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Biological effect measures in fish – application to treated sewage effluent

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Executive summary

It is now well established that effluents from sewage treatment works that receive domestic wastewater can have oestrogenic effects in fish. Exposure to oestrogens may impair reproduction with potentially detrimental consequences for fisheries. The main aim of this programme was to investigate the application of fish bioassays for assessing adverse effects of treated sewage effluents on reproductive health. A secondary benefit was the generation of hazard data for the three test effluents investigated, although it must be noted that as with all effluent testing the variation (diurnal patterns/dilution rates/treatment efficiencies etc) of the target compound(s) must be understood. Another key aim of this work was to investigate how effective specific biomarkers (for example, vitellogenin (VTG) and secondary sexual characteristics (SSC)) were for predicting impacts of the effluents on reproductive output, and thus their potential utility for monitoring environmental impacts of effluent discharges.

The fathead minnow (*Pimephales promelas*) is a member of the carp family and is therefore representative of a major component of the UK freshwater fisheries. It has been shown to be a robust organism for laboratory studies on reproduction and is used in the Organisation for Economic Co-operation and Development (OECD) and United States Environmental Protection Agency (USEPA) standardised regulatory tests. The non-spawning and pair-breeding (spawning) tests for this species have previously been validated for assessing the effects of oestrogens and other endocrine disrupting chemicals (Environment Agency, 2006). The biomarkers measured in these tests include levels of the female egg yolk precursor protein, VTG, and the male SSC. These biomarkers are oestrogen and androgen dependent, respectively, and depending on the stage of sexual development and sex of the fish used, can be used to measure for exposure to oestrogens, anti-oestrogens, androgens and anti-androgens.

Three effluents were selected for this programme of work, based on prior knowledge of their oestrogen content. They were categorised as having a ‘high’ (effluent I), ‘intermediate’ (effluent II) or ‘low’ (effluent III) level of oestrogenic activity. The use of recombinant Yeast Oestrogen Screens (rYES) conducted on each batch of effluent arriving at the laboratory confirmed that effluent I did indeed have a high level of oestrogenic activity (11.1 to 38.2 ng oestradiol equivalents per litre), and effluent III a lower level (7.1 to 12.7 ng oestradiol equivalents per litre). However, on this occasion effluent II was also found to possess a low level of oestrogen (0.7 to 9.4 ng oestradiol equivalents per litre), demonstrating the variation that can be encountered even in previously characterised effleunts. Further rYES analysis on samples taken from the storage tank demonstrated that the oestrogenic potency of these effluents when maintained chilled (8°C) did not differ from that measured on arrival for a period of up to 8 days (longer time periods were not investigated). The oestrogenic activity, therefore, was considered to be stable in the storage facility used.

VTG concentrations measured in fathead minnow exposed to the three effluents supported the relative oestrogenic loads, with effluent I inducing a high concentration of VTG in males (at all concentrations of the effluent tested; 25, 50 and 100%) in both the non-spawning and pair-breeding experiments. Effluents II and III were comparable in their effects on males in the non-spawning experiment, with both effluents inducing significant increases in VTG in males at concentrations of 50% and 100%. Effluent III also induced an elevated VTG concentration in males exposed at 50% and 100% in the pair-breeding experiments, but effluent II was only effective in doing so at the 100% concentration, indicating a lower oestrogenic content in effluent II collected for the pair-

breeding test. Exposure to the effluents increased the relative weight of the male gonads in the non-spawning assays, but there were no clear effects in the pair-breeding experiment. Relative gonad weight was not affected in females exposed to the effluents in either the non-spawning or the pair-breeding experiment. There were no clear concentration-related effects of the effluent on male SSCs.

In the pair-breeding experiments, effluent I (with a 'high' level of oestrogenic activity) reduced total egg production at concentrations of 50% and 100%, while effluent III ('low' oestrogenic activity) reduced egg production at the 100% concentration only. In contrast, effluent II (the 'intermediate' oestrogenic effluent) did not affect egg production over the period of exposure (21 days). The effects of the three effluents on egg production are consistent with their measured oestrogenic potencies. The oestrogen positive control, 17α-ethinyloestradiol (8.2 to 15.7 ng/L) consistently induced higher concentrations of VTG than any of the effluents tested, but did not affect reproduction within the test period. This indicates that the effects of the effluents on reproduction seen within the test may be a consequence of exposure to a complex mixture of chemicals within the effluent that act via multiple mechanisms (additional to acting as oestrogen receptor(s) agonists).

The results from these investigations demonstrate that it is possible to assess the impact on reproduction through direct exposure of fish to treated sewage effluents. However it is important to note that the outcome of these tests will of course depend on the samples of effluent used and this in turn is dependant on the objective of the study. For this reason it is necessary to characterise the variation in oestrogenic loading in the effluent using a cost effective means such as rYES or chemical analysis prior to embarking on the measurement of effects in fish. While such characterisation is required to ensure the samples used are representative, these results alone are not sufficient to understand the potential adverse effects that such effluents might pose to fish health and reproductive competence.

The test system employed here is practicable and through the use of an effluent storage facility (chilled to 8°C) and a flow-through system it is possible to provide a realistic scenario for effluent exposures to fish. The fathead minnow pair-breeding assay was shown to be robust and the test can be conducted over a relatively short time period, thus providing a cost-effective means of assessing effluent exposure effects on reproduction. The importance of using integrative methods such as the fathead minnow pair-breeding test lies in the complex interactions of the various concentrations and potencies of all of the (anti)oestrogenic and (anti)androgenic compounds found in treated sewage effluents on fish hormonal control that ultimately determine the outcome of the test, i.e. the effect on reproduction.

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Contents

1 Background

Extensive surveys of wild fish populations in the UK have identified a high incidence of intersexuality in roach, *Rutilus rutilus* (Jobling *et al*., 1998; Jobling *et al*., 2006), gudgeon, *Gobio gobio* (van Aerle *et al*., 2001) and flounder, *Platicythys flesus* (Kirby *et al*., 2004) in rivers and estuaries that receive effluent discharges from sewage treatment works (STWs). Intersexuality is defined as the simultaneous presence of both testicular and ovarian tissues in the gonads of fish species that are normally single sexed. In populations of roach inhabiting UK Rivers, it has been shown that the severity of the intersex condition is related to the concentration of STW effluent in the river (Jobling *et al*., 1998). Furthermore, in roach, a correlation between the severity of the intersex condition and a reduced reproductive capability (reduced gamete viability in males) has been demonstrated with non-viable gametes produced in the most severe cases (Jobling *et al*., 2002a, 2002b). In some wild intersex fish captured, the gonadal duct was closed, thus preventing gamete release and rendering those individuals infertile (Jobling *et al*., 2002a, 2002b). In has yet to be determined what impact less severe manifestations of intersex, as more commonly found in effluent contaminated rivers in the UK, have on the ability of individuals to breed in natural group spawning scenarios.

The demonstration of a reciprocal relationship between the number of intersex roach caught at any one site and the number of males (Jobling *et al*., 1998), has led to the hypothesis that intersexuality in UK fish populations is a consequence of the feminisation of males, rather than the masculinisation of females. This is supported by observations of elevated concentrations of the vitellogenin (VTG) protein in the blood of wild intersex roach (Jobling *et al*., 1998) and in caged male rainbow trout and roach placed downstream of STW effluent discharges (Harries *et al*., 1999; Tyler *et al*., 2005). VTG is an egg yolk precursor that is produced at high concentrations in mature females in response to increased concentrations of endogenous oestrogens (Van Bohemen *et al*., 1982; Ng & Idler, 1983; Tyler, 1991). Male and immature female fish possess very little, if any, detectable VTG in their plasma but exposure to exogenous oestrogen has been demonstrated to induce production of VTG (van Bohemen *et al*., 1982; Lazier & MacKay, 1993; Sumpter & Jobling, 1995). Attempts to identify the chemical cause of intersexuality have therefore focused on chemicals that affect the oestrogenic pathway.

Fractionation of effluents from STWs has identified the major oestrogenic chemicals to be the natural (oestradiol and oestrone) and synthetic (ethinyloestradiol) steroidal oestrogens and some alkylphenolic chemicals (Desbrow *et al*., 1998; Rodgers-Gray *et al*., 2001; Gibson *et al*., 2005; Liney *et al*., 2006). These chemicals have been shown to be prevalent in the wider aquatic environment and frequently occur as mixtures (Environment Agency, 2004). In laboratory investigations, these oestrogenic chemicals have been demonstrated to induce VTG synthesis at environmentally relevant concentrations (Routledge *et al*., 1998; Panter *et al*., 1998; Thorpe *et al*., 2000, 2001) and to feminise both the male reproductive duct (van Aerle *et al*., 2001) and secondary sexual characteristics (Harries *et al*., 2000; Panter *et al*., 2006). Oestrogens have also been shown to inhibit reproduction (Harries *et al*., 2000; Shioda & Wakabayashi, 2000; Länge *et al*., 2001; Zillioux *et al*., 2001) albeit only at high concentrations in short-term exposures. However, life-long exposure to environmentally relevant concentrations of ethinyloestradiol-17α has been shown to disrupt reproduction in freshwater fish (5 ng/L in zebrafish [Nash *et al*., 2004]; 1 ng/L in fathead minnow [Parrot & Blunt, 2005]). It is also important to realise that oestrogenic chemicals are generally present as mixtures in the aquatic environment, and that as mixtures they have been shown to interact in a concentration additive manner, *in vivo*, to produce a higher effect on the reproductive system than that produced by the individual chemicals (Thorpe *et al*., 2001, 2003; Tinwell & Ashby, 2004; Brian *et al*., 2005, 2007).

Although much of the focus in identifying the chemical cause of reproductive abnormalities in the environment has been on chemicals that mediate their effects via the oestrogenic pathway, laboratory investigations have also demonstrated that chemicals that alter steroid biosynthesis or act as anti-androgens can feminise males and inhibit reproduction (Bayley *et al*., 2002; Ankley *et al*., 2002; Panter *et al*., 2004; Jensen *et al*., 2004). Recent surveys conducted by the UK Environment Agency, in collaboration with WRc-NSF Ltd and the University of Exeter (Environment Agency, 2004) have shown that, in addition to oestrogenic activity, STW effluents throughout England and Wales possess anti-androgenic activity. The chemical(s) responsible for this anti-androgenic activity within STW effluents have yet to be identified. It is not known whether anti-androgenic chemicals are a potential cause of intersexuality in the environment but their ability to feminise males and inhibit reproduction makes them likely contributors to this effect. Therefore the interactive effects of oestrogens and anti-androgens (and other endocrine active chemicals; EACs) should be considered when assessing the potential health effects of STW effluents. Tests developed and applied to STW effluents should, therefore, be capable of capturing the different modes of action that might impact on the health of the fish populations in UK Rivers.

Intersex has proven a valuable endpoint for assessing the degree of sexual disruption in wild fish populations, and for demonstrating a link between exposure to STWs and a reduced reproductive capacity at the individual level. Induction of intersexuality, however, requires exposure to environmentally relevant concentrations of oestrogens for long periods. As an example, in wild roach it has been shown that intersexuality (specifically the presence of oocytes in the testis) may not become evident until the fish are 2 years old (Jobling *et al*., 2006). Experiments investigating long-term exposures are expensive, time consuming and can be difficult to maintain. The induction of intersex is, therefore, not practical for screening the biological impacts of EACs or STW effluents in controlled experiments. Many short-term whole organism tests have been developed that are more readily applied to the assessment of the effects of EACs and STW effluents on the reproductive axis and these range from measuring changes in biomarker responses (e.g. VTG induction in males) to direct assessments on reproductive capacity.

To protect the fish populations of UK rivers it is necessary to have a clear understanding of the biological consequence of any change in the target endpoint for the population. Such a relationship has yet to be clearly demonstrated for the more widely employed biomarkers of reproductive health, (e.g. VTG induction, male secondary sexual characters). For this reason tests that directly assess effects on reproduction offer an improvement as they can demonstrate a clear link between exposure to chemical(s) and a reduced reproductive capacity at the individual level. Where a complete inhibition of reproduction is observed the consequences for the population are easily extrapolated. The adult fish reproduction test (Harries *et al*., 2000; Ankley *et al*., 2001; Thorpe *et al*., 2007a,b) offers considerable promise in this regard, as this highly integrative test assesses effects on reproductive performance (frequency of spawning and number of eggs spawned). In the reproduction test sexually mature fish are established as breeding groups or pairs in the test system for a pre-exposure period of approximately three weeks, to provide tank-specific baseline fecundity data. Once breeding is established for all fish, the chemical or effluent of interest is introduced to the test system and the breeding groups or pairs exposed for three weeks to assess impacts on reproductive performance. This test allows the effects all classes of chemicals, and their mixtures, on the reproductive axis to be determined, irrespective of their mode of action. Information on the mechanisms via which the test chemical is acting can be gained through inclusion of biomarkers that are specific to particular classes of chemicals.

In an earlier research programme sponsored by the Environment Agency and AstraZeneca (Environment Agency, 2006), it was demonstrated that the fathead minnow adult pair-breeding test could be used to assess the reproductive health effects

of chemicals that act by binding the oestrogen or androgen receptors or through disrupting the biosynthesis of the endogenous sex steroids. Furthermore it was shown that measurements of the oestrogenic biomarker, VTG, and the androgen dependent male SSC could be used to elucidate the mechanisms by which the test chemicals affected reproduction. Exposure to an oestrogen (oestrone; 307 and 781 ng/L), an anti-androgen (linuron; 842 and 2074 µg/L) and an aromatase inhibitor (fenarimol; 497 µg/L) were all shown to reduce reproduction. For each test chemical, the reduction in total egg number resulted from a reduction in the number of spawning events over the 21 day study period. Exposure to the oestrogenic chemical induced high levels of VTG in males and females and reduced the prominence of the SSC in males. Exposure to the anti-androgenic chemical also reduced the prominence of the male secondary sexual characters but did not induce vitellogenesis in males or females. Exposure to the aromatase-inhibiting chemical decreased plasma VTG concentrations in females, but did not affect plasma VTG concentrations in males or the prominence of the male SSC. From these results it was proposed that the fathead minnow pair-breeding assay, with VTG measurement and assessment of SSC that signal for specific mechanisms, be considered for wider use in assessing the endocrine activity of chemicals mixtures in effluents and environmental samples.

The primary objective of this programme of work was to determine whether the adult fathead minnow pair-breeding test could be practically applied to assessing the effects of treated STW effluents on reproduction. The project also aimed to provide much needed data on the reproductive health effects of direct exposure of fish to treated STW effluents. Three treated sewage effluents were included within the research programme. The first effluent had been previously well characterised and shown to possess a high level of oestrogenic activity (15 to 220 ng/L of oestrone and 4 to 88 ng/L of oestradiol; Rodgers-Gray *et al*., 2000). Exposure to this effluent had been shown to induce high levels of plasma VTG concentrations in male fish and to feminise the male reproductive duct when exposure occurred during early life (Rodgers-Gray *et al*., 2001; Liney *et al*., 2006). This effluent was selected as a representative 'highly' oestrogenic effluent. The second effluent was selected as an 'intermediate' oestrogenic effluent and was demonstrated in a recent survey coordinated by the Environment Agency, to possess an oestrogenic activity of between 2.2 and 3.0 ng 17β-oestradiol equivalents per litre. The third effluent selected was identified in the same survey as having 'low' oestrogenic activity (between 0.9 and 1.2 ng 17β-oestradiol equivalents per litre).

The research programme was divided into two phases. In the first phase a non-spawning fish screen was used to evaluate the *in vivo* oestrogenic potency of the three effluents and to identify any overt adverse health effects resulting from exposure to the effluent for a period of 14-21 days. Effects on VTG induction, on the relative weight of the gonads (gonadosomatic index; GSI) and on the prominence of the male SSC (SSCs) were measured. In the second phase of the programme the fathead minnow pair-breeding tests were conducted to assess the effects of the three effluents on reproductive performance (egg number) over a 21 day exposure period. Effects on VTG induction and on the SSC were also evaluated to enable a direct comparison with the results from the non-spawning tests. In both phases of the programme, the oestrogenic activity of the effluents was also measured daily, using the recombinant yeast oestrogen screen (rYES), to establish the relative oestrogenic activity of the individual batches of effluent used within each study and to determine how this activity fluctuated as a function of time within the storage system employed. In addition, the actual concentrations of two of the key natural steroidal oestrogens present in STW effluents, 17β-oestradiol and oestrone, were analytically measured in each batch of effluent used in the *in vivo* tests.

2 Materials and Methods

2.1 Test organisms

The fathead minnow used in experiments IA to IIIA were bred in the husbandry unit at Brixham Environmental Laboratory (BEL). For experiments IIA and IIIA, the fish were transported to the fathead minnow facility at the University of Exeter a minimum of 4 weeks prior to the onset of each experiment. The fathead minnow used in experiments IB to IIIB were bred at the University of Exeter from the fish stocks originally supplied by BEL. A minimum of 2 weeks prior to the onset of each experiment male and female fish (differentiated through the presence of SSCs; nuptial tubercles and a dorsal fatpad on the males; an ovipositor on the females) were separated to prevent any spawning activity. Sexually maturing fish that were undergoing rapid gonadal growth were required for the non-spawning tests, therefore, fish with evident but non-prominent SSCs were selected (a relationship between gonadal size and prominence of the male SSCs has previously been demonstrated; Environment Agency, 2006). For the pair-breeding tests, sexually mature fish were required and so fish with well developed SSCs were selected.

Throughout all acclimations and exposures fish were maintained under flow-through conditions in de-chlorinated water at 25 $\pm$ 1°C, with a 16h light: 8h dark photoperiod. The fish were fed adult *Artemia* sp. twice daily and Ecostart 17 1.0 mm fish food pellets (Biomar ltd., Brande, Denmark) once daily.

2.2 Water Supply and Test Apparatus

The water supplied to the laboratory dosing system was prepared using a reverse-osmosis system with the addition of salts as described in OECD Guideline 203. The conductivity of the test water ranged between 201 and 255 μS/cm. Dissolved oxygen concentrations and pH levels were determined in the individual tanks on days 0 and 1 and then at a minimum of twice weekly throughout each experiment. In all experiments, the tanks were gently aerated at the surface, using a glass pipette, to ensure that the dissolved oxygen concentration remained >80% of the air saturation value throughout. Water temperatures were monitored daily and ranged between 23.8 and 25.5 °C in all experiments, while pH levels were checked at least twice per week and ranged between 6.6 and 7.8. Dilution water and test chemical flow rates were checked at least twice per week. Flow-rates (20 mL/min experiments IA to IIIA; 12 mL/min experiments IB to IIIB) to the individual aquaria provided a 75% replacement time of 24 hours. The test vessels had a working volume of 20 L (experiments IA to IIIA) or 12 L (experiments IB to IIIB) and were constructed of glass, with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions.

2.3 Sewage Treatment Works Effluent

For the first experiment (IA) effluent was collected into 40 × 10L carboys from the final effluent stream at the STW on five separate occasions. The effluent was collected between the hours of 8 and 10 am and immediately transported to the testing facility. The effluent was transferred in batches to the testing laboratory, acclimated to a temperature of 18°C, and then dosed to the test aquaria via glass mixing tanks in order to be diluted to the required concentration. The low dosing rates used allowed the

effluent to slowly acclimate to the desired test temperature of 25°C before reaching the test aquaria. The remaining carboys were stored at < 10°C until required.
For the remaining non-spawning (IIA, IIIA) experiments and for the pair-breeding experiments (IB, IIB and IIIB) the effluent was collected from the final effluent stream at each STW by an industrial stainless steel tanker. Approximately 2000 L of effluent was collected on each occasion, at weekly intervals for the non-spawning experiments and three times each week for the pair-breeding experiments. On each occasion, the effluent was collected between the hours of 8 and 10 am and immediately transported to the testing facility where it was transferred into a fully enclosed stainless steel holding tank chilled to 8 °C. Immediately prior to the delivery of each new batch of effluent, the holding tank was drained and cleaned with dilution water to prevent a build up of algae or bacteria.

Conductivity, pH, dissolved oxygen concentration and temperature were checked for each batch of effluent on arrival at the testing laboratory. Conductivity ranged from 1153 to 1450 μS/cm for effluent I, from 608 to 882 μS/cm for effluent II, and from 1040 to 1261 μS/cm for effluent III. Conductivity values were similar for the two experiments conducted with each effluent. The pH values were comparable for all effluents tested and ranged from 7.6 to 8.4. Dissolved oxygen concentrations were above 80% for all effluents on delivery to the laboratory, but temperatures were variable according to the ambient environmental temperatures. Temperatures of the individual batches of effluent collected for the non-spawning experiments ranged from 15.5 to 16.9ºC, 18.4 to 24.6ºC and 20.7 to 22.0ºC for effluents I, II and III, respectively, on arrival at the laboratory. Temperatures of the individual batches of effluent collected for the pair-breeding experiments ranged from 9.8 to 14.0ºC, 15.0 to 16.9ºC and 11.5 to 15.6ºC on arrival for effluents I, II and III, respectively.

2.4 Oestrogen positive controls

For the oestrogen positive controls, 17β-oestradiol (98% purity, Lot 103K1117, experiment IA) and 17α-ethinyloestradiol (98% purity, Lot 024K1196, experiments IIA, IIIA and IB to IIIB) were purchased from Sigma, Poole, Dorset, UK. Solvent free stock solutions of the oestrogen positive control were prepared every three days by adding 1 ml of a concentrated stock solution of the steroidal oestrogen, 17β-oestradiol or 17α-ethinyloestradiol (prepared in HPLC grade acetone; Fisher Scientific), to a 10 L glass vessel. After evaporation of the acetone, at room temperature, 10 L of dilution water was added to the glass vessel and the solution stirred for approximately 2 hours using a magnetic stirrer and follower. The solvent free stock was then dosed to the glass mixing vessels via a peristaltic pump, to mix with the dilution water.

2.5 Experimental design; non-spawning test

Experiment IA was conducted at BEL, while experiments IIA and IIIA were conducted at the University of Exeter. Mixed sex fathead minnow (8 males and 8 females in each of two replicate 20 L glass tanks for each treatment) were exposed for 14 days (IA) or 21 days (IIA and IIIA) to a dilution water control, oestrogen positive control and graded effluent concentrations of 25, 50 and 100% (n = 32 fish/treatment).

2.6 Experimental design; pair-breeding test

All pair-breeding tests were conducted at the University of Exeter. To initiate each test, males and females were placed as pairs into 8 replicate glass test vessels (12 L working volume) for each concentration to be tested. Each vessel contained a

spawning substrate (PVC half guttering tile placed above a stainless steel mesh screened glass egg collection tray; Thorpe *et al*., 2007a). The fish were acclimated to the test conditions for a minimum of 10 days before the onset of the pre-exposure period. During acclimation, the spawning substrates were checked at 10:30 am each day for the presence of eggs to confirm spawning activity. Subsequently, egg number was determined daily for each pair of fish over a pre-exposure period of 3 weeks, to provide pair-specific baseline data for statistical comparison with the subsequent exposure phase. After determination of egg number on the final day of the pre-exposure period, dosing of the DWC, oestrogen positive control or graded effluent concentrations (25, 50 and 100%) to the individual tanks was initiated. The number of eggs spawned by each pair of fish was determined daily, over an exposure period of 3 weeks, to determine the effects of the test solution on the reproductive capacity of the individual pairs of fish.

2.7 Measurement of Oestrogenic Activity

Composite water samples were collected from the replicate tanks for each treatment in experiments IA to IIIA, and from two tanks per treatment in experiments IB to IIIB, into solvent-cleaned flasks. For measurement of oestrogenic activity in the recombinant yeast screen (rYES) a total of 700 mL was collected from each treatment on days 2, 4, 7, 10, 14, 17 and 21 and for the analytical determinants 2.5 L was collected on days 4, 7, 14 and 21. Samples were also collected on a daily basis from the effluent storage tank for analysis in the rYES and on the morning following delivery of each new batch of effluent for the analytical determinants. Immediately after collection, the samples were spiked with 0.05% methanol and extracted onto preconditioned Sep-Pak Classic C18 Cartridges (Waters Ltd, Hertfordshire, UK). To assess the efficiency of the extraction for each procedure spiked dilution water and effluent samples (0.05% of an estrogenic mixture containing 4 μg of 17β-oestradiol, 1.6 μg of ethinyloestradiol-17α, 8 μg of oestrone and 800 μg of nonylphenol prepared in 1L of methanol) were extracted under the same conditions. Dilution water spiked with 0.05% methanol was also extracted under the same conditions. The cartridges were eluted using methanol (5 mL/cartridge) and the eluant stored at –20°C for subsequent analysis.
For measurement of estrogenic activity in the rYES, the methanol was removed under a stream of nitrogen and the extracts resuspended in 5 mL ethanol on the day prior to setting up the yeast assay. The extracts were analysed using the rYES following the methods outlined in Thorpe *et al*. (2006). 17β-oestradiol and oestrone concentrations using GC-MS, the samples were treated as described in Thorpe *et al*. (2006).

2.8 Fish Sampling

At the end of each experiment, all fish were sacrificed using a lethal dose (500 mg/L) of MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt; Sigma), buffered with 1M NaOH to pH 7.4. The total length and wet body weight of the fish were recorded to the nearest 1mm and 0.01 g, respectively. Condition factor was derived by expressing the cube of the total fish length as a percentage of the wet body weight. Blood was collected by cardiac puncture, using a heparinised syringe (1000 Units heparin/mL), then centrifuged (7,000 g; 5 min, 15°C) and the plasma removed and stored at -20°C until required for analysis of VTG using a carp ELISA (Tyler *et al*., 1999). The gonads were removed, wet weighed to the nearest 0.01 mg and the GSI derived by expressing the gonad weight as a percentage of the total body weight. The prominence of the tubercles was recorded using the scoring system given by Thorpe and Tyler (Environment Agency, 2006). The numbers of tubercles on the snout of each fish were recorded and the dorsal fat pad removed and wet weighed to the nearest 0.01 mg.

2.9 Statistical analyses

To investigate the effects of the effluent exposure or the oestrogen positive control, data were compared to the DWC using a commercial statistics package (SPSS version 13.0). Data meeting the assumptions of normality and homogeneity of variance were analysed using one-way analysis of variance (ANOVA) followed by a comparison with the DWC using Dunnett's test. The VTG data was $\log_{10}$ transformed prior to analysis in order to meet the assumptions of both normality and homogeneity of variance. To investigate the effects on reproductive activity, mean cumulative egg production over the 21 day pre-exposure and exposure periods were compared for each treatment group using the Kolmogorov-Smirnov test (KS-test).

3 Results

Results are reported for each of the three effluents tested in turn and include the oestrogenic activity measurements using the rYES; the analytical chemistry for specific oestrogen contaminants (17β-oestradiol and oestrone); the *in vivo* biomarker responses from male and female fish in both the spawning and non-spawning assays and the reproductive output data from the pair-breeding assays.

3.1 Effluent I

In vitro analysis using the rYES, demonstrated that the oestrogenic activity of the effluent used in the non-spawning experiment (38.2 ± 8.4 ng 17β-oestradiol equivalents per litre) was higher than that used in the pair-breeding experiment (11.1 ± 1.3 ng 17β-oestradiol equivalents per litre). Mean measured oestrogenic activities in the 0% and 100% effluent exposure tanks were 2.5 ± 1.0 and 21.2 ± 2.9 ng 17β-oestradiol equivalents per litre, respectively, in the non-spawning experiment and 1.9 ± 0.4 and 16.9 ± 7.4 ng 17β-oestradiol equivalents per litre, respectively, in the pair-breeding experiment. Recovery of total oestrogenic activity, in the rYES, was 86%.

Chemical analysis of the batches of effluent I used in each experiment demonstrated that oestrone was consistently present at a higher concentration than 17β-oestradiol. Mean measured concentrations of 17β-oestradiol and oestrone in the delivered effluent were 7.3 ± 1.7 ng/L and 97.4 ± 22.2 ng/L, respectively, for the non-spawning assay and 4.3 ± 0.4 ng/L and 70.5 ± 15.3 ng/L, respectively, for the pair-breeding assay. Concentrations of 17β-oestradiol and oestrone in the 100% effluent exposure tanks were 2.9 ± 0.9 ng/L and 23.8 ± 2.8 ng/L, respectively, in the non-spawning assay and 6.3 ± 1.9 ng/L and 92.7 ± 64.2 ng/L, respectively, in the pair-breeding assay. Both 17β-oestradiol and oestrone were detected in the DWC exposure tanks at concentrations of 0.5 ± 0.2 ng/L and 2.7 ± 0.9 ng/L, respectively, in the non-spawning assay and at concentrations of 0.9 ± 0.3 ng/L and 2.1 ± 0.6 ng/L, respectively, in the non-spawning assay. Mean recoveries for 17β-oestradiol and oestrone, in the spiked effluent samples were 93% and 89%, respectively.

Exposure to the 'high' oestrogenic effluent (effluent I) did not affect survival, growth or condition of the fish in either experiment. In both experiments, there were concentration-related increases in plasma VTG in the effluent exposed males ($P < 0.01$), with significant increases, relative to the DWC, at all dilutions tested (25, 50 and 100% effluent; $P < 0.01$; Figure 1). The levels of VTG induction in males exposed to the undiluted effluent indicated that the oestrogenic potency of the effluent was higher in the non-spawning experiment (5235-fold increase in male VTG concentrations, relative to the DWC) compared to the pair-breeding experiment (1765-fold increase in VTG, relative to the DWC). Plasma VTG concentrations were elevated, relative to the controls, in females exposed to the undiluted effluent (100%; $P < 0.01$; Figure 2) in the non-spawning experiment, but not the pair-breeding experiment ($p > 0.05$; Figure 2), further confirming the differences in the relative oestrogenic potency of effluent I during the two study periods.

For effluent I, an increase in GSI was observed in non-spawning males exposed to an effluent dilution of 50% ($P < 0.01$; Figure 3), but there was no evidence of an enhanced GSI in males exposed to the undiluted effluent ($p > 0.05$). Exposure of non-spawning females, to this effluent, resulted in a dilution-related ($P < 0.01$) decrease in GSI, but these decreases were not significant, relative to the DWC (Figure 4). There was no

evidence of an effect of the effluent on the GSI in either males or females in the pair-breeding experiment ($p > 0.05$; Figures 3 and 4).

In the non-spawning experiment, males exposed to the 50% effluent dilution appeared to have more prominent SSCs (tubercle number and prominence, fatpad weight; Figures 5 and 6) but this was not significant when compared with the DWC. Exposure to the undiluted effluent (100%) did not affect the appearance of the male SSCs ($P > 0.05$). In the pair-breeding experiment, there was no evidence that exposure to this effluent affected the SSCs in males (Figures 5 and 6).

In the pair-breeding experiment, exposure for 21 days, to the 50 and 100% effluent dilutions resulted in a significant reduction in mean cumulative egg production, relative to the pre-exposure period ($p < 0.05$; Figure 7).

3.2 Effluent II

In vitro analysis using rYES found a higher oestrogenic activity in the batches of effluent II used in the non-spawning experiment (9.4 ± 5.1 ng 17β-oestradiol equivalents per litre) compared with those used in the pair-breeding experiment (0.7 ± 0.1 ng 17β-oestradiol equivalents per litre). Mean measured oestrogenic activities in the 0% and 100% effluent exposure tanks, were 17.6 ± 4.9 and 20.2 ± 4.3 ng 17β-oestradiol equivalents per litre, respectively, in the non-spawning experiment and 3.7 ± 0.5 and 2.0 ± 0.4 ng 17β-oestradiol equivalents per litre, respectively, in the pair-breeding experiment. Recovery of total oestrogenic activity, in the rYES, was 97% in the spiked effluent samples.

Chemical analysis of the batches of effluent II used in each experiment demonstrated that oestrone was consistently present at a higher concentration than 17β-oestradiol; mean measured concentrations of 17β-oestradiol and oestrone in the delivered effluent were 1.4 ± 0.5 ng/L and 6.3 ± 1.6 ng/L, respectively, for the non-spawning assay and 0.9 ± 0.2 ng/L and 1.6 ± 0.1 ng/L, respectively, for the pair-breeding assay. Concentrations of 17β-oestradiol and oestrone in the 100% effluent exposure tanks were 4.0 ± 1.3 ng/L and 23.6 ± 6.1 ng/L, respectively, in the non-spawning assay and 1.1 ± 0.2 ng/L and 2.6 ± 0.3 ng/L, respectively, in the pair-breeding assay. Both 17β-oestradiol and oestrone were detected in the DWC exposure tanks at concentrations of 1.3 ± 0.3 ng/L and 8.4 ± 1.5 ng/L, respectively, in the non-spawning assay and at concentrations of 1.4 ± 0.2 ng/L and 3.8 ± 1.5 ng/L, respectively, in the non-spawning assay. Mean recoveries for both 17β-oestradiol and oestrone, in the spiked effluent samples were 92%.

Exposure to the 'intermediate' oestrogenic effluent (effluent II) did not affect survival, growth or condition of the fish in either experiment. A concentration-related increase in plasma VTG ($p < 0.01$) was observed in males exposed to the effluent in the non-spawning experiment, with significant increases in males exposed to the 50% and 100% effluent ($P < 0.01$; Figure 1). In the pair-breeding experiment, increases in plasma VTG concentrations were only significant in males exposed to the full-strength effluent (100%; $P < 0.05$; Figure 1). Plasma VTG concentrations were elevated in females exposed to the 50% and 100% effluent ($P < 0.01$; Figure 2) in the non-spawning experiment, but there was no effect of the effluent on female VTG concentrations in the pair-breeding experiment ($p > 0.05$; Figure 2). The higher levels of VTG induction observed in fish exposed to the undiluted effluent in the non-spawning experiment (for males a 15-fold increase, relative to the DWC) compared with fish in the pair-breeding experiment (for males a 9-fold increase, relative to the

DWC) are consistent with the differences in the oestrogenic content of this effluent at the different study intervals identified by the rYES.

In both the non-spawning and the pair-breeding experiments, an increase in GSI was observed in males exposed to the full strength effluent (100%; $P < 0.05$; Figure 3). Exposure of the non-spawning females to the 50% and 100% effluent also resulted in an apparent increase in the GSI, but this was only significant in females exposed to the 50% effluent ($P < 0.05$; Figure 4). In the pair-breeding experiment, effluent exposure resulted in a concentration-related increase in female GSI ($P < 0.05$), but no single concentration was significantly different when compared with the DWC ($p > 0.05$; Figure 4).

In the non-spawning males, the increase in GSI was accompanied by an apparent increase in tubercle number in non-spawning males exposed to 100% effluent, but this was not significant when compared to the controls (Figures 5). Exposure to the effluent did not affect the appearance of the male SSCs in the pair-breeding experiment ($p > 0.05$; Figures 5 and 6).

Exposure of the pair-breeding fathead minnow, for 21 days, to effluent II did not affect mean cumulative egg production ($p > 0.05$; Figure 7).

3.3 Effluent III

Analysis using the rYES demonstrated that the batches of effluent III used in the non-spawning experiment had a higher oestrogenic activity (12.7 ± 7.9 ng 17β-oestradiol equivalents per litre) compared with those used in the pair-breeding experiment (7.1 ± 1.5 ng 17β-oestradiol equivalents per litre). Mean measured oestrogenic activities in the 0% and 100% effluent exposure tanks, were 8.7 ± 3.9 and 34.1 ± 7.1 ng 17β-oestradiol equivalents per litre, respectively, in the non-spawning experiment and 8.3 ± 1.5 and 18.0 ± 4.0 ng 17β-oestradiol equivalents per litre, respectively, in the pair-breeding experiment. Recovery of total oestrogenic activity, in the rYES, was 81% in the spiked effluent samples.

Chemical analysis of the batches of effluent III used in each experiment demonstrated that oestrone was consistently present at a higher concentration than 17β-oestradiol; mean measured concentrations of 17β-oestradiol and oestrone in the delivered effluent were 1.2 ± 0.6 ng/L and 3.5 ± 1.9 ng/L, respectively, for the non-spawning assay and 0.8 ± 0.2 ng/L and 3.8 ± 0.7 ng/L, respectively, for the pair-breeding assay. Concentrations of 17β-oestradiol and oestrone in the 100% effluent exposure tanks were 3.9 ± 1.7 ng/L and 12.5 ± 6.8 ng/L, respectively, in the non-spawning assay and 2.5 ± 0.8 ng/L and 4.1 ± 0.7 ng/L, respectively, in the pair-breeding assay. Both 17β-oestradiol and oestrone were detected in the DWC exposure tanks at concentrations of 0.8 ± 0.1 ng/L and 3.8 ± 1.4 ng/L, respectively, in the non-spawning assay and at concentrations of 0.8 ± 0.3 ng/L and 2.1 ± 0.3 ng/L, respectively, in the non-spawning assay. Mean recoveries for 17β-oestradiol and oestrone, in the spiked effluent samples were 75% and 122%, respectively.

Exposure to the 'low potency' oestrogenic effluent (effluent III) did not affect survival, growth or condition of the fish in either experiment. Exposure to the full-strength and 50% effluent significantly increased male plasma VTG ($p < 0.01$; Figure 1) in both the non-spawning and pair-breeding experiments. The higher levels of VTG induction in males exposed to the undiluted effluent in the non-spawning experiment (a 214-fold increase, relative to the DWC) compared with in the pair-breeding experiment (a 46-fold increase, relative to the DWC) reflected the differences in the oestrogenic content

measured in the rYES. Exposure of the females to the effluent did not appear to affect plasma VTG during the non-spawning experiment ($p > 0.05$; Figure 2), however, in the pair-breeding experiment, plasma VTG concentrations were found to be decreased in females exposed to the undiluted effluent (100%; $p < 0.05$; Figure 2). There was no evidence of an effect of exposure to the 25% and 50% effluent dilutions on plasma VTG concentrations in the pair-breeding females ($p > 0.05$; Figure 2).

In the non-spawning experiment, exposure to effluent III resulted in a concentration-related increase in the male GSI ($P < 0.01$), that was significant, relative to the DWC, in males exposed to the undiluted effluent (100%; $P < 0.05$; Figure 3). In the pair-breeding experiment, exposure to the effluent did not affect male GSI ($P > 0.05$; Figure 3). Exposure of the females to effluent III did not affect GSI in either the non-spawning or the pair-breeding experiments ($p > 0.05$; Figure 4).

Exposure to effluent III did not affect the appearance of the SSCs in males in the non-spawning assay ($P > 0.05$; Figures 5 and 6). In the pair-breeding experiment, tubercle number was not affected by exposure to the effluent ($P > 0.05$; Figure 5), however, the prominence of the tubercles was decreased in males exposed to 50% and 100% effluent ($P < 0.05$, data not shown) and the weight of the fatpad was decreased in all effluent exposed males (25, 50 and 100%; $P < 0.05$; Figure 6).

Exposure of the pair-breeding fathead minnow to 100% effluent III for 21 days resulted in a significant reduction in the mean cumulative egg production relative to the pre-exposure period ($p < 0.05$), but there was no evidence for an effect of the diluted effluent on egg production (25%, 50%; $P > 0.05$; Figure 7).

3.4 Oestrogen Positive Controls

Oestradiol equivalent concentrations in the positive control tanks, determined using the rYES, were 26.9 ± 8.4, 65.1 ± 3.1 and 69.2 ± 8.3 ng 17β-oestradiol equivalents per litre in experiments AI, AII, AIII (non-spawning experiments) and 11.3 ± 1.1, 20.6 ± 1.3 and 52.2 ± 3.1 ng 17β-oestradiol equivalents per litre in experiments BI, BII and BIII (pair-breeding experiments), respectively. Mean measured concentrations of 17β-oestradiol in the positive control tanks in experiment AI were 13.8 ± 3.6 ng/L over the 14 day exposure period. Mean measured concentrations of ethinyloestradiol-17α in the positive control tanks in experiment AII, BI, BII and BIII were 18.0 ± 2.0, 8.2 ± 2.8, 14.0 ± 1.0 and 15.7 ± 1.3 ng/L, respectively. Due to technical problems no values were determined for the concentrations of ethinyloestradiol-17α in the positive control tanks used in experiment AIII.

Exposure to the steroidal oestrogen positive controls for 14 or 21 days, did not affect survival, growth or condition of the fish in any experiment ($p > 0.05$). Plasma VTG concentrations were significantly increased in the oestrogen exposed males in all experiments ($p < 0.05$; Figure 1). Plasma VTG concentrations were also significantly increased in the oestrogen exposed females in each of the non-spawning experiments ($p < 0.05$; Figure 2) and in one pair-breeding experiment (IB; $p < 0.05$; Figure 2).
In all experiments, apparent decreases in GSI were observed in males exposed to ethinyloestradiol-17α, however, this was only found to be significant in one non-spawning experiment (IIIA; $p < 0.05$; Figure 3). Exposure to 17β-oestradiol did not affect the GSI in males in experiment IA ($p > 0.05$; Figure 3). Significant reductions in the GSI were observed in all oestrogen (17β-oestradiol and ethinyloestradiol-17α) exposed females in the non-spawning experiments ($p < 0.05$; Figure 4). Apparent decreases in GSI were also observed in the ethinyloestradiol-17α exposed females, in pair-breeding experiments IB and IIB, but these were not significant relative to the DWCs ($p > 0.05$; Figure 4).

Oestrogen exposure (17β-oestradiol and ethinyloestradiol-17α) caused an apparent decrease in the number (Figure 5) and prominence of the male nuptial tubercles in all experiments, but these effects were only significant in the three pair-breeding experiments (IB, IIB, IIIB; $p < 0.05$; Figure 5). Reductions in male dorsal fatpad weight were also apparent in all experiments, but were only significant in the third pair-breeding experiment (IIIB; $p < 0.05$; Figure 6).

There was no effect of the ethinyloestradiol-17α positive control on cumulative egg production in any of the three pair-breeding experiments ($p > 0.05$; Figure 7).

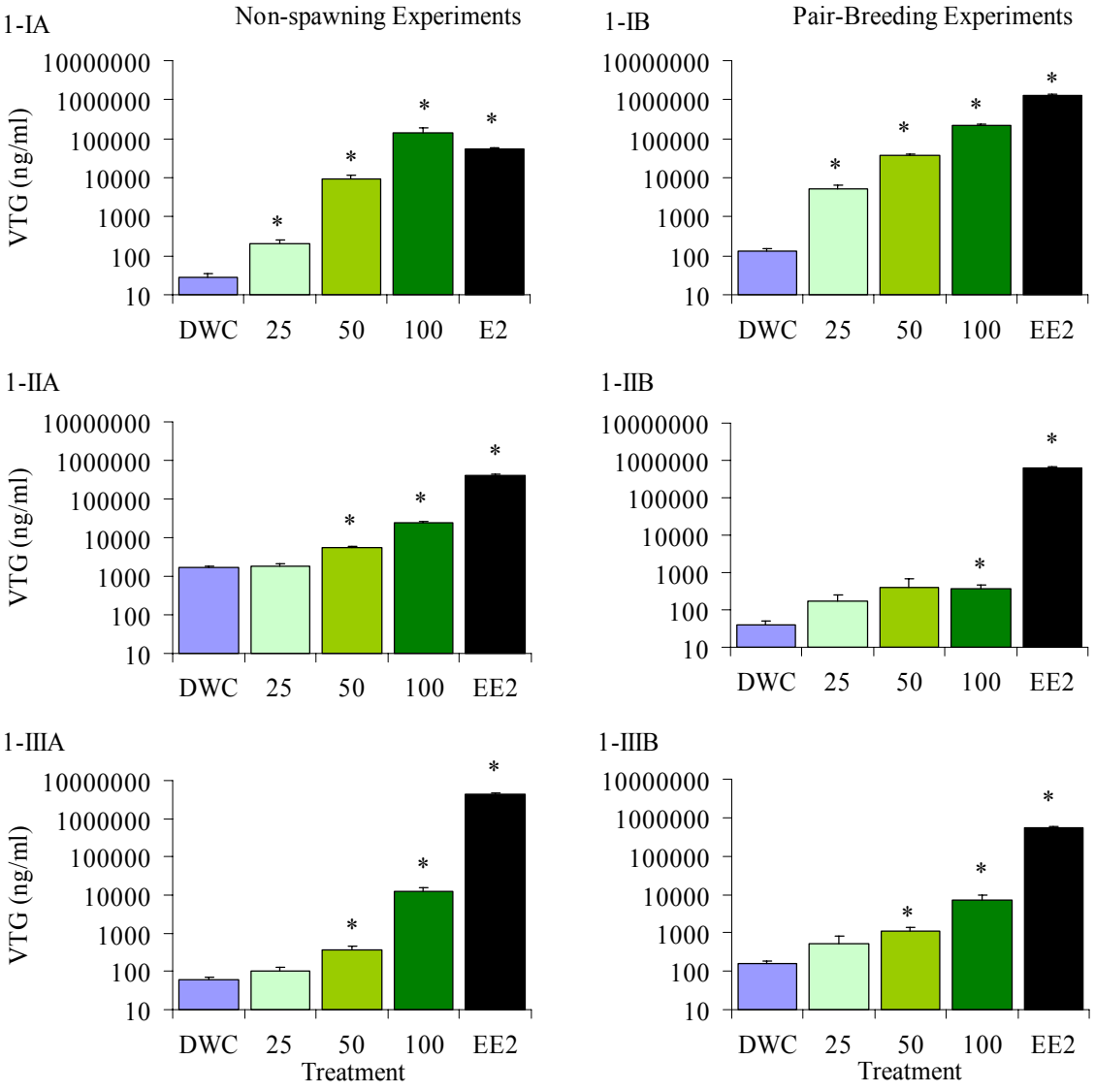

Figure 1. Influence of treated sewage effluents with predicted, high (I), intermediate (II) and low (III) oestrogenic potency on plasma VTG concentrations in male fish exposed in (A) a non-spawning and (B) a pair-breeding bioassay.
Data are shown as mean ± standard error of the mean. *denotes significant differences relative to the dilution water control (DWC; $p < 0.05$).

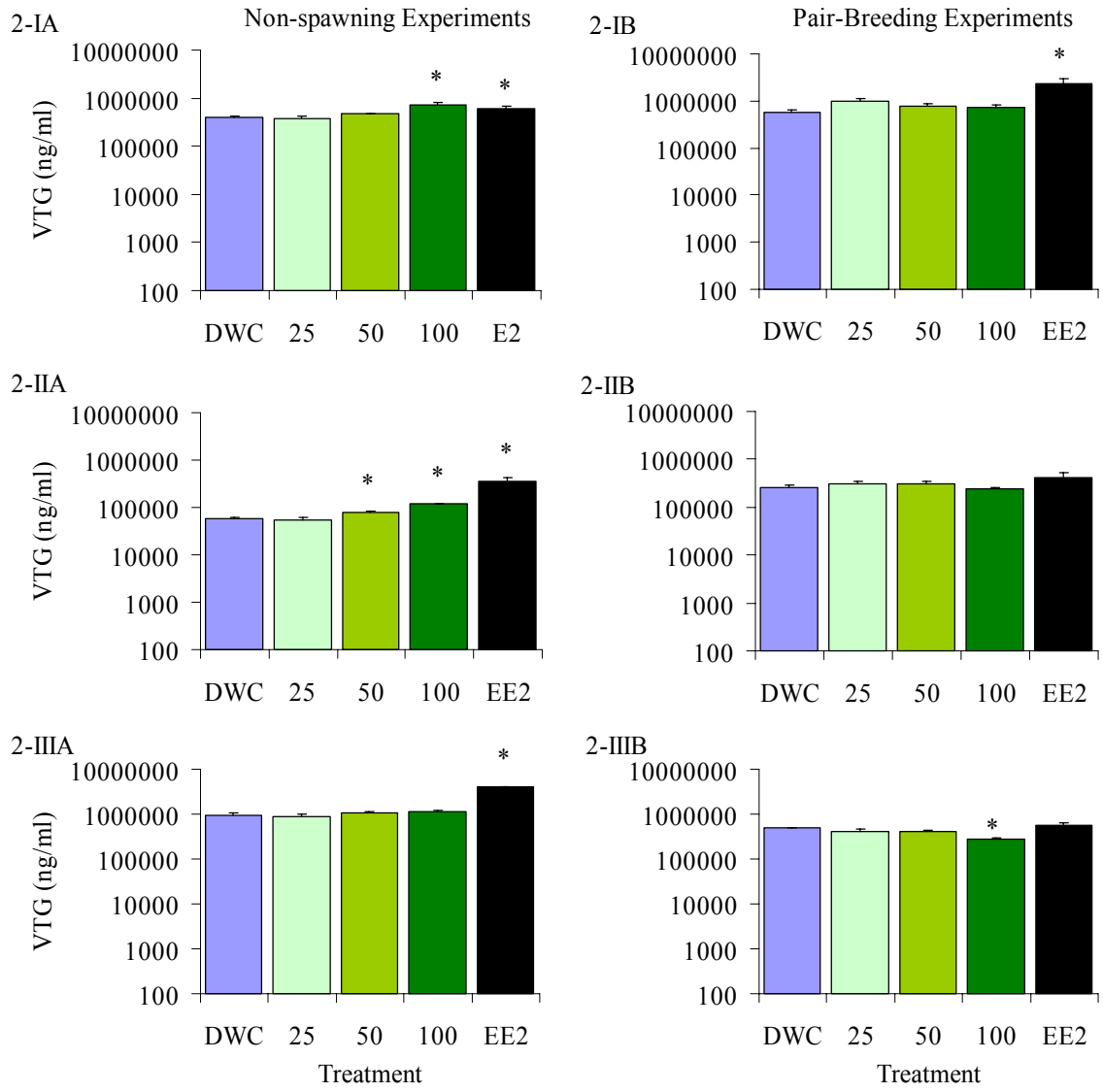

Figure 2. Influence of treated sewage effluents with predicted, high (I), intermediate (II) and low (III) oestrogenic potency on plasma VTG concentrations in female fish exposed in (A) a non-spawning and (B) a pair-breeding bioassay.
Data are shown as mean ± standard error of the mean. *denotes significant differences relative to the dilution water control (DWC; $p < 0.05$).

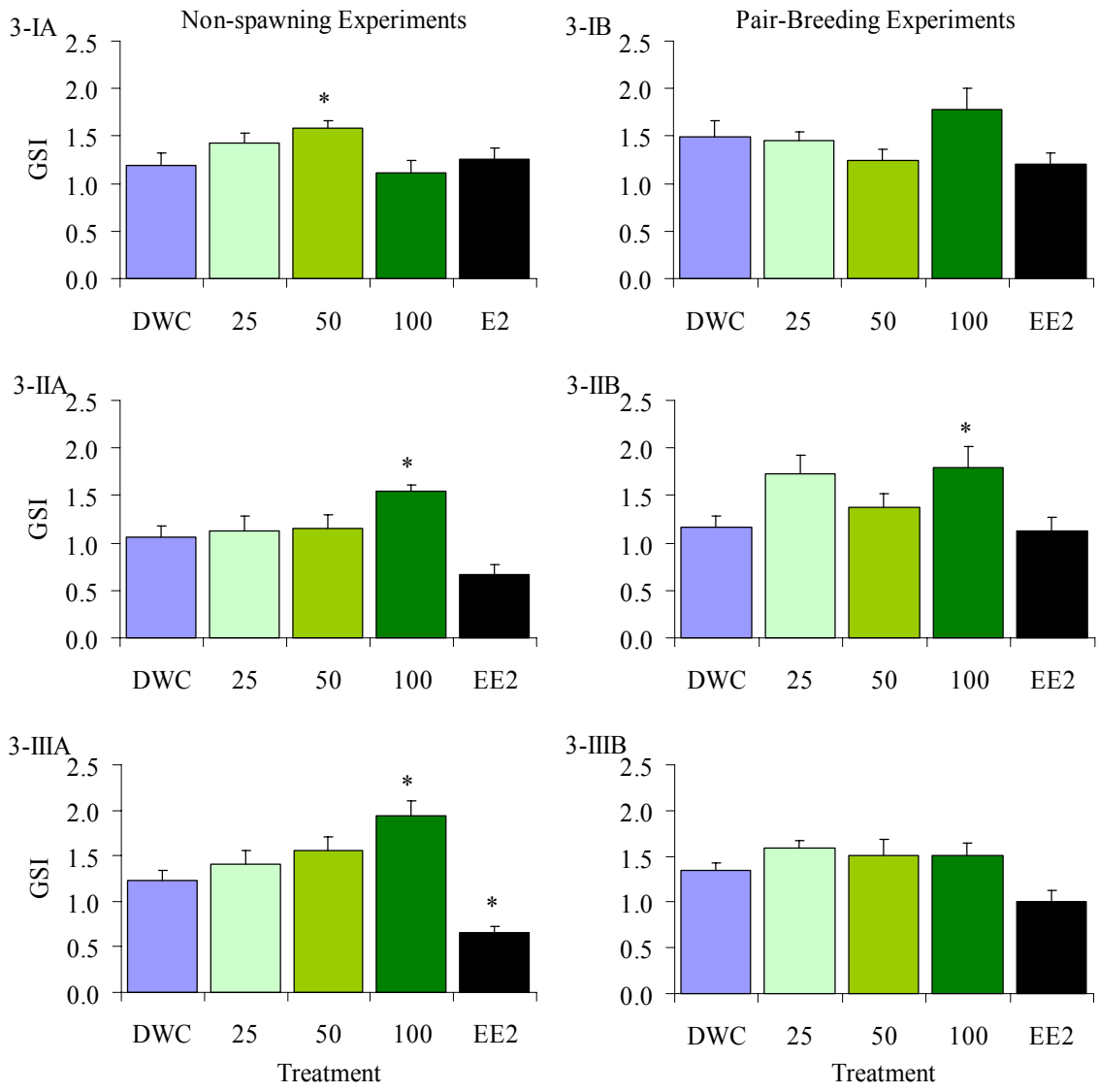

Figure 3. Influence of treated sewage effluents with predicted, high (I), intermediate (II) and low (III) oestrogenic potency on relative gonad weight (GSI) in male fish exposed in (A) a non-spawning and (B) a pair-breeding bioassay.
Data are shown as mean ± standard error of the mean. *denotes significant differences relative to the dilution water control (DWC; $p < 0.05$).

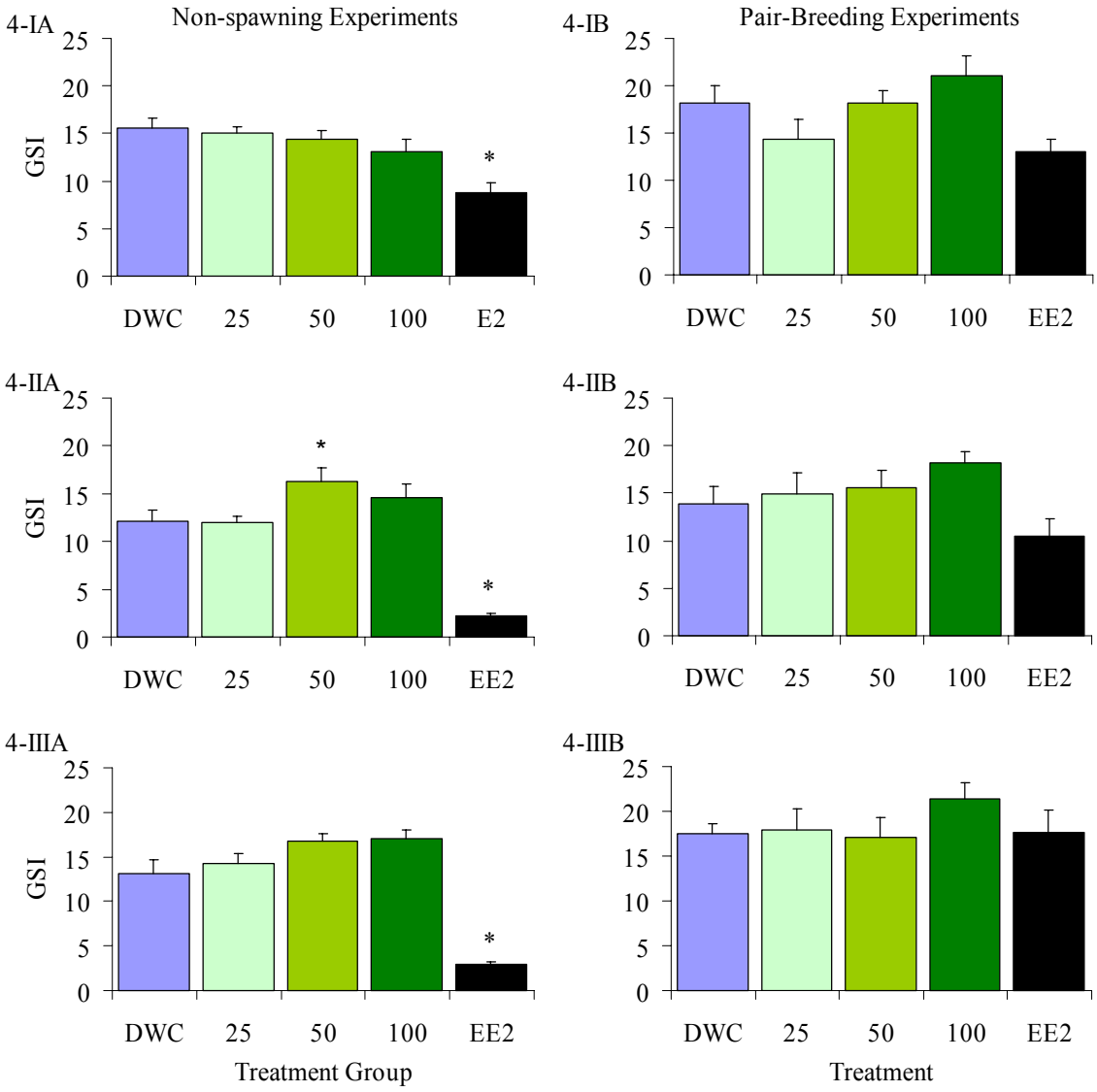

Figure 4. Influence of treated sewage effluents with predicted, high (I), intermediate (II) and low (III) oestrogenic potency on relative gonad weight (GSI) in female fish exposed in (A) a non-spawning and (B) a pair-breeding (B) bioassay.
Data are shown as mean ± standard error of the mean. *denotes significant differences relative to the dilution water control (DWC; $p < 0.05$).

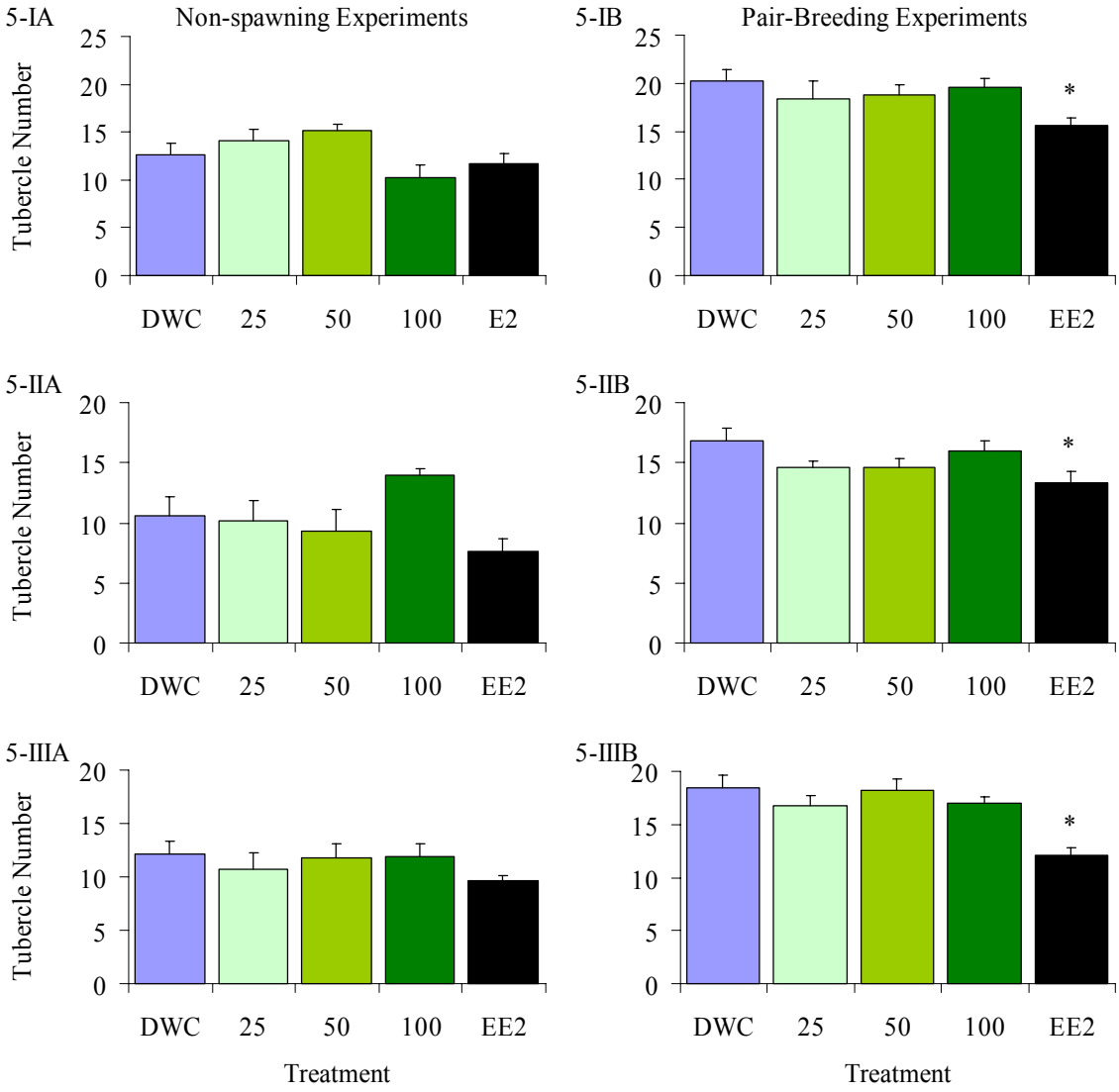

Figure 5. Influence of treated sewage effluents with predicted, high (I), intermediate (II) and low (III) oestrogenic potency on tubercle number in male fish exposed in (A) a non-spawning and (B) a pair-breeding bioassay.
Data are shown as mean ± standard error of the mean. *denotes significant differences relative to the dilution water control (DWC; $p < 0.05$).

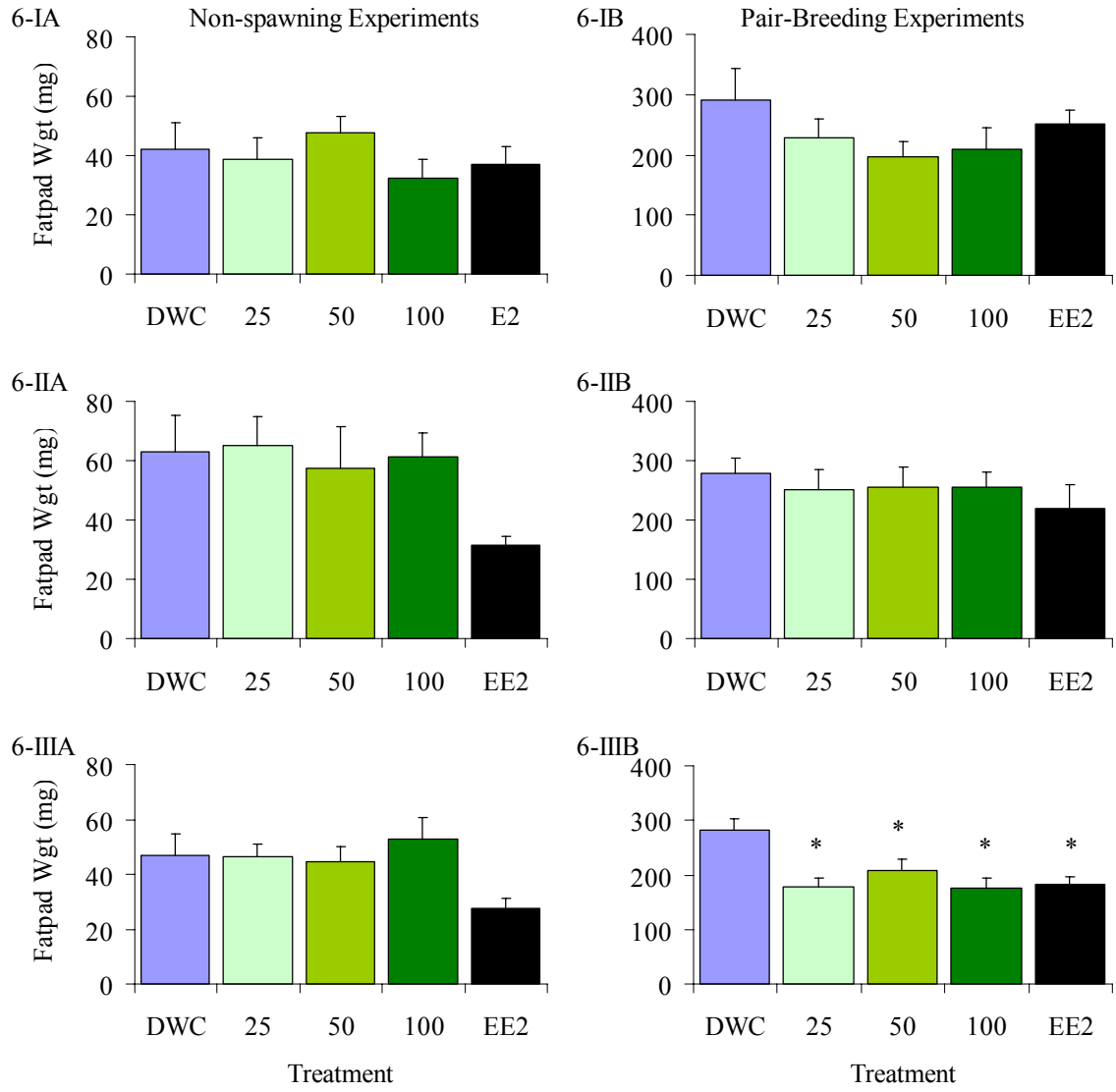

Figure 6. Influence of treated sewage effluents with predicted, high (I), intermediate (II) and low (III) oestrogenic potency on weight of the dorsal fatpad in male fish exposed in a non-spawning (A) and a pair-breeding (B) bioassay.
Data are shown as mean ± standard error of the mean. *denotes significant differences relative to the dilution water control (DWC; $p < 0.05$).

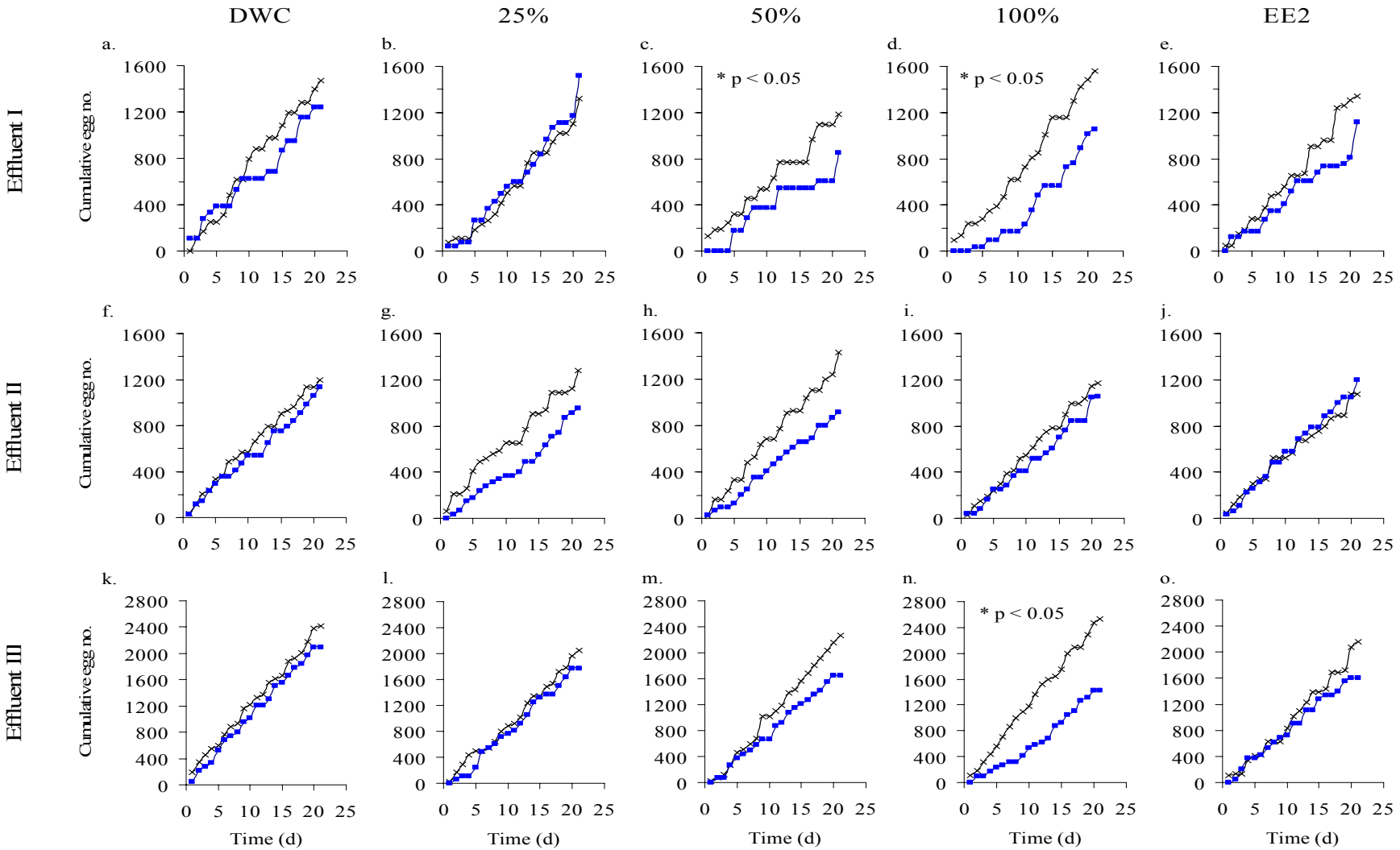

Figure 7. Mean cumulative egg number in pairs of fathead minnow exposed for 21 days to either a dilution water control (DWC), an oestrogen positive control (17β-oestradiol) or graded concentrations (25%, 50% and 100%) of the three effluents (Effluent I, II and III).

*p < 0.05 indicates differences in mean cumulative egg production between the pre-exposure (black lines) and exposure (blue lines) periods for the 8 pairs of fish assigned to each treatment group.

4 Discussion

The series of experiments described here demonstrate the successful application of an *in vivo* laboratory test system for quantifying the reproductive health effects of effluents from STWs that receive primarily domestic waste. The test system enabled the oestrogenic effects of final STW effluent discharges to be evaluated in the fathead minnow under controlled laboratory test conditions. It was demonstrated for the first time that the impact of oestrogenic effluents on reproduction (egg production) could be directly quantified. This information provides an important step for further understanding the health consequences of STW effluent discharges for fish populations.

For each of the three effluent discharges tested in the fish assays, sub-samples of the effluent were extracted for analysis in the rYES, to determine total oestrogenic activity as oestradiol equivalent concentrations, and for analytical determination of the concentrations of two specific oestrogenic contaminants (oestradiol and oestrone). Effluent I was found to possess a relatively high oestrogenic activity in the rYES and high concentrations of 17β-oestradiol and oestrone when compared with effluents II and III. The concentrations of the two natural oestrogens were consistent with those previously reported in studies using effluent discharged from this STW (Rodgers-Gray *et al*., 2001; Liney *et al*., 2006). However, the oestrogenic contents (as measured by the rYES and analytical chemistry for 17β-oestradiol and oestrone) of the effluent discharges collected from STWs II and III for the non-spawning assays were similar to each other and did not give the gradient of oestrogenic potencies envisaged in the original concept. In addition, the rYES results are higher than the measured steroids during these two exposures, suggesting that there are other oestrogenic compounds in the effluent which are unaccounted for.

The total oestrogenicity (in the rYES) of the effluent with 'intermediate potency' (effluent II) was found to be very low in the samples collected for the pair-breeding assay compared with those for the non-spawning assay. This change in effluent quality may be a consequence of an alteration in the treatment process at this STW between the two exposures, or it may relate to differences in influent input to the STWs, levels of microbial activity and/or dilution factors both within the STWs and for the effluent discharge. Clear differences in oestrogenic content were also observed between the different batches of effluent collected for effluents I and III both within and between the different exposures, highlighting the need to consider the effects of effluents at more than one time-point and demonstrating the difficulties in reproducing tests for even a single effluent over time.

Daily analysis of the oestrogenic activity (using the rYES) in sub-samples of effluent collected from the chilled storage tank confirmed that the oestrogenic content of each batch of effluent was stable for the duration of storage (up to 8 days). In the non-spawning experiments, however, there was a poor agreement between the oestrogenic activity of the stored effluents and the levels of oestrogenic activity measured in the 100% exposure tanks. In the first experiment (IA), 3-4 fold lower concentrations of oestrogen were observed in the exposure tanks when compared with concentrations measured in the delivered effluent. However in the second two experiments (IIA and IIIA), the oestrogenic content of the 100% exposure tanks was 3-4 fold higher than that measured in the delivered effluent. The conflicting observations are most likely to be a consequence of the different effluent holding conditions used. In the first experiment, the effluent was transferred to the temperature controlled testing laboratory (air temperature maintained at 25°C) twice per day in quantities sufficient to last for a 12h period. The effluent was then pumped via a mixing tank, held at 25 °C, to the test

system. In the second two experiments the effluent was pumped directly from the effluent storage tank in which it was held at a temperature of 8 °C, via a mixing tank held at 18 °C, to the exposure tanks in the temperature controlled laboratory (25 °C).

The prolonged period during which the effluent was held at a high temperature during the first experiment (IA) most likely resulted in higher levels of biodegradation of the parent oestrogens and consequently reduced the oestrogen content in the exposure tanks. The modified exposure system used in experiments IIA and IIIA appeared to be much more effective in minimising degradation. In these two experiments, the higher oestrogen content observed in the exposure tanks was most likely to be a consequence of the secretion of steroids into the water by the experimental fish (females). The presence of both 17β-oestradiol and oestrone in the DWC tanks in experiments IIA and IIIA, supports this hypothesis. Although the oestrogenic content of the delivered effluent was different from that measured in the 100% exposure tanks, it was observed that the oestrogenic content was stable (as measured using the rYES) in both the storage tank and the 100% exposure tanks. In the pair-breeding studies where the second exposure system was used and replacement of the effluent was more frequent, a good agreement was observed between the oestrogen content of the delivered effluent and the 100% exposure tanks. This is further evidence that the exposure system described can be used to hold effluent samples over periods of several days to enable chronic testing of effluent effects under flow-through laboratory conditions.

Each of the effluents assessed in this study induced significant levels of VTG in the plasma of male fish, which was consistent with the measured oestrogenic content. Comparison of the magnitude of the vitellogenic responses confirmed that the biological response was greater for effluent I, but that effluents II and III were similar in their oestrogenic potency; induction of VTG in males exposed to effluent I occurred at the weakest dilution (25% effluent) and at 50% dilutions for effluent II (in the non-spawning assay only) and for effluent III. These data on the induction of VTG demonstrates the sensitivity of this biomarker in male fish for detecting oestrogenic chemicals, even when they are contained within complex matrices such as effluents.

Effluents contain varied mixtures of chemicals, many of which have the potential to affect the reproductive axis via mechanisms other than (or in addition to) the oestrogenic receptor(s). Measurements of GSI and male SSCs were therefore included within the non-spawning bioassay to provide additional biomarkers of reproductive health that were not specific to the oestrogenic pathway. Exposure to the effluents did not appear to affect ovary growth/size in females (as determined by the GSI) but effects on the gonad (increases in the relative size of the testis) were observed in males exposed to effluents I, II and III in the non-spawning assays and for effluent II in the pair-breeding assay. This contrasted with the effects of the oestrogen positive control, which caused a suppressive effect on gonadal growth/size (a decrease in GSI) in the non-spawning females (significant decrease in experiments IA, IIA and IIA) and in the non-spawning males (significant decrease in experiment IIIA only). This disparity between the effects of the effluent and the oestrogen positive control supports the hypothesis that effluents can affect gonadal development via pathways additional to activation of the oestrogen receptor.

In the fathead minnow pair-breeding assays, exposure to effluents I and III significantly reduced egg production over the 21 day exposure period, but there was no evidence of an effect on egg production from exposure to effluent II. These responses correlated with the measured oestrogenic potencies of the effluents; effluent I increased VTG in males exposed to all dilutions and reduced egg production at 50% and 100% concentrations; effluent III increased VTG in males exposed to 50% and 100% and reduced egg production at 100% concentrations; effluent II did not affect egg

production and only increased VTG in males exposed to the undiluted effluent. This pattern strongly suggests that the oestrogenic components of the effluent are responsible for the reproductive impacts observed. In contrast, exposure to the oestrogen positive controls did not affect egg production in any of the three pair-breeding experiments conducted, despite having more potent effects on both VTG induction and on the male SSCs when compared with all three effluents. Previous investigations have demonstrated that exposure to oestrogenic chemicals can inhibit egg production in the fathead minnow, but in short-term exposures (21 days) these effects are observed only at concentrations of oestrogens that are associated with toxicity to the males (Kramer *et al*., 1998; Seki *et al*., 2002; Thorpe *et al*., 2007b). It was not considered appropriate to use such unrealistic concentrations in these experiments. Instead the chosen concentrations were as high as could be reasonably expected not to elicit toxic effects. This being a research programme, the outcome of the positive control trials does not invalidate the effluent assays. It is likely that the single chemicals used and the complex mixtures in the effluents operate through the expression of different receptor sub-types, and consequently further work is needed to develop an appropriate positive control.

Chemicals that target other pathways within the reproductive axis, such as the through affecting the activity of the aromatase enzymes (Ankley *et al*., 2002, 2005; Thorpe *et al*., 2007b) have also been shown to reduce egg production without an associated overt toxicity. The effects on reproduction observed in the effluent-exposed fish in this study were not associated with any overt toxicity, so it is likely that the effects of the effluent in reducing egg production operate through mechanisms additional to the oestrogen receptor. The observation that the degree of fold induction of VTG in males was less for the effluents than for the positive controls supports this, otherwise a lower VTG response might be expected to have less effect on reproduction were the response mediated by the oestrogenic pathway alone. Furthermore, in the exposures to effluent III, there were concentration-related decreases in plasma VTG concentrations in females that were significant relative to the DWC. This contrasted with the increase in VTG concentrations observed in the females exposed to ethinyloestradiol-17α in the positive controls. Reductions in plasma VTG concentrations have previously been demonstrated in mature females exposed to anti-oestrogenic chemicals (Panter *et al*., 2002) and aromatase inhibitors (Ankley *et al*., 2002, 2005; Panter *et al*., 2004) and in the latter case the reduction in female VTG concentrations was associated with a reduced egg production (Ankley *et al*., 2002; 2005). It is therefore possible that chemicals capable of binding the oestrogen receptor as an antagonist or those inhibiting the action of aromatase were also present within the test effluents.

5 Conclusions

These investigations describe the first attempt to investigate directly the effects of effluents from STWs that treat domestic wastewater on egg production in fish and they provide an important step to furthering our understanding of the potential health effects that treated effluents might pose to our wild fish populations. The fathead minnow pair-breeding assay was shown to be highly robust for the effluent exposures. As the test can be conducted over a relatively short time period, it provides a cost-effective means of assessing effluent exposure effects on reproduction, in comparison with the effort required if a native UK fish species with a much longer life-cycle was used. The test system employed here is practicable and it has been shown that it is possible to use an effluent storage facility (chilled to 8°C) and a flow-through system to provide a realistic scenario for effluent exposures to fish. Daily analysis of the effluents in the storage tank, using the rYES, demonstrated that their oestrogenic potency did not differ from that measured on arrival for a period of up to 8 days (longer time periods were not investigated) and so their oestrogenic activity can be considered to be stable. Overall the data have shown that quantifiable levels of oestrogen persisted throughout the studies.

Differences were observed in oestrogenic potency between different batches of the same effluent used during the experiments, but given that daily fluctuation in the chemical composition of the influent will affect the oestrogenic potency, such variations were expected. Of more consequence, for each of the three effluents studied here, differences were observed in their biological potency between the non-spawning and pair-breeding bioassays; effluents I, II and III were 2-, 10- and 3-fold more potent, respectively, in the samples used for the non-spawning assay than those taken for the pair-breeding assay. This illustrates the difficulty of assessing the impact of effluents on the receiving environment and demonstrates the need for conducting long-term monitoring investigations. Analytical chemical methods and simple *in vitro* bioassays have considerable value in such long-term monitoring and in these investigations good relationships were observed between the results of the chemical measurements, oestrogenic activity in the rYES and induction of VTG. However, the results of the reproductive endpoint in the pair-breeding assay show that focusing on simple methods that are designed to detect individual chemicals or effects on a single receptor system cannot provide a holistic understanding of the potential health effects of such complex mixtures. Integrative test methods that can assess effects on the entire reproductive axis are required to fully comprehend the long-term environmental health effects of effluent discharges. The importance of this is demonstrated by this study, where effluents with a lower measured oestrogenic potency than that expected for the steroidal oestrogen positive controls, impacted negatively on reproductive output, where the single substances alone did not.

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Abbreviations

OECD	Organisation for Economic Co-operation and Development
rYES	recombinant Yeast Oestrogen Screen
SSC	Secondary sex character
STW	sewage treatment works.
USEPA	United States Environmental Protection Agency
VTG	vitellogenin

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