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Assessment of (anti-) oestrogenic and (anti-) androgenic activities of final effluents from sewage treatment works

Science Report – SC020118/SR

SCHO0207BMAX-E-P

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Steve Killeen

Head of Science

Executive summary

Background

In March 2002, the Agency committed itself to furthering the risk assessment and risk management of oestrogenically active effluents. The purpose of this project was to establish the current oestrogenic activity of up to 43 effluents to help inform site selection for an 'Endocrine Disruption Demonstration Programme' being planned in collaboration with the water industry. The Demonstration Programme is being developed to evaluate the efficacy of existing and improved treatment processes at reducing steroid concentrations and endocrine disrupting effects in effluents. Twenty-five of these effluents were previously identified as causing 'feminising' impacts on wild fish.

The objective of the project was to provide information on the oestrogenic chemical profile and activity of 43 priority sewage treatment works effluents. The data collated in this study will be used in a separate risk assessment of the impacts of steroid discharges. That risk assessment will inform the selection of sites for an endocrine disruption demonstration programme to evaluate treatment options.

The objective was achieved by a) analysis of the effluent samples for steroid oestrogens, namely oestrone (E1), 17β-oestradiol (E2) and 17α-ethinyloestradiol (EE2); b) analysis of the effluent samples for alkylphenols (nonylphenol and lower chain ethoxylates [NP1-5EO]); c) screening of the effluent samples for (anti-) oestrogenic and (anti-) androgenic activity (in YES and YAS assays).

Results

We measured steroid concentrations in the ranges of <1 to 100 ng l^{-1} for oestrone, <1 to 22 ng l^{-1} for oestradiol and <1 to 3.2 ng l^{-1} for ethinyloestradiol. There were high levels of steroids, primarily oestrone, in final effluents from a number of locations. We found oestrone concentrations of $\geq$ 20 ng l^{-1} in 13 of 25 final effluents on one or more of the sampling occasions in Phase 1 and in 8 of 18 final effluents on one or more of the sampling occasions in Phase 2. A risk assessment of the effluents, taking into account data on STW effluent flow, steroid loads and river dilution is presented in a separate risk assessment report.

In this report, comparisons of the predicted concentrations for the natural and synthetic steroids with the proposed Predicted No Effect Concentrations (PNECs) for these compounds indicate that a number of these effluents even following dilution, would result in receiving water steroid concentrations that could produce oestrogenic effects in resident fish populations downstream of the discharges.

With regard to the analysis of some final effluents, no quantifiable peaks could be discerned, particularly for the synthetic steroid ethinyloestradiol. This is currently being addressed through method optimisation by the Environment Agency's National Laboratory Service, overseen by a technical working group.

The levels of nonylphenol in the final effluent samples taken in both Phases 1 and 2 ranged from the limit of detection (1 $\mu g\ l^{-1}$) to maximum concentrations of 7.7 $\mu g\ l^{-1}$ and 3.8 $\mu g\ l^{-1}$ respectively. The majority of effluent concentrations were in the range of <1 to 3 $\mu g\ l^{-1}$. Concentrations above 3 $\mu g\ l^{-1}$ were measured at only seven of the 25 sites sampled in Phase 1 and at one of the 18 sites sampled in Phase 2.

Risk assessment using these results suggests that nonylphenol (at least in isolation) is less likely to elicit oestrogenic effects in fish downstream of STWs, once dilution of the effluents is taken into account at the majority of sites.

For NP1EO, no samples in Phase 1 and only two samples in Phase 2 exceeded 10 $\mu g\ l^{-1}$. For NP2EO, one sample in Phase 1 and one sample in Phase 2 exceeded 10 $\mu g\ l^{-1}$. For NP3-5EO, seven samples in Phase 1 and 13 samples in Phase 2 exceeded 10 $\mu g\ l^{-1}$. The significance of the data in terms of oestrogenic responses is, however, associated with considerable uncertainty, due to limited ecotoxicological effect data against which to compare measured concentrations.

All of the samples tested exhibited some oestrogenic activity in the YES assay, with potency equivalents of between 0.4 and 20.5 ng E2 l^{-1} for the Phase 1 samples and between 0.9 and 42.7 ng E2 l^{-1} for the Phase 2 samples. None of the samples tested exhibited any clear anti-oestrogenic activity.

All samples, though, exhibited significant anti-androgenic activity in the YAS assay, with potency equivalents of between 21.3 and 228 μg flutamide l^{-1} for the Phase 1 samples and between 90.7 and 1231 μg flutamide l^{-1} for the Phase 2 samples. None of the final effluent samples from Phase 1 and only final effluent samples from one location in Phase 2 were found to contain any detectable androgenic activity.

Recommendations

The issues associated with the analysis of steroids and the role of interferents in the extraction procedure need to be resolved. Since completion of this project a technical working group has been convened by the Agency to address this and other analytical issues.

Further studies are needed to assess the relationship between the results of *in vitro* assays and chemical concentrations of oestrogenic substances. Since the completion of this project a biological effects technical working group has been convened by the Agency to address this issue and to ensure that extraction methodologies for *in vitro* assays and chemical analysis are consistent.

The substances responsible for the anti-androgenic activity are unknown. The wider biological significance anti-androgenic activity of STW effluents (notably in the generation of intersex) requires investigation. Defra are currently funding a project to determine the biological significance of androgen antagonism in the stickleback as part of their research programme on endocrine disruption in the aquatic environment (EDAQ). The detection of anti-androgenic activity in the current study suggests, that this, and anti-androgenic biological effects should also be taken into consideration when monitoring treatment options during the demonstration programme.

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1 Introduction

1.1 Scientific and policy background to the project

Research over the past 15 years has revealed a widespread occurrence of male intersex fish in English rivers (Environment Agency, 1998a, 2006a). In some cases, the intersex condition has been severe and at a high incidence in the population. Typically, males exhibit female-like characteristics, possessing female oviducts and eggs within the testes. Such effects have been shown to be permanent and progressive, and lead to reduced reproductive performance. These effects have been causally linked to exposure to sewage effluents containing mixtures of a number of 'feminising' chemicals, including natural and synthetic steroid oestrogens, alkylphenols, and alkylphenol ethoxylates.

Initial research programmes focused only on a limited number of highly impacted sites. A recent survey then established a more comprehensive view of the spatial extent and severity of oestrogenic effects in wild fish (roach) in English and Welsh rivers (Environment Agency, 2006a). This latest survey also reviewed predictions of impacts on fish at high, medium and low risk sites. The categorisation of sites into high, medium and low risk was based on comparisons of model predictions of steroid concentrations in the environment (predicted environmental concentrations PECs) with predicted no effect concentrations (Environment Agency, 2006b). Of the sites surveyed, 25 of the medium and high-risk sites downstream of STWs were found to be associated with the greatest incidence and severity of intersex effects in fish. Following presentation of these findings, the Department for Environment, Food and Rural Affairs (Defra) issued guidance for the inclusion of an endocrine disruption demonstration programme as part of the environmental programme to be undertaken by water companies in the period 2005-2010. The Demonstration Programme is being developed to evaluate the efficacy of existing and improved treatment processes at reducing steroid concentrations and endocrine disrupting effects in effluents.

Preliminary work was required to inform the selection of sites for inclusion in the demonstration programme. In discussion with Defra and the Water Industry the Agency agreed to undertake further evaluation of the 25 medium and high risk sites associated with environmental impacts. The effluents from sewage treatment works at these locations were to be sampled and tested to establish their current quality and confirm that they contain oestrogenically active substances. The testing of these 25 effluents constituted Phase 1 of the programme.

Subsequently, a further 18 sites were included for confirmatory analysis. This was to include additional sewage effluents to widen the geographical coverage. Some of these STWs were also identified as high or medium risk from model predictions. The testing of these effluents constituted Phase 2 of the programme.

1.2 Objectives

1.2.1 Overall Objective:

To provide information on the oestrogenic chemical profile and (anti-) oestrogenic / (anti-) androgenic activity of 43 priority sewage treatment works effluents. The data collated in this study was used in a separate risk assessment of the impacts of steroid discharges, which influenced site selection for the endocrine disruption demonstration programme.

This was achieved by:

- Analysing effluent samples for steroid oestrogens (oestrone, 17β-oestradiol, 17α-ethinyl oestradiol);
- Analysing effluent samples for alkylphenols (nonylphenol and lower chain ethoxylates [NP1-5EO]);
- Screening effluent samples for (anti-) oestrogenic and (anti-) androgenic activity (in YES and YAS assays).

2 Sewage treatment works sampled

We sampled the sewage treatment works (STWs) in two phases. Twenty-five STWs were sampled in Phase 1. These were sites that were previously predicted to be high or medium risk and at which the greatest level and severity of effects were found in wild fish in a recent spatial survey of oestrogenic effects in roach (Environment Agency, 2006a). A further 18 STWs were sampled in Phase 2 to widen the geographical coverage. Some of these additional effluents were identified as high or medium risk from model predictions.

2.1 Sampling locations

Table 2.1 summarises the 25 STWs operated by five water companies that were sampled during Phase 1 of the study.

Table 2.1 Summary of the works sampled in Phase 1 of the study programme

Water Company	STW	Population equivalents
A	A1	178000
	A2	17770
	A3	40000
B	B1	7329
	B2	49000
	B3	12911
	B4	88841
	B5	65859
C	C1	98469
	C2	66241
	C3	10982
D	D1	39900
	D2	192000
	D3	280000
	D4	117000
	D5	31600
	D6	75285
E	E1	93503
	E2	55769
	E3	555194
	E4	515864
	E5	285463
	E6	910465
	E7	18780
	E8	573489

Table 2.2 summarises the 18 sewage treatment works that were subsequently added in the second phase of this study (Phase 2) to include works from the remaining water companies. In addition, a few high-risk sites from model predictions were added for some water companies included in the first phase (Phase 1).

Table 2.2 Summary of the works sampled in Phase 2 of the study programme

Water Company	STW	Population equivalents
A	A4	16000
C	C4	46085
F	F1	41327
	F2	18145
	F3	17517
G	G1	320000
	G2	20000
	G3	8000
H	H1	69437
	H2	138875
	H3	61029
	H4	15108
I	I1	11045
	I2	13001
	I3	59836
J	J1	10608
	J2	214800
	J3	13248

2.2 Sampling schedule

We sampled all the works in Phase 1 on two occasions between 1st April and 21st May 2003 (see Table 3.1). We sampled all the works in Phase 2 between 16th July and 19th August 2003 (see Table 3.2).

2.3 Collection and processing of the samples

The objective of the sampling campaign was to obtain, for each final effluent, two spot samples taken at different occasions (but at similar times of the day), to establish the presence of steroids in effluents across many works and provide an indication of potential risk to the environment. The variability of steroid concentrations released from STWs over time will be assessed separately by a series of short-term and long-term studies as part of the demonstration programme.

We sampled the works as groups, depending on their location. Each sample was taken into three separate containers to allow for subsequent analysis for:

- Natural and synthetic steroids (carried out at Brixham Environmental Laboratory in Phase 1 and WRc-NSF in Phase 2);
- Nonylphenol and lower chain nonylphenol ethoxylates (carried out at WRc-NSF);
- Hormonal activity in YES/YAS bioassays (carried out at the University of Exeter).

We collected on-site effluent samples in:

- three 2.5-litre amber bottles which had been previously rinsed with HPLC grade methanol (for the steroid analysis and YES/YAS assays);
- one 1-litre Duran bottle (for nonylphenol and lower chain nonylphenol ethoxylates).

Each bottle was rinsed with final effluent, filled and left to stand for five minutes before being emptied and then refilled. The samples taken on a given day were then returned to either the WRc-NSF laboratory or, for certain campaigns, to the regional laboratory of the Environment Agency where they were extracted (if required).

Table 2.3 summarises the procedures adopted to stabilise the samples for chemical analysis (for steroids and alkylphenols and alkylphenol ethoxylates) and YES/YAS analysis.

For nonylphenol and the lower chain nonylphenol ethoxylates, a 3ml aliquot of 10 per cent sulphuric acid was added to the one-litre bottle on return to the laboratory to lower the sample pH to approximately 2. The sample could then be stored at 4^{o}C for subsequent analysis.

On return to the laboratory, each of the 2 x 2.5 litre samples for steroid analysis was spiked with 125 μl of a mixed deuterated steroid solution (d_4 STR 15 A12 from the Organic Chemistry Group of WRc-NSF) which contained 0.1 ng μl^{-1} of d_4-oestradiol, d_4-oestrone and d_4-ethinyloestradiol. This resulted in an initial concentration of 5 ng l^{-1} of each of the deuterated steroids in each of the sample bottles.

Table 2.3 Summary of the extraction procedures for effluent samples

Determinands	Stabilisation procedure
Steroids	5 litres of each effluent sample which had been collected in 2 x 2.5 litre amber bottles were initially filtered through a combination of a prefilter and 0.2 μm filter which had been conditioned with HPLC grade methanol and double distilled water. The filtrate was stored in 2 x 2.5 litre methanol rinsed amber glass bottle in the cold store overnight. The following morning the filtrate was extracted onto a 5g C_{18} column in the laboratory and kept at 4°C until subsequent extraction with methanol.
Alkylphenols and alkylphenol ethoxylates	1 litre of each effluent sample was acidified to pH 2 in the laboratory and maintained at 4 °C. Nonylphenol was analysed after liquid-liquid extraction, whereas lower chain nonylphenol ethoxylates were extracted onto a C_{18} column.
Samples for bioassays and storage	2.5 litres of each effluent sample which had been collected in a 2.5 litre amber bottle were initially filtered through a combination of a prefilter and 0.2 μm filter which had been conditioned with HPLC grade methanol and double distilled water. The filtrate was then stored in a 2.5 litre methanol rinsed amber glass bottle in a cold store overnight.
Oestrogenicity in YES/YAS assays (Exeter University)	The following morning, a 0.75 litre aliquot of the filtrate was extracted onto a 500 mg C_{18} column in the laboratory and kept at 4°C until subsequent extraction with methanol. The samples were not acidified prior to extraction (i.e. a pH neutral extraction was performed), which would exclude nonylphenol in the analysis.
Stored sample (backup replicate sample)	The following morning, a 0.75 litre aliquot of the filtrate was extracted onto a 500 mg C_{18} column in the laboratory and kept at –20°C until subsequent extraction with methanol.

Table A1 in Appendix A summarises the volumes of each effluent sample extracted onto C_{18} columns for the different procedures during Phases 1 and 2.

2.4 Analysis of the samples

2.4.1 Steroid analysis

The methods used for the conduct of the steroid analysis in Phase 1 are described in the report from the Brixham Environmental Laboratory given in Appendix B. In Phase 2, the steroids were analysed at WRc-NSF using comparable techniques.

2.4.2 Nonylphenol and nonylphenol ethoxylate analysis

Nonyl phenols

Acidified samples (500 ml) were filtered and then extracted with dichloromethane (DCM) (2 x 50 ml). The DCM extracts were dried and then concentrated to 1 ml prior to analysis by GCMS. The GCMS system consisted of a HP 5980 GC directly coupled to a VG Trio-1 mass spectrometer operated in selected ion recording mode with electron impact ionisation. The ions monitored were those at m/z 107,121,135 and 220. The GC column used was a DB-1 capillary column.

Pure 4-n-NP is available as an analytical standard. Commercial NP is, however, a mixture of various isomers. Most of these are 4-NPs, where there is branching in the C-9 alkyl chain, but other isomers, in which there is more than one alkyl chain (in total containing nine carbon atoms) attached at different positions with respect to the phenolic OH group on the aromatic ring, may also be present. All of these isomers can be individually detected using GCMS. For analytical purposes, the assumption has to be made that all of the isomers behave in an identical manner to 4-n-NP. One important consequence of the potential presence of several isomers, though, is that the limit of detection (LOD) for 'total' NP is higher than would be if only a single isomer is determined. For example, if the LOD for a single isomer is 0.5 $\mu g\ l^{-1}$, and if there is a possibility of 10 isomers being present, then the LOD for these considered as a total would be ca. 5 $\mu g\ l^{-1}$. This is because each could occur at a concentration just below 0.5 $\mu g\ l^{-1}$ and would remain undetected.

Quantification of nonylphenols was based on the use of a labelled internal standard ($^{13}C_6$-4-n-NP) which was added to the samples prior to analysis. This allowed any variations in extraction efficiencies to be compensated for, thus providing more accurate quantification of NP isomers. Calibration data was obtained using a technical mixture of nonylphenol (Sigma-Aldrich, Dorset, UK). QA/QC blank and spiked samples were analysed with each batch of samples.

Nonylphenol ethoxylates

Samples (100 ml) were filtered and extracted using solid phase extraction (SPE). The SPE cartridges were eluted with methanol (1 ml) and the extracts placed in an autosampler vial prior to analysis using LCMS. The LCMS system consisted of a HP 1100 LC system coupled to a Micromass Quattro mass spectrometer operated in selected ion recording mode with positive atmospheric pressure chemical ionisation. The ions monitored corresponded to the $[M+Na]^+$ of the various nonylphenol ethoxylate homologues, $NP(EO)_n$, from n=1 to n=15. Quantification was based on the use of external standards and QA spikes were analysed with each batch of samples.

2.4.3 YES/YAS bioassays

The methods used for the conduct of the YES and YAS bioassays are described in detail in the report from the University of Exeter given in Appendix C.

3 Results

3.1 Effluent flows at the times of sampling

Tables 3.1 and 3.2 summarise the effluent flows at different sewage treatment works on the two sampling occasions during Phase 1 and 2. The effluent flow data were required to calculate steroid loads in the risk assessment of steroid discharges from the STWs, which is reported separately in Environment Agency (2006b). For population equivalents of the STWs, please see tables 2.1 and 2.2.

Table 3.1 Summary of the effluent flows at the times of sampling in Phase 1

Sewage treatment works location	Sampling time and date	Effluent flows on each sampling occasion (l s^{-1})
A1	11.30 – 7/4/03	91.2 (E)
	11.00 – 24/4/03	92.4 (E)
A2	10.50 – 9/4/03	486 (E)
	11.10 – 10/5/03	469 (E)
A3	8.45 - 9/4/03	No final effluent meter
	9.30 – 10/5/03	No final effluent meter
B1	11.00 - 14/4/03	20.4
	10.45 - 6/5/03	23.2
B2	12.00 - 14/4/03	106
	11.30 - 6/5/03	101
B3	11.40 - 15/4/03	31
	11.30 - 7/5/03	No data received
B4	12.15 - 15/4/03	No data received
	12.30 - 7/5/03	No data received
B5	14.20 - 15/4/03	348 (E)
	14.30 - 7/5/03	221 (E)
C1	11.00 - 9/4/03	382
	11.55 - 28/4/03	587
C2	11.45 - 10/4/03	185
	10.30 - 29/4/03	70
C3	12.30 - 10/4/03	24.1
	12.15 - 29/4/03	31.4
D1	13.00 - 7/4/03	132
	12.00 - 24/4/03	142
D2	11.00 - 8/4/03	503 (E)
	10.40 - 25/4/03	607 (E)
D3	10.45 - 1/4/03	824
	10.30 - 22/4/03	760
D4	10.50 - 3/4/03	697
	11.05 - 23/4/03	682
D5	11.30 - 3/4/03	90.0
	10.00 - 23/4/03	86.5
	13.30 - 28/4/03	184 (E)

Table 3.1 Continued

Sewage treatment works location	Sampling time and date	Effluent flows on each sampling occasion (l s^{-1})
D6	12.50 - 9/4/03	227 (E)
	13.30 - 28/4/03	184 (E)
E1	15.00 - 21/5/03	390
	11.15 - 22/5/03	588
E2	14.00 - 12/5/03	286
	11.00 - 14/5/03	219
E3	10.45 - 12/5/03	1851
	10.30 - 14/5/03	1352
E4	11.45 - 13/5/03	1537
	9.45 - 15/5/03	1773
E5	10.00 - 12/5/03	1153
	10.00 - 14/5/03	1020
E6	10.30 - 13/5/03	2613
	10.30 - 15/5/03	3053
E7	17.15 - 21/5/03	54
	9.15 - 22/5/03	77
E8	13.10 - 21/5/03	2048
	13.15 - 22/5/03	2402

Notes: E – Estimated from daily flows

Table 3.2 Summary of the effluent flows at the times of sampling in Phase 2

Sewage treatment works location	Sampling time and date	Effluent flows on each sampling occasion (l s^{-1})
A1	13.00 – 16/7/03	62 (E)
	9.15 – 21/7/03	61 (E)
C4	11.30 – 16/7/03	No data received
	11.50 – 17/7/03	No data received
F1	9.20 – 13/8/03	85
	9.00 – 14/8/03	188
F2	12.00 – 13/8/03	66
	11.45 – 14/8/03	42
F3	10.15 – 13/8/03	91
	10.45 - 14/8/03	81
G1	10.50 – 30/7/03	136
	8.45 – 31/7/03	111
G2	11.55 – 30/7/03	36.4
	10.00 – 31/7/03	54.6
G3	9.30 – 30/7/03	40.4
	11.25 – 31/7/03	17.3

Table 3.2 Continued

Sewage treatment works location	Sampling time and date	Effluent flows on each sampling occasion (l s^{-1})
H1	8.15 – 11/8/03	216
	8.35 – 12/8/03	230
H2	8.50 – 11/8/03	456
	9.15 - 12/8/03	462
H3	14.10 – 11/8/03	135
	11.00 – 12/8/03	138
H4	14.45 – 11/8/03	56
	11.45 – 11/8/03	74
I1	11.30 – 22/7/03	No final effluent meter
	10.50 – 23/7/03	No final effluent meter
I2	10.20 – 22/7/03	No final effluent meter
	10.20 - 23/7/03	No final effluent meter
I3	12.55 – 22/7/03	187.5
	12.10 – 23/7/03	237.1
J1	9.30 – 19/8/03	32.2
	9.15 – 20/8/03	31.4
J2	10.50 – 19/8/03	45.4
	10.15 – 20/8/03	63
J3	12.45 – 19/8/03	27 (E)
	11.55 – 20/8/03	25 (E)

3.2 Chemical analysis of final effluent samples

3.2.1 Steroids

The preparative and analytical technique we ultimately used to analyse the steroids was effective in quantifying concentrations of oestrone, oestradiol and ethinyloestradiol in the final effluent samples. We had to modify the initial extraction technique and add an additional clean-up stage to improve the discrimination of the technique (see Appendix B). For certain of the final effluents analysed in Phases 1 and 2, though, it was not possible to identify quantifiable peaks for either the inherent steroids or the internal standards present in the samples. This related particularly to the quantification of ethinyloestradiol that was the steroid present in the final effluent samples at the lowest concentrations. We believe that this may be due in part to the presence of interferents in the samples (such as carboxylic acids) which may bind competitively to the C_{18} SPE column, particularly for substances such as ethinyloestradiol which are present in the final effluent samples only at low concentrations. The causes of the problems with the quantification of steroids in certain samples are currently being investigated. The actual measurement technique was not the cause of the identified problem, as the analysis of the blanks, AQC spikes and standard solutions resulted in concentrations that were consistent with those expected (see Table 3.5).

The data from the steroid analysis in both Phase 1 (Table 3.3) and 2 (Table 3.4) showed that:

- Marked variations in steroid concentrations were observed between the samples for different sewage treatment works final effluents;
- Steroid concentrations were measured in the ranges of <1 to 100 ng l^{-1} for oestrone, <1 to 22 ng l^{-1} for oestradiol and <1 to 3.2 ng l^{-1} for ethinyloestradiol. There were high levels of steroids, primarily oestrone, in final effluents from a number of locations. Oestrone concentrations of $\geq$ 20 ng l^{-1} were found in 13 of 25 final effluents on one or more of the sampling occasions in Phase 1, and in 8 of 18 final effluents on one or more of the sampling occasions in Phase 2. The risk posed to fisheries by the levels of steroids in final effluents will depend on the degree of dilution and dispersion in the receiving water.
- There was general consistency between the results obtained for the two samples taken at a given location. A limited number of final effluents (G3, H2, H3 and I2), though, showed more marked variability (>5 times difference) between the results for the two samples;
- For certain final effluents, no quantifiable peaks could be discerned, particularly for the synthetic steroid ethinyloestradiol. While the absence of easily quantifiable peaks represents a technical difficulty, it is also likely to indicate that the actual concentrations of steroids present in the effluents are low.

Table 3.3 Summary of the results of steroid analysis of the final effluent samples in Phase 1

Sewage treatment works location	Sampling time and date	Concentrations of different determinands		
		Oestrone (ng l^{-1})	Oestradiol (ng l^{-1})	Ethinyloestradiol (ng l^{-1})
A1	11.30 - 7/4/03	5.6	0.9	0.3
	11.00 - 24/4/03	3.1	NQP	NQP
A2	10.50 - 9/4/03	5.2	<1	0.3
	11.10 - 10/5/03	NQP	NQP	NQP
A3	8.45 - 9/4/03	91	NQP	NQP
	9.30 - 10/5/03	48	2.7	1.5
B1	11.00 - 14/4/03	50	6.6	0.5
	10.45 - 6/5/03	19	1.9	NQP
B2	12.00 - 14/4/03	3.8	0.5	<2
	11.30 - 6/5/03	11	0.8	<2
B3	11.40 - 15/4/03	NQP	NQP	NQP
	11.30 - 7/5/03	11	NQP	NQP
B4	12.15 - 15/4/03	11	<0.5	NQP
	12.30 - 7/5/03	26	<1	NQP
B5	14.20 - 15/4/03	NQP	NQP	NQP
	14.30 - 7/5/03	1.9	<0.3	<0.5

Table 3.3 Continued

Sewage treatment works location	Sampling time and date	Concentrations of different determinands		
		Oestrone (ng l^{-1})	Oestradiol (ng l^{-1})	Ethinyloestradiol (ng l^{-1})
C1	11.00 - 9/4/03	17	6.0	0.7
	11.55 - 28/4/03	38	NQP	0.3
C2	11.45 - 10/4/03	8.7	NQP	<2
	10.30 - 29/4/03	21	2.9	<0.5
C3	12.30 - 10/4/03	14	2.9	0.8
	12.15 - 29/4/03	11	<1	<2
D1	13.00 - 7/4/03	25	5.5	0.3
	12.00 - 24/4/03	16	2.1	0.5
D2	11.00 - 8/4/03	3.6	NQP	<0.5
	10.40 - 25/4/03	5.0	<0.5	<1
D3	10.45 - 1/4/03	NQP	NQP	NQP
	10.30 - 22/4/03	NQP	NQP	NQP
D4	10.50 - 3/4/03	<0.5	<0.5	<0.5
	11.05 - 23/4/03	NQP	NQP	NQP
D5	11.30 - 3/4/03	15	1.6	<0.5
	10.00 - 23/4/03	14	NQP	NQP
D6	12.50 - 9/4/03	43	NQP	<1
	13.30 - 28/4/03	25	2.2	<0.2
E1	15.00 - 21/5/03	74	18	1.3
	11.15 - 22/5/03	100	22	1.7
E2	14.00 - 12/5/03	28	<0.5	NQP
	11.00 - 14/5/03	33	<0.5	<1
E3	10.45 - 12/5/03	13	NQP	NQP
	10.30 - 14/5/03	27	NQP	NQP
E4	11.45 - 13/5/03	56	NQP	NQP
	9.45 - 15/5/03	71	NQP	NQP
E5	10.00 - 12/5/03	14	<1	<1
	10.00 - 14/5/03	14	<1	<2
E6	10.30 - 13/5/03	54	6.2	3.2
	10.30 - 15/5/03	57	5.2	2.5
E7	17.15 - 21/5/03	8.1	1.3	1.0
	9.15 - 22/5/03	10	1.5	NQP
E8	13.10 - 21/5/03	35	2.6	NQP
	13.15 - 22/5/03	28	4.0	NQP

Notes: NQP indicates that no quantifiable peak was identified

Table 3.4 Summary of the results of chemical analysis of the final effluent samples in Phase 2

Sewage treatment works location	Sampling time and date	Concentrations of different determinands		
		Oestrone (ng l^{-1})	Oestradiol (ng l^{-1})	Ethinyloestradiol (ng l^{-1})
A4	13.00 – 16/7/03	NQP	NQP	NQP
	9.15 – 21/7/03	NQP	NQP	NQP
C4	11.30 – 16/7/03	34.2	NQP	NQP
	11.50 – 17/7/03	15.5	0.6	<1
F1	9.20 – 13/8/03	24.8	<1	NQP
	9.00 – 14/8/03	39.8	<1	NQP
F2	12.00 – 13/8/03	NQP	NQP	NQP
	11.45 – 14/8/03	0.7	<1	<1
F3	10.15 – 13/8/03	3.7	<1	NQP
	10.45 - 14/8/03	2.9	<1	<1
G1	10.50 – 30/7/03	18.6	<1	NQP
	8.45 – 31/7/03	11.6	<1	NQP
G2	11.55 – 30/7/03	4.3	NQP	NQP
	10.00 – 31/7/03	1.2	<1	NQP
G3	9.30 – 30/7/03	6.6	1.2	NQP
	11.25 – 31/7/03	41.6	<1	<1
H1	8.15 – 11/8/03	23.6	<1	<1
	8.35 – 12/8/03	33.0	NQP	NQP
H2	8.50 – 11/8/03	1.9	<1	NQP
	9.15 - 12/8/03	17.3	<1	<1
H3	14.10 – 11/8/03	5.9	<1	<1
	11.00 – 12/8/03	39.8	<1	NQP
H4	14.45 – 11/8/03	11.3	<1	NQP
	11.45 – 11/8/03	NQP	NQP	NQP
I1	11.30 – 22/7/03	NQP	<1	NQP
	10.50 – 23/7/03	11.2	<1	NQP
I2	10.20 – 22/7/03	0.9	<1	<1
	10.20 - 23/7/03	26.2	<1	NQP
I3	12.55 – 22/7/03	1.9	<1	NQP
	12.10 – 23/7/03	NQP	NQP	NQP
J1	9.30 – 19/8/03	0.9	NQP	NQP
	9.15 – 20/8/03	<1	NQP	NQP
J2	10.50 – 19/8/03	10.0	<1	1.5
	10.15 – 20/8/03	2.2	<1	<1
J3	12.45 – 19/8/03	30.7	<1	NQP
	11.55 – 20/8/03	77.5	<1	NQP

Notes: NQP indicates that no quantifiable peak was identified

Table 3.5 Summary of the quality control data for the analysis of steroids in effluents

Sample type	Range of concentrations of different steroids		
	Oestrone (ng l^{-1})	Oestradiol (ng l^{-1})	Ethinyloestradiol (ng l^{-1})
Groundwater blanks	<1	<1	<1
AQC spike (5 ng l^{-1} of each steroid)	3.7 – 3.8	3.5 – 3.7	3.1
Standard solutions (5 ng l^{-1} of each steroid)	4.8 – 4.9	4.5 – 4.8	5.3 – 6.5

3.2.2 Nonylphenol and lower chain (NP1-5EO) ethoxylates

Nonylphenol

The levels of nonylphenol in the final effluent samples taken in both Phases 1 and 2 ranged from the limit of detection (1.0 μg l^{-1}) to maximum concentrations of 7.7 μg l^{-1} and 3.8 μg l^{-1} (see Tables 3.6 and 3.7). Most effluent concentrations were in the range of <1 to 3 μg l^{-1}. Concentrations above 3 μg l^{-1} were only measured at seven of the 25 sites sampled in Phase 1 (A3, B3, E1, E2, E3, E5 and E6) and at one of the 18 sites sampled in Phase 2 (C4). There was general consistency between the results obtained for the two samples taken at a given location.

Nonylphenol (NP1-5EO) ethoxylates

The levels of lower chain nonylphenol ethoxylates in the effluents (see Tables 3.6 and 3.7) were as follows:

NP1EO: <1 to 2.3 μg l^{-1} in Phase 1 and <1 to 97.5 μg l^{-1} in Phase 2

NP2EO: <1 to 18.8 μg l^{-1} in Phase 1 and <1 to 29.0 μg l^{-1} in Phase 2

NP3-5EO: <1 to 20.3 μg l^{-1} in Phase 1 and <1 to 136.0 μg l^{-1} in Phase 2

For NP1EO no samples in Phase 1 and only two samples in Phase 2 (from F3) exceeded 10 μg l^{-1} while for NP2EO one sample in Phase 1 (from E1) and one sample in Phase 2 (from F3) exceeded 10 μg l^{-1}. For NP3-5EO seven samples in Phase 1 (from A3, B3, C2, E2, E4 and E5) and 13 samples in Phase 2 (from C4, F2, F3, G1, I2, I3 and J3) exceeded 10 μg l^{-1}.

Table 3.6 Summary of the results of nonylphenol and nonylphenol ethoxylate analysis of the final effluent samples in Phase 1

Sewage treatment works location	Sampling time and date	Nonylphenol ($\mu g\ l^{-1}$)	NP1EO ($\mu g\ l^{-1}$)	NP2EO ($\mu g\ l^{-1}$)	NP3-5EO ($\mu g\ l^{-1}$)
A1	11.30 - 7/4/03	2.1	<1	<1	<1
	11.00 - 24/4/03	1.1	NR	NR	NR
A2	10.50 - 9/4/03	1.6	<1	<1	<2.6
	11.10 - 10/5/03	<1	NR	NR	NR
A3	8.45 - 9/4/03	3.7	<1	<1	<1
	9.30 - 10/5/03	2.0	<1	<1	12.9
B1	11.00 - 14/4/03	2.4	<1	<1	<1
	10.45 - 6/5/03	1.1	NR	NR	NR
B2	12.00 - 14/4/03	2.4	<1	<1	2.6
	11.30 - 6/5/03	1.5	NR	NR	NR
B3	11.40 - 15/4/03	3.6	<1	<1	1.5
	11.30 - 7/5/03	2.6	1.5	3.8	20.3
B4	12.15 - 15/4/03	2.3	<1	<1	3.9
	12.30 - 7/5/03	1.1	NR	NR	NR
B5	14.20 - 15/4/03	<1	<1	<1	2.2
	14.30 - 7/5/03	1.2	<1	<1.7	9.1
C1	11.00 - 9/4/03	1.9	<1	1.7	<1
	11.55 - 28/4/03	1.1	NR	NR	NR
C2	11.45 - 10/4/03	2.7	1.2	<1	6.5
	10.30 - 29/4/03	1.8	<1	<1	17.1
C3	12.30 - 10/4/03	2.1	<1	<1	5.2
	12.15 - 29/4/03	<1	<1	<1	5.3
D1	13.00 - 7/4/03	2.3	<1	<1	<1
	12.00 - 24/4/03	1.1	<1	<1	4.2
D2	11.00 - 8/4/03	<1	<1	<1	1.8
	10.40 - 25/4/03	1.2	<1	<1	6.6
D3	10.45 - 1/4/03	2.2	<1	<1	1.6
	10.30 - 22/4/03	<1	<1	<1	7.7
D4	10.50 - 3/4/03	1.5	<1	<1	1.6
	11.05 - 23/4/03	<1	NR	NR	NR
D5	11.30 - 3/4/03	1.8	<1	1.5	<1
	10.00 - 23/4/03	<1	<1	<1	9.4
D6	12.50 - 9/4/03	2.5	<1	<1	1.4
	13.30 - 28/4/03	1.1	NR	NR	NR
E1	15.00 - 21/5/03	4.2	1.9	18.8	3.2
	11.15 - 22/5/03	5.0	2.3	5.7	5.1
E2	14.00 - 12/5/03	5.6	<1	<1	3.6
	11.00 - 14/5/03	7.7	<1	<1	14.0
E3	10.45 - 12/5/03	3.3	<1	<1	2.7
	10.30 - 14/5/03	6.3	<1	<1	7.7
E4	11.45 - 13/5/03	3.3	<1	<1	10.3
	9.45 - 15/5/03	3.6	<1	<1	13.8

Table 3.6 continued

Sewage treatment works location	Sampling time and date	Nonylphenol ($\mu g\ l^{-1}$)	NP1EO ($\mu g\ l^{-1}$)	NP2EO ($\mu g\ l^{-1}$)	NP3-5EO ($\mu g\ l^{-1}$)
E5	10.00 - 12/5/03	7.1	<1	<1	<1
	10.00 - 14/5/03	6.4	1.6	3.0	15.8
E6	10.30 - 13/5/03	5.0	<1	<1	2.3
	10.30 - 15/5/03	5.3	1.3	<1	4.4
E7	17.15 - 21/5/03	1.3	1.4	1.5	6.1
	9.15 - 22/5/03	1.4	2.3	3.3	<1
E8	13.10 - 21/5/03	3.5	<1	3.4	5.1
	13.15 - 22/5/03	3.7	<1	<1	5.6

Notes: NR – no results due to interferences

Table 3.7 Summary of the results of nonylphenol and nonylphenol ethoxylate analysis of the final effluent samples in Phase 2

Sewage treatment works location	Sampling time and date	Nonylphenol ($\mu g\ l^{-1}$)	NP1EO ($\mu g\ l^{-1}$)	NP2EO ($\mu g\ l^{-1}$)	NP3-5EO ($\mu g\ l^{-1}$)
A4	13.00 – 16/7/03	<1	3.4	1.5	13.6
	9.15 – 21/7/03	<1	2.0	0.9	8.3
C4	11.30 – 16/7/03	<1	8.3	2.5	19.2
	11.50 – 17/7/03	3.8	NR	NR	NR
F1	9.20 – 13/8/03	<1	<1	<1	<1
	9.00 – 14/8/03	<1	2.1	2.7	9.5
F2	12.00 – 13/8/03	<1	4.5	1.0	11.2
	11.45 – 14/8/03	<1	3.6	<1	7.9
F3	10.15 – 13/8/03	1.0	31.1	8.0	42.1
	10.45 - 14/8/03	2.3	97.5	29.0	136.0
G1	10.50 – 30/7/03	<1	2.1	<5	17.7
	8.45 – 31/7/03	<1	2.7	2.7	20.6
G2	11.55 – 30/7/03	<1	<1	<1	<1
	10.00 – 31/7/03	<1	<1	<1	<1
G3	9.30 – 30/7/03	<1	<1	<1	<1
	11.25 – 31/7/03	<1	3.1	<1	5.2
H1	8.15 – 11/8/03	<1	<1	<1	<1
	8.35 – 12/8/03	<1	<1	<1	<1
H2	8.50 – 11/8/03	<1	<1	<1	<1
	9.15 - 12/8/03	<1	2.1	<1	9.3
H3	14.10 – 11/8/03	<1	<1	<1	<1
	11.00 – 12/8/03	<1	<1	<1	<1
H4	14.45 – 11/8/03	<1	<1	<1	<1
	11.45 – 11/8/03	<1	<1	<1	<1

Table 3.7 Continued

Sewage treatment works location	Sampling time and date	Nonylphenol (μg l^{-1})	NP1EO (μg l^{-1})	NP2EO (μg l^{-1})	NP3-5EO (μg l^{-1})
I1	11.30 – 22/7/03	<1	1.4	3.4	<1
	10.50 – 23/7/03	<1	1.9	<1	<1
I2	10.20 – 22/7/03	<1	8.7	4.0	23.0
	10.20 - 23/7/03	<1	8.4	6.8	29.0
I3	12.55 – 22/7/03	<1	7.8	2.0	19.2
	12.10 – 23/7/03	NR	<1	<1	17.8
J1	9.30 – 19/8/03	<1	<1	<1	<1
	9.15 – 20/8/03	<1	<1	<1	<1
J2	10.50 – 19/8/03	<1	<1	<1	<1
	10.15 – 20/8/03	<1	<1	<1	<1
J3	12.45 – 19/8/03	<1	2.8	1.6	10.1
	11.55 – 20/8/03	<1	1.8	1.7	14.8

Notes: NR – no results due to interferences

3.3 Assessment of final effluent samples with the YES/YAS assays

Tables 3.8 and 3.9 summarise the results of the analysis of the final effluent samples for Phases 1 and 2 with the yeast screens for oestrogenic and androgenic activity. A report supplied by the University of Exeter describing all the methods used and the data obtained is given in Appendix C.

In Phase 1 of the study, all of the samples tested exhibited some oestrogenic activity with potency equivalents of between 0.4 and 11.7 ng E2 l^{-1} for the first sampling occasions and between 0.9 and 20.5 ng E2 l^{-1} for the second sampling occasions (Table 3.8). None of the samples tested exhibited any clear anti-oestrogenic activity. In addition, none of the samples contained any detectable androgen activity. All samples, though, exhibited significant anti-androgenic activity with potency equivalents of between 21.3 and 228 μg flutamide l^{-1} on the first sampling occasion and between 23.5 and 200 μg flutamide l^{-1} on the second sampling occasion (Table 3.8).

In Phase 2 of the study, all of the samples tested exhibited some oestrogenic activity with potencies equivalent to between 0.9 and 42.7 ng E2 l^{-1} on the first sampling occasion and 1.0 to 42.7 ng E2 l^{-1} on the second sampling occasion (Table 3.9). None of the final effluent samples tested in Phase 2 exhibited any clear anti-oestrogenic activity. Only one of the samples (F2) was found to contain any detectable androgenic activity at each sampling occasion, with a potency equivalent to 72.5 and 70.4 ng DHT l^{-1} on the first and second sampling occasions respectively. Nearly all of the samples exhibited anti-androgenic activity with potencies equivalent to between 90.7 and 764.4 μg flutamide l^{-1} on the first sampling occasion and between 90.7 and 1230.8 μg flutamide l^{-1} on the second sampling occasion (Table 3.9). The final effluent samples A4 and F2 did not exhibit any anti-androgenic activity on either sampling occasion.

Table 3.8 Summary of the results of YES (oestrogenic)/YAS (anti-androgenic) analysis of the final effluent samples in Phase 1

Sewage treatment works location	Sampling time and date	YES (oestrogenic) assay response[1]	YAS (anti-androgenic) assay response[2]
A1	11.30 - 7/4/03	1.5	125.5
	11.00 - 24/4/03	2.5	71.0
A2	10.50 - 9/4/03	1.2	53.3
	11.10 - 10/5/03	2.9	50.0
A3	8.45 - 9/4/03	2.7	112.3
	9.30 - 10/5/03	2.7	76.2
B1	11.00 - 14/4/03	5.5	21.3
	10.45 - 6/5/03	10.9	41.0
B2	12.00 - 14/4/03	2.2	142.2
	11.30 - 6/5/03	3.0	61.5
B3	11.40 - 15/4/03	1.3	81.6
	11.30 - 7/5/03	4.1	115.4
B4	12.15 - 15/4/03	2.4	35.6
	12.30 - 7/5/03	9.5	142
B5	14.20 - 15/4/03	0.4	76.2
	14.30 - 7/5/03	2.0	76.2
C1	11.00 - 9/4/03	4.4	35.6
	11.55 - 28/4/03	16.2	34.0
C2	11.45 - 10/4/03	1.5	66.7
	10.30 - 29/4/03	8.9	76.2
C3	12.30 - 10/4/03	1.0	61.0
	12.15 - 29/4/03	4.3	61.5
D1	13.00 - 7/4/03	8.8	142.2
	12.00 - 24/4/03	7.6	42.1
D2	11.00 - 8/4/03	1.2	177.8
	10.40 - 25/4/03	0.9	100.0
D3	10.45 - 1/4/03	0.8	35.6
	10.30 - 22/4/03	2.9	28.6
D4	10.50 - 3/4/03	0.4	71.1
	11.05 - 23/4/03	0.9	30.8
D5	11.30 - 3/4/03	7.3	21.3
	10.00 - 23/4/03	4.3	200.0
D6	12.50 - 9/4/03	11.7	142.2
	13.30 - 28/4/03	8.2	61.5
E1	15.00 - 21/5/03	11.0	35.6
	11.15 - 22/5/03	20.5	47.1
E2	14.00 - 12/5/03	0.7	133.3
	11.00 - 14/5/03	1.4	61.5
E3	10.45 - 12/5/03	1.0	228.6
	10.30 - 14/5/03	1.5	100.0

Table 3.8 Continued

Sewage treatment works location	Sampling time and date	YES (oestrogenic) assay response[1]	YAS (anti-androgenic) assay response[2]
E4	11.45 - 13/5/03	3.4	224.6
	9.45 - 15/5/03	6.7	41.0
E5	10.00 - 12/5/03	1.2	125.5
	10.00 - 14/5/03	1.4	53.3
E6	10.30 - 13/5/03	9.3	26.7
	10.30 - 15/5/03	10.7	100.0
E7	17.15 - 21/5/03	1.5	53.3
	9.15 - 22/5/03	2.3	53.3
E8	13.10 - 21/5/03	1.8	35.6
	13.15 - 22/5/03	2.3	23.5

Notes: [1] – Oestradiol equivalent concentrations (ng l^{-1}), [2] - Flutamide equivalent concentration ($\mu g\ l^{-1}$)

Table 3.9 Summary of the results of YES (oestrogenic)/YAS (anti-androgenic) analysis of the final effluent samples in Phase 2

Sewage treatment works location	Sampling time and date	YES (oestrogenic) assay response[1]	YAS (anti-oestrogenic) assay response[2]
A4	13.00 – 16/7/03	2.7	No activity
	9.15 – 21/7/03	6.6	No activity
C4	11.30 – 16/7/03	8.6	457
	11.50 – 17/7/03	6.5	1231
F1	9.20 – 13/8/03	17.5	348
	9.00 – 14/8/03	1.8	382
F2	12.00 – 13/8/03	42.7	No activity
	11.45 – 14/8/03	42.7	No activity
F3	10.15 – 13/8/03	1.6	348
	10.45 - 14/8/03	1.0	573
G1	10.50 – 30/7/03	3.4	235
	8.45 – 31/7/03	12.7	500
G2	11.55 – 30/7/03	7.8	281
	10.00 – 31/7/03	2.2	421
G3	9.30 – 30/7/03	5.6	91
	11.25 – 31/7/03	8.6	91
H1	8.15 – 11/8/03	6.6	319
	8.35 – 12/8/03	7.0	319
H2	8.50 – 11/8/03	6.2	280
	9.15 - 12/8/03	4.9	358
H3	14.10 – 11/8/03	2.9	319
	11.00 – 12/8/03	2.8	319
H4	14.45 – 11/8/03	1.4	267
	11.45 – 11/8/03	2.5	302

Table 3.9 continued

Sewage treatment works location	Sampling time and date	YES (oestrogenic) assay response[1]	YAS (anti-oestrogenic) assay response[2]
I1	11.30 – 22/7/03	2.7	229
	10.50 – 23/7/03	3.7	471
I2	10.20 – 22/7/03	8.6	432
	10.20 - 23/7/03	15.3	640
I3	12.55 – 22/7/03	5.1	222
	12.10 – 23/7/03	9.3	246
J1	9.30 – 19/8/03	1.6	209
	9.15 – 20/8/03	1.0	164
J2	10.50 – 19/8/03	1.8	764
	10.15 – 20/8/03	1.5	478
J3	12.45 – 19/8/03	9.0	573
	11.55 – 20/8/03	7.0	382

Notes: [1] – Oestradiol equivalent concentrations (ng l^{-1}), [2] - Flutamide equivalent concentration ($\mu g\ l^{-1}$)

4 Discussion

4.1 Comparison of the analytical chemistry data with previous UK studies and the significance of the findings

4.1.1 Natural and synthetic steroids

Table 4.1 summarises the results for steroids concentrations measured at four sewage treatment works (C2, D4, D5 and E1) during this survey and in previous studies (Desbrow *et al*, 1998; Williams *et al,* 2002). From the data in Table 4.1 it is evident that steroid concentrations measured during this survey were generally consistent with concentrations found at these locations previously. The exceptions were the higher oestradiol and ethinyloestradiol concentrations recorded in the E1 STW final effluent during this study.

Table 4.1 Comparison of steroid data with previous results for four sewage treatment works

Sewage treatment works location	Data source	Sampling dates	Concentrations of different steroids		
			Oestrone (ng l^{-1})	Oestradiol (ng l^{-1})	Ethinyloestradiol (ng l^{-1})
C2	Desbrow *et al* (1998)	30/11/95	6.1	4.9	0.2
		4/12/95	10	5.7	0.6
		15/1/96	12	4.0	0.8
	This study	10-29/4/03	8.7 - 21	2.9	<0.5
D4	Williams *et al* (2002)	29/9/00 – 12/10/00	<0.4 – 2.2	<0.4 – 1.87	<0.5 – 1.07
	This study	3-23/4/03	<0.5	<0.5	<0.5
D5	Desbrow *et al* (1998)	17/7/95	5.2	3.7	<0.2
		24/7/95	8.5	7.1	<0.2
		1/8/95	8.9	4.4	<0.2
	Williams *et al* (2002)	29/9/00 - 12/10/00	3.38 – 12.22	<0.4 - 4.29	<0.5 – 3.38
	This study	3-23/4/03	14 - 15	1.6	<0.5
E1	Sheahan *et al* (2002)	1996	180	5	<0.1
	This study	21-22/5/03	74-100	18-22	1.3-1.7

The significance of the measured steroid concentrations in the final effluents needs to be judged against the Predicted No Effect Concentrations (PNECs) for oestrone, oestradiol and ethinyloestradiol proposed in Environment Agency (2002a) and reviewed in Defra (2003a).

Table 4.2 summarises the proposed PNECs (lower effect thresholds) and the upper effects thresholds derived for each of the steroids. From the available ecotoxicological data, it was evident that the order of oestrogenic potency for the steroids is ethinyloestradiol > oestradiol > oestrone. Given that steroid oestrogens have the same mode of action and will invariably occur as mixtures in sewage effluents and receiving waters, a PNEC for 'total' steroid oestrogens was also derived. This is reflected in a combined PNEC for steroid oestrogens of 1 ng l^{-1}, which takes into account the relative potency of each steroid and their additive effects (Environment Agency, 2002b).

The potency of the natural oestriol was also considered in the review documents. The high exposure concentrations for the lower and upper effects thresholds (100 and 1000 ng l^{-1}) and the marked lower concentrations that are likely to be found in the environment mean that oestriol does not represent a key steroid in terms of causing effects in the receiving water environment. Oestriol can, therefore, be excluded from assessments of the effects of total steroids.

Data on STW effluent flow, steroid loads and river dilution need to be taken into consideration when estimating environmental exposures. This was undertaken as part of a risk assessment, which is reported separately (Environment Agency, 2006b). A basic comparison with the proposed PNECs indicates, though, that a number of STWs, even after dilution, would result in receiving water steroid concentrations that could result in oestrogenic effects in resident fish populations downstream of the discharges.

Table 4.2 Proposed PNECs (lower effects thresholds) and upper effect thresholds for ethinyloestradiol, oestradiol and oestrone

Substance	PNEC (Lower effect threshold) (ng l^{-1})	Upper effect threshold (ng l^{-1})
Ethinyloestradiol	0.1	0.57
Oestradiol	1.0	10
Oestrone	3.0	30

4.1.2 Nonylphenol and nonylphenol ethoxylates

Nonylphenol

Table 4.3 shows historic data for the levels of nonylphenol for certain of the discharges measured in the present survey. The data from Environment Agency (1998b) show that current values are consistent with those recorded in 1996.

Table 4.3 Current and historic nonylphenol concentrations in certain of the final effluents surveyed

Sewage treatment works location	Data source	Sampling dates	Nonylphenol concentration ($\mu g\ l^{-1}$)
E4	Environment Agency (1998b)	1996	1.4 – 3.5 (n=3)
	This study	13-15/5/03	3.3 – 3.6 (n=2)
E3	Environment Agency (1998b)	1996	45.0 (n=1)
	This study	12-14/5/03	3.3 – 6.3 (n=2)
E1	Environment Agency (1998b)	1996	3.5 – 88.0 (n=33)
	This study	21-22/5/03	4.2 – 5.0 (n=2)

In 1998, the Agency derived an Environmental Quality Standard (EQS) for nonylphenol of 1 $\mu g\ l^{-1}$, which was based on acute toxic effects to the freshwater shrimp *Gammarus pulex*. The data available in 1998 indicated that acute toxicity, not endocrine disruption, was the most sensitive endpoint. Since 1998, though, more data on the oestrogenic effects of nonylphenol on fish have become available. A selection is summarised below. Nonylphenol has been identified as a Priority Substance. A Quality Standard will therefore be derived under the Water Framework Directive. It will replace the Agency EQS.

In laboratory studies, Jobling *et al.* (1996) found that nonylphenol concentrations of approximately 20 $\mu g\ l^{-1}$ produce a significant elevation of plasma vitellogenin (VTG) concentrations in male rainbow trout (*Oncorhynchus mykiss*). Miles-Richardson *et al.* (1999) exposed fathead minnows to 4-nonylphenol (and nonylphenol ethoxylates) to determine the effects on secondary sexual characteristics and the gonads of sexually mature fish during 42 days continuous flow exposure. Nonylphenol did not cause effects on female gonads or secondary sexual characteristics at concentrations up to and including 5.5 $\mu g\ l^{-1}$. There was, though, evidence of histological effects on male gonads (changes in number and size of Sertoli cells and large, multi-nucleated germ cells) at concentrations of 1.1 and 3.4 $\mu g\ l^{-1}$.

Thorpe *et al*. (2001) assessed the *in-vivo* potency of a series of substances (including nonylphenol) singly or in combination on VTG concentrations in juvenile rainbow trout (*Oncorhynchus mykiss*). The lowest observed effect concentration of nonylphenol which induced VTG induction was 6.1 – 6.4 $\mu g\ l^{-1}$. Yokota *et al*. (2001) studied the chronic effects of continuous exposure of 4-nonylphenol on the reproductive status of the Japanese medaka (*Oryzias latipes*) over two generations. Exposure to 17.7 $\mu g\ l^{-1}$ resulted in the development of the intersex condition in four of 20 fish in the parental generation. In the F_1 generation, two of 20 and five of 20 fish developed the intersex condition following exposure to 8.2 and 17.7 $\mu g\ l^{-1}$, respectively. In addition, the sex ratio of males to females the F_1 generation was 1:2 in the 17.7 $\mu g\ l^{-1}$, as opposed to the approximate 1:1 ratio in the control or lower concentration treatments. These data are consistent with a

reported LOEC of 11.6 μg l^{-1} for induction of ovotestis in Japanese medaka (Seki *et al.,* 2003). Subsequently, Schwaiger *et al.* (2002) investigated the effects of intermittent exposure of adult rainbow trout of both sexes to nominal concentrations of 1 and 10 μg l^{-1} for four months before spawning. In the study, adult male rainbow trout showed significantly elevated plasma vitellogenin levels (relative to control animals) at 1 μg l^{-1}. In females, reproductive success was reduced at 10 μg l^{-1}, as indicated by decreased hatching rates due to higher mortalities occurring in early development. Hormonal imbalances were detected in the offspring of the exposed adult fish, indicating a trans-generational effect mediated by the endocrine system.

These laboratory studies provide datasets that show that exposure of fish to nonylphenol can induce a range of feminising effects observed in wild fish in English rivers. The range of nonylphenol concentrations (<1 to 7.7 μg l^{-1}) measured in final effluents in this study falls within the range of concentrations at which effects have been reported in the laboratory studies (1 to 20 μg l^{-1}). At most sites, though, nonylphenol concentrations were measured in the range of <1 to 3 μg l^{-1}, and concentrations exceeded 3 μg l^{-1} only at eight sites. These results suggest that nonylphenol (at least in isolation) is not likely to elicit oestrogenic responses in fish downstream, once the effects of dilution of the effluents at most sites is taken into account.

Nonylphenol ethoxylates

The Agency derived an EQS for alkylphenol ethoxylates, recognising that the EQS for nonylphenol, a breakdown product of nonylphenol ethoxylates, does not provide protection or regulatory control for the parent compounds (Environment Agency, 2002b). The EQS is based on a toxic equivalency approach, which takes into account the relative toxicity of each ethoxylate chain length in a mixture. As with the nonylphenol EQS, the alkylphenol ethoxylate EQS is based on data for acute toxicity. The limited data on the oestrogenicity of the ethoxylates was reviewed at the time. It indicated that the oestrogenicity of the ethoxylates increased with decreasing chain length.

Data on the potential endocrine disrupting effects of different lower chain nonylphenol ethoxylates are limited compared to that for nonylphenol. The available data are summarised below. As a result, there is greater uncertainty in interpreting the significance of the levels of NP1EO, NP2EO and NP3-5EO measured in the final effluent samples.

Jobling and Sumpter (1993) used an *in-vitro* fish hepatocyte bioassay to determine the oestrogenic potencies of a range of substances including nonylphenol and nonylphenol ethoxylates (2 carbon and 9 carbon chain length). The results showed that the oestrogenic potency of the ethoxylates decreased with increasing chain length. Nonylphenol was 1.1 times more potent than NP2EO and 5.1 times more potent than NP9EO.

Miles-Richardson *et al.* (1999) exposed fathead minnows to a mixture of primarily 7-11 carbon chain nonylphenol ethoxylates (and 4-nonylphenol) to determine the effects on secondary sexual characteristics and the gonads of sexually mature fish during 42 days continuous flow exposure. The nonylphenol ethoxylates did not

cause effects on male or female gonads or secondary sexual characteristics at concentrations up to and including 5.5 μg $l^{-1.}$

Gordon *et al.* (2002) assessed the relative endocrine disrupting activity of nonylphenol and nonylphenol ethoxylates using a three-month *in-vivo* assay with Japanese medaka. The effects assessment was based on the induction of vitellogenin and histological changes in the gonads of exposed fish. The researchers found oocytes in the testicular tissue of male fish at nominal concentrations of 100 μg nonylphenol l^{-1}. No effects were evident at nominal NP4EO concentrations of 10-1000 μg l^{-1}.

Given the high levels of lower chain nonylphenol ethoxylates found in final effluents from certain sewage treatment works, it would be valuable to undertake an assessment of their relative potencies compared to nonylphenol so that potential effects can be determined.

4.2 Comparison of the YES bioassay data and the analytical chemistry data

Figure 4.1 compares the oestrogenic potency of the final effluent samples as determined by the YES assay and based on the analytical chemistry data. We used the concentration data for steroids to provide a summed oestradiol equivalent for each sample based on the relevant potency of the individual steroids *in vitro*. The relative *in vitro* potencies of the steroids are as follows: ethinyloestradiol is 1.67 times more potent than oestradiol, which is 1.5 times more potent than oestrone (Thorpe, pers comm). Van den Belt *et al.* (2004) evaluated the relative potencies of the steroids in the YES assay and found that ethinyloestradiol and oestrone were of similar potency while oestrone was approximately 2.5 times less potent.

A poor relationship was found between the oestradiol equivalents calculated from the chemistry data and the oestradiol equivalents measured in the YES assay (R^2 = 0.037, p = 0.07), with the gradient of the regression line not differing significantly from 0. For many effluents, the high levels of steroids found (>20 ng l^{-1}) were not reflected in correspondingly high YES assay responses. Since oestradiol equivalents for the chemical data were only calculated for the three steroids (i.e. not including nonylphenol or its lower chain ethoxylates), only the hormones contributed to the calculated oestradiol equivalents.

In published studies that compare measured *in vitro* oestradiol equivalents with calculated oestradiol equivalents from chemical analyses, *in vitro* oestrogenic activity has often been found to be lower than the chemically measured oestrogenic activity (Kinnberg, 2003 and references therein). This discrepancy may have several explanations. Compounds may be present in the samples that interfere with the YES assay. Though the YES assay will detect the oestrogenicity of all the chemicals present in a sample, the assay will also be susceptible to antagonistic effects of mixtures at low exposure concentrations or possibly toxic effects if certain substances are present at higher concentrations in the mixture. Toxic effects of nonylphenol can be excluded, since the extraction procedure, performed at a neutral pH, would have excluded any acidic/phenolic compounds.

However, a few effluents were found to have relatively high concentrations of lower chain nonylphenol ethoxylates. These compounds would not have been excluded through the extraction method, and although generally considered less toxic than nonylphenol (Environment Agency (2002b), could have contributed to any potential toxic effects. Another explanation for the discrepancy in measured and calculated oestradiol equivalents may be that extraction and cleanup methods between chemical and *in vitro* samples often differ, highlighting the need for these to be as similar as possible. These data will be re-examined together with the data that will arise from the proposed demonstration programme. In addition, an Environment Agency chaired technical working group is addressing the extraction methods used and choice of *in vitro* analysis for the proposed demonstration programme.

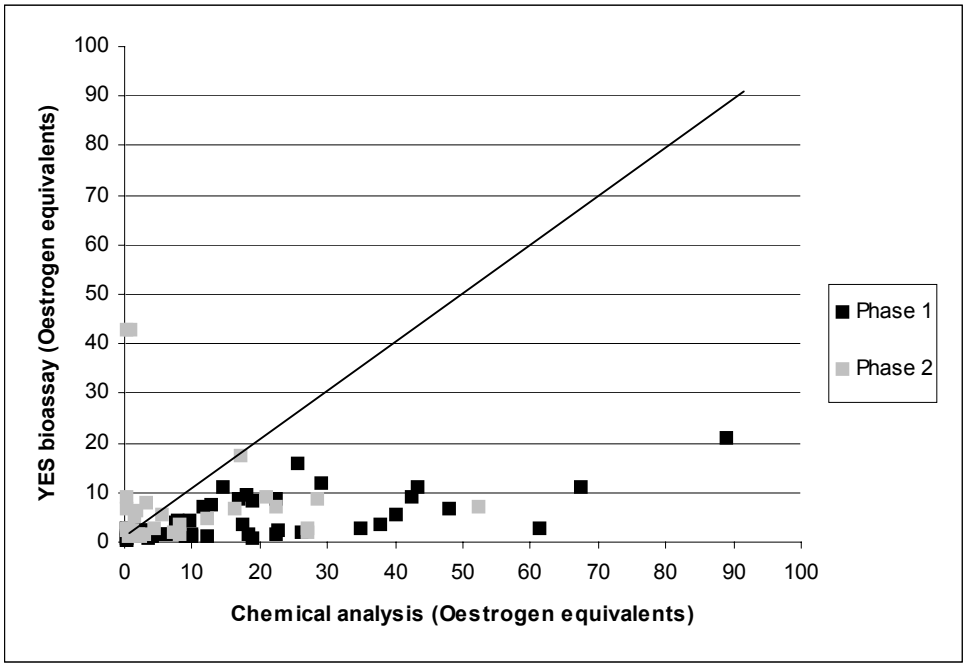

Figure 4.1 Comparison of the oestrogenicity of final effluent samples from Phases 1 and 2 of the study based on chemical analysis data and YES bioassay data. The line in the graph indicates where the data points would lie if the oestrogen equivalents from the YES bioassay and the chemical analysis corresponded to each other.

4.3 Hormone activity in effluents as determined in the YAS bioassay

The widespread nature of the anti-androgenic activity measured in the treated final effluents in this study would suggest that it is domestic in origin, but this has yet to be determined.

It is now well established that some chemicals can act as mimics of more than one hormone. Bisphenol A and some phthalates, for example, can act as both agonists of the oestrogen receptor (ER) and as antagonists of the androgen receptor (AR) in both mammals and fish (Sohoni and Sumpter, 1998). The lack of a clear relationship between the presence of oestrogen activity and anti-androgenic activity in the individual effluents though, suggests that the chemical(s), or the mixtures thereof, mediating these effect at the ER (agonists) and AR (as antagonists) may be different.

The significance of the finding of widespread anti-androgenic activity in the treated effluents has yet to be established, but the measured anti-androgenic activity in some effluents (given in flutamide equivalents) is sufficient to induce biological responses in fish. Recent data in the stickleback have shown that 125 $\mu g\ l^{-1}$ flutamide (for a three-week exposure) totally inhibited the production of 5aDHT-induced spiggin (a protein produced in the kidneys in response to androgens and used by male sticklebacks in the process of nest construction). In females, an exposure to 10 $\mu g\ l^{-1}$ causes a significant reduction in induced spiggin production. In male sticklebacks, the concentrations of flutamide that induced significant inhibitions and a total cessation of spiggin production (for a three-week exposure) were 50 and 250 $\mu g\ l^{-1}$, respectively. Spiggin production has also been shown to be responsive to other environmental chemicals that are known to act as anti-androgens, including linuron, diazinon and ethinyloestradiol (Defra, 2003b). Flutamide has also been shown to cause a regression in male secondary characteristics in fathead minnows (number of nuptial tubercles) at concentrations between 320 and 1000 $\mu g\ l^{-1}$ in three-week exposures. The effective concentrations for inducing a reduction in tubercle prominence in fathead minnows is below a concentration of 320 $\mu g\ l^{-1}$ (Panter pers com). The environmental anti-androgen linuron also caused a reduction in the fatpad size (index) in male fathead minnows exposed to 220 μg linuron l^{-1} for three weeks (Thorpe *et al.*, unpublished data).

4.4 Evaluation of different types of treatment on resulting steroid oestrogen concentrations in effluents

As part of the evaluation of the oestrogenicity of the 43 final effluents, we have considered the effects that different treatment processes have on final effluent steroid concentrations and YES bioassay data. We obtained information on the treatment processes from water companies operating the works. It has not been possible, though, to use the data for all works, most specifically those where different processes treat a proportion of the influent to the works before the different treatment streams are combined to produce the final effluent.

Table 4.4 summarises the steroid (oestrone, oestradiol and ethinyloestradiol concentrations and YES bioassay data (as oestradiol equivalents) for sewage works operating different treatment processes. Data are available for groups of seven works that treat influents using activated sludge and 10 works treating influents in a combination of biological (percolating) filters and humus tanks. From these data, it is evident that there is a marked variability in the effectiveness of the works operating a given process.

Within a given treatment type, steroid concentrations and YES bioassay responses range from low (all steroid concentrations at, or below, limits of detection and YES bioassay response < 1 ng l^{-1} oestradiol equivalents) to high (oestrone concentrations > 20 ng l^{-1} and YES bioassay responses > 5 ng l^{-1} oestradiol equivalents). Preliminary analysis of the data indicate that there is no general difference in the effectiveness of one treatment process over the other. The data from H1 and H2 sewage treatment works are interesting in that the influent is split one third to H1 and two thirds to H2 and treated in a similar manner, with the exception of the addition of ferric chloride or aluminium sulphate dosing at H2. Inclusion of the additional treatment stage does appear slightly to reduce oestrone concentrations and YES bioassay responses, though data are only available on two occasions, and so we cannot draw robust conclusions.

Within the scope of this project it was only possible to collate limited information on the STW process types. Evidence from other research programmes (e.g. COMPREHEND) suggest that process type and management practices can influence the removal of steroids from effluents. This was investigated in further R&D Environment Agency (2006c), and will be investigated in the proposed demonstration programme.

Table 4.4 Summary of the type of treatment processes operated at different works and the final effluent oestrogenicity

Type of treatment	Works	Population equivalents	Measured steroid concentrations (ng l^{-1})			YES assay (Oestradiol equivalents)
			Oestrone	Oestradiol	Ethinyloestradiol	
Activated sludge	B3	11870	*0.5* - 11	*0.5*	*0.5*	1.3 – 4.1
	B4	89291	11 - 26	<0.5 - <1	*0.5*	2.3 – 9.8
	B5	117166	*0.5* - 1.9	<0.3 – *0.5*	*0.5*	0.4 – 2.0
	C4	No data	15.5 - 34.2	*0.5* – 0.6	*0.5* - <1	6.5 – 8.5
	D2	192000	3.6 - 5.0	*0.5*	<0.5 - <1	0.9 – 1.2
	D4[1]	35006	<0.5	<0.5	<0.5	0.4 – 0.9
	E2	69169	28 - 33	<0.5	*0.5* - <1	0.7 – 1.4
	E8[2]	573484	28 - 35	2.6 - 4.0	*0.5*	1.8 – 2.3
Biological (Percolating) filters and humus tanks	A2	15217	*0.5* - 5.2	*0.5* - <1	0.3 - *0.5*	1.2 – 2.9
	A4	16000	*0.5*	*0.5*	*0.5*	2.7 – 6.6
	C1	195000	17 - 38	*0.5* - 6.0	0.3 - 0.7	4.4 – 16.2
	C2	107250	8.7 - 21	*0.5* - 2.9	<0.5 - <2	1.5 – 8.9
	E1	105879	74 - 100	18 - 22	1.3 - 1.7	11.0 – 20.5
	E4[1]	775457	56 - 71	*0.5*	*0.5*	3.4 – 6.7
	E7	18869	8.1 - 10	1.3 - 1.5	*0.5* - 1	1.5 – 2.3
	F1	41327	24.8 - 39.8	<1	*0.5*	1.8 – 17.5
	I1	No data	*0.5* - 11.2	<1	*0.5*	2.7 – 3.7
	I2	No data	0.9 - 26.2	<1	*0.5* - <1	8.6 – 15.3
Primary sedimentation, high rate filters, conventional filters and humus tanks	H1	No data	23.6 - 33.0	*0.5* - <1.0	*0.5* - <1	6.6 – 7.0
Primary sedimentation, high rate filters, conventional filters, humus tanks and ferric chloride or aluminum sulphate	H2	No data	1.9 – 17.3	0.5 - <1	*0.5* - <1	4.9 – 6.2

Notes: Notes: Values in italics are estimated values: steroid concentrations which were not quantifiable (see section 3.2.1) have been estimated to be 0.5 times the limit of detection, 1 – Process also includes tertiary sand filters, 2 – fully nitrifying

5 Conclusions and recommendations

5.1 Conclusions

The data from the analysis of ***steroids*** allowed us to draw the following conclusions:

- Marked variations in steroid concentrations were observed between the samples for different sewage treatment works final effluents;
- Steroid concentrations were measured in the ranges of <0.5 to 100 ng l^{-1} for oestrone, <1 to 22 ng l^{-1} for oestradiol and <1 to 3.2 ng l^{-1} for ethinyloestradiol. There were high levels of steroids, primarily oestrone, in final effluents from a number of locations. Oestrone concentrations of $\geq$ 20 ng l^{-1} were found in 13 of 25 final effluents on one or more of the sampling occasions in Phase 1 and 8 of 18 final effluents on one or more of the sampling occasions in Phase 2. A basic comparison with the proposed Predicted No Effect Concentrations (PNECs) for the natural and synthetic steroids indicates that a number of STWs, even following dilution, would result in receiving water steroid concentrations that would potentially result in oestrogenic effects in resident fish populations downstream of the discharges. A risk assessment of the effluents, taking into account data on STW effluent flow, steroid loads and river dilution is presented in a separate risk assessment report;
- There was general consistency between the results obtained for the two samples taken at a given location. A limited number of final effluents (G3, H2, H3 and I2), though, showed more marked variability (>5 times difference) between the results for the two samples;
- For certain final effluents, no quantifiable peaks could be discerned, particularly for the synthetic steroid ethinyloestradiol. We believe that this may be due in part to the presence of interferents in the samples (such as carboxylic acids) which may bind competitively to the C_{18} SPE column, particularly for substances such as ethinyloestradiol which are present in the final effluent samples only at low concentrations. While the absence of easily quantifiable peaks represents a technical difficulty it may also suggest that the actual concentrations of steroids present in the effluents are low.

The data from the analysis of ***nonylphenol and nonylphenol ethoxylates*** allowed us to draw the following conclusions:

- There was general consistency between the nonylphenol results obtained for the two samples taken at a given location;
- The levels of nonylphenol in the final effluent samples taken in both Phases 1 and 2 ranged from the limit of detection (1.0 μg l^{-1}) to maximum concentrations of 7.7 μg l^{-1} and 3.8 μg l^{-1} respectively. Most effluent concentrations were in the range of

<1 to 3 μg l^{-1}. Concentrations above 3 μg l^{-1} were only measured at seven of the 25 sites sampled in Phase 1 and at one of the 18 sites sampled in Phase 2;

- These results suggest that nonylphenol (at least in isolation) is less likely to elicit oestrogenic responses in fish downstream, once dilution of the effluents is taken into account at the majority of sites;
- For NP1EO, no samples in Phase 1 and only two samples in Phase 2 (from F3 STW) exceeded 10 μg l^{-1}. For NP2EO, one sample in Phase 1 (from E1 STW) and one sample in Phase 2 (from F3 STW) exceeded 10 μg l^{-1}. For NP3-5EO, seven samples in Phase 1 (from A3, B3, C2, E2, E4 and E5 STWs) and 13 samples in Phase 2 (from C4, F2, F3, G1, I2, I3 and J3 STWs) exceeded 10 μg l^{-1}. The significance of the data in terms of oestrogenic activity is, though, uncertain due to limited data against which to compare measured concentrations.

The data from the ***YES and YAS bioassays*** allowed us to draw the following conclusions:

- All of the samples tested exhibited some oestrogenic activity, with potency equivalents of between 0.4 and 20.5 ng E2 l^{-1} for the Phase 1 samples and between 0.9 and 42.7 ng E2 l^{-1} for the Phase 2 samples;
- None of the samples tested exhibited any clear anti-oestrogenic activity;
- None of the final effluent samples from Phase 1 and only final effluent samples from one location in Phase 2 (F2 STW) were found to contain any detectable androgenic activity;
- All samples, though, exhibited significant anti-androgenic activity, with potency equivalents of between 21.3 and 228 μg flutamide l^{-1} for the Phase 1 samples and between 90.7 and 1231 μg flutamide l^{-1} for the Phase 2 samples.

5.2 Recommendations

The findings of this research result in the following recommendations:

- The issues associated with the analysis of steroids and the role of interferents in the extraction procedure, particularly for the synthetic steroid ethinyloestradiol, need to be resolved. Since completion of this project a technical working group has been convened by the Agency to address this and other analytical issues;
- Further studies are needed to assess the relationship between the results of *in vitro* assays and chemical concentrations of oestrogenic substances. These should include a comparison of different *in vitro* assays (e.g. YES and ER-CALUX). The assays will detect the combined effects of mixtures, but they are also susceptible to toxic effects at higher exposure concentrations. It is, therefore, important to understand how different substance types such as steroids and alkylphenols and alkylphenol ethoxylates interact in the assays. Since the completion of this project a biological effects technical working group has been convened by the Agency to address this issue and to ensure that extraction methodologies for *in vitro* assays and chemical analysis are consistent;

- Variations in effluent steroid concentrations need to be better understood, especially with respect to differences resulting from the various treatment processes. This is being addressed within the demonstration programme, so that environmental risk can be evaluated better;
- The limited data currently available indicate that the oestrogenicity of nonylphenol ethoxylates decreases with increasing chain length. The relative potencies of the different lower chain nonylphenol ethoxylates need to be investigated so that their relative contributions to an overall oestrogenic effect can be identified. However, the use of nonylphenol ethoxylates was restricted from January 2005, so their contribution to oestrogenic effects in the environment is expected to diminish.
- The substances responsible for the anti-androgenic activity are unknown and this should be investigated using tools such as Toxicity Identification Evaluation (TIE). Anti-androgenic substances, though, suppress the effect of androgens in male fish, and the overall effect is expected to be similar to oestrogens in leading to a feminising effect. The wider biological significance of the anti-androgenic effects (notably in the generation of intersex) are not known. Defra are currently funding a project to determine the biological significance of androgen antagonism in the stickleback as part of their research programme on endocrine disruption in the aquatic environment (EDAQ). The detection of anti-androgenic effects at this stage does not detract from the focus of removing steroid oestrogens from effluents in the UK demonstration programme. It does, though, suggest that anti-androgenic biological effects should also be taken into consideration when monitoring treatment options.

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Appendix A

Volumes of effluent extracted for different determinands

Table A1 Volumes of effluent extracted for different determinands on the first sampling occasion in Phase 1

Water Company	Sewage Works	Column A (l)	Column B (l)	Column C (l)	Column D (l)
A	A1	0.75	1.0	0.75	5.20
	A2	0.75	1.0	0.75	5.30
	A3	0.75	1.0	0.75	5.20
B	B1	0.75	1.0	0.75	5.10
	B2	0.75	1.0	0.75	5.10
	B3	0.7	0.8	0.7	5.20
	B4	0.75	1.0	0.75	5.20
	B5	0.75	1.0	0.75	5.0 (D1A = 1.95, D1B =3.05)
C	C1	0.75	1.0	0.75	5.20
	C2	0.6	0.75	0.6	4.65
	C3	0.75	1.0	0.75	5.20
D	D1	0.75	1.0	0.75	5.00
	D2	0.75	1.0	0.75	5.30
	D3	0.75	1.0	0.75	5.30
	D4	0.75	1.0	0.75	5.00
	D5	0.75	1.0	0.75	5.30
	D6	0.75	1.0	0.75	5.00
E	E1	0.75	1.0	0.75	5.20
	E2	0.75	1.0	0.75	5.20
	E3	0.75	1.0	0.75	5.20
	E4	0.75	1.0	0.75	5.20
	E5	0.75	1.0	0.75	5.20
	E6	0.75	1.0	0.75	5.20
	E7	0.75	1.0	0.75	5.20
	E8	0.75	1.0	0.75	5.20
Control					5.20

A – YES/YAS bioassays
B – Immunoprecipitation studies (Not conducted)
C – Spare sample
D – Steroid analysis

Table A2 Volumes of effluent extracted for different determinands on the second sampling occasion in Phase 1

Water Company	Sewage Works	Column A2 (l)	Column B2 (l)	Column C2 (l)	Column D2 (l)
A	A1	0.75	1.0	0.75	5.20
	A2	0.65	0.6	0.6	1.6 (D2A = 0.75, D2B =0.85)
	A3	0.75	0.8	0.75	5.0 (D2A=2.2,D2B=2.8)
B	B1	0.75	1.0	0.75	5.20
	B2	0.75	1.0	0.75	3.70
	B3	0.65	0.6	0.7	3.45 (D2A=2.6, D2B=0.85)
	B4	0.65	0.6	0.6	3.45 (D2A=2.6, D2B=0.85)
	B5	0.75	1.0	0.75	4.0 (D2A=2.7, D2B=1.3)
C	C1	0.75	1.0	0.75	5.20
	C2	0.75	1.0	0.75	5.20
	C3	0.75	1.0	0.75	5.20
D	D1	0.75	1.0	0.75	5.10
	D2	0.75	1.0	0.75	5.20
	D3	0.7	1.0	0.7	5.30
	D4	0.75	1.0	0.75	5.10
	D5	0.75	1.0	0.75	5.00
	D6	0.75	1.0	0.75	5.20
E	E1	0.75	1.0	0.75	5.20
	E2	0.75	1.0	0.75	5.20
	E3	0.75	1.0	0.75	5.20
	E4	0.75	1.0	0.75	5.20
	E5	0.75	1.0	0.75	5.20
	E6	0.75	1.0	0.75	5.20
	E7	0.75	1.0	0.75	5.20
	E8	0.75	1.0	0.75	5.20
Control					5.20

Notes:

A – YES/YAS bioassays

B – Immunoprecipitation studies (Not conducted)

C – Spare sample

D – Steroid analysis

Table A3 Volumes of effluent extracted for different determinands on the first sampling occasion in Phase 2

Water Company	Sewage Works	Column A (l)	Column C (l)	Column D (l)
A	A4	0.75	0.8	5.2
C	C4	0.75	0.75	5.2
F	F1	0.75	0.75	5.2
	F2	0.75	0.75	5.2
	F3	0.75	0.75	5.2
G	G1	0.75	0.75	5.2
	G2	0.75	0.75	5.2
	G3	0.75	0.75	5.2
H	H1	0.75	0.75	5.2
	H2	0.75	0.75	5.2
	H3	0.75	0.75	5.2
	H4	0.75	0.75	5
I	I1	0.75	0.75	5.2
	I2	0.75	0.75	5.1
	I3	0.75	0.75	5.2
J	J1	0.75	0.75	5.2
	J2	0.75	0.75	5.2
	J3	0.75	0.75	5.2

Table A4 Volumes of effluent extracted for different determinands on the second sampling occasion in Phase 2

Water Company	Sewage Works	Column A (l)	Column C (l)	Column D (l)
A	A4	0.75	0.8	5.2
C	C4	0.75	0.75	5.2
F	F1	0.75	0.75	5.2
	F2	0.75	0.75	5.2
	F3	0.75	0.75	5.2
G	G1	0.75	0.75	5.2
	G2	0.75	0.75	5.2
	G3	0.75	0.75	5.2
H	H1	0.75	0.75	5.2
	H2	0.75	0.75	5.2
	H3	0.75	0.75	5.2
	H4	0.75	0.75	5
I	I1	0.75	0.75	5.2
	I2	0.75	0.75	5.2
	I3	0.75	0.75	5.2
J	J1	0.75	0.75	5.2
	J2	0.75	0.75	5.2
	J3	0.75	0.75	5.2

Appendix B

Assessment of steroid concentrations in the final effluent samples in Phase 1

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B1 Introduction

The samples were received, pre-extracted, on C_{18} solid phase extraction cartridges. These were processed to give solutions of derivatised steroids in dichloromethane. The processed samples and standards were run using gas chromatography (GC) coupled with ion trap mass spectroscopy (MS) to enable selected reaction monitoring experiments to be carried out.
The initial instrumental analysis was not very successful, and so a clean up procedure using silica solid phase extraction cartridges was therefore used. While not being totally successful on all samples, this allowed data to be obtained for more samples.

B2 Methodology

The samples arrived, pre-extracted, on C_{18} solid phase extraction cartridges. These were eluted with 20ml of 85 per cent methanol 15 per cent water into a 40ml EPA vial. The solvent was reduced to low volume (<5ml) under a gentle stream of nitrogen and then transferred to a 5ml reacti-vial. This was evaporated to dryness under nitrogen.

The residues were derivatised as follows: 0.2ml pyridine and 0.3ml N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide was added and the vial capped. This was heated at 60°C for 30 minutes and then allowed to cool. 0.3ml Bis(trimethylsilyl) trifluoroacetamide was added and the vial heated for a further 120 minutes at 120°C. The vial was allowed to cool and the reagents removed with heating under a gentle stream of nitrogen. The residue was resuspended in 0.5ml dichloromethane, and an aliquot (~0.25ml) transferred to a low volume autosampler vial for analysis by GCMS.

Standards were prepared by transferring a known amount of standard and internal standard to a reacti-vial and derivatising as described for the samples.
The samples and standards were analysed by GCMS using an ion trap for selected reaction monitoring. The molecular ions for the derivatised steroids and internal standards were stored and fragmented in the ion trap. The resultant ions were then scanned out to give daughter ion spectra.
We obtained peak areas from the extracted ion chromatograms of $[M-57]^+$ plus $[M-39]^+$ for estrone and estradiol and $[M-15]^+$ for ethynylestradiol. The data was calculated by reference to the internal standards (deuterated d_4 analogues). The operating conditions are given in Table B1.

Note: The $[M-39]^+$ fragments are from an aduct $[M+H_2O-57]^+$ ion formed as a consequence of the ion storage in an ion trap MS. It was necessary to introduce a post derivatisation clean up. This was done by diluting the remaining, derivatised sample to 2ml with DCM. This was passed through a 500mg Waters Sepak Plus silica solid phase extraction cartridge. Further DCM was applied to the cartridge until ~3ml had been collected. This was evaporated to dryness and resuspended in 250ul DCM. We also prepared derivatised standards using this clean up stage.

Table B1 Gas Chromatograph Conditions

Injection mode	Splitless (100 ml min^{-1} split at 0.75 min)
Injection temperature	300°C
Injection volume	5μl
Carrier gas	Helium at 1.0 ml min^{-1}
Column	30m x 0.25mm DB5-MS (df = 0.25μ)
Initial temperature	65°C for 10min
Ramp 1	15°C min^{-1} to 250°C
Ramp 2	2°C min^{-1} to 285°C hold for 1 min
Transfer line temperature	275°C

Mass Spectrometer Conditions

Ionisation mode	+ve ion electron impact
Source temperature	200°C

Ion trap parameters

Compound	Time (min)	Precursor ion	Product ions
Oestrone	32.5	384	200 – 390
d_4-Oestrone	"	388	200 – 390
Oestradiol	33.45	458	300 – 470
d_4-Oestradiol	"	462	300 – 470
Ethinyloestradiol	35.8	482	300 – 490
d4-Ethinyloestradiol	"	486	300 – 490

B3 Results

A summary of the results obtained is given in Table B2. The values given in brackets are for samples that have not been through the post derivatisation clean up stage.

B4 Discussion

We found the samples to be a challenge for the GC-MS system. The methanol extracts from the cartridges were coloured (dark brown through to dark green) when reduced to low volume and left a significant residue when reduced to dryness. After derivatisation, the final DCM extracts were a very dark brown/black colour. The standards were only a pale straw colour.

After running only a few samples, the response from the GC-MS had deteriorated badly. This was evidenced by an overall drop in response of the standards, resulting in the complete disappearance of some peaks. When we examined the injection liner after a 30 injection (12 actual samples) run, it was found to have a black coating which was likely to be at least part of the problem. The GC-MS could be restored to its initial response conditions only by: changing the injection liner, removing up to two metres from the injection end of the column and completely cleaning the mass spectrometer ion source. As this would be necessary every few injections, a better solution was required.

The indications were that the GC-MS could not cope with the amount of extraneous material that was being injected into the system. We therefore decided to try to clean up the derivatised sample extracts.

As the clean-up stage appeared to be successful with standards, all the samples were processed and re-run on the GC-MS. The silica clean-up could be seen to remove most of the intense colouration from the extracts.

This resulted in a more stable response from the GC-MS, though some degradation in response was still in evidence.

Table B2 Summary of the steroid concentration data

Sample	Oestrone (ng l^{-1})	Oestradiol (ng l^{-1})	Ethinyloestradiol (ng l^{-1})
A1 - Sample 1	5.6 (4.4)	0.9 (0.8)	0.3 (0.5)
A2 - Sample 2*	3.1 (-)	- (-)	- (-)
A2 - Sample 1	5.2 (6.6)	<1.0 (<0.5)	0.3 (0.3)
A2 - Sample 2	- (-)	- (-)	- (-)
A3 - Sample 1	91 (78)	- (-)	- (-)
A3 - Sample 2*	48 (28)	2.7 (-)	1.5 (-)
B1 - Sample 1	50 (48)	6.6 (7.9)	0.5 (1.0)
B1 - Sample 2	19	1.9	-
B2 - Sample 1	3.8 (1.6)	<0.5(<0.2)	<2.0 (<0.2)
B2 - Sample 2	11 (-)	0.8 (-)	<2.0 (-)
B3 - Sample 1	- (-)	-1.3	- (-)
B3 - Sample 2*	11 (-)	NIS (-)	- (-)
B4 - Sample 1	11 (14)	<0.5 (-)	- (-)
B4 - Sample 2*	26 (18)	<1.0 (<0.5)	- (-)
B5 - Sample 1*	- (-)	- (-)	- (-)
B5 - Sample 2*	1.9 (<0.5)	<0.3 (<0.3)	<0.5 (<0.5)
C1 - Sample 1	17 (27)	6 (3.6)	0.7 (<1.0)
C1 - Sample 2	38 (-)	- (-)	0.3 (-)
C2 - Sample 1	8.7 (6.6)	- (-)	<2.0 (-)
C2 - Sample 2	21 (-)	2.9 (-)	<0.5 (-)
C3 - Sample 1	14 (20)	2.9 (2.0)	0.8 (-)
C3 - Sample 2	11 (-)	<1.0 (-)	<2.0 (-)
D1 - Sample 1	25 (30)	5.5 (5.4)	0.3 (0.5)
D1 - Sample 2	16 (17)	2.1 (2.1)	0.5 (<1.0)
D2 - Sample 1	3.6 (2.9)	-0.4	<0.5 (<0.5)
D2 - Sample 2	5.0 (4.6)	<0.5 (<0.5)	<1.0 (<0.5)
D3 - Sample 1	NIS (23)	- (-)	- (-)
D3 - Sample 2	-27	- (-)	- (-)
D4 - Sample 1	<0.5 (0.6)	<0.5 (0.5)	<0.5 (0.3)
D4 - Sample 2	- (<0.2)	- (-)	- (-)
D5 - Sample 1	15 (19)	1.6 (2.3)	<0.5 (<1.0)
D5 - Sample 2	14 (23)	- (-)	- (-)
D6 - Sample 1	43 (NIS)	-13	<1.0 (-)
D6 - Sample2	25 (-)	2.2 (-)	<0.2 (-)
E1 - Sample 1	74	18	1.3
E1 - Sample 2	100	22	1.7
E2 - Sample 1	28 (-)	<0.5 (-)	- (-)
E2 - Sample 2	33 (-)	<0.5 (-)	<1.0 (-)
E3 - Sample 1	13 (NIS)	- (-)	- (-)
E3 - Sample 2	27 (20)	- (<0.5)	- (-)
E4 - Sample 1	56(-)	- (-)	- (-)
E4 - Sample 2	71 (NIS)	-7.4	- (-)
E5 - Sample 1	14 (18)	<1.0 (<0.5)	<1.0 (-)
E5 - Sample 2	14 (16)	<1.0 (-)	<2.0 (-)
E6 - Sample 1	54 (-)	6.2 (-)	3.2 (-)
E6 - Sample 2	57 (NIS)	5.2 (6.3)	2.5 (NIS)
E7 - Sample 1	8.1	1.3	1

E7 - Sample 2	10	1.5	-
E8 - Sample 1	35	2.6	-
E8 - Sample 2	28	4	-

Notes:

*	Sample extracted on two cartridges
( )	Values in brackets are from extracts that have not been through the clean up stage
-	Indicates no quantifiable peak for either the steroid or its internal standard
NIS	Peak Present but internal standard peak not present
<	values are estimated from the response of the internal standard

Appendix C

Assessment of (anti-) oestrogenic and (anti-) androgenic activity in effluents from sewage treatment works using recombinant yeast screens

Work sub-contracted to Exeter University by WRc-NSF for the Environment Agency's National Programme for assessing hormonal activity in UK sewage treatment works effluents

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C1 Background

The intrinsic (anti-) oestrogenic and (anti-) androgenic activity in effluent samples from 43 UK sewage treatment works (STWs), extracted by WRc-NSF were tested in the recombinant yeast oestrogen and androgen receptor assays (for details on the assays see Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998). The assays employ a yeast*, Saccharyomyces cerevisiae*, which is rendered either oestrogen- or androgen-responsive through the integration of the DNA sequence for the human oestrogen (hER) or androgen (hAR) receptor, respectively, into the yeast genome. The yeast also harbours expression plasmids carrying oestrogen- or androgen-responsive sequences, which control the expression of the reporter gene, lac-Z (encoding the enzyme □-galactosidase). In the presence of a receptor agonist, the activated receptor binds to the response element stimulating production of □-galactosidase. This, in turn, breaks down a chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG; yellow colour) in the medium into a red product that can be measured by absorbance. The hormonal activity of the effluent samples can then be determined by directly comparing the concentration-response curves of the individual effluent samples with the concentration-response curves obtained for reference standard chemicals.

C2 Methods

C2.1 Reference Standard Chemicals

The reference standard chemicals for the oestrogen (17β-oestradiol; E2; > 98 per cent pure, and 4-hydroxytamoxifen; > 99 per cent pure), and androgen (dihydrotestosterone; DHT; > 98 per cent pure and flutamide; > 98 per cent pure) assays were bought from Sigma Chemical Company Limited (Dorset, UK). To determine agonist activity, the natural ligands, E2 (oestrogen agonist) and DHT (androgen agonist), were serially diluted in ethanol and 10 μl aliquots transferred to a 96-well flat bottom microtitre plate (Linbro/Titertek, ICN FLOW, Bucks, UK). Antagonist activity was determined by incubating serial dilutions of 4-hydroxytamoxifen (anti-oestrogen) or flutamide (anti-androgen) with the natural ligands (E2 or DHT; at a concentration that produced a sub-maximal response ~ 75 per cent) and determining the ability of the chemicals to inhibit the colour change induced by the natural ligand.

C2.2 Test Samples

Solid-phase extraction columns, containing concentrated effluent samples (nominally 750 ml) were obtained from WRc-NSF, Medmenham, Marlow, Bucks, UK and stored at 4˚C. There were 86 samples in total (duplicates samples from 43 locations sampled at 2 different times). Prior to analysis in the yeast assays, the columns were eluted with 5 ml of methanol, the solvent was then removed under a stream of nitrogen and the extracts were re-suspended in 1 ml of ethanol.

C2.3 Assay Procedure

The yeast assays were performed as described by Routledge and Sumpter (1996). Briefly, test chemicals were serially diluted in ethanol and 10 μl aliquots transferred to 96-well flat bottom microtitre plates. The ethanol was allowed to evaporate to dryness, after which aliquots (200 μl) of assay medium (containing the recombinant yeast and the chromogenic substrate, CPRG) were dispensed into each sample well. The plates were sealed, shaken for two minutes, and then incubated at 32˚C. The androgen screen was incubated for two days and the oestrogen screen for three days. Then we measured colour development in the medium at an absorbance of 540 nm and turbidity of the yeast at 620 nm (using a Spectramax Plus, microtitre plate reader).

C3 Results

Concentration-response relationships for reference standard chemicals in the yeast (anti-) oestrogen (A) and (anti-) androgen (B) assays are shown in Figure C1.

All of the Phase 1 samples tested exhibited some oestrogenic activity with potency equivalents of between 0.4 and 11.7 ng E2 l^{-1} for the first collection point and between 0.9 and 20.5 ng E2 l^{-1} for the second collections (Table C1). None of the samples tested exhibited any clear anti-oestrogenic activity. In addition, none of the samples were found to contain any detectable androgen activity. All samples, though, exhibited significant anti-androgenic activity with potency equivalents of between 21.3 and 228 μg flutamide l^{-1} on the first sampling and between 23.5 and 200 μg flutamide l^{-1} on the second sampling (Table C2).

All of the Phase 2 samples tested exhibited some oestrogenic activity, with potencies equivalent to between 0.9 and 42.7 ng E2 l^{-1} on the first sampling and 1.0 to 42.7 ng E2 l^{-1} on the second sampling (Table C3). None of the final effluent samples tested in Phase 2 exhibited any clear anti-oestrogenic activity. Only one of the samples (from F2 STW) was found to contain any detectable androgen activity at each sampling, with a potency equivalent to 72.5 and 70.4 ng DHT l^{-1} on the first and second sampling, respectively (Table C4). Nearly all of the samples exhibited anti-androgenic activity with potencies equivalent to between 90.7 and 764.4 μg flutamide l^{-1} on the first sampling and between 90.7 and 1230.8 μg flutamide l^{-1} on the second sampling (Table C5). The STW samples taken from A4 STW and F2 STW did not exhibit any anti-androgenic activity on either sampling.

C4 References

Routledge, E.J. and Sumpter, J.P. (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem.* **15**, 241-248.

Sohoni, P. and Sumpter, J.P. (1998) Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **158**, 327-339.

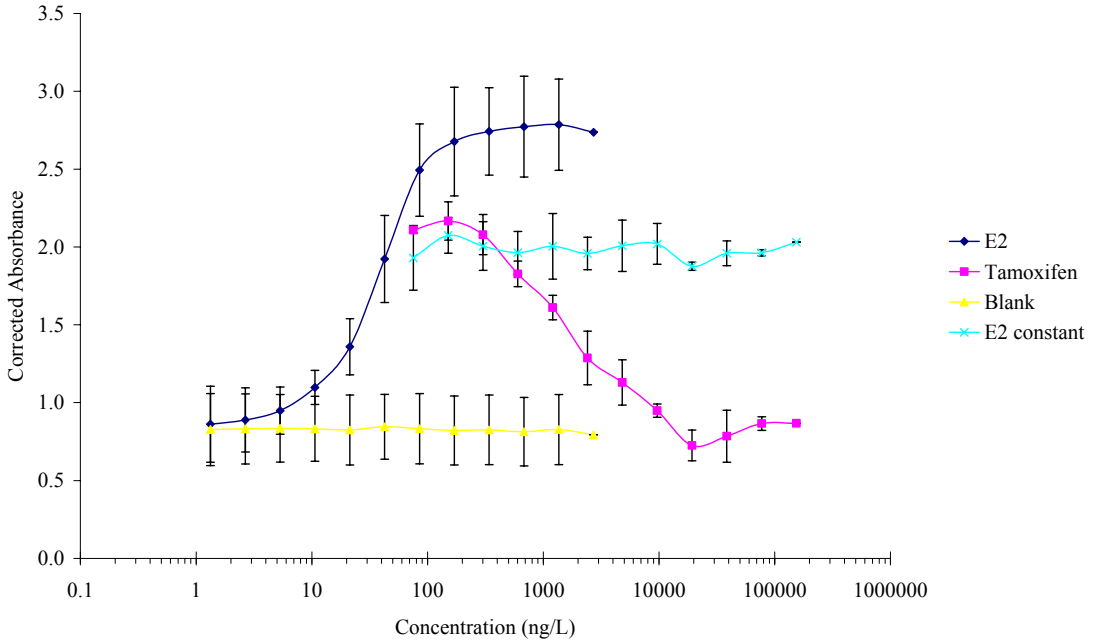

Figure C1 Concentration-response relationships for reference standard chemicals in the yeast (anti-) oestrogen (A) and (anti-) androgen (B) assays. Results shown as the mean corrected absorbance for duplicate assays ± standard deviation

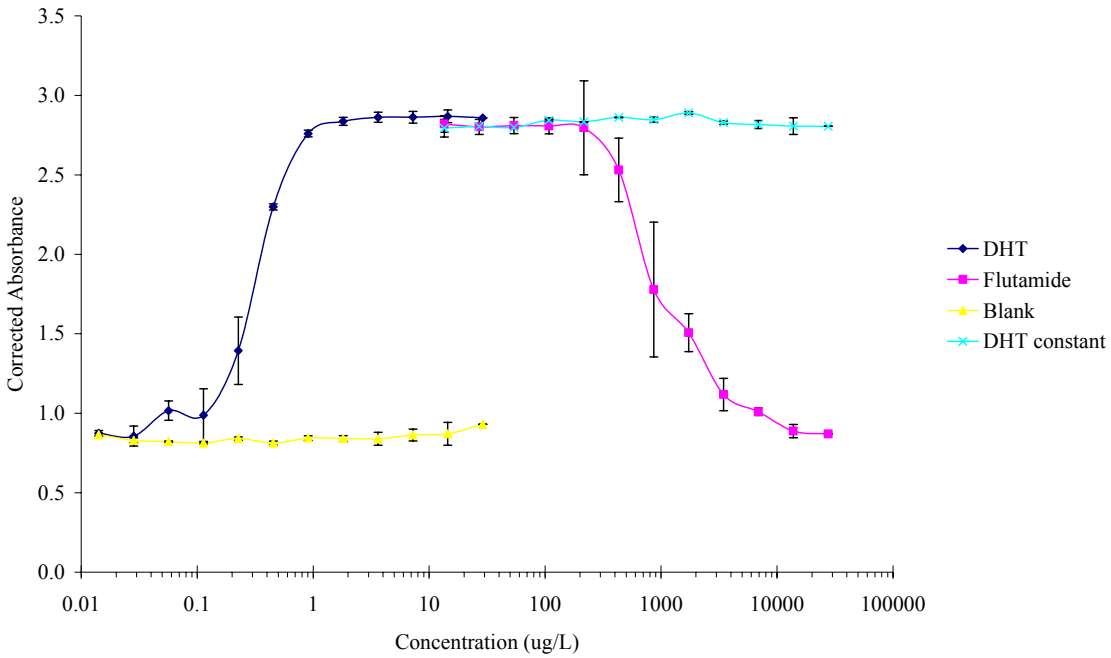

Figure C1 Concentration-response relationships for reference standard chemicals in the yeast (anti-) oestrogen (A) and (anti-) androgen (B) assays. Results shown as the mean corrected absorbance for duplicate assays ± standard deviation

Table C1 Oestradiol equivalent concentration (ng l^{-1}) in effluents from 25 STWs tested in Phase 1

Sample ID	Oestradiol equivalent concentration (ng l-1)		
	Sample 1	Sample 2	Mean
A1	1.5	2.5	2
A2	1.2	2.9	2.1
A3	2.7	2.7	2.7
B1	5.5	10.9	8.2
B2	2.2	3	2.6
B3	1.3	4.1	2.7
B4	2.4	9.5	6
B5	0.4	2	1.2
C1	4.4	16.2	10.3
C2	1.5	8.9	5.2
C3	1	4.3	2.7
D1	8.8	7.6	8.2
D2	1.2	0.9	1.1
D3	0.8	2.9	1.8
D4	0.4	0.9	0.7
D5	7.3	4.3	5.8
D6	11.7	8.2	10
E1	11	20.5	15.7
E2	0.7	1.4	1.1
E3	1	1.5	1.2
E4	3.4	6.7	5
E5	1.2	1.4	1.3
E6	9.3	10.7	10
E7	1.5	2.3	1.9
E8	1.8	2.3	2

Table C2 Flutamide (anti-androgen) equivalent concentration ($\mu g\ l^{-1}$) in effluents from 25 STWs tested in Phase 1

Sample ID	Flutamide equivalent concentration (ug l-1)		
	Sample 1	Sample 2	Mean
A1	125.5	71	98.2
A2	53.3	50	51.7
A3	112.3	76.2	94.2
B1	21.3	41	31.2
B2	142.2	61.5	101.9
B3	81.6	115.4	98.5
B4	35.6	142	88.8
B5	76.2	76.2	76.2
C1	35.6	34	34.8
C2	66.7	76.2	71.4

C3	61	61.5	61.2
D1	142.2	42.1	92.2
D2	177.8	100	138.9
D3	35.6	28.6	32.1
D4	71.1	30.8	50.9
D5	21.3	200	110.7
D6	142.2	61.5	101.9
E1	35.6	47.1	41.3
E2	133.3	61.5	97.4
E3	228.6	100	164.3
E4	224.6	41	132.8
E5	125.5	53.3	89.4
E6	26.7	100	63.3
E7	53.3	53.3	53.3
E8	35.6	23.5	29.5

Table C3 Oestradiol equivalent concentration (ng l^{-1}) in effluents from 18 STWs tested in Phase 2

Sample ID	Oestradiol equivalent concentration (ng l-1)		
	Sample 1	Sample 2	Mean
A4	2.7	6.6	4.7
C4	8.6	6.5	7.6
F1	17.5	1.8	9.7
F2	42.7	42.7	42.7
F3	1.6	1	1.3
G1	3.4	12.7	8.1
G2	7.8	2.2	5
G3	5.6	8.6	7.1
H1	6.6	7	6.8
H2	6.2	4.9	5.6
H3	2.9	2.8	2.9
H4	1.4	2.5	2
I1	2.7	3.7	3.2
I2	8.6	15.3	12
I3	5.1	9.3	7.2
J1	1.6	1	1.3
J2	1.8	1.5	1.7
J3	9	7	8

Table C4 Dihydrotestosterone (androgen) equivalent concentration (ng l^{-1}) in effluents from 18 STWs tested in Phase 2

Sample ID	Dihydrotestosterone equivalent concentration (ng l^{-1})		
	Sample 1	Sample 2	Mean
F2	72.5	70.4	71.5

Table C5 Flutamide (anti-androgen) equivalent concentration ($\mu g\ l^{-1}$) in effluents from 18 STWs tested in Phase 2

Sample ID	Flutamide equivalent concentration (ug l-1)		
	Sample 1	Sample 2	Mean
A4	No activity	No activity	No activity
C4	457.1	1230.8	844
F1	347.5	382.2	364.5
F2	No activity	No activity	No activity
F3	347.5	573.3	460.4
G1	235.3	500	367.7
G2	280.7	421.1	350.9
G3	90.7	90.7	90.7
H1	318.5	318.5	318.5
H2	279.7	358.3	319
H3	318.5	318.5	318.5
H4	266.7	301.8	384.3
I1	228.6	470.6	349.6
I2	432.4	640	536.2
I3	222.2	246.2	234.2
J1	208.5	163.8	186.2
J2	764.4	477.8	621.1
J3	573.3	382.2	477.8

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