ENDOCRINE DISRUPTORS IN THE IRISH AQUATIC ENVIRONMENT

FINAL REPORT

(Project: Estimation of Estrogenic Compounds in Irish Surface and Waste Waters)

Prepared for the Environmental Protection Agency
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EXECUTIVE SUMMARY

The objective of this study was to provide an assessment of the potential risk from endocrine disrupting compounds (EDCs) to Irish freshwaters, their ecosystems and associated drinking water resources. The different elements of the study are integrated in a manner designed to answer the following questions:

1. Do Irish rivers contain EDCs?
2. If so, at what concentrations?
3. Are these levels likely to pose a threat to aquatic ecosystems, particularly wildfish?
4. Is there a risk to drinking water supplies?

Chapter 1 thoroughly reviews the current literature in the area and demonstrates that no research, prior to this study, had been carried out to address these questions in an Irish context.

Chapter 2: Details of the methodologies applied in this study are described.

Chapter 3: An *in vivo* caged fish study was performed to determine the effects of wastewater treatment plant (WWTP) effluent on exposed fish populations in Irish rivers, and any associated risk to drinking water resources. To this end, cages of male rainbow trout were deployed at test and control sites on the river Lee, and levels of plasma vitellogenin were used as a sensitive indicator of exposure to environmental estrogens. No evidence of estrogen exposure was found at any of the test or control sites, including the site at the intake to the Lee Road water treatment works, which supplies drinking water to Cork City.

Chapter 4 presents the results of a survey of feral Brown trout populations in the rivers Liffey, Lee and Bandon, and the Killarney Lakes. There was no evidence of exposure to environmental estrogens in any of the sites surveyed, with the exception of the Liffey site, located immediately downstream of Osberstown WWTP. Raised plasma vitellogenin levels in male fish taken from this site indicated estrogenic activity in this region of the river.

Chapter 5: An *in vitro* bioassay analysis of point sources of estrogens; namely WWTP effluents, and their impact on receiving waters, was performed. Estrogen levels in effluents ranged from 1-17 ng/l while receiving waters were less than 3 ng/l in all cases. These levels compared favourably with similar size plants investigated in other countries, including the UK. A model recently developed (2004) at CEH Wallingford predicts estrogenicity of effluents and receiving waters impacted by domestic waste. This model was applied for the first time in an Irish context. Generally, for effluents, agreement was good between measured and predicted values for estrogenicity. However, the model consistently under-predicted the measured concentration of estrogens in the receiving waters. Given the predominantly rural nature of the catchments involved, it is suggested that the additional estrogenic signal is derived from intensive livestock agriculture.

Chapter 6 presents a summary of the general conclusions from this study.

LIST OF ABBREVIATIONS

4t-OP	4-tert-octyl phenol
ACTH	Adrenocorticotropin
ADC	Aquaculture Development Centre
AFP	Alpha-fetoprotein
Ah	Aryl hydrocarbon receptor
AP	Alkylphenol
AP_2EC	Alkyl phenol ethoxy carboxylate
APEO	Alkylphenol polyethoxylate
AR	Androgen receptor
ARE	Androgen response element
ARNT	Ah receptor nuclear translocator
BBP	Butylbenzyl phthalate
BCF	Bioconcentration factor
BCHP	Butyl cyclohexyl phthalate
BOD-5	5-Day biological oxygen demand
BPA	Bisphenol-A
CBG	Corticosteroid binding globulin
CNS	Central nervous system
CPRG	β-D-Galactopyranoside
CRH	Corticotropin-releasing hormone
DBP	Dibutyl phthalate
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di(ethylhexyl)phthalate
DEP	Diethyl phthalate
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DHT	Dihydro-testosterone
DIBP	Diisobutyl phthalate
DINP	Diisononyl phthalate
DnBP	Di-n-butyl phthalate
DPhP	Diphenyl phthalate
DTDP	Ditridecyl phthalate
E2	17β-Estradiol
$E2_{eq}$	Estradiol Equivalent
E3	Estrone
EA	Environment Agency
EFB	Eastern Fisheries Board
EDC	Endocrine disrupting chemical
EE_2	17α-Ethinylestradiol
EE_2	17α-Ethinylestradiol
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EPA	Environmental Protection Agency

ER	Estrogen receptor
ERE	Estrogen-response element
ER-α	Alpha isoform of the estrogen receptor
ER-β	Beta isoform of the estrogen receptor
ETIA	Energy transfer immunoassay
EU	European Commission
FSH	Follicle stimulating hormone
GC-MS	Gas chromatography-mass spectrometry
GnRH	Gonadotropin-releasing hormone
GSI	Gonadosomatic index
GtH	Gonadotrophin
hAR	Human androgen receptor
hER	Human estrogen receptor
HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
HPT	Hypothalamic-pituitary-thyroid axis
HSI	Hepatosomatic index
hsp	Heat shock protein
IC_{50}	Half-inhibitory concentration
IHBP	Isohexylbenzyl phthalate
IPC	Integrated pollution control
LC-MS	Liquid chromatography-mass spectrometry
LH	Luteinizing hormone
NF-KB	Nuclear factor kappa B
NP	Nonylphenol
NP_1EC	Nonyl phenol mono-carboxylic acid
NP_2EC	Nonyl phenol di-carboxylic acid
NP_2EO	Nonylphenoldiethoxylate
NPEO	Nonylphenol polyethoxylate
$NPEO_{15}$	Nonylphenol polyethoxylate 15
NR	Steroid/nuclear receptor
NTP	National Toxicology Program
OECD	Organisation for Economic Cooperation and Development
OP	Octylphenol
OSPAR	Oslo and Paris Commission
PAH	Polyaromatic hydrocarbon
PCB	Polychlorinated biphenyl
PE	Adjusted population equivalent
PHAH	Polyhalogenated aromatic hydrocarbon
PMSF	Phenylmethylsulfonylfluoride
PR	Progesterone receptor
PRE	Progesterone response element
PVC	Polyvinylchloride
RIA	Radioimmuoassay
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHBG	Sex hormone binding globulin
SWRFB	South Western Region Fisheries Board
T_3	Triiodothyronine

T_4	tetraiodothyronine or thyroxine
TBG	Tetrachlorodibenzo-*p*-dioxin
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
T_{eq}	Testosterone equivalent
TIE	Toxicity Investigation and Evaluation
TIRF	Total internal reflection fluorescence
TOC	Total organic carbon
TR	Thyroid hormone receptor
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone or thyrotrophin
VTG	Vitellogenin
WTW	Water treatment works
WWTP	Waste-water treatment plant
XRE	Xenobiotic response element

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1. Literature Review

1.1 General Introduction

In the last 40 years a wide range of environmental contaminants have been shown to adversely affect wildlife at both the individual and population level by altering the normal functioning of their reproductive, growth and immune systems. Within an organism the endocrine system orchestrates these functions and may be considered as a primary target for many man-made chemicals found in the environment today. The endocrine system is complex and it is this complexity that tends to obstruct the identification of specific mechanisms through which these endocrine disrupting compounds (EDCs) elicit observed biological responses.

The alteration of normal hormonal functions in wildlife is not new – some plants produce natural estrogenic compounds, the phytestrogens – but is seen as an issue of increasing concern. The scientific community has begun to compile a growing list of man-made chemicals (xenobiotics) that have been shown in laboratory studies to have the ability to interact with and to modify the endocrine system. Many of the compounds discovered to have endocrine disrupting effects are chemicals commonly used in a wide variety of applications and therefore have the potential to be widely dispersed throughout the environment.

In the book Silent Spring (1962), the author Rachel Carson demonstrated that wildlife species are excellent sentinels of ecosystem health and human health. Monitoring of the concentrations of environmental pollutants in the tissues of wildlife and/or their biological effects has since become a standard method for assessing ecosystem health. The ideas presented in Silent Spring were recently updated, to specifically discuss the potential effects of endocrine disrupting chemicals on wildlife and human populations (Colborn *et al.*, 1996)

Rivers and estuaries are commonly repositories for large amounts of domestic and industrial waste. The majority of chemicals shown to interact with the endocrine system, particularly the estrogen receptor have been found in sewage works effluents and in untreated industrial and domestic wastes by researchers around the world. Fish living in these riverine and estuarine environments can therefore be considered as sentinel species for assessing the impact of EDCs on the environment.

In the United Kingdom, Italy and the United States studies have shown that disruption of the endocrine system of some wild fish populations is occurring and have in some cases linked these effects to specific EDCs.

This review discusses the current state of knowledge in relation to the mechanisms of endocrine disruption. It details currently used methods for the identification and monitoring of endocrine disrupting chemicals (EDCs) in the environment using *in vivo* and *in vitro* techniques as well as by chemical analysis. The properties, environmental behaviour and environmental concentrations of known endocrine disrupting chemicals are discussed in detail.

As a result of living within the aquatic environment, commonly close to pollution point sources, fish are often the primary organisms exposed to Endocrine Disrupting Chemicals (EDCs). As they may be exposed to relatively high waterborne

concentrations of these chemicals they are often used as sentinel organisms for perceived environmental problems. Aquatic species can be exposed to these relatively high concentrations due to their habitation of watercourses that may receive agricultural run-off or be impacted by domestic and/or industrial effluents either after wastewater treatment or as raw effluents. For these reasons fish have often been used to screen for the presence of EDCs in the aquatic environment using both *in vivo* (see section 1.4) and *in vitro* (see 1.5) techniques. In order to understand how EDCs affect the biological functioning of fish it is first important to understand the general mechanisms of endocrine disruption, and the primary mechanisms controlling the teleost endocrine system.

1.2 General Mechanisms of Endocrine Disruption

The endocrine system is complex with many organs contributing to a multifaceted regulatory system that governs the normal growth, development and reproduction of an organism. This complexity obstructs the identification of the specific mechanisms through which EDCs elicit their responses (Crain *et al.*, 1999). Discovery of the modes of action of EDCs is also made difficult by the fact that a range of factors (e.g.. duration, level and timing of exposure; nutritional status, age and gender of individual; cell or tissue type in which EDC acts) may result in different effects being produced (Lister and Van der Kraak, 2001). The lack of an organised, mechanistic understanding of EDC effects provides perhaps the greatest impediment to this area of research (Rudel, 1997).

Although all aspects of the endocrine system and potentially, all chemical signalling pathways in the organism are susceptible to the effects of endocrine disruption, the focus of this review will be on the three major endocrine axes that affect reproductive development and function. This focus was chosen as many manifestations of endocrine disruption involve the reproductive system, particularly during it's vulnerable developmental period. The particular aspects of the vertebrate endocrine system that will be covered include: the hypothalamic-pituitary-gonadal axis (HPG), the hypothalamic-pituitary-thyroid axis (HPT) and the hypothalamic-pituitary-adrenal axis (HPA).

1.2.1 Overview of the Endocrine System

Hormones are specific chemical products of organs or tissues of the endocrine system (Figure 1.2.1) that are transported by the blood or other body fluids, and elicit a specific regulatory effect on target tissues or organs. Specific actions of hormones may include a whole body response; a regulatory action; a morphogenic action, or a permissive or complementary action.

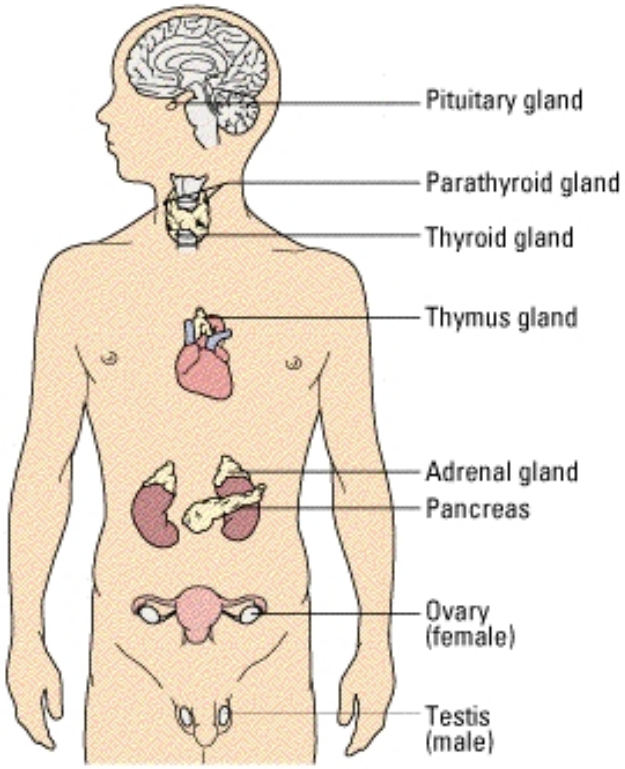

Figure 1.2.1
The human endocrine system

The hypothalamus, located at the base of the brain, releases hormones that both stimulate and inhibit the release of hormones from the pituitary gland. These hypothalamic hormones all have a polypeptide structure and act on the anterior lobe of the pituitary. The anterior pituitary produces seven specific hormones that stimulate (or inhibit) the various "target" glands or tissues associated with the endocrine system, including: adrenocorticotropin (ACTH) that acts on the adrenal cortex; follicle stimulating hormone (FSH) that acts on the ovary and testes; luteinizing hormone (LH) acting on the ovary and testes and thyrotropin (thyroid stimulating hormone (TSH)) acting on the thyroid. The hypothalamus can both stimulate and inhibit the release of pituitary hormones through the action of hypothalamic "releasing factors" or "inhibiting factors". The pituitary then stimulates other endocrine glands and tissues by release of it's hormones The interactions of the major endocrine glands and their target tissues are summarised in Figure 1.2.2.

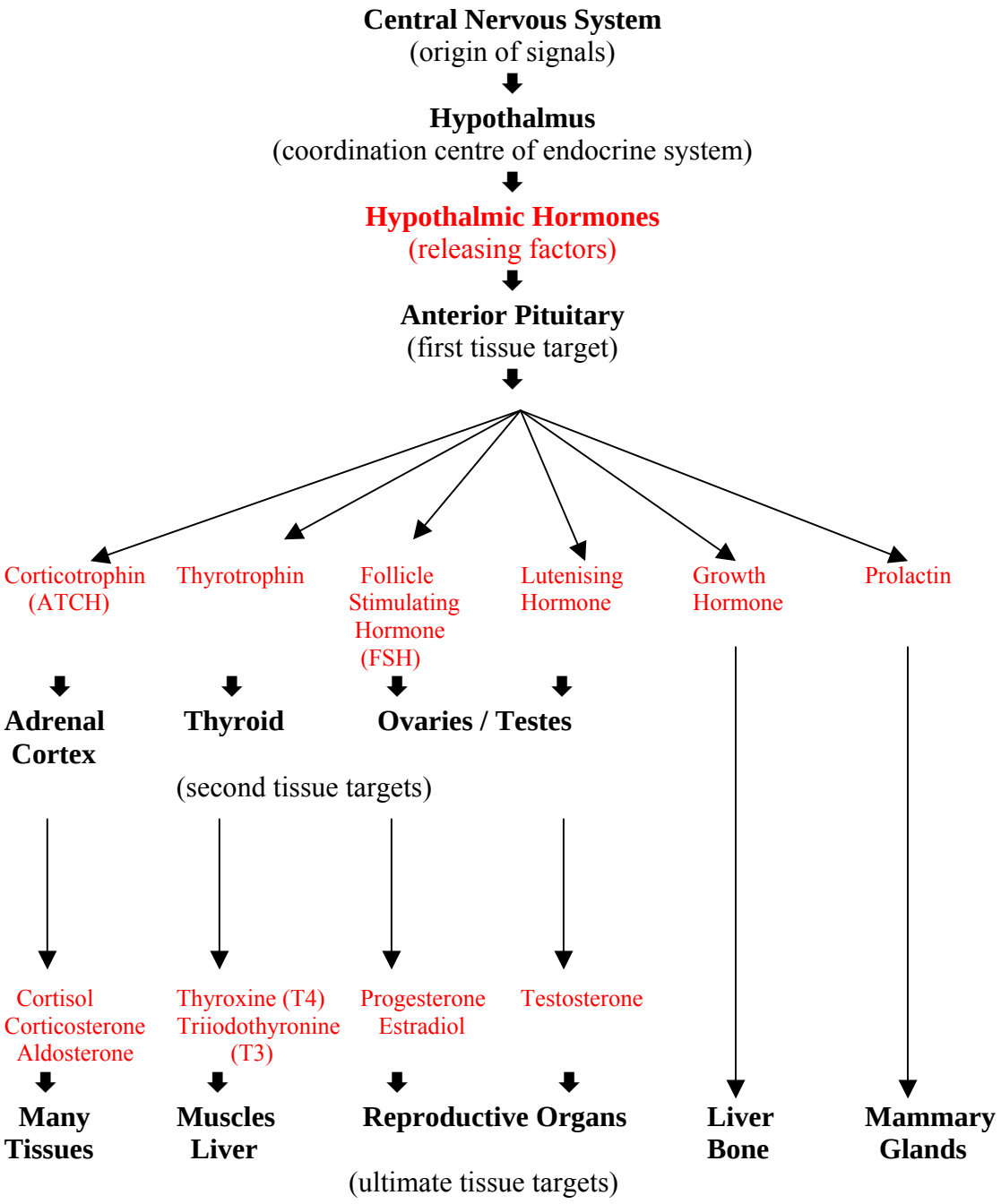

Figure 1.2.2
Selected major endocrine glands and their target tissues (adapted from Nelson and Cox, 2000).
Hormones are indicated in red ink.

(i) The Hypothalmic-Pituitary-Gonadal Axis

The HPG axis involves three component parts: gonadotropin-releasing hormone (GnRH) neurons projecting from the hypothalamus of the brain, gonadotropes in the anterior pituitary gland (adenohypophysis), which secrete the gonadotropins luteinizing hormone and follicle-stimulating hormone and the somatic cells of the gonads (theca and granulosa cells in the ovary; Leydig and Sertoli cells in the testes). GnRH is

secreted in pulses (Kimura and Funabashi, 1998; Terasawa, 1998) from the terminals of GnRH neurons and acts on the gonadotropes to induce secretion of both LH and FSH, which then act on their respective target cells in the gonads (LH on theca/Leydig cells; FSH on granulosa/Sertoli cells). In females, LH stimulates ovulation and the conversion of the ovulated ovarian follicle into an endocrine structure called a corpus luteum. In males, LH stimulates the secretion of male sex hormones (mainly testosterone) from the interstitial cells of Leydig in the testes. FSH stimulates the growth of ovarian follicles in females and the production of sperm in the testes of males. Secretion of GnRH is modified by other neurons, and the action of GnRH on gonadotropin release may be modified by other hypothalamic or pituitary peptides (Evans, 1999). As a consequence, gonadal sex steroids produced in response to FSH and LH are released into the bloodstream and these feedback to the hypothalamus and pituitary gonadotropes to reduce the secretion of GnRH, LH and FSH, with inhibin (a protein hormone produced by the testes) selectively inhibiting FSH and the sex steroids inhibiting LH secretion (Crowley *et al.*, 1991).

The role of the proteins that bind the sex steroids and transport them around the body via the bloodstream is of prime importance. These include albumin, alpha-fetoprotein (AFP) in the fetus/neonate and, most importantly in humans, sex hormone binding globulin (SHBG). Approximately 97-98% of testosterone and estradiol that circulate in blood in humans is bound to SHBG and only 2-3% is free and biologically active (Moore and Bulbrook, 1988; Rosner, 1990). This arrangement has two important consequences : (a) the half-life of the sex steroids is considerably prolonged and (b) a new indirect pathway for regulating sex steroid action becomes evident; i.e. modulation of SHBG secretion (by the liver) can potentially alter levels of bioactive sex steroid without affecting any of the major component parts of the HPG axis (Rosner, 1990).

The level of pituitary hormone production and secretion is, in general, regulated by negative feedback mechanisms. Hormone production and release by target endocrine glands such as the thyroid and adrenal cortex are stimulated directly by the pituitary (and indirectly by the hypothalamus by the release of TSH and ACTH, respectively). When the resultant hormones released by these glands (i.e. thyroxine and cortisol), reach a high blood concentration, stimulation of the target endocrine glands by both the hypothalamus and pituitary is decreased. The “long loop” feedback mechanism is where the blood concentration of hormones feed EDCs back to the hypothalamus or pituitary; the “short loop” is where the trophic hormones of the pituitary act on the hypothalamus in a feedback mechanism.

(ii) The Hypothalmic-Pituitary-Adrenal Axis

Adrenal cortex steroids of major interest include glucocorticoids, mineralocorticoids, androgens, estrogens and progesterone. The HPA axis operates in a similar way to the HPG axis, the major difference being in the regulatory and secretory molecules involved. Corticotropin-releasing hormone (CRH) is secreted from the terminals of hypothalamic neurons and acts on corticotropes in the anterior pituitary gland to regulate the synthesis and secretion of ACTH, which is then transported via the bloodstream to the adrenal glands where it stimulates the secretion of glucocorticoid hormones (cortisol and/or corticosterone). Glucocorticoid functions include enhancement of fat lipolysis and protein catabolism while promoting the production of glucose from non-carbohydrate sources. Thus, abnormally high levels of these steroids

can cause "muscle wasting" together with the redistribution of fat. The glucocorticoids exert negative feedback effects at the hypothalamic and pituitary levels to suppress CRH secretion. Similar to the sex steroid hormones, much of the glucocorticoid in circulation in blood is bound to a binding protein in the human (corticosteroid binding globulin (CBG)), and local release of bioactive hormone from the CBG represents one mechanism of local tissue response to pro-inflammatory changes (Rosner, 1990).

In the context of reproduction, the most important products of the adrenal glands are the weak androgen, dehydroepiandrosterone (DHEA), DHEA sulfate and androstenedione, the secretion of which are also stimulated by ACTH. These adrenal androgens may be converted in target tissues to more potent androgens or to estrogens and can therefore potentially affect functioning of the reproductive endocrine axis and the cell types that are responsive to androgens and estrogens (Simpson and Rebar, 1995).

(iii) The Hypothalmic-Pituitary-Thyroid Axis

In the HPT axis, thyrotropin-releasing hormone (TRH) is secreted from the terminals of hypothalamic neurons and acts on thyrotropes in the anterior pituitary gland to regulate the synthesis and secretion of TSH in mammals (Reed and Pangaro, 1995). TSH is then transported via the bloodstream to the thyroid gland where it acts to stimulate the synthesis of triiodothyronine (T_3) and tetraiodothyronine (thyroxine or T_4) that are released into the bloodstream and act throughout the body to stimulate general metabolic activity. T_3 and T_4 feedback to the hypothalamus and pituitary and reduce the levels of TRH and TSH. There is 70 times more plasma T_4 circulating than T_3. T_3 is more metabolically active than T_4 and is loosely bound to a plasma carrier protein, thyroxine-binding globulin (TBG). If the level of TBG is increased (as seen in pregnancy or women taking birth control pills containing estrogen), the level of free hormone feeding back to the hypothalamus and pituitary is decreased. This event results in increased TRH and TSH, which stimulate the thyroid to increase the total hormone level to a point where the "free" hormone is again within normal limits. Other hormones such as anabolic steroids, lower the TBG levels and increase the total amount of circulating T_4, causing a reverse of the above situation. In the present context, interest in the thyroid endocrine axis stems from: (i) the demonstration that certain polychlorinated biphenyls (PCBs) have anti-thyroidal activity, i.e. they can antagonize the effects of T_3/T_4 levels (Grey *et al.*, 1993; Porterfield and Hendry, 1998), and (ii) the important role that the HPT axis plays in terminal differentiation of various tissues, extending from neurons to muscle and to Sertoli cells in the testes.

1.2.2 Main Classes of EDC

Initially, the concern over endocrine disruption focussed only on environmental estrogens. However, research has led to the recognition of five main classes of EDC: (i) (anti) estrogens; (ii) (anti) androgens; (iii) (anti) progestins; (iv) aryl hydrocarbon receptor agonists and (v) thyroid hormone disruptors. In general, the activity of estrogenic, androgenic and thyroid-like chemicals is mediated by their respective nuclear receptors, although other mechanisms of action such as interactions with binding globulins, inhibition of steroidogenic enzymes and binding to membrane receptors, or other nuclear receptors cannot be excluded (Zacharewski *et al.*, 2002).

Direct acting EDCs interfere with some step in the mechanism of action of the normal hormone, for example by binding to the steroid hormone receptor or by altering subsequent downstream events in signal transduction. Indirect acting EDCs alter the rates of synthesis, secretion, transport, uptake, metabolism or clearance of the steroid hormone (Bunce *et al.*, 2000). Of all the EDCs, those that are direct acting estrogen receptor (ER) agonists or antagonists, or androgen antagonists have received the greatest attention, due in part to their importance in embryonic development. The greatest concerns are (a) the fact that many EDCs may be harmful at levels well below those that are lethal and (b) that exposure to EDCs during critical periods of development may contribute to the manifestation of irreversible adverse effects in adults (Zacharewski *et al.*, 2002 ; Kime, 1999).

(i) Estrogens

EDCs include chemicals that can interact with any hormone receptor. There is emerging evidence that many environmental chemicals have a weak ability to bind to and activate the ER. Compounds that compete with estrogen for binding to the ER have been termed estrogen mimics. Those chemicals that block the response of the natural hormone are referred to as estrogen antagonists or anti-estrogens.

Estrogens are steroid hormones that help to regulate the growth, differentiation and function of reproductive tissues, as well as those of other organs such as the bones, brain and cardiovascular system (Ciana *et al.*, 2003). Estrogen action is mediated by the ER, a ligand-dependent transcription factor. ER belongs to the steroid/nuclear receptor (NR) superfamily (McKenna *et al.*, 1999), a large group of transcription factors whose members share basic structural and functional homology. Estrogens act by binding to specific receptors present in the cell nucleus: ER-α and ER-β. The activated receptors in turn bind to control regions of target genes and trigger their transcription, initiating a cascade of molecular cellular events. Anchoring of ligand in the binding pocket is the first step in ER activation. This allows the cytoplasmic ER to release heat shock proteins (hsp) and become an active and nuclear transcription factor. ER, activated by the presence of ligand binds specific nucleotide sequences called estrogen-response elements (ERE) in target genes, resulting in their expression (Figure 1.2.3) (Brosens and Parker, 2003).

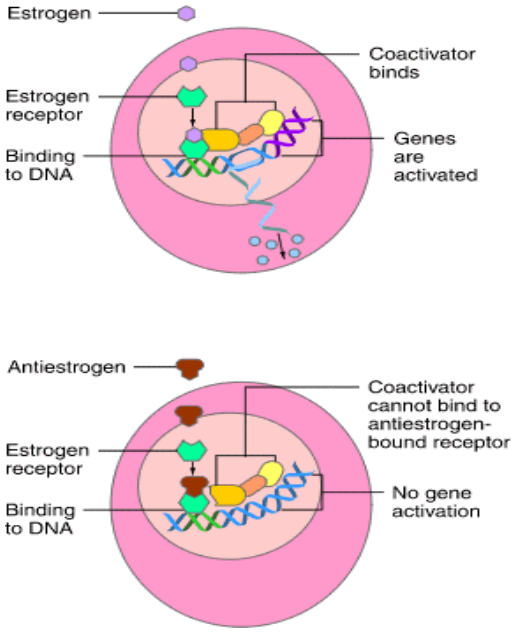

Figure 1.2.3
Action of estrogens/antiestrogens on the ER

Plasticity of the Estrogen Binding Site:

Two isoforms of the ER, arising from two different genes, exist in vertebrates. These are the earlier described ERα (Walter *et al.*, 1985) and the more recently discovered ERβ (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996; Tremblay *et al.*, 1997). The DNA binding domains of these two receptors are highly conserved (96% identity) and contain nearly identical zinc finger motifs and D-boxes, which are involved in (estrogen response element) ERE sequence recognition and dimerization of the ER DNA binding domains respectively (Loven *et al.*, 2001). Crystallographic analyses of ERα and ERβ ligand binding domains (53% identity) demonstrate that both receptors have similar alpha helical configurations and hormone binding pockets (Brzozowski *et al.*, 1997; Pike *et al.*, 1999). In addition, both receptors have similar affinities for a number of agonists including 17β-estradiol (Kuiper *et al.*, 1997; Peterson *et al.*, 1998). However, at the consensus ERE, ERα activates transcription when bound to either agonists or partial agonists but ERβ can activate transciption only when bound to pure agonists, thus demonstrating differences in the activation potential of these isoforms (Vasudevan *et al.*, 2001) and the possibilities for different EDCs interacting with either of the two receptors. Thus the ER is very plastic with respect to it's binding properties, making it impossible to predict estrogenicity of compounds on basis of structure alone.

The tissue-specific distribution of ERα and ERβ (Figure.1.2.4) suggests that the individual receptors may regulate specific subsets of estrogen responsive genes in different cell backgrounds and so a wide range of effects are possible. This level of complexity enables cells harbouring these receptors to exert exquisitely fine-tuned control of cellular functions in response to hormones and antihormones (Loven *et al.*, 2001).

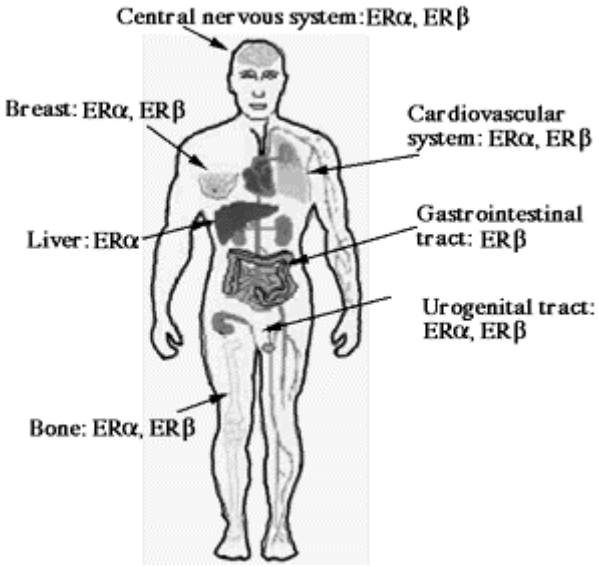

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Figure 1.2.4
Distribution of ERα and ERβ in the human

Endogenous estrogens, in mammals at least, bind to specific plasma proteins and thus only a small fraction of endogenous estrogens in the blood is able to pass into cells and be biologically active. However, many man-made chemicals that have been examined do not bind significantly to plasma hormone binding proteins (Soto *et al.*, 1995). Potentially 100% of these chemicals circulating in the blood could enter cells and exert biological effects (vom Saal *et al.*, 1995). In mammals, estrogens increase the concentration of SHBG in the plasma, thus decreasing the fraction of biologically active estrogens in the blood. It is possible, therefore, that EDCs might have a similar effect. Additionally, in many cases the affinity of EDCs for binding proteins is quite low, meaning the availability of EDCs to hormone receptors is quite high with respect to endogenous steroids (Denison and Helferich, 1998). The biological effects of estrogen mimics depend largely on their availability and concentration within tissues and body fluid, their binding to SHBG, and their binding affinity to the ER relative to that of estradiol.

Some EDCs can affect the number of hormone receptors within a cell. This has an impact on the responsiveness of a tissue to a hormone, which is dependent on the density of receptors within its component cells. For example, the dioxin 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), can act to increase or decrease the expression of the ER (Romkes *et al.*, 1987).

The affinities with which chemicals bind to the ER may vary vastly and the responses induced are not necessarily related directly to binding affinity. Species differentiation must also be considered. Hormone systems may have different developmental functions in different phylogenetic groups. For example, in birds, males are the default phenotype, with estrogen acting to induce feminisation of embryos. In contrast, in mammals the female is the default phenotype in the absence of testosterone (vom Saal *et al.*, 1995). Thus, a compound that acts as an EDC through receptor binding interactions in one species may not necessarily bind to the same receptor or provoke the same response in another species.

The developmental stage at which exposure to environmental estrogens occurs is particularly important. During the period of early differentiation, organs are particularly susceptible to the disruptive effects of chemicals that have hormonal or anti-hormonal activity (vom Saal *et al.*, 1995; Guillette *et al.*, 1995; Kavlock *et al.*, 1990). Such effects have been termed "organisational effects" as they may lead to permanent structural modifications to the reproductive, immune or nervous system (Guillette *et al.*, 1995). Thus, exposure to an EDC does not need to be chronic as transient exposure at a critical time during development is all that is required to cause a permanent adverse or abnormal change. The male children of many women who were prescribed the synthetic estrogen, diethylstilbestrol (DES), during pregnancy were born with genital tract malformations while girls exposed *in utero* have a much increased chance of developing vaginal cancer post pubertally (Lewis *et al*., 1996; Swan, 2000). Although many organisational effects occur before birth, the effects can occur throughout life, but especially prior to puberty (Guillette *et al.*, 1995). The consequences of such exposure are typically not recognised until adulthood (vom Saal *et al.*, 1995). In adulthood, the effects of estrogenic or anti-estrogenic chemicals on the endocrine system are more transitory in action and are referred to as "activational effects". As is the case with endogenous hormones, chemicals that act through the medium of hormone receptors may be capable of profound effects at very low concentrations.

(ii) Androgens

Androgens play a crucial role in the development, maintenance, and regulation of male phenotype and reproductive physiology (Quigley *et al.*, 1995). Androgens exert their effects by means of a specific receptor, the androgen receptor (AR). AR is a member of the NR superfamily. It mediates the effects of testosterone and dihydro-testosterone (DHT) in cells. The binding of these hydrophobic ligands induces AR to assume a configuration that leads to transcriptional activation (or inhibition) and allows transmission of extracellular signals into intracellular responses by targeting promoter response elements and recruiting co-factors (Gobinet *et al.*, 2002). AR, activated in the presence of ligand, translocates to the nucleus (George *et al.*, 1997) and binds specific nucleotide sequences called androgen response elements (ARE) in target genes, resulting in stimulation of their expression (Wong *et al.*, 1993).

Anti-androgens bind to the AR and block it's function. The inhibition of androgen action by androgen antagonists (e.g.. dichlorodiphenyltrichloroethane (o,p'DDT)) provokes deleterious effects: delay or absence of the appearance of the male primary and secondary sexual characters, gigantism and sterility (Massaad *et al.*, 2001). Recent *in vitro* and *in vivo* studies of EDCs, including the fungicide vinclozolin and environmental contaminant dichlorodiphenyldichloroethylene (p,p'-DDE), suggest that environmental antiandrogens that bind to and inhibit AR may be crucial contributors to abnormal male sex development (Kelce and Wilson, 1997). Environmental chemicals that bind to AR and elicit a biological response are referred to as androgens or androgen agonists.

It is established that disorders of development of the male in which androgen production or action are abnormal are associated with a substantial increase in risk of developing testicular germ cell cancer. Both cryptorchidism (testicular maldescent) and hypospadias (abnormal penis development) occur in male infants in whom androgen

production or action is abnormally low (Sharpe and Skakkebaek, 1993). Similarly, both conditions can be induced in animals by exposing the mother, during pregnancy, to chemicals that can block androgen action (i.e. are anti-androgenic) (Imajima *et al.*, 1997; McMahon *et al.*, 1995).

(iii) Progestins

The progesterone receptor (PR) is another member of the steroid receptor superfamily. It is associated with physiological events such as implantation and the maintenance of pregnancy. When progesterone, or related agonists bind to the PR, the receptor is released from associated heat-shock proteins, allowing the formation of a ligand-receptor complex that binds to the progesterone response element (PRE) in the promoters of progesterone responsive genes (Sumida *et al.*, 1988).

Concentrations of the hormone progesterone, which is necessary to maintain pregnancy, must remain high throughout pregnancy to avoid the loss of the developing embryo. Researchers studying women who suffer miscarriages report that they have higher than average PCB levels in their bodies compared to women who have normal pregnancies. Studies in rats and mice indicate that PCBs cause a reduction in progesterone by accelerating it's breakdown in the liver (Colborn *et al.*, 1997).

(iv) Aryl Hydrocarbon Receptor Agonists

The aryl hydrocarbon (Ah) receptor and nuclear factor kappa B (NF-KB) are inducible transcription factors, each governing the expression of distinct sets of genes that are important for normal physiology as well as pathophysiological responses. The Ah receptor plays a pivotal role in mediating detoxification of xenobiotics as well as toxic responses induced by dioxin and related compounds (Tian *et al.*, 2000). NF-KB is a key transcription factoring regulating the immune system and inflammatory responses, combating infections and in the response to cellular stresses such as hypoxia and oxidative stress (Ghosh *et al.*, 1998).

Unliganded Ah receptor is located in the cytoplasm associated with the hsp90, an immunophilin and p23 proteins (Perdew, 1988; Denis *et al.*, 1988; Meyer *et al.*, 1998; Ma and Whitlock 1997; Kazlauskas, 2000). Upon ligand-binding, the receptor translocates into the nucleus and dimerizes with the Ah receptor nuclear translocator (ARNT) protein (Hoffman *et al.*, 1991). The heterodimer binds to the xenobiotic response element (XRE) and alters the expression of genes controlled by XREs.

The immune system is exquisitely sensitive to dioxin toxicity. Both humoral and cell mediated immunities are known to be suppressed by TCDD. A heterodimer of AhR and ARNT mediates most of the toxic effects of dioxins (Sogawa and Fujii-Kuriyama, 1997; Schmidt and Bradfield, 1996). Recently, Ohtake *et al.* (2003) reported that agonist-activated AhR-ARNT heterodimer directly associates with ER-α and ER-β. This association results in the recruitment of unliganded ER and the coactivator p300 to EREs, leading to activation of transcription and estrogenic effects. The study also found that the AhR-ARNT complex was also found to repress ER function when estrogens are present and bound to the receptors, thus providing an explanation for previous reports of anti-estrogenic activities of dioxins (Brosens and Parker, 2003; Ohtake *et al.*, 2003).

(v) Disruptors of Thyroid Hormone

Thyroid hormone receptors (TRs) also belong to the nuclear receptor superfamily. On binding ligand, the receptor acts as a ligand-activated transription factor, regulating the transcription of target genes (Mangelsdorf *et al.*, 1995). TRs play an important role in growth, development and differentiation (Chin, 1991). In adult homeothermic organisms, they are also involved in the regulation of key metabolic processes, such as lipogenesis, lipolysis and thermogenesis (Oppenheimer *et al.*, 1995).

TRs are encoded by two genes, α and β, whose mRNAs are then differentially spliced to give four isoforms (Lazar, 1993). The TR has been shown to bind the consensus ERE, derived from the vitellogenin gene, and consequently interfere with the ability of the ER to transactivate from this ERE (Zhang and Lazar, 2000).

At a physiological level, thyroid hormone elevation has been shown to adversely affect reproductive capacity in birds, sheep and rodents. Indeed, thyroid hormone co-administration to ovariectomised female rats and mice reduced estrogen-mediated lordosis behaviour demonstrating that TR can interfere with an ER-driven behaviour (Dellovade *et al.*, 1999; Morgan *et al.*, 2000).

Normal brain development is heavily influenced by a host of hormonal signalling systems. Thyroid hormones play a major role in this process. The sex steroids contribute to sexual differentiation of brain centres, and thereby, to the development of sexual identity and sexual behaviours. Because of the relative sensitivity of the developing central nervous system (CNS) to normal changes in gonadal hormone secretion, metabolism, and transport to the brain, it is possible that developmental neurotoxicity may be one of the more sensitive consequences of exposure to EDCs (Laessig *et al.*, 1999). Typically, neurobehavioural functions are not directly affected by chemicals, but result from chemical-induced morphological and/or functional alterations in a variety of neuroendocrine pathways. Because of the organisational role of thyroid hormones in general development and in brain development in particular, hypothyroid action of poly halogenated aromatic hydrocarbons (PHAHs) during development could be a mechanism by which neurobehavioural dysfunction is mediated (Porterfield, 1994). Hypothyroid alterations in association with neonatal PCBs/PHAHs at background environmental levels of exposure have been reported fairly consistently in developmental studies in infants. It has been reported that *in utero* exposure to PCBs resulted in adverse effects in neurologic and intellectual function (memory and attention) in young children born to women who had eaten PCB-contaminated fish in the USA (Jacobson and Jacobson, 1996). It has also been speculated that exposure to environmental pollutants with steroidal activity may be influencing human sexual development and sexually controlled behaviour (Shantz, 1996; Spencer and Schaumberg, 2000).

1.2.3 Disruption of the Pathways of Hormone Synthesis, Transport and Metabolism.

The mode of action of EDCs is not limited to those agents that interact directly with steroid receptors, eliciting either an agonistic or antagonistic effect. Because of the prevalence of these studies and the availability of numerous techniques to measure steroid-receptor binding, the phenomenon of receptor interaction has become

synonymous with endocrine disruption. However, there are many sites independent of the receptor that should be considered when assessing endocrine disruption. EDCs may act by altering levels of circulating natural hormones by interfering with steroidogenesis, hormone excretion and biotransformation processes. Therefore accurate assessment of an EDC's potential to alter the endocrine system depends upon consideration of the entire hormone dynamic pathway.

A mechanism that is likely to be involved in contaminant-induced endocrine disruption is an alteration in the rate of steroid biotransformation. Biotransformation may convert chemicals to estrogenic metabolites. Also, many Cytochrome P450 enzymes that are responsible for the detoxification of xenobiotics are also involved in the metabolic and biosynthetic conversion of steroid hormones. Therefore, exposure to environmental contaminants could alter endogenous hormone concentrations by inducing P450 enzymes. PCBs and dioxins are well known for their ability to induce certain iso-enzymes of P450 in the mammalian liver (Goldstein and Safe, 1989).

The timing and duration of steroid hormone production is controlled by an intricate system of receptor-mediated feedback mechanisms. Steroidogenesis is the biochemical synthesis of steroid hormones. It is a system of interlinked pathways consisting of precursors, enzymes and their products. EDCs may obstruct this by altering the availability of cholesterol to begin steroidogenesis (e.g.. dioxin), by altering steroidogenic enzyme expression or activity thereby altering circulating hormone concentration, or by disrupting the feedback loop at any point in the hypothalamic-pituitary-gonadal axis by acting in an agonistic or antagonistic fashion. For example, the phytestrogen β-sitosterol is able to reduce gonadal steroid biosynthesis by either affecting cholesterol availability or by altering the activity of P450-dependent enzymes (Majdic *et al.*, 1996; MacLatchy *et al.*, 1997). Tributyltin inhibits the conversion of androgens to estrogens in neogastropods by inhibiting aromatase or by inhibiting testosterone metabolism and excretion (Bettin *et al.*, 1996).

1.2.4 Dose Sensitivity and the Effect of Chemical Mixtures

Dose-response assessment is the process of characterising the relationship between the dose of an agent administered and the effect in exposed populations. Dose-response relationships are determined graphically by determining the effect of varying the administered dose on the response. Generally, increasing the dose of a harmful agent will result in a proportional increase in both the incidence of an adverse effect as well as the severity of the effect. The issue of dose-response relationships is of central importance to the debate regarding EDCs (McLaughlin, 2001).

EDCs often act by mimicking or antagonising the actions of naturally occurring hormones, which are already present at physiologically functional concentrations. Therefore, the dose-response considerations for EDCs are often different than for other chemicals that are not acting directly on the endocrine system (WHO/PCS/EDC/02.2).
Dose-response relationships are likely to vary for different chemicals and endocrine mechanisms. The issue of the shape of the dose-response curve has garnered specific attention since low doses are not often included in standard toxicological testing paradigms, but are extrapolated from data obtained from high dose exposures (Needham et al., 1995). Traditional toxicology assumes that dose-response curves are always

monotonic (the higher the dose, the greater the effect) and this hypothesis underpins all regulatory testing. Hormone systems are very complex however and do not conform to the assumptions that underlie classical toxicology. In some cases a high dose of an EDC may paradoxically cause less of an effect than a lower dose. Also very low levels of an EDC may be able to trigger a response. Reported low-dose effects of EDCs have come under intense scrutiny regarding the question of the adequacy of traditional toxicology testing paradigms for detecting low-dose effects. A common dose-response relationship for all effects and all endocrine disruption mechanisms should not be expected.

In recent years, concern has been growing that adverse health consequences may result from low-levels of exposure to commonly occurring environmental estrogens such as bisphenol A, nonylphenol and methoxychlor. Further, considerable interest has been generated in the toxicological community by reports of opposite effects occurring at low and high doses (Newbold *et al*., 2004). In 2001, The National Toxicology Program (NTP), within the U.S. Department of Health and Human Services, sponsored a meeting specifically to address low dose issues. At this meeting, low dose was defined as doses below the range typically used in toxicology studies and doses to which humans may be exposed. The meeting addressed numerous issues including statistical methods appropriate for analysing data from low dose exposures and the shape of the dose response curves (Haseman *et al*., 2001). Conclusions drawn from the NTP meeting were that low dose effects were clearly demonstrated in laboratory animals exposed to estradiol, DES, and other environmental estrogens; and the effects were dependent on the compound studied and the endpoint measured (Melnick *et al*., 2002.).

Different EDCs have different potencies, represented by unique dose-response curves. Assessment of the potency of an EDC is necessary for the characterisation of the risk posed following exposure to the chemical. Many factors determine the potency of a given contaminant including bioavailability, affinity for target receptor, metabolism of the contaminant, half-life of the contaminant and the relative potency of endogenous hormones.

Research is required in order to develop knowledge on dose-response relationships for EDCs. Evaluation of the dose-response relationships for health and environmental effects of EDCs will be most credible when information is available from several sources. Credible dose-response relationships will be obtained from several sources including toxicity studies, mechanistic and epidemiological studies, and field studies (McLaughlin, 2001).

Wildlife is very rarely exposed to single chemicals, but instead are much more likely to be exposed to complex, often ill-defined, mixtures of chemicals; WWTP effluent is one such mixture. Mixtures, defined as a combination of two or more chemicals, are one of the huge unknowns in toxicology. Many potential EDCs exist as mixtures of related isomers and congeners. Individual chemicals within these mixtures may vary greatly in potency and may interact with each other in an unpredictable manner. Evidence is emerging that EDCs can act together and that seemingly insignificant quantities of individual chemicals can have a major cumulative effect. Rajapakse et al (2002) demonstrated this using eleven weakly estrogenic compounds and 17β-estradiol. When combined with 17β-estradiol, the xenoestrogens (each at a level where no effect was observable), caused a doubling of the effect of the 17β-estradiol. Chemical interactions

may be additive, inhibitory or synergistic. Synergistic interactions are the most problematic, because they indicate that the effects of multiple chemicals together can be significantly more powerful than might be predicted simply by adding up their effects one at a time and therefore they are hardest to predict. Screening and testing small numbers of compounds in mixtures is currently technically feasible. However, humans and wildlife are exposed to many contaminants simultaneously, most of which are virtually unstudied with respect to specific endocrine impacts. This highlights the need for more research on the effects of chemical mixtures with endocrine disrupting potential.

1.3 Mechanisms of Endocrine Disruption in Fish

1.3.1 The Teleost Endocrine System

The endocrine system is a hormonal network the purpose of which is to regulate the metabolic and growth activities of the various organs in the body of the fish, while maintaining homeostasis. This relies on interaction with the nervous system, which communicates with target tissues via nerve synapse relays. These target tissues respond to certain hormones or hormone analogues via interaction with specific hormone receptors. Receptors are specific protein molecules located in the plasma membrane, cytoplasm, nucleus or other intracellular organelles of the target cell. There are receptors for both androgens and estrogens and these are found in many tissues including the brain, pituitary and accessory sex organs (Tyler *et al.*, 1998).

Signals from the brain, resulting from external seasonal or local cues, are modulated by the hypothalamic-pituitary system as changes in the levels of hormone secretion. Hormones released from the hypothalamus (GnRH; CRH; TRH; see section 1.2.1) stimulate the release of hormones from secretory cells in the pituitary gland (Kime 1998). These peptide hormones (GtH, ACTH, TSH) act on the gonad, adrenal and thyroid glands, respectively, to stimulate the secretion of their respective hormones (see section 1.2.1). Both the amount of endogenous hormones and the timing of their release into the blood are carefully controlled by the endocrine system.

Endocrine disruption is the disturbance, by natural hormones or xenobiotics present in either the habitat or the diet of the fish, of the normal hormonal mechanisms within the fish. This disruption is associated with many different mechanisms, one of which involves binding xenobiotics to the androgen and estrogen receptors, evoking physiological responses within the fish. As the major functions of estrogens and androgens are in sex determination, sexual differentiation, and sexual development, many of the effects of EDCs are associated with the reproductive health of fish (Tyler *et al.*, 1998).

EDCs can affect the endocrine system of fish in several ways; they can disrupt the hypothalamic and pituitary function, reproductive function, eggs embryos, larvae and juvenile fish, liver function and thyroid and inter-renal function.

1.3.2 Disruption of Hypothalamic and Pituitary Function in Fish

The hypothalamic and pituitary function is difficult to examine in isolation because it is functionally part of the brain. Neural function is disrupted by EDCs in the same way as a neurotoxin, which will destroy any nervous tissue and lead to a general malfunction of the animal. The endocrine system acts an amplifier of the signal and relatively minor disruptions of the neural input can result in profound disturbances of endocrine function. Therefore, EDCs can affect the neurosecretory function in the hypothalamus at levels lower than those that induce general neural damage. This could result in disruption of the secretion of releasing hormones into the pituitary. For example, EDCs may inhibit GnRH synthesis in the hypothalamus, and/or gonadotropin synthesis in the pituitary gland. The morphology of the pituitary may be affected by exposure to EDCs during the very early stages of life. This effect may be in the form of reduced size, which can result in delayed sexual maturity and, when the organism is sexually mature, the production of fewer eggs and a shorter reproductive period (Kime, 1998).

1.3.3 Disruption of the Reproductive Function

In common with other vertebrates, reproduction in fish is regulated by gonadal steroids and is under the direct control of two pituitary gonadotropins, GtH I and GtH II (Campbell *et al.*, 1994). One of the GnRH genes and at least one of the gonadotropin genes of fish contain estrogen responsive elements, therefore, it is likely that the expression of these genes and hence the synthesis of GnRH and gonadotrophins is controlled at least in part by estrogen (Jobling *et al.*, 1996). Thus any xenobiotics that mimic these chemicals and are capable of binding to their receptors are most likely a cause of endocrine disruption.

Certain areas of the body such as the reproductive organs are particularly sensitive to physiological levels of estrogen, due to the high concentration of receptor sites in theses target cells (Montaganani *et al.*, 1996). Estrogen is the collective name given to the group of organic molecules, derived from cholesterol, that bind to estrogen receptor sites *in vivo* and evoke physiological responses. The principal natural estrogen is 17β-estradiol (Montagnani *et al.*, 1996).

Decreased gonadosomatic index, GSI (the ratio of gonad weight to whole body weight), in fish exposed to xenobiotics has been reported by many authors (Jobling *et al.*, 1996; Billard *et al.*, 1981). The mechanisms underlying inhibition of testicular growth by estrogenic chemicals are not known. It is not clear if the effect is due to disruption via the piturity-hypothalamic system or due to direct action on the testis itself, but it is possible that these chemicals exert their effects directly on the testis, possibly via the inhibition of androgen synthesis (Jobling *et al.*, 1996)

The EDCs therefore may have direct effects on the testis either (i) cytotoxicologically, where the disruption is caused by damage to the cellular integrity or function of gonadal cells in general or (ii) endocrinologically, in which the functions of specific cells are disrupted due to an endocrine malfunction. This could be due to altered pituitary secretions or to alterations in the chain of enzymes within the testes, which lead to synthesis of the testicular hormones (Kime, 1998).

It is also possible that estrogenic chemicals inhibit spermatogenesis (sperm production) by acting at one or more levels in the cascade of hormones that regulate development of the testes. For example, estrogens may inhibit GnRH synthesis in the hypothalamus, and/or gonadotropin synthesis in the pituitary gland (Jobling *et al.*, 1996).

The principal stimuli for fish spermatogenesis are pituitary gonadotropins and testicular androgens, and the specific role played by each individual hormone has not been clarified. Gonads of developing male animals are very sensitive to estrogens. This was clearly demonstrated by the complete inhibition of gonadal development when maturing male salmonids were exposed to 17β-estradiol via their diet (Billard, 1981). Although mature fish may also be effected, as in viviparous guppies (*Poecilia reticulata*) blocked spermatogonial mitosis were seen in the testis structure after exposure to octyphenol and 17β-estradiol (Kinnberg *et al.,* 2003).

Finally secondary sexual characteristics and courtship behaviour can be disrupted or are absent due to disruption of normal endocrine functioning due to decreased production or absence of normal circulating hormones (Robinson *et al.*, 2003).

1.3.4 Disruption in Eggs, Embryos, Larvae and Juvenile Fish

Few studies have been conducted on sex determination in fish. However, examples of both male and female heterogamety and temperature dependency occur. It has also been noted that when juveniles are exposed to EDCs before or at the time of sex differentiation, sterilization, sexual reversion or hermaphroditism may occur (Johnstone, 1978). EDCs have been seen to cause an increase in egg/embryo production in fathead minnows when administered at low doses (Jobling *et al.*, 2003). Although many teleost species are functionally hermaphroditic, the majority of them have separated sexes. Inappropriate exposure to estrogens (or mimics), at the wrong time in the life cycle, and/or at uncharacteristic concentrations, may cause adverse effects (Tyler *et al.*, 1998).

1.3.5 Disruption of Liver Function

A major function of the liver during ovarian recrudescence is the synthesis of vitellogenin (Kime, 1998). The liver in the mature female teleost, is the primary target tissue of 17β-estradiol. Binding of 17β-estradiol to the estrogen receptor in hepatocytes induces the synthesis of vitellogenin (VTG), a glycolipophosphoprotein that serves as the precursor for yolk proteins (Lazier and MacKay, 1993). Although both male and female fish express the ER, only the female is capable of synthesising the steroid 17β-estradiol, which is produced in the ovaries. Male fish have fewer receptors and in the brown trout (*Salmo trutta*) the liver of a sexually mature female contains more than twice as many estrogen-binding sites as that of the male (Pottinger, 1986). Receptors present in male fish are fully functional and capable of binding to EDCs and, hence, VTG synthesis may be induced. In juvenile fish and mature males, exposure to estrogens can induce synthesis of this protein; therefore VTG expression is a good biomarker of estrogenic activity (Purdom *et al.*, 1994). Most xenestrogens act by a similar mechanism but have less affinity for the ER, thus, less potency than 17β-estradiol (Nimrod and Benson, 1996).

VTG expression is an important biomarker in *in vivo* experiments (section 1.4.4), but high levels of exposure to EDCs can be detrimental to fish health, even causing mortality. This mortality is due to a combination of kidney function impairment, diversion of essential nutrients to excess protein production, calcium deficiency (VTG binds calcium), and other factors (Lee Herman and Kincaid, 1988).

1.4 *In Vivo* Bioassays for the Evaluation of Endocrine Disruption in Fish

Much work has been carried out over the last ten years investigating endocrine disruption in fish. These studies have focused on the effects of endocrine disrupting chemicals on individual fish, fish populations and different fish species.

The effects of EDCs on fish include reduced plasma sex steroid concentrations, reduced pituitary gonadotrophin concentrations, decreased gonadal size, decreased fecundity with age, impaired male maturation, absence of secondary sexual characteristics in males, intersex, induced vitellogenesis in juveniles and males, changes in gonad morphology and increased liver size (Munkittrick *et al.*, 1992; Munkittrick *et al.*, 1994; Munkittrick and Vanderkraak, 1994, Harries *et al.*, 2000; Robinson *et al.*, 2003).

Some of the listed effects, as well as being induced by the presence of EDCs, are also subject to seasonal changes. For example gonad size increases as mature fish approach their spawning period and plasma steroid concentrations fluctuate depending on what stage an individual fish is at in its reproductive cycle. Other indicators are affected by pollutants other than the endocrine disrupting chemicals. Increased liver size is a general indication of exposure to a wide range of environmental contaminants. It is important to know as much as possible about the physiological and biological stage at which fish species are sampled in the wild or used in laboratory experiments. It is also useful to have background information on the types of contaminants, other than EDCs, which may be present within the habitat.

In vivo experiments have concentrated on determining which chemicals are estrogenic and at what levels, and on examining the effects of EDCs on the fish themselves. The studies have focused mainly upon intersex, gonad morphology, liver size and vitellogen synthesis in male and juvenile fish. These are generally the most reliable and easily detected biomarkers of endocrine disruption in fish.

1.4.1 Intersex

Intersex is defined as the simultaneous presence of both male and female gonadal characteristics. Such major gonadal abnormalities have been detected in several species of teleost fish following exposure to pharmacological doses of sex steroids or aromatase inhibitors and act as a biomarker of estrogen exposure on wild populations (Piferrer *et al.*, 1994; Gray and Metcalfe 1997, Jobling *et al.*, 1998, Simpson *et al.*, 2000; Stentiford *et al.*, 2003). Intersex occurs during the labile period, that is, the time prior to morphological sex differentiation, just following hatching or at the juvenile stage when the fish is most susceptible to endocrine perturbation (Jobling *et al.*, 1998).

Intersex associated with estrogen mimics is due to the feminisation of genetically male fish rather than to androgenization of genetic females, although exposure to androgens

results in both androgenic and estrogenic effects (Hornung *et al.*, 2004). When examining a male fish the testes are examined for the presence of female gonadal characteristics, such as the presence of female germ cells or oocytes, within the testicular tissue utilising histological examination (Gray and Metcalfe 1997; Nolan *et al.*, 2001). Histological examination involves fixing and embedding testes in paraffin wax, followed by sectioning and staining with Mayers haematoxylin-eosin. The stained sections are then examined by light microscopy.

Levels of intersex vary from multifocal intersex, in which a few or numerous primary and secondary oocytes are observed throughout the testicular tissue, to focal intersex, in which large areas of ovarian tissue are separated clearly from testicular tissue (Nolan *et al.*, 2001). An intersex index has been devised by Jobling *et al.* (1998) for roach (*Rutilus rutilus*) describing the degree of feminisation in each fish, ranging from zero to seven.

1.4.2 Gonad Weight

The inhibition of testicular growth in male fish is a biomarker of estrogen exposure. Studies on rainbow trout (*Oncorhynchus mykiss*) have shown a correlation between the estrogenic potency of an EDC and the degree of inhibition of testicular growth, with certain chemicals reducing testicular growth by as much as 50% (Billard *et al.* 1981; Jobling *et al.*, 1996). Additionally, Billard *et al.* (1981) found that the inhibitory effects varied according to dose, type of steroid, and stage of testicular development at the time of exposure. The gonad size is expressed in relation to body weight, and GSI is calculated as follows:

GSI = Gonadal weight / (total body weight – gonadal weight).

1.4.3 Liver weight

Another biomarker of estrogen exposure is liver size (Lye *et al.*, 1997). Liver size is measured using the hepatosomatic index (HSI), which is the ratio of liver weight to total body weight. The HSI is seen to increase in fish that have been exposed to estrogenic compounds (Herman and Kincaid, 1988; Van Boheman *et al.*, 1982; Nimrod and Benson, 1996; Lye *et al.*, 1997; Thorpe *et al.*, 2000, Harries *et al.*, 1997). The increased liver weight might be due to resultant hepatocyte proliferation, as it has been found that there is a constant protein concentration during the HSI increase (Van Boheman *et al.*, 1982).

1.4.4 Vitellogenesis

Vitellogenesis in male fish has been shown to be an excellent biomarker for estrogenic contamination of the aquatic environment (Sumpter and Jobling, 1995; Thorpe *et al.*, 2000). Vitellogenesis in fish is the process whereby eggs acquire yolk. It involves the synthesis of VTG by the liver and its uptake by growing oocytes, where it is stored as yolk, to act as a nutrient to support the subsequent development of the embryo (Tyler *et al.*, 1998). The production of VTG is estrogen-dependent and therefore usually restricted to females; little, if any, VTG can normally be detected in plasma of male fish (Tyler *et al.*, 1998). However, males do carry the VTG gene, and exposure to both natural and synthetic estrogens can trigger its expression (Le Guellec *et al.*, 1988). The

exposure to estrogens and their mimics particularly alkylphenolic chemicals, has been shown to cause the synthesis and secretion of VTG (Jobling and Sumpter, 1996).

There are several methods of determining VTG levels in fish blood: radioimmuoassay (RIA), enzyme linked immunosorbent assay (ELISA) or two-antibody sandwich assay and SDS polyacrylamide gel electrophoresis (SDS-PAGE). Both RIA and ELISA methods require blood plasma from the fish being examined and SDS-PAGE requires blood serum. The blood is usually collected from the caudal sinus of the fish, although cardiac puncture and tail removal are also used (Panter *et al.*, 2000; Lye *et al.*, 1997). The syringes used to collect plasma contain heparin and an enzyme inhibitor, to prevent the degradation of intact vitellogenin (Sumpter, 1985a). The plasma and serum are obtained by centrifuging the blood and once obtained can be stored at -70^{o}C until analysis.

(i) Radioimmunoassay for Plasma Vitellogenin Concentration
Sumpter (1985a) originally developed an RIA for measuring VTG levels in rainbow trout. This RIA has subsequently been adapted for use on roach (*Rutilus rutilus*) and carp (*Cyprinus carpio*). In the RIA, blood is taken into syringes containing heparin and the enzyme inhibitor phenylmethylsulfonylfluoride (PMSF). The plasma obtained is then fractionated on a column of Sepharose 6B. Fractions containing VTG are then pooled and applied to a Concanavalin A Sepharose column. After all unabsorbed protein has been eluted, adsorbed protein is displaced with buffer containing glucoside. This eluant contains vitellogenin, free of the presence of other blood proteins.

The isolated VTG is iodinised using the oxidising agent Iodogen. After separating the iodinated proteins from any free iodine, the labelled protein is gel-filtrated on Sepharose 6B. A standard RIA protocol is then adopted using anti-rabbit gammaglobulin as the precipitation agent, with antibodies to trout vitellogenin that have been raised in rabbits. Plasma is usually assayed in triplicate at varying dilutions depending upon the vitellogenin level. RIA techniques are very specific and sensitive (Sumpter, 1985a; Tyler and Sumpter, 1990).

(ii) Enzyme-linked ImmunoSorbent Assay (ELISA) for Plasma Vitellogenin
These assays are used to determine the antigen (VTG) concentration in unknown samples. The assays are rapid, accurate and the antigen does not need to be purified before use as required in RIA (Harlow and Lane, 1988).

An ELISA, using a monoclonal antibody to VTG, has been used to determine VTG levels in channel catfish (*Ictalurus punctatus*) using monoclonal antibodies specific to channel catfish (Nimrod and Benson, 1996; Goodwin *et al.*, 1992). An ELISA, using polyclonal antibody for VTG, has also been developed by Bon *et al.* (1997) for rainbow trout.

The two-antibody sandwich assay requires two antibodies that bind to non-overlapping epitopes on the antigen. These may be either two monoclonal antibodies that recognise discrete sites or one batch of affinity purified polyclonal antibodies can be used. One antibody is purified and bound to a solid phase, and the antigen (VTG) in the test solution is allowed to bind. Unbound proteins are removed by washing, and the labelled second antibody is allowed to bind to the antigen. After washing, the assay is quantified

by measuring the amount of labelled second antibody that is bound to the matrix (Harlow and Lane, 1988).

(iii) SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)
The principles of gel electrophoresis are the same for both protein and DNA studies: the separation of macromolecules in an aqueous solution when exposed to an electric field. In protein gel electrophoresis, proteins in a sample are separated based on differences in their net charge (a function of their amino acid sequence), size and shape. In what can be viewed as a molecular sieving process, small molecules move through the gel relatively quickly, whereas larger molecules are inhibited by the gel matrix and thus move through the gel more slowly. Once the proteins are separated in the gel matrix they can be visualised by colorimetrically using Coomassie Blue or silver staining techniques. Alternatively, the separated proteins can be subjected to Western blot analysis. In Western blot analysis, the separated proteins in the gel matrix are transferred to a membrane and antibodies used to detect the amount of a specific protein (VTG).

Lye *et al.*, (1997) used serum samples from flounder containing approximately 40µg protein, suspended in 10µl Laemmelli sample buffer, vortexed and boiled at 100^{o}C for 5 min and centrifuged at 16000g for 5 min. Protein concentrations in the supernatant are determined by the method of Bradford (1976) using a serum volume of 0.1-0.25µL in 1ml of Bradford reagent. A SDS-PAGE is then performed using the method of Laemmelli and Favre (1973), using a Tris/glycine buffer system (Lye *et al.*, 1997).

1.4.5 *In vivo* Studies to Determine the Effects of EDCs on Fish

(i) Wild Fish Studies
The examination of wild fish populations has been carried out in the UK, USA and Europe, looking at roach (*Rutilus rutilus*), common carp (*Cyprinus carpio*), flounder (*Platichthys flesus*), Europena eel (*Anguilla anguilla*) and barbel (*Barbus plebejus*) (Lye *et al.*, 1997; Jobling *et al.*, 1998; Lye *et al.*, 1998; Allen *et al.*, 1999; Lye *et al.*,1999; Viganò *et al.*, 2001; Jobling *et al.*, 2003; Versonnen *et al.*, 2004). In the UK, it was observed that up to 5% of a roach population living downstream of a waste-water treatment plant (WWTP) showed incidence of hermaphroditism (Jobling *et al.*, 1998). This widespread sexual disruption of a riverine roach population occurred from exposure to ambient levels of endocrine disrupting chemicals in the river studied (Jobling *et al.*, 1998). Lye *et al.* (1999, 1998, and 1997) have investigated adult male flounder in the UK downstream of WWTP in the Solway, Tyne and Tees estuaries. The flounder examined showed varying levels of VTG in their plasma, indicative of different levels estrogenic exposure. Varying occurrence of testicular abnormalities also indicated different levels of estrogenic exposure. In the River Po, Italy, intersex barbel have been observed and 50% of fish sampled below a tributary containing EDCs showed intersex gonads (Viganò *et al.*, 2001). It is not clear whether the intersex barbel examined were feminised males or masculinised females, but it is thought that the most likely case is feminised males, which would suggest exposure of the barbel to EDCs at a very early age (Viganò *et al.*, 2001). In the USA, Denslow *et al.*, (1996) described male common carp, collected from an effluent channel, as having significantly elevated vitellogenin concentrations.

(ii) Tank Studies

Tank studies have been carried out on a variety of fish species, including rainbow trout, fathead minnows (*Pimephales promelas*), goldfish, European eel, sand goby (*Pomatoschistus* minutes), channel catfish, guppies (*Poecilia reticulata*), Japanese medaka (*Oryzias latipes*) and roach (Routledge *et al.*, 1998; Billard *et al.*, 1981; Le Guellec *et al.*, 1988; Van Boheman *et al.*, 1982; Nimrod and Benson, 1996; Gray and Metcalfe 1997; Chen 1982; Thorpe *et al.*, 2000; Bjerselius *et al.*, 2001; Rodgers-Gray *et al.*, 2001; Sohoni *et al.*, 2001; Harries *et al.*, 2000; Robinson *et al.*, 2003; and Kinnberg *et al.*, 2003). The rainbow trout has been the most widely used of these species.

In tank studies, fish are kept in tanks of ranging size including 75, 80, 400, 500, 800, 1200, and 1400 litre tanks. The tanks used have been made of varying materials including steel, glass, fibreglass and plastic. The water quality used has also varied between studies with reservoir, bore-hole/well water/ground water, running fresh water, de-chlorinated, deionised, mains tap water, and filtered (reverse osmosis) all having been used. The tank set-ups have also utilised both flow through and recirculation systems. The fish have been fed, exposed to or injected with estrogenic compounds and the different parameters of endocrine disruption have been measured (Le Guellec *et al.* 1988, Nimrod and Benson, 1996; Rodgers-Gray *et al.*, 2001). Pickford *et al.*, (2003) found that EDCs such as 4-nonylphenol has a higher estrogenic potential when it enters the bloodstream via the gills/skin of a fish compared with exposure through diet. In these *in vivo* experiments the expression of vitellogenin in male and juvenile fish, the GSI index, HSI index and occurrence of intersex are the end points examined (Thorpe *et al.*, 2000; Rodgers-Gray *et al.*, 2001; Sohoni *et al.*, 2001).

Using such tank studies it is possible to identify estrogenic chemicals and examine the effects of exposure on fish (Sheahan *et al.*, 1994; Panter *et al.*, 2000; Thorpe *et al.*, 2001). The chemicals and compouds that have been tested include 17α-methyltestosterone, 17β-estradiol (E_2) and estrone (E_3), 17α-ethinylestradiol (EE_2), 4-nonylphenol (NP), 4-tert-octylphenol (OP), 4-nonylphenoxycarboxylic acid (NP_1EC), nonylphenoldiethoxylate (NP_2EO), technical nonylphenol, 4n-nonylphenol (4n-NP), 4n-octylphenol (4n-OP), and bisphenol-A (BPA). These tank experiments have also been used to test effluents from oil refineries treatment works and WWTP (Knudsen *et al.*, 1997).

(iii) Cage Studies

Cage studies, involving the placement of male fish in effluent stream and rivers have been carried out in the UK and Italy. Previous experiments have looked at estrogenic activity in WWTP effluents (Harries *et al.*, 1999), downstream of WWTP (Harries *et al.*, 1996), and in inland waters (Harries *et al.*, 1996). These studies have used caged rainbow trout, which have been shown to be sensitive to estrogen exposure, as indicators of the levels of estrogenic activity (Purdom, *et al.*, 1994; Harries *et al.*, 1996; Harries *et al.*; 1997).

The cages used are made of galvanised steel and usually have dimensions around (1.24 x 0.5 x 0.5 m). These cages may contain between 10 and 30 fish, usually mature male rainbow trout in the 200-300 g weight category, although mixed stocks have been used. The fish used in the cage trials are obtained from local fish farms and kept in bore hole

or de-chlorinated water for 2 - 3 weeks prior to the trial. This period takes into account that the fish may have been exposed to EDCs previously, allowing for the depuration of any chemicals present as well as the breakdown of any VTG that may be present in the blood of the male fish.

During the trials the cages are left in the watercourse to be investigated for 2-3 weeks. The cages are normally located at the source of the suspected input of EDCs, downstream of the source and upstream of the source. The upstream site is a control site, this is to make sure that there is no other input of EDCs into the watercourse other than that being specifically tested. Several cages may be located downstream of a suspected source to investigate dilution effects.

As with the tank experiments, the end points in these *in vivo* experiments are VTG expression in male fish and any morphological changes in the gonads and/or liver. Samples are usually taken when the fish arrive from the farm, prior to being placed *in situ* and at the end of the trials. Five hundred- to fifty thousand-fold increases in plasma VTG concentrations have been seen if fish are exposed to EDCs and plasma VTG levels as high as 10^4 μg/ml. These effects have been seen as shortly as after the first week of the experiment. Rodgers-Gray *et al.* (2000) has shown that estrogenic responses in wild fish cannot be predicted by short term exposures using caged fish as there are long-term fluctuations in the concentrations of the estrogenic components of WWTP effluents. To combat this, trials are often repeated at a low water flow and a high water flow, and in conjunction with wild fish surveys.

1.5 *In vitro* Bioassays for the Evaluation of EDCs in Environmental Samples

In vitro bioassays are vitally important when an integrated approach (i.e. a Toxicity Investigation and Evaluation (TIE) scheme) is taken to the study of endocrine disrupting compounds in the aquatic environment. While the *in vivo* assays described in section 1.4 are critical for providing evidence for cause-effect relationships, they are generally impractical for routine screening of environmental samples (Snyder *et al.*, 2000).

To this end *in vitro* bioassays such as human cell line, competitive binding, vitellogenin and yeast assays have been developed to measure potential estrogen activity in a variety of environmental and clinical matrices. The rapidity, inexpensiveness and reproducibility of *in vitro* bioassays make them extremely attractive as screening tools. Limitations of *in vitro* bioassays are reflected by an inability to represent the pharmokinetics, biotransformation, and binding to carrier proteins of a compound and they may therefore miss effects that would only take place in intact organisms (Snyder *et al.*., 2000; US National Research Council, 1999). It is impossible, therefore, for one *in vitro* bioassay to be capable of predicting all the potential *in vivo* responses elicited by an (xeno)estrogen. The sensitivity, advantages and limitations of each individual *in vitro* bioassay reviewed in this section, all of which still require validation and standardisation, are described briefly in Table 1.5.1. The *in vitro* bioassays described here represent those most extensively employed, to date, to detect chemicals with estrogenic activity, however, many more bioassays have been developed (for examples see Gray *et al*., 2002 and Mueller, 2002). In addition new techniques for estrogen detection are continuously being developed e.g.. total internal reflection fluorescence (TIRF) and energy transfer immunoassay (ETIA) (Coille *et al.*., 2001).

Table 1.5.1
The sensitivity, advantages and limitations of the individual *in vitro* bioassays for estrogenic endocrine disruption

	Sensitivity	**Advantages**	**Limitations**
Human cell line assays (MCF-7 cell proliferation)	3 x 10^{-11} M of estradiol.	Can detect agonists and antagonists; Easy to perform; Can screen compounds over a concentration range.	Non-estrogenic mitogen interference; Assay duration; Endogenous estrogens; MCF-7 cell metabolic capacity, Reproducibility (multiple sub-clones and different assay conditions).
Vitellogenin assays	1 x 10^{-9} M of estradiol.	Solely directed at wildlife monitoring.	Limited detection of vitellogenin protein; Assay duration; Similarities in responsiveness.
Competitive binding assays	Variable.	Direct assessment of chemical binding to the estrogen receptor; Assay duration.	Does not measure ER activation; Cannot distinguish between agonists and antagonists; Inability to account for metabolism; Unsuitability to automation; Non-competitive displacement at high concentrations.
Yeast screen assays (YES)	1 x 10^{-11} M of estradiol.	Can detect agonists, partial agonists and antagonists; Partial metabolic competence; Monitoring of environmental samples; Reliability; Robustness; Cost-effectiveness; Assay duration.	Toxicity of certain chemicals; Limited ability of some chemicals to cross the yeast cell wall; Chemical sorption to the plastic plates; Creeping of certain chemicals.

1.5.1 Human cell line assays

Cells lines derived from estrogen-responsive tissue of human origin (e.g.. MCF-7, Ishikawa, MDA-MB-231, CL10A, T-47D and HeLa cell lines) can be used to identify chemicals with estrogenic activity. The MCF-7 cell line has recently been recommended for further development and validation as an *in vitro* screening tool by the Organisation for Economic Cooperation and Development (OECD, 2001) and is therefore discussed in detail below.

(i) MCF-7 Cell Lines

MCF-7 cells represent an estrogen-related human cell line screen, which demonstrates the potential effects of suspect estrogenic chemicals in humans and to date is the most commonly used human-derived cell line for the assessment of estrogenic activity. MCF-7 cell line assays are easy to perform and can be used to screen compounds over a range of concentrations. As such a large number of natural and synthetic chemicals have been tested using this cell line, including natural and synthetic hormones, some pesticides, PCBs, plasticisers, phytestrogens and animal foodstuffs (OECD, 2001).

Various workers have developed a range of endpoints to assess the cellular responses of MCF-7 cells to potential estrogenic compounds. Endpoints include measurement of the induction of cell proliferation, levels of estrogen receptor or it's mRNA, levels of specific (estrogen inducible) exoproteins (e.g.. pS2) or even, in a transfected sub-strain of MCF-7, activity of a luciferase enzyme. Endpoints can be measured by using a Coulter counter; enzyme immunoassay kits; gel electrophoresis and a luminometer, respectively (OECD, 2001). MCF-7 cell line assays are very sensitive e.g.. the cell proliferation and transfected luciferase assays can detect 3×10^{-11} M and 5 pg/ml of estradiol or equivalent activity, respectively (OECD, 2001; Beresford *et al.*, 2000).

Cell proliferation is the most established endpoint of assays employing MCF-7 cell lines. The principle of cell proliferation assays is that estrogen induces the proliferation of MCF-7 cells by neutralising the inhibitory effect of anti-proliferation molecules present in human serum. As such, the proliferation response is not dependent on the interaction of estrogens with the estrogen receptor. Non-estrogenic steroids and growth factors do not abolish the inhibitor effect, thus, only xenestrogens and estrogens elicit a proliferation response (Dempsey and Costello, 1998). Soto *et al*. (1992) developed a MCF-7 cell proliferation assay referred to as the E-Screen which compares the number of cells present following a 6-day incubation period in medium supplemented with steroid stripped dextran-coated charcoal serum in the presence or absence of suspect chemicals. By comparing the proliferation effects of the xenestrogen and estrogen, the relative estrogenic potency of a compound can be determined. Some authors have found the endpoint of the cell proliferation assay to be responsive to several non-estrogenic mitogens including bile acids, insulin-like growth factor and epidermal growth factor. As a result of these observations, it has been proposed that the assay suggests, but does not equivocally demonstrate, the estrogenic activity of a substance. The E-Screen can detect either estrogenic agonistic or antagonistic activity, however, the E-Screen is limited as a rapid screening tool as the induction of proliferation takes several days to assess. The transfected MCF-7 luciferase assay, which also can detect agonists and antagonists, can overcome this time constraint as luciferase can be measured 24 hours after incubation (OECD, 2001).

Assays utilising MCF-7 cells require the removal of endogenous estrogens from human and fetal bovine serum, hence the use of dextran-coated charcoal in the E-Screen. Although such treatment may remove greater than 99% of the endogenous estrogen, a sufficient amount may remain to elicit an estrogen-dependant response. Also of concern is the limited information available regarding MCF-7 cell metabolic capacity, which raises the question of the estrogenicity of parent compounds, verses their metabolites e.g.. PCB parent compound is not estrogenically active whereas PCB metabolites are estrogenically active. This limitation of MCF-7 assays can be overcome by using liver microsomes either before the assay or incorporated into the assay procedure (OECD, 2001). The reproducibility of MCF-7 cell line assays is compromised due to the presence of multiple sub-clones (e.g.. MCF-7 BUS, MCF-7 UCL and MCF-7 SOP) and different assay conditions (Payne *et al*., 2000). Indeed the most important variables affecting interlaboratory reproducibility and standardisation of MCF-7 cell line screens are cell stock uniformity and culture conditions (Zacharewski, 1997).

(ii) Vitellogenin Assays

Vitellogenin induction in trout hepatocytes represents an *in vitro* assay, which is solely directed at wildlife monitoring. In oviparous organisms, estrogen secretion by the ovaries activates vitellogenin gene expression in the liver, causing it to produce and excrete vitellogenin proteins. As described in section 1.4.4, vitellogenin is a large lipoglycophosphoprotein that contains one zinc atom and two calcium atoms per molecule. In female fish vitellogenin is normally secreted by the liver in response to endogenous estrogens and is carried by the blood to the ovaries, where it is selectively sequestered by the developing oocytes. In the ovaries vitellogenin forms the major constituent of yolk, supplying part of the energy reserves for the fertilised egg and yearlings. Although males do not produce vitellogenin, they have a functional vitellogenin gene that can be activated by (xeno)estrogens (section 1.4.4) (Gagne and Blaise, 1998; Jobling and Sumpter, 1993).

The *in vitro* vitellogenin bioassay was initially performed by Pelissero *et al.*., (1993) and was subsequently developed by Jobling and Sumpter, (1993 and 1995) and White *et al*. (1994). The bioassay is based on the ability of primary cultures of rainbow trout (*Oncorynchus mykiss*) hepatocytes to respond to estrogenic stimulation by producing vitellogenin protein. The endpoint of the assay is the amount of vitellogenin protein produced, which can be determined by mono- or polyclonal antibody-based methods e.g.. ELISA and RIA. In addition to the detection of vitellogenin protein mRNA can be quantified using molecular methods such as Northern blot analysis using specific DNA probes. The test requires less than a week (approx. 6 days) to perform and the data obtained for a test compound is compared to the system's response to estradiol thereby identifying chemicals with estrogenic activity (Gagne and Blaise, 1998; Jobling and Sumpter, 1993).

The response of the vitellogenin bioassay to a large number of chemicals has been examined and the assay was found to give good sensitivity and specificity. Pelissero *et al*. (1993) reported that 1 x 10^{-9} M of estradiol was the minimal detectable signal whilst 1 x 10^{-6} M was required for a maximal response. However, some similarities in responsiveness between a number of phytestrogens and some androgenic /progestational agents were observed leading to questions over the assay specificity. Also the detection of vitellogenin protein is limited within and across species due to variations in the immunological and structural properties of the protein. However, it may be possible to develop antibodies against conserved protein regions that could be used for a number of species while maintaining acceptable sensitivity (Zacharewski, 1997). These characteristics have lead the OECD (2001) to recommend the assay as being a possibly candidate suitable for further progression.

1.5.2 Competitive Binding Assays

In vitro competitive binding assays for the ER are well established and have been extensively used to investigate estrogen receptor-ligand interactions (Zacharewski, 1997). In competitive binding assays cytosolic ER preparations (derived from estrogen-responsive tissues such as rat uteri) are incubated with a radioactively labelled estradiol and with varying concentrations of an unlabelled test compound (Ashby *et al.*, 2000). Analysis of kinetic-binding data can be performed to determine half-inhibitory (IC_{50})

concentration values, which define the concentration of the test compound required to displace 50% of the radioligand (US National Research Council, 1999).

In vitro competitive binding assays detect the ability of a chemical to bind with a particular receptor with the inference that high binding affinity would potentially result in marked biological activity of some type. However, binding to the ER alone is not sufficient to determine the estrogenic potency of a compound, as potency is dependent not only on affinity for the receptor but also on the ability of the ligand to activate the receptor initialising a cascade of events that culminate in an adverse response. The binding of a substance to the ER, therefore, is only suggestive that it may be estrogenic but does not provide sufficient evidence to conclude that the compound will adversely affect human heath or environmental quality (Schultz *et al.*., 2000; Zacharewski, 1997). This inability of the ER binding assay to address post-receptor interactions also indicates that the assay cannot distinguish between receptor agonists, antagonists and mixed effects. To over come these confines of binding assays additional *in vitro* tests would be required to fully assess the potential risks of a test chemical (Ashby *et al.*., 2000; Zacharewski, 1997).

Additional limitations of binding assays include an inability to account for metabolism or excretory processes that may alter parent chemicals *in vivo*, non-competitive displacement of competitor ligand at high sample concentrations and unsuitability to automation which limits their utility as a screening tool (Jobling, 1998; Zacharewski, 1997). Despite these limitations *in vitro* competitive binding assays can be useful in providing an initial assessment of whether a suspect chemical or its metabolites/degradates are likely to bind to the ER (Jobling, 1998). However, it is questionable if such studies can play a role as a general estrogenic screen and the OECD (2001) reflected this opinion when they deemed it unadvisable to employ binding assays as a screening tool.

1.5.3 Yeast Screen Assays

In recent years, transfection techniques have been developed which permit the controlled insertion of hormonal receptor structures from various animal species, including humans, into yeast cells. This has lead to the development of recombinant receptor-reporter gene yeast screens for estrogenically active chemicals in which the activation of the ER results in the induction of a reporter gene. Various yeast screen models exist which vary with respect to the variety of strain, construct, substrate and culture methodology (for examples refer to Arnold *et al.*., 1996; Connor *et al.*., 1996; Maier *et al.*., 1995; Klein *et al.*., 1994; Berry *et al.*., 1990). ER-expressing transfected yeast models are capable of high levels of sensitivity, can give close correlation with other model systems and have been shown to demonstrate dose-response relationships for a wide range of chemicals (OECD, 2001).

Many potential benefits exist relating to the use of a yeast cell (*Saccharomyces cerevisiae* being the most commonly employed) as a culture system. Firstly the basic morphology, biochemistry, physiology and genetic structure of yeast cells are well established. Secondly yeast is widely available, is easily cultured and has a rapid growth rate. Additionally, yeast has a wide range of suitable plasmids and promoters available, is easily transformed and transformed cells are easily selected and maintained. Also the

absence of sex hormone receptor mechanisms in the untransformed organism overcomes the complex interplay that exists between the ER and receptors of other steroids and growth factors making yeast an attractive model for estrogen studies (OECD, 2001; Dempsey and Costello, 1998; Routledge and Sumpter, 1996).

(i) Yeast Estrogen Screen (YES)

Most of the published examples of yeast estrogen assays are based on yeast cells transformed with the human ER cDNA and an estrogen response element (ERE)-regulated *lac-Z* reporter gene that encodes a β-galactosidase enzyme (Jobling, 1998). One such assay for the detection of estrogenic compounds (i.e. YES) utilises the recombinant yeast strain (Figure 1.5.1) developed by the Genetics Department of Glaxo (Stevenage, Harts, UK). To generate the recombinant yeast the DNA sequence of the human estrogen receptor-α (hERα) was stably integrated into the main chromosome of the yeast (*Saccharomyces cerevisiae*). The ER is a member of a superfamily of transcription factors and activation of ER by estrogen is similar in yeast and mammalian cells (Arnold *et al.*., 1996). At least two different ER exist in humans and other higher organisms, ERα is involved with cell proliferation in breast and uterine tissue, whereas ERβ is involved with osteoblasts (Gutendorf and Westendorf, 2001). Expression plasmids carrying the reporter gene *lac-Z* (encoding the enzyme β-galactosidase) were also integrated into the yeast cells. In YES, the hERα is expressed in a form capable of binding to ERE's which are situated within a strong promoter sequence on the expression plasmid. Upon binding an active ligand, the estrogen-occupied receptor interacts with the ERE's to modulate *lac-Z* gene expression. β-Galactosidase is therefore synthesised and secreted into the medium, where it metabolises the chromogenic substrate, chlorophenol red β-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured spectrophotometrically at 540 nm (Routledge and Sumpter, 1996).

The YES provides a sensitive and specific process for detecting estrogenic activity and the screen has been partially validated using a range of steroids and steroid metabolites. The screen can detect estradiol at concentrations above 1×10^{-11} M and as such contamination by a weak estrogen can significantly affect the results. The assay can be employed to investigate the agonistic, partial agonistic and antagonistic hormonal activities of chemicals. However, standardisation of the assay is required as changes in method can make the difference between labeling a sample a partial agonist or a full agonist. Also the assay system cannot detect all antagonists, as yeast may not contain all the repressors necessary for antagonism. Synergism and additivity between chemicals can also be revealed using the assay (Beresford *et al*., 2000; Schultz *et al*., 2000; Sohoni and Sumpter, 1998; Harris *et al*., 1997).

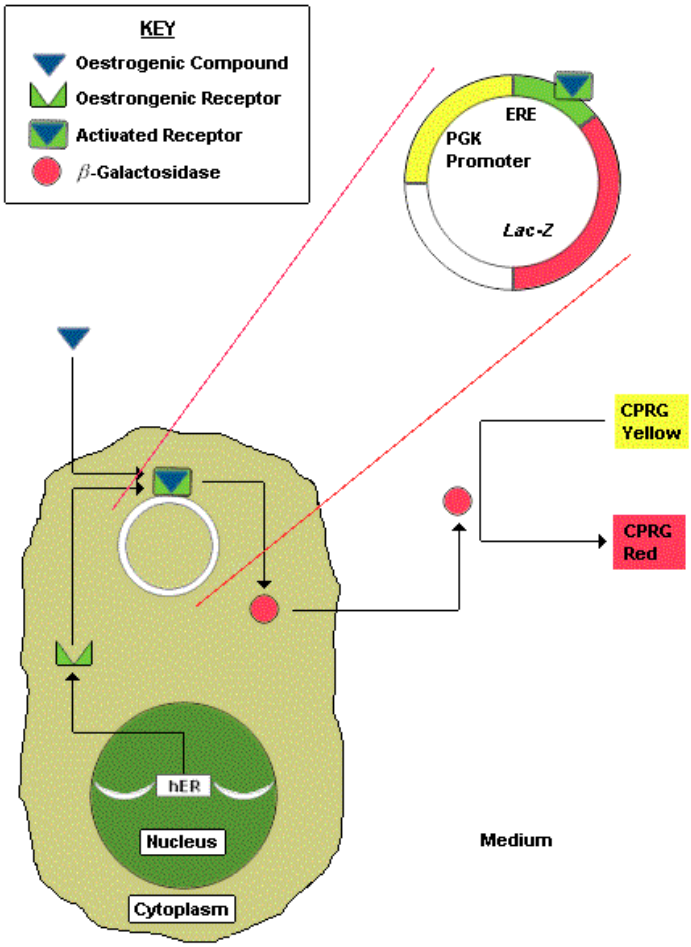


Figure 1.5.1
Schematic of the Yeast Estrogen Screen (YES)
The hER is integrated into the main genome and is expressed in a form capable of binding to EREs within the hybrid promoter on the expression plasmid. Activation of the receptor, by binding of the ligand, causes expression of the reporter gene *lac-Z*, which produces the enzyme β-galactosidase. This enzyme is secreted into the medium and metabolises the chromogenic substrate CPRG (normally yellow) into a red product, measured by absorbance (Routledge and Sumpter, 1996).

Several yeast based assays offer some degree of metabolising competence, but this will be different from that found in animals. The YES fails, therefore, to account for the toxickinetic and toxicodynamic complexity of the whole organism and specifically it lacks the ability to metabolise all chemicals to their active form (Schultz *et al*., 2000). However, the assay does exhibit partial metabolic competence due to the presence of cytochrome P450. Examples of yeast metabolic competence include methoxychlor demethylation and selective esterase activity. Therefore, the YES has some intrinsic metabolic capacity however metabolites should be tested along with parent compounds to remove the possibility of mislabeling prestrogens. Also the combination of liver microsomal incubations either before the assay or incorporated into the assay procedure can introduce metabolism into the final *in vitro* screening assay (Elsby *et al*., 2001; Beresford *et al*., 2000; Harris *et al*., 1997; Odum *et al*., 1997).

The YES is prone to certain disadvantages such as the toxicity of certain chemicals (e.g.. surfactant AE3) to the yeast cells and the established problem of the limited ability of some chemicals to cross into the cell through the specialised cell walls found in yeast. Additional problems include chemical sorption to the plastic plates e.g.. *op'*-DDT and the creeping of certain chemicals e.g.. alkylphenols (APs) and PCBs across the assay plate which leads to underestimation of the potency of the creeping chemical and false positives in adjacent wells (Beresford *et al.*, 2000; Sohoni and Sumpter, 1998; Routledge and Sumpter 1996).

Yeast assays perform better monitoring of environmental samples than animal cells as these samples are usually contaminated with other substances interfering with the growth and viability of animal cells, but not with yeast cells (Graumann *et al*., 1999). The assessment of complex environmental samples with the YES, however, can be subject to considerable interferences e.g.. the presence of humic acids results in a decrease of the estrogen response. Also synergistic activation of the ER is a concern given the biochemical complexity and the wide variety of synthetic chemicals that may be present in environmental samples. Therefore, the assay should be used in combination with chemical screening for estrogen testing of environmental samples (Tanghe *et al*., 1999).

The YES is a powerful estrogen-screening tool due to its high degree of specificity, extreme sensitivity, reliability, robustness, cost-effectiveness and the short duration of the assay period. The assay is capable of detecting agonists, partial agonists and antagonists and to date it has produced no false-negative or false-positive results (Tyler *et al*., 2000; Sohoni and Sumpter, 1998). Indeed the OECD (2001) recommended the YES for further development and validation in a recent report.

The YES has been used to determine the relative estrogenic potency of numerous pure chemicals, many of which have been shown to be present in the aquatic environment. A summary of the estrogenic potency of these chemicals, relative to the model estrogen 17β-estradiol, is provided in Table 1.5.2.

Table 1.5.2
Relative potencies of various chemicals determined by the YES

Chemical	Description	Relative potency*	Reference
Estrone	Natural Estrogen	0.5	
Estriol	Natural Estrogen	0.0033	
17α-Estradiol		0.025	Routledge and Sumpter, 1997
Diethylstilbestrol	Synthetic Estrogen	0.25	
Dihydrotestosterone	Androgen	0.00025	Beresford *et al.*, 2000.
Methyl Paraben		0.0000004	
Ethyl Paraben		0.0000067	
Propyl Paraben	Preservatives	0.000033	Routledge *et al.*., 1998
Butyl Paraben		0.0001	
Dodecyl Paraben		-	
Bisphenol A	Plasticiser	0.0001	Beresford *et al.*, 2000.
Propyl Phenol	Alkyl Phenols	0.00000005	
4-t-Butyl Phenol		0.00000067	
4-t-Amyl Phenol		0.00001	
4-t-Hexyl Phenol		0.00017	Routledge and Sumpter, 1997
4-t-Heptyl Phenol		0.00033	
4-t-Octyl Phenol		0.007 - 0.001	
4-t-Nonyl Phenol **		0.000033	Routledge and Sumpter, 1997
		0.00025	Beresford *et al.*, 2000;Tanghe *et al.*, 1999;
		0.00014	Routledge and Sumpter, 1996
		0.00020	Gaido *et al.*, 1997
Nonyl Phenol Di-Ethoxylate	Alkyl Phenol Derivatives	0.00002	
Nonyl Phenol Mono-Carboxylic Acid (NP_1EC)		0.00004	Routledge and Sumpter, 1996
Nonyl Phenol Di-Carboxylic Acid (NP_2EC)		0.00004	
Methoxychlor	Pesticide	0.0000125#	Routledge and Sumpter, 1996
Diethyl phthalate	Plasticiser	0.0000005	
Dibutyl phthalate		0.0000001	
Diisobutyl phthalate		0.0000001	Harris *et al.*, 1997
Di-tridecyl phthalate		0.0000001	
Butyl benzyl phthalate		0.0000010	
Fenpropathrin	Pyretheroid Insecticides	0.00000005	Tyler *et al.*., 2000
Permethrin			
O,p -DDT	Pesticide	0.00025#	Routledge and Sumpter, 1996.
Genestein	Phytoestrogen	0.0001	

***** *Relative potencies shown are representative of magnitude potencies less than 17β-Estradiol. Variations in measured potencies may occur in the literature these differences can be attributed to varying levels of purity and isomerisation of the chemicals tested.* #*Sub-maximal response achieved. ** Industrial production of Nonyl Phenol results in a mixture of isomeric homologues (see section 1.6.2.2) that have been shown to have different estrogenic potencies. This will inevitably lead to batch-to-batch differences in the estrogenic potency of the final product or standard used for testing with the bioassay.*

1.6 The Environmental Chemistry of Potentially Endocrine Disrupting Chemicals

1.6.1 Introduction

The list of chemicals that are known to or are suspected of producing endocrine disrupting effects *in vivo* or *in vitro* is expanding rapidly (see section 1.2.2). There are however, few cases where endocrine disorders have been unequivocally linked to endocrine disrupting chemicals, primarily due to the biological complexity of living organisms and the environmental systems studied. The definition of what constitutes a hormone-disrupting compound is wide enough to include a large number of chemicals, both organic and inorganic. Many of the contaminants that have historically been examined for endocrine disrupting activity have been banned (e.g.. many organochlorine and organophosphorous pesticides, polychlorinated biphenyls) or their use has been restricted. Those in use are included in the pollution emission registers that must be submitted by users as part of the industrial pollution control (IPC) licensing system overseen by the Environmental Protection Agency (EPA) in Ireland. Due to their environmental stability the residues (either parent compounds or metabolites) of these compounds are now ubiquitous in almost all environmental compartments studied. Of greater concern, perhaps, are those chemicals that have recently been shown to disrupt normal endocrine system functioning but which are still used in large amounts in a wide range of uncontrolled processes. Many have been found to be estrogenic by chance (i.e alkylphenolics, bisphenol A and phthalates) which suggests that there may be many more chemicals currently considered as safe on the basis of standard toxicity tests but which are able to cause an effect on the endocrine system, whose degree of useage and environmental behaviour following release is relatively unknown.

In December 1999 the European Commission (EU) adopted a 'Community Strategy for Endocrine Disruptors' (COM (1999) 706), with a key short term objective being the establishment of a priority list of substances for further evaluation of their role in endocrine disruption. The result of this effort is the establishment of a candidate list of 553 man-made substances and 9 synthetic and natural hormones as detailed in the EUs progress report (COM (2001) 262 final) published in June 2001 and in the main report commissioned by the EU and prepared by BKH Consulting Engineers, The Netherlands (BKH, 2000). Of these 553 candidate substances, 118 were deemed to have evidence of endocrine disruption or potential endocrine disruption. Of these 118, 109 were already subject to bans or restrictions or were being addressed under existing EU legislation, although in many cases for reasons not linked to endocrine disruption.

The main purpose of this section is to provide an overview of the useage, chemistry and environmental behaviour of those chemicals or groups of chemicals that have most commonly been linked to endocrine disruption in wildlife populations. When discussing these chemicals reference will be made to their classification as described in the EU report (COM (2001) 262 final). With regards to endocrine disruption, compounds with estrogenic activity have generally received more attention in the recent scientific literature than compounds that disrupt the normal functioning of other parts of the endocrine system. In the review provided below the focus is mainly upon estrogen mimicking compounds although chemicals that cause other types of endocrine dysfunction are also detailed. The aim is not to cover all aspects of all chemicals which may cause some form of endocrine disruption, but to detail those which have been

designated as 'target compounds' in the Irish context and as originally detailed by Desbrow *et al.*, 1998 and Thomas *et al.*, 2000. The compound groups have been selected using the following criteria: they are shown to be highly estrogenic by a recombinant yeast (YES) assay; they are weakly estrogenic but are likely to be present at relatively high concentrations in Irish wastewaters, leachates and surface waters.

These target compounds are:
Group 1: Alkylphenol ethoxylates and their neutral metabolites
Group 2: Steroid sex hormones and Related Synthetic Compounds
Group 3: Bisphenol A
Group 4: Phthalates
Group 5: Pesticides and Herbicides

Analytical methods within the TIE scheme will be designed to optimise the analysis of the above components. Using such a scheme any compound present in the sample exhibiting estrogenic properties will be identified independent of whether or not it was previously designated as a 'target compound'.

Several reviews on endocrine disrupting chemicals have been published recently and these have been used in part to prepare the following section and where necessary have been updated with more recently published studies. The reader is referred to the following reviews: An Overview of the Endocrine Disrupters Issue (Phillips and Harrison, 1999); A review of Hormonally Active Agents in the Environment (National Research Council, 1999); A Review of the Estrogen Mimicking Chemicals in Relation to Water Quality in Ireland (Dempsey and Costello, 1998); reviews of the environmental occurrence and aquatic toxicology of alkylphenols and alkylphenol ethoxylates (Bennie, 1999; Servos, 1999); a review of the fate and behaviour of Bisphenol A (Staples *et al.*, 1998); a review of the environmental fate and behaviour of phthalates (Staples *et al.*, 1997); a UK Environment Agency (EA) Strategy document on endocrine disruptors (Environment Agency; 1999); an EA review of Human Pharmaceuticals in the Environment (Environment Agency, 2000); and the European Union progress report on its Community Strategy for Endocrine Disruptors (COM (2001) 262 final).

1.6.2 Alkylphenol Ethoxylates

1.6.2.1 Useage and Legislative Status

4-Alkylphenol polyethoxylates (APEOs) are non-ionic surfactants particularly suited for use wherever the interfacial effects of detergency, (de)foaming, (de)emulsification, dispersion or solubilisation can enhance product or process performance (de Voogt *et al.*, 1997). As a result of these properties APEOs have become the second largest group of non-ionic surfactants in production since their initial introduction in the 1940s. The most commonly used APEOs are nonylphenol polyethoxylates (NPEOs) that represent 80-90% of APEO usage with octylphenol polyethoxylates making up the remaining 10-20% (CES, 1993; Renner, 1997). Data from 1990 showed that UK consumption was 16,000 - 19,000 tonnes, while later figures for Ireland suggest that they are used to a lesser extent with 3940 and 2290 tonnes being imported in 1997 and 1998 respectively (CTC, 1999). The main areas of application of NPEOs in the UK market are listed

below in Table 1.6.1. The table also includes an estimate of the proportions of nonylphenol (NP) waste derived from each industrial sector in Europe (CTC, 1999).

Table 1.6.1.
Areas of use of NPEOs and potential for production of NP waste in the UK and European Union.

Area of Use	Approx % of Total NPEO Production *	Approx % of Environmental NP Burden **
Industrial and Institutional Cleaning	50	45
Personal Domestic	- [a]	9
Paint Formulation	12	- [a]
Textile Industry	5	15
Leather Industry	- [a]	6
Agrochemical Industry	12	1
Metal Finishing	5	1
NPE production	- [a]	6
Pulp and paper production	- [a]	2
Wetters in Miscellaneous Industry	16	15

*Key - * UK figures, 1990 (CES, 1993), ** an estimate of the % of nonylphenol waste generated by each sector, a - <1% and therefore included in miscellaneous industry figure.*

A combination of laboratory and field studies on rainbow trout and roach have suggested that the production of the female egg yolk precursor lipoprotein, vitellogenin, and observed abnormal testicular development may be related to the presence of APs and APEOs, in combination with other hormonally active compounds (Purdom *et al.*, 1994; White *et al.*, 1994; Harries *et al.*, 1996; Jobling *et al.*, 1996; Desbrow *et al.*, 1998).

The Oslo and Paris Commission (OSPAR) recommended that the use of APs and APEOs in domestic cleaning products be phased out by 1995. In many countries, including Ireland, these compounds are now no longer used in these products. In the Irish industrial sector NP is used by adhesive manufacturers (383 tonnes in 1996), ink manufacturers (468 tonnes in 1998) and in the production of organic and organometallic chemicals (2356 tonnes per annum). NPEOs, which are not manufactured in Ireland, are predominately used in industrial detergents, with an estimated 200 tonnes being imported for this purpose (CTC, 1999). NPEOs are also used in the manufacture of water based emulsion paints, however no information on the amounts used in this sector is available (CTC, 1999). The OSPAR commission recommended that the use of NPEOs in industrial cleaning products be phased out by 2000. In the Cork area at least two companies are thought to use AP or APEO type compounds in their production processes, although there may be more companies yet to be identified. In the EU candidate substances list, both NP and OP are defined as category 1 endocrine disruptors (at least one study provides evidence of endocrine disruption in an intact organism). Octylphenol (OP) is one of nine substances in the EU list which was highlighted due to the lack of restriction of use or which is not being addressed under current EU legislation. Over the next 12-18 months it is envisaged that further detailed studies of the endocrine disrupting properties of OP will be addressed through the EU research programme (COM (2001) 262).

1.6.2.2 The Chemistry of APEOs

APEOs are synthesised from phenol by alkylation of the carbon para to the hydroxyl group of the phenol with a mixture of either nonene isomers to form NP or 2,2,4 - trimethylpentane to form OP (Theile *et al.*, 1997). The chemical structure of NP and related compounds are given in Figure 1.5.1. In the case of NP the presence of different nonene isomers in the reaction mixture leads to the formation of a complex mixture comprising more than 31 NP isomers each with differently branched structures of the alkyl side-chain. Each isomer may have different estrogenic potency (Yamashita *et al.*, 1999) although few studies have so far been carried out to elucidate structural relationships (Gerhartd, 1979; Bhatt *et al.*, 1992). In contrast, OP is formed as a single isomer product (Renner, 1997). APEOs are formed from the parent alkylphenols (AP) by ethoxylation using a Potassium Hydroxide/Ethanol catalyst and supplying a known ratio of ethylene oxide to AP. The resulting products are mixtures of homologues with varying lengths of the ethoxy chain following a Poisson distribution. The products are named by identification of the mean ethoxylate chain length e.g. Nonylphenol polyethoxylate 15 ($NPEO_{15}$) is a mixture of NPEOs with an average chain length of 15 ethoxylate units.

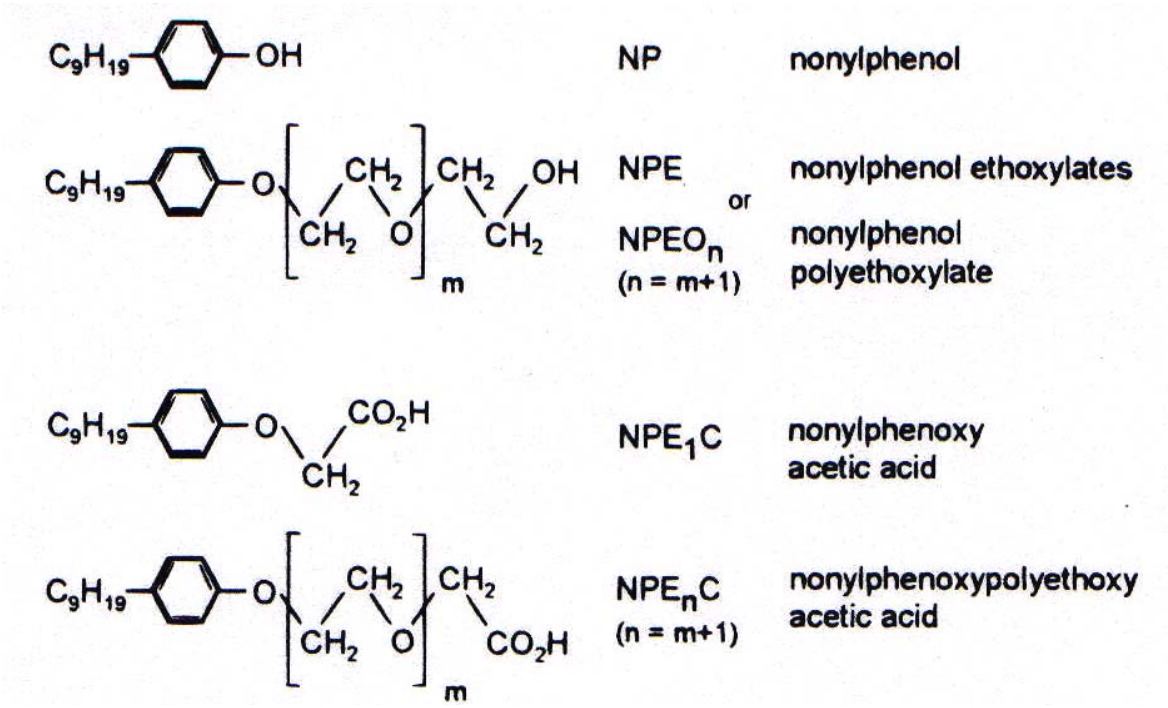


Figure 1.6.1
Structures of nonylphenol and related compounds

1.6.2.3 Environmental Behaviour of APEOs

The relative complexity of APEO formulations and the formation of a range of degradation products makes the chemical analysis and environmental risk assessment of these compounds difficult. As trade effluents containing these chemicals enter wastewater treatment plants or are released directly into the aquatic environment biodegradation, leading to the formation of metabolites with different properties, and fractionation of these metabolites into different environmental matrices will occur. The metabolites of APEOs are often more toxic to aquatic organisms than the original formulations themselves and several have been shown to be estrogenic (Servos *et al.*, 1999; Jobling *et al.*, 1996). The physical behaviour of these compounds within treatment works and subsequently following their release into the aquatic environment (see section 1.6.2.3) is highly dependent on their physico-chemical properties. The most important of these properties are water solubility (S), lipophilicity (Log Kow) and volatility (Henrys Law constant), and these, where available in the literature are shown in Table 1.6.2. The elimination of APEOs from wastewater treatment plant influents is often incomplete (this varies with the type and efficiency of treatment) and the majority of APEOs and their metabolites reach the aquatic environment via this route. A graphical summary of the environmental behaviour of APEOs in treatment works and following their release into the aquatic environment is given in Figure 1.6.3.

Table 1.6.2
Physico-chemical properties of APEOs and related compounds

Compound	Water Solubility S (mg/L) [a]	Lipophilicity Log K(ow) [b]	Volatilty Henrys Law Constant [c]
NP	5.43	4.48	435 Pa.m3/mole
$NPEO_1$	3.02	4.17	-
$NPEO_2$	3.38	4.21	-
$NPEO_3$	5.88	4.20	-
$NPEO_4$	7.65	-	-
$NPEO_5$	9.48	-	-
NPE_1C	-	-	-
NPE_2C	-	-	-
OP	12.6	4.12	-

[a] Ahel and Giger, 1993a, [b] Ahel and Giger, 1993b c

(i) Behaviour of APEOs in Wastewater Treatment Plants

If present in treatment work influents, alkylphenolic compounds will enter either as relatively long chain APEO formulations or less commonly as APs. In countries where the use of these compounds in domestic formulations has been banned, APEOs and their metabolites are only likely to be found in effluents from treatment works receiving a high proportion of industrial wastewater (Blackburn and Waldcock, 1995). Any relatively lipophilic AP components present in the treatment works influent are likely to occur as a result of the degradation of APEOs in the sewer system under anaerobic conditions (Xia *et al.*, 2001). A high proportion of these compounds may be removed during primary (settlement) treatment when settled sludge is drawn off. In primary effluents up to 80% of the total NP and 60% of the total OP is present in the particulate phase (Isobe *et al.*, 2001). Ahel and coworkers (1994) found that NPEOs with > 3 ethoxylate chains made up 82.4 % of the nonylphenolic compounds present in primary effluent samples (effluent which has not undergone biological treatment), with

concentrations as high as 2 mg/L. Similar findings were also reported by Fujita *et al.* (2000) who studied APEOs in 40 wastewater treatment plants throughout Japan. The results showed that NPEOs with 4-8 ethoxylate chains constituted on average 80.4% of all the nonylphenol compounds found in primary effluents. $NPEO_{1-3}$ and $NP_{1-3}EC$ made up 18% and 1% of the total respectively. Concentrations of nonylphenol and octylphenol were higher in untreated septage from Cape Cod, Massachussets, U.S..A than untreated wastewater and were not detected in treated septage and wastewater (Rudel *et al.*, 1998).

Primary treatment effluent is drawn off and undergoes secondary biological treatment where micro-organisms oxidise the settled sludge (Try and Price, 1995). Aerobic degradation processes lead to ethoxylate chain shortening via the sequential removal of C_2 units from the end of the ethoxylate chain to form short chain APEOs, primarily $APEO_2$ (Manzano *et al.*, 1998; John and White, 1998). Subsequent oxidation of the terminal ethoxylate to form the corresponding alkyl phenol ethoxy carboxylate (AP_2EC) may then occur which can be degraded further to AP_1EC. Anaerobic degradation also results in the formation of short chain APEOs, but these are not oxidised and may be biotransformed to the parent AP. Compared to the parent long chain APEOs the metabolites are both more toxic and estrogenic (see Table 1.5.2). The typical degradation behaviour of nonylphenol ethoxylates in sewage treatment plants is shown in Figure 1.6.2.

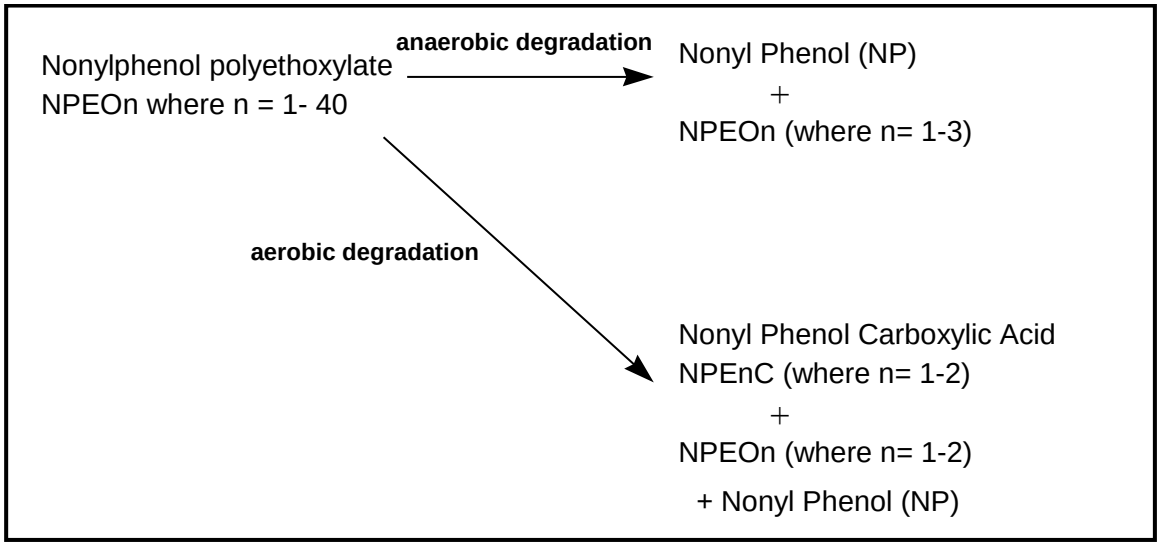


Figure 1.6.2
Degradation Pathways of Nonylphenol Polyethoxylates in WWTP

In a study of Swiss wastewater treatment plants it was observed that following secondary, biological treatment the longer chain NPEOs made up just 28.2% of the total, a reduction of 54.2% from primary treatment effluent concentrations. Short chain $NPEO_{1-2}$ and nonyl phenol carboxylic acids ($NP_{1-2}EC$) composed the majority of the other compounds present in the secondary effluent. Nasu *et al.* (2001) found that concentrations of NP, OP, $NPEO_{1-4}$ and $NPEO_5$ were reduced by up to 98% from treatment influent to effluent samples in Japanese wastewater treatment plants. APEC concentrations were not measured in this latter study. In another Japanese study Isobe *et al.* (2001) showed that the efficiency of NP removal during secondary treatment ranged

from 79-99% with a mean of 93%. OP removal was less effective with an average of 84%. In a study of German wastewater treatment plant Korner *et al.* (2000) described an elimination rate of 85% for nonylphenol and 13% for octylphenol during one sampling period. However, during another sampling period concentrations in the final effluent were higher than the influent, indication wide variations in the input proportions of alkylphenolic compounds in the influent.

The type and efficiency of secondary treatment used in the treatment works is important in determining the relative concentrations of the NPEOx parent components and their metabolites present in effluent. Concentrations of $NPEO_{1-3}$ > NPEC indicate that the predominant degradation route has been under anaerobic conditions, while if $NPEO_{1-3}$ < NPEC then this would indicate that aerobic degradation is predominating. In the U.S., effluents which had undergone secondary oxygenated activated sludge treatment showed concentrations of $NPEO_{1-3}$ > NPEC and where standard activated sludge treatment was used $NPEO_{1-3}$ > NPEC (Barber *et al.*, 2000; Petrovic and Barcelo, 2001).

In a study on the concentrations and relative proportions of NPECs in wastewater effluents entering the Fox River, U.S.., NPE_2C and NPE_3C were found to be the predominant metabolites making up 85% of the total NPEC concentration, with NPE_1C (7%) and NPE_4C (8%) making up the remainder. Pulp mill effluents, however showed different NPEC proportions with NPE_1C (16%), NPE_2C (72%), NPE_3C (10%) and NPE_4C (2%). The reason for the differences between the two effluent types was not clear (Field and Reed, 1996). The types of metabolites formed during wastewater treatment will have a direct influence on the environmental impact of this class of compounds in the receiving water body. It has been reported that up to 90% of NP and NPEO in a wastewater plant is associated with organic rich solid particles due to their relatively high lipophilicity, as measured by their octanol/water (log Kow) coefficient and low water solubility (see Table 1.6.2). NP has been found at high concentrations in the anaerobically digested sewage sludge with up to 90% of the total NP in the treatment works being found in this compartment (Nasu *et al.*, 2001).

In contrast only 10% of total NPEC concentrations were measured in the particulate phase, with the considerably higher concentrations being found in the dissolved fraction. This is due to the ionic character and therefore greater water solubility of these compounds (Ahel *et al.*, 1994; Field *et al.*, 1996; Ahel *et al.*, 2000). The amount of suspended solids in the final effluent and the total organic carbon (TOC) present will have a large effect on the ratios of the different APEO components in the effluent.

As mentioned previously, a high proportion of the total concentration of AP and APEOs present in the treatment works will be found in the sludge compartment with concentrations of nonylphenol (and potentially octyl phenol) up to μg/g levels. Sludge is often dewatered prior to disposal in landfill sites (as at Ballincollig WWTP in the present study). This solid by-product of wastewater treatment has been identified as a major potential source of APs and short chain APEOs to the terrestrial environment following the spreading of sludges on agricultural land (Sole *et al.*, 2000; Xia *et al.*, 2001). Only a small proportion of the total will be released in the treatment works effluent entering the aquatic environment, with a high proportion associated with the particulate phase of the effluent. The levels in the effluent, however, may still be

ecologically signficant. A higher proportion of the total APECs present in the treatment works are likely to be found in the dissolved phase of the effluent sample.

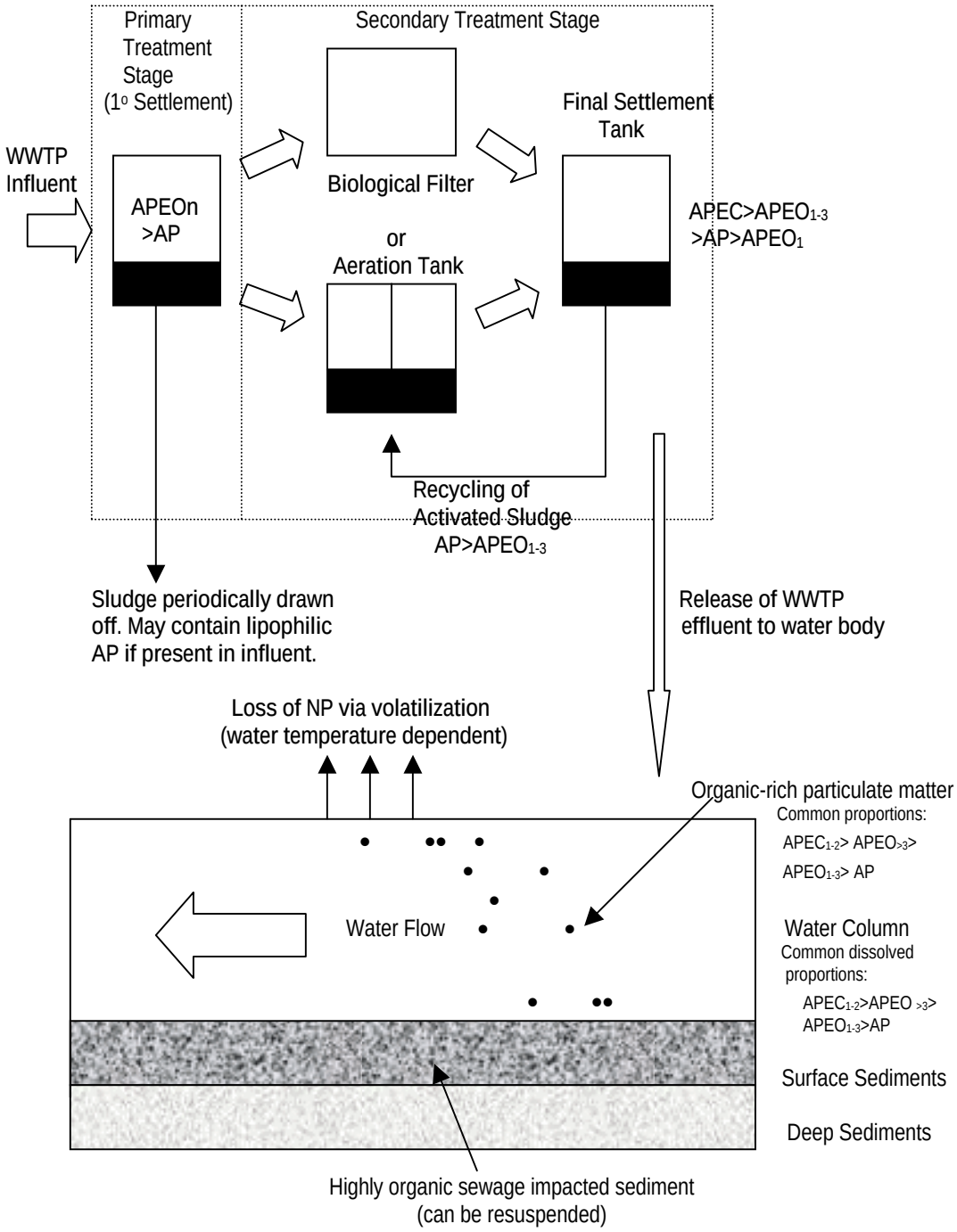


Figure 1.6.3
Behaviour of APEOs and their metabolites during wastewater treatment and following their release into the aquatic environment

Key: NP – Nonylphenol, OP – Octylphenol, APEOn – Alkylphenol polyethoxylate with n ethoxylate chains, APEC – Alkylphenol carboxylic acid, BCF – Bioconcentration Factor (Water to Fish)

(ii) Behaviour of APEOs Following Release into the Environment

Water

Once effluent containing APEOs and APEO metabolites is discharged into the aquatic environment, physical, chemical and biological processes combine to lower the environmental concentrations in the water column. The main influence on concentrations of APEOs and APEO metabolites are the effects of (i) dilution in a larger water body, (ii) the scavenging of dissolved compounds by organic rich particulate material present in the water column, (iii) sedimentation of this particulate bound component and (iv) aerobic degradation.

In a study of the Glatt River in Switzerland by Ahel and coworkers (1994) the most abundant compounds measured in water samples taken between 1984-1986 were the relatively water soluble metabolites NPE_1C and NPE_2C, followed by the considerably more lipophilic short chain $NPEO_1$ and $NPEO_2$ components and NP. The same finding was observed in a follow-up study carried out in the same river during 1997-1998 (Ahel *et al.*, 2000). NPECs were also among the most commonly observed AP related compounds in a recent study of the Anoia and Cardener Rivers in Spain that supply water to Barcelona (Petrovic and Barcelo, 2001). The highest concentrations on both Rivers were measured below wastewater treatment plants. A laboratory-based study by Staples *et al.* (1999) determined that NPECs (also OPECs and NP) could be degraded by aerobic organisms commonly present in surface waters. Laboratory-determined half-lives of these metabolites were of the order of 12 to 22 days, suggesting that NPECs and OPECs present in the dissolved phase should not persist in the environment. Field studies or further studies which more closely reflect field conditions and analyte concentrations are required to verify these results. The same study also suggested that NP in the dissolved phase would not be persistent but this may be irrelevant as the major mechanism of removal from the water column of NP is likely to be particulate scavenging and deposition in sediments (see following discussion of sediments).

Blackburn *et al.* (1999), who did not measure NPECs, found that NP and $NPEO_{1-2}$ were present in equal proportions in water samples from English rivers. In the River Aire, known to be impacted by APEOs from wool scouring plants, a slightly higher proportion of the NP was present in the dissolved phase than the particulate phase, as was the case for $NPEO_{1-2}$. This is surprising as NP and shorter chain NPEOs are relatively lipophillic and would be expected to bind to organic rich particulate matter in the water column. No details of suspended solid or total dissolved organic carbon concentrations were provided for these samples and the results may suggest that there was little suspended matter present. In the Tees and Mersey estuaries, heavily impacted by NPEOs, $NPEO_{1-2}$ were present at considerably higher proportions than NP with the majority of the compounds (> 75%) being present in the particulate phase. Ahel *et al.* (1994) found that longer chain NPEOs were present in much lower proportions than short chain NPEOs or NP, most probably as a result of their degradation in water treatment works prior to release. This ties in with the results of a study of Taiwanese rivers by Ding and Wu (2000). High concentrations of longer chain NPEOs have been detected in these systems due to what the authors describe as '….Taiwans deficient wastewater treatment.' (Ding and Tzing, 1998; Ding *et al.*, 1999; Ding and Fann, 2000).

In the Glatt River study (Ahel *et al.*, 1994) nonylphenolic component concentrations were measured over a year with lower concentrations being found during summer than

in winter. In a study of the seasonal variation of concentrations of NP, $NPEO_1$ and $NPEO_2$ in the Glatt and Thur Rivers, Switzerland significantly higher levels of all components were measured in samples taken in the winter months (Ahel *et al.*, 2000). Such a finding was also observed more recently by Maruyama and coworkers (2000) in water samples taken from three rivers in Tokyo, Japan. Concentrations of all nonyl phenolic compounds were found to decrease with increasing water temperature indicating volatilisation to be a significant factor. The temperature-dependant volatilisation of NPEOs from local water bodies was found to the major input of these compounds into the atmosphere of the Lower Hudson River Estuary in the U.S.. indicating that loss to the atmosphere from surface waters is a relatively important elimination route (Van Ry *et al.*, 2000).

Maruyama *et al.* (2000) reported that the average chain length of NPEOs in winter was longer (5-8) than in summer (2-5) suggesting that higher water temperature and therefore greater bacterial activity may cause faster cleavage of ethoxylate chains. The results from the Glatt River study also indicated that $NPEO_1$ and $NPEO_2$ were being eliminated in the river, whilst NPECs were being formed, indicating that aerobic degradation was occuring within the river system.. The transformation of $NPEO_{1-2}$ to NPEC is the predominant degradation process occurring under anaerobic conditions. Potter *et al.*, (1999) clearly showed a two-stage degradation process with rapid long chain NPEO degradation followed by accumulation and decay of $NPEO_2$ and finally accumulation of NPE_2C. The relative importance of these biotransformations is likely to be dependant on the type of wastewater treatment of effluent prior to release into the river system. In the same study nonyl phenol was found in the greatest proportions in the underlying river sediments. NPECs were also found to be the predominant APEO type compounds in water samples from the Illinois, Des Plaines and Minnesota Rivers in the Northern U.S. (Barber *et al.*, 2000). The vast majority of studies on the distribution of alkylphenol ethoxylates and alkylphenols in the aquatic environment have been related to freshwater or estuarine systems. However, limited information is available for the marine environment. Kannan *et al.* (1998) measured nonylphenols concentrations of 0.002 – 0.150 ng/l in the semi-enclosed Sea of Japan. Concentrations in the German Bight were found to be three orders of magnitude higher: 1-30 ng/l (Bester *et al.*, 2001)

Sediments

Short-chain APEOs and APs are relatively lipophilic and show a tendency to be associated with particulate matter in the water column. This leads to relatively high concentrations of APs and short chain APEOs in river and marine sediments, particularly muddy sediments rich in organic matter and sites heavily impacted by sewage effluents or historical sewage sludge dumping (Ahel *et al.*, 1994; Petrovic and Barcelo, 2001; Ferguson *et al.*, 2001). Sediment samples collected from two Spanish rivers impacted by both domestic and industrial effluents contained NPEO > NP > OP > NPEC with concentrations of NPEO up to 818 mg/kg wet weight (Petrovic and Barcelo, 2001). Heinis *et al.* (1999) showed that nonyl phenol spiked into a littoral enclosure was rapidly dissipated to the sediment compartment in 6-22 days, with no observed degradation of the nonyl phenol in the sediment. Similar findings were described by Shang and coworkers (1999) who found that NPEOs and their metabolites were persistant contaminants of marine sediments with an estimated half-life of up to 60 years. Many APEOs and APEO metabolites in marine sediments were shown to be

primarily associated with the organic rich fine-grained sediment fraction (Ferguson *et al.*, 2001). In this study the more lipophilic components (i.e. $APEO_{0-3}$) constituted > 85% of the total APEOs present in the sediment with $APEO_1$ being present in the highest proportions and longer chain APEOs (n=8-14) made up only 8.5% of the total. These findings are in general agreement with earlier work by Ahel *et al.* (1994) who found that NP and $NPEO_1$ were the predominant components in Swiss River sediments, with the concentrations being positively correlated with the amount of organic carbon present. This data is in contrast to that of Shang *et al.* (1999) who reported that longer chain APEOs (ethoxylate chain length 4-20 made up to 50% of the total concentration of AP and APEOs present in sediments near a municipal outfall in the Georgia Strait, British Columbia. This difference may have been due to differences in the level of treatment associated with the effluents at these different sites (i.e. lower levels of biological treatment in the latter case). In a study of UK river sediments, where present, NP and $NPEO_{1-2}$ concentrations broadly correlated with the concentrations observed in the overlying water with sediment concentrations being 600-1000 times greater than in the water column in general agreement with log organic carbon to water coefficients (Blackburn *et al.*, 1999). In marine sediments from the Barcelona area, concentrations were 50-100 times greater than observed in the overlying seawater (Petrovic and Barcelo, 2001).

John *et al.* (2000) showed that interaction with organic matter is important in the sorption of APs and short chain APEOs with relatively high octanol water coefficients (Log Kow, see Table 1.6.2). As the number of ethoxylate chains increases and lipophilicity decreases association with mineral surfaces becomes the dominant sorption mechanism. This indicates that the sediment type in the environmental system being studied is of great importance with adsorption to sediments being a combination of two interactions – hydrophobic interaction of lipophilic components with organic matter and hydrophilic interaction of more water-soluble components with mineral components of the sediment.

The data of Shang *et al.*, (1999) suggests that no further significant biotransformation occurs once the components become incorporated as minor components of the marine sediments. The sorption of APEOs into sedimentary layers and their apparent slow degradation rate in this medium suggests that the sediment will act as a sink, as observed with other lipophilic organic contaminants such as polychlorinated biphenyls and organochlorine pesticides (Hess, 1998). Concentrations of NP and OP were measured in a dated sediment core from Tokyo Bay, Japan. Concentrations throughout the core were an order of magnitude higher than common organochlorine contaminants and polyaromatic hydrocarbons (PAHs). NP was only detected in samples from the core corresponding to the mid-1960s with concentrations peaking in the mid-1980s and remaining stable throughout the 1990s. OP concentrations were 5-50 times lower than NP (Yamashita *et al.*, 2000). Like these other organic contaminants the sediment may then act as a secondary source of APEO contamination to the water column as a result of resuspension or as a primary source to benthic invertebrates and the demersal fish that feed upon them.

Biota

Several reports have been published on the accumulation and elimination of APEOs in freshwater and marine biota and many are detailed in the review by Servos (1999). In

laboratory experiments with Killifish (*Oryzias latipes*) bioconcentration factors (BCF) of 167 +/- 23 and 261+/- 62 were determined for NP and OP respectively. BCFs for NPl in bluegill sunfish (*Lepomis macrochirus*) have been calculated as 220 and for fathead minnows (*Pimephales promelas*) as 271 (Brooke, 1993 and Ward and Boeri, 1991). In the latter species Snyder *et al.* (2001) also determined an average BCF of 353 over three concentrations of NP treatments. BCFs of 1300 and 282 had previously been determined for NPs in sticklebacks (*Gastorosteus aculeatus*) in the field and by laboratory experiments with salmon, respectively (McLeese *et al.*, 1981; Ekelund *et al.*, 1990). Three freshwater fish species were analysed by Ahel *et al.* (1993) and BCFs determined by comparison with water samples. These ranged from 13-408, 3-300 and 3-326 for NP, NPEO1 and NPEO2 respectively in the three species studied and resulted in total NPEO tissue concentrations in the edible parts of fish up to 5.8 mg/kg dry weight.

In a three-week dosing experiment Blackburn *et al.* (1999) determined BCF factors of between 90 and 125 by the end of the dosing period for rainbow trout muscle. A similar experiment in mussels (*Mytilus edilus*) showed BCFs of 19 and 60 after three and six days, respectively, although an earlier study predicted BCFs of 3400 for NP in the same species (Osborne and Waldock, unpublished data; Ekelund *et al.*, 1990).

The concentrations of NP, OP and $NPEO_1$ were determined in muscle and liver tissues of flounder (*Platichthys flesus*) in two highly urbanised and industrialised estuaries – the Tyne and the Tees – in the North East of England. When present in Tyne samples $NPEO_1$ was found at higher concentrations than NP and OP was not detectable. Concentrations in the Tyne were higher with NP and OP being detected but $NPEO_1$ being below detection limits (Lye *et al.*, 1999). Three freshwater fish species - gudgeon, roach and chub – were caught in an NPEO impacted River Aire in the UK (Blackburn *et al.*, 1999). In all species, $NPEO_{1-2}$ were found at higher concentrations in the fish muscle than NP. These data are in agreement with the relative abundances of the same compounds in barbel (*Squalus squalus*) muscle but not in the liver of the same species ($NP>NPEO_1>NPEO_2$) or liver and muscle of chub ($NP>NPEO_2>NPEO_1$). The distributions may be due to the different lipophilicities of the individual compounds and differences in lipid storage in the different fish species. The highest concentrations were measured in the demersal (bottom feeding) Gudgeon indicating the influence of sediment NPEO concentrations and concentrations present in the benthic dwelling prey items of this species. In another study a variety of freshwater fish species collected near wastewater treatment plants in Michigan, U.S. were found to have higher tissue concentrations of NP relative to more remote sites (Keith *et al.*, 2001).

All of the above studies have been carried out in freshwater or estuarine fish species. A survey of offshore species of commercial importance (plaice, haddock and mackerel) caught around the coasts of Scotland showed concentrations of APEOs below detection limits (0.05-0.1 μg/g NP). These low concentrations can be explained by the much greater dispersion and dilution processes occurring in the open sea than those influencing estuarine or freshwater APEO concentrations (Blackburn *et al.*, 1999).

Metabolism and Tissue Distribution of Alkylphenols in Fish

Until recently little work had been carried out on the metabolism of alkylphenols in fish or the tissue distribution of metabolites. Straight chain and secondary alkylphenols are known to be rapidly metabolised by phase I and II enzymes in rainbow trout and

salmon, however little was known of the behaviour of the environmentally significant tertiary (tert) alkylphenols (Meldahl *et al.*, 1996; Thilbaut *et al.*, 1998a,b; Arukwe, 2000). Ferreira-Leach and Hill (2001) dosed rainbow trout with radio-labelled tert-OP (4t-OP) in an extensive tank study. Fish were sacrificed after 4, 7 and 10 days and a wide variety of tissues were removed for chemical analysis. In some tissues such as bone, brain, eye, fat, gills and muscle only the parent compound (4t-OP) was found. Samples of blood, spleen and heart contained a 4-t-OP glucuronide metabolite, 4-(1,1,3,3,-tetramethylbutyl)phenoxy-β-glucuronide (metabolite I). Such a metabolite was also determined in the bile of Atlantic salmon (*Salmo salmar*) after dietary exposure to 4-n-NP (Arukwe *et al.*, 2000). This metabolite was also found in bile and faeces long with another compound, a 4-octylcatechol glucuronoride metabolite (metabolite II). In liver, intestine and kidney all three compounds were observed. The accumulation of all three compounds was found to reach steady state within 4-10 days, with bioaccumulation of the parent compound found to be tissue dependant (see Table 1.5.3). The highest concentration of total OP compounds (parent 4-t-OP + metabolites) was consistently found in the bile fluid with metabolite I >> metabolite II. High concentrations of these metabolites were also found in the liver, faeces suggesting that biliary excretion via a phase II glucuronidation pathway is an important mechanism of AP elimination. To a lesser extent, phase I (hydroxylation) pathways have also been identified as an elimination route in fish.

Table 1.6.3
The bioconcentration of 4-t-OP in tissues of rainbow trout
(from Ferreirra-Leach and Hill, 2001)

Tissue	Bioconcentration Factor
Gills	259
Skin	187
Fat	1190
Muscle	101
Brain	116

(iii) Concentrations of APEOs and APEO metabolites in WWTP and Environmental Samples.

There are a wide variety of methods available for the analysis of APEOs and their related compounds in a wide range of matrices and it is this variability which often precludes detailed comparisons of data produced by different research groups. The common problems in comparing data are:

(a) Different APEO components are measured in samples by different research groups. The most commonly measured components are the APs and the shorter chain APEOs, while there is little data available on the concentrations of carboxylic acid metabolites of the APEOs (APE_nC). This may be due to the need for differential extraction techniques for these more ionic compounds and the requirement for derivitisation prior to gas chromatography-mass spectrometry (GC-MS) analysis in order to achieve useful detection limits or the requirement for Liquid Chromatography-Mass Spectrometry (LC-MS) systems.

(b) The lack of full sample descriptions in many studies. Water samples may be analysed for APEOs and related compounds in the dissolved (filtered) total extractable (unfiltered) or particulate (filtered-unfiltered) phase. Sediments may

be dried, crushed and sieved to isolate the fine organic-rich fraction or they be analysed without the sieving step or 'as received', results may also be normalised to the moisture content or the total organic carbon (TOC) content. Concentrations in biota may be reported on a wet weight or lipid weight basis.

The number of studies being carried out on the concentrations of APEO and related compounds is growing, particularly following the discovery that many of the compounds have estrogenic properties. Taking into account the caveats outlined above concentrations of these compounds in wastewater treatment works samples are provided in Table 1.6.4a while concentrations in other samples from the aquatic environment are given in Table 1.6.4b. The contents of the tables are not designed to be exhaustive but are provided to give an overview. Further reference data can be found in the reviews by Bennie (1999) and Servos (1999).

The highest concentrations of APEOs are found in the influents and effluents of wastewater treatment works which intake trade effluents from the textile industry and pulp mills (Blackburn and Waldcock, 1995; Naylor *et al.*, 1992; Tanghe *et al.*, 1999; Smith, 2000; Xia *et al.*, 2001). Lower concentrations of most compounds are observed in the effluents released to the aquatic environment. Concentrations in environmental samples from Europe are highest in locations heavily impacted by treatment works effluents dealing with predominantly industrial emmissions. In the U.S. where APEOs are still used in domestic detergents levels are higher in areas near municipal sewage effluent discharges (e.g. Shang *et al.*, 1999; Ferguson *et al.*, 2001). The positive impact of risk reduction measures (i.e the banning of APEOs in laundry detergents, the voluntary phase out of the use of APEOs in household hard-surface cleaners and the improvement of the efficiency of wastewater treatment works) has been clearly shown in Switzerland. NPECs were found at, on average, 30 times lower concentrations following the risk reduction measures and NP, $NPEO_1$ and $NPEO_2$ concentrations were up to 50 times lower than measured in 1984 (Ahel *et al.*, 2000). The only reported analysis for APEOs in Ireland reported a concentration of 0.17 μg/g total APEOs in a sediment sample from Dublin Port (Marine Institute, 1999).

Concentrations measured in the environmental samples listed below are often above the thresholds for vitellogenin induction of 3 μg/L and 10 μg/L for OP and NP respectively. Concentrations have also been measured that exceed acute toxicity thresholds (LC_{50} 130-900 μg/L) in several species of fish including fathead minnows (*Pimephales promelas)*, rainbow trout (*Oncorhynchus mykiss)* and Atlantic salmon (*Salmo salar)* (Servos, 1999).

Table 1.6.4a
Concentrations of APEOs and APEO metabolites in trade effluents and WWTP Samples

Matrix	Location (Sampling Year)	Concentration	Reference
Trade Effluent	Pulp Mill Effluent, U.S.	<0.02 –21 μg/L NP 3.1-730 μg/L $NPEO_{1-17}$	Naylor *et al.*, 1992
	Pulp Mill Effluent, U.S.	17-1200 μg/L NPEC	Field and Reed 1996
	Textile Industry Trade Effluents, East of Scotland (1999-2000)	1 – 220 μg/L NP 1-180 μg/L $NPEO_{15}$	Smith, 2000
	Treated Textile Effluents, Belgium (1997)	122 μg/L NP	Tanghe *et al.*, 1999
Untreated Sewage or WWTP Influent	Cape Cod, U.S. (1996-1997)	25-33 μg/L NP 0.20-0.74 μg/L OP, n.d. $OPEO_1$ 15-21 μg/L $NPEO_1$ 1.3-1.7 μg/L NPEC	Rudel *et al.*, 1998
	WWTP, Kansas, U.S. (2000)	3.39-169 μg/L NP	Xia *et al.*, 2001
	WWTP, Japan (1998-1999)	1.3 – 75 μg/L NP n.d – 2.3 μg/L OP 6.1 – 92 μg/L $NPEO_{1-4}$ 44-810 μg/L $NPEO_5$	Nasu *et al.*,2001
Septage	Cape Cod, U.S. (1996-1997)	1000-1500 μg/L NP 35-42 μg/L OP 8.0-9.8 μg/L $OPEO_1$ 440-550 μg/L $NPEO_1$ 37-57 μg/L NPEC	Rudel *et al.*, 1998
WWTP Effluent	Municipal waste effluent – Fox River, U.S.	0.83 – 14.0 μg/L NP 47 – 350 μg/L $NPEO_{1-17}$	Naylor *et al.*, 1992
	Marley WWTP, River Aire, England (Heavily Industrialised – Textile Industry)	330 μg/L Total Extractable NPEOs, 150 μg/L $NPEO_2$, 0.5 μg/L OP	Blackburn and Waldcock, 1995
	WWTP handling Domestic waste in South-East England	< 1 μg/L all compounds	Blackburn and Waldcock, 1995
	Howdon WWTP, River Tyne, UK (1997)	3 +/- 0.85 μg/L NP 45 +/- 16 μg/L $NPEO_1$ n.d OP	Lye *et al.*, 1999
	Jedburgh Activated Sludge Plant Effluent, Scotland * (1999-2000)	2 – 20 μg/L NP	Smith, 2000
	NE Spain (1999)	6.0 – 343 μg/L NP n.d. – 928 μg/L $NPEO_{4+6}$ 4.0 – 270 μg/L NPEC	Sole *et al.*, 2000
	South Central Michigan (1997)	0.17 – 37 μg/L NP n.d – 0.67 μg/L OP n.d – 332 μg/L NPEO	Snyder *et al.*, 1999
WWTP Effluent	Municipal Waste Effluent –Fox River North U.S. (1995)	140-270 μg/L NPEC	Field and Reed, 1996
	Municipal Wastewater Treatment Plants, Japan (1998-2000)	0.1 – 0.9 μg/L NP n.d – 0.4 μg/L OP 0.1 – 3.2 μg/L $NPEO_{1-4}$ 1.6 – 24 μg/L $NPEO_5$	Nasu *et al.*,2001
	Northern U.S. WWTP (1998)	36-380 μg/L Total APEOs 0.9 – 19 μg/L NP, n.d – 0.20 μg/L OP, 2.3 – 165 μg/L $NPEO_{1-2}$, 1.0 – 51 μg/L $OPEO_{1-3}$, 52 – 188 μg/L $NP_{1-4}EC$	Barber *et al.*, 2000

Table 1.6.4b
Concentrations of APEOs and APEO metabolites in samples from the aquatic environment

Matrix	Location (Sampling Year)	Concentration/ Compound	Reference
Fresh Water	Northern U.S.. (1998)	n.d – 1.1 μg/L NP n.d - 2.1 μg/L $NPEO_{1-2}$ 0.10 – 19.0 μg/L $NP_{1-4}EC$	Barber *et al.*, 2000
	N.E. Spain (1999)	18 – 644 μg/L NP n.d – 100 μg/L $NPEO_{4+6}$ n.d. – 70 μg/L NPEC	Sole *et al.*, 2000
	Barcelona, Spain (2000)	0.5 – 15 mg/L NP <0.1 mg/L OP 2.2 – 41 mg/L NPEO <0.08 – 35 mg/L NPEC	Petrovic and Barcelo, 2001
	South Central Michigan, U.S.. (1997)	<0.01 – 20.7 μg/L NP <0.002 – 0.7 μg/L OP n.d – 20.7 μg/L NPEO	Snyder *et al.*, 1999
	Great Lakes/ St. Lawrence, Canada	<0.01-1.28 μg/L NP <0.01-0.08 μg/L OP <0.02-7.8 μg/L $NPEO_1$ <0.02-10 μg/L $NPEO_2$	Bennie *et al.*, 1997
	Glatt River, Switzerland (1983-1986)	0.3 – 45 μg/L NP <3 – 69 μg/L $NPEO_1$ <0.3 – 30 μg/L $NPEO_2$ <1 – 45 μg/L $NPEC_1$ 2 – 75 μg/L $NPEC_2$	Ahel *et al.*, 1994
	Glatt River, Switzerland (1997-1998)	<0.03-0.48 μg/L NP <0.03-0.26 μg/L NPEO1 <0.03-0.31 μg/L NPEO2 0.5-3.0 μg/L NP1-2EC	Ahel *et al.*, 2000
	River Aire, UK (1993-1994) River Thames, UK (1993-1994) River Lea, UK (1993-1994) River Wye, UK (1993-1994)	<1.6 – 180 μg/L Total NP 0.8 – 2.3 μg/L Total NP 0.5 – 12.0 μg/L Total NP 0.2 - < 2.7 μg/L Total NP	Blackburn and Waldock, 1995
	Industrially impacted Rivers, Belgium (1997)	2.0-42 μg/L NP	Tanghe *et al.*, 1999
	Fox River, U.S.	0.12-0.64 μg/L NP 0.97-8.2 μg/L $NPEO_{1-17}$	Naylor *et al.*, 1992
	Fox River, U.S..	<0.10-13.5 μg/L NPEC	Field and Reed, 1996
	Taiwanese Rivers (1999)	0.5 – 2.4 μg/L NP	Ding and Wu, 2000
	3 Rivers, Japan (1998-1999)	0.14-1.78 μg/L NP 0.01-0.07 μg/L OP	Tsuda *et al.*, 2001
Estuarine Water	New York Harbour – (1998)	0.08 μg/L dissolved NP	Van Rye *et al.*, 2000
	Tees Estuary, UK (1998)	0.77 μg/L NP	Thomas *et al.*, 2000
	Tees Estuary, UK (1993-1994) Mersey Estuary, UK (1993-1994)	0.08 – 3.1 μg/L dissolved NP <0.08 – 0.32 μg/L dissolved NP	Blackburn and Waldock,1995
Fresh water / Estuarine Sediment	River Tyne, UK (1997)	0.03 – 0.10 μg/g NP 0.1 – 1.86 μg/g $NPEO_1$	Lye *et al.*, 1999
	River Tyne, UK (1997)	0.002 – 0.02 μg/g OP 1.28 – 10.2 μg/g NP 0.08 – 5.96 μg/g $NPEO_1$ 0.02 – 0.4 μg/g OP	Lye *et al.*, 1999

Table 1.6.4b (cont).
Concentrations of APEOs and APEO metabolites in samples from the aquatic environment

Matrix	Location (Sampling Year)	Concentration/ Compound	Reference
Marine Sediment	Dublin Port, Ireland (1995)	0.17 μg/g Total APEOs	Marine Institute, 1999
	Jamaica Bay, New York State (1998)	0.05 – 30.0 μg/g $NPEO_{0-3}$ 0.004 – 0.289 μg/g $OPEO_{0-3}$	Ferguson *et al.*, 2001
	Strait of Georgia, British Columbia (1996) Heavily Sewage Impacted Control Site 20km from source	 1.50 +/- 0.13 μg/g total NPEO 0.3 μg/g total NPEO	Shang *et al.*, 1999
	Venice Lagoon, Italy	0.50 – 6.70 μg/g NP+ $NPEO_1$,+$NPEO_2$	Marcomini *et al.*, 1990
	Tokyo Bay, Japan (1995)	4.81 ug/g d/w NP	Yamashita *et al.*, 2000
	Wastewater Impacted (1998) Masan Bay, Korea	0.51 (0.11-3.89) μg/g d/w NP+NPEO1 0.02 (0.004-0.18) μg/g d/w OP	Khim *et al.*, 1999
	Barcelona Municipal Outfall	0.10 – 6.60 μg/g $NPEO_{0-3}$	Valls *et al.*, 1988
	Barcelona and Almeria Harbours (2000)	<15 – 590 μg/g NP <10 μg/g OP 44-325 μg/g NPEO <8 – 92 μg/g NPEC	Petrovic and Barcelo, 2001
Biota	Chriesbach Creek, Switzerland (1984-85) Chub Muscle Chub Liver Barbel Muscle Barbel Liver Rainbow Trout Muscle	*All concs in mg/kg dry weight 0.18-0.46 NP, 0.18-0.34 $NPEO_1$, <0.03-0.13 $NPEO_2$ 1.0-1.4 NP, 1.0-1.8 $NPEO_1$, <0.03-1.4 $NPEO_2$ 0.38 NP, 3.1 $NPEO_1$, 2.3 $NPEO_2$ 0.98 NP, 0.88 $NPEO_1$, 0.14 $NPEO_2$ 0.15 NP, 0.42 $NPEO_1$, 0.05 $NPEO_2$	
	River Tyne, UK (1997) Flounder Muscle Flounder Liver Flounder Liver River Tees, UK (1997) Flounder muscle/liver	 0.005-0.055 mg/kg ww NP 0.010-0.030 mg/kg ww NP 0.19 – 0.94 mg/kg ww $NPEO_1$ 0.02-0.18 mg/kg ww NP	Lye *et al.*, 1999
	Rock Bass Bluegill Sunfish Green Sunfish Smallmouth Bass White Sucker Long-nose Sucker Rainbow Smelt	8.1+/- 5.3 ng/g w/w NP 5.7 +/- 5.2 ng/g w/w NP <3.3 ng/g w/w NP 5.8 +/- 5.2 ng/g w/w NP 7.2 +/- 5.2 ng/g w/w NP <3.3 ng/g w/w NP 7.7 +/- 4.0 ng/g w/w NP	Keith *et al.*, 2001
	Rivers, Japan Whole Ayu Fish (1998-1999)	0.01 – 0.11 mg/kg ww NP	Tsuda *et al.*, 2001

1.6.3 Steroid Sex Hormones and Related Synthetic Compounds

1.6.3.1 Useage

Both humans and animals produce natural estrogens (17β-estradiol, estrone and estriol) and progestogens (progesterone). In humans progestogens prepare the uterus for implantation wheresas estrogens increase myometrial excitability at parturition (Nathanielsz *et al.*, 1995; Lye, 1996). They are transported in the blood, metabolised by the liver and excreted in urine or via the gallbladder and intestines as conjugated metabolites.

Commonly used synthetic or exogenous hormones include the estrogens (e.g.. 17α-ethinylestradiol and its 3-methyl ester, mestronal and synthetic 17β-estradiol) and the progestogens (e.g..levonorgestrel, norethindroneacetate) have been used in animals and humans for a variety of purposes for many years. They are used in humans in birth control pills, for hormone replacement therapy during the menopause and in cancer therapy.

17α-Ethinylestradiol and its 3-methyl ester (mestronal) are the most frequently used estrogens used in contraceptive pills for women (Turan, 1995). The most popular contraceptives are 'combined pills'. These contain two synthetic hormones (an estrogen and progestogen) similar in biochemical action to those produced naturally. The earlier contraceptive formulations contained between 100-150 mg of estrogen, however following evidence that such high doses produced serious side effects newer contraceptive pills were developed containing less estrogen (20–35 mg) and progestogen (1 mg), as was the mini-pill which contains progestogen only (Rathner and Sonneborn, 1979; Gardner, 1983). Modern contraceptive pills often contain 20-35 μg of estrogen.

In general synthetic hormones are more slowly metabolised and have longer half-lives in the body than natural hormones (the 17α-ethynyl group or methyl group is specifically designed to prevent metabolic conversion to the less active estrone or androstenedione (Kime, 1998).

Another synthetic hormone, diethylstilbestrol, was used in Ireland between 1941 and 1975 to prevent spontaneous abortion in pregnant women. Its use during pregnancy was stopped following the development of cancers in the daughters of patients prescribed the drug. DES has also been used in agriculture as a growth promotion agent in livestock and to a lesser extent as an antiabortive in dogs (Toppari *et al.*, 1995).

Of all the compounds determined to have estrogenic activity many studies have found two of the above mentioned compounds to account for the majority of estrogenic activity observed in environmental samples, particularly sewage effluents – estrone and 17β-estradiol (Desbrow *et al.*, 1998; Rodgers-Gray *et al.*, 2000)

1.6.3.2 The Chemistry of Steroid Sex Hormones and Related Synthetic Compounds

Natural estrogens have an aromatic ring A and a phenolic hydroxy group at position 3 (Figure 1.6.4). Hepatic metabolism of both natural and synthetic estrogens occurs via phase I and phase II processes by 2-hydroxylation and 16α-hydroxylation leading to the

formation of estriol from 17β-estradiol followed by conjugation by sulphuric and glucuronic acids (Christensen, 1998; Orme *et al.*, 1983). In the case of 17α-ethinylestradiol a significant portion of the parent compound is directly conjugated and excreted. The human excretion of both natural and synthetic estrogens and progestogens is thought to be the principal source of these compounds in the aquatic environment. In a study on the excretion of natural hormones in urine, 17β-estradiol (2.4μg), estrone (7μg) and estriol (4.6μg) were excreted daily by cycling women, while pregnant women may excrete up to 30mg estriol per day. These conjugates have no biological activity, however they may act as precursors for biologically active de-conjugated compounds following biotransformation by bacteria in the environment (Turan *et al.*, 1995). Several authors suggested that deconjucation of these estrogens by the enzyme β-glucoronidase found in *Escherichia coli*, commonly the most abundant bacterium strain in domestic sewage, may take place in sewers prior to entry into sewage treatment plants (Dray *et al.*, 1972; Panter *et al.*, 1999; Hirsch *et al.*, 1999, Ternes *et al.*, 1999b; Larsson *et al.*, 1999; Baroni *et al.*, 2001). By the same processes, up to 30% of unbound estrogens and their metabolites may be excreted in the faeces, possibly as a result of deconjugation in the colon (Orme *et al.*, 1983).

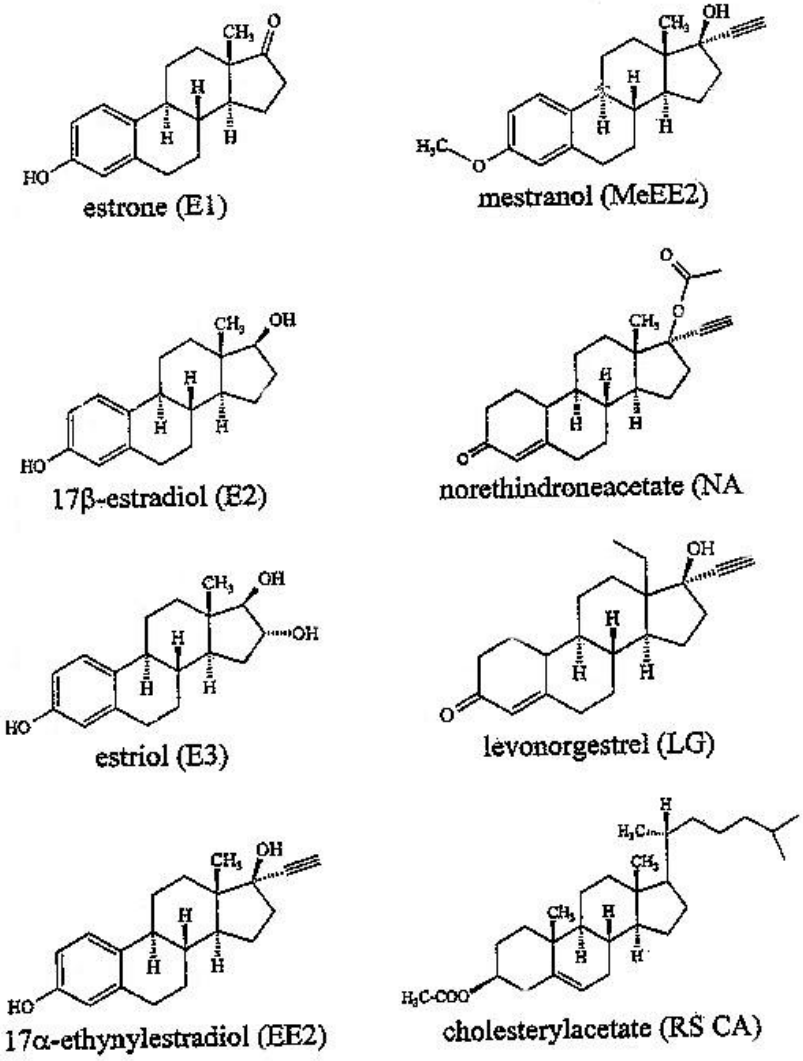


Figure 1.6.4
Structures of naturally occurring steroid sex hormones and related synthetic compounds

1.6.3.3 Environmental Behaviour of Steroid Sex Hormones and Related Synthetic Compounds

A comprehensive 'review of the fate of human pharmaceuticals in the environment, including steroid sex hormones', was published by the Environment Agency, UK (2000). In addition, a review of the behaviour of endocrine chemicals in activated sludge treatment works is in preparation (Johnson and Sumpter, 2001). A brief overview of the area is provided below.

(i) Behaviour of Steroid Sex Hormones and Related Synthetic Compounds in WWTP

Until recently there have been relatively few reliable studies on the concentrations of steroid sex hormones and related synthetic compounds in wastewater influents and effluents. This is due to difficulties in achieving the analytical detection limits required for these compounds following the discovery that the estrogens are capable of inducing effects at concentrations in the ng/L range. To date, there have been very few studies on the occurrence of progestogens (levonorgestrel and norethinidrone) in wastewaters. Concentrations of these compounds in wastewater treatment plant effluents if NE Spain were below detection limits in all samples taken although the achievable detection limits were only 20 ng/L (Sole *et al.*, 2000). Lower detection limits of 1 ng/L were achieved by Kuch and Ballschmiter (2000), however, levonorgestrel and norethinidrone acetate were only observed in a single treated effluent at 1 ng/L.

In theory, the behaviour of natural and synthetic hormonal components in wastewater, as with the alkylphenol ethoxylates and their metabolites discussed in the previous section, is dependent on the physical properties of each compound. The water solubility (S), lipophilicity of an estrogenic compound (Log Kow) and its water:organic carbon coefficient (Log Koc) will determine the degree to which the compound is removed from wastewaters by adsorption onto sludge. As the Log Kows of the majority of estrogens are relatively high, many unconjugated compounds are removed from the effluent during settling and activated sludge treatment. 17α-ethinylestradiol is considerably more water-soluble than 17β-estradiol and this is likely to be an important factor in the determination of the environmental behaviour of this compound. The water solubility of conjugated estrogens is higher than the unbound components and this may also influence their behaviour in treatment works (Furhacker *et al.*, 1999).

In a Dutch study, raw effluents and wastewater treatment plant influents from domestic sources contained higher concentrations of estrogens than those from industrial sources (Belfroid *et al.*, 1999) and contained higher concentrations of estrogens than effluents that had undergone biological treatment indicating efficient removal processes. Estrone is often found at the highest concentrations while 17β-estradiol >17α-estradiol = 17α-ethinylestradiol. This pattern is explained by the relatively rapid (3-4 hrs) degradation of 17β-estradiol to estrone in wastewater (Ternes *et al.*, 1999a). Levels of hormones in treated effluents are also dependent of the type of treatment available (Shore *et al.*, 1993). Concentrations of these compounds measured in wastewater treatment plant samples are detailed in Table 1.5.4. Measured concentrations of 17β-estradiol in influents ranged from 15-260 ng/L (Ternes *et al.*, 1999a; Sole *et al.*, 2000; Nasu *et al.*, 2001) while concentrations in effluents ranged from 1.0-76 ng/L (Desbrow *et al.*, 1998; Ternes *et al.*, 1999a; Belfroid *et al.*, 1999; Thomas *et al.*, 2000; Nasu *et al.*, 2001). Although concentrations of the synthetic estrogen 17α-ethinylestradiol are, when measured, commonly lower than those of the naturally occurring hormones, it should be noted that the synthetic compound is more potent at promoting vittelogenisis and is more likely to persist in the environment. In a study of a small sewage treatments works in Sweden which had both primary and secondary treatment facilities, Larsson *et al.* (1999) noted that concentrations of hormones in the effluent followed the order estrone > 17α-ethinylestradiol > 17β-estradiol. They suggested that the relatively higher amount of the synthetic hormone was due to the more rapid degradation of the natural

hormones during wastewater treatment. This theory is strengthened by early studies that showed that the microbiological degradation of 17α-ethinylestradiol in sewage sludges is considerably slower than the degradation of the natural hormones (von Rathner and Sonneburn, 1979; Tabak *et al.*, 1981)

Table 1.6.4
Concentrations of naturally occurring steroid sex hormones and related synthetic compounds in WWTP and other environmental matrices

Matrix	Location and Sampling Year	Concentration	Reference
Sewage Influent	Tel Aviv, Israel (1991-1992)	48-141 ng/L 17β-estradiol 208-320 ng/L testosterone	Shore *et al.*, 1993
	Japanese WWTP (1998-1999)	30-90 ng/L 17β-estradiol	Nasu *et al.*, 2001
	Brazilian Raw Sewage (dissolved fraction) German Raw Sewage (dissolved fraction)	21 ng/L 17β-estradiol 40 ng/L estrone 15 ng/L 17β-estradiol 27 ng/L estrone	Ternes *et al.*, 1999a
	UK treated waters	13.6 – 24 ng/L 17β-estradiol *	Johnson *et al.*, 1999 *
	NE Spain (1999)	n.d – 263 ng/L estriol n.d.-43 ng/L diethylstilbestrol	Sole *et al.*, 2000
Sewage Effluent	Japanese WWTP (1998-1999)	32-55 ng/L 17β-estradiol	Nasu *et al.*, 2001
	Canadian WWTP (dissolved fraction) German WWTP (dissolved fraction)	64 ng/L17β-estradiol 48 ng/L estrone 3 ng/L 17β-estradiol 70 ng/L estrone	Ternes *et al.*, 1999a
	Netherlands WWTP	1-12 ng/L 17β-estradiol up to 47 ng/L estrone	Belfroid *et al.*, 1999
	Howdon WWTP, Tyne Estuary, U.K. (1998)	19 ng/L 17β-estradiol	Thomas *et al.*, 2000
	Chelmsford WWTP, U.K. (1997-1998)	4-88 ng/L 17β-estradiol 15-220 ng/L estrone 1.7 – 3.4 ng/L ethinylestradiol	Rodgers-Gray *et al.*, 2000
	UK Treated Waters	3.0-48 ng/L 17β-estradiol 1.0-76 ng/L estrone <1.0-7.0 ng/L 17α-ethynyl estradiol	Desbrow *et al.*, 1998
Freshwater	Northern U.S. Wastewater Treatment Plants (1998)	< 58 ng/L 17β-estradiol	Barber *et al.*, 2000
	South Central Michigan, U.S.. (1997)	n.d -3.7 ng/L 17β-estradiol n.d-0.8 ng/L 17α-ethynyl estradiol	Snyder *et al.*, 1999
Estuarine	Dabholm Gut, Tees Estuary, U.K. (1998)	0.73 ng/L 17β-estradiol	Thomas *et al.*, 2000

** Estimated influent concentrations calculated from population equivalent, excretion rate data and concentrations in effluent determined by Desbrow et al., 1998.*

The effluent enters a wastewater treatment works and undergoes primary (filtering and settling) treatment. Concentrations of free (unconjugated) estrogens in the effluents were found to rise at this stage due to bacterially mediated estrogen-glucoronide breakdown (Ternes *et al.*, 1999b). During secondary (biological) treatment the vast majority of estrogens present (both conjugated and free) are removed and the progestogen, norethisterone was removed completely (see Table 1.6.5 for details) although the processes of removal are not well understood. Belfroid *et al.* (1999) measured higher conjugated estrone levels than free estrone levels in treated domestic

effluents, suggesting that in some treatment works 'activation' of secreted hormones was not complete. Failure to measure the inactive, bound hormonal fraction may therefore lead to an under-estimation of the estrogenic potential of effluents as inactive estrogen-glucoronide metabolites may still be transformed by bacterial action in sewage impacted rivers following the release of effluents into the aquatic environment.

Of greater potential estrogenic significance are those effluents that do not undergo any kind of secondary (biological) treatment.

Table 1.6.5
Efficiency of removal of natural and synthetic hormones in wastewaters in WWTP with secondary treatment

Compound	**Author**				
	Rathner and Sonneborn, 1979	Tabak *et al.*, 1981	Ternes *et al.*, 1999b	Baroni *et al.*, 2001	Nasu *et al.*, 2001
Estrone	-	50-70%	50%	-	-
17β-Estradiol	-	50-70%	95%	87 +/- 9%	64 (7-99)%
Estriol	-	50-70%	-	95 +/- 6%	-
17α-Ethinyl estradiol	0%	20-40%	20%	85 +/- 14%	-
Norethisterone	100%	-	-	-	-

(ii) Behaviour of Steroid Sex Hormones and Related Synthetic Compounds in the Aquatic Environment

In comparison to studies relating to wastewater treatment works samples there have been relatively few studies focussed on the environmental behaviour of steroid sex hormones in surface waters. Jurgens *et al.* (1999) performed laboratory studies showing that 17α-ethinylestradiol was more persistent to aerobic degradation (half-life 46 days) in river water than 17β-estradiol (half-life 4-27 days) that was converted to estrone (half-life 4-27 days) before full degradation. Under anaerobic conditions persistence in water was in the order 17β-estradiol > estrone > 17α-ethinylestradiol.

In field studies on Dutch surface waters by Belfroid *et al.* (1999), the majority of samples had concentrations of natural and synthetic sex steroids below detection limits or up to a maximum of 5ng/L. Estrone was the most commonly detected compound. In a study of German rivers by Ternes *et al.* (1999) detected estrone in only three of fifteen rivers (0.1-7.6 ng/L). 17α-ethinylestradiol and 17β-estradiol were not detected in any samples.

Using immunoassay techniques, Aherne *et al.* (1985) measured the progestogens norethisterone and progesterone in a few UK rivers at concentrations of 17 and 6 ng/L respectively. The majority of river samples did not contain concentrations of these compounds above the analytical detection limits (10 and 5 ng/L respectively).

1.6.4 Bisphenol A

1.6.4.1 Chemistry, Useage and Legislative Status of Bisphenol A

2,2-(4,4-dihydroxydiphenyl) propane or as it is commonly known, Bisphenol A (BPA), is an industrial chemical formed by the acid-catalysed condensation of phenol with acetone. The structure is composed of two unsaturated phenol rings and is shown in Figure 1.6.5. BPA is used in the production of polycarbonate plastics (65% of total useage) and for producing epoxy resins via a reaction with epichlorohydrin (25%) for powder paints and other coatings, electrical laminants, composites and adhesives. The remainder of the BPA produced is used in the production of speciality unsaturated polyester, polysulphone, polyethrimide and polyarylate resins, flame-retardants (tetrabromobisphenol A), in the packaging industry (e.g.. in the lining of tin cans containing food products) and in the paper industry. As a result of its incorporation into a wide variety of production processes BPA is found in many everyday applications. These include powder paints, compact discs, optical lenses, reusable bottles, tooth fillings and as linings in food and drink containers (Chemical Market Reporter; Staples *et al.*, 1998; Furhacker *et al.*, 2000). The general structure of polycarbonates made from BPA monomers is shown in Figure 1.6.5. The carbonate linkages are relatively stable but may be broken (hydrolysed) at high temperatures and neutral to alkaline pHs resulting in the release of BPA (Lazear, 1995).

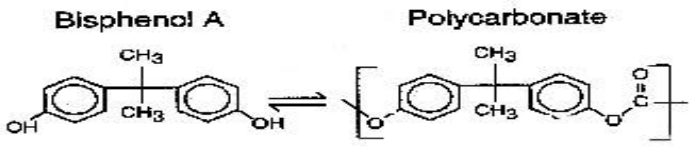


Figure 1.6.5
Structure of BPA monomer and after polymerisation (polycarbonate)

BPA is manufactured in large quantities in the United States with demand from industry increasing steadily with the demand by 2002 being projected to exceed 1 billion kg/year. In the European Union consumption of BPA was estimated to be 640,000 tonnes per year, with approximately 830,000 tonnes being produced at plants in Belgium, Germany, The Netherlands and Spain per year (Chemical Market Reporter). There are no Bisphenol A production facilities in Ireland. In the EU candidate endocrine substances list BPA is defined as a category 1 endocrine disruptor (at least one study provides evidence of endocrine disruption in an intact organism), although there is considerable controversy, primarily from chemical manufacturers, over its inclusion in this category.

1.6.4.2 Environmental Behaviour of Bisphenol A

Stables *et al.* (1998) provided a review of the environmental fate of bisphenol A, the main points of which, and details of experimental fieldwork carried out since, are detailed below.

BPA has been shown to enter the environment both through the degradation of plastic products (particularly from landfill sites where hydrolytic and leaching processes cause the release of BPA) and via the direct discharge from BPA processing or manufacturing plants (Yasuhara *et al.*, 1997; Yasuhara *et al.*, 1999; Furhacker *et al.*, 2000; Yamamoto *et al.*, 2001). The levels of BPA in landfill site leachates has been directly associated with the types of waste being disposed of at a particular site. Landfill leachates from sites taking large amounts of waste plastics in Japan were found to contain very high (6.96 and 17.2 mg/L) concentrations of BPA. This is acceptable where landfills are operated as closed systems and the leachate is treated in a wastewater treatment plant prior to release (see section i below) into the aquatic environment, but is obviously of great concern if leachates are released without treatment.

(i) Occurrence and Behaviour of BPA in WWTP and Industrial Wastewaters

In an extensive study of the occurrence of BPA in Canadian municipal wastewater influents and effluents, Lee and Peart (2000b) found that wastewaters from chemical coatings and resin manufacturers, packaging and paper product industries and commercial dry-cleaning facilities contained the highest concentrations of BPA, while those from plastics manufacturers were relatively low (see Table 1.6.6 for details). BPA was higher in industrial influents and effluents than in domestic waste. In a similar study carried out recently in Germany the paper industry was found to be the largest contributor to observed bisphenol concentrations in WWTP influents (Furhacker *et al.*, 2000).

In the Canadian study, the removal efficiency of BPA during wastewater treatment ranged from <1-99% with a median of 68%. These values are in agreement with removal efficiencies described by other authors that ranged from 80-100% degradation following aerobic and anaerobic treatments (see Staples *et al.*, 1998 for references and Yamada *et al.*, 1999; Petrovic and Barcelo, 2001). Several studies have noted that in plants incorporating secondary treatment and which showed continuous and higher BPA inputs, removal efficiency was considerably higher than in plants with the same level of treatment but which had non-continuous and lower BPA inputs. They suggest that in the former case bacteria in the activated sludge were acclimatised to BPA and performed more efficient biodegradation of the chemical than in sludge that had non-continuous and lower BPA inputs (Furun *et al.*, 1990; Lee and Peart, 2000b). By way of confirmation, Ike *et al.* (2000) studied the biodegradation of BPA by microcosms typically found in activated sludges from (a) domestic treatment plants, (b) a laboratory activated sludge set up and (c) from an industrial treatment works whose influent was known to contain BPA. Sludges (a) and (b) removed all the BPA present after a 48–56 hour lag period after which rapid degradation took place. The BPA acclimatised sludge (c) showed no lag period and BPA was removed completely within 24 hours. As the typical hydraulic residence time in many wastewater treatment works is approximately 5 hours, microcosms in treatment plants which are not acclimatised by continuous BPA inputs would be unlikely to remove a significant proportion of BPA, leading to a release into the aquatic environment. Where the BPA was biodegraded a number of stable metabolites were observed including 2,2-bis(4-hydroxyphenyl)-1,2-propanediol and p-hydroxyphenacyl alcohol. In a study of river water microcosms BPA degrading bacteria were found to be relatively widespread, particularly in BPA impacted areas and were responsible for metabolising the compound in the aquatic environment.

(ii) Behaviour of BPA in the Aquatic Environment

As with the other endocrine disrupting chemicals described in this review, several processes act to disperse BPA following its release in to the aquatic environment. The major factors influencing BPA concentrations are (i) dilution in the water column, (ii) microbial or enzymatic degradation, (iii) adsorption to suspended particulate material in the water column and subsequent deposition in sediments and (iv) photodegradation.

BPA is relatively water-soluble (250-300 mg/L), is less volatile than water, has a log organic carbon to water partitioning coefficient (Koc) between 2.5 and 3.32 and has a Log Kow value between 2.0 and 4.0. These physical properties were used to construct a fugacity model that described the likely partitioning of BPA between environmental compartments. Following release of BPA into the environment it would partition mainly into sediments (23%), soils (25%) and water bodies (52%) (Dorn *et al.*, 1987; Staples *et al.*, 1998; www.bisphenol-a.org, Lee and Peart, 2000). These measurements were made independent of the effect of any biodegradation processes, although as detailed in section 4.4.2.1 above BPA appears to be readily metabolised by microbial populations in surface waters (West *et al.*, 1998) and may not accumulate to a high degree in aerobic environments. Any undegraded BPA in the water column is likely to be bound to suspended solids which will later be absorbed onto sediments where further degradation may occur. Bioconcentration experiments giving bioconcentration factors of 5.1-68 and theoretical calculations using water solubility and Log Kow values giving BCFs of 49 and 196 indicate that BPA is unlikely to accumulate in biota inhabiting affected waters to the same degree as many of the other compounds detailed in this review (Staples *et al.*, 1998). In a study of the biodegradation of BPA in Japanese rivers Ike *et al.* (2000) found that BPA-degrading bacteria were relatively widely and abundantly distributed. The study concluded that BPA could be easily removed during wastewater treatment processes and that BPA could be easily degraded even if it is discharged into the aquatic environment. Two metabolites, 2,3-bis(4-hydroxyphenyl)-1,2-propanediol and p-hydroxyphenacyl alcohol, were found to be formed

(iii) Concentrations of BPA in WWTP, Industrial Wastewaters and Other Environmental Samples

Considering the extent to which BPA is used in industrial processes there are relatively few studies detailing environmental concentrations. In their recent review article, Staples *et al.* (1998) detail five studies carried out on BPA in surface waters between 1973 and 1996 in which concentrations were in the range 0.01-1.9 μg/L, with the levels in most samples being below instrumental detection limits (~ 0.01 μg/L). The concentrations of BPA in a variety of environmental samples from more recent studies are detailed in Table 1.6.6. Concentrations of BPA ranged from 0.01–17200 μg/L with the highest levels being measured in Japanese landfill site leachates (Yamamato *et al.*, 2001) and untreated wastewater from Canadian paper mills (Lee and Peart, 2000b). The lowest concentrations are generally found in biologically treated effluents and unimpacted surface waters.

Many of the studies show that environmental concentrations can exceed levels shown to cause induction of progesterone receptors and cell proliferation in cultured MCF-7 cells (Krishnan *et al.*, 1993). In the case of the landfill leachate samples the concentrations exceed LC_{50} for rainbow trout: 3000-7000 μg/L, fathead minnows: 4600-4700 μg/L and other fish species; 4000 – 15,000 μg/L (see Staples *et al.*, 1998 for references).

Table 1.6.6
Concentrations of BPA in WWTP and other environmental matrices

Matrix	Location (Sampling Year)	Concentration	Reference
WWTP Influent (untreated)	Canadian WWTP (1999) Canadian WWTP (1999-2000)	0.193 – 2.44 μg/L 0.329 (0.08-4.98) μg/L	Lee and Peart, 2000a Lee and Peart, 2000b
WWTP Effluent	Austria (1998-1999)	1.5 (n.d – 2.5) μg/L	Furhacker *et al.*, 2000
	NW U.S. (October 1997) NW U.S. (Feb-March 1998)	0.16 μg/L 0.38 – 2.7 μg/L	Barber *et al.*, 2000
Industrial Wastewater	Canadian WWTP (1999) Canadian WWTP (1999-2000)	0.031 – 0.233 μg/L 0.01-1.08 μg/L	Lee and Peart, 2000a Lee and Peart, 2000b
	Austria (1998-1999)	n.d – 50 μg/ L	Furhacker *et al.*, 2000
Paper Mill Wastewater	Toronto, Canada (1999-2000)	0.23 – 149.2 μg/L	Lee and Peart, 2000b
	Toronto, Canada (1999-2000)	Primary effluent: 0.753 (0.026-139) μg/L Secondary Effluent: 0.092 (<0.005-0.406) μg/L	Lee and Peart, 2000b
Raw Sewage Sludge	WWTP, Austria (1998-1999)	41 (28-72) μg/L	Furhacker *et al.*, 2000
Digested Sewage Sludge	Canadian WWTP (1999) Canadian WWTP (1999-2000)	0.20 – 8.73 μg/g dry weight 0.20 – 8.75 μg/g dry weight	Lee and Peart, 2000a Lee and Peart, 2000b
Landfill Leachate	Japanese Landfill sites (1994) Japanese Landfill sites (1995) Japanese Landfill sites (1995) 3 Swedish Landfill sites (1999)	0.35 (0.15-12.3) μg/L 61.4 (6.32-2980) μg/L 269 (1.3-17200) μg/L 4 – 136 μg/L	Yasuhara *et al.*, 1997 Yasuhara *et al.*, 1999 Yamamoto *et al.*, 2001 Paxeus, 2000
River Water	Taiwan (1999)	<0.05 – 3.0 μg/L	Ding and Wu, 2000
	Netherlands (1997)	0.004 - 0.065 μg/L	Belfrod *et al.*, 1999
	Canadian WWTP (1999) Canadian WWTP (1999-2000)	0.268-12.5 μg/g dry weight 0.033-36.7 μg/g dry weight	Lee and Peart, 2000 Lee and Peart, 2000b
Surface Waters	NW U.S. (October 1997) NW U.S. (Feb-March 1998)	0.06 μg/L 0.20 μg/L	Barber *et al.*, 2000
	Japanese Surface Waters (1996)	0.01 – 0.268 μg/L	Japanese EPA, 1997
River Sediment	NW Spain (2001)	<10 – 40 μg/kg	Petrovic and Barcelo, 2001
Marine Sediment	Masan Bay, Korea (1998)	0.012 (0.003-0.050) μg/g dry weight	Khim *et al.*, 1999

1.6.5 Phthalates

1.6.5.1 Useage and Legislative Status

Esters of phthalic acid, commonly known as phthalates, are in widespread use as additives that impart flexibility in polyvinylchloride (PVC) resins, (in which the phthalate concentration may reach 30-40% of the material). They are also used in other resins such as polyvinyl acetates, cellulosics and polyurethanes. They are used in a wide variety of commercial products such as food packaging, in the manufacture of vinyl floor tiles, adhesives, industrial paints, dielectric fluids (as replacements for PCBs), and synthetic leathers. In fact they are among the most widely used group of industrial chemicals in existence (Harris *et al*., 1997) They are produced in large volumes with global production of the most prolific phthalate, di(ethylhexyl)phthalate (DEHP) alone amounting to 3-4 million tonnes per annum (Wams, 1987). Ballpark European

consumption figures for 35 phthalates were detailed by Harris *et al.* (1997) and those which were found to be weakly estrogenic using a recombinant yeast screen in the same study are reported in Table 1.6.7 below. The chemical structures of selected phthalates are given in Figure 1.6.6.

Table 1.6.7
European and Ireland only consumption data for phthalates shown to be estrogenic in a recombinant yeast screen (taken from Harris *et al.*, 1997; CTC, 1998b,c)

Phthalate Name	Abbreviation	European Consumption (tonnes/annum)	Imports into Ireland (tonnes/annum)
Di(ethylhexyl)phthalate	DEHP	400,000 – 500,000	6,000 *
Diisononyl phthalate	DINP	100,000 – 200,000	n.a
Butylbenzyl phthalate	BBP	20,000 – 50,000	1100
Di-n-butyl phthalate	DnBP	20,000 – 40,000	6000 *
Diisobutyl phthalate	DIBP	20,000 – 40,000	6000 *
Ditridecyl phthalate	DTDP	3,000 – 10,000	n.a
Diethyl phthalate	DEP	10,000 – 20,000 (with dimethyl phthalate)	n.a
Butyl cyclohexyl phthalate	BCHP	Negligable	n.a
Diphenyl phthalate	DPhP	Negligable	n.a
Isohexylbenzyl phthalate	IHBP	Negligable	n.a

** sum of all dioctyl phthalates imported (DEHP + DBP + DIBP + di-n-octyl phthalate + dioctyl isophthalate)*

There is limited information about the quantities and types of phthalates used by different industrial sectors in Ireland. The Clean Technology Centre (1999b, 1999c) have provided information on two of the most widely used compounds: DEHP and BBP. The heaviest users of DEHP appear to be those companies manufacturing paints and vinyl floor coverings, although other industries including the manufacture of man made fibres and upholstery, mouldings, rubber, rigid and flexible PVC and in the dyeing, treatment and finishing of textiles are thought to use phthalates in their processes.

In the U.S., four phthalates (DMP, DEP, DnBP and BBP) are included in the Toxics Release Inventory, which requires manufacturers producing or using these chemicals to report estimates of the amounts of these chemicals released to surface waters (Staples *et al.*, 2000).

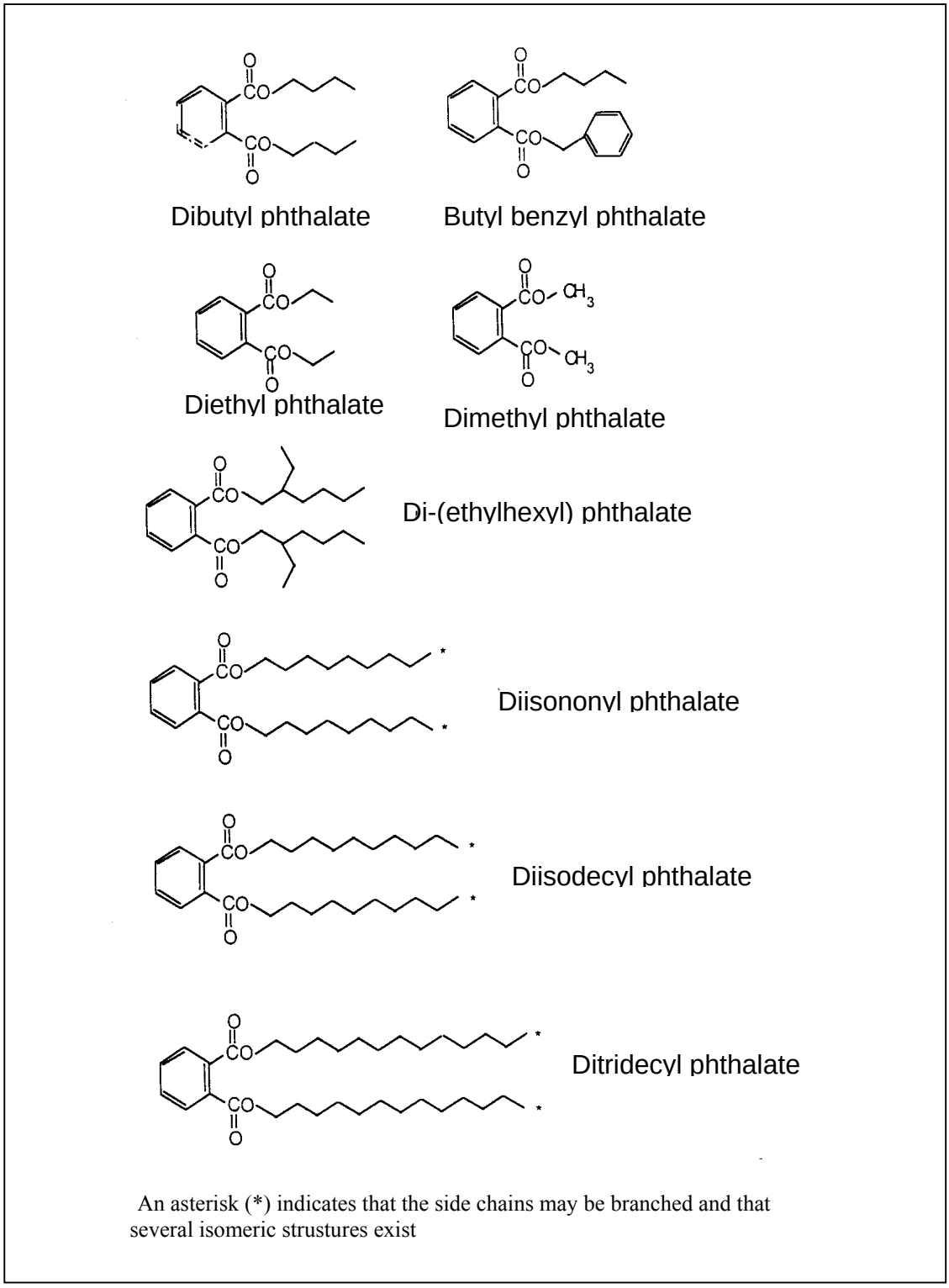


Figure 1.6.6
The chemical structures of selected phthalates

1.6.6.2 Environmental Behaviour of Phthalates

In many applications, phthalates are not chemically bonded to the plastic polymers to which they have been added and therefore can be leached from those products into the

environment. It is for this reason, and the widespread use of these chemicals in a vast array of products, that many phthalates are now ubiquitous environmental contaminants (Rhodes *et al.*, 1995; Harris et al., 1997). Despite the constant input of these chemicals into the aquatic environment, their rapid degradation prevents their serious environmental accumulation. The environmental behaviour of individual phthalates is heavily influenced by their physicochemical properties. These properties were reviewed by Dempsey and Costello, 1998 and Staples *et al.*, (1997) and are summarised in Table 1.6.8.

(i) Behaviour in WWTP

The biodegradability of phthalates has been extensively reviewed (Staples *et al.*, 1997). In acclimated activated sewage sludge biodegradation of DMP, DEP, DBP and BBP was >99% (Sugatt *et al.*, 1984). All subsequent data have supported the assumption that WWTP conservatively remove these phthalates at least by 90%. Phthalates with longer alkyl side chains have been shown to remain intact in anaerobic digestor sludge (Shelton *et al.*, 1984). These latter compounds will, however degrade, albeit at a slower rate than the smaller molecules, in aerobic activated sludge systems.

The behaviour of phthalates in WWTP will be controlled by their physico-chemical properties. Those phthalates with longer alkyl chain lengths and therefore greater lipophilicity, sediment sorption coefficients and low water solubility will be bound to the particulate phase and will eventually end up in the sewage sludge. Reported removal rates of BBP and DEHP in WWTP is > 90% with active coal and activated sludge treatments (Riwa, 1998). This has been confirmed by the high concentrations of one such phthalate, DEHP, found in dried sewage sludge from municipal WWTP throughout the world that ranged from 3.9 – 661 mg/kg dry weight. (NSEPB, 1992; DEPA, 1995, Schnaak *et al.*, 1997; Staples *et al.*, 1997, Cheng *et al.*, 2000). Once in the sewage sludge, there is evidence of biodegradation of DEHP by indigenous micro-organisms but this process appears to be relatively slow (Madsen *et al.*, 1999; Cheng *et al.*, 2000). The application of sewage sludge to soil is a potential source of phthalates into the terrestrial and aquatic environment (Harris et al., 1997).

Table 1.6.8
Physicochemical properties and general environmental behaviour of selected phthalates (adapted from Staples *et al.*, 1997 and BKH, 2000)

Name*	Alkyl Chain Length	Log K_{ow} **	Aqueous Solubility (mg/L)	Vapour Pressure (mm Hg ~25°C)	Henry's Constant (atm-m^3/mol) ***	K_{oc} Soil/ sediment (L/kg)	General Comment
DMP	1	1.61	4200	2×10^{-3}	1.22×10^{-7}	55-360	Very water-soluble. Relatively volatile. Weak absorption onto sediments. Low bioaccumulation expected
DEP	2	2.38	1100	1×10^{-3}	2.66×10^{-7}	69-1726	Very water-soluble. Relatively volatile. Weak absorption onto sediments. Low bioaccumulation
DIBP	4	4.11	20	1.8×10^{-6} - 5.8×10^{-4}	1.83×10^{-7}	-	-
DnBP	4	4.45	11.2	2.7×10^{-5}	8.83×10^{-7}	1375-14900	Poorly soluble in water with low volatility. Strong sorption onto sediments. Possible bioaccumulation at low trophic levels.
BBP	4	4.59	2.7	5×10^{-6}	7.61×10^{-7}	9000-17000	Poorly soluble in water with low volatility. Strong sorption onto sediments. Possible bioaccumulation at low trophic levels.
DHP	6	6.30	0.05	5×10^{-6}	4.50×10^{-5}	52600	Very low water solubility. Strong sorption onto sediments. Possible bioaccumulation at low trophic levels.
DnOP	8	8.06	0.0005	1×10^{-7}	1.03×10^{-4}	-	Practically insoluble in water, low volatility although is known to evaporate from products. Possible bioaccumulation at low trophic levels.
DEHP	8	7.50	0.003	1×10^{-7}	1.71×10^{-5}	-	Practically insoluble in water, low volatility although is known to evaporate from products. Possible bioaccumulation at low trophic levels.
DINP	9	9.0–9.4	<0.001	$< 5 \times 10^{-7}$	-	-	-
DTDP	13	13.1–13.4	<0.001	$<5 \times 10^{-7}$	-	1200000	-

** see Table 1.6.7 for full chemical name, ** - Log octanol/water coefficient as a measure of lipophilicity *** Henry's Law constant is the ration of vapour pressure to molar water solubility and estimates the tendency of a substance to escape from water to air.*

(ii) Behaviour in the Aquatic Environment

The physico-chemical properties of the individual phthalates will dictate their behaviour in the aquatic environment.

Water

There are few reliable studies (containing full quality assurance information) relating to the determination of phthalates in water samples. Those that are reliable include reports from the Rhine River (Furtmann, 1993, Hendriks *et al.*, 1994; RIWA, 1998) and from the Niagra River (DIG, 1988-1995). Concentrations of DMP, DEP, DBP and BBP generally ranged from 0.01 to 0.1 μg/L. No reliable information is available on phthalate concentrations in the riverine environment in Ireland (Dempsey and Costello, 1998). Recent studies on the Lough Ree and Derg catchments in central Ireland reported concentrations of DEHP and BBP, however there was considerable doubt over the results due to poor quality assurance data and lack of sample contamination controls (Kirk, McLure, Morton, 2002). Of 10 Irish groundwater samples collected, 3 had measurable concentrations (0.28-0.45 μg/l) of phthalates (Cullen and Co, 1994).

The adsorption of the more hydrophobic phthalates onto particulate material in the water column is enhanced in the presence of salt and this is likely to play a major role in the distribution of these compounds in the estuarine environment. As a result of this mechanism more than 50% of DEHP discharged to a catchment may be retained in estuarine sediment (Turner and Rawling, 2000).

Landfill leachates

The majority of phthalates, especially those with longer alkyl chains, are hydrophobic and would not be expected to leach into groundwater and surface water from landfilled phthalate containing material, however they are often found at relatively high concentrations in this matrix. As with other hydrophobic organic contaminants (such as polychlorinated biphenyls and organochlorine pesticides) phthalates may be mobilised through interaction with dissolved organic carbon and as undissolved lipid-rich particles. In a study of leachates collected from German landfill sites, the solubilising effects of dissolved organic carbon meant that up to 85% of the lipophilic phthalates were present in the dissolved fraction of the leachate (Bauer and Hermann, 1998). In a more recent study of organic contaminants in landfill leachates from Sweden, Paxeus, (2000), measured five phthalates: DEP, DnBP, BBP, DEHP and DnOP. DEHP was present in the highest concentrations in all three sites studied (see Table 1.6.9).

Sediments

As observed in Table 1.6.8 many phthalates are expected to bind strongly with particulate matter (this will also be dependent on the amount of dissolved organic carbon present in the water column (see above (ii)) and sediments would be expected to act as both a sink and secondary source of these compounds to the aquatic environment. Generally, in sediments as well as other environmental matrices DEHP is present at the highest concentrations as this compound is the most commonly used phthalate in many industrial processes and products. High concentrations have been found in sediments around wastewater treatment plant outfalls with concentrations decreasing with distance from the discharge point (McDowell and Metcalfe, 2001)

Biota

Many studies have been carried out on the bioconcentration and bioaccumulation of phthalates and are summarised in the review of Staples *et al.* (1997). In general bioaccumulation of these chemicals appears to be limited by biodegradation (enzymatic metabolism) in aquatic organisms that increases with increasing trophic level (Wofford *et al.*, 1981).

(iii) Environmental Concentrations

Table 1.6.9
Concentrations of phthalates in different environmental matrices

Matrix	Location (Sampling Year)	Concentration	Reference
Sewage Sludge	Tapei, Taiwan (1999)	105 – 153 mg/kg d.w. DEHP	Cheng *et al.*, 2000
	Sweden	25-661 mg/kg d.w. DEHP	NSEPB, 1992
	Denmark	3.9 – 170 mg/kg d.w. DEHP	DEPA, 1995
	Germany	170 mg/kg d.w. DEHP	Schnaak *et al.*, 1997
	USA	136-578 mg/kg d.w. DEHP	Staples *et al.*, 1997
Landfill Leachates	Bavaria, Germany (1996)	0.1– 7.7 µg/L DMP dissolved 0.1 – 5.9 µg/g DMP particulate 0.1 – 4.0 µg/L DEP dissolved 0.3 – 8.6 µg/g DEP particulate 0.2 – 62.7 µg/L DnBP dissolved 3.5 – 126 µg/g DnBP particulate 0.3 – 4.7 µg/L BBP dissolved 0.1 – 26.9 µg/g BBP particulate 0.6 – 236 µg/L DEHP dissolved 0.4 – 168 µg/g DEHP particulate	Bauer and Hermann, 1998
River Water	River Aire (UK) River Trent (UK)	0.36 – 21.0 µg/L DEHP 0.74 – 18.0 µg/L DEHP	Long *et al.*, 1998
	Rivers Etherow and Irwell (UK)	0.4 – 1.9 µg/L DEHP	Fatoki and Vernon, 1990
	Central Italy	0.3 – 31.2 µg/L DEHP	Vitali *et al.*, 1997
River Sediments	River Aire (UK) River Trent (UK)	7.89 – 115 µg/g DEHP 0.84 – 31.0 µg/g DEHP	Long *et al.*, 1998
	Central Italy	0.06 – 0.49 µg/g DEHP	Vitali *et al.*, 1997
Coastal Waters	Tees Bay (UK) Plymouth Sound (UK)	0.98-2.20 µg/L DEHP 0.010-0.28 µg/L DEHP	Law *et al.*, 1991
Sewage Impacted coastal sediments	Hamilton Harbour, Ontario Canada (1997)	6.5-29.7 µg/g d.w DEHP <0.30 µg/g d.w DBP <0.11 µg/g d.w BBP DEP N.D DINP N.D	McDowell and Metcalfe, 2001

1.6.6 Other Potential Endocrine Disruptors

There are many more potentially endocrine disrupting chemicals than those TIE target compounds detailed above: these include (i) phytestrogens and mycestrogens (estrogenic), (ii) pesticides (estrogenic), (iii) PCBs, (estrogenic and anti estrogenic) and their hydroxylated metabolites (estrogenic), (iv) dioxins and furans (anti-estrogenic), and (v) cadmium and organotin compounds. The estrogen-mimicking potential of

compounds within these groups has been reviewed by Dempsey and Costello (1999), the U.S. National Research Council (1999) and Harries (1997). A representative, qualitative (i.e. the list does not give an indication of the absolute estrogenic potency) list of compounds with estrogenic properties is provided in Table 1.5.2. Chemicals with the potential to disrupt the endocrine systems of fish are reviewed by Kime (1999), and in other biological systems by Rooney and Guillette (2000).

2. Development of Methodology and Validation

2.1 Introduction

This Chapter provides a discussion of each of the methodologies used in the execution of this body of work, and of any validation studies that were carried out prior to using these techniques for the purposes of the current study.

2.2 *In Vitro* Bioassay and Toxicity Identification and Evaluation (TIE) Methodologies

2.2.1 Semi-quantitative Detection of Salmonid Vitellogenin in Wild Brown Trout Plasma by Enzyme Immunoassay (EIA).

The Salmonid Vitellogenin Enzyme Immunoassay (EIA) was performed using a kit from Biosense Laboratories (Norway). The kit contains a complete set of EIA reagents designed for use in the semi-quantitative detection of vitellogenin in plasma samples from fish. The assay is based on the detection of vitellogenin by a monoclonal antibody, BN-5, raised against vitellogenin from Atlantic salmon (*Salmo salar*). Due to cross-reactivity of the antibody with vitellogenin of other species, the assay may be used for the detection of vitellogenin in plasma samples from a variety of other species including the *Salmoniformes* of which Brown trout (*Salmo trutta*) is a member. The assay may be considered equally sensitive for vitellogenin from both Brown trout and Atlantic salmon (Table 2.2.1).

Table 2.2.1
Cross-reactivity of the monoclonal antibody BN-5 with vitellogenin from different fish species in EIA (adapted from Biosense Laboratories product description)

Fish Species	Relative cross-reactivity
Salmoniformes	
Atlantic salmon (*Salmo salar*)	+++
Brown trout (*Salmo trutta*)	+++
Rainbow trout *(Oncorhynchus mykiss)*	+++
Arctic charr *(Salvelinus alpinus)*	+++
Pleuronectiformes	
Turbot *(Scophthalmus maximus)*	++
Flounder *(Platichthys flesus)*	++
Halibut *(Hippoglossus hippoglossus)*	++
Gadiformes	
Atlantic cod *(Gadus morhua)*	None
Cypriniformes	
Zebrafish *(Danio rerio)*	+

The EIA is a semi-quantitative assay for vitellogenin, and it is not suitable for measuring absolute amounts of vitellogenin. There is no simple, direct correlation between absorbance values and vitellogenin concentrations. The level of vitellogenin is measured as absorbance values, the higher the absorbance value the higher the level of vitellogenin in the plasma sample. In each assay performed the positive control, negative control and samples were assayed in duplicate. The absorbances were imported into Microsoft Excel and the average of each duplicate set was calculated. Sample

absorbances significantly higher than that of the negative control were considered to be vitellogenin positive (Figure 2.2.1).

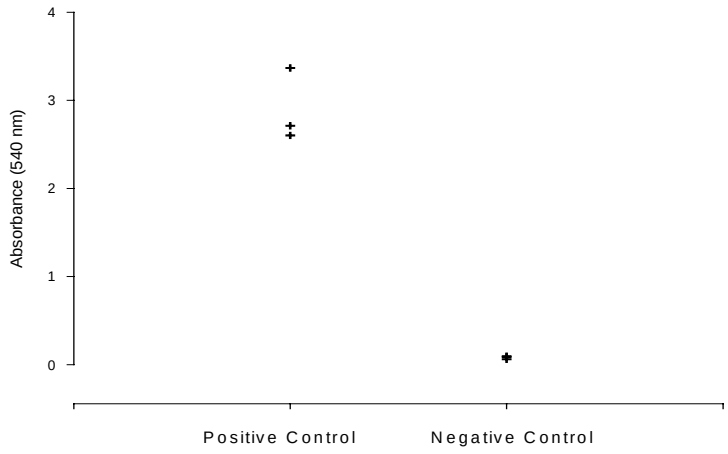


Figure 2.2.1
Cross-reactivity of BN-5 antibody with purified Atlantic salmon vitellogenin (postive control) and coating buffer (negative control).
Positive and negative control values from three separate experiments are shown. Each data point represents the mean of duplicate determinations.

2.2.2 Quantitative Detection of (Anti)Estrogenic Chemicals using a Recombinant Yeast Bioassay

The recombinant yeast strain was developed by the Genetics Department of Glaxo Wellcome, plc. (UK). To generate the recombinant yeast the DNA sequence of the human estrogen receptor (hER) was stably integrated into the main chromosome of the yeast *Saccharomyces cerevisiae*. Expression plasmids carrying the reporter gene *lac*-Z (encoding the enzyme β-galactosidase) were also integrated into the yeast cells.

The hER is expressed in a form capable of binding to estrogen-responsive sequences (EREs) which are situated within a strong promoter sequence on the expression plasmid. Upon binding an active ligand, the estrogen-occupied receptor interacts with the EREs to modulate *lac*-Z gene expression. β-galactosidase is therefore synthesised and secreted into the medium, where it metabolises the chromogenic substrate, chlorophenol red β-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorbance at 540 nm.

In addition to detecting estrogenic chemicals, this yeast estrogen screen (YES) can also be used to detect anti-estrogens. In this case the natural estrogen 17β-estradiol induces a sub-maximal response and the ability of chemicals to inhibit this response is determined. Estradiol equivalent ($E2_{eq}$) calculations can also be performed using the YES. These calculations can be employed to determine the $E2_{eq}$ concentration of any sample tested using the bioassay by relating the absorbance of the sample to a 17β-estradiol standard curve.

Quality control requirements necessitated that each YES plate contained at least one row of blanks (solvent and assay medium), and that each bioassay performed included a

control plate containing a 17β-estradiol standard curve. The absorbances of each well were measured at 540 nm (optimum absorbance for CPRG is approximately 575 nm) and 620 nm (for turbidity) and a simple calculation to correct for turbidity was applied to the absorbance data.

The sensitivity and reproducibility of the YES (Figure 2.2.2) were assessed by measuring the response of the yeast to triplicate dilutions of the natural estrogen 17β-estradiol compared to triplicates containing ethanol (blank). The response of the yeast over the concentration range 1 x 10^{-8}M to 4.8 x 10^{-12}M was highly reproducible, with a concentration of 3.7 x 10^{-11} M (10 ng/L) 17β-estradiol producing a detectable increase in β-galactosidase production.

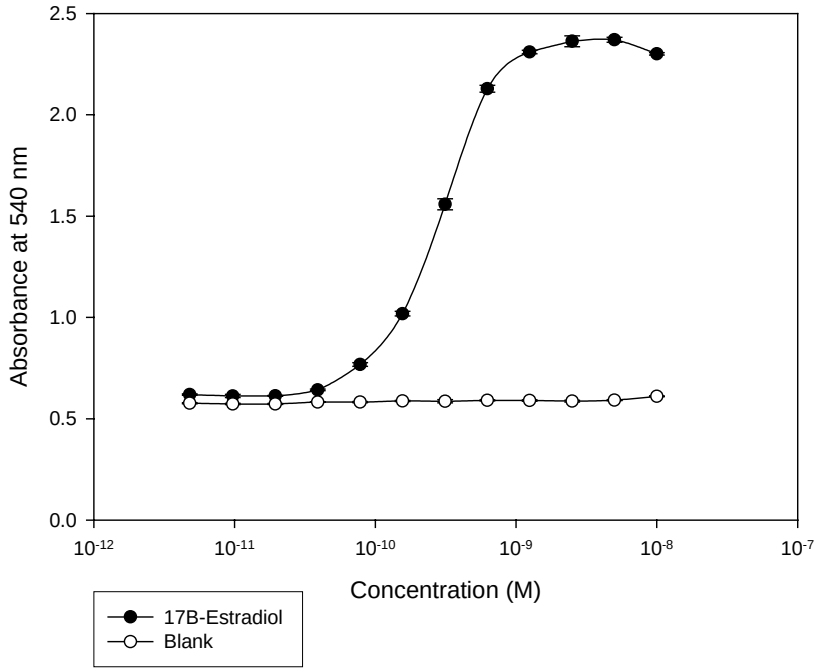


Figure 2.2.2
The sensitivity and reproducibility of the YES.
17β-estradiol concentration is plotted against the absorbance of the medium after 3 days incubation. Values represent the mean ± SEM (n = 3).

The specificity of the YES (Figure 2.2.3) was assessed by determining the ability of a range of steroids to stimulate β-galactosidase production. The natural estrogen 17β-estradiol produced a full dose-response curve over the concentration range tested. Bisphenol A and genestein induced β-galactosidase synthesis and produced full dose-response curves. Testosterone, BBP and o,p'-DDT produced sub-maximal dose-response curves of varying potency. DEHP induced a slight response at concentrations higher than 1 x 10^{-5} M. The results of the YES specificity assessment presented here are comparable to those previously reported (Beresford *et al*., 2000; Sohoni and Sumpter, 1998; Routledge and Sumpter, 1996).

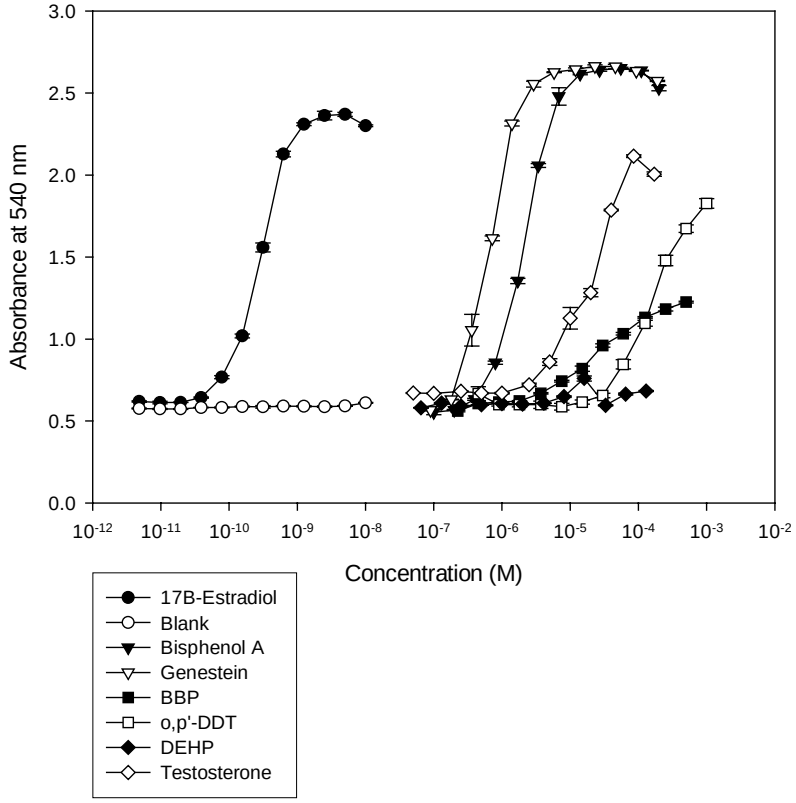


Figure 2.2.3
The estrogen specificity of the YES
The effect of selected steroids on the YES was investigated. Values represent the mean ± SEM (n = 3).

2.2.3 Quantitative Detection of (Anti)Androgenic Chemicals using a Recombinant Yeast Bioassay

The recombinant PGKhAR yeast strain was developed by the Genetics Department of Glaxo Wellcome, plc. (UK). To generate the recombinant yeast the DNA sequence of the human androgen receptor (hAR) was stably integrated into the main chromosome of the yeast strain *Saccharomyces cerevisiae*. Expression plasmids carrying the reporter gene *lac*-Z (encoding the enzyme β-galactosidase) were also integrated into the yeast.

The hAR is expressed in a form capable of binding to androgen-responsive sequences (AREs) that are situated within a strong promoter sequence on the expression plasmid. Upon binding an active ligand, the androgen-occupied receptor interacts with the AREs to modulate *lac*-Z gene expression. β-Galactosidase is therefore synthesised and secreted into the medium, where it metabolises the chromogenic substrate, CPRG, which is normally yellow, into a red product measured by absorbance at 540 nm.

In addition to detecting androgenic chemicals the hAR bioassay can also be used to detect anti-androgens. In this case the androgen testosterone induces a sub-maximal response and the ability of chemicals to inhibit this response is determined. Testosterone equivalent (T_{eq}) calculations can also be performed using the hAR bioassay. These

calculations can be employed to determine the T_{eq} concentration of any sample tested using the bioassay by relating sample absorbance to a testosterone standard curve.

Quality control requirements necessitated that each hAR bioassay plate contained at least one row of blanks (solvent and assay medium), and that each bioassay performed included a control plate

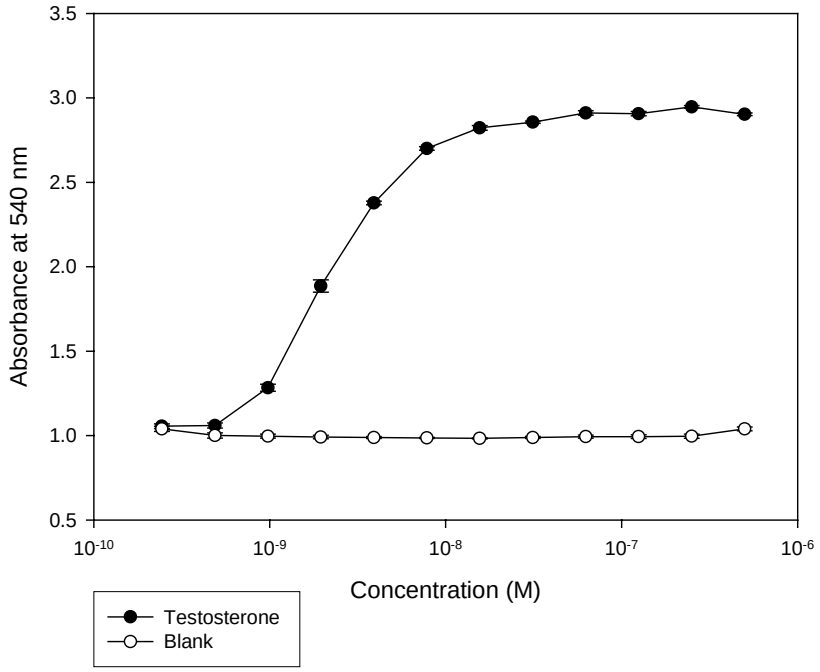


Figure 2.2.4
The sensitivity and reproducibility of the hAR bioassay.
The concentration of testosterone is plotted against the absorbance of the medium after 2 days incubation. Values represent the mean ± SEM (n = 3)

containing a testosterone standard curve. The absorbance of each well was measured at 540 nm (optimum absorbance for CPRG is approximately 575 nm) and 620 nm (for turbidity) and a simple calculation to correct for turbidity was applied to the absorbance data.

The sensitivity and reproducibility of the hAR bioassay (Figure 2.2.4) was assessed by measuring the response of the yeast to triplicate dilutions of the natural androgen testosterone compared to triplicates containing ethanol (blank). The response of the yeast over the concentration range 5×10^{-7} M to 2.44×10^{-10} M was highly reproducible, with a concentration of 4.88×10^{-10} M (140 ng/L) testosterone producing a detectable increase in β-galactosidase production.

The specificity of the hAR bioassay (Figure 2.2.5) was assessed by determining the ability of a range of steroids to stimulate β-galactosidase production. The androgen testosterone produced a full dose-response curve over the concentration range tested. 17β-Estradiol was less potent than testosterone and produced a sub-maximal dose-response curve. Progesterone and estrone induced β-galactosidase synthesis at

concentrations above 10^{-8} M, but not at lower concentrations. Hydrocortisone was inactive in the hAR bioassay at the concentration range tested. The results of the hAR bioassay specificity assessment presented here are comparable to those previously reported (Sohoni and Sumpter, 1998).

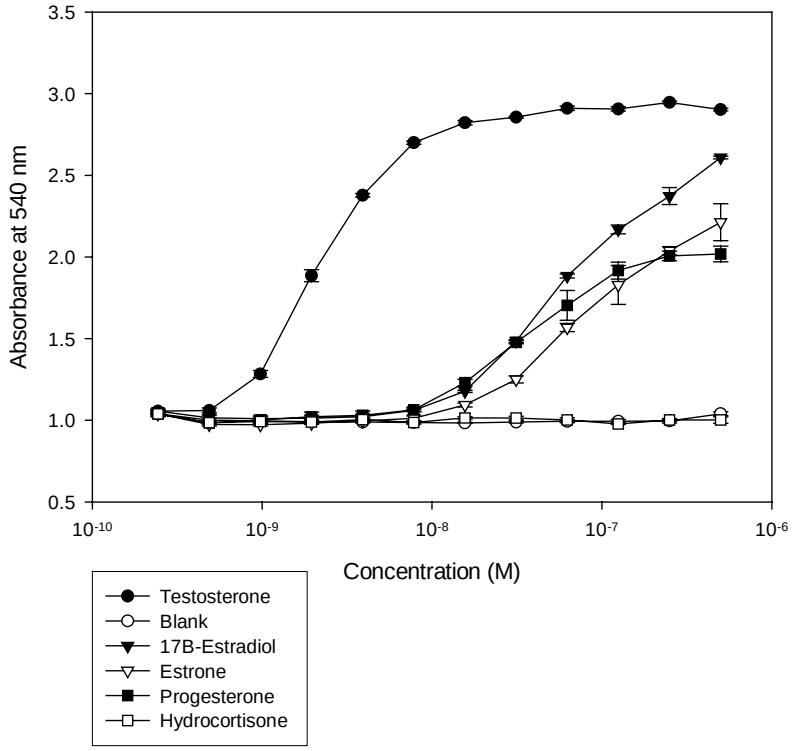


Figure 2.2.5
The androgen specificity of the hAR bioassay.
The effect of selected steroids on the hAR bioassay was investigated.
Values represent the mean ± SEM (n = 3).

The anti-androgen specificity of the hAR bioassay (Figure 2.2.6) was assessed by determining the ability of certain chemicals to inhibit the activity of testosterone. The clinical anti-androgen, flutamide, was successful at inhibiting the activity of testosterone in a dose-dependent manner. The chemicals' BPA, BBP and vinclozolin were also successful at inhibiting the activity of testosterone in a dose-dependent manner with a potency equal to that of flutamide. The results of the hAR bioassay anti-androgen specificity assessment presented here are comparable to those previously reported (Sohoni and Sumpter, 1998).

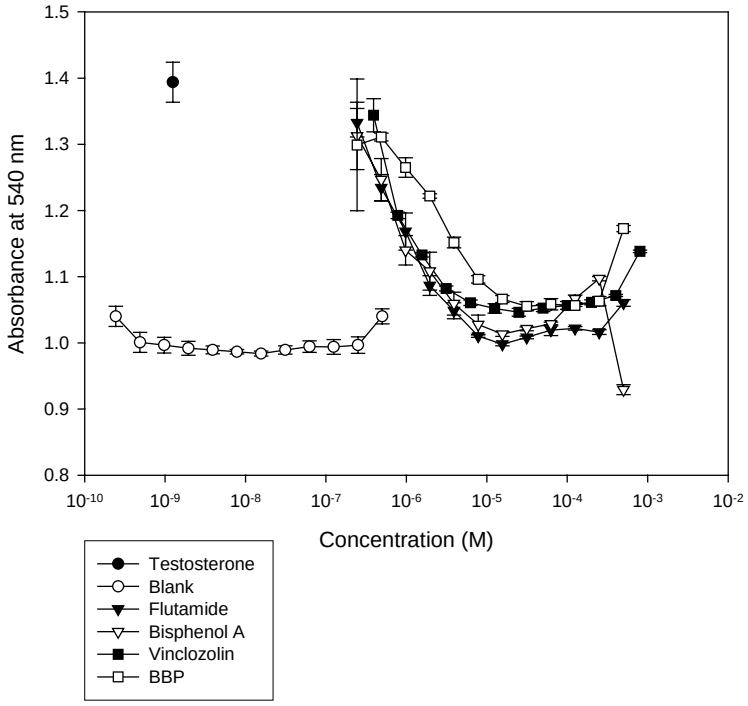


Figure 2.2.6
The anti-androgen specificity of the hAR bioassay
The effect of selected anti-androgens on the hAR bioassay was investigated. Values represent the mean ± SEM (n = 3).

2.2.4 Toxicity Identification and Evaluation Methods

The analytical methodologies described in the following pages form a relatively novel technique for the identification of estrogenic substances in a variety of aqueous environmental matrices. The reasons for choosing a TIE type method over a targeted analyte approach have been reviewed and discussed in the literature review section of this report. The performance of the method has been determined using a number of estrogenic substances most likely to be present in the aquatic environment, listed in Table 2.2.2.

Table 2.2.2
Target compounds and Calibration Standard Components
(List 1 and List 2 are calibration mixtures).

List 1	
Dimethyl Phthalate	Plasticiser
Diethyl Phthalate	Plasticiser
Di-n-butyl Phthalate	Plasticiser
Butyl Benzyl Phthalate	Plasticiser
Bis 2-(ethylhexyl) Phthalate	Plasticiser
Di-n-octyl phthalate	Plasticiser
Hexachlorobenzene	Fungicide
Atrazine	Pesticide
4-tert-Octylphenol	Detergent breakdown product
Chlorinated biphenyl 153	Industrial chemical indicator compound
Bisphenol A	Plastic additive
p,p'-DDT	Organochlorine Pesticide
Endrin	Organochlorine Pesticide
Lindane (γ-HCH)	Organochlorine Pesticide
17β-Estradiol	Natural/Synthetic Hormone
Estrone	Natural Hormone
17α-Ethinyl estradiol	Synthetic Hormone

List 2	
Nonylphenol isomers	Industrial Detergent breakdown product
Nonylphenol monoethoxylate	Industrial Detergent component / breakdown product
Nonylphenol diethoxylate	Industrial Detergent component / breakdown product

The technique uses a mixture of analytical chemistry techniques (solid phase extraction, liquid/liquid extraction, semipreparative HPLC fractionation, GC-MS) in combination with a yeast-based estrogen screening bioassay to isolate estrogenic substances.

Any environmental water sample will contain a vast number of organic compounds, many of which have deleterious effects on the aquatic environment but which do not exhibit estrogenic effects. The aim of the TIE method, therefore, is to separate those chemicals present in the environment that do have an estrogenic effect from those which don't. Many of the compounds that exhibit estrogenic effects are well known (see the literature review in this report, section 1.6), however, there may well be chemicals in the environment which elicit these effects but which are as yet unknown.

The entire method is relatively complex and an overview of the entire process is provided in Figure 2.2.7

In brief water samples up to 20 L are collected, filtered and extracted using C18 solid phase extraction cartridges. The particulate phase collected on the filters are extracted ultrasonically in hexane, tested for estrogenic activity and if positive are tested by GC-MS following addition of internal standards to allow quantitation.

Dissolved organic compounds extracted onto the SPE cartridges are sequentially eluted with solvent mixtures of decreasing polarity (from 75:25 methanol:water to 100% hexane). Each fraction (coarse fractions) is tested using the YES bioassay.

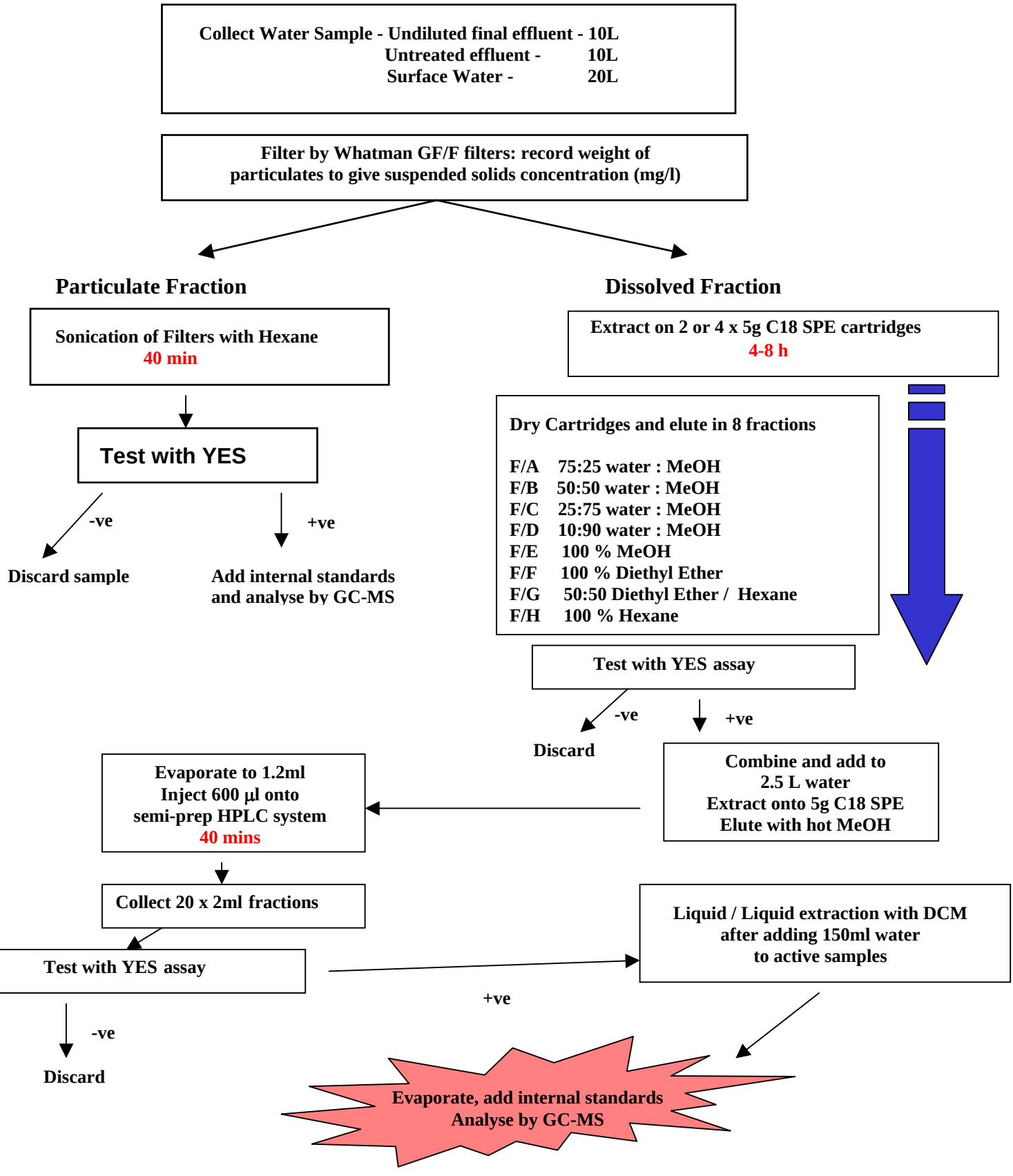
Any of the coarse fractions that give a positive estrogenic response are recombined, dissolved in 2.5 L of ultrapure water and re-concentrated on a C18 SPE cartridge. All of the re-extracted compounds are eluted from the cartridge with hot methanol. The sample is evaporated to 1.2ml and 600 µl of the extract is injected onto a pre-calibrated semi-preparative HPLC system. The sample components are eluted using a solvent gradient from 60:40 water:methanol to 100% methanol over 40 minutes. Fractions (fine fractions) are collected every 2 minutes using a Waters Fraction collector.

Each of these fractions is tested for estrogenic activity using the YES bioassay. Fractions not showing any estrogenic activity are discarded. Samples which do show activity are diluted with 300 ml ultra pure water and liquid/liquid extracted with DCM.

The samples are then evaporated and a known amount of deuterated PAH internal standards are added. The sample is evaporated to a known final volume and is ready for analysis by GC-MS.

The GC-MS system is calibrated using a 5 level calibration curve for each of the compounds listed in Table 2.2.2. Samples are analysed in single ion monitoring mode for the target compounds and in full scan to provide more information on other compounds present in the active fraction.

Figure 2.2.7 Overview of the Toxicity Identification and Evaluation (TIE) Method for Endocrine Disrupting Chemicals in Raw, Potable and Waste Waters



2.3 *In vivo* Studies Methodology

2.3.1 Site Selection for *In Vivo* Studies

2.3.1.1 Caged Fish Study

The water system chosen for the *in vivo* caged fish study was the River Lee. A stretch of the river between Ballincollig and Cork City was the main focus of the study. Ballincollig WWTP (adjusted population equivalent (PE) 18,700) discharges into this section of the river, which is 4 km upstream of the intake to the Lee Road water treatment works (WTW), which provides drinking water to Cork City. A weir immediately downstream of the WTW prevents the tidal nature of the lower reaches of the river from affecting the quality of the drinking water.

This location was selected on the basis of the Ballincollig PE value, output of the WWTP and the flow regime of the River Lee, which suggested, based on the work of Jobling *et al*. (1998), that during periods of low flow the River Lee is likely to be estrogenically activity below the WWTP outfall at Ballincollig (see Chapter 5).

The study was performed using caged male Rainbow trout (minimum of 20 male fish per cage) located at test sites immediately upstream (Ordnance Survey grid reference: W591716) at the Ballincollig WWTP outfall (W 592715) (Figure 2.3.1) and 4 km downstream at the input of the Lee Road WTW (W 649715).

Figure 2.3.1
River Lee upstream of Ballincollig WWTP outfall, Co. Cork.
This site serves as a control for the effect of the Ballincollig WWTP effluent on the river Lee. The fish cage was placed in the river at the position indicated by the house, which is approximately 50 m upstream of the WWTP outfall. The cage visible in the foreground is located at the WWTP outfall.

A cage was also placed at a control site located at Gougane Barra (headwaters of the river Lee: W 095661) that served as an internal control for the Lee river system. The primary control (reference) site was at Lough Barfinnihy, a highland oligotrophic lake in Co. Kerry with no anthropogenic inputs (V 850766) (Figure 2.3.2). These sites allowed determination of the effects (if any) of background levels of estrogens on fish populations.

Figure 2.3.2
Lough Barfinnihy, Kenmare, Co. Kerry
Lough Barfinnihy is an oligotrophic lake that served as the primary control (reference) site for the caged fish study.

In addition, positive control studies were set-up in flow-through tanks at the UCC Aquaculture Development Centre, Lee Maltings, Cork (see section 2.3.4).

2.3.1.2 Wild Brown Trout Survey

Samples were taken from the Rivers Liffey, Lee and Bandon, as well as from Lough Leane and Lough Guitane in the Killarney Lakes. Each of these water systems was chosen for study because of the potential for pollution from EDCs from several different sources including domestic, agricultural and industrial.

On each river/lake system several sites were sampled; these sites were chosen for different reasons. On each system the control site was chosen to give a balance between a site minimally impacted by human activity and a site that has a wild fish population that could sustain sampling. In the case of the River Liffey, a control site was not included in the sampling programme (see below). The other study sites on each system were chosen to incorporate a site downstream (same water body for Lakes) of potential EDCs input and a site upstream of potential EDCs input.

(i) River Liffey:
The sampling programme in the River Liffey was designed to determine the effect (if any) of the effluent from the Osberstown WWTP (PE 68,000) on the wild Brown trout population. Thus, all sampling took place in the vicinity of the Osberstown sewage treatment works, Co. Kildare (N 867203) (Figure 2.3.3). The upstream site, which is unaffected by the Osberstown WWTP effluent, was at Carragh Bridge, Co. Kildare (N 854208).

Figure 2.3.4
The Leinster Aqueduct, Co. Kildare.

The second sampling site was situated just downstream of the outfall from the WWTP at Osbertown (N 869207) and ended at the Leinster Aqueduct (N 877227) (Figure 2.3.3).

Figure 2.3.3

River Liffey sampling sites, upstream and downstream of the Osberstown WWTP.

(ii) River Lee

Ballincollig WWTP (PE 18,000) was considered the most significant potential source of EDCs to the River Lee (see Chapter 4 for further discussion of Ballincollig WWTP as a source of EDCs). Thus, the study sites were chosen to be upstream and immediately downstream of the outfall from the Ballincollig WWTP, while Ballyvourney was chosen as the control site for the River Lee system. This control site comprised a 100m stretch bellow Milleeny Bridge (OS grid reference W 162759), which is located above

the town of Ballyvourney. This site was chosen as it is upstream of any potential EDCs input and has a population of fish suitable to be sampled. All fish at this site were caught by electro-fishing.

Figure 2.3.5.
River Lee sampling sites, upstream and downstream of the Ballincollig WWTP.

The site upstream of Ballincollig WWTP comprised a 150m stretch of river between OS grid reference W 580714 and 581714. This site is located 1 km upstream of the Ballincollig WWTP outfall pipe. All fish at this site were caught by electro-fishing (Figure 2.3.5).

The site downstream of Ballincollig WWTP comprised two stretches of the river: one above OS grid reference W 605718 to 606715 and one below Bannow Bridge (OS grid reference W 610718 to 614717). The further upstream of these two locations is 1.5 km downstream of the Ballincollig WWTP outfall pipe (Figure 2.3.5). Fish at this site were caught by both electro-fishing and rod and line angling.

(iii) River Bandon

The River Bandon has potential for significant EDC input from three sources: Bandon WWTP, the agricultural mart and a food processing facility. These three potential sources of EDCs all enter the River Bandon below Bandon town. Thus, the study sites were chosen to be upstream and immediately downstream of Bandon town, while Ardcahan Bridge (OS grid reference W 243557) was chosen as the control site for the River Bandon. It is located upstream of any major urban areas, so there is little risk of EDCs entering the river. At this location the River Bandon is large enough to support a Brown trout population suitable to be sampled. At Ardcahan Bridge all fish were obtained by electro-fishing. Fish were taken from a stretch 100m above to 100m below the bridge.

Figure. 2.3.6
River Bandon sampling sites upstream and downstream of Bandon town.

The site upstream of Bandon town comprised a stretch of river between Baxter's Bridge (OS grid reference W 442546) and Bandon Park (OS grid reference W 474546), which is located above the Bandon town weir (Fig. 2.3.6). The weir acts as a partial natural barrier hindering fish movement between this site and the downstream site although fish can migrate downstream and a fish pass allows fish movement upstream. All fish from this site were caught using rod and line.

The site downstream of Bandon town comprised a stretch of river between Rough Hole (name of a pool) (OS grid reference W 509562) and O'Driscoll's Bridge (OS grid reference W 516569). This site is downstream of all potential EDCs input from the town (Figure 2.3.6). All fish at this site were caught using rod and line.

(iv) Killarney Lakes

Lough Guitane, was chosen as the control site for the Killarney lakes system. It is located upstream of any major urban areas, so there is little risk of EDCs entering the lake. Lough Guitane supports a large Brown trout population suitable to be sampled, fish were caught by rod and line.

Lough Leane was the chosen test site for the Killarney Lakes as it receives potentially significant EDC input from the Killarney WWTP effluent (PE 34,800 increasing to 70,000 during the tourist season) that enters the Lough in the Ross Bay area. In Lough Leane all fish were caught by rod and line during the angling competitions held on the 24/8/01, 29/9/01, 30/9/01 and the 12/10/01. The site upstream of the potential EDC input from Killarney town comprised a stretch of the River Gearhameen in the Black Valley. All fish from this site were caught using rod and line.

2.3.2 Sampling Methodologies

2.3.2.1 Fishing Methods

Handling: The trout that were retained for sampling were all greater than 13 cm in length. All smaller trout were returned unharmed as well as all other fish species encountered which included: salmon parr, stone loach (*Barbatula barbatula*), European eel (*Anguilla anguilla*), bream (*Abramis brama*), perch (*Perca fluviatilis*) minnow

(Phoxinus phoxinus) and gudgeon (*Gobio gobio*). Retained fish that were to be sampled were kept in a large bucket until sampling the area was complete, when they could be sacrificed.

Sacrificing the Fish: The trout were all sacrificed using a priest, a fishing implement made of brass that is used to kill the fish with a sharp rap to the head. Other methods of sacrificing the trout could have been used but were not for several reasons. Use of chemicals, such as benzocaine, was considered inadvisable for the purposes of this study, as they might interfere with any subsequent liver, bile duct and gall bladder analysis that might be required. CO_2 saturated water was not used, as it was impractical to bring compressed CO_2 to the study sites for the wild fish survey.

Fishing Methods: A large proportion of the wild Brown trout sampled were obtained by means of electrofishing. Portable electrofishing kits owned by UCC and the South Western Region Fisheries Board were employed for sampling on the rivers Lee and Bandon. Such equipment is usually only suitable for use in small streams and thus could only be used on the rivers Lee and Bandon during very low water conditions. The rivers Lee and Bandon were also sampled by rod and line. This electrofishing equipment was not suitable for use on lakes, and so all samples obtained from the Killarney Lakes were taken by means of rod and line. Samples taken from Lough Leane were obtained by means of angling competitions, held on 24/08/01, 29/09/01 and 12/10/01. However, in these cases the fish were dead several hours before tissue sampling could commence, and therefore no blood samples were obtained for fish from the Lough Leane.

Boat-operated electrofishing equipment, suitable for use on large rivers, was available to rent from the Eastern Fisheries Board, and this was used to catch native Brown trout from the two study sites on the river Liffey (Figure 2.3.7). The sampling was performed on two consecutive days and there were three boats in the river both days, each boat containing two electrofishers. As it was not possible to access the bank at all points of the river, the samples were collected at the end of the fishing period. The fish were kept in black bins in the boats until they could be delivered to the bank. It was necessary to keep the fish alive in order to take the blood samples, so it was essential that the water in the bins was kept oxygenated. All of the fish were sacrificed on the bank by means of a sharp blow to the head.

Figure 2.3.7

Electrofishers fishing the stretch from Carragh Bridge to Osberstown WWTP.

2.3.2.2 Tissue Sampling

(i) Blood:

Blood was taken from each fish, immediately after sacrificing, from the caudal sinus just behind the ventral fin (Figure 2.3.8). The sample was taken using a 2ml syringe that had been rinsed with a protease inhibitor (aprotinin), to prevent any degradation of the blood sample. A minimum of 1ml of blood is required to carry out analysis of the vitellogenin content of the blood; so 1-2ml of blood was sampled from the caudal sinus of each fish. The blood was stored in 5ml lithium heparin tubes on ice. The blood was stored in the lithium heparin tubes for at least one hour after the sample was taken. In some cases it was not possible to keep the fish alive until a blood sample was taken (e.g. fish obtained from angling competitions on Lough Leane) and in these cases vitellogenin analysis could not be performed (see section 2.2.1).

Figure 2.3.8
Collecting blood from the caudal sinus of a Brown trout using a syringe

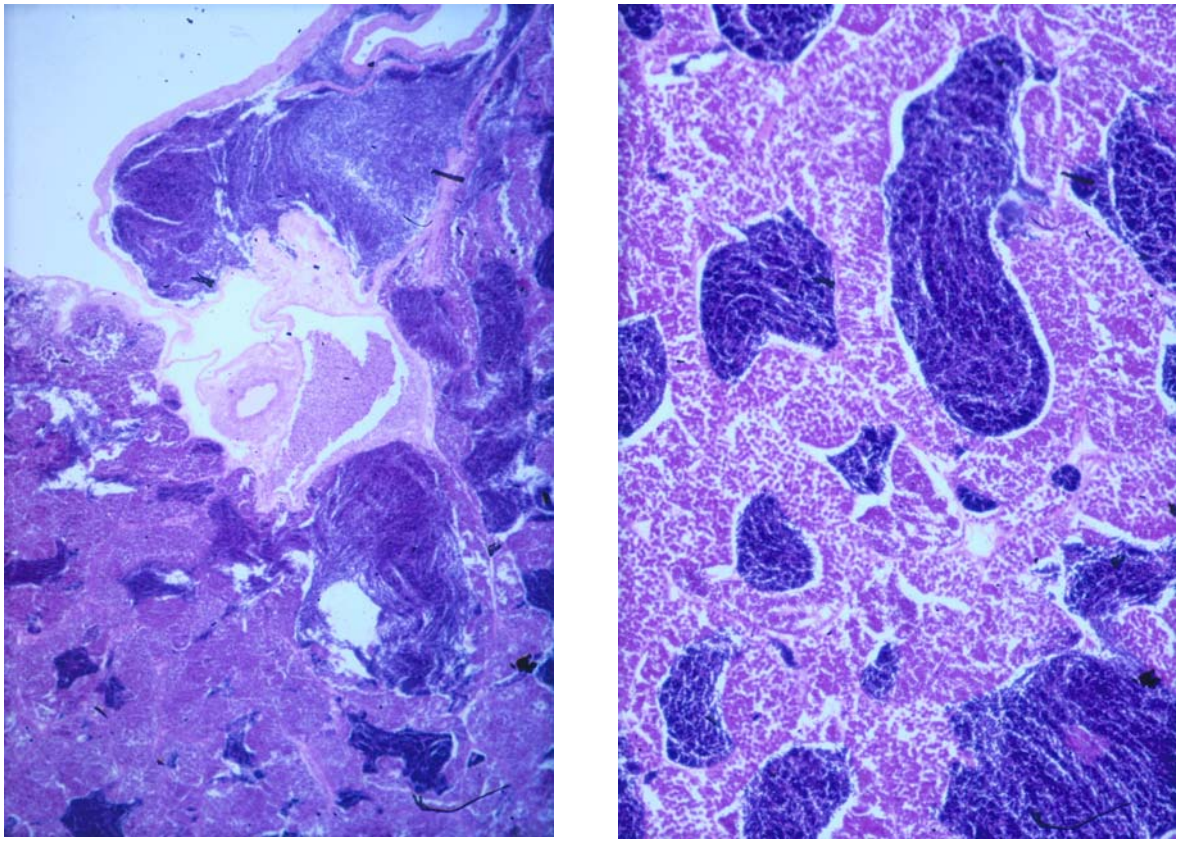
On return to the laboratory each blood sample was transferred from the lithium heparin tubes into 2ml centrifuge tubes. The tubes were placed in a centrifuge at 3,000 rpm for 15 minutes to separate the plasma from the red blood cells. The plasma was removed from the red blood cells using a 2ml pipette and placed in a new 2ml centrifuge tube for storage. The plasma samples were then frozen in liquid nitrogen until they were returned to the laboratory (Aquaculture Development Centre, UCC), where they were stored in the freezer at -20ºC, for subsequent vitellogenin analysis (see section 2.2.1).

(ii) Length and Weight: The length and weight of each fish was recorded before any dissections were carried out.

(iii) Liver: The liver was dissected out of all of the male fish. The liver weight was noted, so that the hepatosomatic index could be calculated at a later stage. Each liver was then wrapped in a tinfoil square that had been cleaned with hexane to remove any possible contaminants. The package was then frozen in liquid nitrogen and stored at -20ºC for future analysis.

(iv) Testes: The testes (Figure 2.3.9) were dissected out from all male fish. The two testes are located in the body cavity of the fish. They lie in parallel running from the head end to the vent either side of the vertebrae. The testes were weighed and placed in 20ml tubes, and fixed and stored with 10% buffered formalin solution until they were processed for histology. The testes may be stored indefinitely in the 10% formalin solution, as long as the tissue is completely covered by the solution. If the tissue is not totally immersed in the solution then tissue degradation may occur. The testes were subsequently examined histologically (see section 2.3.3.1) for the presence of female germ cells and/or the presence of ovarian tissue, which would show the presence of intersex.

(v) Ovaries: The ovaries (Figure 2.3.10) were removed from one mature and one immature female, and fixed, stored and sectioned in the same manner as the testes. These two samples were merely used as reference when the testes were examined for female reproductive tissue.



(a) **(b)**

Figure 2.3.9
Transverse sections of healthy maturing male Brown trout testes, showing (a) the location of the sperm duct and (b) the different spermatogenic cell types, and spermatozoa

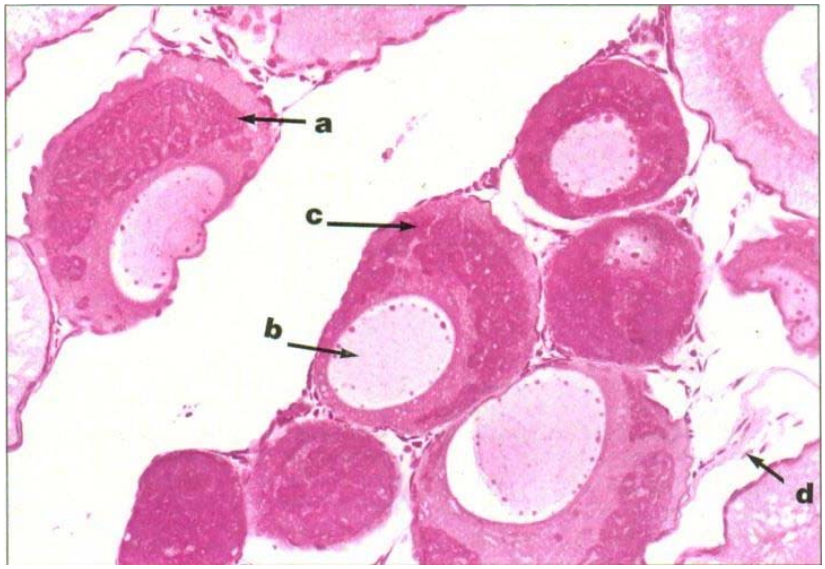


Figure 2.3.10

Ovaries of a mature female; (a) Oocyte, (b) Nucleus, (c) Cytoplasm, (d) Connective tissue. (Taken from Amin *et al.*, 1992)

2.3.3 Tissue Analysis

2.3.3.1 Histology

(i) Sample Processing:

Each gonad sample was sectioned into three sections: anterior, middle and posterior. (For each fish, both gonads were processed for histological analysis). Three 5-10 mm sections were cut from each of these pieces and placed in individual histology cassettes. The cassette was labelled with all the necessary information from the fish, e.g. A 135 D (A = Anterior, 135 = Number of the fish, D = Downstream of WWTP). The cassettes were placed in a 50% alcohol bath for at least one hour. From the 50% alcohol bath, the cassettes were placed in a Shandon tissue processor, which sequentially immerses the histology cassettes into six alcohol and two wax baths over a period of 20 hours (Table 2.3.1). This removes all of the water from the gonad sample and replaces it with wax, preparing the sections for the wax embedding process.

Table 2.3.1
Series of baths through which the histology cassettes pass in the Shandon Tissue Processor.

Bath No.	Contents	Time (hr)
1	30% Alcohol	1
2	30% Alcohol	1
3	70% Alcohol	1
4	80% Alcohol	1
5	95% Alcohol	1
6	95% Alcohol	1
7	Absolute Alcohol	2
8	Absolute Alcohol	2
9	Butanol	2
10	Butanol	2
11	Wax	3
12	Wax	3

(ii) Embedding the Tissues:

The cassettes were the transferred into a wax bath. The cassettes were removed from the wax bath one at a time, opened, and the tissue removed and placed into position in an embedding dish. Paraffin wax was then poured into the embedding dish. The base of the cassette was placed on top of the embedding dish (this allowed the cassette to be identified and held in placed during sectioning). The embedding dish was left for 1 minute to allow the surface of the wax to set, then placed on ice for a further 5-10 min to set fully. When the wax was completely set, the wax block was removed from the embedding dish. The embedded samples were then stored in a cool room (4^{o}C) for 48 h, to allow the paraffin wax to set completely. A sample that has been embedded in wax may be stored indefinitely.

(iii) Sectioning the Tissues:

When the blocks were to be sectioned, the excess paraffin wax was removed using a razor blade, and the face of the block was trimmed. This aided the sectioning and allowed more sections of tissue to fit on an individual slide. The cassette was mounted in the microtome and sectioned at 3-5 μm intervals.

(iv) Mounting Tissue Samples onto Slides:
The sections cut by the microtome were laid out and the best three chosen, each consisting of between three and six individual sections. These were placed into a 10% alcohol bath for 1 minute. Using the slide the sections were lifted into a warm water bath. The warm water bath removed any creases that had formed on the sections. The slides were then removed with the sections in place. For each cassette three slides were made (a, b, and c). The slides were dried for 48 h in an oven and for a further 48 h at room temperature.

(v) Staining with Mayers Haematoxylin Eosin:
The slide to be stained, usually the best of the three slides a, b or c, was placed into a slide rack which in turn was placed into the Shandon stainer. The Shandon stainer is an automated stainer that places the slides into a series of baths containing xylene, methylated spirits, distilled water, haematoxylin, tap water, eosin, and absolute alcohol. The whole staining process took about 70 min (Table 2.3.2). Once the staining was complete the slides were left in the xylene bath. One at a time they were removed and DPX mountant was dropped onto the slide, using a glass pipette and a cover slip was then lowered. The covered slides were left for a further 48 h at room temperature to dry, before they were viewed through a microscope.

Table2.3.2
Solutions used to stain the slides, and the time taken in each solution

No.	Solution	Time (min)
1.	Xylene	25
2.	Methylated Spirits	5
3.	Distilled Water	3
4.	Haemotoxylin	8
5.	Tap water	10
6.	Methylated Spirits	3
7.	Eosin	7
8.	Absolute alcohol	2
9.	Absolute alcohol	3
10.	Xylene	5

(vi) Examination of the Slides:
The examination of the slides was carried out using a binocular microscope, between 40 and 500 x magnification. Structures of interest were photographed using a Nikon FX-35WA and the images optimised using Nikon Coolscan.

The sectioned testicular tissue was examined for the incidence of intersex. The forms of intersex examined for were multifocal, focal, and duct malformations as seen by (Nolan *et al.*, 2001) in roach.

Multifocal Intersex (oocytes in the testes) was the most common form of intersex found in the roach and may be defined as the presence of a few primary oocytes or numerous primary and secondary oocytes randomly distributed throughout the testicular tissue. In most cases it has been found that primary oocytes occurred singly or in clusters sometimes with secondary oocyte (Nolan *et al.*, 2001).

Focal intersex (areas of ovarian tissue) may be defined as large areas of ovarian tissue separated clearly from testicular tissue, and often occupying large areas of the male tissue sections. These areas of focal intersex contain both primary and secondary oocytes (Nolan *et al.*, 2001).

Duct malformations are found in both focal and multifocal types of intersex, the most common malformation is the presence of both sperm duct and an ovarian cavity in the same individual, in one or both gonads. Almost all intersex individuals have a female like reproductive duct (ovarian cavity). In the most severe cases the sperm duct is absent or vestigial (Nolan *et al.*, 2001).

2.3.3.2 Vitellogenin Analysis of Blood Samples

(i) Quantitative Vitellogenin Determination:

A sensitive radioimmunoassay (limit of detection: 10 ng/ml) was developed by Sumpter (1985) for quantitation of vitellogenin in Rainbow trout. In the current study, quantitative vitellogenin determination of plasma samples obtained in the caged fish study was performed by Professor Sumpter's group in the Dept.of Biological Sciences, Brunel University.

(ii) Semi-quantitative (Qualitative) Vitellogenin Analysis:

Semi-quantitative vitellogenin analysis of wild Brown trout plasma samples was performed at CIT. The assay was performed using a kit from Biosense Laboratories, which contains a complete set of EIA reagents designed for semi-quantitative detection of vitellogenin in plasma samples from a variety of fish species including the *Salmoniformes* of which Brown trout (*Salmo trutta*) is a member (see section 2.2.1).

2.3.3.3 Condition Index

The condition index for fish is the relationship between the length and the weight of the fish and is used as a general indication of fish health. In extreme cases of endocrine disruption liver damage, and even liver failure, occurs and this level of stress is likely to reduce the condition of a fish. Each fish species has a different formula to determine the condition value. For Salmonids Fulton's Salmonid condition index is used which is $K_c = 100W/L^3$, where W is the weight and L is the length. A K_c value of around 1 indicates a healthy fish while K_c values less than 1 represent fish in poor condition, i.e. long and skinny. The condition index was used to show the general health of the fish populations examined and to see if there were any differences between sites. Differences in condition are to be expected due to natural differences between sites such as different diets due to variable habitat, but these differences are expected to result in fish having a high condition value rather than one below the 1 threshold. Fish of a very poor condition are more likely to be associated with disease and high stress levels that may be a result of pollution.

2.3.3.4 Gonado Somatic Index

The GSI is the gonad size expressed in relation to body weight, and it was calculated as follows: GSI = Gonadal weight / (total body weight – gonadal weight). The GSI was taken for each fish. The inhibition of testes growth is a biomarker of exposure to EDCs, thus, the GSI is an accurate method of determining whether the testes sizes are different between the sites, and an indicator of exposure to EDCs.

2.3.3.5 Liver Analysis and Hepatosomatic Index

EDCs such as nonylphenol and alkylphenols have been found to bioaccumulate in fish tissue, particularly the liver tissue, with a bioconcentration factor of between 13 to 410 for NP (McLeese, 1981, Ahel *et al.*, 1993, Blackburn, 1999). For this reason, the liver, bile duct and gall bladder were dissected out of all the fish and weighed, prior to being wrapped in hexane-washed aluminium foil and stored below –20ºC for subsequent analysis as part of this, or future, projects. The liver was weighed so the hepatosomatic index could be calculated using the following formula:

HSI = {Liver weight / (total body weight – liver weight)}*100

2.3.3.6 Statistical Analysis

The data obtained from the wild Brown trout survey on the Rivers Lee and Bandon, and in the Killarney Lakes, was analysed for homogeneity of variance and, where necessary, $\log^{10}$ transformed HSI. Condition and HSI were tested by a two-way analysis of variance (2-way ANOVA $p<0.05$) followed by Tukeys-tests. SYSTAT package and Excel were used for all statistical calculations.

The HSI data collected from the wild fish survey on the River Liffey was tested for normality using the Kolmogorov-Smirnov test. A one-way analysis of variance was used to compare the HSI values between the sites upstream and downstream of the Osberstown WWTP. Analysis of variance was also used to compare the HSI values between the mature and immature males. A Chi-squared test ($p < 0.01$) was used to determine the significance of the numbers of male fish showing raised plasma vitellogenin downstream of the Osberstown WWTP, relative to those obtained from the upstream site.

2.3.4 Experimental Design for Tank Study

Tank experiments were performed as a positive control for the caged fish study described in Chapter 3. Estrogenic compounds (17β-estradiol and 17α-ethynylestradiol) known to be present in WWTP effluent were used to elicit a vitellogenesis response in adult male Rainbow trout. A flow-through tank system was constructed (see below) and the fish were exposed to known concentrations of the estrogens dissolved in the water, as this route of exposure has previously been shown to be extremely effective (Pickford *et al*., 2003; Panther *et al*., 1998; Routledge *et al*., 1998). At the beginning and at the end of the 21-day exposure period blood was taken from the fish. Plasma vitellogenin concentration was then determined by quantitative radioimmunoassay.

Prior to commencing the study, the trout were kept in de-chlorinated water in a flow-through tank system for 21 days (approximate water flow rate of 400 ml/min). The fish were not fed during this acclimation period to limit exposure to any unknown sources of EDCs. Any plasma vitellogenin that may already have been present in the male trout (due to previous exposure to EDCs) should hve been metabolised during this period, as its plasma half-life is estimated as 44 h in Rainbow trout (Schulz *et al*., 2001). A random sample of the fish was taken at the beginning and the end of the acclimation period for comparison with the control fish at the end of the study.

After the depuration period, the trout were moved into the treatment tanks in the flow-through system. The flow-though tank system was constructed using eight circular tanks (approximately 1m diameter by 0.7 m height). Two were used as header tanks and six as treatment tanks. Using 5 cm piping and valves, each treatment tank received de-chlorinated water from the header tank at a flow rate of 400 ml/min. A water de-chlorination system was used to provide the de-chlorinated water for fish depuration and the flow-through system. Stock solutions (1000 mg/L in acetone) of each of E2 and EE2 were used to spike Elga water, yielding 2.5L treatment solutions at a final concentration of 200 μg/L and 800 μg/L, respectively. The treatment solutions were stored in darkened glass bottles and silicone tubing and flow control pinch valves were used to deliver the material to the treatment tanks at a flow rate of 0.2 ml/min (Figure 2.3.11). This resulted in nominal final tank concentrations of 100 ng/L E2 and 400 ng/L EE2. In summary, the treatment tanks were as follows: 2 tanks receiving treatment 1 (E2 to maintain a nominal concentration of 100 ng/L), 2 tanks receiving treatment 2 (EE2 to maintain a nominal concentration of 400 ng/L), 1 tank receiving treatment 3 (50:1, H_2O:Acetone) and 1 tank receiving treatment 4 (de-chlorinated water). Activated charcoal filters were fitted to the outflow pipes from the treatment tanks, to ensure that all estrogenic chemicals were bound and removed from the wastewater prior to discharging to the municipal sewerage system.

Fish sampling was performed (i) at the beginning of the 21 day acclimation period, (ii) at the end of the 21 day acclimation period directly prior to the fish being moved to the treatment tanks, and (iii) after 21 days in treatment tanks.

Blood and tissue (testes, liver, bile duct and gall bladder) samples were taken, prepared for analysis and stored as described in section 2.3.2.4. Plasma samples were stored at –20°C prior to transport to Brunel University for analysis by quantitative radioimmunoassay (section 2.3.3.2).

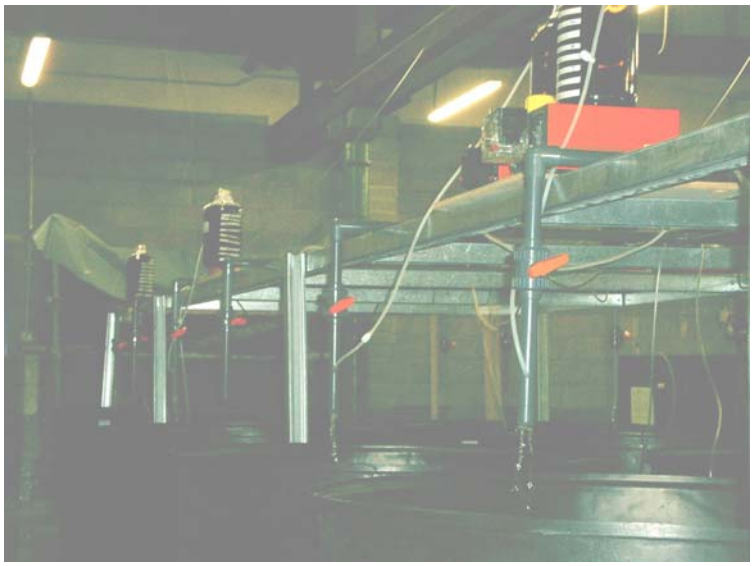


Figure 2.3.11

Flow-through tank system used in the demonstration of an estrogenic response in male Rainbow trout exposed to E2 and EE2.

2.3.5 Experimental Design for Caged Fish Study

Caged fish studies, using male Rainbow trout, have been widely employed to elucidate the effects of WWTP effluents on exposed fish populations in the receiving waters (for a review see section 1.4.5 of this report). During the current trial, cages were placed in the watercourse to be investigated and the fish were exposed for 3 weeks. The cages were located at the source of the suspected input of EDCs (Ballincollig WWTP), with control sites located downstream of the source (at the intake to the Lee Road WTW) and immediately upstream of the putative EDC source. As with the tank experiments (section 1.4.5, section 2.3.4), the end points in these *in vivo* experiments are vitellogenin expression in male fish and any morphological changes in the gonads and/or liver. A series of control fish are sacrificed (i) when the fish arrive from the farm, (ii) prior to being placed *in situ* and (iii) at the end of the trials. In the current study, a stretch of the river Lee between Ballincollig and Cork City was investigated (rationale for site selection: section 2.3.1.1 and 3.1).

Prior to commencing the study, the trout were held in de-chlorinated water in circular tanks (approximately 1 m diameter by 0.7 m height) in a flow-through system. The flow rate was approximately 400 ml/min and the depuration period lasted for 21 days. The fish were not fed during this time to limit exposure to any unknown sources of EDCs and to allow for the breakdown of any plasma vitellogenin that may have been present. A random sample of the fish was taken at the beginning and the end of the acclimation period for comparison with the control fish at the end of the caged fish study.

Five cages were construced of galvanised steel (final dimensions: 1.5m * 1m * 1m) and flotation collars were constructed for each cage using 5 m of 110 mm sewage pipe and four elbow joints. The cages were moved to the study location using a flat bed truck and manoeuvred, once in location, using a ribbed inflatable dingy. The cages were placed *in situ* on the river and in the lakes, several days prior to the study to ensure that any unforeseen problems could be dealt with before the fish were placed *in situ*. Where

necessary, attachment points were added to the bank or bank structures, and a rope and chain was used to secure the cages to the riverbank or lakeside. Each cage was fitted with a lock to prevent theft of the cage or its contents (Figure 2.3.12).

Figure 2.3.12
Fish cage located at Ballincollig WWTP outfall on the river Lee, Ballincollig, Co. Cork.

The study fish were moved to the cage location after 21 days in the acclimation tanks. The fish were moved in an oxygenated tank. The tank was oxygenated using compressed oxygen. When moving the fish into a cage a large hand net was used, and the fish were individually counted into each cage. During the experiment the cages were visited on a regular basis (approximately every 1 to 3 days) and examined for any signs of damage, build-up of debris and change of position. Fish sampling was performed (i) at the beginning of the 21 day acclimation period, (ii) at the end of the 21 day acclimation period directly prior to the fish being moved to the study location, and (iii) after 21 days *in situ*.

Blood and tissue (testes, liver, bile duct and gall bladder) samples were taken, prepared for analysis and stored as described in section 2.3.2.4. Plasma samples were stored at –20°C prior to transport to Brunel University for analysis by quantitative radioimmunoassay (section 2.3.3.2).

3. An Investigation of the Potential for Estrogenic Contamination of the River Lee and its Associated Drinking Water Resources Using Caged Male Rainbow Trout.

3.0 Introduction

Contamination of the aquatic environment by estrogenic chemicals has become an issue of global concern over the past decade. Studies have shown that many of these endocrine disrupting chemicals (EDCs) cause exposed male fish to display physiological responses normally associated with high systemic concentrations of estrogens (Thorpe *et al*., 2003; Pedersen *et al*., 2003). Effluent from sewage treatment works has been shown to contain many of these EDCs (Routledge *et al.*, 1998), and a number of studies have demonstrated the "feminising" effects of exposure to WWTP effluent (Rodgers-Grey *et al*., 2001; Solé *et al*., 2001), as determined by the presence of raised plasma levels of the female egg yolk precursor protein, vitellogenin. The major estrogenic chemicals identified in treated WWTP effluents of domestic origin are the female steroid hormones, both natural (17β-estradiol and estrone) and synthetic (17α-ethinylestradiol, a component of the contraceptive pill), which are present in UK WWTP effluents at concentrations sufficient to cause vitellogenesis in exposed roach populations (Desbrow *et al*., 1998; Routledge *et al*., 1998).

Rainbow trout (*Oncorhynchus mykiss*) have been widely used as a sentinel species in studies on WWTP effluents as salmonids are more estrogen-sensitive than cyprinid species (Purdom *et al*., 1994). Exposure of captive male Rainbow trout to known concentrations of estrogenic compounds has been shown to cause specific physiological responses. These responses, which include raised plasma vitellogenin, are well documented (Routledge *et al*., 1998; Jobling *et al*., 1996; Routledge *et al*., 1996 and Sheahan *et al*., 1994) and, therefore, can be compared with similar responses seen in fish where concentrations of estrogenic chemicals are unknown. Routledge *et al*. (1998) demonstrated that vitellogenin synthesis in male Rainbow trout is extremely sensitive to estrogens, and the threshold concentration for a response to 17β-estradiol is between 1 and 10 ng/L. Also, at the same exposure (100 ng/L E2) levels, the magnitude of the vitellogenin response in Rainbow trout was shown to be 30-fold greater than in roach.

Plasma vitellogenin is a sensitive and widely used biomarker of exposure to estrogens in male fish (Sumpter and Jobling, 1995). Vitellogenin is an egg yolk precursor normally produced in oviparous females as a response to circulating estrogens. Male fish do not normally synthesize this protein because of the very low level of estrogens in the blood. However, they do have the capacity to synthesize it to the same degree as females as a consequence of exposure to xenoestrogens, providing a sensitive bioassay with a response range that spans several orders of magnitude, and with a lower and less variable base line than that in female fish. A sensitive radioimmunoassay (limit of detection: 10 ng/ml) was developed by Sumpter (1985) for quantitation of vitellogenin in Rainbow trout.

To date, there have been no published studies documenting the effects of WWTP effluents on exposed fish populations in Irish rivers, or on any associated risk to drinking water supplies and, hence, the Irish population. The current study was designed to address this knowledge gap.

3.1 Characteristics of the Site for the *In Vivo* Caged Fish Study

The water system chosen for this *in vivo* caged fish study was the river Lee. A stretch of the river between Ballincollig and Cork City was the main focus of the study. Ballincollig wastewater treatment plant (WWTP) discharges into this section of the river. The outfall is 4 km upstream of the intake to the Lee Road water treatment works (WTW), which provides drinking water to Cork City. A weir immediately downstream of the WTW prevents the tidal nature of the lower reaches of the river from affecting the quality of the drinking water.

Based on influent PE values, Ballincollig is the largest WWTP, discharging to freshwaters, in County Cork. The PE loading of the Ballincollig WWTP influent is 15,000, where one PE is defined as the amount of organic biodegradable load that has a 5-day biological oxygen demand (BOD 5) of 60 g of oxygen per day. The PE loading of the influent was calculated on the basis of the maximum average weekly load entering the plant during the year (2003 figures used throughout this report), excluding unusual situations such as heavy rain. The adjusted PE, representing the average dilution of the effluent in the river, is 194 for Ballincollig WWTP, while Jobling and co-workers (1998) have previously reported a significant level of intersex in the wild fish population of the river Rea (UK) downstream of a WWTP with an adjusted PE of 119. The Ballincollig WWTP influent is almost entirely domestic in origin (see Chapter 5, Table 5.1), which makes it comparable to similar UK studies investigating effects of natural and synthetic estrogens on fish populations (Harries *et al*., 1999). Women between the ages of 15 to 44 years represent 25% of the Ballincollig population (2002 census, Central Statistics Office).

Fermoy is the next largest WWTP discharging to freshwaters (river Blackwater) in Cork, with an influent PE loading of 12,600 and an adjusted PE of 41. The waste from Cork City (PE 328,000), at the time of this study, did not receive any treatment and discharged directly into the Lee estuarine waters in Cork Harbour.

Thus, Ballincollig WWTP, the Lee Road WTW and the Lee river presents a suitable system in which to assess the impact of Irish WWTP effluents on the receiving freshwaters, and their associated wildlife and human populations. Consideration of the following combination of factors made this system the location of choice for the current investigation;

(a) PE (and adjusted PE) load of WWTP influent,

(b) Domestic origin of the influent (natural and synthetic female steroids have been shown to represent the main source of estrogenic compounds in most UK WWTP effluents), and

(c) Location of the WWTP outfall (4 km upstream of the intake to the Lee Road WTW which supplies drinking water to Cork City).

The study was performed using caged male Rainbow trout (approximately 20 male fish per cage) located at test sites immediately upstream of Ballincollig WWTP (OS grid reference: W 591716), at the WWTP outfall (OS grid reference: W 592715) (Figure 3.1) and 4 km downstream at the intake of the Lee Road WTW (OS grid reference: W 649715).

Figure 3.1
River Lee upstream of Ballincollig WWTP outfall, Co. Cork.
This site serves as a control for the effect of the Ballincollig WWTP effluent on the river Lee. The fish cage was placed in the river at the position indicated by the house, which is approximately 50 m upstream of the WWTP outfall. The cage visible in the foreground is located immediately underneath the WWTP outfall pipe.

Figure 3.2
Lough Barfinnihy, Kenmare, Co. Kerry
Lough Barfinnihy is an oligotrophic lake used as a reference (negative control) site for the cage fish study.

A cage was also placed at a control site located at Gougane Barra (headwaters of the river Lee: W 095661) that served as an internal control for the Lee river system. The primary control (reference) site was at Lough Barfinnihy, a highland oligotrophic lake in Co. Kerry with no anthropogenic inputs (V 850766) (Figure 3.2). These sites allowed determination of the effects (if any) of background levels of estrogens on fish populations. In addition, positive control studies were set-up in flow-through tanks at the UCC Aquaculture Development Centre, Lee Maltings, Cork.

3.2 Results and Discussion

3.2.1 Impact of Ballincollig WWTP on Estrogen Levels in the River Lee

Caged fish studies, using male Rainbow trout, have been widely employed to elucidate the effects of WWTP effluents on exposed fish populations in the receiving waters (for a review see section 1.4.5 of this report). In the present study, two separate *in vivo* bioassays were performed, in March and August 2002, to assess the impact (if any) of effluent from Ballincollig WWTP (PE 15,000) on estrogen levels in the river Lee. Adult male Rainbow trout (*Oncorhynchus mykiss*) were placed in cages at the outfall of Ballincollig WWTP, the intake to the Lee Road WTW and at each of the above described control sites (section 3.2). At the beginning and the end of the 21-day exposure period, blood was taken from the fish and plasma vitellogenin concentrations were determined, thus providing a reliable measure of the level of exposure to estrogens during the study period (see section 2.3.5 for experimental details). The experimental design was in keeping with published *in vivo* studies investigating the impact of WWTP effluents on the river Lea in the UK (Harries *et al*., 1996, 1997, 1999).

There was no evidence of raised plasma vitellogenin in any fish located at either the test or negative control sites in either of the two experiments. In each case, vitellogenin concentrations were in the range of 10 to 100 ng/ml. There was no difference (within the limits of experimental error) between vitellogenin concentration in the fish located at the outfall of Ballincollig WWTP or downstream at the intake to the Lee Road WTW, relative to the control site immediately upstream of the WWTP or the negative control sites at Lough Barfinnihy or in the river Lee headwaters at Gougane Barra (Fig. 3.3).

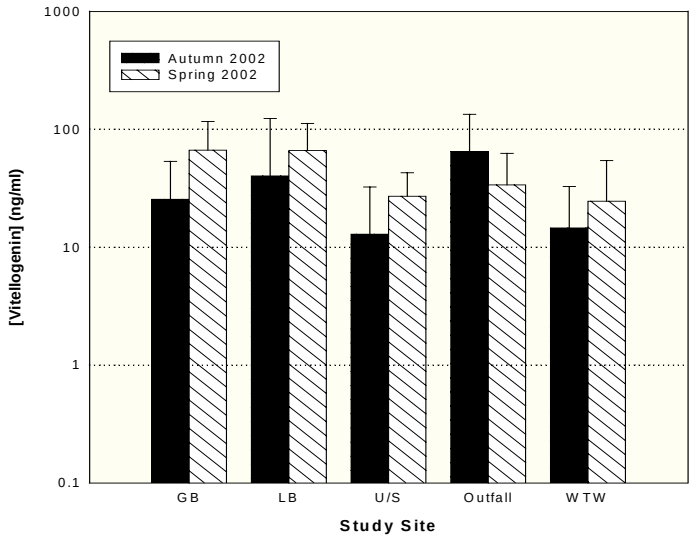


Fig. 3.3
Absence of Vitellogenesis in Male Rainbow Trout (*Oncorhynchus mykiss*) Exposed at Ballincollig WWTP Outfall in the River Lee
Adult male Rainbow trout (205 ± 45 g) were located at test and control sites for 21 days. Plasma vitellogenin concentration (ng/ml) ± SD was determined, n = 14-28 male trout per cage. (GB: Gougane Barra; LB: Lough Barfinnihy; U/S: immediately upstream of WWTP outfall; WTP: Lee Road WTP).

The vitellogenin concentrations detected in this study were in agreement with literature values for background (pre-estrogen exposure) levels in male Rainbow trout. Routledge *et al*. (1998) conducted a series of tank studies exposing male Rainbow trout to known

concentrations of estrogens (E2 and EE2) and reported pre-exposure vitellogenin levels of approximately 50 ng/ml, while the threshold for induction of vitellogenin synthesis was between 1 and 10 ng/L E2 for a three week exposure period. Depending on the level of estrogen exposure, a positive vitellogenin response ranged from 10^3 to 10^6 ng of vitellogenin per ml of plasma.

Prior to starting the main study, preliminary *in vivo* and *in vitro* tests indicated that the undiluted effluent from Ballincollig WWTP is estrogenic to fish;

(a) An aerated flow-through tank system was set up on the WWTP site and a small number (3) of male Rainbow trout were exposed to undiluted effluent for a 15-day period. The male fish exhibited significantly elevated plasma vitellogenin levels (results not shown). However, a full-scale experiment set up in conjunction with the main study was unsuccessful, as the fish did not survive for the 21-day study period in the undiluted effluent.

(b) Subsequently, the *in vitro* yeast estrogen screen (YES) estimated estrogen levels (17β-estradiol equivalents, $E2_{eq}$) in the undiluted effluent of 3.2 ± 1.1 ng/L (Chapter 5, Table 5.2). This is a concentration that previous studies have indicated should be estrogenic to male Rainbow trout (Routledge *et al*., 1998). However, the WWTP effluent is significantly (77-fold) diluted upon discharging to the river Lee, which reduces the concentration of estrogens contributed by the effluent to a level below that required to induce vitellogenesis in fish. Interestingly, the actual estrogenicity of the river water at the outfall pipe (1.5 ± 0.6 ng/L, as measured by the YES) is considerably higher than that predicted from diluted effluent values alone (0.04 ng/L), suggesting that there are other sources of estrogens contributing significantly to the estrogenic activity of the Lee (Chapter 5; section 5.4.4). This level of activity is at the lower end of the range known to be estrogenic to fish under continuous exposure conditions. As significant diurnal variations in the estrogenic burden of Ballincollig WWTP effluents have been demonstrated (data not shown), it may be concluded that the caged fish in this study were not exposed to the same constant estrogenic environment as fish in, for example, tank studies. It is therefore probable that exposure to concentrations of steroid estrogens in the upper portion of the ranges described as active in the literature (1-10 ng/L E2 and 0.1-1 ng/L EE2) would be required to induce feminising effects in exposed caged and wild fish populations.

In conclusion, the results of this *in vivo* study have demonstrated that the effluent from Ballincollig WWTP is not estrogenic to fish in the river Lee. Indeed, at no point in the river (until it enters Cork City) is the water estrogenic to fish, as determined by vitellogenin bioassay, even when the fish were located at the apparent estrogenic "hotspot" directly beneath the outfall pipe from the WWTP. It may, thus, be concluded that estrogenic compounds from Ballincollig WWTP are not emitted in quantities sufficient to pose a threat to the quality of the Lee waters or its associated drinking water supplies.

3.2.2 Induction of Vitellogenesis in Male Rainbow Trout by 17β-Estradiol and 17α-Ethinylestradiol in Flow-Through Tank Studies

A series of tank experiments were performed, in which natural (E2) and synthetic (EE2) steroid estrogens (the main estrogenic components of domestic WWTP effluents; Routledge *et al*., 1998) were used to induce vitellogenesis in exposed adult male Rainbow trout (*Oncorhynchus mykiss*). The ability of the fish to respond, within the 21 day study period, to these estrogens with raised vitellogenin levels served as a positive control to the above described caged fish study (3.2.1).

Acclimatised fish, from the same batch used in the cage study, were placed in de-chlorinated water in a flow-through tank system and exposed to known concentrations of 17β-estradiol and 17α-ethinylestradiol dissolved in the water, as this route of exposure has previously been shown to be extremely effective (Pickford *et al*., 2003; Panther *et al*., 1998; Routledge *et al*., 1998). The nominal concentrations of E2 and EE2 were 100 ng/L and 400 ng/L, respectively. A control tank that did not receive any known estrogen exposure was also included. Plasma vitellogenin levels were determined at the beginning and at the end of the 21-day exposure period (see section 2.3.4 for experimental details).

The male Rainbow trout responded as expected to the potent estrogens, E2 and EE2, with plasma vitellogenin levels of between 5 and 7 orders of magnitude (10^6 - 10^8 ng/ml) greater than the control group (10 - 100 ng/ml) (Fig. 3.4).

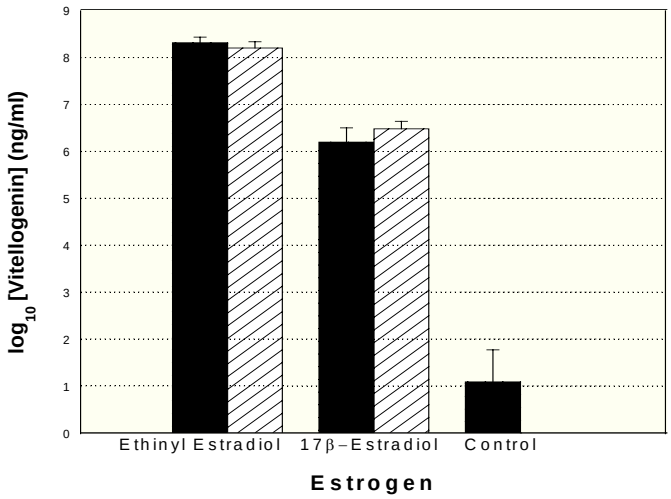


Fig. 3.4
Induction of Vitellogenesis in Male Rainbow Trout (*Oncorhynchus mykiss*) by the Steroid Estrogens; 17β-Estradiol and 17α-Ethinylestradiol.
Adult male Rainbow trout (205 ± 45 g) were placed in a flow-through tank system (400 ml/min) and exposed to nominal concentrations of (a) 100 ng/L E2 or (b) 400 ng/L EE2 for 21 days. A control tank with no known estrogen exposure was also set up. Data represents mean vitellogenin concentration (ng/ml) ± SD, where n = 17-27 male fish per tank. Tanks containing estrogens were performed in duplicate. Vitellogenin concentrations are plotted on a logarithmic scale due to the wide range of values measured.

The magnitude of the vitellogenic response was in agreement with values reported in the literature, with Routledge and co-workers reporting vitellogenin levels of 10^6 ng/ml in response to a three week exposure to 100 ng/L E2.

3.2.3 Conclusions

Many previous studies have investigated the occurrence of estrogenic compounds in surface waters, and WWTP effluents have been cited as a major source of environmental estrogens. A German study carried out by Kuch *et al.* (2001) showed that a similar spectrum of estrogenic compounds (steroids, Bisphenol A and alkylphenols) appeared in surface water and drinking water, though their concentration was measurably lower in drinking water. For example, the concentration of steroids was reduced from 0.2-5 ng/l in surface water to 0.1-2 ng/l in drinking water. The study concluded that environmental estrogens that enter the aquatic environment from WWTP effluent may eventually be found in drinking water supplies. However, to date, the greatest concern regarding estrogens in surface water is their effects on the health of fish populations as measured by raised plasma vitellogenin and an increased incidence of intersex in male fish (Ertmans *et al.*, 2003).

No work has been published, to date, addressing these concerns in an Irish context. Thus, the current study was designed to elucidate the effects of Irish WWTP effluents on receiving waters, and determine the risk (if any) on the exposed fish populations in Irish rivers, or on any associated risk to drinking water supplies and, hence, the Irish population.

Based on the results of this *in vivo* study, it may be concluded that environmental estrogens are not present in the river Lee in concentrations capable of eliciting vitellogenesis in exposed male Rainbow Trout. Indeed, at no point in the river (until it enters Cork City) is the water estrogenic to fish, as determined by vitellogenin bioassay, even when the fish were located at the apparent estrogenic “hotspot” directly beneath the outfall pipe from the WWTP. It may, thus, be concluded that estrogenic compounds from Ballincollig WWTP are not emitted in quantities sufficient to pose a threat to the quality of the Lee waters or its associated drinking water supplies. A positive control tank study, conducted concurrently with the main *in vivo* study, demonstrated that the assay system was responsive to steroid estrogens, as reported in the literature.

4. A Survey of the Irish Wild Brown Trout Population for Evidence of Endocrine Disruption

4.1 Introduction

The possibility that wildlife populations are being affected by exposure to EDCs is supported by a growing body of evidence demonstrating reproductive and developmental abnormalities in various groups of organisms, including birds, fish, invertebrates and reptiles (Damstra *et al.*, 2002). Of particular concern are those species susceptible to exposure to EDCs in or via the aquatic environment. Rivers and estuaries are commonly repositories for large amounts of domestic and industrial waste. The majority of chemicals shown to interact with the endocrine system have been found in WWTP effluents and in untreated industrial and domestic waste. Thus, fish living in these riverine and estuarine environments can be considered as sentinel species for assessing the impact of EDCs on the environment.

In 1994, Purdom *et al.* (1994) reported that effluents from many WWTPs in the UK were estrogenic to male Rainbow trout (*Oncorhynchus mykiss*) held in cages in WWTP discharges. Subsequent studies of the wild fish population living downstream of major UK WWTPs showed a high incidence (16 – 100%) of intersexuality among male roach (*Rutilus rutilus*). In comparison, levels of intersex males ranged from 4 to 18% at control sites, and from 12 to 44% in the upstream sites studied (Jobling *et al.*, 1998). The severity of the condition ranged from the occasional oocyte in otherwise normal testicular tissue, to large regions of mature ovarian tissue interspersed with abnormal testicular tissue. Blood vitellogenin concentrations, in male fish sampled downstream of the WWTPs, were significantly higher than those of males from either upstream sites or from control sites. An elevated blood level of the female egg yolk protein, vitellogenin, in male fish, is one of the most sensitive and widely accepted biomarkers of estrogenic contamination of the aquatic environment (Sumpter and Jobling, 1995; Tyler *et al.*, 1999).

Further investigation of endocrine disruption in roach demonstrated feminisation of gonadal ducts in juvenile roach exposed to WWTP effluent during the period of gonadal differentiation (Rogers-Grey *et al.*, 2001) and altered sexual maturation and gamete production in wild roach living in UK rivers receiving WWTP effluent (Jobling *et al.*, 2002). Furthermore, wild intersex roach have been shown to have reduced fertility, thus demonstrating a link between the morphological effects of endocrine disruption and the reproductive capabilities of the affected fish population (Jobling *et al.*, 2002).

Additional UK studies on another cyprinid species, gudgeon (*Gobio gobio*), revealed that sexual disruption in wild fish living downstream of WWTPs is not a species-specific phenomenon (Van Aerle *et al.*, 2001). Nor is it limited to freshwater fish, as wild male European flounder (*Platichthys fleus*) with raised vitellogenin levels and testicular abnormalities were discovered at a series of heavily polluted estuaries in the UK (Lye *et al.*, 1997; Allen *et al.*, 1999a, 1999b). Parallel studies in other countries have revealed that sexual disruption of wild fish populations, and indeed other wildlife populations, is a world-wide phenomenon (for a comprehensive review see Damstra *et al.*, 2002).

Tank studies combined with fractionation and chemical analysis of WWTP effluents has shown that, in the majority of cases, the estrogenic chemicals causing fish feminisation are the natural female steroids estradiol (E2) and estrone (E1), and the synthetic steroid ethinyl estradiol (EE2) used in oral contraceptive formulations (Desbrow *et al.*, 1998;

Routledge *et al.*, 1998). In agricultural areas, these natural and synthetic steroids may, in part, be contributed by animal waste products (Orlando *et al*., 2004; Soto *et al*., 2004; Lange *et al*., 2002). These steroids are present at very low concentrations (ng/L) in effluents but are extremely potent. They are of significance at sites where WWTP effluents make up a considerable proportion of the river flow, particularly when fish receive continuous exposure via the water in which they live. These hormones enter the sewerage system as inactive conjugates but can be deconjugated into active compounds by bacterial enzyme activity (Belfroid *et al.*, 1999; Panther *et al.*, 1999, D'Ascenzo *et al.*, 2003). However, in locations with heavy industrial activity, xenoestrogens, such as phthalates, Bisphenol A, nonylphenol and related compounds, may contribute significantly to the estrogenic burden of effluents (Lye *et al.*, 1999; Larsson *et al.*, 1999; Thomas *et al.*, 2001; Sheahan *et al.*, 2002a, 2002b).

To our knowledge, there has been no published investigation of possible endocrine disruption in Irish feral fish populations, to date. The survey performed in the present study: an assessment of wild Brown trout in the Rivers Lee, Liffey and Bandon, and in the Killarney Lakes, was performed to address this knowledge gap. Employing standard end-points for exposure to estrogens (raised plasma vitellogenin levels in male fish and the presence of intersex) and general indicators of fish health (hepatosomatic index, length, weight and condition), this study investigated the possibility that Irish rivers contain estrogenic chemicals at levels capable of affecting the reproductive health and success of exposed fish populations.

4.2 Selection of Sites for the Wild Brown Trout Survey.

The water systems chosen for study were the rivers Liffey, Lee and Bandon, as well as Lough Leane and Lough Guitane in the Killarney Lakes. They were chosen to investigate the potential for pollution from EDCs from several different sources including domestic, agricultural and industrial.

On each river/lake system several sites were sampled; on each system the control site was chosen to give a balance between a site minimally impacted by human activity and a site that has a wild fish population that could sustain sampling. In the case of the River Liffey, a control site was not included in the sampling programme for logistical reasons. The other sampling sites on each water system were chosen to incorporate a site upstream and downstream (same water body for Lakes) of a potentially substantial EDCs input.

The sampling programme in the river Liffey was designed to determine the effect (if any) of the effluent from Osberstown WWTP (PE 68,000) on the wild Brown trout population. Thus, all sampling took place in the vicinity of the Osberstown sewage treatment works, Co. Kildare. The upstream site, which is unaffected by the Osberstown WWTP effluent, was at Carragh Bridge, Co. Kildare (N 854208) while the second sampling site was situated just downstream of the WWTP outfall at Osbertown (N 869207) and ended at the Leinster Aqueduct (N 877227).

Ballincollig WWTP (PE 18,000) was considered the most significant potential source of EDCs to the River Lee (see Chapter 3 for further discussion of Ballincollig WWTP as a source of EDCs). Thus, the study sites chosen were upstream and immediately

downstream of the outfall from the Ballincollig WWTP, while Ballyvourney was chosen as the control site for the River Lee system. This control site comprised a 100m stretch below Milleeny Bridge (W 162759), which is located above the town of Ballyvourney. This site was chosen as it is upstream of any potentially significant EDCs input and has a population of fish suitable to be sampled.

The River Bandon has potential for significant EDC input from three main sources: the WWTP, the agricultural mart and a food processing facility in Bandon town. Effluents from these sources enter the river Bandon immediately below Bandon town. Thus, the Bandon river study sites were chosen to be upstream and immediately downstream of Bandon town, while Ardcahan Bridge (W 243557) was chosen as the control site for the river Bandon. This control site is located upstream of any major urban areas, so there is little risk of any significant EDC input at this site. At this location the river Bandon is large enough to support a Brown trout population suitable to be sampled. The site upstream of Bandon town comprised a stretch of river between Baxter's Bridge (W 442546) and Bandon Park (W 474546), which is located above the Bandon town weir. The weir acts as a partial natural barrier hindering fish movement between this site and the downstream site although fish can migrate downstream and a fish pass allows fish movement upstream. The site downstream of Bandon town comprised a stretch of river between Rough Hole (name of a pool) (W 509562) and O'Driscoll's Bridge (W 516569). This site is downstream of all potential EDC inputs from the town.

Lough Guitane, was chosen as the control site for the Killarney lakes system. It is located upstream of any major urban areas, so there is little risk of EDCs entering the lake at this point. Lough Guitane supports a large Brown trout population suitable to be sampled. Lough Leane was the chosen test site for the Killarney Lakes as it receives potentially significant EDC input from the Killarney WWTP effluent (PE 34,800 increasing to 70,000 during the tourist season) that enters the Lough in the Ross Bay area. The site upstream of the potential EDC input from Killarney town comprised a stretch of the River Gearhameen in the Black Valley. For further details on the study sites and the sampling methods used at each site, refer to Chapter 2 of this report.

4.3 Results of the Survey of the Wild Brown Trout Population in the Rivers Lee and Bandon, and the Killarney Lakes.

4.3.1 River Bandon

On the river Bandon 110 trout were sampled. The length ranges, mean length, weight ranges and mean weights for the three sample sites can be seen in Table 4.3.1. The control site fish obtained were of a smaller mean length and weight than the fish from the two sites upstream and downstream of the potential EDC input from Bandon town.

Table 4.3.1.
Length range, mean length, weight range and mean weight for Brown trout at the three sample sites on the river Bandon.

Site	Length range (cm)	Mean length (cm)	Weight range (g)	Mean weight (g)
Control	13.7 - 29.6	20.2	27.8 - 258.1	95.5
Upstream	18.4 - 32.0	23.6	73.2 - 400.0	161.6
Downstream	17.4 - 30.2	24.3	68.3 - 297.9	178.2

The sex and maturity of the trout from the three sample sites on the river Bandon can be seen in Table 4.3.2. A greater proportion of the fish, sampled from the sites upstream and downstream of the potential EDC input, was mature fish. At the control site, although there were still more mature fish than immature fish, the difference was not as great.

Table 4.3.2.
Sex and maturity of the 110 trout sampled from the three sites on the river Bandon.

Site	Mature Male	Mature Female	Immature Male	Immature Female	Total
Control	9	11	4	7	31
Upstream	13	18	6	6	43
Downstream	18	13	2	3	36
Total	40	42	12	16	110

(i) Sex Ratio:

The sex ratios for the fish taken from the three sites on the river Bandon are given in Figure 4.3.1. The sex ratios show that there is a reasonably balanced fish population in the river, with approximately the same number of male and female fish present at each site.

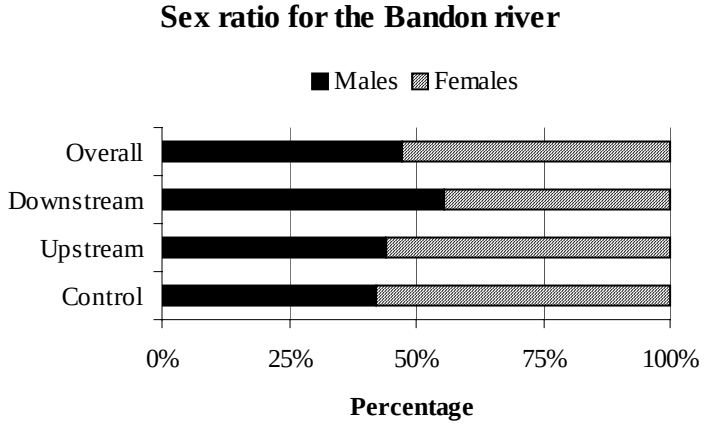


Figure 4.3.1.
Sex ratio for 110 trout sampled at three sites on the river Bandon,with the overall sex ratio.

(ii) Condition:

The condition of the trout from the three sites on the River Bandon was determined using Fulton's Salmonid condition function K_c (Figure 4.3.2). The average condition values (K_c) for the control site are significantly smaller than those for the trout taken

from the sites upstream and downstream of Bandon town (2-way ANOVA). This shows that the fish from the control site, although in good condition ($k_c > 1$), were thinner than the fish sampled from either the upstream or the downstream site.

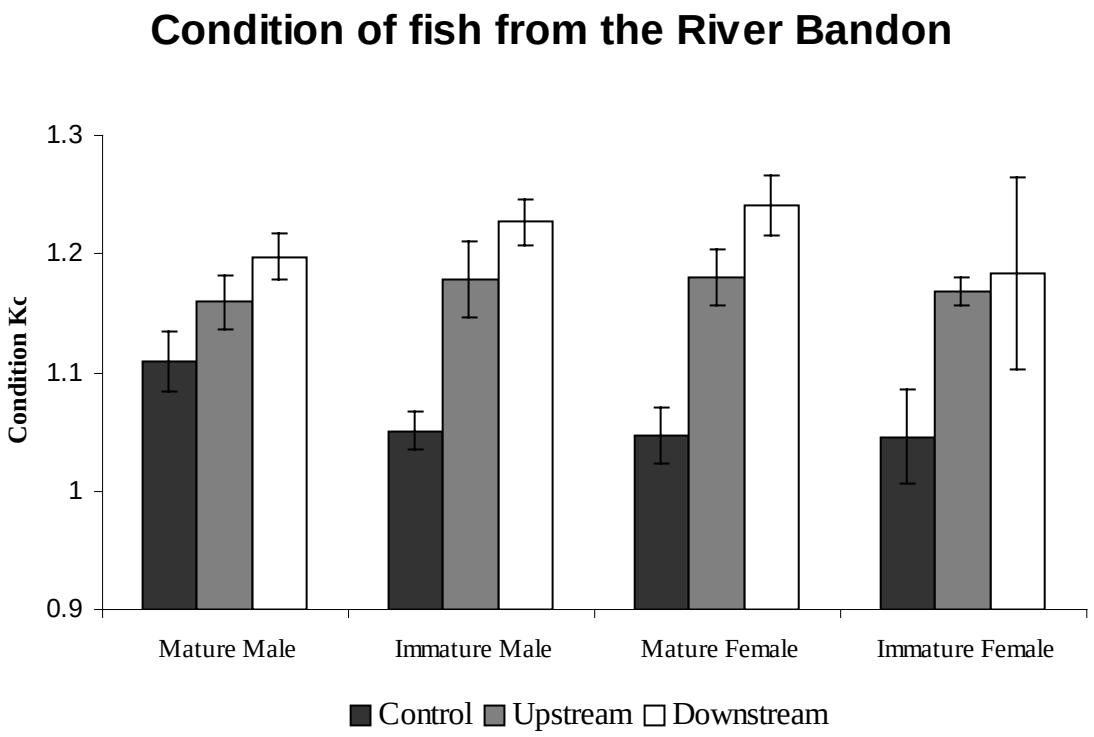


Figure 4.3.2
Mean (SE) condition values k_c (Fulton's Condition of Salmonids) of mature male, immature male, mature female and immature Brown trout from three sites on the river Bandon.

(iii) Hepatosomatic Index:

Liver weights were recorded for all fish taken from the Bandon river. The HSI values for the immature males, males, immature females, and mature females are shown in Figure 4.3.3. At all three sites on the Bandon river, the mean HSI-values for the mature females were found to be significantly different to those of the mature males, immature males and immature females (2 way ANOVA). This difference was a significantly lower $\log_{10}$ HSI value, which indicates an increased liver size in the mature female trout. Differences between the three sites on the river Bandon were not significant (Tukey's 0>0.05).

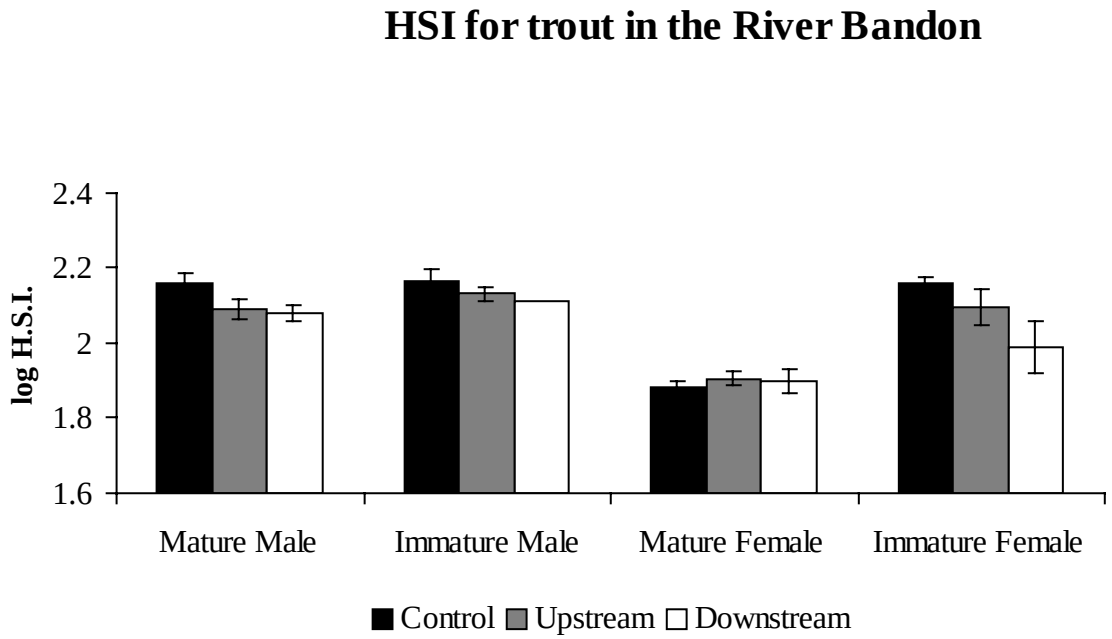
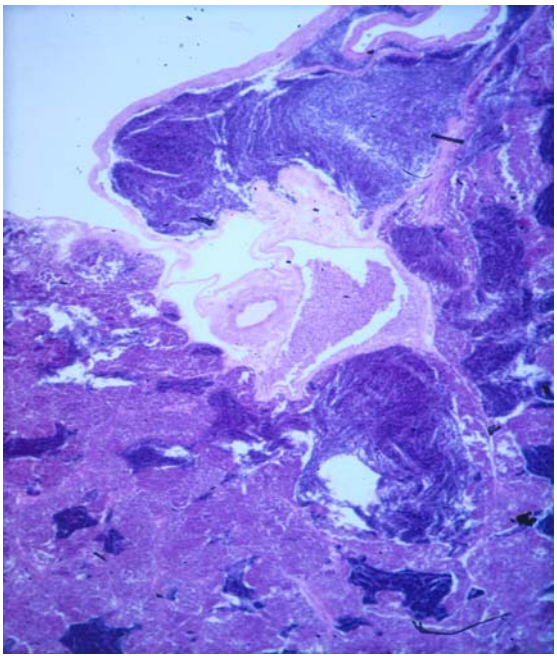


Figure 4.3.3
Mean (±SE) hepatosomatic index (HSI; liver weight (g)/body weight (g)) of mature male, immature male, mature female and immature Brown trout, from three sites on the Bandon river.

(iv) Intersex:

The testes of all male fish, sampled from the three sites on the river Bandon, were examined macroscopically and no abnormalities in either morphology or colouration were detected. All testes were examined microscopically with sections from the anterior, middle and posterior being viewed. None of the sections examined showed the presence of intersex tissue, yielding a 0 % occurrence of intersex in the fish sampled. Sections of healthy testes from Bandon sites are shown in Figure 4.3.4.



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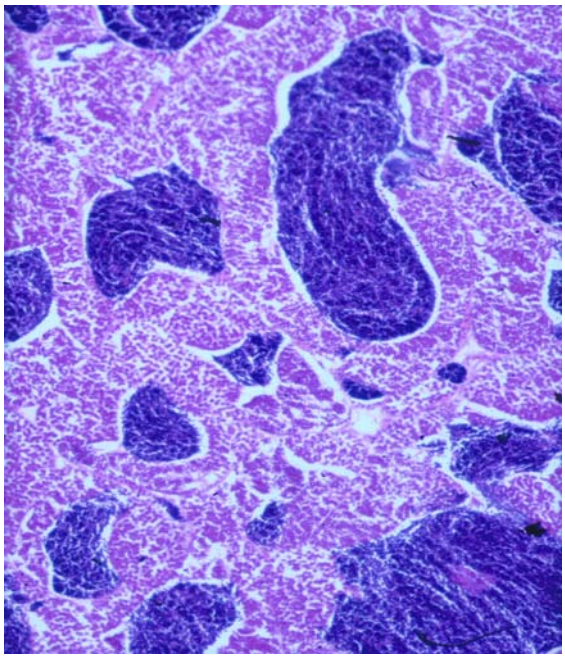
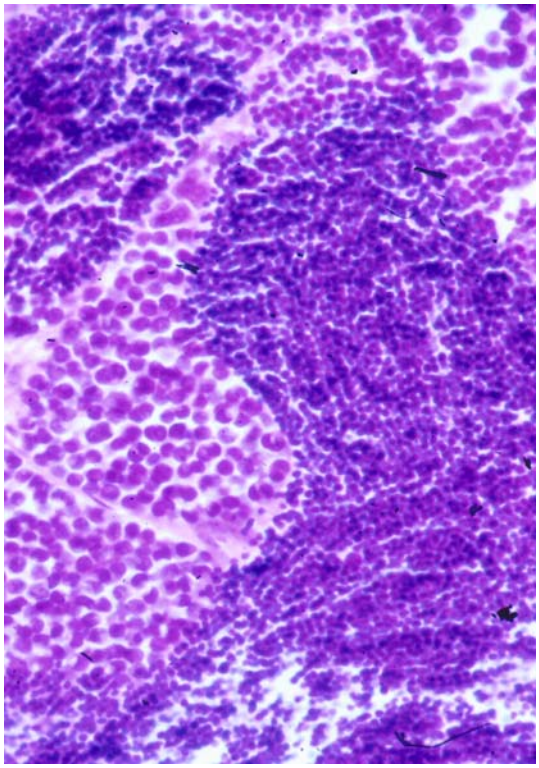
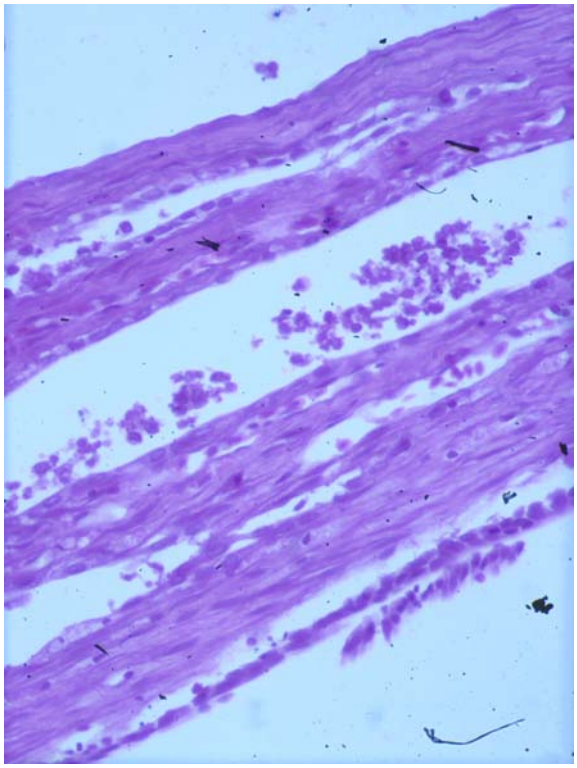


1 (b)



1 (c)



1 (d)

Figure 4.3.4
Transverse sections (a), (b), (c) of healthy maturing male Brown trout testes, showing (a) the location of the sperm duct and (b) (c) the different spermatogenic cell types, and spermatozoa. Longitudinal section of a sperm duct (d).

4.3.2 River Lee

On the river Lee 84 trout were sampled. The length range, mean length, weight ranges and mean weights are given in Table 4.3.3. The control site fish were of a smaller mean length and weight than the fish from the two sites upstream and downstream of the EDC input at Ballincollig WWTP.

Table 4.3.3.
Length range, mean length, weight range and mean weight for the trout sample from three sites on the river Lee

Site	Length range (cm)	Mean length (cm)	Weight range (g)	Mean weight (g)
Control	13.5 - 21.2	16.1	25.1 - 109.8	46.4
Upstream	16.3 - 30.1	21.2	59.8 - 301.5	125.9
Downstream	14.4 - 34.6	21.0	38.9 - 600.0	138.5

The sex and maturity of the trout sampled from the three sites on the river Lee is summarised in Table 4.3.4. No trend was noted in the numbers of mature, immature, male or female from the three sites on the river Lee, which may be due to the small sample size.

Table 4.3.4
Sex and maturity of the 84 fish sampled from the three sites on the river Lee.

Site	Mature Male	Mature Female	Immature Male	Immature Female	Total
Control	5	7	7	18	37
Upstream	3	4	4	9	20
Downstream	3	7	8	9	27
Total	11	18	19	36	84

(i) Sex Ratio:

The sex ratio for the fish sampled from the three sites on the river Lee shows a higher ratio of females to males at each site, with only 32 % of the control population being male fish (Figure 4.3.5).

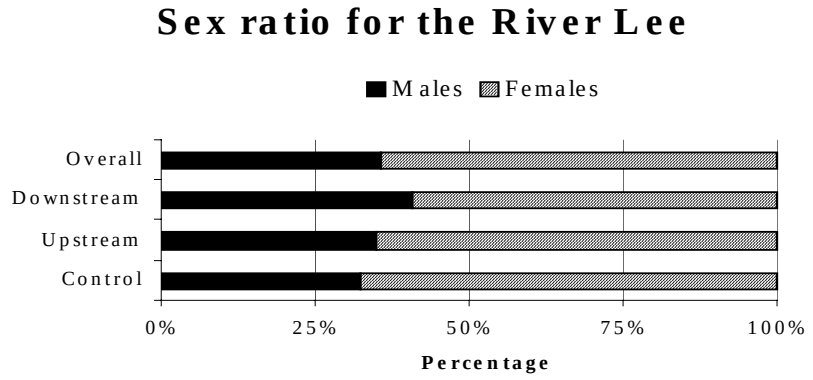


Figure 4.3.5
Sex ratio for 110 trout sampled at three sites on the river Lee, with the overall sex ratio.

(ii) Condition:

The condition of the trout from the three sites on the river Lee was determined using Fulton's Salmonid condition function K_c (Figure 4.3.6). The condition values (K_c) for the control site are significantly smaller (2-way ANOVA) than the condition values for mature males, immature males, mature females and immature females at the sites upstream and downstream of Ballincollig WWTP. Thus, for the river Lee, as for the river Bandon, the trout from the control site were in good condition ($K_c > 1$) but were thinner than fish from the upstream and downstream sites.

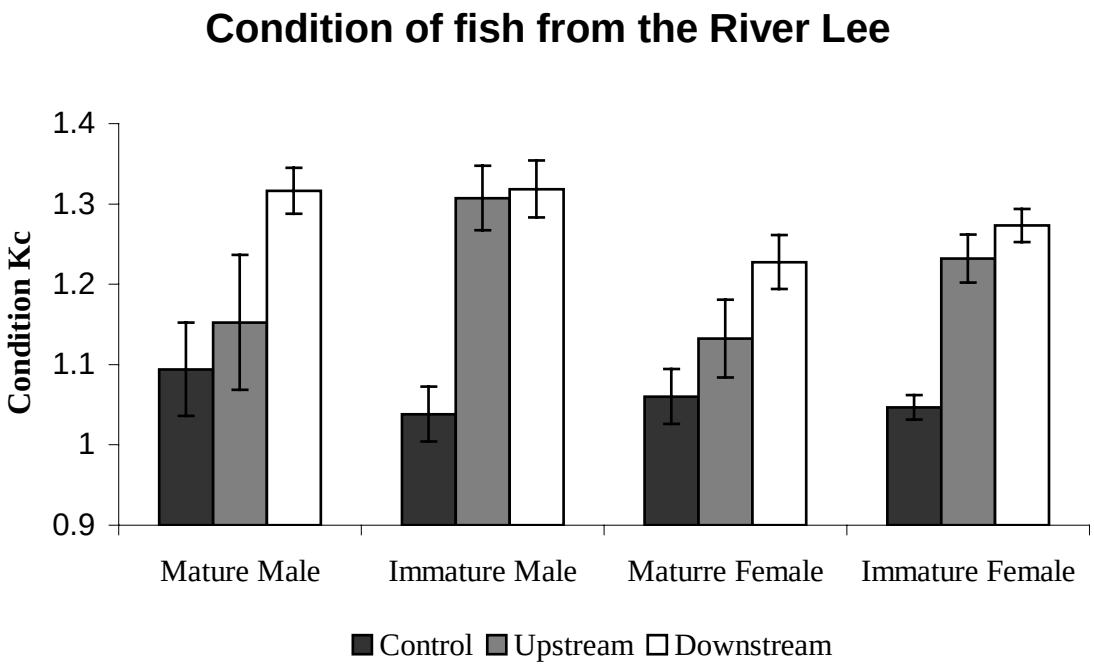


Figure 4.3.6
Fulton's Condition of Salmonids for trout sampled from three sites on the river Lee.

(iii) Hepatosomatic Index (HSI):

At all three sites on the river Lee the mean HSI-values for the mature females were found to be significantly different to those of the mature males, immature males and immature females (2 way ANOVA, Figure 4.3.7). This difference was a significantly lower $\log_{10}$ HSI value that indicates an increased liver size in the mature female trout. A significant difference was found between the control site HSI-values and the upstream and downstream sites, but no difference between the upstream and downstream sites. The difference seen was a significantly higher $\log_{10}$ HSI value, which signifies a decreased liver size in all fish found at the control site.

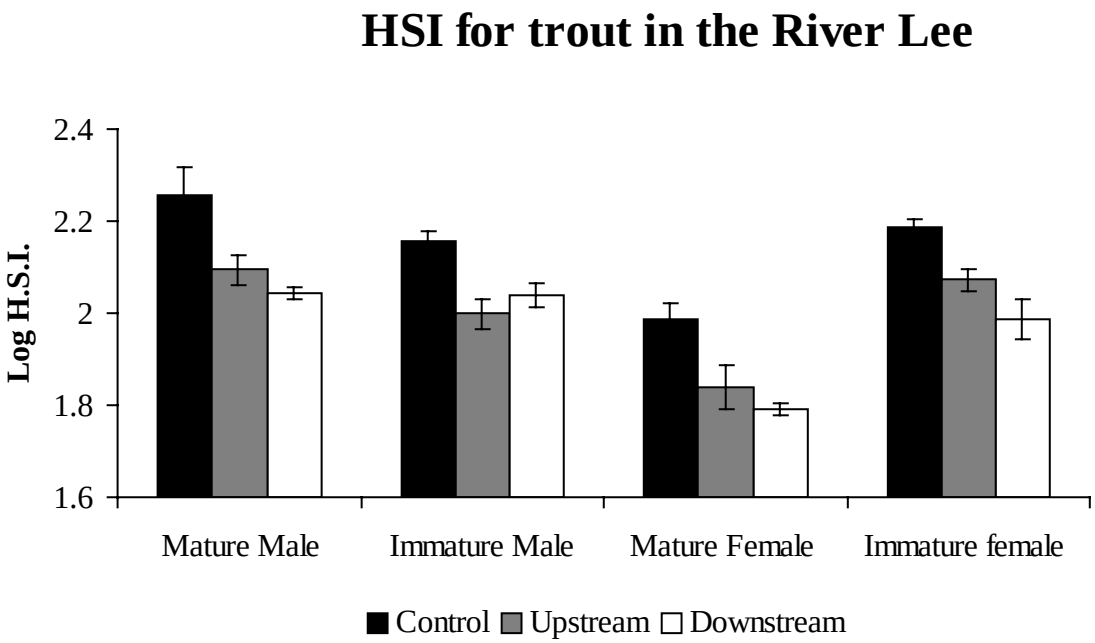


Figure 4.3.7
Mean (±SE) hepatosomatic index (HSI; liver weight (g)/body weight (g)) of mature male, immature male, mature female and immature Brown trout, from three sites on the river Lee.

(iv) Intersex:
The testes of all male fish, sampled from the three sites on the river Lee were examined macroscopically and no abnormalities in either morphology or colouration were seen. The testes were also examined microscopically with sections from the anterior, middle and posterior being viewed from each fish. Microscopic examination of the testes showed a 0% incidence of intersex in the fish sampled.

4.3.3 Killarney Lakes

A total of 24 fish were sampled from the three locations in Killarney: Lough Guitane, Black Valley, and Lough Leane. The length range, mean length, weight ranges and mean weights are summarised in Table 4.3.5.

Table 4.3.5
Length range, mean length, weight range and mean weight for the Brown trout sampled from the three sites on the Killarney lakes.

Site	Length range	Mean length	Weight range	Mean weight
Guitane	27.2 - 27.6	27.4	184.0 - 221.9	203.0
Black Valley	27.0 - 28.5	27.8	212.7 - 247.4	230.1
Leane	21.6 - 33.0	26.1	96.6 - 462.0	125.9

The sex and maturity for the trout obtained from the Killarney lakes are given in Table 4.3.6.

Table 4.3.6
Sex and maturity of the 24 fish sampled from the three sites in the Killarney lakes region.

Site	Mature Males	Mature Females	Immature Males	Immature Females	Total
Guitane	1	1	0	0	2
Black Valley	2	0	0	0	2
Leane	11	6	2	1	20
Total	14	7	2	1	24

These fish were all obtained from fishing competitions as netting and electro-fishing techniques could not be used on the lakes. Many anglers only donated the male fish to the study, retaining the females, and hence a sex ratio has not been calculated. Insufficient numbers of fish were obtained to allow a statistical analysis of condition (K_c) or HSI values.

(i) Intersex:
The testes of all male fish, sampled from the three locations in the Killarney lakes examined macroscopically and no abnormalities in morphology, or colouration were detected. All testes were examined microscopically with sections from the anterior, middle and posterior being viewed. None of the sections examined showed the presence of intersex tissue, yielding a 0 % occurrence of intersex in the sampled population.

4.3.4 Other Indices of Reproductive Health

(i) Gonadosomatic Index (GSI):
The GSI was calculated for the male fish from each of the sampling sites on the Lee. However, the fish were sampled over a period of three months, and as the gonad size of the fish increases as the fish approach their breeding season the testes will have been increasing in size over the sampling period. Thus, no conclusions can be drawn from any GSI differences noted between the sites.

(ii) Semi-Quantitative Determination of Male Brown Trout Plasma Vitellogenin:
Wherever possible, blood samples were taken from the sampled fish and semi-quantitative determination of plasma vitellogenin levels was performed by enzyme immunoassay (see Chapter 2). Blood samples were obtained from all fish caught by electrofishing, and from most fish caught by rod and line, with the exception of those obtained from angling competitions (i.e. all fish obtained from the Killarney Lakes).

The enzyme immunoassay is based on detection of vitellogenin by a monoclonal antibody, BN-5, raised against vitellogenin from Atlantic salmon (*Salmo salar*). Due to cross-reactivity of the antibody with vitellogenin of other species, the assay may be used for the detection of vitellogenin in plasma samples from a variety of other species including the *Salmoniformes* of which Brown trout (*Salmo trutta*) is a member.

There was no evidence of raised plasma vitellogenin in any of the male wild Brown trout taken from the rivers Lee and Bandon (Figure 4.3.7). In contrast, raised plasma vitellogenin was detected in a significant proportion of the fish sampled from the river

Liffey, downstream of Osberstown WWTP (Figure 4.3.8, Table 4.3.7). This result is described in detail in the following section (section 4.4).

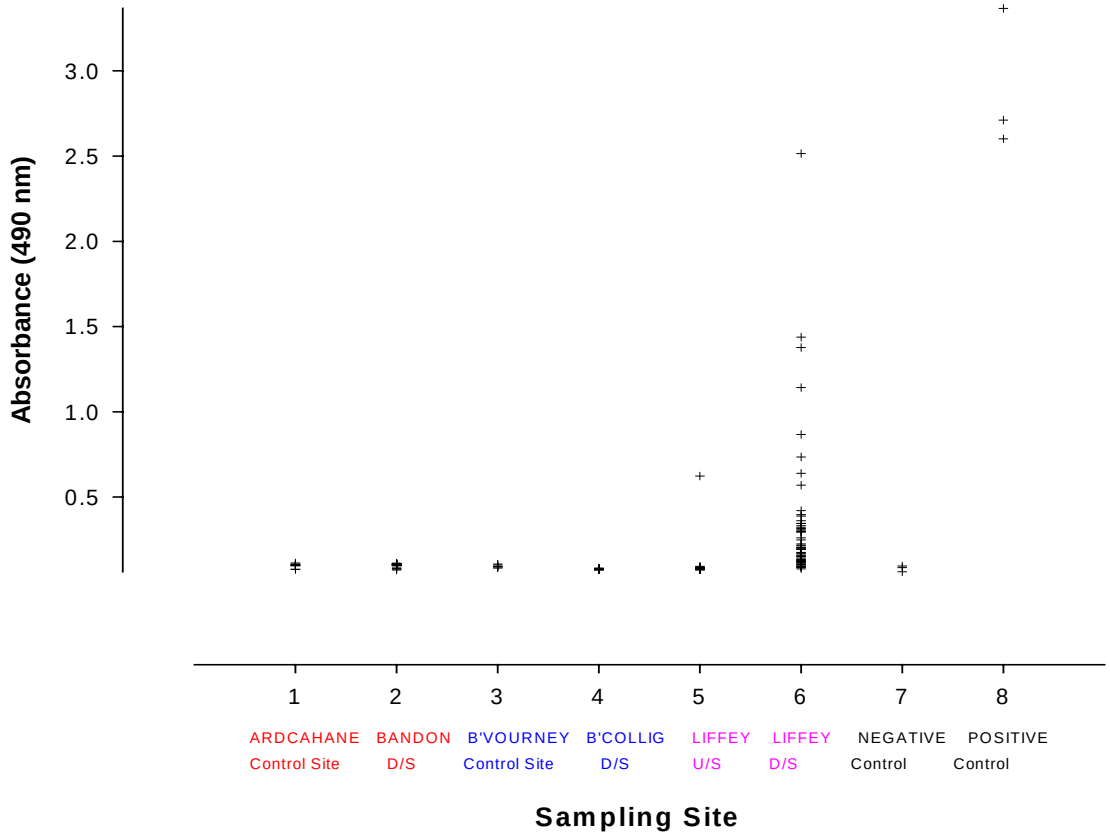


Figure 4.3.7
Plasma vitellogenin levels in the male wild Brown trout in the rivers Bandon, Lee and Liffey.
Semi-quantitative determination of plasma vitellogenin in male wild Brown trout was performed by enzyme immunoassay (Salmonid Vitellogenin Enzyme Immunoassay kit: Biosense Laboratories, Norway). The numbers of fish with raised vitellogenin levels at each sample site are as follows: Ardcahane (Bandon river internal control) = 0 of 10; Bandon town = 0 of 35; Ballyvourney (Lee river internal control) = 0 of 6; Ballincollig town = 0 of 14; Liffey (upstream Osberstown) = 1 of 47; Liffey (downstream Osberstown) = 15 of 57. The results shown are from three separate assays, each of which included a negative and positive control.

Table 4.3.7
Characteristics of the Wild Fish Sampling Sites
One population equivalent (PE) is defined as 60g BOD 5. The river dilution factors were calculated using the dry weather flow data for the WWTPs and 95 percentile river flow values. Adjusted PE values represent the PE value divided by appropriate the river dilution factor. n.d. = not detected, U/S: upstream, D/S: downstream.

River	Site	Type of Site	Vitellogenin Detected	Incidence of Intersex	PE	River Dilution Factor	Adjusted PE
Liffey	Osberstown	a. U/S WWTP outfall	a. Positive (1 of 47)	n.d.			
		b. D/S WWTP outfall	b. Positive (15 of 57)	n.d.	66,100	24.65	1,704
Bandon	Ardcahane	Headwaters	Negative (0 of 10)	n.d.	N/A		
	Bandon	D/S WWTP Outfall	Negative (0 of 35)	n.d.	6,200	34.18	292
Lee	Ballyvourney	Tributary Headwaters (R. Sullane)	Negative (0 of 6)	n.d.	N/A		
	Ballincollig	D/S WWTP Outfall	Negative (0 of 14)	n.d.	15,000	77.12	195

4.4 Results of the Survey of the Wild Brown Trout Population in the River Liffey, Upstream and Downstream of the Osberstown WWTP.

A total of 325 fish were sampled from the River Liffey: 160 were obtained from the site upstream of Osberstown WWTP and 165 were sampled from the site downstream of the WWTP outfall (Figure 4.4.1).

Upstream of the WWTP outfall, there were a total of 56 mature (or nearly mature) males caught, 16 immature males, 83 mature (or nearly mature) females and 5 immature females. Downstream of the WWTP outfall, there were a total of 47 mature (or nearly mature) males caught, 10 immature males, 79 mature (or nearly mature) females and 29 immature females (Figure 4.4.1).

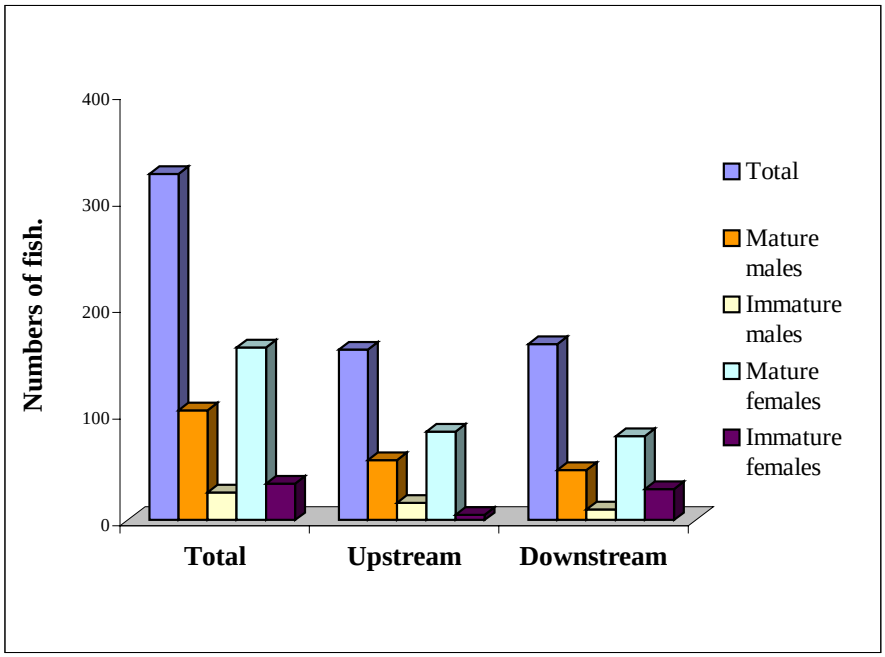


Figure 4.4.1
Numbers of fish caught upstream and downstream of the outfall of Osberstown WWTP.

(i) Intersex

Histological analysis was performed on the testes of all male fish sampled from the river Liffey. No abnormalities in either morphology or colouration were seen. The testes were also examined microscopically with sections from the anterior, middle and posterior being viewed from each fish. None of the sections examined showed the presence of intersex tissue yielding a 0% incidence of intersex in the fish sampled from both upstream and downstream of the outfall from Osberstown WWTP.

(ii) Semi-Quantitative Determination of Male Brown Trout Plasma Vitellogenin:

In as many cases as possible, blood samples were taken from the sampled fish and semi-quantitative determination of male trout plasma vitellogenin levels was performed by enzyme immunoassay (see Chapter 2). As the assay is semi-quantitative for vitellogenin, the results are presented as negative, positive or weakly positive for raised vitellogenin in the male fish plasma. The positive and weakly positive samples are considered together in the following analysis of results.

Of the 57 male fish caught downstream, 15 of them showed raised vitellogenin levels i.e. 26.32% of the male fish sampled (Figure 4.3.7, Table 4.3.7).

In contrast, 72 males were sampled upstream of the WWTP, 47 of which yielded plasma samples suitable for analysis. Only one fish (2.1%; 1 of 47) was positive for raised plasma vitellogenin (Figure 4.3.7, Table 4.3.7). Although Brown trout are not considered to be a highly migratory species, it is possible that the one fish with raised vitellogenin upstream of Osberstown WWTP had migrated upstream from downstream of the treatment plant.

Analysis of variance was carried out to investigate the significance of the results that showed raised vitellogenin levels in the two sites. Chi-squared analysis showed that there was a significant difference between the results from the two sites ($p < 0.01$) P=0.000).

(iii) Hepatosomatic Index (HSI):

The HSI was calculated for all of the male fish (both mature and immature) (Figure 4.4.3). The livers were not retained from the mature females and therefore the HSI could not be calculated for these fish.

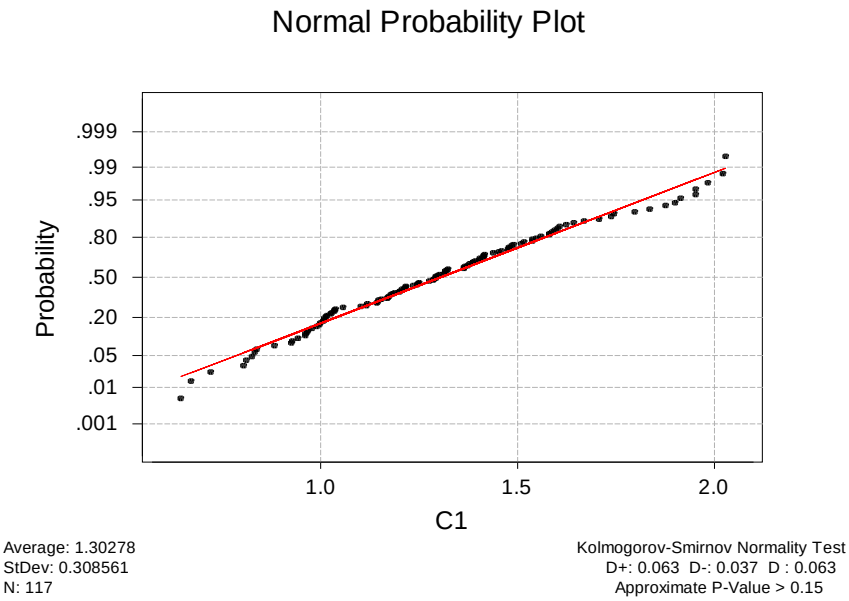


Figure 4.4.3
The results of the normality test on the HSI values.

The mean values for the HSI indices from all of the male trout caught at the two sites were calculated (Table 4.4.1). Overall the immature males had higher HSI values than the mature male fish. But the downstream fish livers comprised a greater percentage of the fish body, both in the mature and immature trout.

Table 4.4.1
Mean HSI values for male fish caught upstream and downstream of the WWTP (HSI values are % values).

Upstream		Downstream	
Mature Males	1.21 ± 0.04	**Mature Males**	1.32 ± 0.05
Immature Males	1.38 ± 0.06	**Immature Males**	1.64 ± 0.08

Before the HSI could be statistically analysed it had to be transformed into a suitable format because HSI values are percentages. The data was transformed by dividing the HSI value by 100, then getting the square root of this value and then calculating the sine of the answer. It was these figures that were used in the statistical analysis.

The data was tested for normality using the Kolmogorov-Smirnov test (Figure 4.4.3) and did not differ significantly from the normal ($p > 0.15$) (Dytham, 1999).

Analysis of variance (One-way) was carried out to examine the differences in HSI values upstream and downstream and also the differences between mature and immature males. As might be expected there was a very significant difference between HSI values from the immature and mature males ($F_{116}=11.57$, $P=0.01$). There was also a significant difference between HSI values from all male fish caught upstream and downstream ($F_{116}=6.22$, $P=0.014$).

4.5 Discussion

This survey of feral Brown trout populations was designed to investigate the possibility that Irish freshwaters contain estrogenic chemicals at levels capable of affecting the reproductive health and success of exposed fish populations. To date, the majority of endocrine disrupting effects reported in wildlife populations have been of a feminising nature and, thus, bioindicators of estrogen exposure were chosen as the tools for the current investigation. The presence of vitellogenin in the blood of male fish is the most widely accepted biomarker of estrogen exposure, providing a sensitive bioassay with a response range that spans several orders of magnitude (Sumpter and Jobling, 1995). Although not a specific indicator, decreases in the gonadal somatic index (GSI) of male fish have been reported in connection with exposure to estrogens (Jobling *et al.*, 1998). In the current study, these two parameters were investigated with a view to establishing whether the Irish wild fish population is exposed to significant levels of estrogens while sexually mature. In addition, evidence for wild fish exposure to estrogens during critical periods of development (i.e. sexual differentiation) was sought from histological analysis of gonadal tissue for intersex, and by determining the sex ratio of the sample population.

Sample Size:
This study aimed to collect a minimum of 100 male Brown trout from each of the 11 sampling sites (section 4.2), to enhance the statistical validity of the study's findings. However, in practice it proved impossible to obtain samples of this size despite the large number (41) of sampling trips made between August 2001 and August 2002. Sampling on the rivers Lee, Bandon and the Killarney Lakes employed a range of fishing techniques; angling, net and electrofishing. Much of the sampling on the rivers Lee and Bandon was carried out using the portable electrofishing apparatuses owned by the ADC and the South Western Region Fisheries Board (SWRFB). This equipment is suitable for small streams and may also be used on sections of large rivers during low water conditions, although it is not suitable for use on lakes. The remainder of the sampling on these water bodies was performed by rod, using angling competitions as a means to increase the number of samples obtained. Some samples from Lough Leane were obtained in cooperation with the Central Fisheries Board (CFB), which was performing a survey of Lough Leane using net fishing during this period.

All sampling on the river Liffey was performed using boat-operated electrofishing equipment that was hired from the Eastern Fisheries Board (EFB), which is a

considerably more efficient and effective method of obtaining large sample numbers in a single fishing operation. Such equipment was not available for hire from the SWRFB.

Sex Ratio:
An excess of female fish was present in the samples from 9 of the 11 sites. This difference was insignificant in the case of the river Bandon, with male fish accounting for 47% of the overall sample (Table 4.3.2, Figure 4.3.1). The sex ratio for the river Lee showed a higher ratio of females to males at each site, with male fish accounting for only 36% of the overall sample population. As this finding was consistent for each of the three sites on the river, and was most pronounced at the control site (32% male), it would not appear to be linked to exposure to EDCs from Ballincollig WWTP (Table 4.3.4, Figure 4.3.5). It should be noted that the rivers Lee and Bandon have not been subject to any Brown trout restocking programme within the past 10 years (O'Connell, 2004; Pers. Comm.) and so all trout sampled were considered wild fish.

The river Liffey also showed a higher ratio of females to males at each of the two sites sampled. This difference was not considered significant (45% male) at the site upstream of the putative EDC input (Osberstown WWTP), although the downstream site did show a decreased percentage of male Brown trout (35% male). The critical period of sex determination in salmonid species is just a few days around the time of hatching and exposure to physiologically relevant concentrations of natural or synthetic estrogens during this period will result in the fish all developing as functional females (Campbell and Hutchinson, 1998). Thus, it could perhaps be argued that exposure to significant levels of estrogens during this critical period resulted in an increased number of females relative to males in the feral Brown trout population downstream of Osberstown WWTP. However, the complete absence of intersex in any of the male fish sampled, which would be expected to accompany such a significant level of estrogen exposure, argues strongly against this hypothesis.

The river Liffey has been restocked with Brown trout on a number of occasions over the past 10 years. However, a 1999 survey of the status of salmonid populations in the river noted stocked fish (64% of the sampled population) at only one site; Clane, Co. Kildare, which is approximately 8 km downstream of Osberstown WWTP (Ní Chochláin *et al.*, 1999). None of the other Liffey sites sampled in the 1999 study, including Caragh Bridge, Co. Kildare (the upstream site in the present study), recorded any stocked fish. The Liffey was restocked with Brown trout again in 2001-2002, but only at Clane (Miley, 2004; Pers. Comm.). Our study did not record whether the sampled fish were of wild or stocked origin. However, as Brown trout are not a highly migratory species it is unlikely that many of the fish caught at the Lenister Aquaduct (approximately 2.5 km downstream of Osberstown WWTP) were stocked fish. This conclusion is supported by the findings of Ní Chochláin *et al.* (1999), as discussed above.

Intersex:
There was no evidence of ovotestis (intersex) in any of the male Brown trout obtained, at any of the sites on the rivers Liffey, Lee, Bandon or the Killarney Lakes. It is known that exposure to exogenous estrogens can cause feminization of male salmonids if exposure occurs during a critical window spanning about 10 days either side of when the eggs hatch (Routledge et al., 1998). Thus, the result of the wild fish survey indicates that the Irish feral Brown trout population has not been exposed, during critical periods

of development, to concentrations of estrogens that can affect gonadal development in any of the rivers or lakes studied. However, with the exception of the river Liffey, it could be argued that a low incidence of intersex in the population might not be detected within the small samples obtained in the Lee and Bandon rivers (Tables 4.3.2, 4.3.4 and 4.3.6).

Many of the studies reporting intersex in feral fish populations refer to cyprinid species such as roach, carp, gudgeon and bream, which are present in greater numbers than the salmonid species in the large slow-flowing rivers that characterise these countries. However, the native Irish ichthyofauna is typically salmonid in nature, with coarse fish showing a more sparse and localised distribution. Thus, Brown trout, which are ubiquitous in Irish freshwaters and begin maturing at approximately one year, were chosen as the indicator species rather than more minor species such as roach and bream. In addition, previous studies have indicated that salmonids (specifically, Rainbow trout (*Oncorhynchus mykiss*)) are more sensitive indicators of estrogen exposure than cyprinids (Routledge *et al.*, 1998; Purdom *et al*., 1994).

Several Swiss studies have reported both raised plasma vitellogenin levels (Wahli *et al*., 1998) and intersex (Bernet and Wahli, 2000) in feral Brown trout populations. It is of interest to note that the intersex was detected in sample sizes similar to those obtained from the Lee and Bandon rivers in the present study.

Plasma Vitellogenin Levels:

There was no evidence of raised vitellogenin levels in any of the male Brown trout obtained, at any of the sites on the rivers Lee and Bandon. This indicates that the wild Brown trout population of these water systems has not been exposed to concentrations of estrogens that can induce vitellogenesis (Figure 4.3.7, Table 4.3.7). No plasma samples were available for fish sampled from the Killarney Lakes (see section 4.3.4) and, thus, no definite conclusion can be drawn regarding levels of vitellogenesis (if any) in male fish taken from this water body.

In contrast, raised vitellogenin levels were detected in male Brown trout from the river Liffey, indicating estrogenic activity in some stretches of this river (Figure 4.3.7, Table 4.3.7).

As the purpose of the Liffey sampling programme was to investigate the effect of effluent from Osberstown WWTP (PE 68,000) on the feral fish population, both sample sites were located in the vicinity of the WWTP (Section 4.2). The upstream site yielded a sample population of 72 male fish, 47 of which yielded plasma samples suitable for vitellogenin testing. One fish (2.1 %; 1 of 47) showed raised plasma vitellogenin levels (Figure 4.3.7, Table 4.3.7). However, 26.3 % (15 of 57) of male Brown trout from the downstream site, showed raised vitellogenin levels (Figure 4.3.7, Table 4.3.7). Thus, the level of vitellogenesis in male fish from the downstream site (a 2.5 km stretch of the river, from the WWTP outfall to the Leinster Aquaduct) is significantly different from the upstream site at Carragh Bridge, Co. Kildare, which is unaffected by the WWTP effluent (Chi-squared test; $p < 0.01$).

This result indicates that environmental estrogens are present at environmentally significant concentrations in this stretch of the River Liffey, a finding that is supported by YES *in vitro* bioassay analyses (Chapter 5, Table 5.2) that place the estrogenicity of this reach of the river into the extreme lower end of the concentration range of E2 required to induce vitellogenesis in male Rainbow trout. YES analysis placed the effluent at 17.2 ± 3.8 ng/L estradiol equivalents ($E2_{eq}$), and the downstream receiving waters at 1.3 ± 0.8 ng/L $E2_{eq}$ (a value that is somewhat higher than that predicted from diluted effluent values alone; 0.7 ng/L).

However, the relatively low incidence (26%) of raised vitellogenin in the downstream Brown trout population, combined with the variability in the level of vitellogenin response in those fish testing positive (Fig. 4.3.7), is suggestive of exposure to a weakly estrogenic effluent: some fish are more sensitive to the estrogens, and respond, whereas others are less sensitive and do not (J.P. Sumpter, Pers. Comm., 2003). This conclusion is further supported by the histological analysis of the gonads of these fish, which did not reveal any evidence of intersex. This indicates that the estrogen exposure was intermittent and did not occur at a critical period of development for any of the fish, reducing the potential impact on reproductive health and development of the fish population.

Previous studies (see introduction) have demonstrated conclusively that treated final effluent, of domestic, or domestic and industrial, origin is a source of estrogens. Where the effluent is predominantly of domestic origin, the main source of estrogenicity has been attributed to the natural (17β-estradiol, estrone) and synthetic steroid estrogens (17α-ethinylestradiol) (Desbrow *et al*., 1998; Johnson and Sumpter, 2001). These compounds are estrogenic at levels of the order of ng/L. For example, Routledge *et al*. (1998) reported 10 ng/L estradiol to be the threshold concentration for induction of the vitellogenin response in male Rainbow trout, while 0.1 ng/L of 17α-ethinylestradiol is sufficient to cause a significant increase in the plasma vitellogenin concentration after relatively brief exposure (Purdom *et al*., 1994; Sheahan *et al*., 1994).

It is of considerable interest to compare the rivers Liffey and Lee, at the Osberstown and Ballincollig study sites, in terms of endocrine disruption of the wild fish population and the corresponding estrogen levels in the river waters (as measured by YES: Chapter 5, Table 5.2). The only evidence for endocrine disruption of fish was obtained downstream of Osberstown WWTP, although the estrogenic burden of the receiving waters at both Osberstown (1.3 ng/L) and Ballincollig (1.5 ng/L) was similar. In light of this apparent discrepancy it is worth considering that Osberstown WWTP is somewhat unique in that a component of its industrial PE (Table 5.2) comprises waste from a pharmaceutical company that manufactures the contraceptive pill and, thus, possibly contained an amount of EE2 (Chapter 5, section 5.2.3). This is an important consideration as the nature of the chemicals giving rise to the estrogenic activity in the effluents is a significant factor when trying to predict the effect of an estrogenic effluent on fish populations. For example, when measured using the *in vitro* YES bioassay, EE2 and E2 are equipotent, although in terms of the *in vivo* vitellogenin response EE2 is an order of magnitude more potent than E2 (Metcalf *et al.*, 2001; Thorpe *et al.*, 2003). This increased potency of EE2 *in vivo* depends on the 17α-ethinyl moiety, which increases EE2's persistence in organisms by reducing its rate of metabolism compared to the endogenous steroids (Routledge *et al.*, 1998). Thus, if the Osberstown WWTP effluent

contained elevated levels of EE2 (for example, as a result of waste from the pharmaceutical plant) then this may have posed a heightened risk to the fish population in the Liffey, relative to other effluents which are of similar estrogenic activity in the YES. (It should be noted that from January 1 2006, the pharmaceutical company will be subject to further substantial reductions in chemical waste discharge levels, including EE2). This may explain why the Osberstown receiving waters (with estrogen levels of 1.3 ng/L) were associated with vitellogenin induction in wild fish whereas the Ballincollig receiving waters were not, despite slightly higher estrogen levels of 1.5 ng/L.

This incidence of endocrine disruption in wild male Brown trout, downstream of Osberstown WWTP, is of added significance given its location upstream of the point of intake of Liffey water to Leixlip WTW. It would be of considerable importance to further characterise this reach of the river with respect to the presence of EDCs. For example, the use of YES bioassay-directed chemical fractionation of water samples would allow the identification and quantification of the specific estrogenic chemicals that are contributing to the observed estrogenic effects in Liffey Brown trout. It would also be of interest to perform further *in vivo* studies, for example by placing cages of male Rainbow trout in the vicinity of the point of intake to Leixlip WTW, in order to determine if estrogenic chemicals are present, in environmentally-relevant concentrations, in this area of the river.

4.6 Conclusions

Ovotestis (intersex) and raised plasma vitellogenin in male fish are widely employed biomarkers of estrogen contamination in the aquatic environment, and were used in this study to investigate the possibility that Irish rivers contain estrogenic chemicals at levels capable of affecting the reproductive health and success of exposed fish populations.

There was no evidence of intersex in any of the male Brown trout obtained, at any of the sites on the rivers Lee and Bandon, or the Killarney Lakes. This indicates that the feral Brown trout population of these water bodies has not been exposed, during critical periods of development, to concentrations of estrogens that can affect gonadal development. However, it could be argued that a very low incidence of intersex in the population might not be detected within the small samples obtained. Neither was there any evidence of raised plasma vitellogenin indicating that the fish were not exposed to environmental estrogens at concentrations sufficient to induce vitellogenesis within the few weeks prior to sampling. When these two results are taken together, it may cautiously be concluded that environmental estrogens are not present in the rivers Lee (upstream of the Lee Road WTW) and Bandon, or in the Killarney Lakes, at levels likely to pose a threat to the reproductive health and development of their fish populations.

In contrast, raised vitellogenin levels were detected in male Brown trout from the river Liffey, indicating estrogenic activity in a stretch of the river downstream of the outfall of the Osberstown WWTP. This result is supported by *in vitro* bioassay studies that place of the estrogenicity of this reach of the river into the extreme lower end of the concentration range of E2 required to induce vitellogenesis in male Rainbow trout (see

Chapter 5 for further discussion). However, histological analysis of gonads and the absence of any evidence of intersex suggests that this estrogen exposure did not occur at a critical period of development for any of the fish, and that the fish are intermittently exposed to raised estrogen levels, thus reducing the potential impact on reproductive health and development of the population.

5. *In Vitro* Bioassay Analysis of WWTP Effluents and Receiving Waters for Estrogenic Chemicals.

5.0 Introduction

This chapter describes the application of the yeast estrogen screen (YES) *in vitro* bioassay to the determination of the estrogenic potency of WWTP effluents and their receiving waters, in a number of sites in the South and East of Ireland.

There is now unequivocal evidence that a wide variety of chemicals, capable of disrupting the endocrine system, are present in the aquatic environment. Studies in the UK have demonstrated that WWTP effluent can be estrogenic to fish (Purdom *et al*. 1994). Three sterols (17β-estradiol, 17α-ethinylestradiol and estrone) have been identified as the major estrogenic chemicals in WWTP effluent of predominantly domestic origin (Desbrow *et al*., 1998). Subsequent studies have confirmed that these natural and synthetic estrogens are present in domestic WWTP effluents at concentrations similar to those reported by Desbrow and co-workers (Kirk *et al*., 2002).

Differences in WWTP technology are thought to be responsible for variation in the extent of endocrine disturbances reported in fish, in UK and US studies. Thus, each WWTP must be considered in terms of its own characteristics, and variables, such as the technical standard of the plant and the dilution ratio of the discharge, will play a defining role in determining the final impact of the effluent on exposed wildlife populations (Burkhardt-Holm *et al*., 2002).

To date, no published data on the estrogenic load of Irish WWTP effluents and their receiving waters exists and so the results of the present study will go some way towards filling this knowledge gap.

5.1 Characteristics of Sample Sites

A survey of the estrogenic potency of treated final effluents from ten WWTP in the South and East of Ireland was carried out. The sites were selected so that the population equivalent (PE) loading of the influents ranged over two orders of magnitude (12,960-2,186,808) and the influent composition varied from that of predominantly domestic origin (e.g. Ballincollig, Killarney, Fermoy) to that containing a substantial industrial input (e.g. Kilkenny, Leixlip, Osberstown) (Table 5.1). All plants included in the survey provided secondary treatment of the effluents. As one of the major factors determining the general quality, and estrogenic activity, of final effluents is the treatment technology applied (Burkhardt-Holm *et al*., 2002), plants providing secondary treatment only were selected for this survey. This increases the comparability of the data obtained from the different plants surveyed. Although some of the WWTP did possess tertiary treatment facilities (UV-irradiation), these were not in operation on any of the sampling dates in this study.

Samples were collected and analysed by YES *in vitro* bioassay as described previously (Chapter 2, section 2.2.2). In some cases, samples were collected from upstream and downstream of the WWTP outfall to examine the impact of the WWTP effluent on the receiving waters.

Table 5.1: WWTP Sample Sites

A selection of WWTP, representative of small, medium and large agglomerations, was made. The population equivalent (PE) loading of the WWTP influents spanned two orders of magnitude (12,960-2,186,808) and in each case the effluent received secondary treatment. Three plants (Killarney, Leixlip and Osberstown) also provide nutrient reduction of the final effluent. The plant discharge was to freshwater systems in all cases, except Tralee and Ringsend WWTP which discharge to coastal waters and estuarine waters, respectively. The Urban Waste Water Treatment Regulations define one population equivalent (PE) as the amount of organic biodegradable load that has a 5-day biological oxygen demand (BOD 5) of 60 g of oxygen per day. The regulations prescribe that the PE loading of a WWTP's influent is calculated on the basis of the maximum average weekly load entering the plant during the year (2003 in this table), excluding unusual situations such as heavy rain.

WWTP Site	Sanitary Authority	PE	Influent Composition (Municipal:Industrial)	Receiving Waters	n
Ringsend	Dublin Corporation	2,186,808	66:33	Liffey Estuary	3
Kilkenny	Kilkenny Co. Council	110,000	33:66	River Nore	4
Osberstown	South Kildare Co. Council	66,100	65:35	River Liffey	5
Leixlip	North Kildare Co. Council	64,539	60:40	River Liffey	2
Clonmel	South Tipperary Co. Council	40,000	50:50	River Suir	3
Carlow	Carlow Co. Council	36,000	33:66	River Barrow	2
Killarney	Kerry Co. Council	32,814	90:10	Lough Leane	3
Tralee	Kerry Co. Council	24,633	80:20	Tralee Bay	3
Ballincollig	South Cork Co. Council	15,000	100:0	River Lee	10
Fermoy	North Cork Co. Council	12,960	80:20	River Blackwater	3

5.2 Determination of the Estrogenicity of WWTP Effluents and Receiving Waters

5.2.1 Dose-Response Curves of Extracts of WWTP Effluents in the YES

The estrogenic potencies of the treated final effluent and receiving water samples were determined by YES. Each site was sampled on a minimum of two separate occasions. Sample extracts were assayed in the YES at a minimum of six dilutions (1-40 µL) in an effort to obtain full dose-response curves for each extract. As described by Kirk *et al.* (2002), the estradiol equivalent concentration ($E2_{eq}$) for each volume of extract may then be calculated by extrapolation from the intra-assay 17β-estradiol standard curve.

The mean of these individual potencies may then be taken as the estrogenic potency of the sample. This allows most of the data from each extract to be used to provide the $E2_{eq}$ value for each sample (Kirk *et al*., 2002).

In the present study, however, the extract dose-response curves were not parallel to those of the bioassay positive control, 17β-estradiol (Fig. 5.1). The clarity of the dose-response curves was increased by assaying each extract at 12 dilutions over the same volume range (1–40 µl) (Fig 5.2). Because the dose-response curves were so shallow, it would have been necessary to assay a larger range of extract volumes in order to achieve full dose-response curves. However, the total volume of extract tested was not increased beyond 40 µl for reasons discussed below. In other cases, the estrogenicity of the WWTP effluent was sufficiently high to elicit a maximal assay response at all volumes tested (Fig. 5.3). In these cases the extracts were diluted and re-analysed using the YES (Fig 5.4).

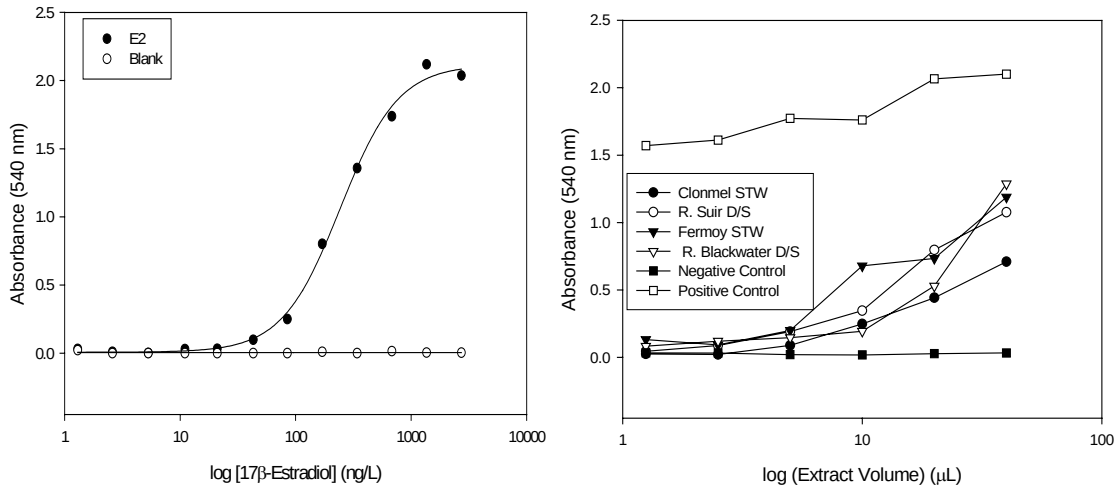


Figure 5.1 Estrogenic Activity of Fermoy and Clonmel WWTP Effluents: Six point Dose-Response Curve

On 06.10.03, 5 L grab samples were taken at Fermoy and Clonmel WWTP. Grab samples (5 L) were also taken from the river Blackwater and river Suir, immediately downstream (D/S) of the WWTP outfalls. Immediately after collection, methanol (50 ml) was added to the sample to slow bacterial growth. The samples were filtered immediately upon return to the laboratory (within 6 h) and stored at 4°C, prior to SPE on 5g C18 cartridges (5L/5g cartridge) within a 24 h period, and subsequent elution with methanol (15mL x 2). The estrogenic activity of the extracts was determined, using the YES, over a range of six dilutions (1.25-40 µL/well) within four days of sample collection. Negative (5L water) and positive (5L water spiked with 150ng/L E2) controls were processed together with the study samples. Each data point represents the mean of duplicate determinations. Panel (a) shows the standard curve of the intra-assay positive control 17β-estradiol; panel (b) shows the dose-response data of the samples collected.

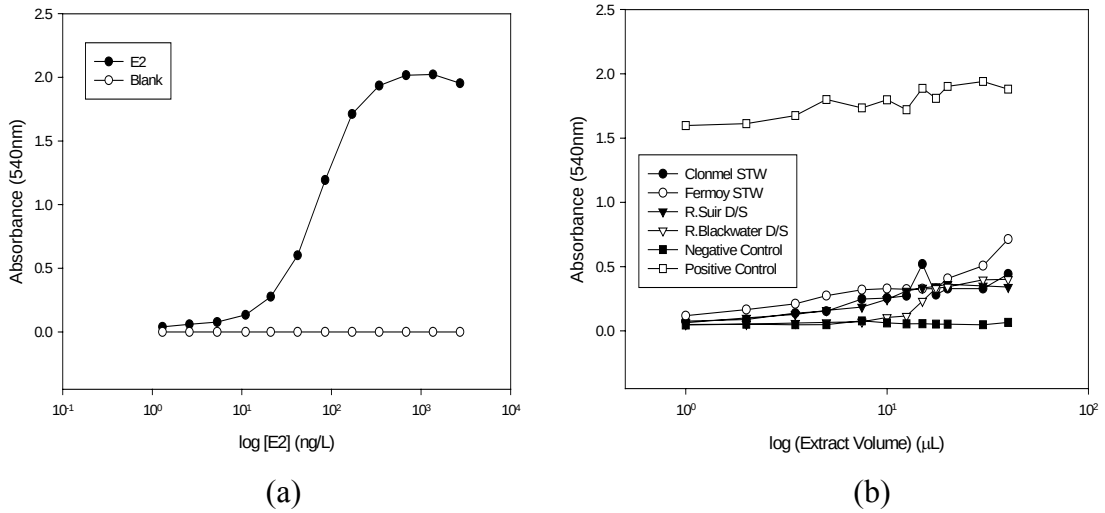


Figure 5.2 Estrogenic Activity of Fermoy and Clonmel WWTP Effluents: 12 point Dose-Response Curve

On 16.02.04, 5 L grab samples were taken at Fermoy and Clonmel WWTP. Grab samples (5 L) were also taken from the river Blackwater and river Suir, immediately downstream (D/S) of the WWTP outfalls. The estrogenic activity of the extracts was determined, using the YES, over a range of twelve dilutions (1-40 µL/well) within four days of sample collection. Negative (5L water) and positive (5L water spiked with 150ng/L E2) controls were processed together with the study samples. Each data point represents the mean of duplicate determinations. Panel (a) shows the standard curve of the intra-assay positive control 17β-estradiol; panel (b) shows the dose-response data of the samples collected.

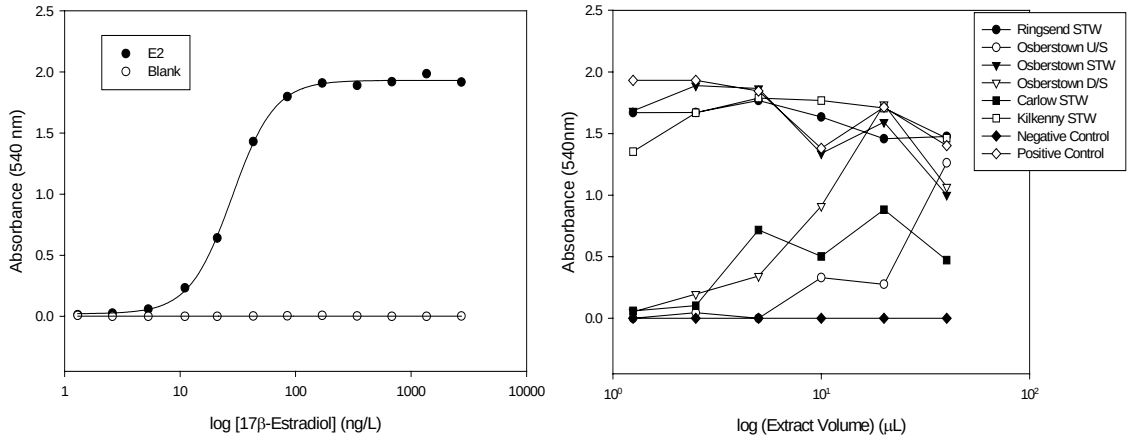


Figure 5.3
Estrogenic Activity of Extracts of WWTP Effluents from Ringsend, Osberstown, Carlow and Kilkenny.

On 13.10.03, 5 L grab samples were taken at Ringsend, Osberstown, Carlow and Kilkenny WWTP. Grab samples (5 L) were also taken from the river Liffey, upstream (U/S) and downstream (D/S) of Osberstown WWTP. The estrogenic activity of the extracts was determined, using the YES, over a range of six dilutions (1.25-40 µL/well) within four days of sample collection. Negative (5L water) and positive (5L water spiked with 150ng/L E2) controls were processed together with the study samples. Each data point represents the mean of duplicate determinations. Panel (a) shows the standard curve of the intra-assay positive control 17β-estradiol; panel (b) shows the dose-response data of the samples collected.

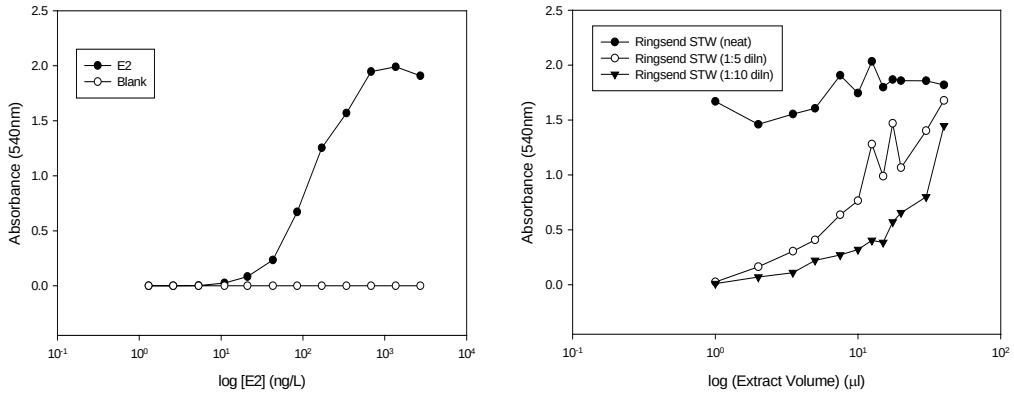


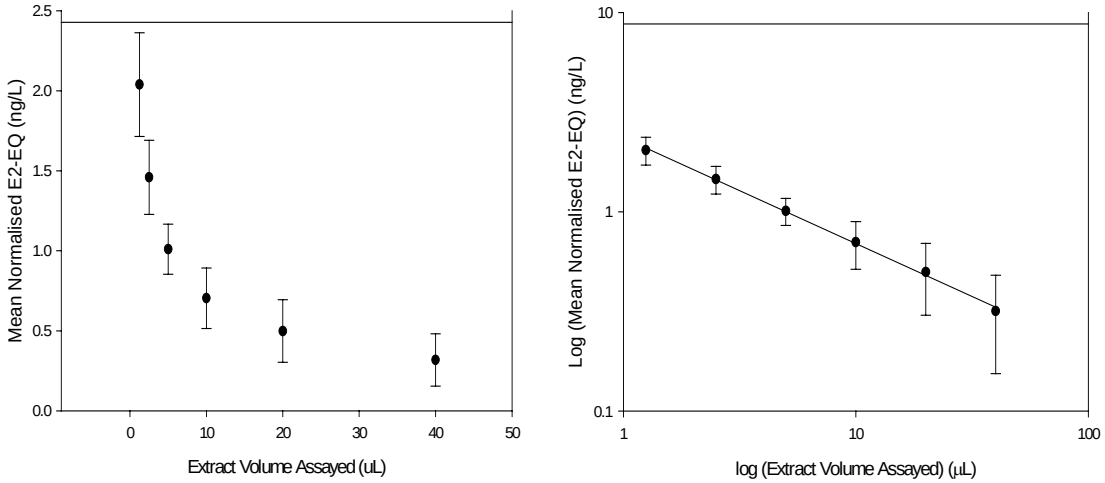
Figure 5.4
Effect of 5-Fold and 10-Fold Dilution on Estrogenic Activity of Extracts of WWTP Effluents from Ringsend WWTP.

On 13.10.03, a 5 L grab sample of final effluent was taken from Ringsend WWTP. The estrogenicity of extracts of WWTP effluent from Ringsend WWTP was sufficiently high to elicit a maximal assay response at all volumes tested in the YES (see Fig. 5.3). The extracts were therefore diluted 1:10 and 1: 5 and re-analysed using the YES.

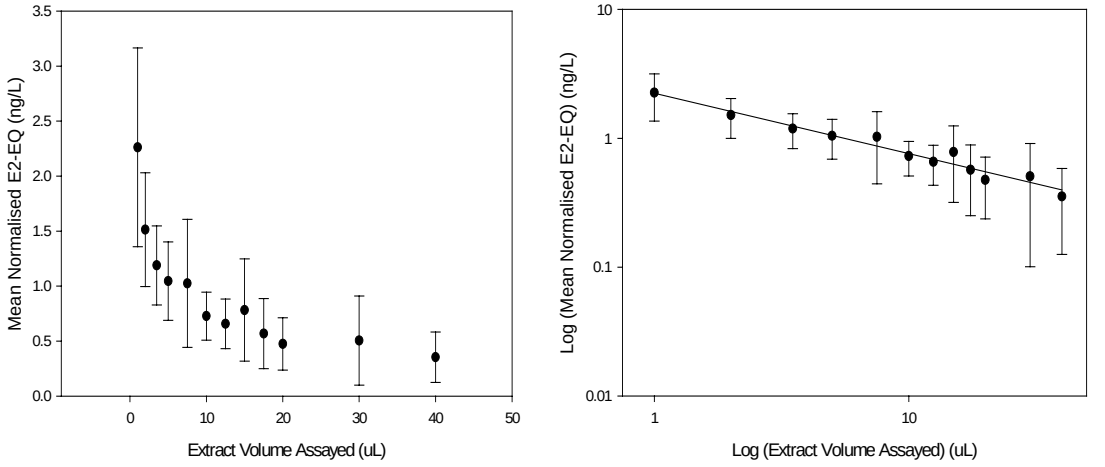
5.2.2 Non-Parallel Dilution of Sample Extracts in the YES

Unexpectedly, calculation of the $E2_{eq}$ values across the range of dilutions assayed for each sample extract (6-12 per extract) revealed that the $E2_{eq}$ values were highly dependent upon the extract volume assayed. For example, 5 L of treated effluent was taken at Leixlip WWTP on 26.10.2004 and the $E2_{eq}$ values calculated for this sample extract were 4.5, 3.2, 1.7, 1.2, 0.5 and 0.3 ng/L, as determined from 1.25, 2.5, 5, 10, 20 and 40 µL volumes of the extract, respectively**.**

This effect was not specific to a single sample but rather, was present to an equal degree in all samples taken during the study. This was demonstrated by normalising the $E2_{eq}$ values calculated for all different sample extracts (n=28 for the extracts assayed in 6 volumes; n=23 for the extracts assayed in 12 volumes) and subsequently determining the mean of these normalised $E2_{eq}$ values for each extract volume across all data sets. When plotted as a function of extract volume assayed in the YES, a relationship between these two parameters was very evident, of the type $E2_{eq}$ = (Extract Volume Assayed)^{m}, where m is a constant defining the slope of the curve (Fig. 4.5).



(a)



(b)

Figure 5.5
Demonstration of a Relationship between Extract Volume Assayed and $E2_{eq}$ in the YES

The $E2_{eq}$ values calculated for all sample extracts (n=28 for the samples assayed in 6 volumes; n=23 for the samples assayed in 12 volumes) were normalised as follows: each individual $E2_{eq}$ value ((a) 6 or (b) 12 values per sample) was divided by the mean $E2_{eq}$ value for that sample. This yielded a set of normalised $E2_{eq}$ values for each of the extract volumes assayed (1-40 µL) that could be directly compared with the other samples. Across the (a) 28 and (b) 23 data sets, the mean of the normalised $E2_{eq}$ values was determined for each extract volume. When plotted as a function of extract volume assayed, the relationship between these two parameters was of the type $E2_{eq}$ = (Extract Volume Assayed)^m, where m defines the slope of the curve. The r^2 values achieved were 0.998 for the extracts assayed over (a) 6 volumes and (b) 0.957 for the extracts assayed over 12 volumes.

Only one reference to a relationship between extrapolated $E2_{eq}$ values and the volume of extract assayed in the YES could be found in the literature (Witters *et al.*, 2001), although other laboratories using the YES have noted a similar effect (M. Jürgens, Pers. Comm., 2004).

The complex nature of WWTP effluent means that a huge variety of chemicals are present, some of which may inhibit the response of the yeast in the YES. However, this phenomenon of non-parallel dilution of extracts was seen in all samples; WWTP

effluents, receiving waters, negative control (pristine) sites and in the YES positive (E2 spiked) control water samples.

Thus, it was considered possible that the effect observed was due to an inhibitory substance entering the extracts during the laboratory preparation of the samples, for example, from the SPE cartridges used to extract the samples or from methanol used in the elution step. This was briefly investigated by determining the effect of aliquots of (a) methanol and (b) a methanol extract of a blank cartridge on the E2 standard curve (Fig. 5.6). No inhibitory effect was observed; on the contrary the extract from the blank cartridge appeared to potentiate the effect of the E2, while the methanol had no marked effect on the overall shape of the E2 dose-response curve (Figure 5.6).

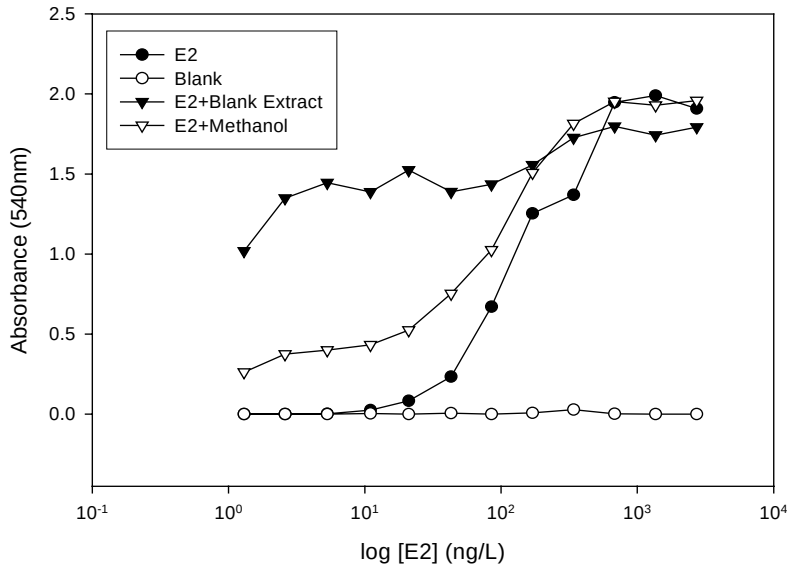


Figure 5.6
Investigation of the effect of the blank extract and methanol on the response of the YES to E2
Microtitre plate wells containing seeded assay medium (200 µl) and a range of dilutions (1.3-2724 ng/L) of E2 (10 µl), were incubated (32°C, 72 h) (a) alone, (b) in the presence of a methanol extract of a blank cartridge (10 µl) and (c) in the presence of methanol (10 µl). Blank (solvent control) measurements were performed by exposing seeded medium to aliquots (10 µL) of ethanol. The magnitude of the estrogenic response was determined by measuring the absorbance at 540 nm. Each data point represents the mean of quadruplicate determinations.

In a separate experiment, a sample extract from Ringsend WWTP was diluted (5- and 10-fold) and estrogenic potency determined using the YES (Fig. 5.4). Non-parallel dilution of the diluted sample extracts was observed in this case also, although it was not possible to determine if the phenomenon was present to the same degree as in the undiluted extracts (Fig.5.7).

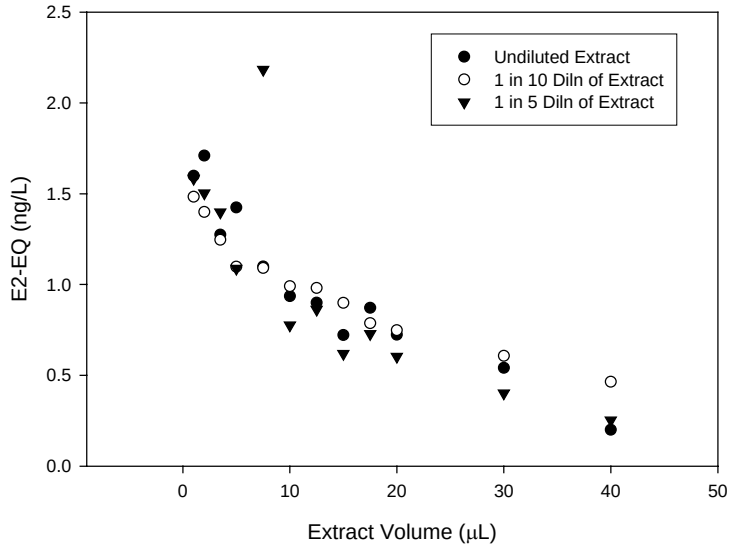


Fig. 5.7
Dilution of WWTP Sample Extracts does not Eliminate the Relationship between Extract Volume and Extrapolated Estrogenicity
On 13.10.03, a 5 L grab sample of final effluent was taken from Ringsend WWTP. The sample was taken and processed as described in section 2.1-2.4. The estrogenic activity of the extract was determined, using the YES, over a range of six dilutions (1.25-40 ml/well) within 4 days of sample collection. The estrogenicity of the extract was sufficiently high to elicit a maximal assay response at all volumes tested. The extract was diluted 1:10 and 1:5 and re-analysed using the YES. The normalised $E2_{eq}$ values were plotted as a function of extract volume assayed, a relationship between these two parameters remained evident for the undiluted, 5-fold and 10-fold diluted extract.

This effect was not investigated further. As the extrapolated $E2_{eq}$ value of the positive control sample was at or close to 100% recovery when the absorbance data obtained from the highest extract dilution (1 μl) was used (decreasing to ≤ 10% when determined from the lowest dilution (40 μl)), all $E2_{eq}$ values presented in this study are derived from the absorbance data obtained using the lowest extract volume giving a positive response above background (usually 1 μl) in the YES.

5.2.3 Estrogenicity of Treated Final Effluents from 10 Irish WWTP and their Receiving Waters

The estrogenic potency of treated final effluents from 10 WWTP, as determined by the YES, is presented in Table 5.6. For six of the ten locations, the estrogenicity of the receiving waters was also determined. The estrogenic activity of each sample was determined by extrapolation from a 17β-estradiol standard curve (as described earlier in this chapter) and, thus, the results are expressed as estradiol equivalents ($E2_{eq}$, ng/L).

The estrogenicity of the WWTP effluents were in the low ng/L range, specifically from 1.1 to 17.2 ng/L, while the receiving waters ranged from 0.9 to 2.9 ng/L. Ringsend and Osberstown WWTP showed comparatively elevated estrogen levels (16 and 17.2 ng/L, respectively) relative to the other plants surveyed (1.1-6.8 ng/L). Background levels of estrogens in the aquatic environment were determined at Gougane Barra (headwaters of the river Lee) and Lough Barfinnihy, an oligotrophic lake in the Kerry mountains remote from anthropogenic inputs.

Table 5.2 Estrogenicity ($E2_{eq}$) of WWTP Final Effluents and Receiving Waters.
Over the period October 2003 to June 2004, $E2_{eq}$ values for 10 WWTP in the South and East of Ireland were determined using the YES. 5L grab samples were taken from each site on a minimum of two separate days (n = number of times the site was sampled). Background levels of estrogens were determined at Lough Barfinnihy and Gougane Barra. $E2_{eq}$ values represent the mean ± SEM ($n \geq 3$) or mean ± range (n = 2) of the samples taken from that site. Estrogenicity was not determined (n.d.) in the case of all receiving waters. Standardised effluent values represent the measured $E2_{eq}$ divided by the appropriate river dilution factor. Effluent dilution factors were calculated using the dry weather flow data for the WWTP and 95 percentile river flow values. One Population Equivalent (PE) is defined as the load resulting from 60g BOD_5 (5 day Biological Oxygen Demand). The influent PE loading was calculated on the basis of the maximum average weekly load entering the plant during 2003.

WWTP Site	PE	Influent Composition (Municipal:Industrial)	Receiving Waters	Effluent Dilution Factor	Effluent $E2_{eq}$ (ng/L)	Receiving Waters $E2_{eq}$ (ng/L)	Effluent $E2_{eq}$ (ng/L): standardised w.r.t. river dilution	n
Ringsend	2,186,808	66:33	Dublin Bay	Infinite	16.0 ± 5.6	n.d.	~ 0	3
Kilkenny	110,000	33:66	Nore	37	6.8 ± 0.2	n.d.	0.18	4
Osberstown	66,100	65:35	Liffey	27	17.2 ± 3.8	0.9 ± 0.4 (upstream) 1.3 ± 0.8 (downstream)	- 0.63 (D/S)	5
Leixlip	64,539	60:40	Liffey	32	2.8 ± 1.4	0.9 ± 0.1	0.09	2
Clonmel	40,000	50:50	Suir	119	2.9 ± 1.0	1.7 ± 0.8	0.02	2
Carlow	36,000	33:66	Barrow	62	1.1 ± 0.2	n.d.	0.02	3
Killarney	32,814	90:10	Lough Leane	~ Infinite	3.7 ± 1.8	1.8 ± 1.5	~ 0	3
Tralee	24,633	80:20	Tralee Bay	Infinite	5.7 ± 0.4	n.d.	~ 0	3
Ballincollig	15,000	100:0	Lee	77	3.2 ± 1.1	1.5 ± 0.6	0.04	10
Fermoy	12,960	80:20	Blackwater	255	5.0 ± 1.1	2.9 ± 2.4	0.02	3
Lough Barfinnihy	-	-	-	-	-	1.1 ± 1.1	-	2
Gougane Barra	-	-	-	-	-	0.9 ± 0.9	-	2

Estrogenic activity at these control sites ranged from undetectable to 1.1 ng/L, on different sampling occasions (Table 5.2). These results indicate that a low level of estrogenic activity may often be present in surface waters, even in isolated regions where anthropogenic activity is minimal. These estrogens may have originated from plant sources (phytoestrogens) or, in the case of Gougane Barra, may be partly due to effluents from the septic tanks of isolated dwellings, or from animal husbandry activities. The wild fish survey and caged fish study (Chapters 3 and 4) have demonstrated that these waters are not estrogenic to fish.

When placed in an international context, the estrogen levels measured in Irish WWTP and receiving waters compare favourably with those derived from similar studies conducted elsewhere in Europe (Johnson and Williams, 2004; Carballa *et al*., 2004). Such studies have suggested that the total estrogenic activity of effluents, from WWTP receiving mostly domestic input, is in the low to medium ng/L range.

The higher level of estrogen activity measured at Osberstown and Ringsend WWTP is worthy of further consideration. Ringsend WWTP is the largest wastewater treatment facility in the country and the measured $E2_{eq}$ of its effluent (16.0ng/L) was also comparatively elevated. The plant has recently been upgraded from primary to secondary treatment. From discussions with the plant manager, there appears to have been a number of operational problems that coincided with the effluent sampling period (October 2003 – April 2004). It is likely that the problems may have hampered the efficient commissioning of the upgraded system and impaired the treatment efficiency of the plant leading to elevated levels of steroids in the plant effluent (see 5.3.4).

The highest estrogenic load was found in effluent from Osberstown WWTP, discharging to the river Liffey, where the measured $E2_{eq}$ was 17.2 ng/L. This value is approximately six times greater than that measured at Leixlip WWTP (2.8ng/L), a WWTP with a comparable influent in terms of PE and the ratio of municipal to industrial waste received. One possible explanation for the unexpected estrogenic potency of the Osberstown WWTP effluent may be that the plant receives industrial waste from a nearby pharmaceutical company that manufactures the contraceptive pill. This company is allowed to release a significant amount of chemical waste to Osberstown WWTP, a component of which is likely to have contained EE2. Therefore, it is conceivable that a small fraction of the 42% of the industrial PE treated by the WWTP may be more estrogenic than is normal for industrial waste, and this may account for the elevated estrogenicity of the Osberstown effluent relative to all of the other WWTP surveyed (section 5.3.4-5 for further discussion). It should be noted that from January 1 2006, the company will be subject to further substantial reductions in chemical waste discharge levels, including EE2.

Based on influent PE values, Ballincollig is the largest WWTP, discharging to freshwaters, in county Cork. The PE loading of the plant's influent is 15,000. The adjusted PE, representing the average dilution of the effluent in the river, is 194 for Ballincollig WWTP, while Jobling and co-workers (1998) have previously reported a significant level of intersex in the wild fish population of the river Rea (UK) downstream of a WWTP with an adjusted PE of 119. The Ballincollig WWTP influent is almost entirely domestic in origin, which makes it comparable to similar UK studies investigating effects of natural and synthetic estrogens on fish populations (Harries *et*

al., 1999). Women between the ages of 15 and 44 years represent 25% of the Ballincollig population (2002 census, Central Statistics Office). Fermoy is the next largest WWTP discharging to freshwaters (river Blackwater) in Cork, with an influent PE loading of 12,600 and an adjusted PE of 41. It should be noted that the waste from Cork City (PE 328,000), at the time of this study, did not receive any treatment and discharged directly into the Lee estuarine waters in Cork Harbour.

The measured $E2_{eq}$ from Ballincollig WWTP effluent (3.2 ng/L) was at a concentration that previous studies have indicated should be estrogenic to fish (Routledge *et al*., 1998). However, the effluent is significantly (77-fold) diluted upon discharging to the river Lee, which reduces the concentration of estrogens contributed by the effluent to a level below that required to induce vitellogenesis in fish. Interestingly, the measured estrogenicity of the river water downstream of the outfall pipe (1.5 ng/L) is considerably higher than that predicted from diluted effluent values alone (0.04 ng/L) (see section 5.3.4-5) and may be considered within the extreme lower end of the concentration range of E2 required to induce vitellogenesis in male Rainbow trout (Routledge *et al*., 1998). However, the *in vivo* studies performed as part of this work (Chapters 3 and 4) have demonstrated that the river Lee is not estrogenic to fish (see section 5.3.4-5 for further discussion).

5.3 Modelling of effluent and receiving water concentrations of estrone, estradiol and ethinylestradiol at selected WWTP

A number of studies have shown that the key chemicals responsible for the endocrine disruption of aquatic ecosystems, particularly wild fish, are the natural and synthetic human steroid hormones E1, E2 and EE2. This has presented those responsible for the management of river catchments with a particular problem in assessing the state of rivers with respect to endocrine disruption, as the costs and complexity of the chemical analysis of sewage effluent and receiving waters for steroids, together with physiological and histological examinations of wild fish, are significant. In light of this problem, Andrew Johnson and Richard Williams of the Centre of Ecology and Hydrology, Wallingford, UK, have developed a chemically-based deterministic model to predict steroid concentrations in WWTP influent, effluent and receiving waters (Johnson and Williams, 2004). The model has since been used by the UK Environment Agency to identify WWTP that require measures to increase steroid treatment efficiency to comply with the Agency's Environmental Quality Standards described below.

The fundamental aspects of the model, in terms of human steroid metabolism, excretion, in-sewer and WWTP process, are somewhat complex and beyond the scope of this text and for further information the reader is recommended to consult the publication cited above. Essentially the model estimates the likely concentration of E1, E2 and EE2 in WWTP effluents and receiving waters, based on the human population served by the works, the treatment efficiency of the sewage works and the dilution available in the river, typically, under low flow conditions. The concentrations are then expressed as an EE2 and E2 equivalent ($EE2_{eq}$ and $E2_{eq}$) based on the relative estrogenic potencies of the compounds in relation to their estrogenic effect on fish and on the YES bioassay using the following formulae:

$$EE2_{eq} = EE2 + E2/10 + E1/30 \quad (1)$$

$$E2_{eq} = EE2 + E2 + E1/3 \quad (2)$$

The $E2_{eq}$ value was derived for comparison with the experimental YES assay data for WWTP effluent and receiving waters; and the $EE2_{eq}$ value was derived to assess the risk of sexual disruption to wild fish, and for comparison with wild fish and caged fish studies. The $EE2_{eq}$ value is also compared with a range of target values for river estrogen concentrations obtained through methods that are similar to those used for setting Environmental Quality Standards (EQS) by the UK Environment Agency.

A wild fish capture site is defined as low risk if the $EE2_{eq}$ concentration is less than the predicted no-effect concentration (PNEC), medium risk if it is greater than the PNEC but lower than the lowest observed effect concentration (LOEC) and high risk if it is greater than the LOEC:

Risk	$EE2_{eq}$ (ng/l)
High	Concentration > 0.57 ng/l
Medium	0.1 ng/l < Concentration < 0.57 ng/l
Low	Concentration < 0.1 ng/l

5.3.1 Sources of data used for modelling

WWTP PE and dry weather flow (DWF) data for 2003, and 95 percentile (Q95) river flow data were obtained from the Irish EPA. Information on the human and industrial composition of the WWTP PE, plant design, plant operating efficiency and updates on DWF were obtained through liaison with plant managers.

5.3.2 WWTP modelled

Table 5.3 shows details of the WWTP and receiving waters modelled, sampled and analysed using the YES assay, as described in Chapter 2. All plants were based on secondary treatment only, with the older plants utilising a biological or a trickling filter design and, in the case of Ballincollig, extended using a conventional aeration (activated sludge) system. Newer plants incorporated extended aeration designs—essentially conventional aeration with longer hydraulic retention.

Table 5.3 Details of the WWTP and receiving waters modelled and sampled.

WWTP	Plant type	Total PE	Human PE	% Human PE	Adjusted Total PE	DWF m^3/d	DWF/PE Total (l)	DWF/PE Human (l)	Receiving water	Q95 River flow m^3/day	Effluent dilution in river
Osberstown	EA	66100	38558	58	2470	16154	244	419	Liffey	416189	26.8
Ballincollig	CA&BF	15000	15000	100	194	3700	247	247	Lee	281664	77.1
Leixlip	CA	64539	38723	60	2005	13848	215	358	Liffey	432000	32.2
Killkenny	EA	100000	20300	20.3	2716	9045	90	446	Nore	324000	36.8
Tralee	CA	34400	27520	80	na	8661	252	315	Tralee Bay	na	Infinite
Carlow	EA	36000	11904	33	581	8500	236	714	Barrow	518400	62.0
Clonmel	CA	40000	20000	50	336	7500	188	375	Suir	885600	119.1
Killarney	EA	32814	29533	90	na	6540	199	221	Lough Leane	na	~Infinite
Ringsend	EA	2186808	1443293	66	na	365153	167	253	Liffey Estuary	na	Infinite
Fermoy	EA	12960	10368	82	51	3279	253	316	Blackwater	832032	254.7

WWTP – Waste Water Treatment Plant, PE – Population Equivalent, DWF – WWTP dry weather flow, Adjusted PE - PE taking into account river dilution, Q95River flow – flow rate exceeded for 95% of the time; na – not available

Table 5.3 also gives values for the percentage human composition of the PE, typically purely residential conurbations were rated as 100% human PE, whereas in some cases industrialised centres had human PE values as low as 20%, as was the case with Kilkenny due the discharges from the Diageo brewery. The dry weather flow (DWF) value describes the WWTP effluent flow rate that is due solely to human or industrial inputs, in the absence of rainfall.

If the PE is composed solely of human input then the PE/DWF value equates to the daily waste-water output of one individual. Across Western Europe the values ranges from 200 to 300 litres/day primarily depending on the factors such as toilet design, number of baths or showers taken and use of water in the kitchen for washing machines and dish washers. In Table 5.3 major deviations from the range are due to significant industrial composition of the WWTP PE that tends to skew the value. The adjusted PE is also given, which standardises the perceived strength of the WWTP effluent with respect to dilution once it has entered the receiving water; obviously these data were not available where the WWTP discharges into open waters or the sea.

5.3.3 Results for sewage effluent modelling

Table 5.4 shows the predicted EE2, E2 and E1 concentrations for selected WWTP, as well as predicted $EE2_{eq}$ and $E2_{eq}$ concentrations and the corresponding $E2_{eq}$ obtained from the YES bioassay analysis of the WWTP effluent.

It is noticeable that the higher values correspond to plants treating primarily domestic waste water e.g. Ballincollig $EE2_{eq}$: 1.43 ng/l and $E2_{eq}$: 9.51 ng/l (100% domestic PE) and Killarney $EE2_{eq}$: 1.595 ng/l and $E2_{eq}$: 10.60 ng/l (90% domestic PE); whereas the plants treating waste water with a high component of industrial effluent tend to be associated with lower values e.g. Carlow $EE2_{eq}$: 0.49 ng/l and $E2_{eq}$: 3.29 ng/l (33% domestic PE) and Kilkenny $EE2_{eq}$: 0.86 ng/l and $E2_{eq}$: 5.71 ng/l (20.3 % domestic PE).

Figure 5.8 shows the predicted $E2_{eq}$ concentrations, and corresponding $E2_{eq}$ concentrations obtained using the YES bioassay. Comparing the results for each WWTP, overall the model generally predicts the measured data with the margins of error, at worst within an approximate of 3 for Osberstown and at best within a factor of 1.2 for Kilkenny.

This overall result is excellent, and particularly interesting considering the numerous parameters and assumptions made within the model, which could produce major errors. For example, the cultural and religious differences between Ireland and the UK may affect the number of females using the contraceptive pill, which the model assumes to be 17% of the total female population.

Other factors that influence the accuracy of the model's predictions include variations in WWTP design and efficiency and the accuracy of the input data, particularly the exact human component of the plant PE value and dry weather flow value. The input data used for this modelling exercise were average values for 2003, which may not have been current during the period (October 2003 to May 2004) when the WWTP effluent was sampled. To improve the accuracy of the prediction it would be desirable to use input data relevant to the precise period of the effluent sampling.

Given the likely unaccounted for error associated with the predicted $E2_{eq}$ values, an exhaustive plant-by-plant examination that may account for the discrepancies between the measured and modelled data presented in Table 5.4 may be inappropriate. However, knowledge gained during liaison with WWTP managers during the course of the project may explain some of the significant discrepancies between modelled and measured data.

The measured $E2_{eq}$ for Osberstown (17.2 ng/l) was three times greater than that modelled (5.12 ng/l). As previously mentioned (section 5.2.3), a possible explanation for the unexpected estrogenic potency of the effluent may be the fact that the plant receives industrial waste from a nearby company that manufactures the contraceptive pill. Under the conditions of the Integrated Pollution Control (IPC) licence awarded by the EPA, the company is allowed to release an amount of chemical waste to Osberstown WWTP. Therefore it is conceivable that a small fraction of the 42% of the industrial PE treated by the plant may be more estrogenic than is normal for industrial waste and, thus, elevates the overall estrogenic load of the plant effluent. This is significant since the model assumes the estrogenic signal delivered to a given plant to be of human origin only.

The measured $E2_{eq}$ for Ringsend WWTP (16.0 ng/l) was approximately two times greater than that modelled (9.28 ng/l). The plant has recently been upgraded from primary to secondary treatment. From discussions with the plant manager there appears to have been a number of operational problems that coincided with the effluent sampling period. It is likely that the problems may have hampered the efficient commissioning of the upgraded system and impaired the treatment efficiency of the plant leading to unexpectedly elevated levels of steroids in the plant effluent.

Conversely, the measured $E2_{eq}$ concentrations for Killarney and Ballincollig WWTP (3.7 and 3.2 ng/l) were approximately three times lower than predicted by the model (10.6 and 9.51ng/l). The discrepancy for Killarney WWTP may be explained by the fact that it is a very popular tourist centre with a human population that varies significantly over a relatively short time scale. The PE value used for modelling was an average figure supplied by the plant manager that may not accurately reflect the PE during the period of the sampling, which occurred outside of the peak tourist season. The discrepancy for the Ballincollig WWTP is particularly interesting since the measured $E2_{eq}$ value is a mean of ten samples and, thus, is likely to be the most accurate effluent value. Therefore the discrepancy is likely to be caused by the use of incorrect in-put data into the model and needs further investigation. Preliminary studies (data not shown) have indicated that a significant fraction of the steroid estrogens in the Ballincollig effluent are present in a conjugated (inactive) form. This may account, to some extent, for the above-described discrepancy between predicted and YES-measured $E2_{eq}$ values.

Finally, the measured $E2_{eq}$ data compares favourably with data sets derived from similar studies on the estrogenicity of sewage effluent conducted elsewhere in Europe (Johnson *et al*, 2005; Carballa *et al*, 2004). As well as providing additional internal method validation, this outcome adds further credibility to the quality of the YES data produced during this study.

Table 5.4 Predicted steroid concentrations, $E2_{eq}$ and $EE2_{eq}$ values of selected WWTP effluent

WWTP	Predicted steroid oestrogen concentrations (ng/l) [mean and range: 2 x SD]			$E2_{eq}$ (ng/l) [mean and range: 2 x SD]		Predicted $EE2_{eq}$ (ng/l) [mean and range: 2 x SD]
	EE2	E2	E1	Predicted	Measured [ ]= n	
Osberstown	0.31 (0.19-0.45)	1.43 (0.48-2.86)	11.70 (6.92-17.19)	5.12 (3.07 – 9.08)	17.2 (13.4 – 21.0) [5]	0.84 (0.47 – 1.31)
Ballincollig	0.53 (0.32–0.77)	2.43(0.81-4.86)	19.98(11.76-29.19)	9.51 (5.01 – 15.27)	3.2 (2.1 – 4.3) [10]	1.43 (0.80 – 2.23)
Lexlip	0.36 (0.22-0.53)	1.68 (0.56-3.36)	13.70 (8.11-20.13)	6.56 (3.46-10.53)	2.8 (1.4 - 4.2) [2]	0.98 (0.55-1.54)
Killkenny	0.32 (0.19-0.46)	1.46 (0.49-2.92)	11.92 (7.05-17.51)	5.71 (3.01-9.16)	6.8 (6.6 – 7.0) [4]	0.86 (0.48-1.34)
Tralee	0.41 (0.25-0.60)	1.91 (0.64-3.81)	15.57 (9.21-22.88)	7.46 (3.93-11.97)	5.7 (5.3 – 6.10) [3]	1.12 (0.62-1.75)
Carlow	0.18 (0.11-0.27)	0.84 (0.28-1.68)	6.86 (4.06-10.08)	3.29 (1.73-5.27)	1.1 (0.9 – 1.3) [3]	0.49 (0.27-0.77)
Clonmel	0.35 (0.21-0.51)	1.6 (0.53-3.2)	13.07 (7.73-19.20)	6.26 (3.30-10.04)	2.9 (1.9-.3.9) [2]	0.94 (0.52-1.47)
Killarney	0.59 (0.36-0.86)	2.71 (0.90-5.42)	22.13 (13.10-32.51)	10.60 (5.59-17.01)	3.7 (1.90 – 5.5) [3]	1.595 (0.89-2.48)
Ringsend	0.51 (0.32-0.75)	2.37 (0.79-4.74)	19.37 (11.46-28.46)	9.28 (4.89-14.89)	16.0 (10.4 – 21.6) [3]	1.40 (0.77-2.17)
Fermoy	0.41 (0.25–0.60)	1.9 (0.63-3.79)	15.49 (9.17-22.77)	7.42 (3.91 – 11.91)	5.0 (3.9-6.1) [3]	1.12 (0.62- 1.73)

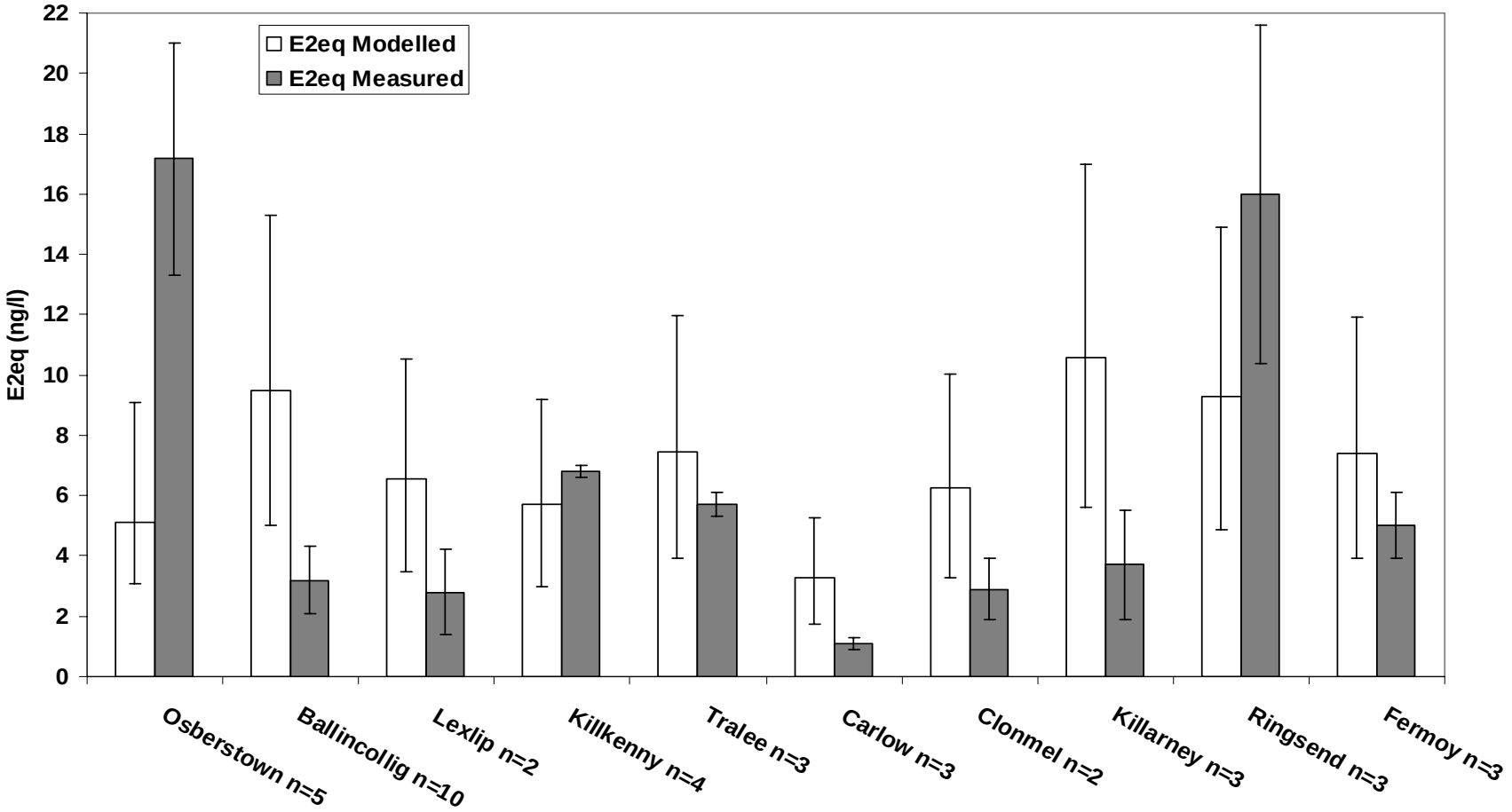


Figure 5.8 WWTP Effluent estrogenicity: Modelled $E2_{eq}$ values compared with measured $E2_{eq}$ values obtained using the YES

(E2eq = EE2 + E2 + E1/3; Error bars represent 2x standard deviation of the mean, X axis n=x refers to sample replicates for YES bioassay).

5.3.4 Results for receiving waters

Table 5.4 and Figure 5.9 show the predicted steroid concentrations for EE2, E2 and E1, and the $E2_{eq}$ and $EE2_{eq}$ concentrations in WWTP effluent receiving waters. For every WWTP, the measured $E2_{eq}$ is significantly greater than the modelled $E2_{eq}$. To explain this trend it may be suggested that, rather than run the model using 95 percentile river flow data, which represents low flow conditions, it may be more realistic to use higher values representing average flows. However, if this approach was to be employed then the modelled $E2_{eq}$ concentrations would be even lower than the data generated using the Q95 river flow data, due to greater effluent dilution.

Table 5.4 Predicted steroid concentrations (EE2, E2, E1), and $E2_{eq}$ and $EE2_{eq}$ concentrations for receiving waters

WWTP	Predicted steroid oestrogen concentrations (ng/l) [mean and range (2 x SD)]			$E2_{eq}$ (ng/l)		Predicted $EE2_{eq}$ (ng/l)
	EE2	E2	E1	Predicted	Measured [ ]=n	
Osberstown	0.012 (0.007-0.016)	0.053 (0.018-0.11)	0.430 (0.26-0.64)	0.21 (0.11– 0.34)	1.3 (0.5-2.1)[5]	0.031 (0.018 – 0. 05)
Ballincollig Upstream	na	na	na	na	0.61	na
Ballincollig	0.03(0.01-0.063)	0.259(0.15-0.37)	0.12(0.07-0.20)	0.012 (0.06 –0.20)	1.5 (0.9-2.1)[10]	0.02 (0.01 – 0.03)
Lexlip	0.01 (0.008-0.019)	0.062 (0.021-0.12)	0.24 (0.13-0.39)	0.24 (0.12-0.38)	0.9 (0.8-1.0)[2]	0.04 (0.02-0.05)
Killkenny	0.008 (0.005-0.013)	0.040 (0.013-0.08)	0.16 (0.08-0.25)	0.16 (0.082-0.25)	n.d	0.023 (0.013-0.036)
Carlow	0.003 (0.002-0.004)	0.014 (0.005-0.027)	0.11 (0.07-0.163)	0.053 (0.028-0.085)	n.d	0.008 (0.0044-0.0124)
Clonmel	0.003 (0.002-0.004)	0.013 (0.004-0.026)	0.11 (0.07-0.161)	0.053 (0.028-0.084)	1.7 (0.9-2.5)[2]	0.008 (0.004-0.010)
Fermoy	0.0016(0.001-O.002)	0.0076(0.003-0.015)	0.0625 (0.04-0.09)	0.045 (0.021-0.065)	2.9 (0.5-5.3)[3]	0.004 (0.002-0.007)

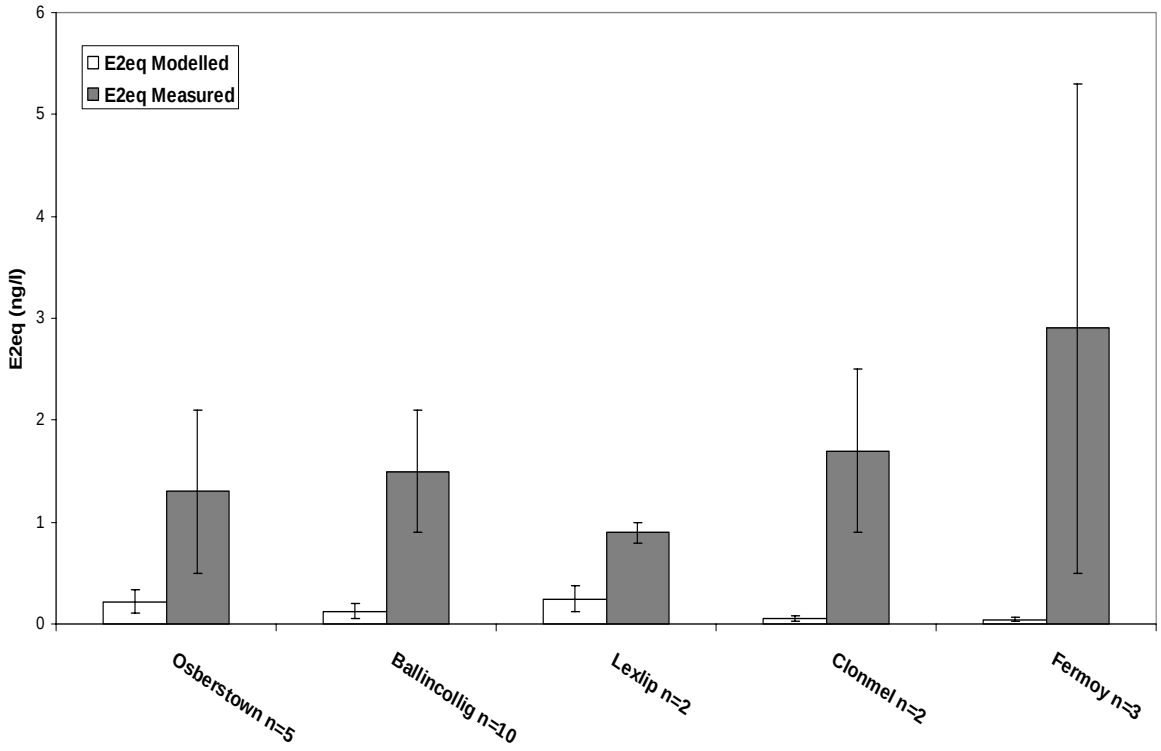


Figure 5.9
Receiving water estrogenicity: Modelled receiving water $E2_{eq}$ compared with YES measured $E2_{eq}$
(E2eq = EE2 + E2 + E1/3; Error bars represent 2x standard deviation of the mean, X axis n=x refers to sample replicates for YES bioassay)

A very similar trend is observed if the YES-measured receiving water $E2_{eq}$ data are compared with the corresponding YES-measured effluent $E2_{eq}$ data, after standardising for the effect of river dilution (Table 5.6 and Figure 5.10). This approach circumvents issues relating to the correct input data for the model, since empirical data are compared that were sampled during the same period. The only factor that was not empirically measured during the sampling period is river flow. However, as previously discussed, the Q95 data used favours the convergence of the discrepancy between modelled and YES-measured river $E2_{eq}$ concentrations.

Hence, it may be tentatively concluded that the steroid estrogens discharged into the receiving waters are not the only chemicals that are contributing to the receiving water $E2_{eq}$ values. If this is indeed the case, then what chemicals are contributing to the estrogenic signal and where do they come from? As reviewed in Chapter 1, there are a great number of chemicals possessing estrogenic properties, the majority of which are of industrial origin and are tens of thousands times less estrogenically potent than the steroid estrogens, e.g. alkylphenols. If such chemicals were causing the observed estrogenicity of the receiving waters one would expect the catchments of the Rivers Liffey, Lee, Suir and Blackwater to be significantly industrialised on a level with the River Aire in North East England — this is not the case (Blackburn and Waldcock, 1995; Blackburn *et al*, 1999).

On examination, the reverse situation appears to prevail. Interestingly, the two WWTP with the lowest discrepancies are those at Osberstown and Leixlip, both discharging

treated effluent into the river Liffey. When compared with the other river catchments the Liffey could be characterised as relatively non-agricultural when compared to the catchments of the Lee, Suir and Blackwater, which are relatively dominated with livestock and diary units.

Table 5.6
Measured receiving water $E2_{eq}$ and measured effluent $E2_{eq}$ standardised with respect to river dilution

WWTP	Receiving water	Effluent dilution in river	Measured $E2_{eq}$ in river (ng/l)	Effluent $E2_{eq}$ standardised w.r.t. river dilution (ng/l)
Osberstown	Liffey	26.8	1.3	0.643
Ballincollig	Lee	77.1	1.5	0.041
Leixlip	Liffey	32.2	0.1	0.087
Carlow	Barrow	62.0	1.1	0.018
Clonmel	Suir	119.1	1.7	0.024
Fermoy	Blackwater	254.7	2.9	0.020

Hence, it is postulated that the unexpectedly high $E2_{eq}$ concentrations found in the receiving waters investigated may be caused by steroid hormones excreted by livestock and dairy herds. In the case of dairy herds, this may be natural excretion as well as excretion of veterinary medicines supplemented with natural steroid hormones to promote milk production. The potential for livestock to contribute to endocrine disrupting effects in receiving waters is currently receiving much attention in the USA and Europe (Hanselman *et al*, 2003; Lange *et al*, 2002). Although livestock can be a significant source of steroid estrogens, it is generally considered that their normal effect on aquatic ecosystems is minor, due to their route to watercourses being attenuated by affinity with the organic fraction of soil materials, and subsequent degradation. However, this assumption may be invalidated if farmyard runoff bypasses the soil buffer through direct runoff into a stream feeding to a watercourse. Also, steroids may be less attenuated by the soil if the field is frequently at near saturation conditions, as a result of pronounced or prolonged rainfall promoting relatively rapid surface runoff into nearby watercourses. Dr Peter Matthiessen (Centre for Ecology and Hydrology Lancaster, UK) is currently leading UK-DEFRA research on the role of livestock agriculture on the estrogenic load to receiving waters and is investigating the scenarios described above. Whilst the project is at is early stages and yet to report, he is of the opinion, with respect to these data, that the elevated rainfall in Ireland compared to the UK may potentially account for the extra estrogenic load measured in the monitored rivers, being sourced from rapid runoff from livestock units (P. Matthiessen, Pers. Comm., 2005).

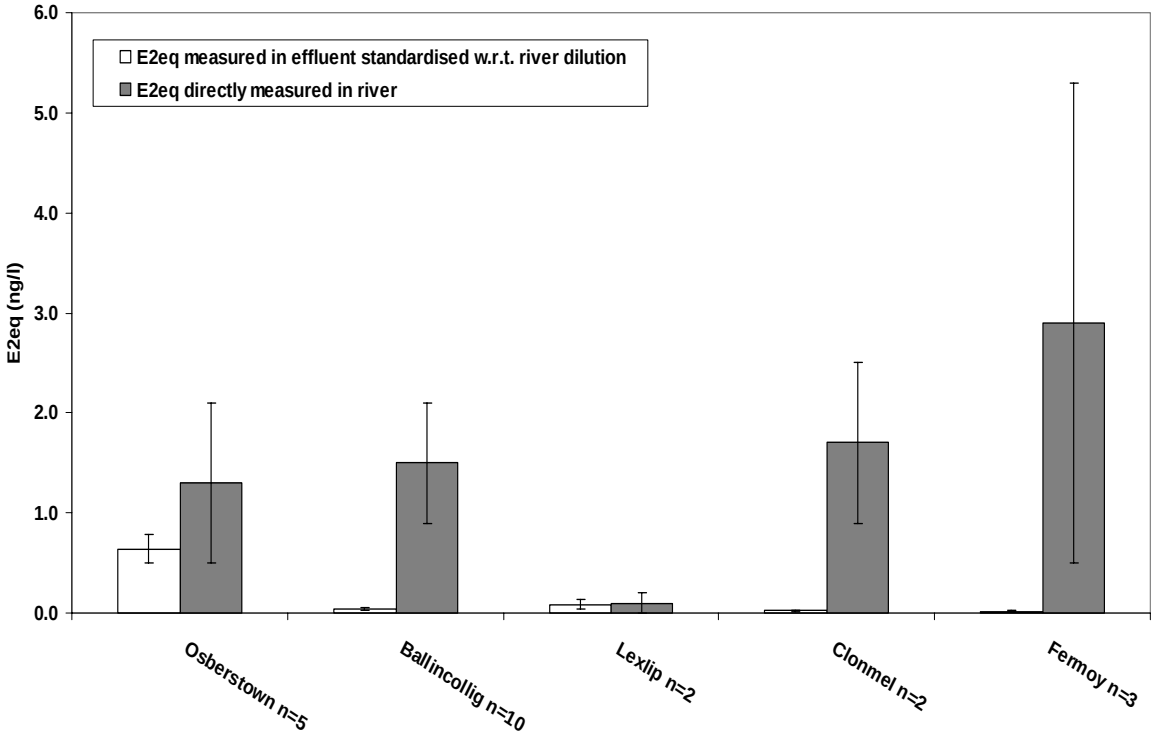


Figure 5.10
Receiving water: Measured river $E2_{eq}$ compared with measured effluent $E2_{eq}$ standardised with respect to river dilution.
(E2eq = EE2 + E2 + E1/3; Error bars represent 2x standard deviation of the mean, X axis n=x refers to sample replicates for YES bioassay)

5.3.5 Link between receiving water estrogenicity and effects in caged and wild fish

It is generally accepted, from constant exposure tank studies, that fish exposed to 0.1 to 1 ng/l of EE2 and 1 to 10 ng/l of E2 will exhibit elevated levels of vitellogenin (Harries *et al*., 1997; Jobling *et al.*, 2002a&b, 2003). Little data exist to show the diurnal fluctuation of steroid estrogen concentrations present in sewage effluent; however Williams *et al*. (2003) report for a single WWTP, the daily E1 removal performance can appear extremely erratic showing a day-to-day variation in effluent concentrations of a factor of ten. Thus, it is unlikely that wild fish or caged fish downstream of WWTP discharge would be exposed to the same constant estrogenic environment as fish in a tank study. Therefore, to incur elevated plasma vitellogenin levels and the physiological and morphological changes indicative of intersex, it is probable that wild and caged fish would need to be exposed to concentrations of EE2 and E2 in the upper portion of the ranges described above.

As discussed in Chapter 4, the Liffey at Osberstown WWTP and Lee at Ballincollig WWTP were investigated for evidence of endocrine disruption of wild fish through assessment of plasma vitellogenin levels and physiological and morphological changes indicative of intersex.

The results showed that the only positive data were at Osberstown where predominantly downstream Brown trout exhibited raised vitellogenin levels compared with fish sampled upstream of the works. How does this result compare with the fish data for

Ballincollig in terms of measured $E2_{eq}$ concentrations? Osberstown gave positive result for vitellogenin induction with an $E2_{eq}$ of 1.3 ng/l (0.5-2.1, n=5); whereas Ballincollig gave a negative result for vitellogenin induction with a slightly higher $E2_{eq}$ value of 1.5 ng/l (0.9-2.1, n=10). At this point, it is worth recalling that Osberstown is somewhat unique in that a component of its industrial PE is likely to have contained an amount of EE2. When measured using the *in vitro* YES bioassay EE2 and E2 are equipotent, each giving a similar contribution to the $E2_{eq}$ value; however, in terms of the *in vivo* vitellogenin fish bioassay, EE2 is an order of magnitude more potent than E2 (Metcalf *et al.*, 2001; Thorpe *et al.*, 2003). This may explain why the Osberstown $E2_{eq}$ value of 1.3 ng/l was associated with vitellogenin induction whereas the Ballincollig was not.

5.4 Conclusions

Model predictions for effluent $E2_{eq}$ concentrations measured using the YES bioassay are excellent and generally within limits of error. Some deviations can be explained by local factors. Predictions could be improved with more accurate input data for WWTP plants and river flows. Measured $E2_{eq}$ data compares favourably with data sets derived from similar studies on the estrogenicity of sewage effluent conducted elsewhere in Europe.

Model predictions for receiving water $E2_{eq}$ concentrations are up to two orders of magnitude lower than the measured $E2_{eq}$ value. Differences are greater for rivers with catchments characterised by intensive livestock agriculture and are less for relatively urbanised catchments. The difference could be accounted for by estrogenic input/runoff from livestock/dairy agriculture.

Comparison of the predicted $EE2_{eq}$ concentrations with the UK EA EQS values shows that wild fish in the rivers investigated are not at risk. This corroborates the results of the wild and caged fish study.

6. Concluding Remarks

Many studies have investigated the occurrence of estrogenic compounds in surface waters, and WWTP effluents have been cited as a major source of environmental estrogens. Some workers have concluded that environmental estrogens that enter the aquatic environment from WWTP effluent may eventually be found in drinking water supplies (Kuch *et al.*, 2001). However, to date, the greatest concern regarding estrogens in surface water is their effects on the health of fish populations, as measured by raised plasma vitellogenin and an increased incidence of intersex in male fish (Eertmans *et al.*, 2003).

No work has been published, to date, addressing these concerns in an Irish context. Thus, the current study was designed to elucidate the effects of Irish WWTP effluents on receiving waters, and provide an assessment of the risk (if any) on the exposed fish populations in Irish rivers, or on any associated risk to drinking water supplies and, hence, the Irish population.

A number of representative aquatic ecosystems were investigated; levels of estrogens in these waters were quantified by *in vitro* bioassay while *in vivo* studies were applied to determine the incidence of endocrine disruption in the wild fish population and any implied threat to the drinking water resources associated with these water systems.

The water systems studied were selected so that both rural and urban catchments were represented; putative significant EDC inputs were domestic/municipal and industrial waste from WWTP discharges, septic tank effluents from isolated dwellings and run-off from agricultural land (in particular, animal husbandry activities). Brown trout were chosen as the indicator species in the wild fish survey, as salmonid species are of great ecological importance in the Irish context. Thus, although the results obtained from this study are specific to the aquatic ecosystems investigated and to the wild Brown trout populations of those systems, the study provides a scientific basis from which to assess the likely levels of environmental estrogens in other Irish freshwater systems and, to some extent, the probable effects on exposed wildlife and fish populations.

The results of this study allow us to conclude that environmental estrogens are not present in the river Lee in concentrations capable of eliciting vitellogenesis in exposed male Rainbow trout. Indeed, at no point in the river (until it enters Cork City) is the water estrogenic to fish, as determined by vitellogenin bioassay, even when fish were located at the apparent estrogenic “hotspot” directly beneath the outfall pipe from Ballincollig sewage treatment works. Fish located at the intake to the Lee Road water treatment works, which supplies drinking water to Cork City, showed no evidence of exposure to environmental estrogens. It may, thus, be concluded that estrogenic compounds are not emitted in quantities sufficient to pose a threat to the quality of the Lee waters or its associated drinking water supplies. A positive control tank study, conducted concurrently with the main *in vivo* study, demonstrated that the assay system was responsive to steroid estrogens, as reported in the literature.

Ovotestis (intersex) and raised plasma vitellogenin in male fish are widely employed biomarkers of estrogen contamination in the aquatic environment, and were used in this study to investigate the possibility that Irish rivers contain estrogenic chemicals at levels capable of affecting the reproductive health and success of exposed feral fish populations.

There was no evidence of intersex in any of the male Brown trout sampled, at any of the sites on the rivers Lee and Bandon, or the Killarney Lakes. This indicates that the feral Brown trout population of these water bodies has not been exposed, during critical periods of development, to concentrations of estrogens that can affect gonadal development. However, it could possibly be argued that a very low incidence of intersex in the population might not be detected within the small samples obtained. Neither was there any evidence of raised plasma vitellogenin, indicating that the fish were not exposed to environmental estrogens at concentrations sufficient to induce vitellogenesis within the few weeks prior to sampling. When these two results are taken together, it may be inferred that environmental estrogens are not present in the rivers Lee (upstream of the Lee Road water treatment works) and Bandon, or in the Killarney Lakes, at levels likely to pose a threat to the reproductive health and development of their fish populations.

In contrast, raised plasma vitellogenin levels (a sensitive and reliable marker of estrogen exposure) were detected in male Brown trout from the river Liffey, indicating estrogenic activity in a stretch of the river downstream of the outfall of the Osberstown WWTP. Subsequent YES *in vitro* bioassay studies placed the estrogenicity of this section of the river ($E2_{eq}$: 1.3 ng/L) into the extreme lower end of the concentration range of E2 required to induce vitellogenesis in male Rainbow trout. However, histological analysis of gonads, and the absence of any evidence of intersex, suggests that this estrogen exposure did not occur at a critical period of development for any of the fish, and that the fish are intermittently exposed to raised estrogen levels reducing the potential impact on reproductive health and development of the population.

Quantitative determination of the estrogenic potency of WWTP effluents, and their receiving waters, was performed using the highly sensitive and reproducible yeast estrogen screen. In our hands, the YES limit of detection is 10 ng/L E2, and this sensitivity may be increased 1000-fold by SPE of water samples prior to performing the bioassay.

The highest estrogenic load was found in effluent from Osberstown WWTP, discharging to the river Liffey, where the measured $E2_{eq}$ was 17.2 ng/L. This value is approximately six times greater than that measured at Leixlip WWTP (2.8ng/L), a plant with a comparable influent in terms of PE and the ratio of municipal to industrial waste received. One possible explanation of the higher estrogenic potency of the Osberstown effluent may be that the WWTP receives industrial waste from a nearby pharmaceutical company that manufactures the contraceptive pill. Under the conditions of the IPC licence awarded to this company from the EPA, the company is allowed to release an amount of chemical waste to Osberstown WWTP. (At this point it should be noted that from January 1 2006, the company will be subject to further substantial reductions in chemical waste discharge levels, including EE2). Therefore, it is possible that a small fraction of the 42% of the industrial PE treated by the WWTP may be more estrogenic than is normal for industrial waste and this may account for the elevated estrogenicity of the Osberstown effluent relative to all of the other WWTP surveyed.

This hypothesis is supported by the presence of feral Brown trout with raised plasma vitellogenin in this stretch of the Liffey (see above). YES analysis provided an $E2_{eq}$ of

1.3 ng/l at this Liffey site, whereas the river Lee at Ballincollig gave a negative result for vitellogenin induction despite a slightly higher $E2_{eq}$ value of 1.5 ng/l. The unique composition of the Osberstown WWTP effluent may help to account for this apparent discrepancy; as mentioned above, a component of its industrial PE may have contained an amount of EE2. When measured using the *in vitro* YES bioassay, EE2 and E2 are equipotent, each giving a similar contribution to the final estrogenic activity of the sample; however, in terms of the *in vivo* vitellogenin fish bioassay, EE2 is an order of magnitude more potent than E2. This may explain why the Liffey at Osberstown $E2_{eq}$ value of 1.3 ng/l was associated with vitellogenin induction whereas the Lee at Ballincollig was not.

In addition, a chemically-based deterministic model described by Johnson and Williams (2004) was used to predict the estrogen levels in the WWTP effluents and receiving waters surveyed. The predicted estrogen values were then compared to the actual values obtained by YES bioassay.

Overall, the model generally predicted the YES bioassay data for effluent estrogen levels within a reasonable margin of error, and the predictions could be further improved with the availability of more accurate data for the composition of WWTP influents and river flows. Measured $E2_{eq}$ data compared favourably with data sets derived from similar studies on the estrogenicity of sewage effluent conducted elsewhere in Europe. In contrast, it was noted that the model's predictions for estrogen levels in the receiving waters were up to two orders of magnitude lower than those measured by YES. These differences were greater for rivers with catchments characterised by intensive livestock agriculture and were less for relatively urbanised catchments. Thus, it is tentatively suggested that the difference could be accounted for by estrogenic input/runoff from livestock/dairy agriculture.

A comparison of the model's predicted $EE2_{eq}$ concentrations with the UK Environment Agency EQS values indicates that wild fish in the Irish rivers investigated are not at risk. This prediction is in agreement with the results of the wild and caged fish studies.

This predictive model has been fully validated in the UK and in Italy, and it has already been usefully employed by the UK Environment Agency to identify WWTP that require measures to increase steroid treatment efficiency to comply with the Agency's Environmental Quality Standards. However, this is the first time that the model has been applied to Irish data. Further validation and refinement of the model for use in the Irish context would provide a helpful tool in the identification of potential estrogenic "hotspots" in the Irish environment.

In summary, with the caveat that estrogenic "hotspots" are more likely in densely populated urban and/or industrialised areas (as demonstrated by the Liffey findings), it may cautiously be concluded that:

i. Irish WWTP effluents are estrogenic, although levels compare favourably with other European countries and the USA (Chapter 5).
ii. Irish rivers and lakes do not appear to be at general risk from significant concentrations of environmental estrogens, (Chapters 3-5).
iii. In general, wild fish populations do not appear to be at risk from estrogenic chemicals, (Chapter 4) and

iv. Judging from the limited number of sites examined in this study, Irish drinking water resources do not appear to be at significant risk from estrogenic chemicals (Chapter 3, 5).

The apparently low level of risk to Irish freshwater systems from environmental estrogens may, in part, be attributed to the coastal locations of most of the heavy centres of population and industry in Ireland. Thus, Irish rivers are not generally receiving high domestic and industrial effluent loads. This is in contrast to the situation in other countries, for example the UK, and is probably a significant factor in explaining the general absence of endocrine disruption in fish populations in Irish freshwaters.

It is recommended that the following work should be performed in the future:

i. Further characterisation of the Liffey is of importance, especially in the region downstream of the Osberstown WWTP and at the point of intake to Leixlip WTW. Investigations in other water bodies, in areas of similar sensitivity, should also be considered.
ii. A review of company IPC licences should be conducted with the aim of reducing the emissions of known EDCs (especially potent estrogens such as synthetic steroids) to Irish fresh and marine waters. The YES (or equivalent bioassay) could be usefully employed to quantify and, subsequently, monitor estrogen levels in relevant industrial effluents.
iii. Investigate levels of EDCs in the marine environment; in particular, estuarine waters receiving high effluent loads at the sites of major agglomerations and in environmentally-sensitive areas of Irish coastal waters. Considering the hydrophobic nature of many EDCs, both water and sediment sampling should be performed to determine EDC levels in the estuarine/marine environment as a whole.
iv. By applying Geographic Information System (GIS) modelling to the whole of Ireland, potential pollution "hotspots" for steroids, pharmaceuticals and other EDCs may be identified and, subsequently, characterised and remedial action taken where necessary. The presence of estrogenic activity in the Liffey at Osberstown indicates the need to introduce methods for removal of estrogens from final effluent, especially for WWTP with higher PE values or discharging into sensitive waters.
v. Investigate agriculture as a source of estrogens (e.g. animal steroids, pharmaceuticals, pesticides and herbicides) in Irish freshwaters. In addition, EDC loads in sewage sludge deposition and land application areas should be investigated.

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