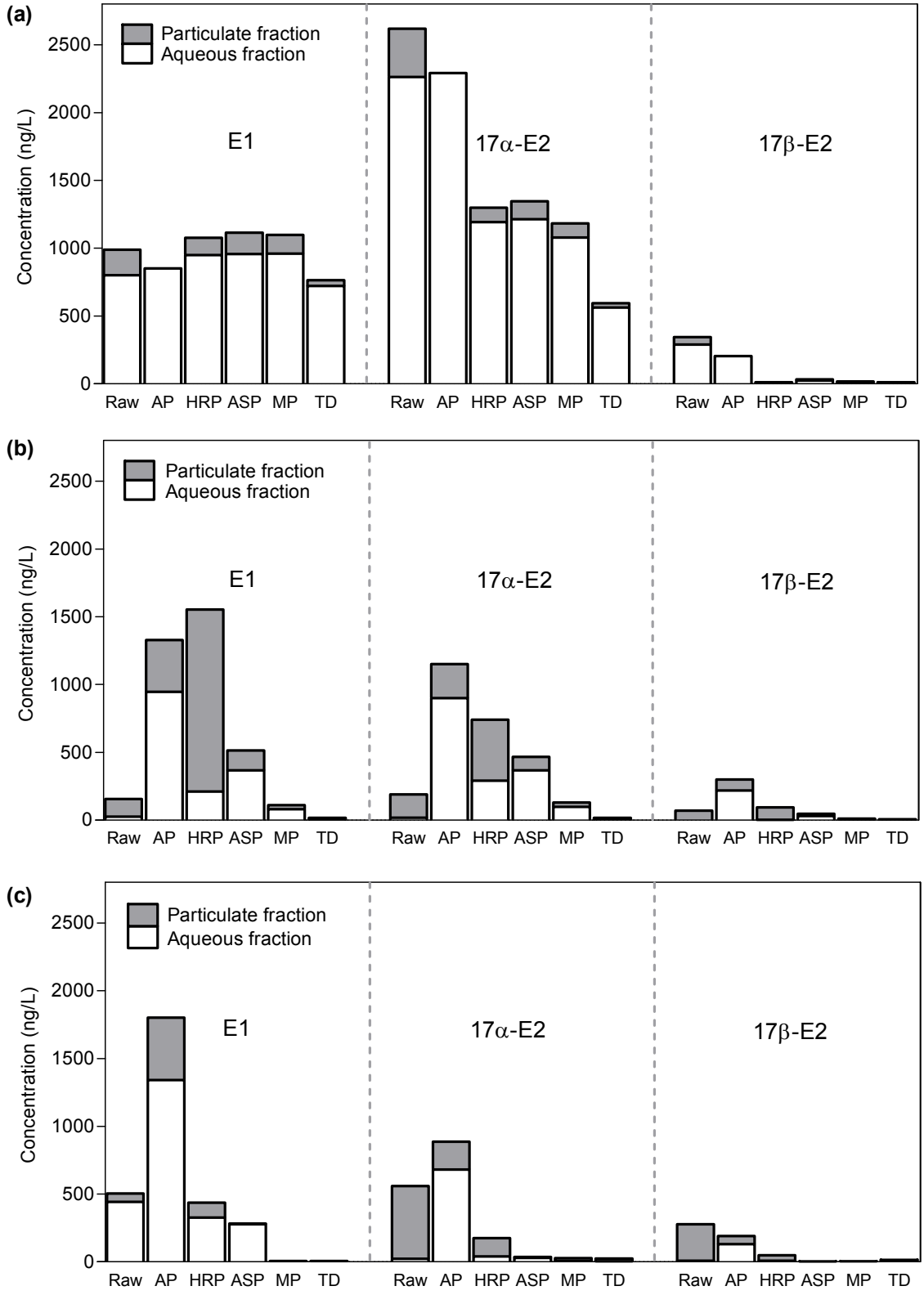


Figure 5.6: Steroid estrogen concentrations in aqueous and particulate fractions at each location in the APS for the three times sampled ((a) August; (b) November; (c) December). Raw effluent was collected from dairy shed drain, AP after anaerobic pond, HRP after High Rate Pond, ASP after Algal Settling Pond, MP after maturation pond and TD after tile drain.

Concentrations and proportions of each steroid were lower in the particulate fraction in August compared to November and December. Higher proportions of 17β-E2 were found in the particulate fraction (53%) compared to E1 (37%) and 17α-E2 (40%), consistent with laboratory sorption studies which indicate typically higher sorption to solids (sludge and soils) for 17β-E2 than E1 (172,191,192). Nonetheless, Hutchins et al. (43) found no difference in the phase distribution of steroids in samples from a dairy lagoon, with 50-52% of each steroid (E1, 17α-E2 or 17β-E2) associated to particulate matter.

Conjugated Estrogens in Effluent through the Advanced Pond System

E1-3S was detected in 2 out of 3 samples from the input to the APS at 138 and 239 ng/L in December and November respectively and 17β-E2-17-G,3S was measured once in the outlet at 26 ng/L (November). No conjugates of 17α-E2 were detected at any location nor were any conjugates detected in the sample collected from the anaerobic pond input in August.

The presence of conjugated estrogens in samples collected in December was also assessed by enzymatic hydrolysis and solvolysis of samples. The amounts of free estrogens released by enzymolysis were minor (Table 5.4) compared to the concentrations of free estrogens measured in the samples, but they were detected in all samples. In contrast, no further steroids were released by solvolysis. The enzyme hydrolysis step did not distinguish between glucuronide and sulfate forms, as the enzyme used had sulfatase activity as well as glucuronidase activity. This was demonstrated by hydrolysis of 17α-E2-3S when tested as a pure standard, though this was not complete. It is likely that the conjugates in these effluent samples were sulfate forms, as *E. coli* is expected to be in the effluent at high levels (this releases a form of β-glucuronidase, which can cleave glucuronides but has little or no sulfatase activity (16,97,98)).

Table 5.4: Concentration of free estrogen conjugates measured by enzymatic hydrolysis of samples from the APS in December (ng/L).

Sample Location	E1	17α-E2	17β-E2
Raw	ND [a]	1.3	1.0
AP	1.9	0.3	0.2
HRP	6.8	ND	1.7
ASP	2.1	ND	0.2
MP	0.9	ND	ND
TD	4.9	ND	1.7

Notes: [a] Not detected.

Estrogenic activity in Effluent through the Advanced Pond System

All analysed samples elicited an estrogenic response in the E-Screen assay, with 17β-E2 equivalents from 4.1 – 382 ng/L (Figure 5.7a). As with the two-pond samples, APS samples that did not show cytotoxicity at higher concentrations demonstrated full agonism (RPE > 90%) in the assay. At most locations, the estrogenic activity of the DSE was higher in August compared to November and December.

The estrogenic activity at the APS inlet was 382 ng/L in August, substantially higher than in November and December (both at 32 ng/L, Figure 5.7a). In August, estrogenic activity decreased through the APS, showing an 80% reduction after the aerobic pond (HRP). Though a slight increase at the ASP and MP was observed, this was within the range of error for the assay. In November and December, estrogenic activity increased from the input to the anaerobic pond outlet, similarly to the trend observed in the two pond system. Again the largest decrease in estrogenic activity in the APS was observed after aerobic treatment, in this case the HRP, with a 92% decrease in estrogenic activity in November (84 to 7 ng/L) and a 94% decrease in December (249 to 14 ng/L). The 4-fold increase observed at the ASP in November was consistent with the increase in steroid concentrations (Figure 5.6); however, activity decreased immediately after this stage to a level comparable to that measured at the HRP. The final outlet consistently measured the lowest concentrations, at 43, 4.1 and 3.2 ng/L respectively.

Predicted estrogen equivalents based on known potencies in the E-Screen for the identified steroid estrogens demonstrated a close agreement to the EEq measured in the assay for many of the samples, particularly those from the August sampling (Figure 5.7).

This indicates steroid estrogens are likely to be the dominant source of observed estrogenic activity in the raw and treated effluent samples.

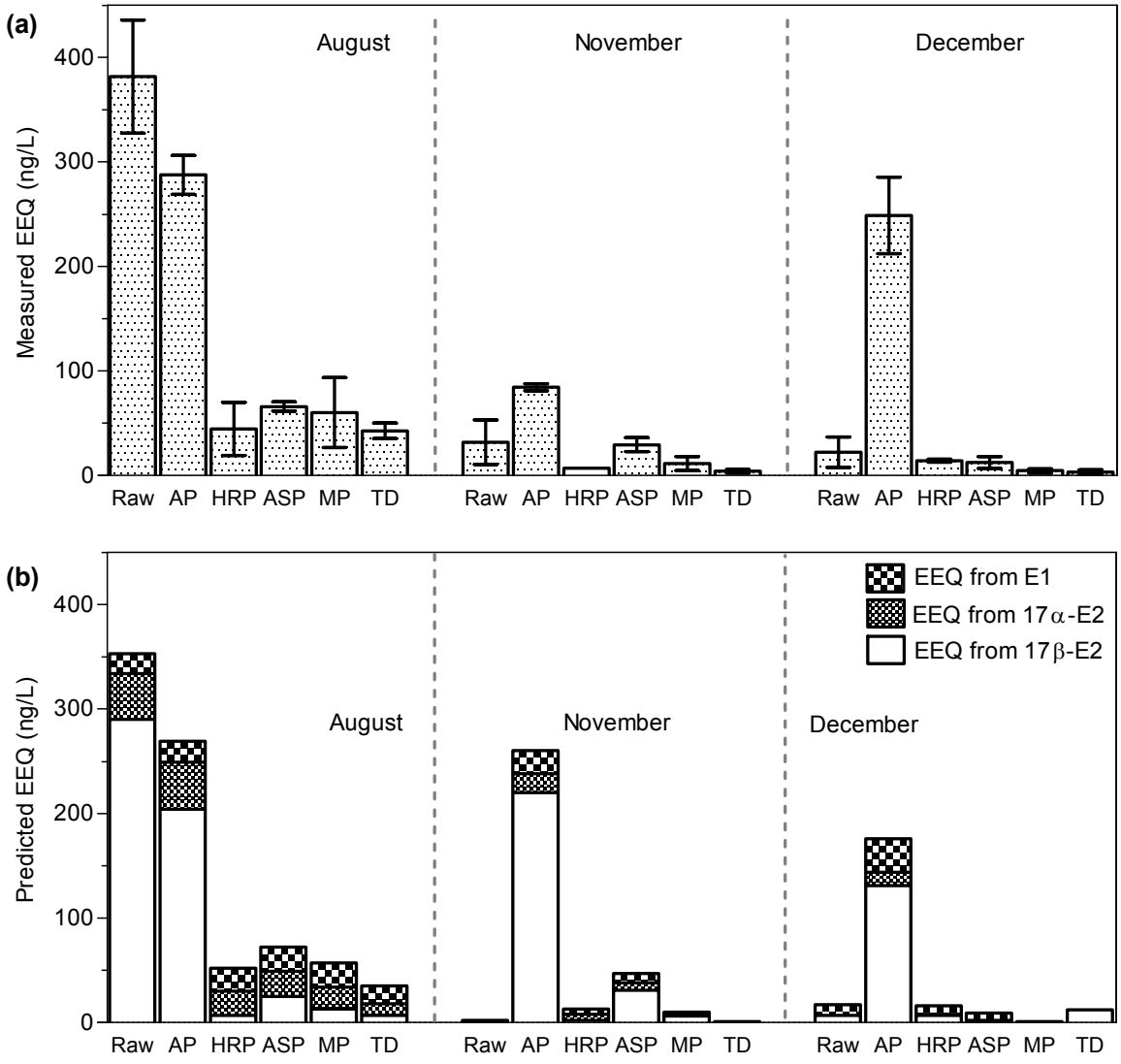


Figure 5.7: (a) EEQ measured by E-Screen throughout the advanced pond system (error bars represent standard error of 2 or 3 measurements). (b) EEQ predicted for each steroid hormone (E1, 17a-E2, 17β-E2) throughout the advanced pond system. Raw effluent was collected from dairy shed drain, AP after anaerobic pond, HRP after High Rate Pond, ASP after Algal Settling Pond, MP after maturation pond and TD after tile drain. December samples not analysed by E-Screen.

The compounds responsible for the observed estrogenic activity appear to change as effluent progressed through the system in August (Figure 5.7b). For samples collected from the raw effluent and anaerobic pond (AP), the majority of estrogenic activity was

due to 17β-E2, while following the HRP, activity was primarily due to E1 and 17α-E2. This pattern was not repeated in samples obtained in November or December.

Estrogenic activity was also measured for the conjugated estrogen fraction (as eluted from Oasis HLB cartridges) in samples obtained in August, and demonstrated a decrease from the raw effluent (13.2 ng/L) through to the final effluent (3.7 ng/L). The activity in this fraction was minor compared to the free estrogen fraction (2-8% of total) and similar to that obtained in samples from the 2PS (1-3%), excluding the raw effluent (15%).

Samples from November fractionated through florisil also indicated that the majority of the activity was retained within the moderately-polar fraction, which contains the free steroid estrogens. Minor estrogenic activity was measured in some samples within the non-polar fraction at 0.6-0.7 ng/L and the polar fraction at 0.3-0.7 ng/L, though activity was higher in the polar fraction of the AP sample at 2.4 ng/L.

Conversion and Removal of Estrogens in the Advanced Pond System

Overall, the APS provided variable treatment for the period monitored, with only 65% of total estrogens removed in August compared to >90% in November and December (Table 5.5). Most of the increased removal in November and December occurred between the ASP and the tile drain. Total estrogen concentrations reduced 96% between the ASP and tile drain in November and 86% in December, compared with only 45% in August. There are many possible reasons for this difference in treatment performance, including the differences in initial concentrations, changes in pond operation, and climatic factors. These are discussed below.

Table 5.5: Reduction in total steroid estrogen concentrations through the APS (% reduction [a]).

	August	November	December
Anaerobic Pond			
E1	14%	-749% [b]	-257%
17α-E2	12%	-504%	-58%
17β-E2	41%	-329%	31%
Sum	15%	-566%	-115%
EEQ	22%	-163%	-667%
High Rate Pond			
E1	-26%	-17%	76%
17α-E2	43%	36%	80%
17β-E2	94%	68%	74%
Sum	29%	14%	77%
EEQ	80%	92%	94%
Algae Settling Pond			
E1	-4%	67%	35%
17α-E2	-4%	37%	79%
17β-E2	-173%	51%	95%
Sum	-4%	57%	51%
EEQ	-13%	-335%	12%
Maturation Pond			
E1	1%	78%	98%
17α-E2	12%	72%	25%
17β-E2	41%	74%	-87%
Sum	8%	75%	88%
EEQ	-21%	63%	65%
Tile Drain			
E1	31%	84%	4%
17α-E2	50%	85%	9%
17β-E2	43%	54%	-205%
Sum	41%	83%	-17%
EEQ	46%	62%	26%
Total Removal			
E1	23%	89%	99%
17α-E2	77%	90%	96%
17β-E2	97%	92%	95%
Sum	65%	90%	97%
EEQ	89%	87%	90%

Note: [a] Reduction from aqueous and particulate fractions, does not include contribution from conjugated estrogens. [b] Negative value indicates increase in concentration.

The largest reduction in total estrogens in August was observed after the aerobic pond treatment stage of APS (HRP, Table 5.5), consistent with results obtained for the 2PS. 17α-E2 decreased by 43% while 17β-E2 decreased by 94%. Conversely, E1 concentrations increased by 26%, mainly due to an increase in concentration in the particulate phase. E1 degradation was lower compared with the facultative pond of the 2P system, which may be due to the difference in hydraulic retention time between the HRP (8 days) and the 2PS

(>30 days). The timeframe for complete degradation of estrone can be up to 10 or 14 days (186,187), longer than the retention time of the HRP. E1 remained at a steady concentration throughout the system in August (Figure 5.6), while a slight increase in 17β-E2 was observed after the ASP. Anaerobic production of 17β-E2 from E1 has been observed in laboratory tests using cultures from lake water and sediment under methanogenic, nitrate-, sulfate-, and iron-reducing conditions (193), and may account for the apparent rise of 17β-E2 observed in this pond.

In November and December, the free estrogen concentrations and the estrogenic activity increased after the anaerobic pond at the APS, consistent with the results obtained for the two-pond system. Unlike the two-pond system, the observed increase does not appear to result from changes in the phase distribution, as the total (aqueous + particulate) concentration of estrogens also increased after the anaerobic pond.

The increase could neither be fully explained from the measurement of conjugated estrogens. E1-3S, the most common conjugate in cattle excreta (99), was detected in the raw effluent, but the concentrations (238 and 138 ng/L) were substantially lower than the observed increase in E1 concentration (920 and 899 ng/L). 17β-E2-conjugates were not fully quantified and may be the source of the increased concentration of free steroids measured after the anaerobic pond. In addition, conjugated estrogens were not measured in the particulate fraction as they are generally considered to be water soluble; however, it is possible that the low pH conditions applied prior to sample extraction increased their sorption to the solid phase, lowering the aqueous concentrations.

The HRP was not as effective at removing steroids in November, with a total removal of only 14%, compared to 29% in August. As noted above, the paddle-wheel was not working during this period and is required to maintain algae populations and consistent treatment (171). Despite this, 77% of total estrogens were removed in the HRP in December, possibly due to warmer temperatures increasing aerobic degradation, and/ or the presence of adapted microbial populations, as discussed above.

Total steroid concentrations reduced by 51-57% after the ASP in November and December. TSS measurements in December indicated a 75% reduction in suspended solids in this pond, which is designed to remove solids and algae through gravity. The

results suggest that adsorption to, and settling of, solids is a significant removal mechanism for steroids.

The maturation pond further reduced steroid concentrations, with 75% and 88% reductions in November and December. The shallow depth and long residence time (15 days) maximises solar-UV radiation in order to reduce bacteria numbers, and the radiation can also be expected to reduce steroid estrogen concentrations. Estimates for the photolytic half-life of 17β-E2 vary from 41 hours (194) to 10 days in clear, colourless waters (195). As photodegradation follows pseudo-first-order kinetics (196), degradation increases with longer UV exposure and may explain the increased removal in the MP in November and December compared to August. The observed reduction may not be solely from photodegradation, as there is also potential for continued biodegradation in this aerobic pond. The tile drain further reduced the concentration of total estrogens in August and November, but not in December, when concentrations were already lower.

5.3.3 Biodegradation of 17α-Estradiol in Laboratory Test

Only 20% of the 17α-E2 spiked at 25,000 ng/L was recovered from the sample immediately after spiking, suggesting either immediate degradation or rapid sorption to solids in the incubation vessel and/or during centrifuging. Immediate sorption of 17α-E2 is supported by a study showing that 87-97% of E1 and 17β-E2 was sorbed onto activated sludge within 30 mins when it was added to water samples at 1 g/L (172), a concentration comparable to the mass of solids in the DSE. The practice of acidifying samples prior to centrifuging and filtering will likely further enhance the sorption of estrogenic steroids to organic particulates.

In the spiked sample, 17α-E2 concentrations decreased over time from an initial mean concentration of 5,420 ng/L to a final mean concentration of 4,100 ng/L after 216 hours (Figure 5.8). It has previously been suggested that 17α-E2 degrades to E1 (43,48); however, in this test, the reduction in 17α-E2 was not associated with a clear or quantitative increase in either E1 or 17β-E2. E1 and 17β-E2 increased by 110 ng/L and 220 ng/L respectively, much less than the concurrent decrease in 17α-E2 of 1320 ng/L.

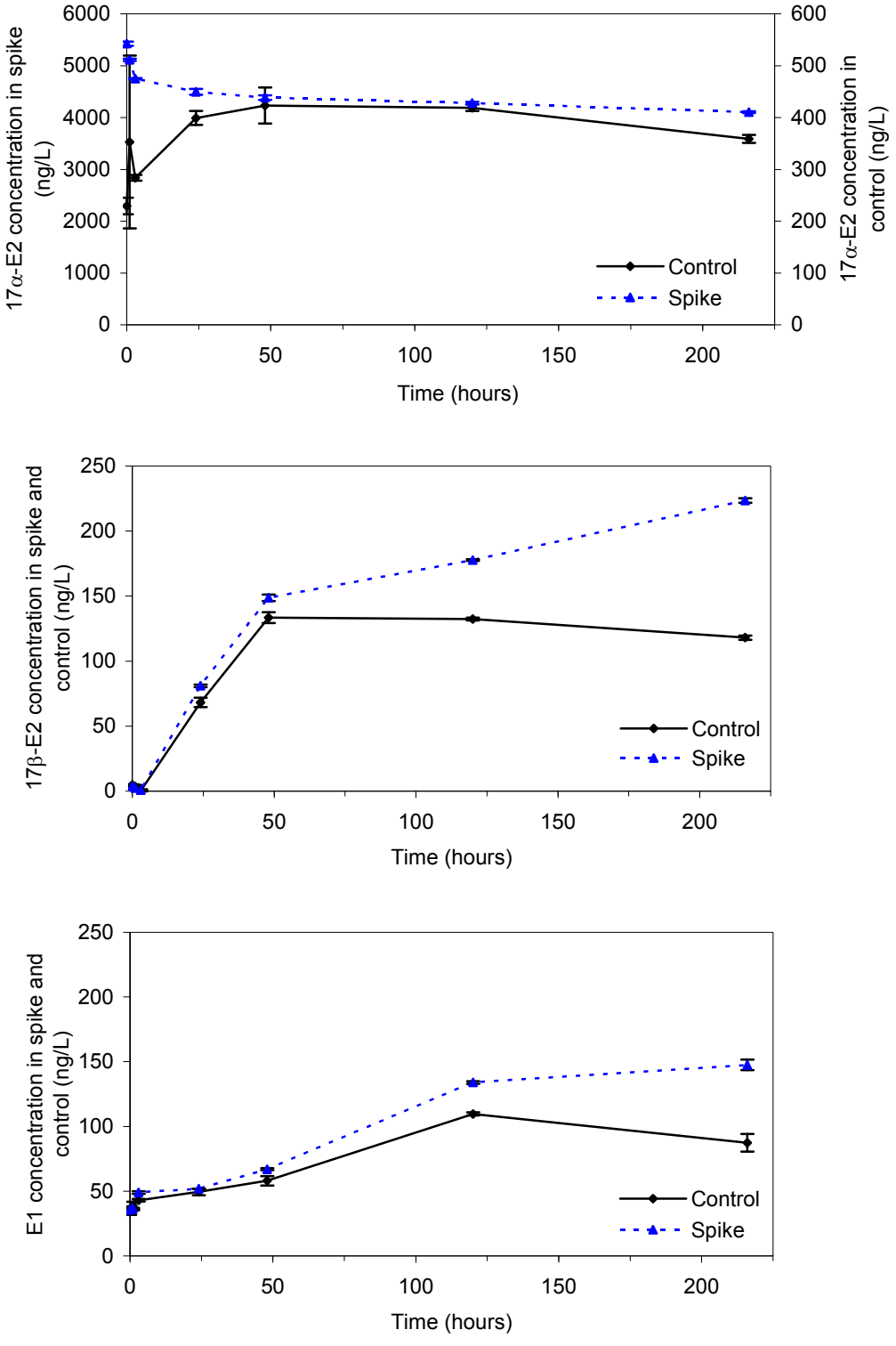


Figure 5.8: Concentration of steroids over time in spiked and control DSE samples (points are mean of three replicates, error bars represent ± 1 standard deviation).

Quantitative degradation of 17β-E2 to E1 has been observed in sewage effluents, river sediments and aquifer materials (98,186,187), followed by slower degradation of E1. It is possible that 17α-E2 did quantitatively degrade to E1 which then subsequently sorbed to solids in the DSE or further degraded. Sorption is likely, as these DSE samples had high solids content; the organic carbon content of effluent sludges is also high (0.75-1.7% wet weight (197)), increasing the sorptive capacity for steroid estrogens (198). This would need to be confirmed by analysis of the particulate fraction of the samples. Concentrations of both E1 and 17β-E2 were slightly higher in the spiked samples than the control, suggesting possible formation of these from 17α-E2. This may occur through initial oxidation of 17α-E2 to E1, followed by formation of 17β-E2 in anaerobic conditions (193). If this can occur, it could increase the potential risk to aquatic environments due to the higher estrogenic potency of 17β-E2, although the extent of this transformation is likely to be relatively low (193).

In the control sample, 17α-E2 concentrations approximately doubled between 0 and 48 hours after sample collection, then decreased slightly. 17β-E2 also increased substantially between 0 and 48 hours, reaching a peak concentration of 130 ng/L, then decreased slightly during the period to 216 hours. By contrast, E1 increased only slowly between 0 and 48 hours, but then increased to a peak concentration of 110 ng/L at 120 hours, probably due to degradation of 17β-E2 and possibly 17α-E2, before decreasing again.

The observed increases in 17α-E2 and 17β-E2 within 48 hours in the control samples are likely to be due to hydrolysis of 17α-E2 and 17β-E2 glucuronides. This has been shown to occur within about 20 hours for 17β-E2 in domestic wastewater and activated sludge solutions (16,98) and is mediated by β-glucuronidase enzymes released by faecal bacteria, including *E. coli* (16). As *E. coli* are expected to be present in DSE at high levels (based on faecal coliform measurements of 3×10^5 – 16×10^5 (199)), enzyme-mediated hydrolysis processes are expected to be rapid in the DSE. Glucuronide forms of E1 are expected to be at lower concentrations, as the sulfate form dominates in cattle urine (20,99).

Overall the results indicated slow degradation of the estrogens, contrasting with results reported previously for activated sludge, soils, river sediments and aquifer sediments (184-187,190,192), where estrogens completely degraded within 3-14 days. This is unlikely to be due to a lack of bacteria, but may be due to the high organic loading of the effluent

or lower oxygen levels maintained in this test system. As mentioned, in experiments with activated sludge, E1 degradation rates were substantially slower with high organic loadings in the influent.

5.3.4 Comparison of Treatment Systems and Potential Improvements for Estrogen Removal

At each sampling occasion, the final effluent from each treatment system contained measurable steroids and demonstrated estrogenic activity above levels known to cause adverse effects in fish (132). There was little difference in the overall removal of total free estrogens between the two systems (Table 5.6). Furthermore, removal through both systems was poorer in August than in November or December, indicating neither system was more robust than the other.

Table 5.6: Reduction in steroid estrogen concentrations in the systems studied.

	Final concentrations in effluent (ng/L [a])				Reduction throughout system (%) [a]			
	Particulate	Aqueous	Total [b]	EEQ [c]	Particulate	Aqueous	Total [b]	EEQ [c]
				Two-pond system				
August	0	295	295	25	100%	-15% [e]	63%	-69%
November	0	20	20	5.5	100%	-24%	99%	-29%
December	0	17	17	3.3	100%	91%	96%	91%
				Advanced Pond system				
August	78	1290	1370	43	87%	62%	65%	89%
November	24	18	42	4.1	94%	60%	90%	87%
December	29	16	45	3.2	97%	97%	97%	90%

Note: [a] Sum of free estrogens, does not include conjugated estrogens. [b] Total of aqueous and particulate fractions. [c]Aqueous fraction only. [d] ND = Not detected. [e] Negative value indicates increase in concentration.

A comparison of the nutrient removal data for each treatment system indicates the two-pond system was producing a higher quality final effluent than the APS (Tables 5.1 & 5.3). No relationship was seen between the concentration of nutrients in the effluent and individual steroids, total estrogens, or estrogenic activity in individual samples. This indicates that effluent composition cannot be used to predict final steroid concentrations. On the other hand, the total removal rate of steroid estrogens and estrogenic activity was similar to that of TSS and TOC for both systems, suggesting that a system designed for improvement of these parameters may have secondary beneficial effects in reducing steroid estrogens levels and estrogenic activity.

The laboratory degradation experiment demonstrated the persistence of the estrogens in the DSE, with significant concentrations remaining after 8 days. Degradation was much slower than had been previously reported for activated sludge, where 17β-E2 and E1 were completely removed after 3 days. This occurred even when the sludge was spiked at 1 μg/L, within the same order of magnitude of 17α-E2 in the unspiked DSE samples used in the experiment reported here.

These results suggest that the retention time of an aerobic pond must be considerably longer than 8 days to provide effective removal of estrogens. The higher removal rates in the facultative pond of the 2PS compared to those for the high rate pond of the APS most likely reflects the longer retention time of the former. Despite this, the subsequent ponds of the APS provided effective treatment, ensuring that the overall removal rate was similar for the two systems studied.

An improved system for removal of steroid estrogens could be to incorporate an aerobic pond with a long retention time into APS. The potential overall reduction that may be achieved with that kind of configuration could be estimated from the final effluent quality of the 2PS and the further removal currently achieved in the latter ponds of the APS (45-96% removal from the ASP to the tile drain outlet). Based on a total estrogen concentration of 20 ng/L in November and subsequent reduction of 96%, a final estrogen concentration of 0.8 ng/L could be achieved. Alternatively, based on the December results of 17 ng/L after the facultative pond and a subsequent reduction of 86%, a final concentration of 2.4 ng/L could be achieved. These would represent a great improvement on the current concentrations in the final treated effluent and would be similar to the concentrations typically measured in effluent from sewage treatment plants. An alternative option could be to improve the efficiency of the aerobic ponds of each system by ensuring that they are fully oxygenated, for example, using a mechnical aerator.

5.4 CONCLUSIONS

Estrogenic activity and steroid estrogens were found at variable levels in the raw DSE samples, with a large proportion of the steroid estrogens adsorbed to particulates in the samples. In both effluent treatment systems, free estrogen concentrations typically increased between the raw effluents and effluent exiting the anaerobic ponds. This is most likely due to hydrolysis of conjugated estrogens and possibly due to desorption of free estrogens from the solid phase. Conjugated estrogens were detected in some samples, particularly in raw effluent. Results obtained using enzymolysis and solvolysis suggested the presence of further conjugated estrogens that were not identified using LC-MS.

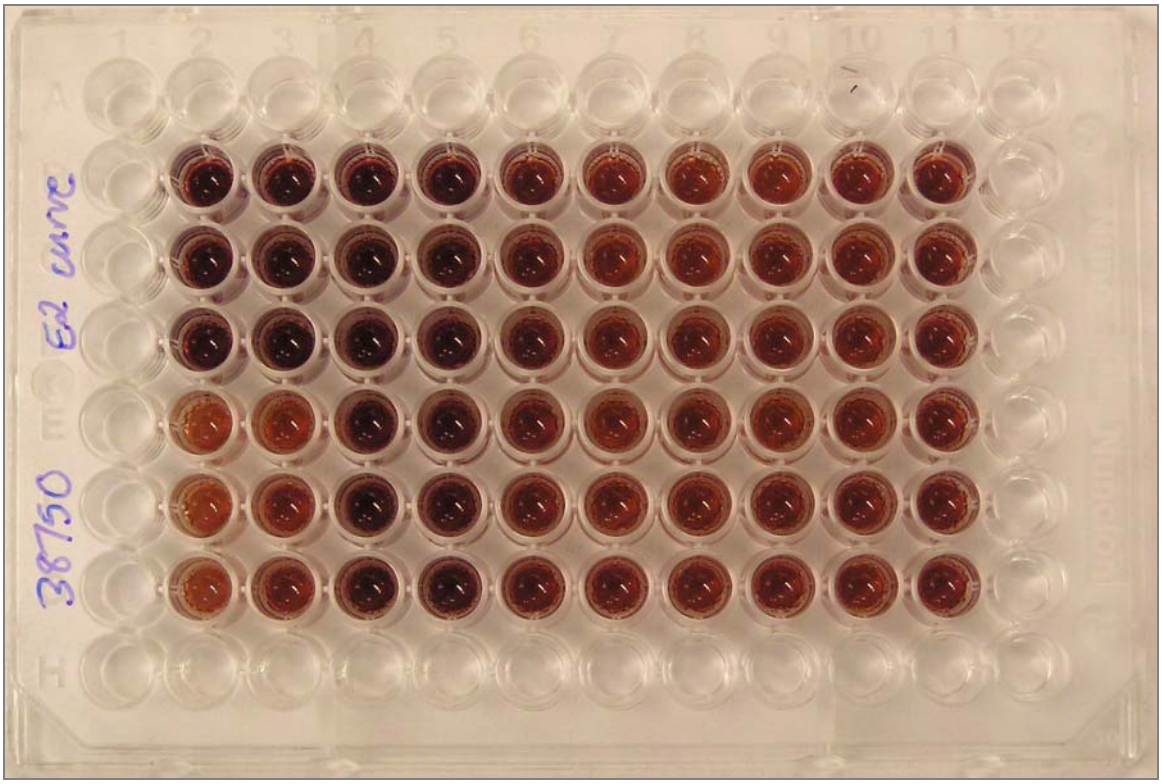
Most of the estrogen removal occurred within the aerobic pond of each treatment system. The inclusion of an aerobic pond therefore appears to be the most important consideration in designing effluent treatment systems to remove steroids. Although it has been suggested that aerobic degradation of 17α-E2 may increase the concentration of E1, this could not be concluded from the results from either pond system or from the laboratory degradation test.

Estrogen concentrations also increased in the first days of the laboratory degradation in the unspiked samples, again suggesting the cleavage of conjugated estrogens to free forms. The degradation study also indicated that steroid estrogens remained at high concentrations after 8 days, contrasting with results for activated sludge and other matrices.

As neither pond system completely removed estrogens from the dairy effluent, the effluents released to the environment from these treatment systems remain a source of estrogens to the environment and a potential risk to aquatic life living downstream of these discharges.

CHAPTER SIX

EVALUATION OF THREE IN VITRO BIOASSAYS TO ESTIMATE THE ESTROGENIC ACTIVITY OF DAIRY SHED EFFLUENTS



Chapter 6: Evaluation of Three *In Vitro* Bioassays to Estimate the Estrogenic Activity of Dairy Shed Effluents

6.1 INTRODUCTION

Steroid estrogens and estrogenic compounds can affect exposed biota by disrupting normal hormonal processes. Biological approaches have been developed to assess the potential effect of estrogenic compounds on fish, amphibians and mammals (200). To reduce time and ethical costs of animal testing, a range of *in vitro* assays has been developed, based on well-characterised mechanisms of action for initial screening of compounds for estrogenic activity (12,200).

These *in vitro* assays developed to evaluate potential estrogenic activity of pure compounds have also been extensively applied to assessing environmental samples. Because bioassays measure the total estrogenic activity of samples without requiring any information regarding chemicals present (68), the results indicate the potential exposure of aquatic biota to mixtures of estrogens and estrogenic compounds. *In vitro* assays have been used to assess estrogenic activity in river waters (e.g., (166,201)), sediments (202), sewage treatment plant influents and effluents (reviewed in (68)) and more recently, agricultural wastes (41,44,49).

The E-Screen assay has been widely used for analysis of estrogenic activity of environmental samples. As it is a cell proliferation assay, it represents a higher level of biological complexity than other *in vitro* assays, and takes account of non-ER mediated effects (70). Furthermore, as the E-Screen has been extensively used to screen compounds for estrogenic activity, potency data is available for a wide range of compounds (32). However, progesterone and other pregnanes can cause cell proliferation in some MCF-7 strains (203,204). As dairy cattle excrete progesterone naturally, and from implants or injections used to bring the cows into synchronised estrus, samples of DSE are likely to contain elevated concentrations of progesterone. This may result in false positives when measuring estrogenic activity with the E-Screen assay.

Receptor-reporter gene assays measure estrogenic effects mediated through the estrogen receptor (67) and have been widely used internationally to assess activity of environmental samples (45,64,205,206). A yeast-based receptor-reporter gene assay has recently been developed in two strains: one incorporating the human ERα (hER) and the other the ERα from the Japanese medaka (*Oryzias latipes,* medER). The use of these two strains in combination can be used to suggest causes of estrogenicity, as the medER has higher affinity for xenoestrogens than the hER (207). A larger estrogenic response in the medER can suggest the presence of xenoestrogens in samples, while a similar response in each assay suggests the activity is more likely due to steroid estrogens.

In vitro assays have only recently been applied to assessing estrogenic activity in agricultural wastes and a small number of samples have been assessed compared to sewage effluents and surface water samples. To date, few of the studies including agricultural samples have supported the bioassay results with chemical analyses of known estrogenic compounds (44,45,166). While *in vitro* assays can be sensitive and rapid, there is potential for them to be affected by other compounds present in the samples that may cause cytotoxicity or anti-estrogenic activity. A comparison of assay and chemical results can help to elucidate non-estrogenic effects.

The aim of the investigation described in this chapter was to confirm the estrogenic activity of DSE samples observed using the E-Screen by testing samples in receptor-reporter gene yeast assays. Potential contributors to observed activity were assessed by comparing assay responses and from predictions of 17β-estradiol equivalents (EEQ) based on known chemical constituents and relative potencies.

6.2 METHODS

6.2.1 Sample Preparation

Grab samples of DSE were collected in MQ water-rinsed amber glass bottles with Teflon-lined lids and immediately preserved by the addition of sulfuric acid (to pH ≤ 2). Samples were transported on ice and extracted within 24 hours of collection. Samples were first centrifuged at up to 1780 *g* for 20 min at 4 °C, then filtered through glass fibre filters

(GF/C) with the assistance of a layer of filter aid. Centrifuged and filtered samples were loaded onto Oasis HLB cartridges (preconditioned with 10mL of methanol followed by 10 mL of MQ water) at a flow rate of 5-10 mL/min. For 500 mL samples, 500 mg Oasis HLB cartridges were used while 1 g cartridges were used for 1 L samples. Oasis HLB cartridges were dried under vacuum to remove residual water then eluted with 30 ml of DCM: DEE: MeOH (40:10:1).

6.2.2 E-Screen

The E-Screen assay was conducted as described in Chapter 4. At least two independent assays were performed for each sample with the average value reported.

6.2.3 Two-hybrid Yeast Assay

The two-hybrid yeast assay followed the procedure described in detail in Shiraishi et al. (208). This assay system used yeast cells (*Saccharomyces cervisiae* Y190) incorporating an estrogen receptor (either the human ERα or Japanese medaka (*Oryzias latipes*) ERα), the coactivator TIF2, and the β-galactosidase reporter gene. Yeast cells were grown overnight at 30 °C in a modified synthetic dextrose medium. The yeast was diluted in growth media to achieve an absorbance of 0.175-0.185 at 595 nm using a spectrophotometer, after a 1 in 10 dilution. Serial dilutions of sample extracts (in DMSO) were prepared in the wells in 60 µL of growth medium with a constant volume of DMSO in each (2%). The yeast mixture was added to the 96-well plates (60 µL per well) and then plates were incubated for 4 hours at 30 °C.

A mixed solution for inducing chemiluminescence and for enzymatic digestion was added at the ratio of 5:3 (v/v) Zymolase 20T and GalactLux substrate buffer (Aurora Gal-XE kit; ICN Biomedicals, California, USA), then plates were incubated at 37 °C for 1 h. A light-emission accelerator reagent was added immediately before measurement, The chemiluminescence produced by released β-galactosidase was measured with a 96-well plate luminometer (FLUOstar OPTIMA, BMG Lab Tech). Estrogenic activity was recorded as the ECx10, which was defined as the concentration of test solution producing a chemiluminescent signal 10 times that of the blank control. EEQ concentrations were calculated by the formula:

EEQ = ECx10 [17β-E2] / ECx10 [sample]

6.2.4 Calculation of EEQs based on Chemical Analysis

Free estrogens in the DSE samples were analysed by GC-MS as described in Chapter 3, then EEQs were calculated for each sample following a toxicity equivalents approach. This has been demonstrated to be appropriate for mixtures where each component acts through the same pathway and the dose-response curves are parallel for individual compounds (137). The concentration of each steroid measured by GC-MS was multiplied by its relative potency (RP) obtained by E-Screen and two-hybrid yeast assays. The EEQs for each compound measured in a sample were summed to obtain the total predicted EEQ for that sample.

$$Total\ predicted\ EEQ\ for\ mixture = \sum_i \{C_i \times RP_i\}$$

6.2.5 Statistical Analyses

Regression analyses used to examine differences between assays and between measured and predicted values were undertaken with the statistical software package JMP v6 (SAS Institute Incorporated).

6.3 RESULTS AND DISCUSSION

6.3.1 Performance of Assays

DSE samples indicated a concentration-dependent response in all assays although cytotoxic effects were evident at high concentrations with a reduction in response. As described in the methods, these data points were removed from the linear regression for the E-Screen assay. Because cytotoxicity occurred only at higher concentrations and the EEQs for the two-hybrid yeast assays were calculated from the EC10, cytotoxicity did not affect the calculation of EEQs in those assays.

The limit of quantitation (LOQ) for the E-Screen assay was typically 5 ng/L, but ranged from 0.3-19 ng/L, depending on the cytotoxicity of samples in the assay. The LOQ for the

hER assay was slightly lower and ranged from 0.9 to 5.3 ng/L. The LOQ for the medER was slightly higher, ranging from 6.6 ng/L to 10 ng/L.

The variation of the E-Screen assay was assessed by repeated analyses of three samples over six to seven assays. Coefficients of variation were determined as 33%, 29% and 17% (n=6, 7 and 7 respectively). Variation within each assay run was lower, with an average difference in duplicate measurements of 12%. This variation should be considered when comparing between assays and with EEQ predicted from chemical analysis.

6.3.2 Comparison of EEQs of DSE Samples in Each Assay

There were 28 DSE samples analysed by both E-Screen and the two-hybrid yeast assays. All samples elicited an estrogenic response in the E-Screen assay, while one sample was below the limit of quantitation in the hER assay and another in the medER assay. This confirmed that the DSE samples were estrogenic and that the estrogenicity was mediated through the estrogen receptor.

The three assays gave very different EEQs, with a median of 28 ng/L with the E-Screen assay, 69 ng/L with the hER assay and 343 ng/L with the medER assay (Figure 6.1). Although different results are not surprising, due to differences in receptors, assay mechanisms and transport of compounds through the cell membrane, the order of magnitude difference between the E-Screen and medER assays was larger than expected.

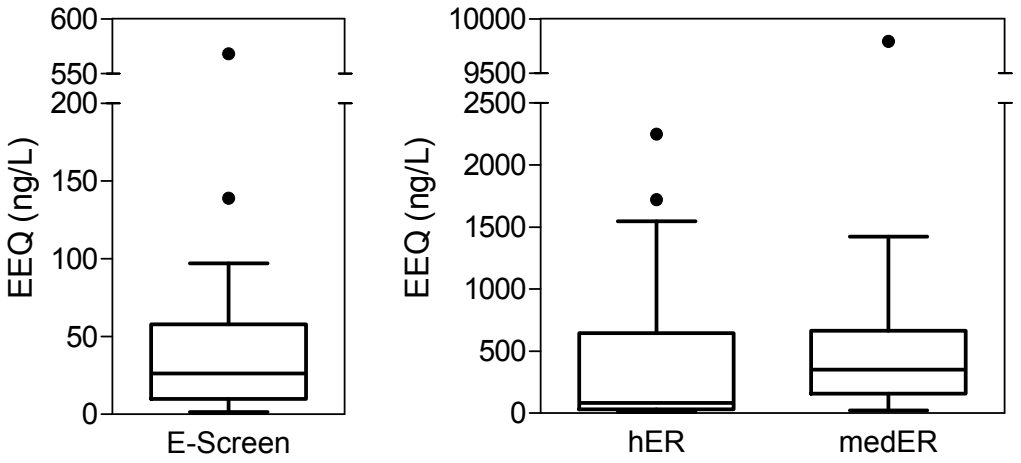


*Figure 6.1: Summary of estrogenic activity for 28 effluent samples in the three assays. Note: The box indicates the interquartile range (IQR), the bar the median value, whiskers = 1.5 * IQR and dots indicate outlier values.*

Previous studies of STP influents and effluents have shown similar EEQs when comparing assays: the ER-CALUX and YES assays (72); four receptor-reporter gene assays in MCF-7 or HeLa cells (209); and the YES assay, a zona radiata protein production assay based on trout hepatocytes and a receptor-reporter gene assay in rainbow trout gonad cells (160). Order of magnitude differences have only been demonstrated previously when comparing receptor binding assays (RBA) with receptor-reporter assays (72) or the E-Screen (144). This can be expected as the RBA measures both ER agonists and antagonists and does not require compounds to cross the cell membrane (72).

The assays used in this study were compared by regression analysis of the EEQs obtained from each assay. The E-Screen assay was weakly correlated to the medER assay results (Figure 6.2, R^2=0.58, p-value < 0.001) but not well correlated to hER data (R^2=0.28, p-value = 0.0043). The hER and medER data were weakly correlated, with R^2 = 0.54 (p-value < 0.001).

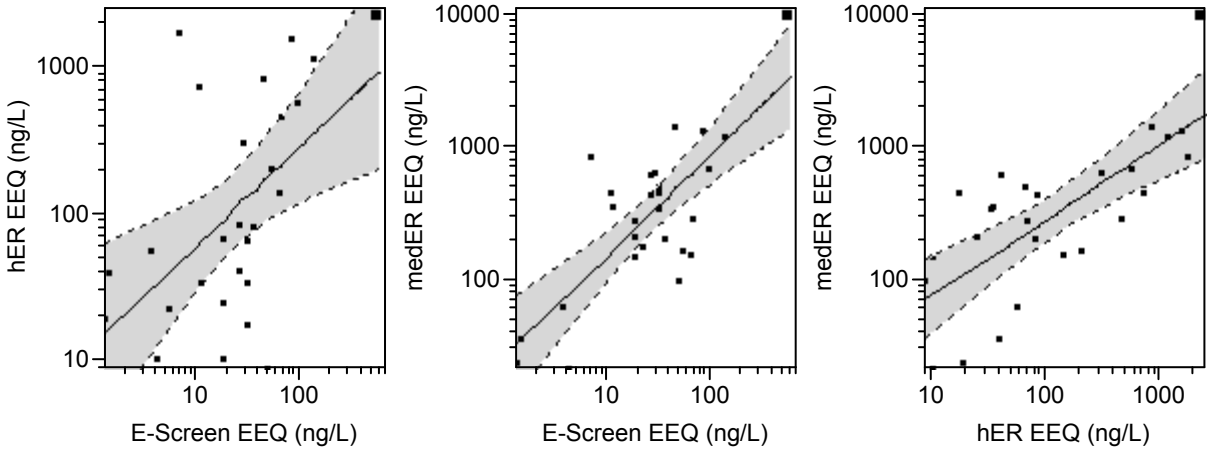


Figure 6.2: Correlation of EEQ measured in the three assays. Solid line is regression line and dotted lines are 95% prediction intervals for new data points.

The regression analysis indicates that although EEQ values were much higher with both yeast assays compared to the E-Screen, the samples followed a broadly similar pattern. A potential reason for the higher EEQs in the yeast assay is the presence of cytotoxic compounds in the E-Screen, which would suppress cell proliferation and result in lower calculated EEQs. Further research is required to assess the presence of cytotoxic chemicals that may affect the E-Screen results. An alternative explanation is related to the mixtures of compounds contained in the DSE samples. Differences in estrogenic potencies of the

compounds in each assay can result in higher or lower overall EEQ for a sample. This is explored further in the next section.

6.3.3 Comparison of Bioassay and Predicted Estrogenic Activity

The estrogenic potency of chemicals differs between *in vitro* assays (132,210,211) resulting in different results for the relative estrogenic potential of samples containing mixtures (29). A comparison of the relative potencies of chemicals in the E-Screen assay and the yeast assays indicates that the steroid estrogens have much higher potency in the yeast assays. Both strains of the yeast assay exhibit higher relative potencies for E1 (0.50-0.63, Table 6.1) and 17α-E2 (0.1 – 0.051) compared to potencies from the E-Screen (E1 0.024; 17α-E2 0.02). For the weakly estrogenic compounds, higher potencies were measured in the medER assay than the E-Screen or hER. The largest differences were observed for genistein (19-fold higher in medER than E-Screen) and the anthropogenic compounds, NP, OP and BPA (38, 19, 14-fold respectively). The few exceptions to this are 17α-E2 and E1 (which both had highest potency in the hER assay) and E3 (which was highest in the E-Screen).

Table 6.1: Comparison of steroid estrogens and estrogen mimics in the E-Screen and Yeast Assay.

Compound	RPP Escreen	RPP hER [a]	RPP medER [a]
17β-E2	1	1	1
17α-E2	0.020 [b]	0.11	0.052
E1	0.024 [b]	0.73	0.67
E3	0.054 [b]	0.0043	0.0019
Genistein	0.00015 [c]	0.00038	0.0028
4-NP	0.000045 [c]	0.0000051	0.0017
4-OP	0.00012 [c]	0.00075	0.0022
Bisphenol A	0.000027 [c]	0.000017	0.00037

Note: [a] L. Hamilton, pers. comm. [b] This study, Chapter 4. [c] Mean of data from (29-32,138).

The difference in potencies may explain why EEQs were higher when measured by the yeast assays, as E1 and 17α-E2 are found at higher concentrations in DSE than 17β-E2. Higher values in the medER compared to the hER assay can suggest the presence of xenoestrogens in samples, while a similar response in each assay suggests the activity is more likely to be due to steroid estrogens.

Calculated EEQs for individual samples were based on GC-MS analysis of steroid estrogens and their estrogenic potencies as measured in the E-screen and two-hybrid yeast assays (Table 6.1). This was not possible for two of the DSE samples (where either the assay or GC-MS results were below quantitation limits), but was possible for the remaining 26 samples.

The predicted EEQs for the effluent samples were compared to the measured EEQs in each assay. The median ratios of measured to calculated EEQ were 1.2 for E-Screen and hER assays and 5.1 for medER. Generally values within the same order of magnitude are considered to be within the precision of assay and chemical analyses (138,160), which suggests a reasonable agreement overall for the E-Screen and hER assays. The EEQs measured with the medER assay were systematically higher than those calculated from steroid concentrations. This contrasts with many previous studies comparing chemical and biological analyses (138,139,160,212,213), where measured EEQ was lower than predicted EEQ.

The predicted EEQs for the effluent samples are compared to the measured EEQs for each assay in Figure 6.3. A positive correlation was observed between the measured and calculated EEQ for each assay but there was considerable variation for the individual values (log-transformed fits for E-Screen, R^2 = 0.66; hER, R^2 = 0.72; medER, R^2 = 0.65; p-values < 0.001 for all fits). The regression line for the medER assay indicates a substantial difference between the measured and predicted values (Figure 6.3), while the values for the hER are similar overall (regression line is close to 1:1 line). For the E-Screen, there was overall a reasonable relationship between measured and predicted EEQs, though values were under-predicted at high EEQs and over-predicted at low EEQs (Figure 6.3). The results suggest that lower EEQs in the E-Screen compared to the hER assay were due to lower potencies of the steroid estrogens in the E-Screen assay than in the hER assay.

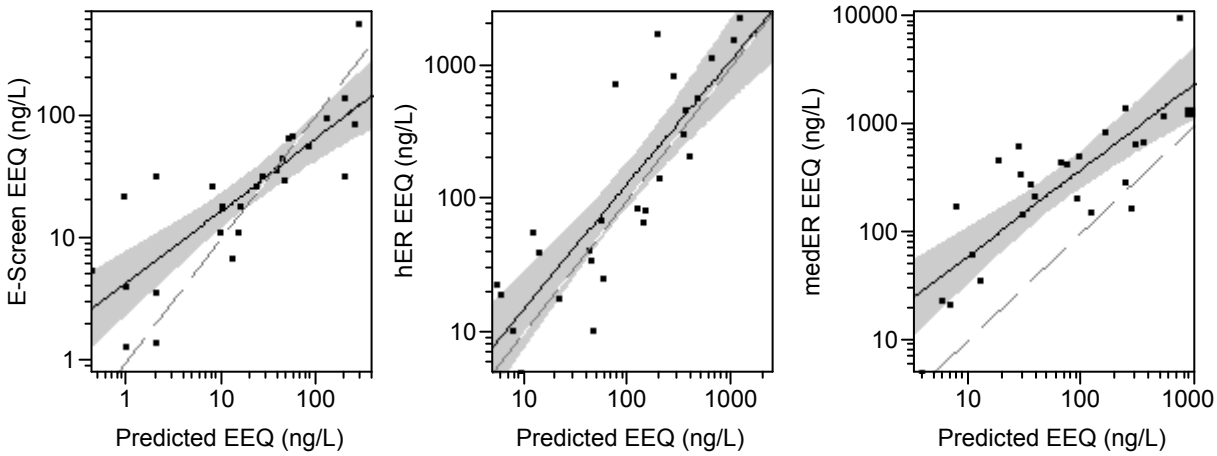


Figure 6.3: Comparison of EEQ measured in assay to that predicted from chemical analyses and relative potencies for each steroid in the assay. Shaded area represents 95% confidence interval around the regression line. Dotted line represents 1:1 regression between predicted and measured EEQ.

There are several potential reasons for the difference between predicted and measured estrogenic activity in the medER assay. The first is the method used to calculate predicted concentrations. The toxicity equivalence approach used only produces reliable estimates when dose-response curves for individual chemicals are parallel in the assay. When this is not the case, a different model, such as the concentration addition model, may be more appropriate (137,214).

A second explanation is the presence of unmeasured estrogenic compounds in the samples, as chemical analysis is targeted at specific compounds, in this case steroid estrogens. The medER assay is substantially more responsive (RP > 100-fold higher) to anthropogenic compounds such as nonyl- and octylphenols and bisphenol A, and to phytoestrogens such as genistein, when compared to the hER assay (Table 6.1). The higher EEQs measured in the medER assay, coupled with the discrepancy between predicted and measured concentrations, suggests that anthropogenic compounds or phytoestrogens may be present in the samples.

Bisphenol A, 4-nonylphenol and other nonylphenols were detected in some samples; however, their contribution to overall estrogenic activity of the sample would be low (<0.5 ng/L), even in the medER assay where they are more potent. Although phytoestrogens were not measured in this study, equol has previously been identified as a contributor to estrogenic activity in a hog manure sample (74) and the mycoestrogen

zearalenone has been measured in cattle manures (162,163). If these or similar compounds are present in the DSE at concentrations high enough to cause a response, this may account for the systematic difference between predicted and measured activities for the medER.

The relative contribution of each of the measured steroid estrogens was assessed for each effluent sample and the mean is shown in Figure 6.4. This indicates that for the E-Screen, the calculated EEQ is dominated by 17α-E2 and 17β-E2, whereas for both yeast assays, E1 and 17α-E2 dominate and 17β-E2 is minor (<20%). The difference between assays is due to the higher relative potency of E1 and 17α-E2 in the yeast assays compared to the E-Screen.

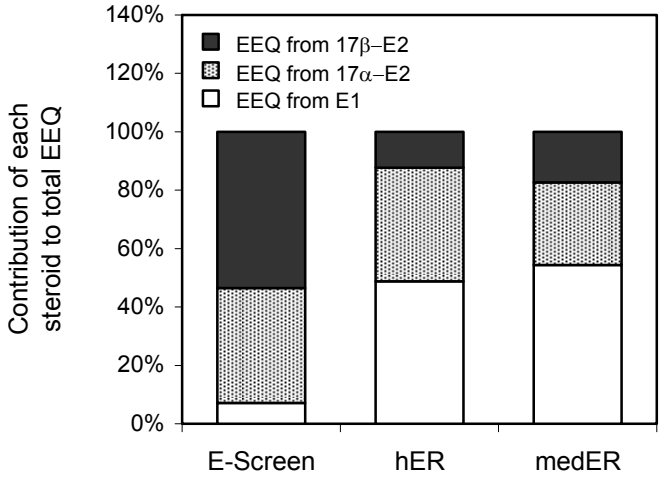


Figure 6.4: Relative contribution of individual steroid estrogens to total calculated EEQ (median % of 26 DSE samples).

Despite the lower relative potency of 17α-E2 (0.020), the concentrations in effluent samples are substantially higher than those of 17β-E2. The mean contribution was 40% of total EEQ in the E-Screen (range 0–71%), 35% in the hER (9-69%) and 25% in the medER (6-57%). The contribution of E1 was much higher in the hER (mean 53%, range 15-7%) and medER assays (56%, 18-86%) than the E-Screen (12%, 1.6-39%). This indicates that both 17α-E2 and E1 are environmentally important when assessing the overall estrogenic potential of dairy wastes.

The results presented in Figure 6.1 demonstrate that differences in relative potency of estrogenic compounds in *in vitro* assays lead to substantial differences in the measured

estrogenic activity of samples containing mixtures. The relative potency of estrogens in *in vitro* assays can also be substantially different to those measured *in vivo* (210,211,215), where chemical uptake, induction of binding compounds such as sex hormone binding globulins, metabolism and inactivation can increase or decrease the potential estrogenic effect (67). The relative potency of E1 *in vivo* is 0.2-0.3 (132), closer to the potency in the yeast assays (0.67-0.73) than the E-Screen (0.024). This suggests that the E-Screen assay may under-estimate, and the hER and medER assays may over-estimate, the potential effect of the dairy effluents *in vivo*. There is no published information on the *in vivo* potency of 17α-E2 and therefore it is not known whether its potential effects would be better predicted by either the E-Screen or the hER and medER assays.

At this stage it is not known whether the presence of 17α-E2 in the DSE would be of significance in terms of adverse effects in exposed biota. Comparisons of the estrogenicity of individual estrogenic compounds *in vitro* and *in vivo* indicate that *in vitro* tests may underestimate potential effects, as the higher level of biological organisation is associated with higher complexity (210,211,215). Predictions of *in vivo* effects using information from *in vitro* assays must therefore be done with caution.

6.4 CONCLUSIONS

Bioassays are an important tool in assessing potential sources of estrogenic activity at least at a qualitative level when working with samples from complex matrices such as DSE. Analysis of DSE samples using the two-hybrid yeast assays confirmed the estrogenicity as measured by E-Screen assay, and indicated estrogenic activity in the samples was mediated through the estrogen receptor.

The E-Screen assay was more sensitive than the two-hybrid yeast assays indicating the advantage of this assay in analysing samples with low estrogenic activity. When samples contain cytotoxic compounds, the two-hybrid yeast assays offer an advantage over the E-Screen. Firstly, yeast-based assays tend to be less sensitive to cytotoxic compounds than the mammalian cells. Secondly, the quantitation method used for the two-hybrid yeast assay is not affected by cytotoxicity at higher doses, while the E-Screen can be.

The estrogen equivalents determined by E-Screen were much lower for than those measured by hER or medER assays. For hER this could be mostly explained by the higher relative potency of E1 and 17α-E2 in that assay compared to E-Screen. Measured values and those calculated from chemical analysis were in general agreement for E-Screen and hER, but not for medER. Much higher EEQs measured in the medER assay may be due to the presence of additional estrogenic compounds in the samples, potentially phytoestrogens or mycoestrogens. The E-Screen assay may underestimate the potential effect of the DSE in aquatic environments. Proper risk characterisation of the presence of estrogenic compounds in the environment should include an *in vivo* component to assess effects at higher levels of biological organisation.

CHAPTER SEVEN

INVESTIGATION OF STEROID ESTROGENS AND ESTROGENIC ACTIVITY IN AQUATIC RECEIVING ENVIRONMENTS OF DAIRY CATCHMENTS

Chapter 7: Investigation of Steroid Estrogens and Estrogenic Activity in Aquatic Receiving Environments of Dairy Catchments

7.1 INTRODUCTION

Estrogenic contamination of waterways is of considerable concern, with numerous studies indicating adverse effects on aquatic life, particularly freshwater fish (216). Field studies demonstrate that the effects are widespread, ranging from increased vitellogenin in male fish in the US (215), Germany (201) and Japan (217) to intersex development in fish in UK rivers (2,6). The effects are most clearly demonstrated downstream of sewage treatment plant effluents, where natural and synthetic estrogens have been established as causative compounds (9,10), producing effects at concentrations as low as 10-100 ng/L (reviewed in (132)).

Recent research, including this thesis, has demonstrated the high concentrations of steroid hormones and estrogenic activity in agricultural wastes (e.g., (39,40,43,44)). Further, steroids have been measured in streams and ponds receiving discharges of treated agricultural effluents (127,218), in runoff from fields after application of animal wastes (100,219-221), in streams draining fields with stock grazing (128,222) and where stock have access to streams (128). Groundwater contamination has also been reported adjacent to dairy wastewater ponds (127) and in agricultural areas (223). Steroids from agricultural sources have also been linked to demasculinisation of male and defeminisation of female fathead minnow (224) and vitellogenin induction in painted turtles (225).

In New Zealand, it is widely recognised that agricultural land use (226,227) and dairy farming in particular (228) has an adverse effect on stream water and groundwater quality. Despite this, endocrine disrupters, and steroids in particular, have not yet been investigated in agricultural waterways. Dairy farming practices in New Zealand could lead to estrogenic contamination of stream waters through several routes: stock having direct access to waterways, particularly minor headwater streams; DSE is discharged

directly to waterways after minimal treatment in many regions; and inappropriate land application of effluent can result in contaminants leaching into groundwater or being transported to surface waters (228-230).

This chapter presents the results of investigations into steroid estrogens in streams within dairy catchments. Firstly, samples were collected to measure estrogen concentrations and activity from streams and groundwater in three catchments where DSE had been sampled. One of the reasons for including these catchments in the DSE study was the willingness of farmers to allow sampling of their effluent and provide general information about their farm, owing to a good prior relationship between the farmers and scientists and regulators. Because of the efforts by farmers in these catchments to reduce the impact of their farm effluents on waterways, and their high level of environmental awareness, it was recognised that results from these stream catchments may not reflect conditions predominating throughout each region.

Secondly, the potential for groundwater contamination was further investigated in an intensive dairying catchment located above a shallow unconfined aquifer. This catchment uses border-dyke irrigation, a method involving the regular flooding of paddocks during dry periods. Because water is applied unevenly with this method, there is potential for significant macropore or bypass flow where cracks and other macropores exist in the soil profile (231). This bypass flow decreases filtration and the retention time in soil and vadose zones. Previous studies in this catchment indicated groundwater was contaminated with pathogens and bacteria originating from dairy farming (231). There is also potential for steroid estrogens from dairy effluents to be transported to the aquifer, thereby resulting in groundwater contamination.

Thirdly, a wider survey was undertaken in the Waikato Region to investigate the extent of estrogen contamination from treated effluents discharged to waterways. Sampling sites were selected from information supplied by Environment Waikato regarding streams thought to be heavily impacted by dairy farming (R. Wightman, pers. comm.) and from maps of dairying in the region. Sites were region-wide and included streams receiving water discharges of treated effluent, and those with few or no water discharges but having numerous discharges to land in the catchment. Three rivers draining

predominantly dairy catchments were also investigated to determine whether estrogenic steroids in dairy effluents had an effect on water quality on a regional scale.

7.2 METHODS

7.2.1 Sampling Sites

Catchment Survey

Samples were collected twice (in August 2006 and May 2007) from several locations in the Toenepi Stream including two tributuaries (Figure 7.1). Samples were collected once each from the Waikuku Stream and Pahau River (March and April 2007, Figures 7.2 and 7.3). Groundwater samples were also collected in the Pahau River catchment in March 2008 (Figure 7.3), and an additional sample was collected from the Pahau River at this time.

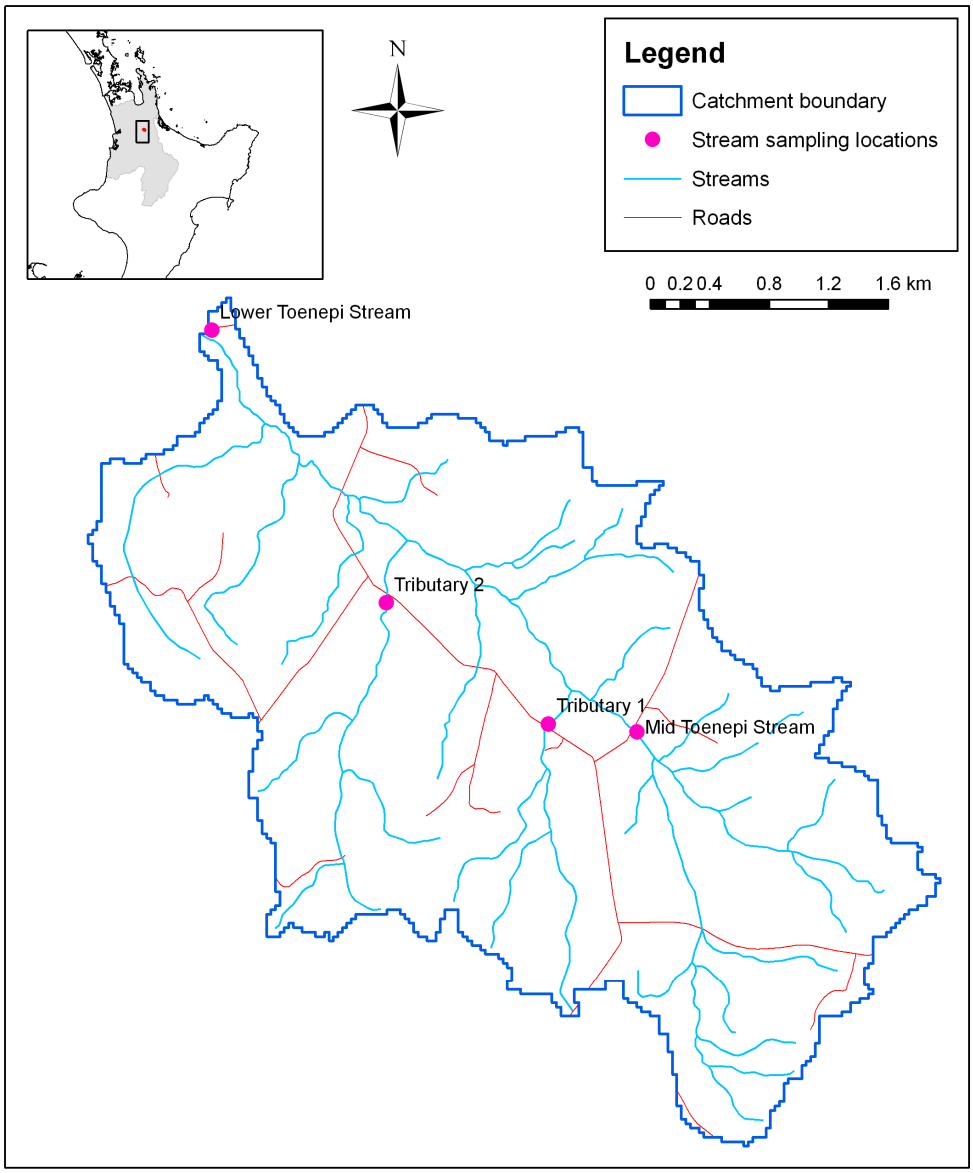


Figure 7.1: Sampling locations in the Toenepi catchment.

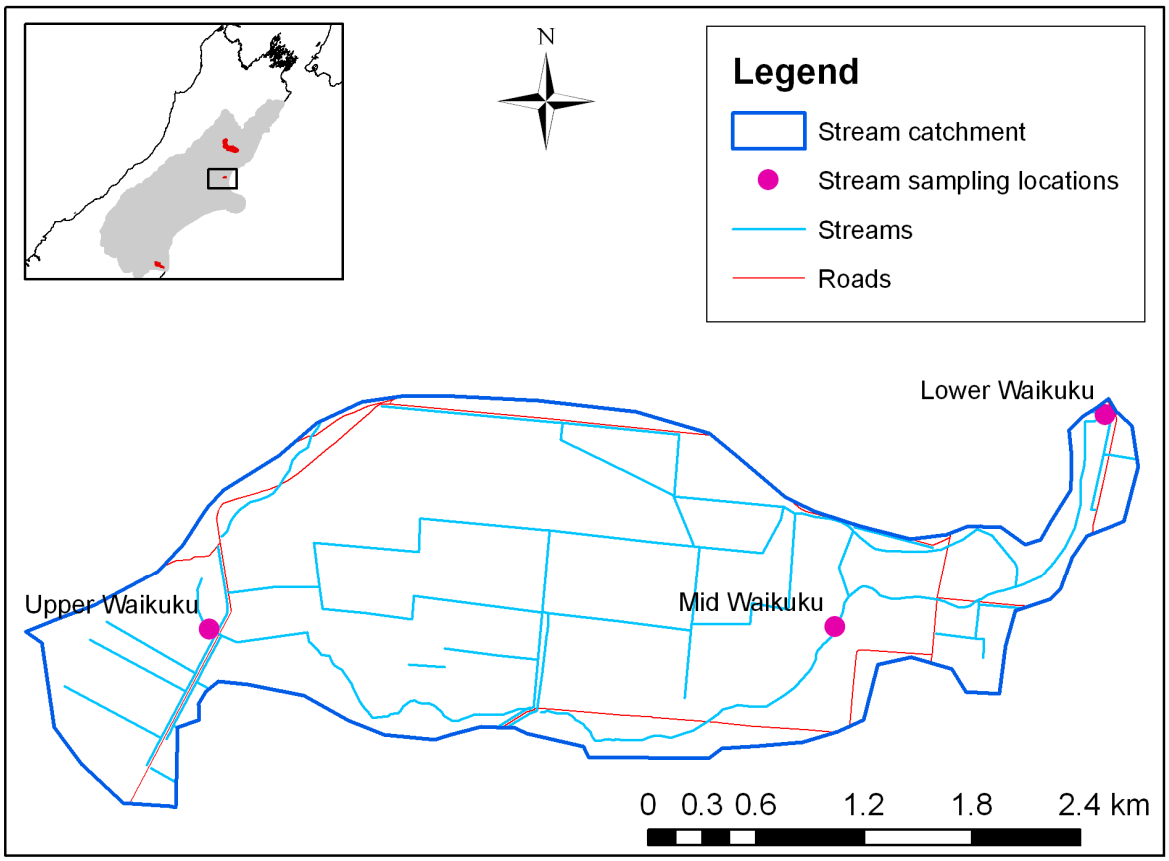


Figure 7.2: Sampling locations in the Waikuku catchment.

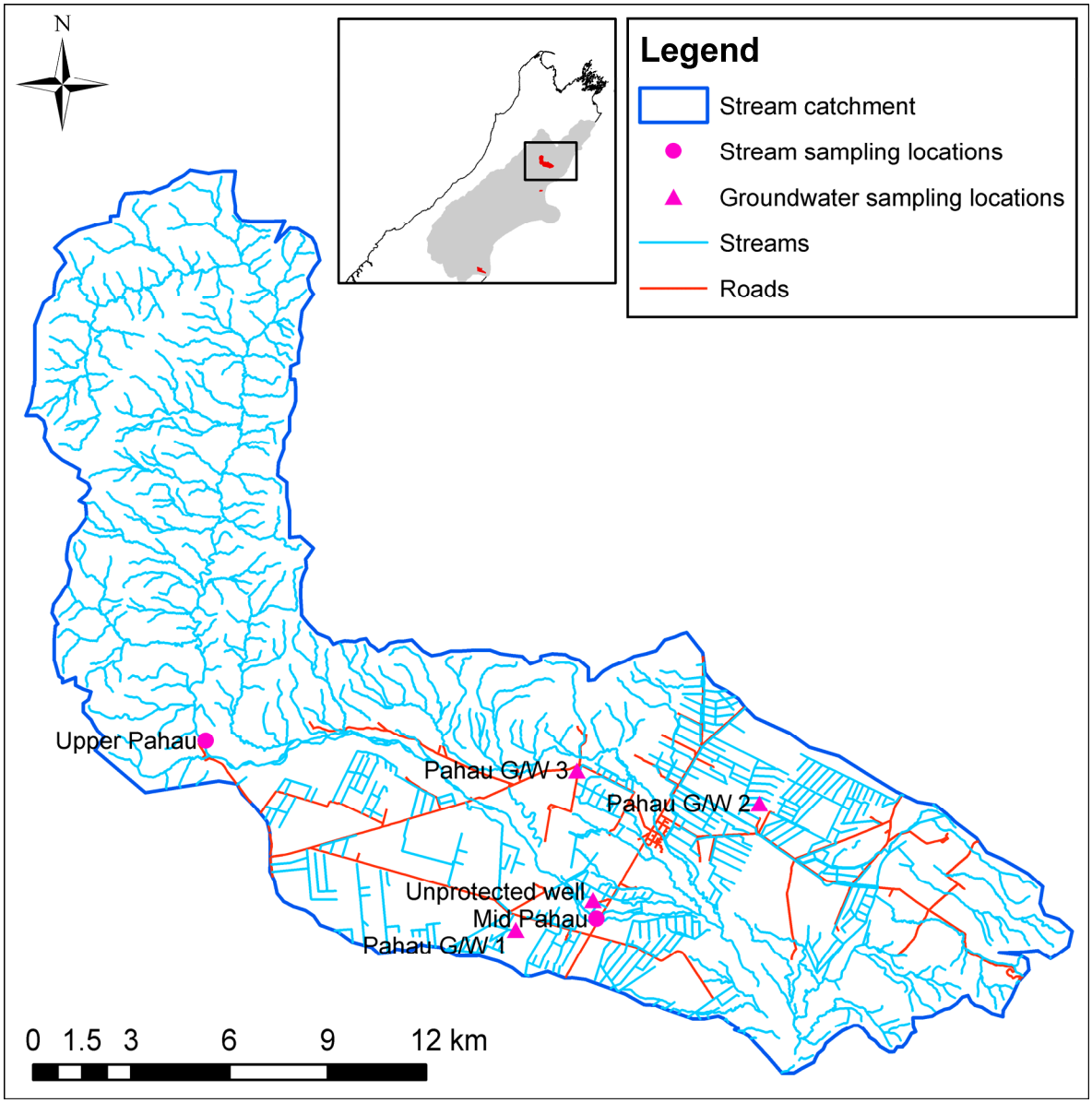


Figure 7.3: Sampling locations in the Pahau catchment.

Waikakahi Border-Dyke Irrigation Catchment

Sampling was conducted during late summer, when irrigation is at its peak. Because the irrigation water is sourced from a nearby river, stream flows are high in summer due to the additional input from irrigation. Samples were collected from five groundwater wells, where groundwater depths were 1.2-7.3 m below ground level. Samples were also collected from three locations along the length of the Waikakahi Stream (Figure 7.4).

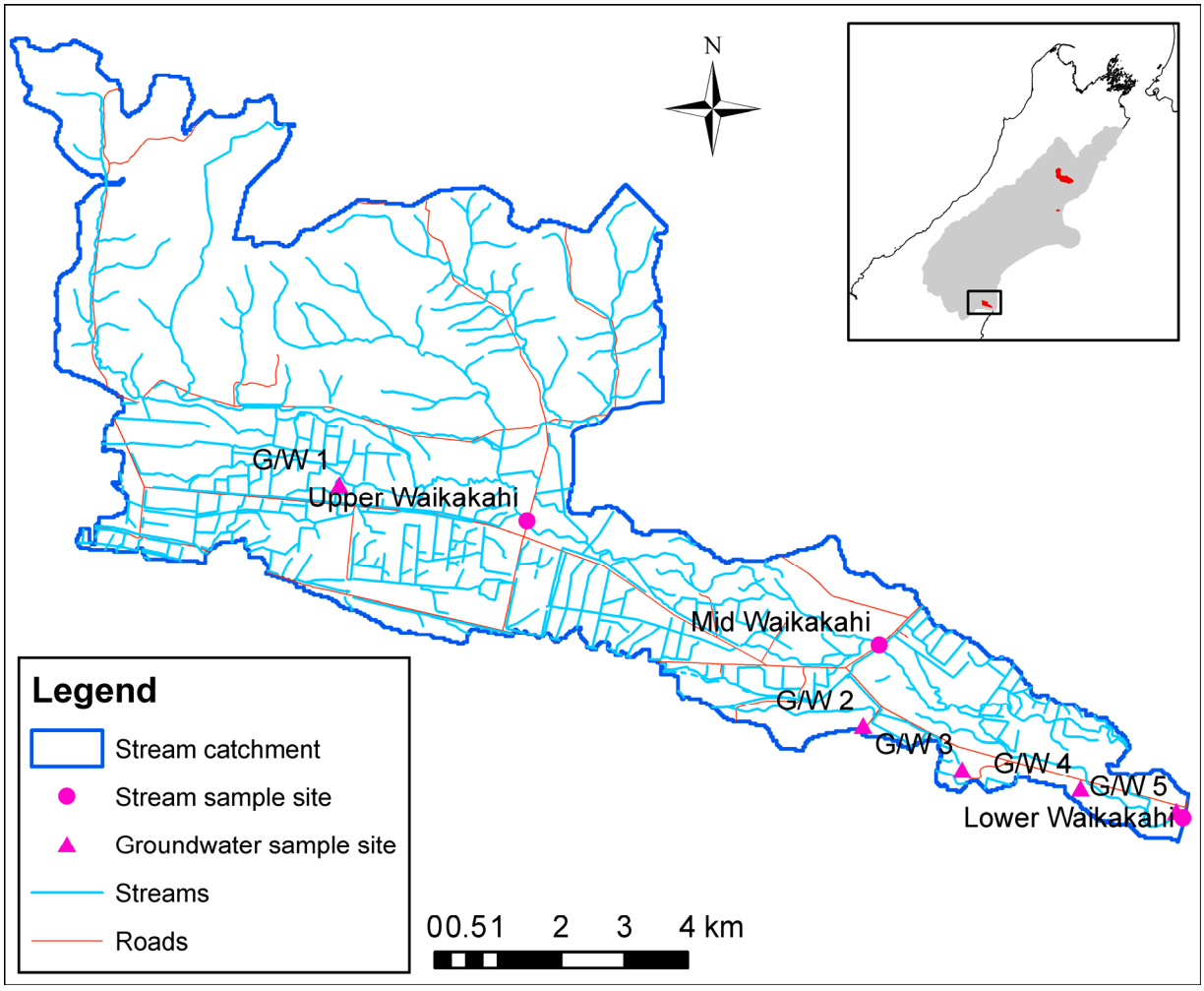


Figure 7.4: Sampling locations in the Waikakahi catchment.

Waikato Regional Survey

Samples were collected from 12 streams distributed throughout the Waikato Region to provide further information on the effect of effluent discharges. Sampling was conducted during summer baseflow conditions to ensure that the influence of effluent discharges dominated through reduced potential for runoff from diffuse sources of estrogens (i.e. from urine and faeces deposited during grazing). In addition, at this time there was lower dilution of effluent directly discharged into waterways for most streams. Streams were located throughout the region (Figure 7.5). Specific details on each stream are provided in Appendix B.

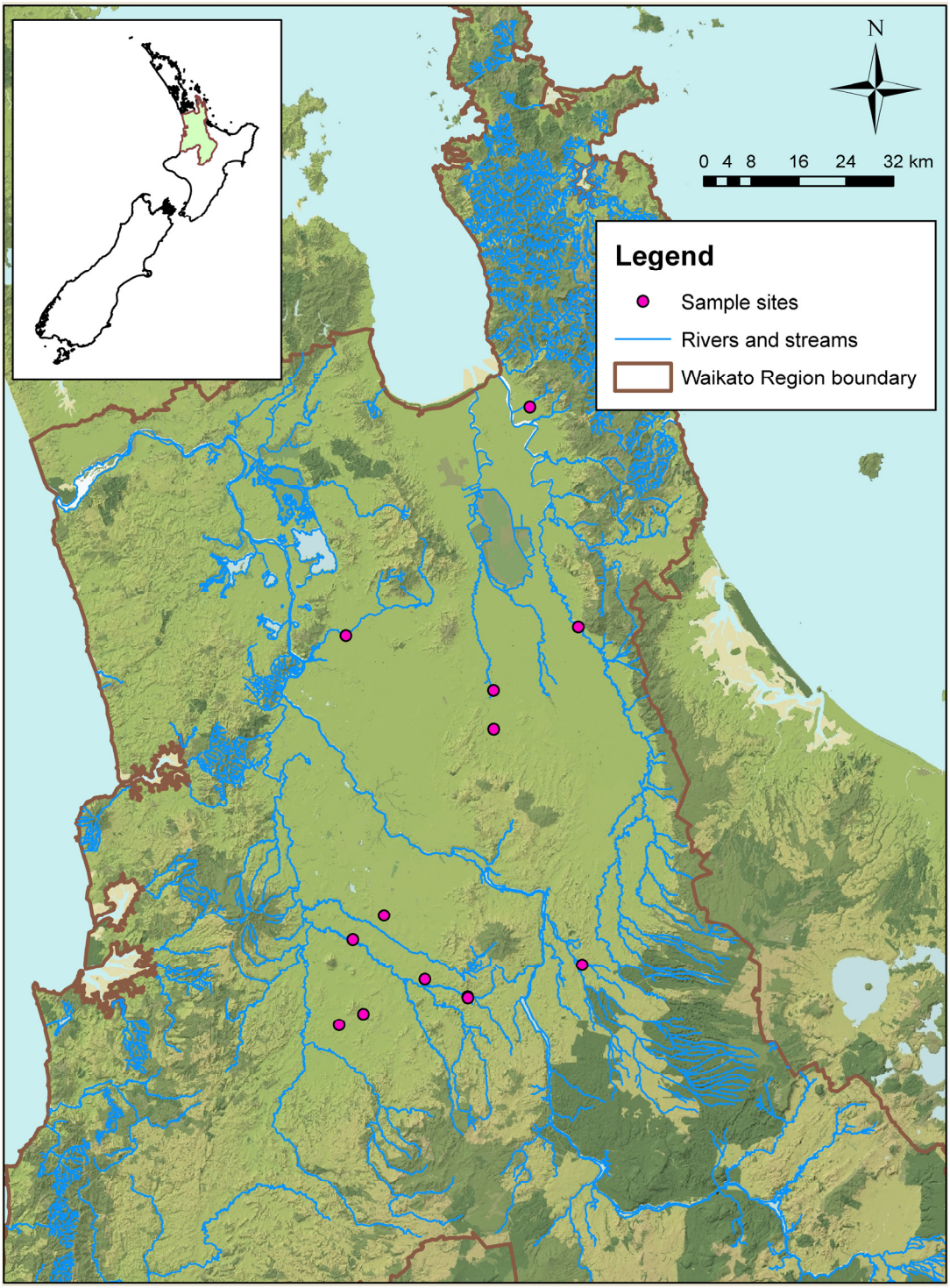


Figure 7.5: Sampling locations around the Waikato Region.

7.2.2 Sample Collection and Extraction

Grab samples were collected from below the water surface in the middle of the stream where possible, or within a flowing section. Groundwater samples were collected after purging the wells for at least three well volumes and once conductivity and pH measurements had stabilised.

Water samples were collected in solvent- and Milli-Q water (MQ)- rinsed 4 L amber glass bottles with Teflon-lined lids. Samples were immediately preserved by the addition of sulfuric acid (to pH ≤ 2) and transported on ice to the laboratory. Sample processing began immediately on return to the laboratory and extraction was completed within 36 hours. Samples were stored at < 4 °C during this period where necessary.

Samples were filtered through glass fibre filters (GF/C, pore size 1.2 μm Whatman). Filtered samples were extracted in duplicate (2 L each) for i) chemical analysis of steroid estrogens, and ii) estrogenic activity using the E-screen assay. Samples for chemical analysis were spiked with surrogate standards (100 ng each E1-d_4, 17β-E2-d_4, E1-3S-d_4 and 17β-E2-3S-d_4). In the spring 2006 sampling, samples were spiked only with 17β-E2-d_4 (250 ng). All samples were loaded onto 500 mg Oasis HLB cartridges (preconditioned with 10 mL of methanol followed by 10 mL of MQ water) at a flow rate of 5-10 mL/min.

7.2.3 Chemical Analysis

Samples for chemical analysis were eluted from Oasis HLB cartridges in two fractions to separate conjugated estrogens and free estrogens as described in detail in Chapter 3 and summarized briefly here. Cartridges were washed with methanol water mixtures then free estrogens were eluted with 30 ml of DCM: DEE: MeOH (40:10:1). TFAA was added to the extract to derivatise estrogens to their trifluoroacetate derivatives for GC-MS analysis in SIM/Scan mode (as fully described in Chapter 3).

Data collected in SIM mode was used for quantitation of the steroid estrogens E1, 17α-E2 and 17β-E2. These were quantified by isotope dilution, based upon the relative response factors for the following pairings: 17α-E2/17β-E2-d_4, 17β-E2/17β-E2-d_4, E3/E2-d_4, E1/E1-d_4.

For the initial samples collected in the Toenepi catchment (spring 2006), estrogens were not quantified by isotope dilution as described above. Estrogens were quantified against E2-17Ac which was added to the purified sample extracts as an internal standard. Estrogens were recovery corrected against the concentration of the surrogate standard 17β-E2-d_4 that was added to raw samples and subsequently measured.

7.2.4 E-Screen Analysis

Samples for analysis by E-screen assay were eluted from the Oasis HLB cartridges with 30 ml of DCM: DEE: MeOH (40:10:1), dried down to approximately 1 mL and solvent exchanged into DMSO (250 µL for samples collected 2006-2007, 50 µL for 2008). Samples were stored at <20°C in the dark until analysed by E-screen assay.

The E-Screen assay was performed as described in Chapter 4. EEQs were calculated from EC50 [17β-E2, ng/L] / EC50 [sample]. The quantitation limit ranged from 0.012-0.20 ng/L depending on sample volume, concentration factors and cytotoxicity in the assay. Samples were considered below detection where the maximum proliferation was less than 20% of the maximum proliferation of the standard curve and where the calculated EC50 was not within the range of actual measurements. Method quality control procedures for the E-Screen assay included analysing a standard and a duplicate sample for each 10 samples; and performing two independent assays for each sample (with the average value reported).

7.2.5 Quality Assurance and Quality Control

Quality assurance and quality control procedures included analysing a 2 L MQ blank and MQ spike (12.5 ng/L) for each sampling event. No steroids were detected in the blank samples. Recovery of the MQ spikes ranged from 77-105% for the Waikato sampling. Spike recovery was lower for samples collected in Waikakahi and Pahau catchments at 55-73%.

7.2.6 Statistical Methods

Regression analysis was used to investigate relationships between estrogenic activity and catchment characteristics. Dairy land use, number of DSE discharges to water and land and the number of stock in each catchment were provided by Environment Waikato (D. Borman, pers. comm.). These data were normalized to the catchment area of each stream. Best subsets linear regression analysis was undertaken using the statistical software package Minitab v15.1.0.

7.3 RESULTS AND DISCUSSION

7.3.1 Catchment Sampling

Samples from the Toenepi, Waikuku and Pahau Streams indicated low to very low concentrations of steroid estrogens and estrogenic activity (Table 7.1). E1 was the only steroid estrogen detected in the water samples, and was identified in 13 out of 18 stream and groundwater samples at concentrations up to 3.3 ng/L. Despite detection limits of ~0.1 ng/L for 17α-E2 and 17β-E2 (based on a signal:noise ratio of 3:1), these compounds were not detected in any stream or groundwater samples.

Eleven of 18 samples tested elicited an estrogenic response as measured by the E-Screen assay. All samples collected from the Toenepi catchment in spring 2006 had measurable activity, consistent with the detection of E1 in these samples. All samples from the Waikuku Stream and the Pahau River demonstrated estrogenic activity by E-Screen assay in Autumn 2007 sampling, while only one sample from the Toenepi Stream or its tributaries was above the quantitation limit.

Table 7.1: Estrogenic activity and steroid estrogen concentrations in stream and groundwater samples from the Toenepi, Waikuku and Pahau catchments.

	Spring 2006		Autumn 2007		Summer 2008	
Stream / Well	**EEQ (ng/L)**	**E1 (ng/L)**	**EEQ (ng/L)**	**E1 (ng/L)**	**EEQ (ng/L)**	**E1 (ng/L)**
Toenepi						
Mid Toenepi Stream	0.88	1.7	0.25	ND [a]	NM	NM
Lower Toenepi Stream	0.28	1.4	ND	ND	NM	NM
Tributary 1	0.38	3.3	ND	ND	NM	NM
Tributary 2	0.31	0.38	ND	0.9	NM	NM
Waikuku						
Upper Waikuku Stream	NM	NM [b]	0.08	ND	NM	NM
Mid Waikuku Stream	NM	NM	0.10	ND	NM	NM
Lower Waikuku Stream	NM	NM	0.07	0.37	NM	NM
Pahau						
Upper Pahau River	NM	NM	0.32	1.4	NM	NM
Mid Pahau River	NM	NM	0.20	0.40	ND	ND
Unprotected g/w well	NM	NM	0.04	ND	NM	NM
Groundwater well 1	NM	NM	NM	NM	ND	ND
Groundwater well 2	NM	NM	NM	NM	ND	ND
Groundwater well 3	NM	NM	NM	NM	ND	ND

Notes: [a] ND = Not detected, less than detection limit of 0.1-0.2 ng/L for E1 and 0.01-0.04 ng/L for EEQ. [b] NM = Not measured.

There was some estrogenic activity (0.04 ng/L) in a shallow groundwater sample collected from an unprotected well adjacent to a dairy shed. This activity was not confirmed in further sampling of groundwater from three wells in the catchment. All steroids were below detection limits and no estrogenic activity was measured by the E-Screen assay at a quantitation limit of 0.02 ng/L. The initial groundwater sample is not expected to reflect groundwater concentrations throughout the catchment as the well was located adjacent to a dairy shed, was not covered and was not purged prior to sampling. Later samples were from protected wells, purged for three well volumes prior to sampling, according to standard groundwater sampling protocols.

Overall there were several unexpected findings in these results. Firstly, the highest concentration measured from the Pahau River was in a sample from a site upstream of all the dairy farms. The upper catchment is predominantly used for grazing by dry stock (beef) and sheep, which can also be sources of estrogen (128). An alternative source may be leachate from septic tanks (232) or waterfowl activity. The results suggest that rural streams with other land use in the catchment may also be at risk of contamination with estrogens.

Secondly, the highest E1 concentration measured (3.3 ng/L) was from a sample collected from a tributary of the Toenepi Stream that had no consented water discharges of treated dairy effluent. Further, higher concentrations (0.38-3.3 ng/L) were measured in samples collected under high flows (391 L/s, spring 2006) in the Toenepi Stream than under low flows (ND-0.9 ng/L, flow of 13 L/s, autumn 2007).

The singular detection of E1 in all samples is likely due to the persistence of E1 relative to 17β-E2, and possibly 17α-E2. Similarly only E1 was detected in waterways in the Netherlands and the U.S. (128,166). The higher concentrations during high stream flows suggest that overland runoff and sub-surface flows transport steroid estrogens to the river. This is supported by the detection of estrone in the tributary with no direct discharges of dairy effluent into the waterway. Steroid estrogens may be sourced from land applications of effluent, from estrogens bound to soil from prior effluent applications or from diffusely deposited manure.

During wet weather increased discharge volumes from treatment ponds may increase the concentrations of estrogenic steroids in the Toenepi main-stem. Although effluent treatment ponds should exclude rainwater from the surrounding farmland or dairy shed (152), not all systems are rigorously designed. During wet periods, increased flow into the ponds results in increased discharge volumes and decreased treatment efficiency due to reduced retention time.

7.3.2 Border-Dyke Irrigation Catchment

Samples collected from the Waikakahi Stream and five groundwater wells in the catchment demonstrated the presence of steroid estrogens and estrogenic activity in the waterways (Table 7.2). All samples elicited an estrogenic response in the E-Screen assay, with EEQs of 0.05-0.34 ng/L (Table 7.2). All three steroids were detected in stream samples, contrasting with the results obtained for the stream samples from the other three catchments. E1 concentrations were highest, measuring 0.61-1.2 ng/L, followed by 17α-E2 (0.21-0.68 ng/L) and 17β-E2 (0.12-0.16 ng/L).

Table 7.2: Estrogenic activity and steroid estrogen concentrations in streams sampled from the Waikakahi catchment.

Stream / Well	EEQ (ng/L)	Steroid estrogen concentrations (ng/L)			
		E1	17α-E2	17β-E2	Total estrogens
Groundwater well 1	0.07	ND [a]	ND	ND	ND
Groundwater well 2	0.08	ND	ND	ND	ND
Groundwater well 3	0.07	ND	ND	ND	ND
Groundwater well 4	0.15	ND	ND	ND	ND
Groundwater well 5	0.05	ND	0.21	ND	0.21
Waikakahi Stream 1	0.10	0.61	0.22	0.12	0.95
Waikakahi Stream 2	0.34	1.2	0.26	0.12	1.5
Waikakahi Stream 3	0.15	0.72	0.68	0.16	1.6

Notes: [a] ND = Not detected, less than detection limit of 0.1-0.2 ng/L for E1 and 0.1 for 17α-E2 and 17β-E2.

Interestingly, 17α-E2 was detected in one groundwater sample. This well was downgradient of most dairy farms but significantly, the neighbouring (and immediately upgradient) farm has swine manure spread on it. Application of piggery manure has been shown to result in leaching of E1 and 17β-E2 into tile drains (233). Although swine do not excrete 17α-E2, it has been detected in lagoons treating swine wastes (39,43) and it may be produced from 17β-E2 and E1 under anaerobic conditions (193), suggesting that the 17α-E2 may have originated from the piggery wastes.

The remaining groundwater well and stream samples were collected upgradient and upstream of this manure spreading operation, indicating that it was not the sole source of steroid estrogens and estrogenic activity in the catchment. Colloidal facilitated transport is thought to enhance leaching of steroids through soil, resulting in environmental concentrations above those predicted by soil sorption experiments (234). As dairy effluents contain high concentrations of dissolved and colloidal organic carbon, this is likely to enhance leaching of estrogens into groundwater and stream water through sub-surface flows.

7.3.3 Waikato Regional Survey

Eleven of the 12 streams demonstrated estrogenic activity by E-Screen assay at 0.02 to 1.44 ng/L (Table 7.3). Steroid estrogens were detected at trace concentrations in five samples. E1 and 17α-E2 were the most frequently detected (each in 3 samples), with 17β-E2 also detected in one sample. Estrone concentrations were somewhat higher than 17α-E2 and 17β-E2, ranging from 0.34 to 4.2 ng/L, while 17α-E2 measured 0.12 to 0.31 ng/L and 17β-

E2 0.58 ng/L. The presence of 17β-E2 in water samples suggests that the steroid estrogens may have been recently excreted, as 17β-E2 is less persistent than E1 in the aquatic environment (195).

Table 7.3: Estrogenic activity and steroid estrogen concentrations in streams sampled from Waikato.

Stream	EEQ (ng/L)	Steroid estrogen concentration (ng/L)			
		E1	17α-E2	17β-E2	Total estrogens
Mangakawaru Stream	0.35	ND [a]	ND	ND	ND
Mangaorongo Stream	0.15	ND	ND	ND	ND
Mangapiko Stream	0.40	ND	ND	ND	ND
Matatoki Stream	0.18	ND	0.12	ND	0.12
Mellsops Drain	0.16	ND	ND	ND	ND
Owairaka Stream	0.19	ND	0.15	ND	0.15
Owairaka tributary	0.29	ND	ND	ND	ND
Piako River	0.28	0.65	ND	ND	0.65
Pokaiwhenua	ND	0.34	ND	ND	0.34
Puniu River	0.05	ND	ND	ND	ND
Toenepi Stream	NM [b]	0.48	ND	ND	0.48
Waihou River	0.02	ND	ND	ND	ND
Waihuka Stream	1.44	4.2	0.31	0.58	5.1

Notes: [a] ND = Not detected, less than detection limit of 0.1-0.2 ng/L for E1, 0.1 for 17α-E2 and 17β-E2 and 0.01 ng/L for EEQ. [b] NM = Not measured.

Highest estrogenic activity and steroid concentrations were measured in the Waihuka Stream. This is a small stream (less than 1 m wide) receiving a single discharge of treated DSE. The second highest concentration of total estrogens was measured in the Piako River. In addition to receiving dairy effluent discharges throughout its catchment, the Piako River also receives discharge from the Morrinsville township sewage treatment plant, servicing a population of ~6000 people.

7.3.4 Comparison to Literature Values

This is the first study detecting estrogenic activity within waterways in New Zealand using an *in vitro* assay and chemical analysis. A previous study on the Waikato River did not find evidence of estrogenic activity, though that study was somewhat limited by the small number (3) of river samples analysed and by methodological issues (235). The Waikato River is a very large river by New Zealand standards, with a median flow in Hamilton of 321 m^3/s (236), at least ten-fold greater than the waterways examined in the current study (median flows of a few L/s to 32 m^3/s). The greater flow therefore provides

much greater dilution of any estrogens entering it from STP effluents and DSE discharges when compared to the small streams and rivers examined in this study.

Estrogenic activities measured in the streams from the dairy catchments were similar to values reported in international literature for streams impacted by agricultural activities, and particularly dairy farming. In this study, 24 of 29 stream samples demonstrated estrogenic activity in the E-Screen assay. The mean EEQ was 0.32 ng/L, with a maximum of 1.4 ng/L. This is comparable with values up to ~1 ng/L (E-Screen assay) in a drain receiving effluent from a beef cattle feedlot (218), up to 2.41 and 1.75 ng/L (YES and ER-CALUX assays respectively) measured in streams in dairy catchments in Australia (45), and up to 1.1 ng/L (YES assay) in Denmark in drains from fields where cattle and pig manure was applied (64). Estrogenic activity (YES assay) was measurable in 25 of 130 samples collected from streams and lakes in animal husbandry catchments (cattle and pigs) in Denmark, with values typically between 1 and 2 ng/L but up to 8.8 ng/L (64). The EEQ measured in drains from cattle breeding areas in the Netherlands were lower, with a maximum of 0.2 ng/L (ER-CALUX) (166). Conversely, higher average EEQs (average 1.4 ng/L, YES assay) were estimated in streams draining mainly dairy farms in the United Kingdom, using passive samplers (222) deployed for an average of 39 days. These samplers integrate concentrations over the deployment period and would incorporate any transient peaks in concentration due to storm runoff, or cattle excreting directly into the streams, potentially leading to overall higher values compared to grab samples. Estrogenic activities measured in the current study and internationally in streams in dairy catchments, are similar to those measured in streams and rivers receiving STP discharges (68), though maximum values are often higher downstream of STPs (5-20 ng/L, (68,237)).

In this study, E1 was measured above the quantitation limit in 15 of 30 samples, while 17α-E2 was measurable in 6 and 17β-E2 in 4 samples. Concentrations were typically less than 1 ng/L, lower than expected based on the measured EEQs. However, the data is within the range reported for rural streams in dairy catchments in North America (127,128,218), the Netherlands (166) and Denmark (64,233). Williams et al. (45) reported substantially higher concentrations of E1 and 17β-E2 in streams draining stock grazing and dairy land use in Australia, including a stream receiving wash-down water from a

dairy shed, drains collecting irrigation runoff from dairy operations, and streams where dairy cattle had access. Using ELISA, they measured E1 concentrations from 1.57 to 18.71 ng/L and 17β-E2 from 0.81 to 3.37 ng/L. E1 and 17β-E2 concentrations were higher in these dairy-impacted streams than in streams in areas where sheep and beef cattle grazed. Results obtained by ELISA need to be treated with caution as the presence of natural organic matter in samples can interfere with measurements, resulting in false positives (79,103) and, as stated by the authors, these results need to be confirmed with a more selective analytical measurement (45). Despite this, they do highlight the greater potential for estrogen contamination in dairy catchments compared to other agricultural uses.

In the current study, estrogenic activity was low in groundwater and steroid estrogens were detected in only one sample, indicating that the steroids in dairy effluents are either strongly retained by soil and/or degraded before they reach groundwater. This contrasts with concentrations of 6-20 ng/L of 17β-E2 measured in karst aquifer springs in Arkansas under baseflow conditions and up to 66 ng/L following recharge (223). These springs also contained *E. coli* measured at 10^2 to 10^4 cfu/100 mL, indicating contamination from animal effluents and/or wastes. Although *E. coli* was not measured in this study, previous measurements of groundwater from these wells indicated values typically $<10^1$ to 10^3 MPN/100 mL (231), suggesting that, although vulnerable, this groundwater is at less risk to contamination from animal wastes applied to soil than the afore-mentioned karst aquifers.

7.3.5 Sources and Routes of Estrogen Contamination

A linear regression analysis was undertaken to examine predictors of estrogenic activity in the streams from the Waikato Region. The Piako River was excluded from this analysis, due to the input of treated sewage upstream of the sampling location. The regression analysis indicated that the normalised number of DSE discharges to water could predict 64% of the variability in EEQ. The inclusion of the number of cattle within the catchment improved the fit of the model, accounting for a further 11% of the variability. Discharges of DSE to land, and the area of land in dairy land use, were not important in predicting EEQ, (each contributing < 1% variability).

Stock numbers were used to examine the potential influence of other stock on estrogenic activity in the Waikato Streams. The number of dairy cattle in the catchments of Waikato streams explained 29.5% of the variation using a best subsets regression. Pig numbers added an additional 16%, while sheep numbers added only 7%. Deer and beef cattle numbers did not appear to influence the estrogenic activity of the streams, adding only a further 1.7%. This analysis suggests that dairy cattle are the likely and predominant source of estrogens measured in the Waikato catchments.

There are several potential routes for estrogens sourced from dairy cattle to enter waterways, including direct discharges of effluent, stock access to streams and overland runoff. The relationship between estrogenic activity measured in streams and discharges of DSE to water, seen in the results of the Waikato Region survey, indicates the potential importance of this route under baseflow conditions. On the other hand, steroid estrogens and estrogenic activity were also detected in streams with no effluent water discharges, indicating that diffuse runoff is an additional route of estrogen contamination. As demonstrated in the Toenepi catchment, this route is likely to be important under wet weather conditions. Similarly, Kolodziej et al. (128) measured highest concentrations of estrone following a storm event when sampling waterways within dairy farm catchments.

The ability of stock to access the streams for watering or grazing was not quantified in this study, though it is expected that stock would have access to most, if not all streams surveyed, thereby permitting direct excretion into the water. Streams were not always fenced in the sampling locations and cattle were observed on the stream bank immediately downstream of the sampling location at one site. Even in the 'best practice catchments' stock had access to the streams: the Toenepi Stream was unfenced along 54% of its reach and the Waikakahi Stream was unfenced along 22% (238).

Recent studies indicate that leaching of steroids applied to land is likely to be greater than predicted from standardised soil sorption experiments, due to macropore transport and colloidal facilitated transport (233,234). Furthermore, degradation may be lower than experimentally predicted due to several reasons. Firstly, biological degradation of estrogens is reduced at cooler soil temperatures (189,233). Secondly, transport through the soil profile reduces exposure to microbial populations which are primarily located in the rhizosphere near the soil surface (239). Thirdly, at greater depths soils may be anaerobic

and estrogens are less readily degraded under anaerobic conditions (182). This would result in greater persistence of estrogens and increased potential for further transport into groundwater (233). These processes may have resulted in the estrogenic activity observed in the Waikakahi Stream, as this stream is largely fed by groundwater and sub-surface flows from summer irrigation.

7.3.6 Potential for Effects on Wildilife

The results indicate widespread estrogenic contamination in these waterways in dairying catchments, with estrogenic activity and/or steroid estrogens measured in 83% of streams, albeit at low levels. Freshwater fish are considered to be the most at-risk of adverse effects from steroid estrogens, and the effect of low concentrations of steroids has been investigated in many laboratory studies (132). Young et al. (132) used these studies to derive proposed Predicted No Effect Concentrations (PNECs) for 17β-E2 and E1, at 1 ng/L and 3-5 ng/L respectively.

The PNEC for 17β-E2 was not exceeded in any of the samples measured in this study, with a maximum concentration of 0.58 ng/L (Waihuka Stream). E1 was measured at a concentration above the suggested PNEC of 3 ng/L in two streams, at 4.2 ng/L in the Waihuka Stream and 3.3 ng/L in a tributary of Toenepi.

Because steroid estrogens can act additively, Young et al. (132) suggested a 'toxic equivalents' approach for assessing water samples containing mixtures, with a maximum acceptable value of 1, based on the following equation:

Equation 7.1:
$$\frac{[17\alpha\text{-}ethynylestradiol]}{0.1} + \frac{[17\beta\text{-}estradiol]}{1} + \frac{[estrone]}{3}$$

Using this equation, total estrogens in Waihuka Stream measured 1.98, exceeding the suggested maximum value of 1. Total estrogens measured 1.1 in the tributary of Toenepi Stream, also exceeding the suggested maximum value of 1. Values derived for all other streams remained below 1, but were 0.5 or above at locations in the Waikakahi and Toenepi Streams. This approach indicates potential for adverse effects in these streams in intensive dairying catchments.

A PNEC for 17α-E2 has not been developed as there is insufficient *in vivo* data. Although this is the predominant steroid in dairy wastes, it was not prevalent in the stream samples, possibly due to its lower persistence compared to E1. Concentrations were less than 1 ng/L in all stream samples and based on its lower potency compared to 17β-E2, its presence alone is unlikely to result in adverse effects. The presence of 17α-E2 would, however, increase the total exposure to steroid estrogens due to additive effects, and if incorporated into Equation 7.1, depending on the derived PNEC, may result in exceedance of a total estrogen concentration of 1.

The streams sampled in the Waikato Region are considered representative of the streams throughout the region, with flows ranging from a few L/s to 32 m^3/s. However, as each stream was sampled only once, this data can be considered no more than a snapshot of the concentration of steroid estrogen and estrogenic activity at one time. Sampling in the Waikato Region was undertaken during an exceptionally dry period, when seepage of steroids through soil can be expected to be lower than during frequent rainfall and saturated soil conditions. Although in-stream dilution would be larger under high flow conditions, sampling in the Toenepi Stream suggests this may be outweighed by the increased load of steroids entering the stream, through either overland flow, seepage or increased discharge from treatment ponds. As in-stream concentrations were close to proposed PNECs, it is likely that under wet-weather conditions the PNECs would be exceeded, suggesting potential for adverse effects in fish.

7.4 CONCLUSIONS

Estrogenic activity and steroid estrogens were widespread in the waterways studied within dairy catchments, particularly in the intensively farmed Waikato Region. Estrogenic activity was measurable in 83% of streams and in 75% of groundwater samples, albeit at low levels. E1 was the predominant steroid measured in the streams, in spite of its lower concentration in dairy effluents compared to 17α-E2, presumably due to more rapid degradation of 17α-E2 in aquatic environments.

E1 and estrogenic activity were also measured in a stream receiving a municipal sewage discharge and in streams with grazing in the catchment. This suggests that while dairy land use may be a major risk factor for estrogen pollution of waterways in rural areas, other estrogen sources should not be ignored.

Estrogenic activity and steroid concentrations were within the range measured internationally in agricultural and particularly dairying catchments. Under baseflow conditions, direct discharges of dairy effluent to waterways may be the most important source of the steroids. However, limited data collected under high flow conditions suggests that higher concentrations may be measured during periods of heavy rainfall and floods, possibly due to overland runoff of steroids in soil.

The data collected indicate potential for adverse effects on fish in two of the streams studied where the estrogen concentrations measured exceeded PNECs. These PNECs were also approached in another two streams. The results suggest that further research, including *in vivo* testing, is warranted to assess whether or not these concentrations are having an adverse effect on stream biota.

CHAPTER EIGHT

FINAL CONCLUSIONS

Chapter 8: Final Conclusions

8.1 OVERVIEW

The previous chapters have each presented the main conclusions of the research described within them. This chapter aims to integrate the findings of each chapter in a short discussion of potential implications, assess whether the research objectives have been met, present the key findings of the research, and suggest recommendations for further research.

8.2 IMPLICATIONS FOR AQUATIC BIOTA OF DSE DISCHARGES TO LAND AND WATER

8.2.1 Water

The presence of residual steroid estrogens in dairy effluents following treatment poses a risk to aquatic receiving environments. A comparison of estrogen concentrations and estrogenic activity in treated DSE and in STP effluents (Table 8.1) demonstrates the importance of these wastes as sources of estrogen pollution. 17β-E2 concentrations are typically much higher in the treated DSE than in STP effluents, with the mean concentration of 17β-E2 in the DSE samples 20-fold higher than the mean in STP effluents worldwide. The estrogenic activity of DSE was also much higher than STP effluents, except where measured using the ERBA. As discussed in Chapter 3 and 5, higher EEQ is expected when using ERBA, as this also measures antagonists.

The volume of DSE discharge to water is typically much lower than volumes of STP effluent. The farms examined in this study discharged between 8 and 15 m^3/day of treated effluent, much less than the maximum limits of 2,000-310,000 m^3/day for the NZ STP effluents. Despite the lower volume, in many cases treated DSE is discharged into minor streams or farm drains that provide low dilution. Furthermore, in catchments where dairy is the predominant land use, the cumulative effects of numerous discharges result in higher in-stream concentrations and an increase in the potential for adverse

Table 8.1: Free estrogens and estrogenic activity in dairy effluents discharging to waters compared with Sewage Treatment Plant effluents (mean ng/L (range)).

	Steroid estrogen concentrations (ng/L)			Dilution		Bioassay	
	E1	**17α-E2**	**17β-E2**	**required [a]**	**EEQ**	**used**	**Ref.**
Dairy effluents [b]	240 (5.2-720)	330 (0.69-880)	61 (0.30-310)	78 (<1-310)	109 (3.2-670)	E-Screen	This study
NZ STP effluents							
Pukete (NZ)	T [c]	ND [d]	T	-	BQL [e]	ERBA	(40)
Taupo (NZ)	84.7	9.5	14.8	28	32	ERBA	(40)
Temple View (NZ)	19.0	T	ND	6	22	ERBA	(40)
NZ & Australia	NM	NM	NM	-	<4–6.4	ERBA	(56)
International STP effluents							
Australia	24 (3.1-39)	NM	3.8 (0.05-6.3)	8 (<1–13)	0.60 (0.03-2.2)	YES	(45)
Canada	17 (1-96)	NM	1.8 (0.2–14.7)	6 (<1-32)	50 (ND-106)	ERBA	(240)
Germany	1.5 (<0.1–70)	NM	0.55 (<0.05–15)	0.55 (<1-23)	1.6 (0.2–7.8)	E-Screen	(201,241)
Netherlands	3.4 (<0.3-11)	<0.4	<0.8	1.1 (<1-4)	ND-2.2	ER-CALUX	(166)
Japan	12 (ND – 180)	NM	ND (ND-11)	4 (<1-60)	NM	NM	(88)
Mean ± std. dev.	15 ± 15	NM	3 ± 3	3	NM	NM	(159)

Notes: [a] Maximum dilution required to reduce median steroid concentrations to below suggested PNECs for E1 (3 ng/L) and/or 17β-E2 (1 ng/L) (132). [b] Mean (range) of data for sites discharging to water. [c] Trace concentrations detected (below quantitation limit). [d] Not detected. [e] Below quantitation limit.

effects in aquatic biota. Stream sampling in the Waikato Region demonstrated widespread potential for elevated concentrations of estrogens in waterways receiving effluents.

8.2.2 Land

Land disposal of DSE is favoured in most regions to reduce impacts of nutrients on waterways. In most cases, effluent is immediately irrigated directly onto paddocks with no form of treatment. Raw effluents are likely to contain E1-3S, which binds to soil much more weakly than free estrogens do (242). There is therefore potential for E1-3S to be transported in runoff to surface waters or to leach through soil into groundwater. Thus, E1-3S may serve as a reservoir for estrogen contamination, which can be subsequently hydrolysed into E1 in favourable conditions.

The free estrogens have greater potential to sorb to soil than the sulfate conjugates and should therefore be retained in the soil. Despite this, free estrogens have, in fact, been measured in soil drainage water (233). DSE has high concentrations of dissolved organic carbon and colloidal organic carbon. This is thought to enhance the transport of free estrogens through soil and result in lower sorption than predicted from soil testing in the laboratory (233,234). The current trend in New Zealand towards larger and more intensive dairy farming may increase the loads of estrogens applied to land.

Estrogens have also been detected in runoff after application of dairy wastes to land (243). There may be increased potential for steroids in runoff where effluent is irrigated near waterways or results in ponding, common occurrences in New Zealand (244-246) despite regulations to the contrary. Furthermore, estrogens deposited diffusely during grazing, a route not examined during this research, may enter waterways through runoff during high rainfall events. Any increase the amount of estrogen deposited diffusely due to increases in dairy cattle numbers and /or increases in the proportion of dairying within a catchment will likely increase the concentrations entering a waterway through runoff.

Anaerobic pond treatment increased the aqueous estrogen concentrations compared to raw effluent. On some Waikato farms, effluent is irrigated from pond systems. The use of holding ponds (RT ≥ 3 days) is increasing in the Canterbury region due to regulatory

pressure. These ponds, likely to be anaerobic due to the high organic loading of DSE, will increase the aqueous concentrations of free estrogens prior to irrigation.

As aerobic and facultative ponds greatly reduced the concentrations of estrogens in the effluent, the ideal disposal method for DSE would appear to be anaerobic treatment to cleave conjugated estrogens, aerobic pond treatment to reduce concentrations, then disposal via irrigation onto land.

8.3 THESIS OBJECTIVES REVISITED

The overall aim of this thesis was to establish whether dairy farming is a contributor of steroid estrogens to NZ's aquatic environment. There were four specific objectives and the outcomes related to each of these are discussed in the following sections.

Objective 1: To develop a method to analyse steroid estrogens and conjugated estrogens in dairy shed effluents and environmental samples.

A GC-MS method was developed through improvements on a previously developed method. The method incorporated SPE extraction, clean-up on aminopropyl and florisil sorbents and through GPC, derivatisation to trifluoroacetates then analysis by GC-MS. Isotopically-labelled standards were used for quantitation and the overall method detection limit was 0.8 ng/L for free estrogens in DSE samples, comparable to previously published methods. This method was applied to the analysis of 17β-E2, 17α-E2 and E1 in dairy effluents and environmental water samples at the ng/L level and over a concentration range of several orders of magnitude. The robustness of the method was further demonstrated through its application to the analysis of extracted samples of dairy effluent solids.

An LC-MS method was developed to analyse sulfate conjugates using LC-IT-MS in SRM mode. This provided suitable sensitivity for the analysis of dairy effluent samples, which contain conjugated estrogens at higher concentrations, but did not provide the sensitivity to analyse environmental water samples, where concentrations are expected to be much lower. Methods based on enzyme hydrolysis and solvolysis were also evaluated but poor recovery was achieved for standards and results for dairy effluent samples conflicted with

LC-MS results. Detection limits for the glucuronide conjugates were not satisfactory and these compounds could not be accurately measured in the DSE samples. Co-eluting compounds in the DSE samples interfered with the quantitation of 17β-E2-3S. The conjugated estrogens 17α-E2-3S and E1-3S were reliably measured in a number of raw and treated DSE samples with method detection limits of ~15 ng/L.

Objective 2: To determine the levels of estrogens in dairy effluents by chemical analysis and compare results with those generated using biological assays.

Steroid estrogens and estrogenic activity were examined in DSE samples from 18 different farms collected at two different periods in the milking season. Steroid estrogens were detected in all DSE samples collected with 17α-E2 measured at the highest concentrations. The results also demonstrated that DSE has high estrogenic activity with a median EEQ of 46 ng/L using the E-Screen assay. Estrogenic activity measured using the E-Screen assay showed generally good agreement with the results from chemical analysis. For most samples, the majority of the activity measured in the assay could be attributed to 17α-E2, 17β-E2 and, to a lesser extent, E1. Selected samples were also tested using ERBA and two-hybrid yeast assays to corroborate estrogenic activity and confirmed that activity was mediated through the estrogen receptor.

Objective 3: To assess whether estrogens are removed in treatment systems used for dairy effluent prior to discharge to waterways.

Analysis of samples collected from the exit point of DSE oxidation pond treatment systems (Chapter 4) indicated that estrogens were present after treatment. More detailed investigations of two systems, a commonly used two-pond system and a newer Advanced Pond System, indicated that estrogens initially increased in concentration then reduced significantly through each system. Between 63% and 99% of total estrogens were removed from the particulate and aqueous phases. The estrogenic activity reduced by up to 89% in the aqueous phase. Despite these large reductions, residual steroid estrogens and estrogenic activity remained in the final effluent of both treatment systems and was discharged into receiving waters.

Objective 4: To investigate the presence of steroid estrogens and estrogenic activity in aquatic receiving environments of dairy catchments.

Steroid estrogens and estrogenic activity were widespread in streams and in groundwater, with measurable estrogenic activity in 83% of stream samples and 75% of groundwater samples. E1 was the predominant steroid measured; at up to 3.3 ng/L. Estrogenic activity and steroid concentrations were within the range measured internationally in agricultural and particularly dairying catchments. The results indicated potential for adverse effects on fish in two of the streams studied where the estrogen concentrations measured exceeded PNECs.

8.4 KEY RESEARCH FINDINGS

The key findings of the research were:

- Steroid estrogen concentrations were elevated in dairy shed effluents and were dominated by the steroid 17α-E2. Despite the lower potency of this compound compared to 17β-E2 and E1, results from the E-Screen showed that it contributed a significant proportion of the overall activity from DSE due to the much higher concentrations. Conversely, results obtained using a two-hybrid yeast assay indicated that 17β-E2 and E1 caused the majority of measured activity. This was due to relative differences in the potencies of E1 and 17α-E2 in each *in vitro* assay. As potencies differ from *in vitro* to *in vivo* assays, *in vivo* testing will be required to determine the true significance of 17α-E2 to cause adverse effects in aquatic biota.

- Much higher EEQs were measured in the two-hybrid yeast assay based on a fish estrogen receptor (medER). This may be due to the presence of estrogenic compounds not measured in this study, such as phytoestrogens or mycoestrogens. If this assay better reflects the effect in exposed fish, the E-Screen assay may underestimate the potential effects in aquatic environments.

- Conjugated estrogens were measured in several raw effluent samples, at concentrations that were similar to the concentrations of free estrogens. In particular, E1-3S (13-180 ng/L) was present at similar concentrations to free E1 in those samples (10-480 ng/L). Although the conjugated estrogens demonstrated only

weak activity in the E-Screen assay, their presence is of importance as they can degrade to the highly active free estrogens. Conjugated estrogens therefore represent a significant source of estrogens that has previously been neglected.

- Enzymolysis and solvolysis methods provided inconsistent results. Recovery was poor for the pure compounds. Furthermore, in some cases, the free estrogen produced from cleavage was different to that in the parent conjugate form.

- In this study, free estrogens were measured at relatively high concentrations in the solids phase of DSE. This may be due to the much higher content of solids in these wastes compared to sewage influent and effluent, and perhaps due to the practice of acidification of samples. Most studies internationally have focussed purely on the aqueous phase of wastewater samples. Future studies of wastes with high solids content should bear this in mind and, if centrifuging and/or filtering samples, measure both particulate and aqueous phases.

- Steroid estrogens were not completely removed by currently used DSE treatment systems. Steroid estrogen concentrations and estrogenic activity increased during anaerobic treatment, due to either desorption processes or hydrolysis of conjugated forms. Aerobic treatment appears to be the most important step to decrease estrogen concentrations in DSE effluent. Residual concentrations in the treated effluent are typically higher than reported concentrations in sewage effluents.

- Steroid estrogens were detected in several waterways in predominantly dairying catchments, and were highest in a minor stream receiving a direct discharge of treated dairy effluent. Steroid estrogens (17β-E2, 17α-E2 and E1) were all detected in at least one waterway. Most waterways demonstrated measurable estrogenic activity. Concentrations of E1 and 17β-E2 were above proposed PNECs in two streams and approached this value in two others. Potential exists for higher concentrations in these streams under different environmental conditions.

- Steroid estrogens and estrogenic activity were also detected in groundwater in an intensive dairying catchment. This suggests that the steroids have potential to leach through soils when applied to land either as dairy effluent, or diffusively during grazing.

8.5 RECOMMENDATIONS FOR FUTURE RESEARCH

The method developed in this study was not sensitive enough to allow quantitation of glucuronides. Further work should be undertaken to improve the quantitation method for this group of compounds to enable their measurement in raw DSE samples. It is likely that they do not survive long in these samples, due to the rich microbial environment; however, this needs to be confirmed by laboratory batch tests of DSE samples spiked with glucuronides.

One of the major findings of this research was that 17α-E2 dominated the steroid profile of effluents and had a major contribution to the overall estrogenic activity of samples. There is currently little information on the fate and effects of 17α-E2. It has been suggested that it may degrade to E1, which may be more a potent estrogen that 17α-E2. Furthermore, at this stage, there are no published studies evaluating the effects of 17α-E2 *in vivo* and no guidelines for maximum safe concentrations in aquatic environments. Future research should be applied to elucidating these questions, given the importance of dairy effluents as a source of estrogens, particularly in New Zealand.

As estrogens were not completely removed in the DSE treatment systems studied, the efficacy of existing treatment systems needs to be improved. Further research into the current mechanisms of removal in these systems, incorporating a mass balance approach and examining bottom sludges from the ponds, would provide much needed information to improve the systems and further reduce the concentrations of estrogens in the final effluent discharged into waterways.

Stream samples collected under flood conditions contained higher concentrations of steroid estrogens than those under baseflow conditions, suggesting that runoff or sub-surface leaching may be a more important route in some catchments than direct discharges. Further sampling under wet-weather conditions, including sampling of runoff, may assist in identifying the processes of steroid transport. This may be particularly important in ephemeral streams, where all the water is sourced from runoff. Alternatively, passive samplers could be deployed in streams within dairy catchments, to provide an integrated estimate of exposure to steroid estrogens.

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APPENDICES

Appendix A: DSE composition

DSE composition varies due to differences in volumes of wash-down water used, feed type and quality, and the time of year (247). Further differences are observed with timing and depth of sampling (39). The composition of the DSE samples collected from the Waikuku catchment was at the lower end of the range observed throughout New Zealand for farm dairy effluents (Table A.1).

Table A.1: Composition of DSE from Waikuku catchment (all data mg/L).

	Total Suspended Solids	**Total Organic Carbon**	**Total Kjeldahl Nitrogen**	**Total Phosphorus**
Wku1	4080	1330	203	41.5
Wku2	3290	1390	246	51.2
Wku3	2110	1030	138	29.4
Wku4	1430	690	149	23.5
Wku5	1790	1630	180	40.2
Wku6	2980	854	184	32.2
Average	**2610 ± 1010**	**1150 ± 360**	**183 ± 39**	**36 ± 10**
NZ Literature [a]				
Mean [b]	9400 ± 3200	2700 ± 830	205 ± 45	65 ± 20
Range [c]	400 – 52,000	700 - 6550	80 – 506 †	21 - 123
Raman et al. (39)	NM	NM	589 ± 364	106 ± 11
Hutchins et al. (43)	718	576	185	30.3

Notes: [a] Data from (156,176,248,249) and references therein. [b] Weighted mean of means ± std deviation of means. [c] Range in mean concentrations.

Appendix B: Stream Sampling Locations and Catchment Characteristics

Table B.1: Stream sampling locations and catchment characteristics.

Stream Name	Sampling location		Total area of catchment (ha)	Area of dairy land use (ha)	No. dairy farms	DSE discharges in catchment		No. of stock in catchment				
	Easting	Northing				No. to water	No. to Land	Dairy cattle	Beef cattle	Pigs	Deer	Sheep
Mangakawaru Stream	2710239	6401370	4038	353	64	6	18	24220	215	0	0	41
Mangaorongo Stream	2709123	6335547	12766	7821	135	17	47	51242	1286	75	1	1964
Mangapiko Stream	2716693	6354091	13624	8126	131	4	66	45439	1411	414	0	125
Matatoki Stream	2741272	6439828	815	156	15		0	3726	366	1	0	220
Mellsops Drain	2723552	6343266	245	106	5		0	2013	17	3	0	0
Owairaka Valley Stream tributary	2730797	6340322	NA [a]	NA	1	0	1	NA	NA	NA	NA	NA
Owairaka Valley Stream	2730824	6340174	4771	2720	33		6	14517	574	4	15	20
Piako River	2735138	6392078	39227	23323	354	31	158	445742	2598	5967	156	629
Pokaiwhenua Stream	2750104	6345734	1178	696	26	1	7	7298	50	0	0	10
Puniu River	2711431	6349998	51907	21981	299	15	111	150790	2928	52	15	5417
Toenepi Stream	2735192	6385475	1493	1118	18	8	10	4529	NA	NA	NA	NA
Waihou River	2749435	6402794	110294	47158	769	35	381	263484	3218	401	1	1279
Waihuka Stream	2713216	6337419	391	376	10	3	3	4034	28	0	0	0

Notes: [a] Data not available.