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Investigation of the estrogenic risk to feral male brown trout (*Salmo trutta*) in the Shannon International River Basin District of Ireland

Miriam A. Kelly, Antoinette M. Reid, Kathryn M. Quinn-Hosey, Andrew M. Fogarty, James J. Roche, Concepta A. Brougham *

Endocrine Disruption Group, Department of Life and Physical Sciences, School of Science, Athlone Institute of Technology, Athlone, Co. Westmeath, Ireland

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ABSTRACT

The estrogenic potential of sewage treatment effluents and their receiving waters in the Shannon International River Basin District (SIRBD) of Ireland was investigated. An integrated approach, combining biological and chemical methods, was conducted to assess 11 rivers adjacent to sewage treatment plants (STPs) and their possible interference with the endocrine system of feral brown trout (*Salmo trutta*). Hepatosomatic index, gonadosomatic index, condition factor, histological (intersexuality) and endocrine (vitellogenin induction) parameters were assessed in a sample size of 10 at each location. The estrogenic burden was determined using an *in vitro* recombinant yeast assay containing the human estrogen receptor (YES assay). In addition, endocrine disrupting chemicals (EDCs) were quantitatively identified through a selection of pre-concentration techniques combined with chromatographic analysis at or near the selected locations.

Chemical analysis of representative site samples identified phthalates and an alkylphenol in water and sediments in μg/L and mg/kg concentrations, respectively. There were no significant difference in somatic indices or the condition factor between upstream control and downstream test sites, and there was no evidence of reproductive alterations or the presence of intersex in studied male brown trout. However, raised vitellogenin (vtg) levels were detected in the blood plasma samples of male brown trout at 8 of the 11 sites. Significant levels were reported at 3 of the positive sites ($p \leq 0.05$). In one particular location, vtg induction was observed in 100% of the male brown trout sampled downstream. These findings were supported by the YES assay, where estrogenic activity was detected in the same upstream and downstream sites giving 17β-estradiol equivalency factor (EEF) values of up to 2.67 ng/L. This study represents an integrated assessment approach, confirming the presence of estrogens in rivers of the SIRBD of Ireland, thus suggesting a cause–effect relationship to prolonged EDC-exposure in fish.
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1. Introduction

The impact of domestic and industrial sewage effluents on the endocrine system of a number of fish species and other aquatic organisms has been well documented worldwide (Adewolu et al., 2009). Studies have correlated exposure to sewage effluent with alterations in sex steroid hormone levels (Folmar et al., 1996), impairment of gonad development (Jobling et al., 1998; Rodgers-Gray et al., 2000) and induction of the yolk precursor protein, vtg, in male fish (Folmar et al., 1996; Jobling et al., 1998; Tarrant et al., 2008). Previous studies have linked the main estrogenic activities of such effluents to steroidal estrogens such as 17β-estradiol (E_2), 17β-ethinylestradiol (EE_2) and estrone (E_1) (Desbrow et al., 1998) at concentrations of up to tens of ng/L (Belfroid et al., 1999; Körner et al., 2001; Ternes et al., 1999). Anthropogenic compounds such as phthalates, alkylphenols and synthetic estrogens have also been associated with endocrine disruption studies confirming reproductive toxicity in humans (Latini et al., 2006; Park et al., 2002) altered gene expression in amphibians (Crump et al., 2002) and the development of adenocarcinomas in humans (Adam et al., 1977), respectively. Such chemicals can disrupt normal endocrine function and as a result, may compromise reproductive health in both humans and wildlife (Jobling et al., 2002). However, the effects are often difficult to detect in feral fish populations as they tend to manifest only after certain periods of exposure (Jobling et al., 2006). Such scenarios have triggered an establishment of sensitive biomarkers such as vtg induction, to reflect potentially adverse biological responses towards these environmental contaminants (Bucheli and Fent, 1995).

Testing methods and guidelines for the screening of endocrine-disrupting effects are currently being developed. The Organization for Economic Cooperation and Development (OECD) has identified

* Corresponding author. Fax: +353 9064 24492.
E-mail address: cbrougham@ait.ie (C.A. Brougham).

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two core endpoints that potentially contribute to the detection of endocrine disruption: secondary sexual characteristics and vtg induction. OECD Phase 3 validation studies support the relevance and specificity of both endpoints (OECD, 2007), and although many studies have demonstrated that each of the selected core endpoints can effectively detect EDCs, their mechanisms together with their biological significance in the context of endocrine disruption, have not been fully elucidated (Lin et al., 2009). In addition, these method recommendations by the OECD are currently taken as proposals, and therefore, may or may not form the policy on the development of future Test Guidelines (OECD, 2007).

Purdom et al. (1994) first reported estrogenic effluents in rivers in the UK, observing their ability to cause feminizing effects in male fish. The occurrence of hermaphrodite fish in wild fish populations living downstream of major UK STPs prompted surveys, which revealed high incidences (16–100%) of intersexuality among male fish and a realization that reproductive and developmental effects do result from exposure to ambient levels of chemicals present in rivers (Jobling et al., 1998). Moreover concentrations of vtg in the blood of male fish sampled downstream of the STPs were significantly higher than those of males from upstream control sites (Purdom et al., 1994). The use of this biomarker to detect endocrine disruption in fish is important for monitoring the effects of environmental contaminants on aquatic inhabitants. Vtg is an estrogen-inducible phosphoprotein that is normally synthesized by the liver of female oviparous vertebrates during oogenesis. It is the precursor of egg yolk proteins, but both males and juveniles can synthesize vtg if estrogens or xenoestrogens are present. Accordingly, this protein can be used as a biomarker for estrogenic exposure in male and juvenile fish (Folmar et al., 1996). To date, the estrogenicity of waterways has been highlighted in many countries using vtg induction as a biomarker of endocrine disruption (Purdom et al., 1994; García-Reyero et al., 2004; Tarrant et al., 2008; Randak et al., 2009).

Brown trout were selected as the environmental indicator species to assess the potential estrogenic burden in Irish waters. The condition factor (CF) was calculated to determine the effects at the organism level, while hepatosomatic (HSI) and gonadosomatic (GSI) indices were used to determine effects at the organ level. In addition, the induction of vtg in male fish was used to assess potential exposure to estrogenic chemicals. The YES assay assessed the estrogenic burden, while quantitative chemical analysis permitted the interrogation of EDC concentrations. Tarrant et al. (2008) focused their study on three rivers in Ireland: the Liffey (Co. Dublin), Lee and Bandon (Co. Cork), only observing vtg induction in male fish sampled downstream of the Liffey at Osberstown STP. To date, no studies regarding the effects of endocrine disruption in wild fish populations in the SIRBD of Ireland have been reported. The purpose of this study was to further characterise Irish freshwater habitats, using an integrated risk assessment approach combining biological and chemical methods. Close collaboration across disciplines is necessary for such an assessment, as there is an increasing societal pressure to move toward *in vitro* and *in vivo* testing (Vermeire et al., 2007). The application of such an assessment to EDCs is presently being investigated in EU funded multi-laboratory collaborative studies (Bridges and Bridges, 2004).

2. Materials and methods

2.1. Study sites and sampling of feral fish

Adult male brown trout were collected from upstream and downstream sites on 11 rivers within the SIRBD of Ireland (Fig. 1). The collection target at each site was 10 male trout and each site was sampled once. All fish were captured by electrofishing in conjunction with members of the Shannon Regional Fisheries Board (SRFB) during the pre-spawning period of late summer. Test sites were approximately 1–2 km downstream of discharge points, representing a range of contaminant sources as determined by general land use (e.g., proximity to STPs, industrialized or agricultural activity). The majority of these sites was selected because they were located near multiple sources of EDCs. The reference control sites were approximately 1–2 km upstream in areas identified as devoid of such sources. Due to the absence of physical barriers, the migration of fish between upstream and downstream locations could not be ruled out. Experiments were conducted in accordance with national and international guidelines (Directive 2007/526/EC of the European Commission), for the protection of animal welfare.

2.2. Quantitative chemical analysis

For chemical analysis, sites were strategically selected to give an estimate of the estrogenic loading of the test sites carried out for vtg studies. Athlone Lock was positioned downstream of tributaries: Camlin, Hind and Inny. The Banagher site was positioned below the rivers Clodiagh and Brosna. Testing was also carried out in the river Nenagh. An inert stainless steel telescopic rod and cup (Telescoop, Reagecon, Ireland) was utilized to procure both liquid and sediment samples. Solid samples were transferred into solvent (organic)/acid (inorganic) washed jars and were dried to constant weight, then stored at 4 °C until analysis. Grab liquid samples were collected into 2.5 L amber glass Winchesters with 5% modifier for preservation and transported to the laboratory in cool bags with icepacks, where immediate sample treatment was performed. Recovery experiments for analytes were conducted according to Reid et al. (2007) for solid phase extraction (SPE) analyzing liquid samples and pressurized liquid extraction (PLE) analyzing sediment and sludge samples. For the majority of analytes tested, the Log P_{ow} (octanol–water partition co-efficient) was above 3.5, therefore the addition of an organic modifier assisted partitioning of analytes into sample solution, maximizing the extraction onto the sorbent phase. For an SPE analysis, a C18 reversed phase was found to be the most suitable for the quantitative extraction of both steroidal and non-steroidal estrogens from environmental samples. Phthalates and alkylphenols were chosen for this study due to their wide occurrence in the environment. The phthalates were dibutyl phthalate (DBP), di(2-ethylhexyl) phthalate (DEHP), di-isononyl phthalate (DINP) and diisodecyl phthalate (DIDP) and the alkylphenol was 4-nonylphenol (NP).

2.3. Preparation of tissue samples

Specimen collection was carried out on-site, where sampled fish were terminally anaesthetised using Tricaine (Sigma-Aldrich, Ireland) and their body length and weight recorded. Fish were bled by caudal puncture into heparinised syringes and the protease inhibitor, aprotinin, was added (3–7 TIU/mg from bovine lung, Sigma). The plasma samples were centrifuged at 4 °C at 10,000*g* for 5 min, transferred to cryogenic tubes, frozen in liquid nitrogen and stored at −80 °C until vtg analysis was carried out. Liver samples were excised, weighed and snap frozen in liquid nitrogen on site. Gonads were also excised and fixed in Bouins fixative solution until histological analysis, conducted according to Nolan et al. (2001), was carried out. Gonadal intersex uses the testis–ovum as an endpoint measure and as such, is characterized by the development of oocytes within the testicular tissue of feminized males. This particular endpoint is the most common method to date for the screening and testing of potential EDCs (Lin et al., 2009). The HSI and GSI were calculated for each brown trout by expressing liver or gonad weight as a percentage of the total body weight. The CF was determined by [total body weight (g)/length (mm)^3] × 100,000.

2.4. Vitellogenin analysis

Semi-quantitative vtg analysis of male brown trout plasma was performed, using an ELISA technique. Antibodies and reagents were purchased from Biosense Laboratories in Norway (product no. V01002401). The vtg detecting monoclonal antibody, BN-5, was raised against Atlantic salmon (*Salmo salar*). Due to the cross-reactivity of the antibody with vtg from other fish species, the antibody may also be used for analysis of plasma samples from a variety of other species, including *Salmoniformes*, *Pleuronectiformes* and some *Perciformes* species. Each plasma sample was tested in triplicate in addition to both negative and positive (purified vtg from Atlantic salmon) controls. Using o-phenylenediamine as a substrate, optical density was measured with a microplate reader at 492 nm (Anthos Labtect Instruments, Salzburg). Due to the semi-quantitative nature of this assay, it was not possible to measure absolute amounts of the biomarker. Therefore, the level of vtg in plasma samples of brown trout was reflected in the absorbance values; the higher the absorbance value, the higher the level of the biomarker in the sample (Biosense Laboratories, Norway).

2.5. Yeast estrogen screen assay

The yeast estrogen screen (YES) was conducted according to Routledge and Sumpter (1996) and all media used for the assay was prepared according to the original protocol.

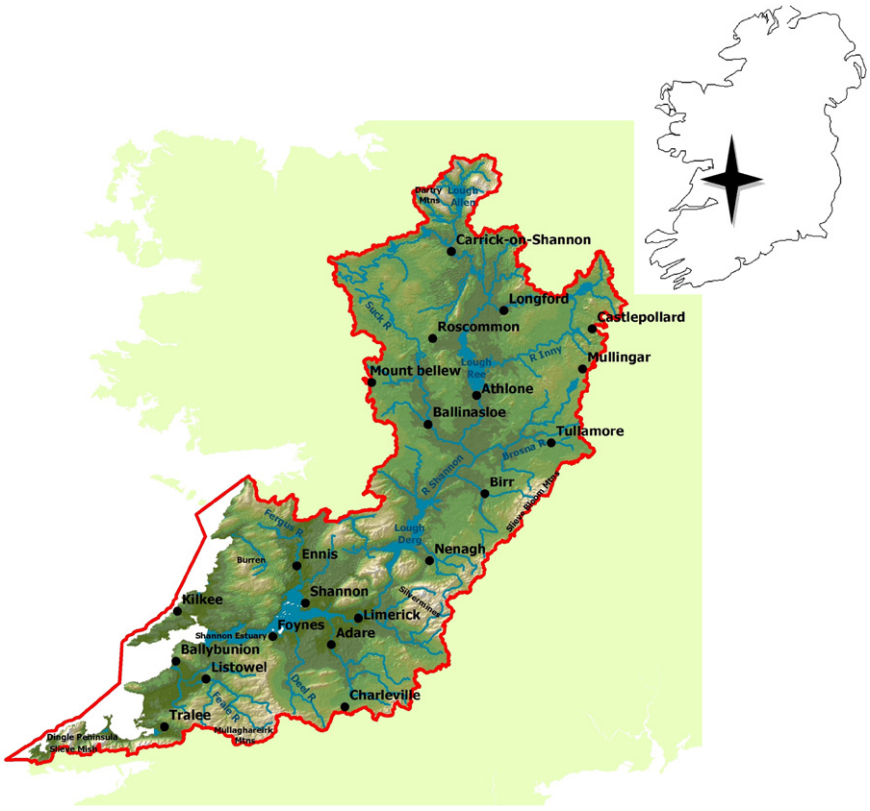

Fig. 1. The Shannon International River Basin District of Ireland.

A volume of 125 μL of yeast from the yeast stock stored at −20 °C was added to the growth medium and grown on an orbital shaker for 24 h. The natural estrogen, E_2 was used as a positive control. A volume of 10 μL of each test concentration was transferred into microtiter plates and the assay medium, containing 2 mL yeast with an absorbance at 640 nm of 1.0 and the chromogenic substrate, chlorophenol red-β-D-galactoside (CPRG) was then added to the wells in 200 μL aliquots. The plates were sealed and shaken on a plate shaker for 2 min. After incubation for 24 h at 32 °C, the plates were shaken for 2 min and incubated for a further 1 h. The plates were then read at 560 nm for color development and at 698 nm for turbidity with a plate reader. At least two independent experiments were performed in triplicate for each sample.

2.6. Statistical analysis

A Two Proportion Hypothesis Test was used to analyze the vtg data obtained from electrofishing and significant differences ($p \leq 0.05$) are indicated where applicable. The CF, HSI and GSI were analyzed for site significance using the two sample *t*-test with MINITAB release 13.2 (MINITAB Inc., PA, USA). Significant differences ($p \leq 0.05$) are indicated where applicable. Data from the YES assay were tested for normality using the Anderson–Darling test. For data that was not normally distributed, the differences between the equality of population medians and diverse treatment groups were assessed using the Kruskal–Wallis test and the Mann–Whitney test. One-way analysis of variance (ANOVA) was used for normally distributed data and the 2-sample *t*-test was used to compute the difference between the means of the diverse treatment groups ($p \leq 0.05$).

3. Results

3.1. Chemical analysis

Endocrine disrupting chemicals were identified in both water and sediment samples collected at the selected locations. Sampling at Banagher captured waters from the Clodiagh and Brosna rivers. Here, 4 phthalates and 1 alkylphenol were identified in μg\L concentrations. The levels reported were DBP at 7.79 μg\L, DEHP at 62.39 μg\L, DINP at 0.52 μg\L, DIDP at 1.81 μg\L and NP at 1.83 μg\L (Table 1). Sediment analysis at the same location identified EDCs in mg/kg concentrations: DBP 11.8 mg/kg, DEHP at 25.27 mg/kg, DIDP at 10.26 mg/kg and NP at 0.78 mg/kg (Table 2). Sampling at Athlone Lock captured waters from the Camlin, Inny and Hind rivers. Four phthalates and 1 alkylphenol were identified in μg\L concentrations. The levels reported were DBP at 11.52 μg\L, DEHP at 73.13 μg\L, DINP at 1.19 μg\L, DIDP at 0.81 μg\L and NP at 5.32 μg\L (Table 1). Sediment analysis also identified EDC's in mg/kg concentrations: DBP 19.44 mg/kg, DEHP at 10.48 mg/kg, DINP at 0.13 mg/kg, DIDP at 7.46 mg/kg and NP at 0.56 mg/kg (Table 2). Additional water and sediment samples were collected from the Hind river. EDCs were detected in water at DBP at 7.93 μg\L, DEHP at 60.81 μg\L, DINP at 1.89 μg\L, DIDP at 1.23 μg\L and NP at 20.91 μg\L (Table 1) and in sediment at DBP 9.46 mg/kg, DEHP at 7.09 mg/kg, DINP at 6.16 mg/kg, DIDP at 0.10 mg/kg and NP at 0.82 mg/kg (Table 2).

Table 1
River water concentrations—recovery adjusted average values from a 12 month period.

Analyte	Athlone lock	Banagher	Hind	Nenagh	Camlin	MRF (%)[a]	RSD[b] (%)
DBP	11.52[c]	7.79	7.93	1.88	0.49	73	12.1
DEHP	73.13	62.39	60.81	2.24	0.77	84	17.6
DINP	1.19	0.52	1.89	0.85	0.39	115	22.7
DIDP	0.81	1.81	1.23	0.49	0.14	84	19.2
NP	5.32	1.83	20.91	0.03	0.66	97	18.9

[c]Limit of detection; DBP=dibutyl phthalate; DEHP=di(2-ethylhexyl) phthalate; DINP=di-isononyl phthalate; DIDP=diisodecyl phthalate; NP=4-nonylphenol.

[a] Method recovery factor.
[b] Relative standard deviation.
[c] Concentrations in μg/L.

Table 2
River sediment concentrations—recovery adjusted average values from a 6 months period.

Analyte	Athlone lock	Banagher	Hind	MRF (%)[a]	RSD(%)[b]
DBP	19.44[c]	11.8	9.46	92	3.4
DEHP	10.48	25.27	7.09	87	4.2
DINP	0.13	< LOD	6.16	84	7.9
DIDP	7.46	10.26	0.10	92	9.6
NP	0.56	0.78	0.82	82	5.2

[d]Limit of detection; DBP=dibutyl phthalate; DEHP=di(2-ethylhexyl) phthalate; DINP=di-isononyl phthalate; DIDP=diisodecyl phthalate; NP=4-nonylphenol

[a] Method recovery factor.
[b] Relative standard deviation.
[c] Concentrations in mg/kg.

Table 3
Biological characteristics of brown trout (*Salmo trutta*) in the studied rivers.

River system	Site	CF[c]	HSI[d]	GSI[e]
River Hind	Control	1.23 ± 0.25	1.24 ± 0.30	0.23 ± 0.24
	D/S	1.18 ± 0.24	1.41 ± 0.24	0.88 ± 1.44
River Camlin	Control	0.43 ± 0.24	1.23 ± 0.48	0.36 ± 0.34
	D/S	0.91	1.77	2.50
River Inny	Control	0.74 ± 0.19	1.18 ± 0.27	1.05 ± 0.47
	D/S	0.87 ± 0.18	0.84 ± 0.16	1.05 ± 1.52
River Clodiagh	Control	0.99 ± 0.30	0.98 ± 0.25	0.77 ± 0.63
	D/S	0.93 ± 0.33	1.13 ± 0.44	0.29 ± 0.52
River Camcor	Control	1.04 ± 0.17	0.85 ± 0.35	2.91 ± 1.30
	D/S	0.98 ± 0.23	0.97 ± 0.19	4.14 ± 0.92
River Little Brosna (Site 1)	Control	0.75 ± 0.14	1.64 ± ± 0.64	0.05 ± 0.04
	D/S	0.54 ± 0.009	2.03 ± 0.10	0.02 ± 0.001
River Big Brosna	Control	0.71 ± 0.04	1.03 ± 0.26	0.03 ± 0.004
	D/S	0.91 ± 0.13	1.02 ± 0.09	0.03 ± 0.009
River Nenagh	Control	1.20 ± 0.40	0.98 ± 0.21	0.10 ± 0.18
	D/S	0.96 ± 0.31	1.45 ± 0.96	0.07 ± 0.07
River Ballyfinboy[a]	Control	0.72 ± 0.17	1.64 ± 0.28	0.17 ± 0.29
River Little Brosna (Site 2)	Control	1.38 ± 0.24	1.16 ± 0.57	0.50 ± 0.33
	D/S	1.14 ± 0.04	0.94 ± 0.20	0.59 ± 0.82
River Tullamore	ND[b]	-	-	-

[a] D/S data for the River Ballyfinboy could not be calculated due to logistical problems encountered during sampling.
[b] ND=not determined for the River Tullamore—data could not be calculated because sampling was carried out at the control location only and all fish sampled were female; D/S=downstream.
[c] CF=condition factor.
[d] HSI=hepatosomatic index.
[e] GSI=gonadosomatic index.

Water samples from the Nenagh river identified EDC levels at DBP at 1.88 μg\L, DEHP at 2.24 μg\L, DINP at 0.85 μg\L, DIDP at 0.49 μg\L and NP at 0.03 μg\L (Table 1), while the Camlin river concentrations were DBP at 0.49 μg\L, DEHP at 0.77 μg\L, DINP at 0.39 μg\L, DIDP at 0.14 μg\L and NP at 0.66 μg\L (Table 1).

3.2. Somatic indices and condition factor

The biological characteristics of fish are given in Table 3. The HSI, GSI and CF were used as general indicators of overall fish health. The HSI values of male brown trout were not significantly affected between upstream control and downstream locations, although differences were observed. In fish sampled downstream of the Clodiagh, Camcor, Little Brosna, Nenagh, Hind and Camlin rivers, HSI values were raised when compared to the control site (Table 3). In addition, GSI values did not differ significantly but were considered to be low at the downstream site on the Nenagh, Little Brosna and Clodiagh rivers (Table 3). Differences were also observed in the CF values, although none were significant. The CF was considered low in the downstream sites on the Camcor, Camlin, Clodiagh, Nenagh and Little Brosna rivers (Table 3).

3.3. Intersex

Gonadal intersex, using the testis–ovum as an endpoint measure, was used to screen for potential EDC-exposure. Gross histological examination of the gonads revealed no apparent major effects on gonadal tissue in any of the male brown trout sampled from the selected rivers.

3.4. Vitellogenin induction

Vitellogenin was detected in male brown trout at 8 of the 11 rivers with significant levels reported at 3 of those rivers. The downstream sampling of fish yielded a considerably higher percentage of positive samples, ranging 14–100%, which would indicate extensive exposure to estrogenic compounds (Fig. 2). In addition, vtg induction was detected at 4 upstream locations ranging 16–33%. While migration of brown trout could not be ruled out at any site, contaminant sources were identified which might explain the detectable vtg levels in males fish. No plasma samples were available for the downstream sites in the rivers Tullamore and Ballyfinboy. The Tullamore river has been identified, in a recent water quality report by the Environmental Protection Agency (EPA), as being seriously polluted either continuously or intermittently for many years (EPA, 2008). Sampling of the Ballyfinboy river yielded only female brown trout. Significant estrogenic activity was detected in the river Hind, where raised vtg levels were recorded in 16% of the fish sampled upstream and in 80% sampled downstream. Sampling in the river Camcor detected vtg in 20% of the fish sampled upstream; however, no evidence of vtg induction was observed at the downstream site. The Clodiagh River was selected due to its close proximity to an area of extensive agricultural activity. Here, 30% of the fish were positive for vtg induction, while 14% was reported downstream. In the river Brosna, sampling upstream and downstream of the nearby STP indicated raised vtg levels in 34% and 67% of the fish sampled, respectively. In the Little Brosna, vtg induction was significantly elevated in 100% of the male fish sampled downstream, while there was no evidence of estrogenic activity at the upstream site. The Little Brosna was also assessed at a second STP and raised vtg levels were detected in 50% of the fish sampled downstream only. In the river Nenagh, vtg was also significantly raised in 67% of the male fish sampled downstream, while upstream was negative. The downstream site for the river Inny was positive for detectable vtg levels in 16% of male fish sampled; however, the upstream location was negative. The river Camlin was assessed, but there was no evidence of vtg induction in male brown trout sampled upstream or downstream of the nearby STP. Although extensive electrofishing was carried out on the river, brown trout sample sizes were small. As a result, further characterization of this river would therefore be recommended. These results suggest that the use of vtg induction in male fish as a specific biomarker of EDC-exposure may be more sensitive than the measured reproductive effects.

3.5. YES assay analysis

The potential estrogenicity at each site was measured by the YES assay and the EEF was calculated to estimate an activity relative to the positive control, E_2 (Table 4). In the Hind river, an activity was reported at 1.05 ng/L EEF downstream. However, there was no sample available for the upstream site due to sampling difficulties. Estrogenicity in the Camcor river was

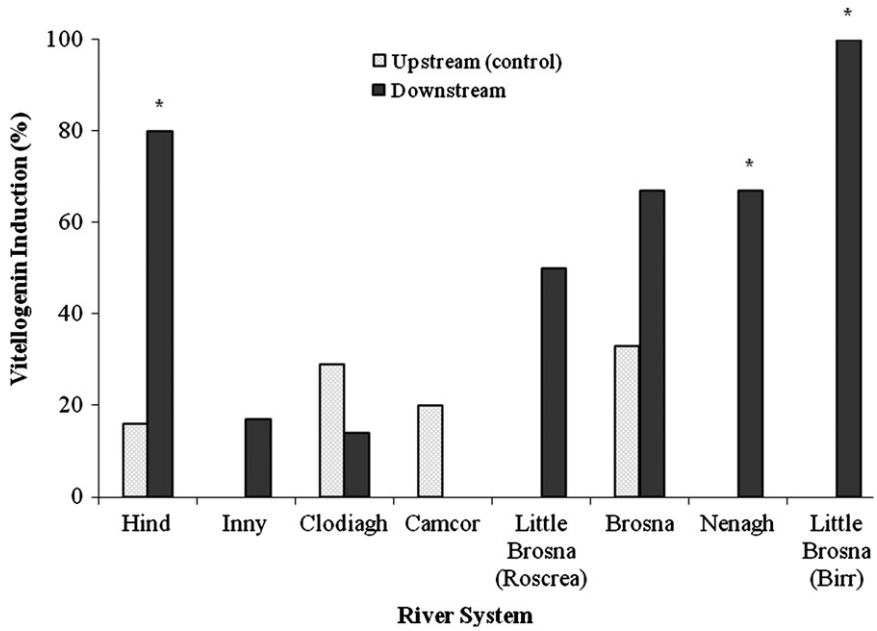

Fig. 2. Vitellogenin induction (%) determined in male brown trout (*Salmo trutta*) in the Shannon International River Basin District of Ireland. Asterisks denote significant difference ($p \leq 0.05$).

Table 4
Estrogenic activity of river systems within the SIRBD region in the Yeast Estrogen Screen, expressed as E_2 equivalency factor (EEF).

River system	Site	EEF
Hind	D/S	1.05 ± 0.03[a]
Clodiagh	D/S	1.42 ± 0.93
Camcor	Control	0.53 ± 0.12
	D/S	0.94 ± 0.31
Brosna	D/S	1.45 ± 0.93
Nenagh	Control	2.67 ± 0.84
	D/S	1.02 ± 0.09

[a] Concentrations in ng/L; D/S=downstream.

confirmed upstream and downstream at 0.53 and 0.94 ng/L EEF, respectively. In the Clodiagh river, there was a significant induction of β-galactosidase activity at the downstream location with 1.42 ng/L EEF (Table 4) but due to sampling difficulties, a value could not be calculated for the upstream site. A significant induction was confirmed at the Brosna river downstream site only, where β-galactosidase activity was calculated at 1.45 ng/L EEF (Table 4). Sampling was not possible on the Little Brosna river. YES assay analysis on the Nenagh river reported estrogenic activity upstream and downstream at 2.67 and 1.02 ng/L EEF (Table 4), respectively.

4. Discussion

4.1. Chemical analysis

Phthalates and alkylphenols have been implicated in many studies as having estrogenic properties (Knudsen and Pottinger, 1999; Streck, 2009); therefore, 4 phthalates and 1 alkylphenol were chosen for this study. The selected analytes were detected in water at μg\L concentrations and in sediments at mg/kg levels, with DEHP occurring at the highest concentration in water samples (0.77–92.84 μg\L). Fromme et al. (2002) reported comparable DEHP levels in surface waters at 97.8 μg\L, but lower sediment levels, ranging 0.21–8.44 mg/kg. In 2000, DEHP was listed as a hazardous substance to be controlled in surface water by the European community (DCE 2000/60/CE) and in 2007; the European Directive proposed a norm for environmental quality (NQE) of 1.3 μg/L DEHP in surface water (Dargnat et al., 2009). The second most abundant phthalate was DBP, which was detected at concentrations similar to those found in Swedish waters at 6–22 μg\L (Paxéus, 1996). Clara et al. (2010) also confirmed the presence of DBP in Austrian wastewaters, but at a lower concentration of 2.4 ng/L. The use of DIDP and DINP increased by almost 50% between 1999 and 2006, due to EU restrictions on the use of DEHP (Clara et al., 2010). Since then, attention has increased towards their environmental impact. This study detected both compounds in notable concentrations; however, Björklund et al. (2009) detected DINP up to 85 μg\L and DIDP up to 17 μg\L in stormwater.

The impacts of an NP in the environment include feminization of aquatic organisms (Milnes et al., 2006), a decrease in male fertility (Kinnberg et al., 2000) and the survival of juveniles at concentrations as low as 8.2 μg/L (Yokota et al., 2001). A critical review of literature by Soares et al. (2008) reported an NP water concentrations in rivers, oceans and estuaries at levels up to 158 μg\L. Sediments were also reviewed with the highest concentrations adjacent to urbanised areas. As the majority of these findings were attributed to STP discharges, this may also be the reason for the levels reported in our study.

4.2. Somatic indices and condition factor

Parameters such as HSI, GSI and CF are widely used as indicators of physiological changes in organisms. Evaluation of these endpoints in this study indicated that although changes were not significant, variances did occur between control and test sites. HSI values in brown trout from 6 rivers were raised when compared to control sites. Vtg was also detected at these rivers. A common consequence of increased vtg production is a corresponding increase in HSI. However, this increase may also be attributed to enhanced detoxification processes in the liver (Andersson et al., 2007). Decreased GSI scores were evident in 3 rivers, where vtg was also confirmed. Laboratory studies have

observed inhibited testicular growth in three-spined sticklebacks exposed to high concentrations of EE_2 (Andersson et al., 2007) and in fathead minnow and common carp accompanied with a reduction in sperm count (Gimeno et al. 1998). CF values were slightly decreased in 5 rivers. Studies have reported significantly lower CF in fish from contaminated sites when compared to control sites (Jenkins, 2004). Although the biological parameters do not differ significantly, these findings may suggest that effluents from Irish STPs could potentially alter gonad growth and liver function of feral male brown trout populations and consequently the general well-being of the species.

4.3. Intersex

Electrofishing was conducted prior to the spawning period and within a 3 week period to minimize variations, due to seasonal change, however, inherent changes may have occurred during this period. Although, this study does not report any evidence of intersex brown trout in the SIRBD district in Ireland, a recent study carried out in Athlone Institute of Technology (AIT) has demonstrated the presence of intersex in male roach (*Rutilis rutilis*) sampled from 2 of the sites investigated in the present study (McGee, personal communication). Many studies have reported intersex fish in STP-contaminated waters (Jobling et al., 2002; Diniz et al., 2005; Bjerregaard et al., 2006). In addition, Tyler et al. (2005) investigated the difference in estrogenic responses between rainbow trout and roach. It was confirmed that estrogenic contaminants had the ability to bioconcentrate in the bile of fish and that the level of accumulation affected the endocrine responsiveness of the fish. Therefore, life-stage may account for the absence of intersex in brown trout in the SIRBD region in Ireland.

4.4. Vitellogenin induction by EDCs

Research to date has shown that the biggest problem with the presence of estrogens in surface water is their effects on fish populations (Matthiessen et al., 2002). Eleven river systems were evaluated for estrogenicity in this study and vtg was employed for the assessment of estrogenic exposure in feral fish populations. Endocrine disruption was detected in male fish sampled throughout the region, where 8 of the 11 rivers selected were positive for vtg induction, including 3 sites, which were significantly raised. In contrast, Tarrant et al. (2008) focused their study on three Irish rivers: the Liffey, Lee and Bandon and the only evidence of endocrine disruption was obtained downstream of the Liffey at Osberstown STP. Their findings observed detectable vtg levels in 2% of the fish upstream and 26.3% downstream. In the present study, vtg was detected in both control and test locations, identifying 33% as the highest incidence upstream and 100% as the highest incidence downstream. Many other studies have observed endocrine disruption in fish exposed to STP discharges (Purdom et al., 1994; Jobling et al., 1996; Harries et al., 1997). Purdom et al. (1994) reported that male rainbow trout (*Oncorhynchus mykiss*) caged in STP discharges in England had elevated plasma vtg levels (up to 147 mg/ml) that were equal to or exceeded the levels found in mature females. In Eastern Europe, the river Elbe is reported as one of the most polluted aquatic ecosystems in the Czech Republic. Studies indicate reduced gonad size, EROD induction, vtg induction and intersex in male chub (*Leuciscus cephalus*) as some of the harmful effects of aquatic pollutants, such as EDCs (Randak et al., 2009). In addition, an AIT study has confirmed the feminization of genetically male roach sampled from the rivers Brosna and Inny (McGee, personal communication).

Estrogenic activity was detected at a number of upstream control locations in this study: the rivers Hind, Clodiagh, Camcor and Brosna. At each of these sites, indicators were present which caused concern. The control site on the river Hind was in close proximity to a disused effluent pipe which may have contained residual contaminants and as such, may have led to the disruption of normal biochemical functions of the native brown trout. The control site for the river Clodiagh was also in an area of concern, where extensive agricultural activity was located. Increased vtg in male fish has been confirmed in areas affected by agricultural runoff (Bjerregaard et al., 2009). Green algae, identified as *Cladophera glomerata*, were also present at the control site and it has been reported to bloom especially well at wastewater disposal sites (US EPA, 2007). These observations may have been responsible for the anomaly observed at the Clodiagh upstream site. The control site for the river Camcor was located approximately 1.6 km upstream from an STP and was the only site, where vtg levels were detected upstream but none downstream. General observations at the downstream site included a brown discolouration of the water, a pungent odor and the presence of slime on the river bed. Sewage fungus, which is often observed as slime, is associated with untreated or poorly treated sewage effluent (Gray, 2004). In addition, heavy rainfall had preceded the sampling date, so it may be realistic to conclude that this combination of events may have caused irregular discharges from the STP, thus causing the induction of vtg in male fish and possibly the migration of these fish to the upstream location. Heavy rainfall has been known to increase upstream migration of brown trout (Høgåsen, 1998). Lastly, vtg induction was reported at both upstream and downstream locations on the river Brosna. An SRFB confirmed the presence of a municipal dump, which was no longer in use but which was in close proximity to the selected control site. Additional studies carried out at an AIT have investigated the negative impact of disused dumps at various locations in the Midlands region in Ireland. High levels of known EDCs were reported to leach from the dump sites into surrounding waters, and as a result may present an environment that may adversely affect the reproductive nature of exposed aquatic inhabitants (Reid et al., 2007). Moreover vtg induction is supported by the YES assay findings, where 5 rivers were positive for both vtg induction and β-galactosidase activity. A number of studies have reported a good correlation between these assays (Huggett et al., 2003; Ying et al., 2009), including Tarrant et al. (2008) who assessed 3 rivers in Ireland. To date, the implications of these responses in fish in terms of reproductive success and viability are still undetermined (WHO, 2002).

The findings in this study confirm the susceptibility of Irish waterways and their inhabitants to the ever-increasing levels of EDCs. The consequences of such an estrogenic burden are highlighted with the biochemical and reproductive anomalies identified in upstream and downstream locations, but the physiological significance of unnatural vtg production in male fish remains unclear. However, it has been shown that synthesis of unnaturally high concentrations in response to E_2 can lead to failure of vital organs and death (Herman and Kincaid, 1988).

4.5. YES assay analysis

The yeast estrogen screen (YES) has been identified as a suitable tool to detect estrogenic activity of individual chemicals and mixtures of chemicals, including environmental samples, which interact with the human estrogen receptor (Routledge and Sumpter, 1996; Pawlowski et al., 2004). In the present study, the YES assay was used as a screening system to detect the estrogenic activities of environmental water samples from locations within

the SIRBD of Ireland. Estrogenicity was evident in 5 rivers at downstream sites, but also in 2 control locations. The YES assay placed the estrogenicity of these rivers in the concentration range of E_2 required to induce vitellogenesis in male rainbow trout (Routledge et al., 1998). Calculated EEF values ranged 0.53–2.67 ng/L in this study, which were higher than the E_2 equivalents reported by Tarrant et al. (2008). These findings clearly document the presence of elevated levels of estrogenic activity in Irish rivers.

5. Conclusion

In the past number of decades, research has led to more significant knowledge regarding the vast number of chemicals entering our waterways. EDCs have received considerable attention worldwide and have been identified as emerging substances that are currently not included in routine monitoring programmes at the European level. However, they may become candidates for future regulation (Tilghman et al., 2009). The findings of this integrated biological and chemical assessment of sewage effluent receiving waters, highlights the SIRBD of Ireland as an area of concern for endocrine disruption. The widespread induction of vtg in male fish sampled in this region in addition to irregularities in somatic indices, suggests that chemicals entering the aquatic environment, have the ability to alter normal biological processes. The YES assay confirms estrogenic activity in addition to chemical analysis identifying known EDCs in high concentrations. These observations, coupled with the recent discovery of intersex fish in Irish waters, indicate that EDC levels are sufficient to cause an endocrine disruption. Such findings highlight the need to re-examine existing monitoring strategies. A combined approach is therefore recommended, where chemical identification and a correlation between concentration and the resultant effect may prompt the restructuring of monitoring strategies in Ireland.

Conflict of interest

The authors have no conflicts of interest

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