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Statistical Modelling Suggests That Anti-Androgens in Wastewater Treatment Works Effluents are Contributing Causes of Widespread Sexual Disruption in Fish Living in English Rivers.

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Abbreviations:

E1: estrone

E2: 17 β -estradiol

EE2: 17 α -ethinyloestradiol

ER: estrogen receptor

NP: nonylphenol

NP1-nEO: nonylphenol ethoxylates (1-5 ethoxylate chain length)

SPE: solid phase extraction

WWTW: waste water treatment works

YAS: yeast androgen screen

YES: yeast estrogen screen

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Abstract

Background: The occurrence of feminised male fish downstream of some wastewater treatment works has led to substantial interest from ecologists and public health professionals. This concern stems from the view that the effects observed have a parallel in humans, and that both phenomena are caused by exposure to mixtures of contaminants that interfere with reproductive development. The evidence for a “wildlife human connection” is, however, weak: Testicular dysgenesis syndrome, seen in human males, is most easily reproduced in rodent models by exposure to mixtures of anti-androgenic chemicals. In contrast, the accepted explanation for feminisation of wild male fish is that it results mainly from exposure to steroid estrogens originating primarily from human excretion.

Objectives: We sought to further explore the hypothesis that endocrine disruption in fish is multi-causal, resulting from exposure to mixtures of chemicals with both estrogenic and anti-androgenic properties.

Methods: Hierarchical generalized linear and generalized additive statistical modeling were used to explore the associations between modeled concentrations and activities of estrogenic and anti-androgenic chemicals in 51 UK rivers and feminised responses seen in wild fish living in these rivers.

Results: In addition to the estrogenic substances, anti-androgenic activity was prevalent in almost all treated sewage effluents tested. Further, the results of the modelling demonstrated that feminizing effects in wild fish could be best modelled as a function of their predicted exposure to *both* anti-androgens and estrogens *or* to anti-androgens alone.

Conclusion: The results provide a strong argument for a multi-causal aetiology of widespread feminisation of wild fish in UK Rivers involving contributions from both steroidal estrogens and xenoestrogens and from other (as yet unknown) contaminants with anti-androgenic properties. They may add further credence to the hypothesis that

endocrine disrupting effects seen in wild fish and in humans are caused by similar combinations of endocrine disrupting chemical cocktails.

Introduction

Wildlife populations associated with the aquatic environment can be exposed to concentrations of endocrine-disrupting pollutants that are high enough to compromise their reproductive capacity (Reviewed by Vos et al. 2000) which may, in turn, have population-level consequences (Kidd et al. 2007). The widespread nature of these abnormalities has led to substantial interest from scientists and the general public. This concern stems, in part, from the hypothesis that reproductive diseases seen in humans are also caused by exposure to the same chemical contaminants (Skakkebaek et al. 2001). The actual evidence to support the wildlife-human connection is, however, weak. Moreover, in most cases there is little evidence to link cause and effect in even a single species, let alone multiple species. Some of the best evidence has been found in riverine fish populations where feminization of wild male fish (e.g. Jobling et al. 1998) is thought to be caused predominantly by exposure to steroidal estrogens in WWTW effluents originating from human and animal excretion (Desbrow et al. 1998; Routledge et al. 1998) with more minor contributions from other estrogenic chemicals found in WWTWs effluents, such as bisphenols and phthalates, nonylphenols (NP) and their ethoxylates and carboxylates (Gibson et al. 2005; Harries et al. 1997; Vajda et al. 2008; Vethaak et al. 2005). Supporting the role of these steroidal estrogens in the feminization of wild fish, recently, a very strong correlation was shown between the predicted steroid estrogen content of UK rivers and feminisation in wild fish (Jobling et al. 2006). Reproductive disorders also seen in human males are, however, best induced by exposing laboratory rodents to environmentally relevant concentrations of anti-androgens and estrogens rather than to estrogens alone (Skakkebaek et al. 2001; Sharpe and Skakkebaek, 2005), thus suggesting that the aetiology of human and fish –

induced reproductive diseases likely differ. Notwithstanding this, the fact that there are more than 100 000 substances in wastewater effluents (not including the different isomers of chemicals, or their products of degradation), many of which have endocrine disrupting properties other than estrogenic, makes it highly likely that the feminizing responses seen in male fish also have a multi-causal aetiology involving chemicals with mechanisms of action other than estrogenic. The objective of this study, therefore, was to further explore this possibility by challenging the hypothesis that steroid estrogens are solely responsible for widespread sexual disruption seen in wild fish in UK rivers. This was done by using data on hormonal (estrogenic, anti-estrogenic, androgenic, and anti-androgenic) activities and concentrations of known endocrine disruptors in WWTW effluents together with hydrological data, to predict hormone and anti-hormone concentrations in receiving waters, over a wide geographical range. We then explored their relationships with sexual disruption in the wild fish living in these waters using statistical modelling. The results suggest that anti-androgenic chemicals of unknown identities are widespread in UK effluents and receiving waters and that, in addition to the steroid estrogens, these constituents of WWTW effluents are likely to play a major role in causing endocrine disruption in wild fish.

Materials and Methods

Data Sources:

Effluent Hormonal Activity and Chemistry

The Environment Agency's survey of hormonal activity in effluents (2007) provided data on effluent chemistry from the results of a U.K. national survey of sewage treatment works effluents (see Figure 1 for locations). Each sample had been analyzed for estradiol-17 β (E2) estrone (E1), 17 α ethinylestradiol (EE2), 4-tert-nonylphenol (NP) and lower NP ethoxylates (NPnEO where n=1-5 and indicates ethoxylate chain length) and for total estrogenic, anti-estrogenic, androgenic, and anti- androgenic activity in

recombinant yeast screens (rYES for [anti-]estrogenic and rYAS for [anti-] androgenic activities). The rYES and rYAS were supplied by J.Sumpter, Brunel University and the assays were ran as described by Routledge and Sumpter (1996) and Sohoni and Sumpter (1998). The detailed methods for the chemical analysis are fully described in Environment Agency (2007) and in other published papers in which these data have also been examined (Johnson et al. 2007; Thorpe et al. 2006,). Steroid estrogens were detected in all effluents at concentrations measured consistent with previous observations, the relative persistence of the three steroid estrogens and differences in human excretion rates.

Estimations of (anti-)Androgenic and (anti-) Estrogenic Activity or Steroid Estrogen and Alkylphenol Concentrations in the River water at the Fish Capture Sites.

30 sites were identified where modeled predictions of exposure to steroid estrogens, nonylphenols and hormonal activities in the receiving environment could be made and where fish were also captured. The sites sampled covered a wide geographical range and had a wide variation in the proportion of the flow of the river composed of sewage effluent.

At each site, the concentrations of various estrogenic chemicals and hormonal activities in the effluents of sewage treatment works located upstream were divided by the dilution factor in the river at the point of fish capture to obtain estimated concentrations of each parameter in the river. Full methods and supporting references are given in Jobling et al. (2006).

Measurements of Sexual Disruption in Fish

The Environment Agency's spatial survey of sexual disruption in fish (Jobling et al. 2006) provided data on the location and prevalence of male fish with elevated plasma vitellogenin levels (VTG), a feminized reproductive duct ("fem.duct") or with developing eggs ("oocytes") in the testes, and on the severity of this condition (mean relative number of oocytes in the testes; "*fem.index*", Nolan et al. 2001) in "male" roach from

each of the 30 sites (Table 1). There were 1083 fish in total (between 12 and 71 from each location). Feminized male fish (fish with feminized ducts and/or feminised germ cells) were present at many of these sites.

Statistical Methods

Because all of the covariates had skew distributions, they were transformed by $x \rightarrow \ln(x + 1)$, the 1 being added to x to avoid difficulties with $\ln(0)$ and also so that 0 maps to 0. Principal components analysis was used to establish the patterns of variation in individual contaminants and hormonal activities in effluent samples collected. Models describing the relationship between each contaminant (alone and in combination) and each of the biological responses were then constructed. These were fitted in a step-wise manner first accounting for the effects due to estrogens and then allowing for additional effects that could be explained by the anti-androgens and NP. Logistic regression was used for the analysis of the binary response variables *oocytes*, *fem.duct* and *VTG*. Generalized linear models (GLM) with gamma distributed errors (35) were found to fit the response *fem. index* well. For all responses, the data had a hierarchical structure with varying numbers (12 – 71) of fish sampled from the 30 sites. The concentrations of each pollutant were at site level, while the response variables were at fish level. A consequence of the data structure was that correlations between fish within sites could be anticipated and needed to be accounted for in the analysis. This was accomplished by first fitting hierarchical GLMs (Gelman and Hill 2007) with random effects for sites. For some responses, variation between sites turned out to be not significant and subsequent analyses were then simplified to ordinary non-hierarchical GLMs. An example of the general form of hierarchical model for a binary response is: $\text{logit}(\theta_{ik}) = \beta_0 + \beta_{1k} \text{age}_{ik} + \beta_2 x_k + \varepsilon_{ik}$, where θ_{ik} is the probability of response for fish i in site k , and x_k is the concentration of one of the pollutants at site k .

This example is represented graphically in Figure 2, in which each rectangle is a level of variation.

Once important covariates were established using these models, smoothed estimates of the relationships were obtained using generalized additive models (GAMs) (37). The aim was to describe the way that covariates interacted with each other in their effect on a response. Surface plots of the fitted models indicate whether pollutants combined in an additive, synergistic or antagonistic way in their joint effect on the response. Two covariates either (a) act *additively*, in the sense that they affect the response independently of each other and then the joint effect is just the sum of their separate effects; or (b) *interact* with each other in their effect on the response. In the latter case, the interaction can be either *synergistic* or *antagonistic*.

The statistical computations were done using the R software (R Development Core Team 2007).

Results

Exposure predictions:

In vitro hormonal (rYES/rYAS) activity

All of the river waters were predicted to contain estrogenic activity and almost all also anti-androgenic activity (see Figure 1 for a map showing the predicted spatial distribution of hormonal activity across the rivers sampled and Table 1 for hormonal activities). Estrogenic and anti-androgenic activities predicted to be present in the rivers ranged from 0.04 to 23.21ngEEQ/L and from 0 to 100.12µg flutamide equivalents/L, respectively.

Concentrations of estrogenic chemicals

After dilution, predicted steroid concentrations in the rivers receiving the effluents were between 24.09 and 0.01 ng/L for E1 and at much lower concentrations for the other two steroids. For some final effluents quantifiable peaks could not be identified for

either the steroids in the effluent extracts or the internal standards in the spiked samples, particularly for EE2 (present at the lowest concentrations). These samples were noted as missing values (NQP). For samples where the analyte was noted as present but at a concentration below detection, a value of half the detection limit was assigned to the effluent. When adjusted to allow for dilution in the river, these values were close to zero. NP and NPnEO (nonylphenol polyethoxylates were n-1-5) were also predicted to be present in river water; concentrations of NP ranged from 0.003 to 2.079 $\mu\text{g/L}$. At only 5 of the sites, the concentration of NP was predicted to exceed 1 $\mu\text{g/L}$ in river water.

Statistical Analysis of the Distribution of the Chemicals

A statistical investigation of the distributions of the various pollutants and hormonal activities present at the sites sampled revealed that many of them were co-occurrent (table 2). A consequence of the multicollinearity seen in the measurements of the various contaminants was that if the relative proportions of estrogens and anti-androgens were similar across the sites, it would have been difficult to distinguish their separate effects on fish. Fortunately, however, the results of the principal components analysis (PCA; Figure 3) revealed that the variation in the chemical composition of the sample sites could be separated into three main components or gradients, including one component (Comp 2; explaining 24% of the variation in the data) that differentiated the sites with high relative proportions of estrogens from those where anti-androgens predominated. Together, the three components accounted for 87.5% of the variation in the data: Component 1(50.3%) separated contaminated waters from background and Component 3 (12.4%) was mainly indicative of the concentration of EE2 compared with the other steroid estrogens

Statistical Associations Between the Chemical Exposure and the Biological Response Variables

The results of the PCA analysis indicated that it may be possible to separate the modeling of the associations between the feminizing effects seen in the fish and the anti-androgen exposure from those associated with estrogens. The hypothesis that anti-androgens contribute to feminisation in wild fish could then be tested using statistical modeling approaches. This was first done by first fitting models for each of the biological responses accounted for by estrogens and then estimating any additional effects that could be explained by anti-androgens.

Response: oocytes

There were 94 cases of fish with *oocytes* in their testes. The probability of *oocytes* in the testis of roach was correlated positively with the age of the fish ($p < 0.0001$) with a sharp increase in the age related effect when the fish were 3+ years old. Multiple logistic regressions on E1, E2, EE2, controlling for age, revealed that E1 was the most important predictor ($p = 0.004$) of *oocytes* and that no additional significant variation in the response could be explained by E2 and EE2 (for EE2, there were only 58 cases from sites with reliable estimates of EE2 concentration) Because NP was highly correlated with E1, it accounted for no additional variation in the response either. Interestingly, there was no correlation between the total estrogenic burden (YES) and the *oocytes* response. After allowing for E1 and age, however, there was a significant correlation between the anti-androgenic activity (anti-YAS) and the *oocytes* response ($p = 0.01$). The surface plot suggested an additive effect of E1 and anti-YAS on the probability of *oocytes* (figure 4). This was confirmed by the non-significant E1 x anti-YAS interaction term ($p = 0.37$) in the logistic regression model.

Response: *fem.index*

Of the 94 cases of fish with *oocytes* in their testes (*fem index* > 0), there were only 58 cases for which there were robust measurements of EE2 in the WWTW effluents which was insufficient for use in further statistical analysis. Disregarding EE2, multiple logistic regressions on E1 and E2 revealed that E2 was the best predictor of *fem.index*

($p=0.02$; averaged over all values of the anti-YAS variable) and there was no effect of NP ($p=0.78$) or YES ($p=0.77$) on this response variable. As with the *oocytes* response, after allowing for the effects of E2, the additional effect of anti-YAS over E2 on the *fem. Index* was, however, significant ($p=0.01$). The surface plot suggested a somewhat non-additive effect of E2 and anti-YAS on the *fem.index* (figure 5). This was confirmed by a significant negative E2 x anti-YAS interaction term ($p=0.02$) in the logistic regression model.

Response: fem.duct

Significant between-site variation ($p<0.0001$) was found for the response *fem.duct*. As explained in the methods, this inter-site variation was accounted for before testing for covariate effects. Multiple logistic regressions on E1, E2 and EE2 showed that, as with the *oocytes* response, the overall effects of steroid estrogens on the probability of *fem.duct* was best explained by E1 ($p<0.002$) and again, as NP was highly correlated with E1, it accounted for no additional variation in the response. The additional combined effects of both YES and anti-YAS over E1 were, however, significant ($p=0.006$). The surface plot suggested that there was an increased probability of *fem.duct* with increased anti-YAS but that increased YES might partially suppress this response (figure 6; See Supplemental Material). This was confirmed by a significant negative YES x anti-YAS interaction term ($p=0.01$) in the logistic regression model.

Response: VTG

Significant between-site variation ($p<0.0001$) was found for the response VTG. This was mainly due to the fact that the fish were sampled throughout the year and that VTG is known to vary with sampling month. After accounting for this, however, multiple logistic regressions on the steroidal estrogens, E1, E2 and EE2 showed that the VTG response was best explained by E1 alone ($p<0.004$). Over and above the steroid estrogens, NP was a good predictor of the VTG response ($p=0.0002$). Moreover, there

was a very significant effect of anti-YAS on the VTG response ($p < 0.0001$). A comparison of models fitted with all possible subsets of the three variables, NP, E1 and anti-YAS, suggested that NP and anti-YAS were jointly the best predictors of the VTG response, although the contribution of NP was marginal ($p = 0.09$) over the overwhelming effect of anti-YAS on its own ($p = 0.008$). The surface plot suggested that, in general, the VTG response increased with increasing anti-YAS (figure 7).

When taken together, the results of the statistical analyses suggested that male roach likely exposed to the highest concentrations of anti-androgens and/or steroid estrogens exhibited the highest prevalence of both ovotestes and oviducts and the highest vitellogenin concentrations. Moreover, the number of developing ova in the testes of the intersex fish (defined by the feminisation index) was also the greatest in these fish. A further important consideration is that, with the exception of the feminisation index, the responses seen in the fish did not correlate with the total estrogenic activity present in the water samples, as measured using the yeast estrogen screen; YES. Models of the interactions between the total estrogenic activity and the total anti-androgenic activity for each of the responses suggested that estrogenic components of the mixture sometimes appeared to antagonise or reduce the responses in the fish that were associated with anti-androgen exposure.

Discussion

These findings support the hypothesis that a *combination* of steroid estrogens, nonylphenolic chemicals and anti-androgens are most likely to cause widespread sexual disruption in wild fish populations in nature. By statistical modeling of the associations between each of the suspected causal factors and the suite of biological effects seen in fish, the likely influence of anti-androgens versus estrogens, both alone and in combination, on each response variable was established. Although these statistical analyses further support the role of steroid estrogens in the causation of

feminization of wild fish in UK rivers, they also suggest that anti-androgens are strong causal factors, necessary for severe effects to occur. Indeed, the likely influence of anti-androgenic chemicals on each of the measured responses is clearly demonstrated using a modeling strategy that allows for the effects of steroid estrogens first before interrogating the data for the existence of additional causal factors. This approach further strengthens the hypothesis that feminization results from the effects of both anti-androgens and estrogens acting in concert.

Sometimes, the anti-androgens appear to act additively with the estrogens to increase a particular response (for oocytes and feminised ducts), whilst in other examples, the effect of the anti-androgens appears greater than that of the estrogens (VTG in the blood plasma of males). For the response *feminised ducts* there is an interaction between the steroid estrogens and the anti-androgenic activity, the estrogens acting to decrease the response due to the anti-androgens. This does not necessarily imply that all of the factors were interacting to produce a particular response at the same time. Some of the responses (e.g. fem.duct) are known to be induced during early development (e.g. Rodgers-Gray et al. 2001), whilst others (e.g. oocytes) are known to manifest themselves throughout life (Jobling et al. 2006). It is conceivable, that when additive relationships are seen, they could be the result of a concentration related effect of an initiator (acting during early life) and a promoter (acting during adult life).

The estrogenic activity of the water samples (as measured in the YES bioassay) did not correlate well with any of the biological responses, or with the concentrations of individual steroid estrogens measured in the effluents. In most cases, the combined estrogenic activity of the steroid estrogens present in the effluents was predicted to be higher than that actually measured using the YES bioassay. This lack of correlation between the YES assay results and the individual concentrations of steroid oestrogens could well have been due to the existence of anti-estrogenic compounds in some of the effluents which would reduce the response seen in the YES assay. Indeed the widespread existence of anti-estrogenic benzotriazoles in STW

effluents, potent in the YES bioassay, has recently been reported (Giger et al. 2006). Moreover, a recent publication, showed that benzotriazoles were not anti-estrogenic in fish, even though they were potent anti-estrogens in the YES bioassay (Harris et al. 2007), thus providing a possible explanation for the mis-match between the fish responses and the YES screen response. Indeed, the strong positive correlations of the biological responses with the steroid estrogen concentrations but not the YES assay results add credence to this suggestion.

Although the PCA indicated heterogeneity of anti-androgens and estrogens across sites, there were still correlations between some of the covariates and the multicollinearity exhibited by these co-occurrent contaminants sometimes confounded the interpretation of the statistical analyses. For example, NP was always highly correlated with E1 (Table 2) and so its association with any of the biological effects could rarely be separated from that of E1. However, when the strength of the association between one of these parameters and a response was stronger than that of the other, this was at least an indicator that the former was a more likely cause than was the latter. Intuitively, strong associations are more likely to be causal than weak ones (Bradford Hill, 1965). Moreover, the statistical modelling strategy adopted ensured that additional likely causal factors (anti-androgenic components) were identified only after accounting for the effects of the main causal factors (steroid estrogens).

Multicollinearity could also account for the possibility that none of the covariates were causes of feminisation in wild fish and that they were masking the identity of an as yet unidentified chemical cause. In most cases, however, this possibility seems highly unlikely, as the association between the anti-androgenic activity and the responses would appear strong enough to rule out hypotheses that the associations are entirely due to one weak unmeasured confounder or other source of modest bias. Moreover, given the fact that laboratory experiments clearly show that exposure to anti-androgens (e.g. Kiparissis et al. 2003; Makynen et al. 2000) or steroid or

xenoestrogens (e.g. Seki et al. 2002; Yokota et al. 2001) can cause sexual disruption in fish, it would seem plausible that chemicals with these mechanisms of action could also cause effects on wild fish. For example, it has been shown many times that intersexuality and vitellogenin induction can be seen in fish exposed to concentrations of steroid estrogens in the low ng/L range. Moreover, at least with the vitellogenin response, combinations of steroid (and other) estrogens have been shown to act additively to cause this effect (Brian et al. 2005; Thorpe et al. 2003).

As with the estrogenic activity, the anti-androgenic activity (given in flutamide equivalents) predicted to be present in the rivers, was often sufficient to induce biological responses in fish (Katsiadaki et al. 2006; Kiparissis et al, 2003). In addition, molecular approaches studying changes in gene expression have shown that the feminizing effects of estrogens and anti-androgens in fish share both common and distinct gene pathways (Filby et al. 2007a, 2007b,). It would seem likely, therefore, that mechanisms exist via which combinations of estrogens and anti-androgens could act together when they are dosed in combination (Kortenkamp 2008), thus offering further support to some of the cause-effect associations postulated in this paper. .

These results are a clear demonstration that induced reproductive health effects in fish in UK rivers likely involves factors other than environmental estrogens. They also provide an interesting parallel with the results of studies performed in rodent models to investigate the suspected environmental causation of testicular dysgenesis syndrome in humans; this is also thought to be mediated primarily by anti-androgenic but also estrogenic mechanisms rather than by estrogenic mechanisms alone (Christiansen et al. 2008; Sharpe and Skakkebaek 2008; Skakkebaek et al. 2001; Wolf et al. 1999). Whilst analysis of the human data by itself has so far failed to provide firm evidence of direct causal associations between low level exposure to specific endocrine disrupting chemicals and endocrine disorders in humans, studies such as ours linking endocrine effects seen in wildlife to exposure to estrogens and anti-androgens present in human domestic waste water may add further credence to the hypothesis that both the effects

seen in wild fish and those seen in humans are caused by similar combinations of endocrine disrupting chemical cocktails to which both fish and humans are exposed to.

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Table 1. Exposure predictions and biological impacts for 30 river sites around the UK. Concentrations of E2=estradiol 17 β , E1=estrone, EE2=17 α -ethinylestradiol, and nonylphenol (NP) as well as total estrogenic activity (in estradiol equivalents; EEQ) and total anti-androgenic activity (in flutamide equivalents; flutamide Eq) were predicted (from effluent concentrations and dilution factors)

Site	E2 ng/L	E1 ng/L	EE2 ng/L	YES EEQ ng/L	Anti-YAS Flutamide Eq μ g/L)	NP (μ g/L)	Ovo testes (n)	Ovi-ducts (n)	Mean VTG male	Mean VTG intersex	Mean Intersex index
1	NQP	0.42	NQP	0.14	9.39	0.15	0	1	-	25	-
2	0.3	5.2	<0.25	2.1	51.7	1.05	3	0	25	32	2.28
3	0.366	9.42	0.203	0.37	12.77	0.386	2	6	-	39	1.25
4	<0.066	5.69	<0.039	23.21	0	0.345	1	0	188	ns	1.33
5	<0.021	0.1	<0.012	1.24	0	0.09	5	0	496	2332	1.54
6	<0.25	<1	<0.15	1.95	0	0.2					
7	1.308	3	0.099	1.63	6.18	0.318	4	3	310	305	1.42
8	0.115	2.13	<0.043	0.75	29.29	0.542	7	2	273	793	1.90
9	NQP	1.26	NQP	0.31	11.31	0.344	3	1	84	525	1.79
10	NQP	14.7	NQP	4.77	70.63	1.353	5	9	202	125	1.44
11	0.198	1.26	0.331	0.79	50.41	0.553	1	2	142	25	1.17
12	<0.08	5.03	<0.05	7.96	0	0.851	5	1	42	25	1.70
13	<0.005	0.01	<0.003	0.04	0	0.003	0	0	16	-	-
14	0.881	4.56	0.116	1.71	5.77	0.247	6	6	34	43	1.67
15	0.991	4.96	0.159	1.07	24.26	0.557	6	3	477	487	2.05
16	NQP	15.9	NQP	45.1	0	0.70	2	3	81	10617	1.17
17	NQP	2.53	NQP	0.67	0	0.072	2	0	37	75	2.52
18	NQP	0.95	NQP	2.94	5.65	0.053	1	0	22	10	1.33
19	0.548	2.06	0.058	1.18	13.30	0.251	3	3	25	51.8	3.28
20	<0.179	3.1	<0.108	0.79	100.12	0.618	3	8	69	334	1.5
21	<0.152	15.2	<0.091	1.1	19.55	0.82	7	11	7022	20907	2.36
22	2.799	24.0	<0.106	7.09	72.21	1.303	7	1	41	186	3.43
23	<0.0013	0.44	<0.0008	0.85	0	0.023	0	0	25	-	-
24	1.086	9.84	0.1	3.94	75.14	0.796	4	6	422	272	2.17
25	<0.092	0.24	<0.0923	1.1	0	0.094	1	0	25	27	1.17
26	<0.052	3.42	<0.031	0.12	10.93	0.739	0	3	25	246	-
27	<0.063	3.56	<0.038	0.33	22.74	1.723	1	8	208	426	1.33
28	0.23	1.6	0.177	0.34	9.436	0.255	6	5	25	37	1.77
29	NQP	18.1	NQP	1.15	17	2.079	8	13	179	203	1.52
30	<0.25	2.0	<0.15	5.1	0	0.7	0	0	122	-	-

Notes: NQP denotes no quantifiable peak (and hence no data). Values with < sign were for effluent samples in which the concentration of the desired analyte was less than the detection limit. The detection limit in each case was divided by the dilution factor of the effluent in the river at the point where the fish were captured.

Table 2. A statistical investigation of the co-occurrence of the various pollutants and hormonal activities present in the effluents sampled. The steroid estrogen E2 and its metabolite E1 were highly correlated (E2 is oxidised to E1). EE2 (the contraceptive pill hormone) was also associated with E2, as expected. There was no correlation between the total estrogenic (YES) and total anti-androgenic (anti-YAS) activities, indicating that the chemicals inducing these two hormonal activities are likely to be different.

	E1	E2	EE2	NP	YAS
E2	0.72 (***) n = 28				
EE2	0.35 (*) n = 22	0.56 (**) n = 22			
NP	0.77 (***) n = 30	0.45 (*) n = 28	0.26 (ns) n = 22		
YAS	0.48 (**) n = 30	0.22 (ns) n = 28	0.00 (ns) n = 22	0.62 (**) n = 30	
YES	0.49 (**) n = 30	0.44 (*) n = 28	0.51 (*) n = 22	0.15 (ns) n = 30	-0.25 (ns) n = 30

Note: Significance levels: (***) P < 0.001
 (**) P < 0.01
 (*) P < 0.05
 (ns) not significant

Figure Legends

Figure 1. Map showing the overlap in spatial distribution of estrogenic and anti-androgenic activity in the U.K waste water treatment works sampled. Red=present; green =not present. Large circles are for anti-androgenic activity and small circles are for estrogenic activity.

Figure 2. An example of the general form of hierarchical model for a binary response is:

$\text{logit}(\theta_{ik}) = \beta_0 + \beta_{1k} \text{age}_{ik} + \beta_2 x_k + \varepsilon_{ik}$, where θ_{ik} is the probability of response for fish i in site k , and x_k is the concentration of one of the pollutants at site k .

Figure 3. Principal components analysis of the chemical and (anti-) hormone composition of the sample sites. The plot illustrates the first two components only. The numbers on the plot are the site codes (also listed in table 1). Comp. 1, on the x-axis, is overall level of contamination. So, for example site 9 is the dirtiest and site 24 the cleanest. Comp. 2 on the y-axis is high for predominantly estrogens, low for predominantly anti-androgens. The extremes on this component are 18 (anti-androgens) and 29 (estrogens). The arrows, labelled E1, NP, etc, represent the variables and two arrows pointing in similar directions means they are correlated. The top and right axes are standardised scores for the variables; the bottom and left axes are scores for the sites.

Figure 4. Surface plot illustrating the results of the statistical modelling of the association between E1 and anti-YAS and the probability of oocytes in the testes of male fish. The lower and upper surfaces represent 95% confidence limits and the middle surface is the fitted mean. The suggested additive effects were confirmed by the non-significant E1 x anti-YAS interaction term ($p=0.37$) in the logistic regression model.

- 2 -

Figure 5. Surface plot illustrating the results of the statistical modelling of the association between exposure to E2 and anti-YAS on the feminisation index in intersex fish. The lower and upper surfaces represent 95% confidence limits and the middle surface is the fitted mean. The plot indicates a somewhat non-additive effect of E2 and anti-YAS on the *fem.index*. This was confirmed by a significant negative E2 x anti-YAS interaction term ($p=0.02$) in the logistic regression model.

Figure 6. Surface plot illustrating the results of the statistical modelling of the association between exposure to estrogenic and anti-androgenic chemicals. The lower and upper surfaces represent 95% confidence limits and the middle surface is the fitted mean. The plot indicates the additional combined effects of both YES and anti-YAS ($p=0.006$) over E1 on the probability of feminisation of the reproductive ducts in wild male fish. The surface plot suggested that there was an increased probability of *fem.duct* with increased anti-YAS but that increased YES might partially suppress this response (See Supplemental Material, figure 6a for two dimensional plot). This was confirmed by a significant negative YES x anti-YAS interaction term ($p=0.01$) in the logistic regression model.

Figure 7. Surface plot illustrating the results of the statistical modelling of the association between exposure to estrogenic and anti-androgenic chemicals on the VTG response in male and intersex fish. The lower and upper surfaces represent 95% confidence limits and the middle surface is the fitted mean. The modeling suggested that NP and anti-YAS were jointly the best predictors of the VTG response, although the contribution of NP was marginal ($p=0.09$) over the overwhelming effect of anti-YAS on its own ($p=0.008$).

Figure 1.

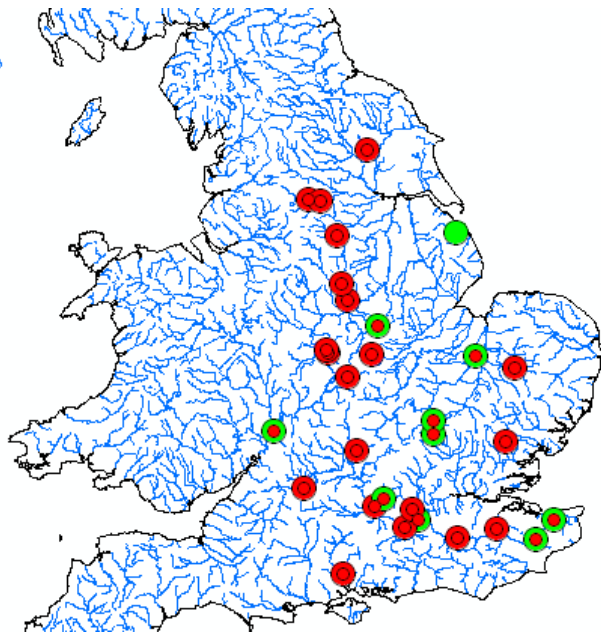
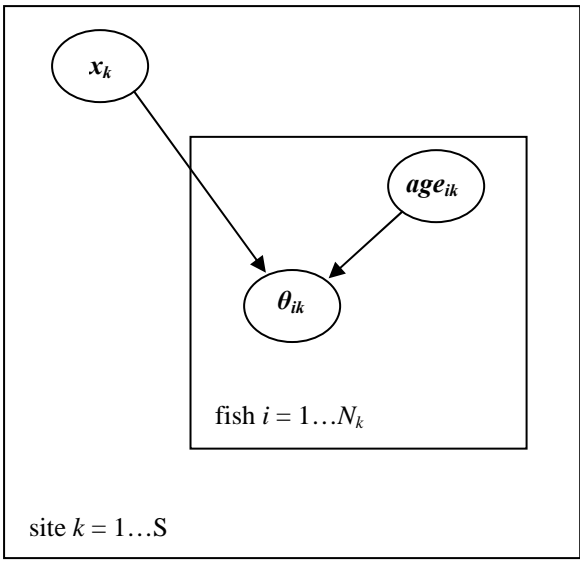
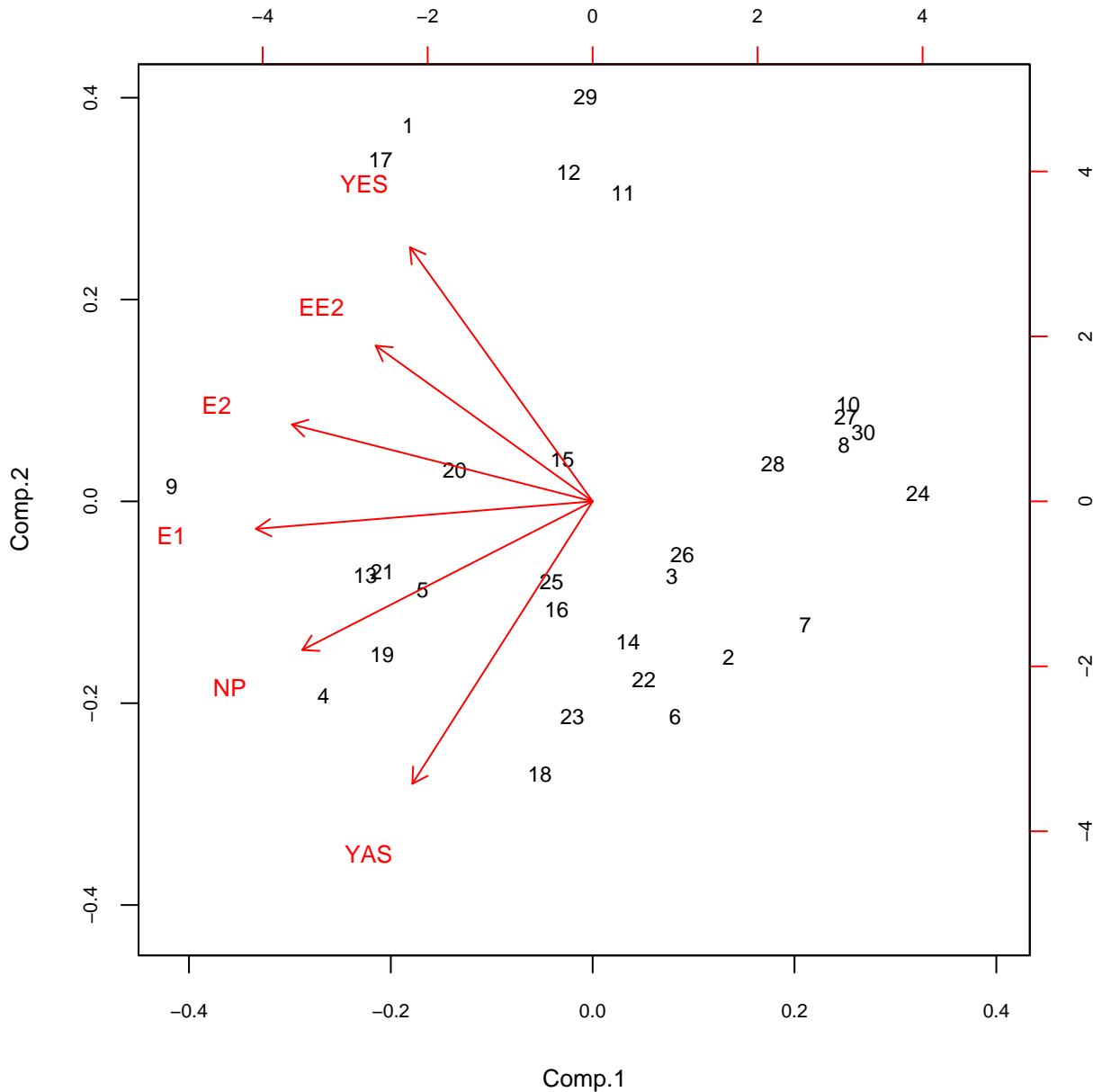


Figure 2.





Supplemental Material, Figure 6a