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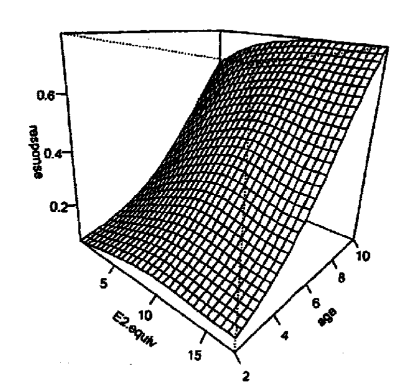
(56) Documents Cited:
Environmental Science and Technology Vol. 38, Issue 13, July 2004 (Washington, DC), A C Johnson and R J Williams, "A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works", pages 3649 to 3658.

(continued on next page)

(54) Abstract Title: **Device and method for predicting effects of steroid oestrogens on aquatic organisms**

(57) A device and method for evaluating the levels of steroid oestrogens such as oestradiol, oestrone and ethinylestradiol in sewage effluents to obtain a pressure assessment. A biosensor or flow rate meter is used for evaluating the levels of steroid oestrogens. The levels of each steroid oestrogen are converted into oestrogen equivalents (EEQ). The EEQ value is compared with a general model to obtain an impact assessment. The pressure and impact assessments are used to predict the overall risk posed by steroid oestrogens to freshwater organisms, such as fish.

Figure 4a



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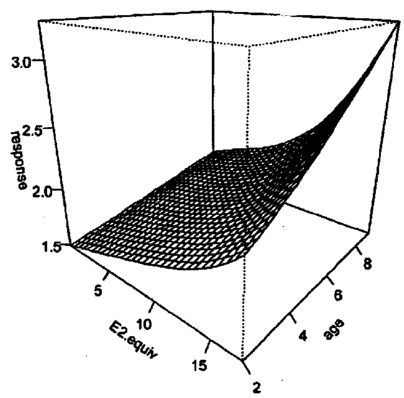

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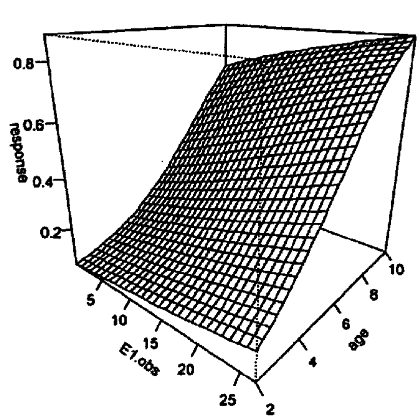

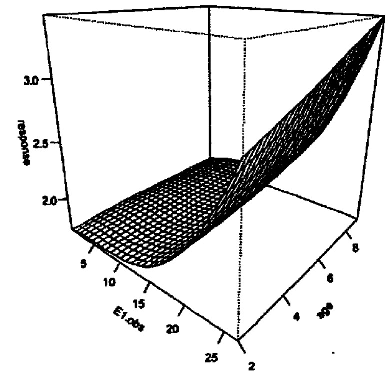

4d

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Chemosphere Vol. 59, Issue 4, April 2005 (Amsterdam), A D Vethaak et al., "An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands", pages 511 to 524.

(58) Field of Search:
INT CL **A61K, C02F, C07J, G01N**
Other: **Online: EPODOC, WPI, TXTE, NPL**

2448682

Working Title: Virtual Fish

Formal title: A device for predicting the effects of pollution in aqueous environments

The invention relates to a device for analysing the levels of steroid oestrogens in freshwater and predicting the risk posed by steroid oestrogen pollutants to the reproductive health of the fish contained in freshwater bodies.

Oestrogenic contaminants are pollutants that have been implicated in the feminisation of freshwater fish in rivers and as possible contributors to the increased incidence of human infertility in males and to breast, prostate and testicular cancers.

The main source of steroid oestrogens is from sewage effluent discharge thus they represent a widespread risk to freshwater bodies all over the World.

Steroidal oestrogens of human origin have been identified in virtually all sewage effluents which have been analysed throughout the World see for example Desbrow et al. 1998; Johnson et al, 2000; Niven et al. 2001; Komori et al. 2004, Johnson et al, 2005.

Farm animals (cattle, swine and poultry) also excrete steroid oestrogens in significant amounts and also contribute to the levels of steroid oestrogens in the aquatic environment via run off from agricultural land.

In the laboratory, following exposure to critical concentrations of steroid oestrogens, male fish respond by producing a female specific blood protein, vitellogenin and by developing a female reproductive tract. In many cases, they also develop eggs within their testes, producing ovotestes. These feminising effects can be so severe that they result in impairment of fertility or complete infertility. Moreover, high concentrations of vitellogenin in the plasma of fish can lead to alternations in kidney development, disruptions of kidney function and a loss of calcium from the scales.

Inappropriate synthesis of vitellogenin by very young fish results in a diversion of valuable energy resources away from growth and survival, and increases their susceptibility to disease and/or predation. When taken together, exposure of fish to steroid estrogens causes many potentially harmful effects on individual fish and on species as a whole. Even more worrying are the long term and unforeseen effects these changes may have on overall population levels and aquatic ecosystems in general.

The occurrence of feminised, hermaphrodite or intersex fish in UK and European rivers and estuaries has been clearly demonstrated and has been found to be a universal phenomenon which is neither species nor habitat specific.

The incidence and severity of ovotestes is positively correlated with the proportion of treated sewage effluent in the rivers in which the fish reside Jobling et al, 1998 Environ. Sci. Technol. **1998**,32,2498-2506.

Current methods for analysing the effects of steroid estrogens on freshwater bodies rely on sampling and analysing steroidal oestrogen concentrations in water (ie.measuring the pressure) using complex, expensive and slow analytical chemistries and the examination of physiological, anatomical and biochemical changes in individual fish (ie. measuring the impact).

The impact assessment involves obtaining the necessary permits, mobilizing a fisheries team to catch the fish, collecting blood, scale and testes samples from the fish. The blood is then centrifuged on site to obtain plasma and stored on dry ice for transportation.

Quantification of vitellogenin in plasma samples is achieved using an established homologous carp radioimmunoassay that has been validated for use with a wide variety of cyprinid fishes. This analysis takes approximately 2 weeks to obtain numerical results from approximately 50 fish.

The assay involves removing the testes and preserving them in fixative and then placing them in 70% alcohol in preparation for histological processing. The fixed gonads are then divided into three equal portions. Representative transverse sections, 3-5 mm thick, are taken from the center of each portion to provide a total of 6 sections per fish; one section from each of the anterior, mid, and posterior regions of each gonad. The sections are then processed histologically, embedded in paraffin wax, and sectioned at 3 μm thickness. All sections are stained with Mayers Haematoxylin-eosin, mounted, and examined by light microscopy. This process takes approximately 1 month to process and analyse the material from 50 fish.

As intersex is age dependent the scales are also collected from each fish. The scales are then analysed to determine the age of the fish (by counting the annuli on the scales, like counting rings on the bark of a tree).

A need exists for a fast, simple means of assessing the effects of sewage effluent discharges and more specifically of steroid estrogens contained in sewage effluent discharges on existing and future fish populations and stocks.

The device enables sewerage companies and regulators to characterize the risk posed by steroid estrogen contaminants on fish living in freshwater bodies. It obviates the need for expensive and slow analytical chemistry or animal tests.

It offers considerable advantages over current analytical methods such as the possibility of portability and on-site working and the ability to measure and/or predict pollutant levels in complex matrices with minimal sample preparation. It is fast, simple and economical to use.

In a first aspect the invention relates to a device for predicting the biological effects of steroidal oestrogens from sewage effluents on aquatic organisms at a specific site comprising:

a) means for evaluating the steroidal oestrogen concentration in a water body to obtain a pressure assessment;

b) means for converting the steroidal oestrogen concentration data determined by a) to an oestrogen equivalent value (EEQ) using the following formula:

$$Cr\ (E2)+(0.3*Cr\ [E1]) + (10 * Cr\ [EE2]) = EEQ$$

where Cr is the concentration of each steroid oestrogen in the water being sampled; and

c) means for comparing the EEQ value obtained in b) with one or more generalised additive models to obtain an impact assessment for the site under investigation.

d) means for combining the pressure assessment and impact assessment results to obtain an overall risk assessment for said site.

Preferred features of the device are defined in claims 2 to 3.

In another aspect the invention relates to a method of predicting the biological effects of steroidal estrogens from sewage effluents on aquatic organisms using the device claimed in claims 1 to 3

Description of the drawings:

Figure 1: shows Table 1 which details the 6 categories used to describe the impact of steroidal oestrogens on freshwater bodies.

Figure 2: shows a Table 2 which details a steroid oestrogen assessment matrix which combines exposure pressure and impact pressure to provide an inter-risk assessment for any water body which falls below a good assessment.

Figure 3: shows Table 3 which shows how confidence and inter-risk relate to the 4 categories used in the UK TAG (from Storey, 2004). Development of a summation engine for providing an overall assessment of the risk of water bodies failing to achieve good status. Environment Agency Report) classification system or the 2 category system currently in use in the rest of Europe.

Figure 4 (a,b,c,d): shows plots of the generalized additive models defined by Equations 4, and 5

Detailed description

The main steroidal oestrogens present in freshwater as a result of sewage effluent discharges are oestrone (E1), 17β–oestradiol (E2) and 17α-ethinylestradiol(EE2). All three are of human origin hence their effects on individual species and on aquatic environments are entirely the result of human populations.

The levels of these steroidal oestrogenic pollutants have been measured in rivers by for example Vethaak AD, Lahr J, Kuiper RV, Grinwis GCM, Rankouhi TR, Giesy JP, *et al.* 2002. Estrogenic effects in fish in The Netherlands: some preliminary results. Toxicology 181:147-150; Williams RJ, Johnson AC, Smith JJL, Kanda R, 2003. Steroid estrogens profiles along river stretches arising from sewage treatment works discharges. Environ Sci Tech 37(9):1744-1750; and estuaries by Thomas, KV, Hurst, MR, Matthiessen, P, Waldock, MJ, 2001. Characterization Of Estrogenic Compounds In Water Samples Collected From United Kingdom Estuaries. Environ. Toxicol. Chem. 20, 2165-2170 and have been found to be in the sub to low nanogram per litre range.

E1 and E2 are natural steroid oestrogens excreted by human beings in varying amounts depending on their age, sex and whether the females are pregnant.

EE2 is used in the contraceptive pill and in hormone replacement therapy drugs and is excreted by women using these products.

The major mechanism of action for all steroid oestrogens is via binding to the oestrogen receptor present in target tissues and organs. Other oestrogenic compounds also bind to this receptor. The potencies of each of these three steroid oestrogens in fish are different; EE2 is the most potent, followed by E2 and then E1. It is increasingly acknowledged that the combined effects of steroid estrogens are likely to be additive and hence are better predicted using a toxic equivalency approach Silva E . Rajapaksse N, Kortenkamp A. 2002. Something from "nothing"-eight week estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ Sci Tech 36:1751-1756; Thorpe KL, Hutchinson TH, Hrtheridge MJ, Scholze M, Brighty GC, Sumpter JP, et al. 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ Sci Tech 37(6):1142-1149; Brian JV, Harris CA, Scholze M, Backhaus T, Booy P, Lamoree M, *et al.* 2005. Prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environ Health Perspect 113:721-728).

The toxic equivalency approach is based on the common mechanism of action for E2 and related compounds in which the oestrogen equivalents (EEQs) of any mixture are equal to the sum of the concentration of individual (i) oestrogens times their potencies (EEFi) relative to the 17β–oestradiol , EEF = 1.0. The EEQ can be readily calculated from analytical data and provides an estimate of the oestrogenicity of any mixture containing oestrogens.

Using this equivalency approach compensates for the difference in potency of different steroid oestrogens: Currently it is generally considered that
a) 1 Oestradiol equivalent (EEQ) corresponds to 0.02-0.2ng/l of EE2 or
b)1 Oestradiol equivalent (EEQ) corresponds to 1-3ng/l E1 and
c)1 Oestradiol equivalent (EEQ) corresponds to 1ngE2/L.

The exact EEFi for each steroid depends on the specific biological endpoint being affected by the oestrogen and may change as our understanding of the potencies of the different steroidal oestrogens on various biological endpoints improves.

The induction of a female-specific protein, vitellogenin, in caged or wild male fish and the induction of intersex in wild male fish exposed to sewage effluents demonstrates that when fish are exposed to oestrogenic substances feminisation occurs Purdom *et al.* Chem Ecol. 1994, 8, 275-285; Harries JE *et al.* 1996. A survey of oestrogenic activity in UK inland waters. Environ Toxicol Chem 15:1993-2002; Jobling S *et al.* 1998. Widespread sexual disruption in wild fish. Environ Sci Tech 32(17):2498-2506).

The weight of evidence for endocrine disruption in fish is considered sufficient to develop a risk management strategy to protect the aquatic environment from endocrine-disrupting chemicals. The focus of this strategy is on chemicals present in sewage effluents, and more specifically on steroidal oestrogens present in sewage effluents as these pollutants have been linked to feminisation in fish.

Under the new European Water Framework Directive http://ec.europa.eu/environment/water/water-framework/index_en.html; http://www.euwfd.com/index.html both chemical surveillance and biological impact surveillance are required to safety assess polluted waters and wastewaters.

Using the claimed invention, the safety assessment for steroid oestrogens is performed by analysing two or more parameters:

a) Chemical surveillance comprises analysing the steroid oestrogen concentrations within the water body termed exposure pressure; and

b) Biological impact surveillance comprises analysing the occurrence of feminisation in roach communities termed impact pressure, which takes into account the incidence of male fish that are intersex and the severity of feminisation.

The likely incidence of feminisation is expressed as a percentage of the total population. Severity takes account of a range of anatomical and biochemical changes which have been found to occur in feminised fish such as the number of eggs within the testes, and the presence of the female yolk protein, vitellogenin, in the blood of male and intersex fish .

Exposure pressure is measured, calculated or estimated for each steroid oestrogen and converted to a steroid oestrogen equivalent value (EEQ) for the site sampled using the following formula:

$$Cr\ (E2)+(0.3*Cr\ [E1]) + (10 * Cr\ [EE2]) \qquad \text{Equation 1}$$

Sites where the combined steroid oestrogen equivalent is less than or equal to 1 ng/l are designated as "low pressure". Sites exceeding 1 ng/l but less than or equal to 5.7ng/l are designated as moderate pressure and sites exceeding 5.7ng/l are designated "high pressure".

Impact is predicted using a series of generalised additive models which describe the effects of steroid oestrogens in wild fish in UK rivers using either EEQ or measured E1 as predictors. These predictions are combined and the overall impact is assigned to one of 6 categories (A-F) which are described in detail in Table 1.
Table 1 details the progression of intersex characteristics in roach in each of these 6 groups.

Classes A and B are considered to be good/ no impact/ not at risk; class C results in the water body being "at risk" as the incidence and severity of intersex in the older intersex fish at the higher end of this category could result in a failure of good status and is therefore used to indicate moderate impact; and classes D, E and F are all used to indicate a high impact.

The results of the pressure and impact assessments are combined to produce an inter-risk assessment value. Table 2 details a steroid oestrogen assessment matrix into which exposure pressure and impact are input. The result for "risk" and "confidence" obtained from table 2 are then input to table 3 to give an overall assessment of the risks posed by steroid oestrogens in the freshwater body to the fish at the site under investigation.

This overall risk assessment can be expressed in a number of ways. By way of example only: using one of the four categories; no pressure, low pressure, moderate pressure and high pressure specified by the UK Technical Advisory Group (TAG), according to System B in Storey(2004). Development of a summation engine for providing an overall assessment of the risk of water bodies failing to achieve good status. Environment Agency Report; or using one of the two categories "at risk"(which correspond to high and moderate risk in the UK TAG system) and "not at risk" (which correspond to no or low risk in the UK TAG system) currently used in the rest of Europe for reporting the overall risk assessment.

In a first embodiment the virtual fish device comprises: (a) one or more oestrogen biosensors; and (b) means for converting the measurements generated by a) into an overall risk assessment for the site under investigation.

The biosensor or biosensors can be selected from by way of example from the BIACORE ® 3000, RIANA (river water analyzer) and the ENDOTECT biosensor.

In a preferred embodiment the one or more biosensors measure the concentration of each individual steroid oestrogen present in the water separately.

If all three measurements are available, these are used to calculate EEQs for the site sampled using the following formula:

$$Cr\ (E2)+(0.3*Cr\ [E1]) + (10 * Cr\ [EE2]) \qquad \text{Equation 1}$$

If only oestrone can be measured, the VIRTUAL fish can use this as a predictor of the other two oestrogens (and therefore the EEQ) using information on the relative concentrations of the other two steroids compared with E1.

These data are compared against several generalized additive models (GAMs) (where measured oestrogen concentrations rather than predicted oestrogen concentrations are used to generate the models) to predict the impact of steroid oestrogens present in the water on the aquatic environment.

The device can be used for risk assessments which are carried out on site using real time measurements from the biosensor or at a laboratory bench (using a dip test type sensor).

In another preferred embodiment the virtual fish device comprises: (a) a single biosensor which is based on a fish oestrogen receptor; and(b) means for converting the measurements generated by a) into an overall risk assessment for the site under investigation.

As all estrogens act by binding to and activating the oestrogen receptor, the use of a biosensor which mimics the oestrogen receptor, enables the total oestrogenic activity present in the water to be measured when the biosensor is immersed in the water from the site of interest.

The binding of any oestrogenic chemicals present in the water is detected by such a biosensor and a value of the total estrogenic activity at the sensor surface generated.

These data are then compared against one or more GAM model (where oestrogenic activity rather than predicted oestrogen concentrations are used to generate the models) to predict the impact of steroid oestrogens present in the water on the fish living in the aquatic environment.

This risk assessment can be carried out on site using real time measurements from the biosensor or at a laboratory bench (using a dip test type sensor).

In another embodiment the virtual fish comprises: (a) an automatic flow measurement element; and (b) means for converting the measurements generated by a) into an overall risk assessment for the site under investigation.

Sewage-effluent discharge and river water concentrations of steroidal oestrogens (E1, E2 and EE2) are estimated using the method of Johnson AC, Williams RJ. 2004. A model to estimate the influent and effluent concentrations of oestradiol, oestrone and ethinylestradiol at sewage treatment works. Environ Sci Tech 38 (13):3649-3658.

The method is based on estimates of oestrogens in effluent discharges which are calculated as follows:

The load of each of the three principal human steroidal oestrogens arriving at a sewage treatment works (STW) is a linear function of the population served by that works. Johnson and Williams (2004) reviewed the literature on excretion values of these steroid estrogens by various cohorts of the human populations and calculated that on average each person excreted 0.89 μg/day of EE2, 3.3 μg/day of E2 and 13.8 μg/day of E1 (either as free hormone, or in forms likely to be transformed into the free hormones).

Thus, by multiplying the domestic population served by a sewage treatment works an indication of the load of these chemicals in the influent to the sewage treatment works (STW) can be calculated.

The sewage treatment process will remove a significant proportion of the steroid estrogens. From a review of the published data, Johnson and Williams (2004) suggested removal efficiencies of 85%, 65% and 82% for EE2, E1 and E2 respectively would be expected from activated sludge treatment works (commonly used for large conurbations). Expressed mathematically, the average daily load of an individual steroid delivered to the receiving water, L_e, is given by the formula:-

$$L_e = (1-r_e)P\alpha_e \qquad \text{Equation 2}$$

Where r_e is the fraction of the steroid removed by the STW, P is the domestic population served by the STW and α_e is the *per capita* steroid load excreted which varies for each steroid oestrogen and reflects the amount excreted by males and females at various times in their lives.

For trickling filter sewage treatment works, the fractions of oestrogen removed may differ. These variations in removal rates can be accommodated by the virtual fish if the type of sewage treatment works an/or the exact removal rates are known.

In this embodiment the virtual fish computes estimated steroid oestrogen equivalent (EEQ) concentrations based on measurements of the flow rates in the sewage effluent channel and in the receiving river.

The automatic flow measurement element can be any one of a range of conventional devices such as the Handheld Doppler current meters (such as the FlowTracker), shallow water flow and level meters (such as the Argonaut-SL) or complete integrated catamaran systems for open channel flow measurement (such as the RiverCAT) which are well known to the skilled addressee.

The automatic water flow measurement element is ideally placed in the receiving river, directly below the sewage treatment plant or at any point downstream from the plant (but upstream of the next sewage treatment plant) where the risk assessment is required.

The concentration of each steroid oestrogen in the river being sampled, C_r, is then given by:

$$C_r = L_e / (F_{cs}) \quad \text{Equation 3}$$

Where: L_e is derived from equation 2; F_{cs} is the river flow rate at a point of measurement immediately downstream of the sewage works.

If the flow ,measurement is made upstream of the works, a further automatic water flow measurement element must be placed in both the sewage effluent channel and in the receiving river. Readings from both places are needed to compute estimated oestrogen equivalents (in nanograms per litre) and their likely impacts on fish in the receiving river.

The concentration of each steroid oestrogen in the river being sampled, C_r, is then given by:

$$C_r = L_e / (F_{cs} + F) \quad \text{Equation 3}$$

Where: L_e is derived from equation 2; F_{cs} is the river flow rate at a point of measurement immediately upstream of the sewage works; and F is the sewage treatment works effluent flow rate.

As before EEQs are calculated for the site sampled using the following formula:

$$Cr\,(E2) + (0.3 * Cr\,[E1]) + (10 * Cr\,[EE2]) \quad \text{Equation 1}$$

Telemetry can be used to communicate these values to an off-site computer which enables the VIRTUAL FISH to predict the pressure and impact in real time. . The impacts are calculated using mathematical models that describe the relationship between predicted or measured EEQs or E1 and various effects seen in wild fish exposed to steroid oestrogens.

The statistical models have been created by using Markov Chain Monte Carlo (MCMC) to explore the causal relationships between estimated EEQ or oestrone concentrations and the real effects seen in 1,457 wild fish from 46 sites all over the UK.

The relationships between pressure (EEQ or E1) and impact (incidence of intersex, severity of intersex and plasma vitellogenin concentrations) have then been explored in greater depth using generalised additive models (GAMS; Wood, 2006 Generalized Additive Models: An Introduction with R" (Chapman Hall/CRC);). These GAMS are incorporated into the virtual fish modelling software. They predict the relationships between pressure and impact for different age groups of fish with good (70-75%) statistical confidence

e.g.

For binary responses:

Logistic regressions of Y_I on *age* and

E1concentration (E1obs) or EEQ: Equation 4

$$\text{logit}(\theta_i) = \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2i},$$

where $\theta_i = \Pr(Y_1 = 1)$, $x_{1i} = age$, $x_{2i} = E1.obs$ or EEQ

For non-binary responses:

$$\text{logit}(\theta_i) = \beta_0 + s(x_{1i}, x_{2i}),$$

where $\theta_i = \Pr(Y_i = 1)$, $\text{logit}(\theta_i) = \log\left(\frac{\theta_i}{1-\theta_i}\right)$, and $s(x_{1i}, x_{2i})$ Equation 5

is a bivariate cubic spline: smooth function of *age* (x_1) and *EEQ or E1obs* (x_2).

The model is $\text{inv}(y_i) = \beta_0 + s(x_{1i}, x_{2i})$, where $\text{inv}(y_i) = \frac{1}{y_i}$ and $s(x_{1i}, x_{2i})$

is a bivariate cubic spline smooth function of *age* (x_1) and *EEQ or E1 obs* (x_2).

Impact predictions for each biological endpoint and each age group of fish are collated by the virtual fish software and then combined in order to assign a single impact category (A-F) to the sample site. This overall impact is obtained by comparing the impact predictions with by way of example only one or more of the descriptions given in table 1 starting with description F and ending with description A. The computer tries each description in turn until it finds one that adequately describes the impact predictions. The WFD impact category (High, medium low or no risk) is then evaluated against the risk assessment matrix (Table 2) together with the pressure assessment. The device then provides a prediction of risk (high, medium or low) and confidence (high, medium or low) for the site under investigation. The risk and its associated confidence value are input into Table 3 to give an overall risk assessment which can be expressed in a number of ways. By way of example only: using one of the four categories; no pressure, low pressure, moderate pressure and high pressure specified by the UK Technical Advisory Group (TAG), according to System B in Storey(2004). Alternatively the overall assessment of the risk can be expressed using one of the two categories "at risk"(which correspond to high and moderate risk in the UK TAG system) and "not at risk" (which correspond to no or low risk in the UK TAG system) currently used in the rest of Europe for reporting the overall risk assessment.

<u>The output from the virtual fish:</u>

In its simplest form, the user interface consists of a set of traffic lights which will move between green through amber to red to provide the user with information on the pass or failure of the water body with respect the concentrations and effects of oestrogens, without ever having to sample the fish (a lengthy and costly process). The invention can also provide the user with more detailed information on impact and pressure and on short and long term health outcomes of being exposed to the water. This more detailed output will be in the form of a series of images that will illustrate the nature and the magnitude of the biological effects caused by mixtures of oestrogens in the water.

Table 1.

Assessment class	WFD Impact Category	Intersex index (measured in fish with ovotestes)	Effects and impacts
A	None		Level of intersex zero in all age groups. Indicative of no exposure to oestrogen. No statistically significant increase in male vitellogenin concentrations relative to reference males
B	Low	<2 in all year classes	The level of ovotestes in roach (approximately 0-15%) of the roach have ovotestes and none of these have feminised reproductive ducts) indicates exposure to steroid oestrogen concentrations probably below the PNEC derived for oestradiol or its equivalent. Severity index in all intersex fish must be less than 2, regardless of their age; i.e. reproductive capability not likely to be impaired in any year class. Mean VTG concentrations in sampled male and intersex fish not statistically significantly different from reference males.

C	Medium	>2 in fish in one or two older year classes	The level of intersex in roach indicates probable exposure to steroid oestrogen concentrations higher than the PNEC derived for steroid estrogens. E.g. there is a risk of adverse effects, such as reduced fertility, in individual older male roach caused by long-term exposure to steroid estrogens (index >/= 2 in some older fish of 6 years old and older indicating reduced sperm counts and fertility). The average severity index across all age groups of fish is <2. Mean VTG concentrations in sampled male and intersex fish may be statistically significantly different from reference males
D	High	Mean index > 2 in two or more year classes. >4 in at least one year class. Average index across all year classes>2	Ovotestes likely to be present in fish from all year classes, including 2 year olds. The reproductive capacity, in these populations of roach, *may* be affected as a result of a high proportion (>50%) of sub-fertile(index >2) /sterile feminised male fish (index>4) in two or more year classes, but some reproductively capable males remain (primarily in the younger year classes). E.g. there is evidence of adverse reproductive health effects in individual fish, which can be directly associated with the exposure to steroid oestrogens. VTG concentrations are higher than in reference males
E	High	Average index>/= 4 across all year	In populations of roach in all year classes >/= 70% of the "male" fish are intersex. Severe feminisation and consequent impairment of male gamete quality is likely in all

		classes	individuals from more than 2 year classes. VTG concentrations higher than in reference males. Sex ratio may be skewed in the female direction
F	High	-	The populations of roach are absent/expired. Populations of roach are 100% female

Table 2

Impact Assessment	No data	No Pressure	Low Pressure (Pass)	Mod Pressure (Fail in river)	High Pressure (Fail in water body)
No data		Low(LC)	Low(MC)	Low(LC)	High(MC)
No impact	Low(LC)	No(HC)	No(MC)	Low (HC)	High(LC)
Low impact		Low(HC)	Low(MC)	Low(MC)	High (LC)
Mod impact		Mod(MC)	Mod(MC)	Mod(MC)	High(HC)
High impact		High(MC)	High (MC)	High (MC)	High (HC)

Table 3

<table>
<tr><th>Risk</th><th>Confidence</th><th>UK TAG</th><th>Europe</th></tr>
<tr><td>High Risk</td><td>High</td><td rowspan="3">High Risk</td><td rowspan="6">At Risk</td></tr>
<tr><td>High Risk</td><td>Medium</td></tr>
<tr><td>Mod Risk</td><td>High</td></tr>
<tr><td>High Risk</td><td>Low</td><td rowspan="3">Moderate Risk (Probable risk)</td></tr>
<tr><td>Mod Risk</td><td>Medium</td></tr>
<tr><td>Mod Risk</td><td>Low</td></tr>
<tr><td>Low Risk</td><td>High</td><td rowspan="3">Low Risk</td><td rowspan="6">Not At Risk</td></tr>
<tr><td>Low Risk</td><td>Medium</td></tr>
<tr><td>Low Risk</td><td>Low</td></tr>
<tr><td>No Risk</td><td>Low</td><td rowspan="3">No Risk</td></tr>
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<tr><td>No Risk</td><td>High</td></tr>
</table>

Claims:

1. A device for predicting the biological effects of steroidal oestrogens from sewage effluents on aquatic organisms at a specific site comprising:

   a) means for evaluating the steroidal oestrogen concentration in a water body to obtain a pressure assessment;

   b) means for converting the steroidal oestrogen concentration data determined by a) to an oestrogen equivalent value (EEQ) using the following formula:

   $$Cr\,(E2)+(0.3*Cr\,[E1]) + (10 * Cr\,[EE2]) = EEQ$$

   where Cr is the concentration of each steroid oestrogen in the water being sampled; and

   c) means for comparing the EEQ value obtained in b) with one or more generalised additive model to obtain an impact assessment for the site under investigation.

   d) means for combining the pressure assessment and impact assessment results to obtain an overall risk assessment for said site.

2. A device as claimed in claim 1 wherein the means for evaluating the steroidal oestrogen concentration in the water body comprises one or more steroidal oestrogen biosensor.

3. A device as claimed in claim 1 wherein the means for evaluating the steroidal oestrogen concentration in the water body comprises:

   i) one or more automatic flow rate meters for measuring the fluid flow rate in the water body which receives the discharge from the effluent sewage discharge channel and/or in the discharge channel itself; and

   ii) means for converting the data obtained by i) to an oestrogen concentration, using the following formulae:

   $$C_r = L_e /(F_{ds})$$

   where: L_e is the average daily load of an individual steroid delivered to the receiving water, F_{ds} is the river flow rate at a point of measurement downstream of the sewage works. or

   $$C_r = L_e /(F_{us}+F)$$

where: F_{us} is the river flow rate at a point of measurement upstreamof the sewage works and F is the sewage effluent flow rate.

4. A method of predicting the biological effects of steroidal estrogens from sewage effluents on aquatic organisms using the device claimed in claims 1 to 3.

5. A device as described herein ...

6. A method as described herein.

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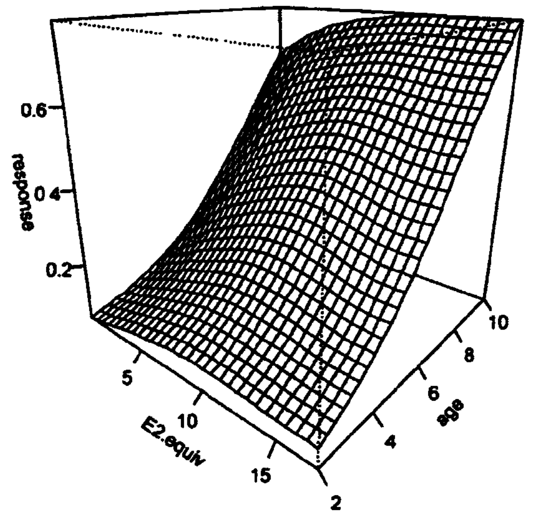
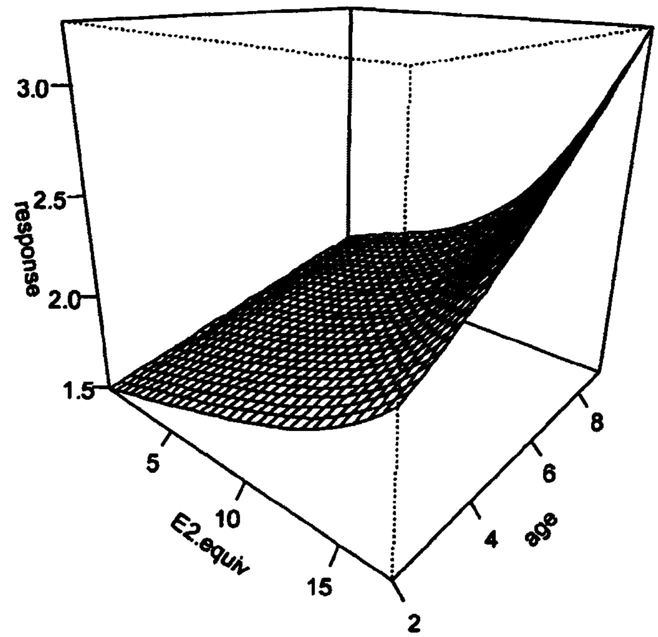
Figure 4a



4b



4c

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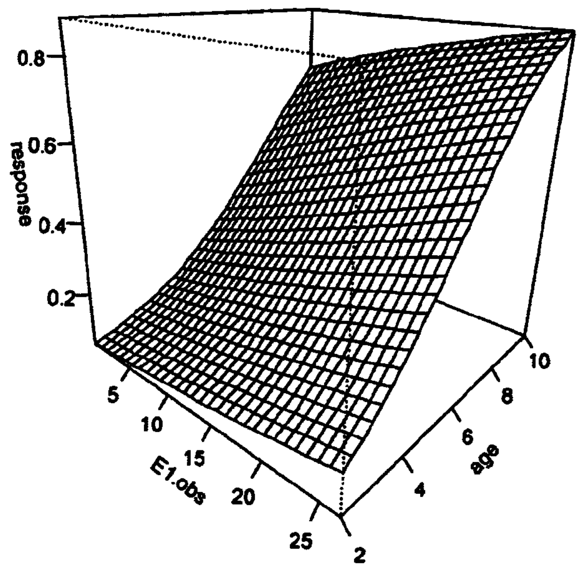
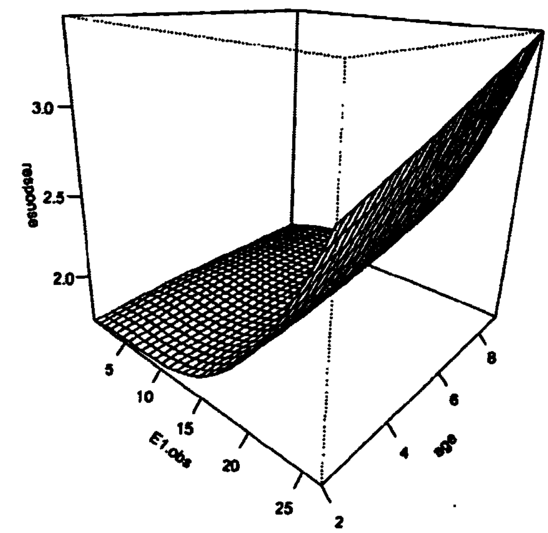
4c





4d

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Application No:	GB0705985.0	Examiner:	Mr Peter Philip Holck
Claims searched:	1-6	Date of search:	18 January 2008

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
A	-	Environmental Science and Technology Vol. 38, Issue 13, July 2004 (Washington, DC), A C Johnson and R J Williams, "A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works", pages 3649 to 3658.
A	-	Chemosphere Vol. 59, Issue 4, April 2005 (Amsterdam), A D Vethaak et al., "An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands", pages 511 to 524.

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^X :

Worldwide search of patent documents classified in the following areas of the IPC

A61K; C02F; C07J; G01N

The following online and other databases have been used in the preparation of this search report

EPODOC, WPI, TXTE, NPL

International Classification:

Subclass	Subgroup	Valid From
G01N	0033/74	01/01/2006
A61K	0031/565	01/01/2006
C07J	0001/00	01/01/2006
G01N	0033/18	01/01/2006