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Endocrine Disruptors in the Irish Aquatic Environment

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Synthesis Report

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1 Introduction

The endocrine system is the hormone control network that exists in animals and humans to co-ordinate all of the fundamental life functions, including regulation of growth, sexual development and reproduction. Over the past two decades it has been recognised that a rapidly growing list of man-made chemicals, or their degradation products, are capable of interfering with this system. Such chemicals are called Endocrine Disrupting Compounds (EDCs). The science of endocrine disruption has arisen from concerns about the effects of these environmental contaminants on the health and reproductive success of exposed human and wildlife populations.

As the endocrine system is exquisitely sensitive, many EDCs are biologically active at extremely low doses (i.e. parts per billion and parts per trillion range), in some cases at a concentration many thousands of times lower than was previously thought to be safe. It is very difficult to predict the effects of EDCs on an exposed organism, as the effects will vary depending upon a number of factors. These include the life stage during which exposure occurs, as well as the duration and amount of exposure, and the precise mixture of chemicals involved. Genetic variability will also modulate the response from one individual to another. In particular, the various life-cycle stages of an organism have very different levels of vulnerability to EDCs. Disruption during foetal and neonatal development results in a series of irreversible 'organisational' changes that remain with the individual for life, such as reduced fertility, genital dysmorphology and even alterations of brain organisation and behaviour. In contrast, adult life stages are far more resilient to the effects of EDCs and the 'activational' changes induced are often transient and reversible.

Many of these chemicals are used in a broad range of applications (industrial, agricultural, pharmaceutical, etc.) and it is now accepted that there is substantial global contamination by EDCs. The majority of known EDCs have been identified in untreated industrial and domestic wastes and in wastewater treatment plant (WWTP) effluents. As rivers and estuaries are repositories for large amounts of domestic and industrial waste, the fish populations of river and estuarine systems can be considered as sentinel species for assessing the impact of EDCs on the environment.

A number of European countries (including the UK, France, Spain, Italy, Denmark, Sweden, Switzerland and the Netherlands), as well as the USA, have documented endocrine disruption in their wild fish populations; in most cases, these effects were of a feminising nature, such as the presence of female ovarian tissue in the testes of male fish, a condition known as 'intersex'. In addition, UK studies have demonstrated that WWTP effluent can be feminising to fish, linking these estrogenic effects to the presence, in such effluents, of the natural female steroids 17β-estradiol (E2) and estrone (E1), and the synthetic steroid 17α-ethinyl estradiol (EE2) used in oral contraceptive formulations. In agricultural areas, these natural and synthetic steroids may, in part, be contributed by animal waste. These steroid estrogens are present at very low concentrations (ng/l) in WWTP effluents but are extremely potent, in many cases contributing greater than 80% of the estrogenic burden of the effluent (Desbrow *et al.*, 1998; Stuer-Lauridsen *et al.,* 2004). They are of significance at sites where WWTP effluents make up a considerable proportion of the river flow. Estrogenic effluents have been reported in many other countries, including Brazil, Belgium, Canada, Denmark, Germany, Israel, the Netherlands, Switzerland and Japan. (For a detailed review of the science of endocrine disruption, the reader is referred to Damstra *et al.,* 2002.)

1.1 Scope and Objectives

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 integrated design of the current study addresses this knowledge gap and answers the following questions:

i. Do Irish rivers contain estrogenic compounds?

ii. If so, at what concentrations?

iii. Are these levels likely to pose a threat to aquatic ecosystems, particularly wild fish?

iv. Is there a risk to drinking water supplies?

To this end, a series of WWTPs was surveyed and the level of estrogenic activity was quantified in the final effluents and, in some cases, the receiving waters. In addition, cages of male rainbow trout were placed at a series of sites in the River Lee, upstream and downstream of potential estrogenic ‘hotspots’ and at the intake to the Lee Road Water Treatment Works (WTW), which supplies drinking water to Cork City. After a 3-week exposure period the fish were examined for signs of endocrine disruption. Finally, a survey of wild male brown trout populations in the Rivers Liffey, Lee and Bandon was conducted and the fish were examined for signs of exposure to environmental estrogens. Taken as a whole, the results of this study allow a first assessment to be made of the risk posed by environmental estrogens to the Irish freshwater environment.

2 Methodology

This study employed a suite of *in vitro* (cell-based) and *in vivo* (whole animal-based) bioassays to (i) quantify the estrogenic activity of WWTP effluents, and (ii) determine their impact on fish (brown trout and rainbow trout) in the receiving waters.

2.1 *In Vitro* Bioassay

The *in vitro* bioassay used was the Yeast Estrogen Screen (YES), which is based on a yeast cell transformed with the human estrogen receptor DNA. Activation of the estrogen receptor by an EDC causes the yeast to produce a marker enzyme that results in a colour change in the assay medium. The intensity of the colour response is quantitatively related to an E2 standard curve and the result is expressed as Estradiol Equivalents ($E2_{eq}$) (Routledge and Sumpter, 1996). The YES has been widely applied by research laboratories to the quantification of estrogens in environmental samples as it is a rapid, inexpensive and reproducible screening tool, capable of detecting as little as 3 ng/l E2 in a water sample. Extraction of chemicals in the water sample, using solid-phase extraction technology, introduces a 1000-fold concentration of estrogens prior to analysis, further increasing the sensitivity of the detection system.

2.2 *In Vivo* Bioassays

Salmonid species were used as the indicator organisms in both the wild fish survey and the caged fish study.

The primary objectives of the wild fish survey were to determine the impacts, if any, of EDCs in native Irish fish populations across the country and the more local impacts on communities upstream and downstream of WWTPs. 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 salmonids 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 (*Salmo trutta*), which are ubiquitous in Irish freshwaters and begin maturing at approximately 1 year, were chosen as the indicator species rather than more minor species such as roach and bream. Endocrine disruption has been reported in feral brown trout populations in both Swiss and Danish studies (Wahli *et al*., 1998; Bernet and Wahli, 2000; Stuer-Lauridsen *et al.,* 2004).

Rainbow trout (*Oncorhynchus mykiss*) have been widely used as a sentinel species in caged fish studies on WWTP effluents, as salmonids are more estrogen sensitive than cyprinid species (Purdom *et al*., 1994). Routledge *et al*. (1998) demonstrated that vitellogenin synthesis (see (i) below) in male rainbow trout is very sensitive to estrogens, and the threshold concentration for a response to E2 is between 1 and 10 ng/l. Also, the magnitude of the vitellogenin response in rainbow trout was shown to be 30-fold greater than in roach for an E2 exposure of 100 ng/l.

Two standard biomarkers of estrogen exposure were chosen as end points for the wild and caged fish studies.

i. ***Vitellogenin***: the presence of this female egg protein in the blood of male fish is one of the most sensitive and widely accepted biomarkers of estrogenic contamination of the aquatic environment (Sumpter and Jobling, 1995). Vitellogenin is produced in response to activation of the estrogen receptor. As male fish will not normally have significant concentrations of estrogens circulating in the blood, the presence of vitellogenin is a reliable indicator of exposure to environmental estrogens. Indeed, continuous exposure of male rainbow trout to E2 (1–10 ng/l), over a 3-week period, gives a measurable increase in vitellogenin concentrations (Routledge *et al*., 1998). However, this is a transient response, and in the absence of estrogens vitellogenin synthesis is discontinued and concentrations drop to undetectable levels after a period of days to weeks. In this study, vitellogenin levels were determined quantitatively for the caged fish study (radioimmunoassay; performed by Dept. of Biological Sciences, Brunel University, London, as described by Sumpter, 1985) and qualitatively for the wild fish survey (Salmonid Vitellogenin Enzyme Immunoassay kit from Biosense Laboratories, Norway).

ii. ***Intersex***: the presence of intersex is a widely accepted biomarker of estrogen exposure in wild fish populations and it is an irreversible 'organisational' effect. It occurs when male fish are exposed to environmental estrogens during the critical period prior to sexual differentiation (just following hatching), or at the juvenile stage when the fish are most susceptible to endocrine disruption, and results in the simultaneous presence of both male and female gonadal characteristics. In this study, the testes of wild male brown trout were fixed and embedded in paraffin wax. The tissue specimens were then sectioned, stained and examined by light microscopy for the presence of female gonadal characteristics, such as the presence of eggs (oocytes).

2.3 Study Sites

The wild fish survey was carried out on the Rivers Liffey, Lee and Bandon, as well as on Lough Leane and Lough Guitane in the Killarney Lakes. Potential estrogenic pollution 'hotspots' were identified and fish were sampled upstream and downstream of these sites. Sample sites in the rivers' headwaters provided internal controls for each water system, and allowed determination of the effects (if any) of background levels of estrogens on fish populations. These internal control sites were chosen to achieve a balance between minimal anthropogenic inputs while containing a wild fish population that could sustain sampling (Table 2.1). Sampling was not performed in the Liffey headwaters for logistical reasons specific to this project.

The caged fish study was conducted entirely on the River Lee, with an additional primary control (reference) site at Lough Barfinnihy, Co. Kerry, an oligotrophic mountain lake with no anthropogenic inputs.

Levels of estrogenic activity at many of these sites were also quantified by the YES *in vitro* bioassay (Table 2.1).

Table 2.1. Summary of *in vivo* study sites. Wild brown trout (*Salmo trutta*) were sampled at a series of sites (*) on each of the water systems listed below. Adult male rainbow trout (*Oncorhynchus mykiss*) were placed in cages at sites (§) on the Lee and in Lough Barfinnihy. All test sites were located downstream of potentially significant EDC inputs, and responses compared with upstream control sites. For each water system (excluding the Liffey), background estrogen activity was determined in a region minimally impacted by human activity and upstream of potentially significant EDC inputs, serving as an internal control for that water system. Lough Barfinnihy served as a primary control (reference site). Levels of estrogenic activity at some sites were also quantified by the YES *in vitro* bioassay (‡). WWTP: wastewater treatment plant; WTW: water treatment works.

Water system	Sample sites	OS grid reference	Site characteristics (control/test)	Potentially significant EDC input
Liffey	Carragh Bridge, Co. Kildare	N 854208	* ‡ Control for Osberstown WWTP	Osberstown WWTP
	Leinster Aqueduct, Co. Kildare	N 869207 to 877227	* ‡ Test	
Lee	Gougane Barra, Co. Cork	W 095661	§ ‡ Internal control (Lee headwaters)	Ballincollig WWTP
	U/S Ballyvourney, Co. Cork	W 162759	* Internal control (Lee headwaters)	
	U/S Ballincollig, Co. Cork	W 580714 to 581714	* Control for Ballincollig Town	
	U/S Ballincollig, Co. Cork	W 591716	§ Control for Ballincollig Town	
	Outfall Ballincollig WWTP	W 592715	§ ‡ Test	
	D/S Ballincollig, Co. Cork	W 605718 to 614717	* Test	
	Lee Road WTW, Cork	W 649715	§ Test	
Bandon	Ardcahane Bridge, Co. Cork	W 243557	* Internal control (Bandon headwaters)	Bandon WWTP, agricultural mart, food-processing facility
	U/S Bandon Town, Co. Cork	W 442546 to 474546	* Control for Bandon Town	
	D/S Bandon Town, Co. Cork	W 509562 to 516569	* Test	
Killarney Lakes	L. Guitane, Co. Kerry	W 015853 to 035853	* Internal control (upstream of L. Leane)	Killarney WWTP
	Ross Bay, L. Leane, Co. Kerry	V 942889 to 949890	* ‡ Test	
Finnihy	L. Barfinnihy, Co. Kerry	V 850766	§ ‡ Primary control (Reference) site	Oligotrophic highland lake, remote from anthropogenic inputs

3 Results and Discussion

3.1 Estrogen Levels in Irish WWTP Effluents and Receiving Waters

A survey of the estrogenic activity of final effluents from ten WWTPs in the south and east of the country 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 3.1). 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 the sampling dates in this study.

In some cases, samples were collected upstream and downstream of the WWTP to examine the impact on the receiving waters. All samples were analysed by *in vitro* bioassay (YES) and the results are summarised in Table 3.1.

Effluent estrogen levels 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 WWTPs showed comparatively elevated levels (16 and 17.2 ng/l, respectively) relative to the other plants surveyed (1.1–6.8 ng/l) (Table 3.1). Background levels of estrogens in the aquatic environment were determined at Gougane Barra and Lough Barfinnihy (Table 2.1).

When placed in an international context, the estrogen levels measured in Irish WWTPs and receiving waters compare favourably with those derived from similar studies conducted elsewhere in Europe (Carballa *et al*., 2004; Johnson and Williams, 2004). Such studies have suggested that the total estrogenic activity of effluents, from WWTPs receiving mostly domestic input, is in the low to medium ng/l range. Background levels of estrogens, determined in the Lee headwaters and at Lough Barfinnihy, ranged from undetectable to 1.1 ng/l, on different sampling occasions (Table 3.1). 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 (Sections 3.2 and 3.3) have subsequently demonstrated that these waters are not estrogenic to fish.

The higher level of estrogen activity measured at Osberstown and Ringsend WWTPs is worthy of further consideration. Ringsend WWTP is the largest wastewater treatment facility in the country and the estrogenic activity of its effluent (16.0 ± 5.6 ng/l) was comparatively elevated. The plant has recently been upgraded from primary to secondary treatment. From discussions with the plant manager, there appear to have been a number of operational problems that coincided with the effluent sampling period. It is likely that these problems may have impaired the treatment efficiency of the plant leading to elevated levels of steroids in the plant effluent, as differences in WWTP technology are known to affect the efficiency of extraction of environmental estrogens from effluents (Burkhardt-Holm *et al.,* 2002; Stuer-Lauridsen *et al.,* 2004).

The highest level of estrogenic activity was found in the Osberstown WWTP effluent (17.2 ± 3.8 ng/l). When considering this result, it is of interest to note that the plant receives industrial effluent from a nearby pharmaceutical company that manufactures the contraceptive pill. Under the conditions of its IPC licence, this company is permitted to release an amount of chemical waste to Osberstown WWTP, some of which may have been EE2. It is therefore possible that a small fraction of the industrial PE treated by the WWTP may have been more estrogenic than average industrial waste, possibly resulting in the comparatively elevated estrogenicity of the final effluent. It should be noted that from 1 January 2006, the company

Table 3.1. Estrogenicity ($E2_{eq}$) of WWTP final effluents and receiving waters. Over the period October 2003 to June 2004, $E2_{eq}$ values for ten WWTPs in the south and east of Ireland were determined using the YES. Grab samples (5 l) were taken from each site on a minimum of 2 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 WWTPs and 95 percentile river flow values. One population equivalent (PE) is defined as the load resulting from 60 g 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	Very high	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 (U/S) 1.3 ± 0.8 (D/S)	– 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	High	3.7 ± 1.8	1.8 ± 1.5	~0	3
Tralee	24,633	80:20	Tralee Bay	Very high	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

will be subject to further substantial reductions in chemical waste discharge levels, including EE2.

The estrogenicity of the Osberstown WWTP effluent is at a level that would be expected to exert feminising effects, such as increased vitellogenin synthesis, on continually exposed fish populations. However, the effluent is diluted approximately 27-fold upon discharging to the River Liffey, which reduces the estrogenic burden of the effluent to a theoretical value of 0.6 ng/l. The actual (YES-measured) estrogenic activity of the downstream receiving waters is somewhat higher (1.3 ± 0.8 ng/l), although it agrees with the predicted value within the limits of experimental error (Table 3.1). This level of activity is at the lower end of the range known to be estrogenic to fish under continuous exposure conditions although it is unlikely that fish in the Liffey, or indeed in any of the other receiving waters examined, are constantly exposed to these levels of estrogenic activity. Significant diurnal variations in the steroid estrogen concentration of WWTP effluents are known to occur in tandem with household activity, and thus it is unlikely that wild fish downstream of a WWTP outfall would be 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 (0.1–1 ng/l EE2 or 1–10 ng/l E2) (Nash *et al.*, 2004) would be required to induce feminising effects in exposed wild fish populations.

However, it is important to consider the nature of the chemicals giving rise to the estrogenic activity in the effluents, as this is also a significant factor when trying to predict the effect of an estrogenic effluent on fish populations. For example, EE2 and E2 are equally estrogenic in the YES, but EE2 is considered at least 10-fold more potent than E2 in fish. This increased potency is, at least in part, due to EE2's resistance to biological and environmental degradation, so that it persists in living organisms for a relatively long time compared to the natural female steroids. Thus, if the Osberstown WWTP effluent contained elevated levels of EE2 (for example, as a result of effluent from the pharmaceutical plant) then this could pose a heightened risk to the fish population in the Liffey, relative to other effluents which are of similar estrogenic activity in the YES.

In light of these considerations, it is of considerable interest to note that wild male brown trout, downstream of Osberstown WWTP, displayed evidence of exposure to environmental estrogens (Section 3.3.1). In contrast, fish sampled from upstream of the WWTP, and from other water systems (Lee, Bandon, Lough Leane), which were of similar estrogenic activity in the YES, displayed no evidence of endocrine disruption. This finding is discussed in Sections 3.2.1 and 3.3.1.

In addition, a chemical-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. 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.

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 WWTPs 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.

3.2 Impact of WWTP Effluent on the River Lee, Exposed Fish Populations and Drinking Water

The impact of WWTP effluents on their receiving waters, exposed fish populations and associated drinking water resources was investigated by an *in vivo* caged fish study located in the River Lee. A stretch of the river between Ballincollig and Cork City was the main focus of the study. Ballincollig WWTP discharges into this section of the river, which is 4 km upstream of the intake to the Lee Road 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, while the adjusted PE, representing the average dilution of the effluent in the river, is 194 for Ballincollig WWTP (Table 3.1). Previous UK studies have reported a significant level of intersex in the wild fish population of the River Rea downstream of a WWTP with an adjusted PE of 117 (Jobling *et al.,* 1998). The Ballincollig WWTP influent is almost entirely domestic in origin, making it comparable to similar UK studies investigating effects of natural and synthetic estrogens on fish populations (Harries *et al.*, 1999), while 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. 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.

In summary, consideration of the following combination of factors made the Lee River system the location of choice for this investigation:

i. PE (and adjusted PE) load of WWTP influent

ii. 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

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

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. In the present study, two separate *in vivo* bioassays were performed, in March and August 2002. Adult male rainbow trout were placed in cages (~20 per cage) at test sites immediately upstream of Ballincollig WWTP, at the WWTP outfall and 4 km downstream at the intake of the Lee Road WTW (Table 2.1). Cages were also placed at a control site located in the Lee headwaters at Gougane Barra and at the reference site in Lough Barfinnihy. These control sites were considered unlikely to be significantly impacted by any of the main sources of EDCs in the environment. At the beginning and the end of the 21-day exposure period, blood was taken from the fish and vitellogenin concentrations were determined by radioimmunoassay, thus providing a reliable measure of any estrogen exposure during the study period (Section 2.2). The experimental design was closely modelled on published *in vivo* studies investigating the impact of WWTP effluents on the river Lea in the UK (Harries *et al.*, 1997).

In addition, positive control studies were performed in which male rainbow trout were exposed to known concentrations of E2 and EE2 using a flow-through tank system at the UCC Aquaculture Development Centre, Lee Maltings, Cork.

There was no evidence of raised vitellogenin concentrations in any fish located at either the test or control sites in either of these two experiments. In each case, vitellogenin concentrations were in the range of 10–100 ng/ml (background levels). There was no difference (within 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 control sites in the Lee headwaters (Gougane Barra) and at Lough Barfinnihy (Fig. 3.1).

The vitellogenin concentrations detected in this study (10–100 ng/ml) were in agreement with literature values for background (pre-estrogen exposure) levels in male rainbow trout. Depending on the level of estrogen exposure, a positive vitellogenin response would be expected to range from 10^3 to 10^6 ng of vitellogenin per ml of plasma (Routledge *et al*., 1998).

A positive control study was also conducted in August 2002, to demonstrate the responsiveness of the rainbow trout to known concentrations of steroid estrogens, which account for the main component of WWTP estrogenic activity. The male rainbow trout responded, as expected,

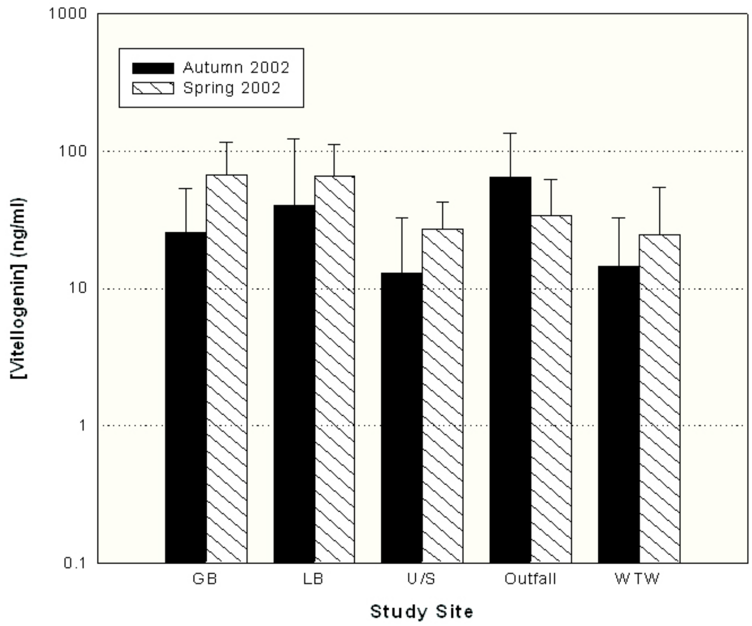

Figure 3.1. No increase in vitellogenin synthesis in male rainbow trout (*Oncorhynchus mykiss*) after exposure for 21 days at a range of sites in the River Lee. Adult male rainbow trout (205 ± 45 g) were located at test and control sites for 21 days, after which time the fish were sacrificed and plasma vitellogenin concentrations (ng/ml ± SD) determined, n = 14–28 male trout per cage. (GB: Gougane Barra (internal control site); LB: Lough Barfinnihy (primary control site); U/S: immediately upstream of WWTP outfall; Outfall: discharge point for effluent from Ballincollig WWTP; WTW: Lee Road Water Treatment Works).

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. 3.2).

It is of interest to note that the YES bioassay placed estrogen levels in the undiluted Ballincollig WWTP effluent at 3.2 ± 1.1 ng/l (Table 3.1). This is a concentration that previous studies have indicated may be estrogenic to male rainbow trout (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 vitellogenin synthesis in fish (Table 3.1). However, the actual estrogenic activity 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 (Section 3.1). This level of activity is at the lower end of the range known to be estrogenic to fish under continuous exposure conditions (Section 3.1). 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 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 (0.1–1 ng/l EE2 or 1–10 ng/l E2) (Nash *et al.*, 2004) 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 (up to the point where 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 consistently emitted in quantities sufficient to pose a threat to the quality of the Lee waters or its associated drinking water supplies.

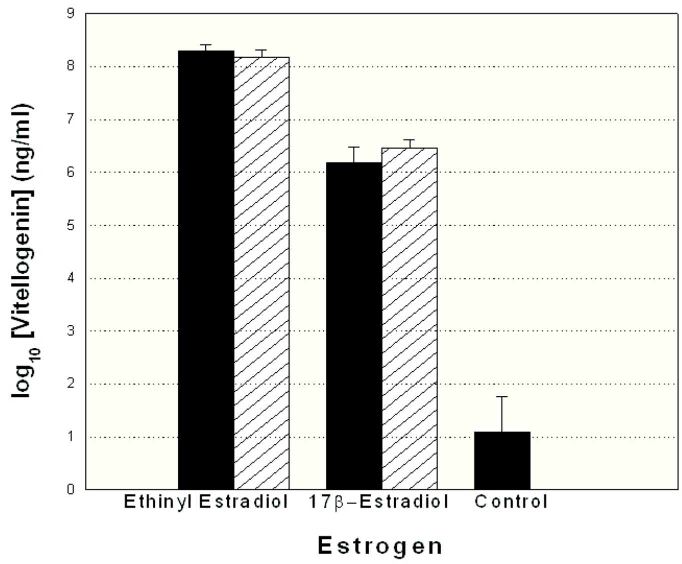

Figure 3.2. *In vitro* bioassay control experiment: steroid estrogens induce vitellogenin synthesis in male rainbow trout (*Oncorhynchus mykiss*). Adult male rainbow trout (205 ± 45 g) were placed in a flow-through tank system (400 ml/min) and exposed to nominal concentrations of (a) 100 ng/l 17β-estradiol or (b) 400 ng/l 17α-ethinylestradiol for 21 days. A control tank (no estrogen exposure) was also set up. Data represent mean vitellogenin concentration (ng/ml) ± SD, where n = 17–27 male fish per tank. Tanks containing estrogens were performed in duplicate (solid and hatched bars). Vitellogenin concentrations are plotted on a logarithmic scale due to the wide range of values measured.

3.3 Survey of the Irish Wild Brown Trout Population for Evidence of Endocrine Disruption

The survey of feral brown trout was designed to investigate the possibility that Irish freshwaters contain estrogenic chemicals at levels capable of affecting the reproductive health and success of exposed wildlife populations. The presence of vitellogenin in the blood of male fish was investigated with a view to establishing whether Irish wild fish populations are 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.

All sampling trips were 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 electro-fishing. Some samples from Lough Leane were obtained in cooperation with the Central Fisheries Board, 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 electro-fishing equipment (courtesy of the Eastern Fisheries Board).

3.3.1 Endocrine disruption in wild brown trout in the River Liffey

There was no evidence of 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 environmental estrogens can cause feminisation 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 Irish feral brown trout populations have 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 these populations might not be detected within the small samples obtained in the Lee and Bandon rivers, and in the Killarney Lakes. In support of our data, however, it should be noted that 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 using sample sizes similar to those obtained from the Lee and Bandon rivers in the present study.

Similarly, 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 (Fig. 3.3). This indicates that the wild brown trout population of these water systems has not been exposed to concentrations of estrogens that can induce vitellogenesis. No plasma samples were available for fish sampled from the Killarney Lakes as these fish were obtained by means of angling competitions and, thus, no definite conclusion can be drawn regarding levels of vitellogenesis (if any) in male fish taken from this waterbody.

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 Osberstown WWTP (Fig. 3.3).

As the purpose of the Liffey sampling programme was to investigate the effect of effluent from Osberstown WWTP on the feral fish population, both sample sites were located in the vicinity of the WWTP (Table 2.1). The upstream site yielded a sample 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 (Fig. 3.3). In contrast, 26.3% (15 of 57) of the male brown trout from the downstream site, showed raised vitellogenin levels. 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 Aqueduct) is significantly different from the upstream site at Carragh Bridge, Co. Kildare, which is unaffected by the Osberstown WWTP effluent (Chi-squared test; $P < 0.01$).

This result indicates that estrogens are present at environmentally significant concentrations in this stretch of the River Liffey. In support of this finding, *in vitro* bioassay analyses (YES) placed the estrogenicity of this part of the river into the extreme lower end of the concentration range of E2 required to induce vitellogenesis in male rainbow trout (Section 3.1). However, the relatively low incidence (26%) of raised

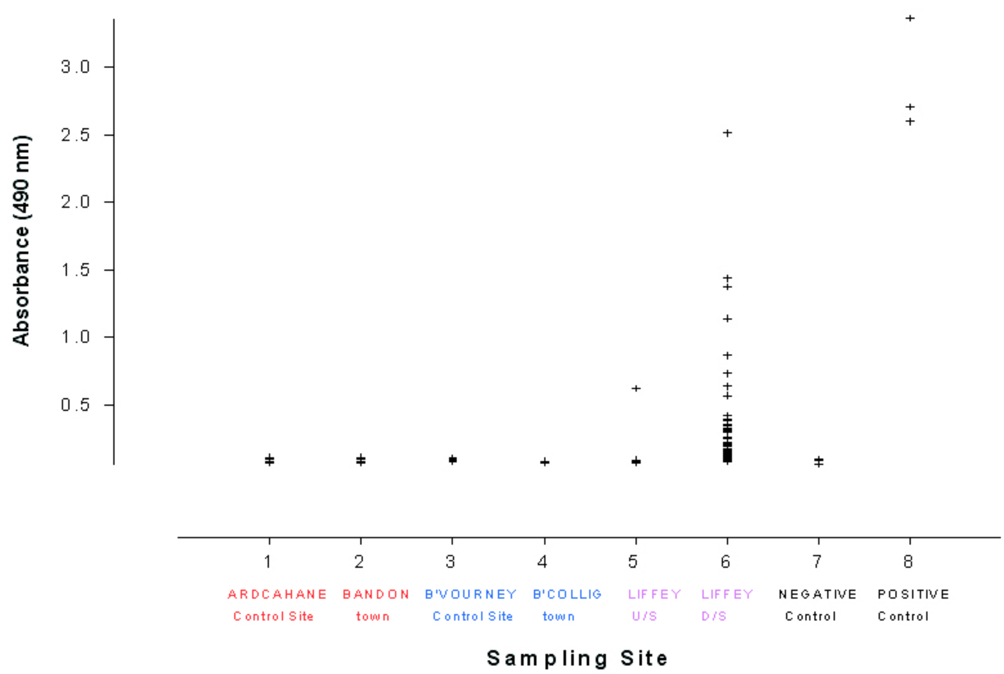

Figure 3.3. Plasma vitellogenin levels in male wild brown trout in the Rivers Bandon, Lee and Liffey. Semi-quantitative determination of plasma vitellogenin in male wild brown trout was performed by enzyme immunoassay (Salmonid Vitellogenin Enzyme Immunoassay kit: Biosense Laboratories, Norway). The 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.

vitellogenin in the downstream brown trout population, combined with the variability in the level of the vitellogenin response in those fish testing positive (Fig. 3.3), 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, Department of Biological Sciences, Brunel University, Uxbridge, UK, personal communication, 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.

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 the YES, Table 3.1). 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 is likely to contain an amount of EE2 (see Section 3.1). 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. 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 Conclusions

This study provides an integrated assessment of the levels of estrogenic chemicals in Irish freshwater systems, and of their effects on freshwater fish populations. 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.

Thus, 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 (Section 3.1)

ii. Irish rivers and lakes do not appear to be at general risk from significant concentrations of environmental estrogens (Sections 3.2 and 3.3)

iii. In general, wild fish populations do not appear to be at risk from estrogenic chemicals (Section 3.3), 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.

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 waterbodies, 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 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. Levels of EDCs in the marine environment, particularly estuarine waters at the sites of major agglomerations and environmentally sensitive areas of Irish coastal waters, should be Investigated. 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.

v. Agriculture as a source of estrogens (e.g. animal steroids, pharmaceuticals, pesticides and herbicides) in Irish freshwaters should be Investigated.

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List of Abbreviations

BOD_5	5-Day biological oxygen demand
E1	Estrone
E2	17β-Estradiol
EE2	17α-Ethinyl estradiol
$E2_{eq}$	Estradiol equivalent
EDC	Endocrine disrupting compound
GIS	Geographic information system
ng/l	Nanograms per litre
OS	Ordnance Survey
PE	Population equivalent
SD	Standard deviation from the mean
SEM	Standard error of the mean
WWTP	Waste water treatment plant
WTW	Water treatment works
YES	Yeast Estrogen Screen