Investigations into the Biochemical and Reproductive Effects of Endocrine Disruptors In Fish (BREEDIF)

Final Report

for

Defra

CEFAS Contract report

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COMMERCIAL IN CONFIDENCE

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Contract A1140

Final Report

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EXECUTIVE SUMMARY

It is important to understand the extent to which biological systems interact when two or more systems are being stimulated by contaminants. A knowledge of the amount of 'crosstalk' taking place allows more confident interpretation of biomarker monitoring data. This is particularly important in multi-contaminant areas, such as estuaries. This study has focussed on the two biomarkers most widely used in estuarine areas, ethoxyresorufin-*O*-deethylase (EROD) and plasma vitellogening (VTG). Using the European flounder (*Platichthys flesus*) as the test species this study has established, under standard laboratory conditions, the potency of several key contaminants in terms of their ability to either induce the mixed function oxygenase (MFO) system, as measured by hepatic EROD concentrations, or act as oestrogens, as measured by the presence and concentrations of VTG in male plasma.

Several organic pollutants were assessed for their ability to induce EROD activity including the polycyclic aromatic hydrocarbons (PAH) dibenz[a,*h*]anthracene (DbA), benzo[*k*]fluoranthene (BkF) and benzo[*a*]pyrene (BaP). DbA was found to give the greatest response with BaP being the least potent EROD inducer. The three main oestrogenic chemicals tested were estradiol (E2), ethynylestradiol (EE2) and a technical mix of nonylphenol (NP). The threshold ranges of VTG induction in male flounder after 10 days exposure were found to be 1 –10 ng/l, 100 – 150 ng/l and 100 – 333 µg/l for EE2, E2 and NP respectively. The information from the single chemical exposures allowed the setting of standard active concentrations for each chemical which were subsequently used in binary mixture experiments of DbA with each of the three oestrogens in order to investigate the potential for up or down regulation in biomarker responses to occur.

All three oestrogenic chemicals were able to suppress PAH mediated EROD induction. A very significant finding was that, although both EE2 and NP had threshold concentration of EROD inhibition at similar points to that at which they also induce VTG production, E2 showed an ability to suppress EROD at concentrations very much lower than that at which it induces VTG. To this end it was established that although EE2 is a more potent VTG inducer it is less potent than E2 in its ability to inhibit EROD activity.

No effects of PAH on the ability of the oestrogenic chemicals to induce plasma VTG was observed other than with NP at the threshold concentrations for VTG induction. This was somewhat surprising in light of the evidence in the literature that suggests that certain PAH have antioestrogenic properties.

The use of a second exposure route, that of intraperitoneal injection, confirmed the potency rankings of the three oestrogens in terms of both their ability to induce VTG and to inhibit PAH-induced hepatic EROD. However, this method did not predict the level by which E2 inhibited EROD compared to its VTG induction threshold. Therefore, though the IP method is recommended as a screening tool for investigating interactions, more appropriate exposure routes are required to meaningfully investigate environmental effects.

Emerging cDNA microarray techniques to measure gene expression in flounder tissue were also applied to one set of samples from a DbA/EE2 mixture experiment. The results confirmed that the genes for vitellogenin and choriogenin showed elevated expression in the oestrogen exposed fish and that the CYP1A gene was expressed in those fish exposed to the PAH. The technique also showed that the VTG and choriogenin genes showed lower expression in co-exposures with DbA confirming the *in vivo* results. A range of other genes were up or down regulated as a results of the exposures and these techniques may prove useful in interpretation and mechanistic determination in the future.

Finally, some hepatic EROD monitoring results from estuarine flounder were reassessed in light of the project findings. It was hypothesised that published EROD monitoring data may be an underestimation of effects if it is assumed that oestrogen mediated MFO suppression is occurring in wild populations. It was recommended that a greater understanding of system interaction and the other factors, some genetically determined, that influence biomarker response to contaminants is required to enable a greater level of confidence in the interpretation of biomarker monitoring data.

1. INTRODUCTION

The use of sub-lethal biological indicators, or biomarkers, as a means of assessing contaminant effects and/ or exposure has become widespread and integral to many national marine monitoring programmes. In the United Kingdom certain biomarkers have been routinely used to assess the biological effects of pollution on fish at a wide range of estuarine and marine sites.

Two prognostic biomarkers that have seen wide usage in fish monitoring and are therefore viewed with great interest are the measurement of hepatic ethoxyresorufin-*O*-deethylase (EROD) activity and the determination of plasma vitellogenin (VTG) concentrations.

Cytochrome P4501A1 (CYP1A1) is the terminal component of the MFO system, and is of pivotal importance in the detoxification of many PAHs and some PCBs. EROD activity is CYP1A1 dependent and is measured as a marker of induction of the MFO system. MFOs are induced by exposure to a number of planar, organic contaminants, specifically PAH and some PCBs, and therefore EROD has been employed extensively as a marker of exposure to these important contaminants in the UK (Kirby *et al.*, 1999; Kirby *et al.*, 2004a) and in other European countries (Beyer *et al.*, 1996; Eggens *et al.*, 1996).

Plasma VTG levels have, in recent years, been deployed as the main assay for the determination of exposure to endocrine disruptors such as oestrogens and their mimics. The use of this biomarker in the European flounder, *Platichthys flesus*, for determination of oestrogenic exposure has been particularly successful (Allen *et al.*, 1999; Kirby *et al.*, 2004b) and is now under consideration for incorporation into monitoring programmes.

Recent studies (Matthiessen *et al.*, 1998; Kirby *et al.*, 1999) have highlighted the high potential for interaction between the systems responsible for EROD induction (MFO – detoxification) and VTG production (oestrogenic - hormonal) and the substances that induce responses in them. The potential for this to occur with MFO and oestrogenic responses is particularly high due to the fact that endogenous substrates and/or xenobiotics that impact each system can also act strongly on the other. For example, certain PCBs and PAHs are known to cause endocrine disruption (oestrogenic or anti-oestrogenic and anti-androgenic effects) and certain endocrine disrupters, for example ethynylestradiol and nonylphenol, have been shown to inhibit EROD activity. Furthermore, components of the endocrine system, such as steroids, are substrates for the MFO system so the possibility of inter-system regulation exists.

Although the scientific literature includes some specific examples of up and down regulation of these responses in fish (Hasselberg *et al.*, 2004; Navas *et al.*, 2004) little research has focussed on the extent to which these biological systems can influence each other when both are stimulated. The investigation of these interactions, or so-called ‘crosstalk’, was one of the main objectives of the current study.

This research is particularly relevant in the context of improving the quality of biological effects monitoring in the UK. There is no doubt that, in areas where MFO inducers and endocrine disrupters are present at active levels, inter-system interactions (cross-talking) could significantly affect both monitoring results and their interpretation. Consequently, in marine areas such as estuaries and the near shore, where active levels of both MFO inducers and endocrine disruptors are present, the biomarker results obtained from monitoring activities may be misinterpreted if each is evaluated in isolation. At a time when the results from the use of these biomarkers may be highly influential in the development of policy and in influencing the direction of research it is essential that we are able to interpret the monitoring results with greater confidence. This study has aimed to understand in greater depth the potential for these two biological systems to influence each other, especially in multi-contaminant areas, and to use this knowledge to aid the interpretation of monitoring data.

In the design and conduct of the project, a core aim was to select both test species and exposure chemicals that would allow the production of both meaningful and environmentally relevant data. To that end, the European flounder (*Platichthys flesus*) was the outstanding candidate as the model species. This species had already been used successfully in laboratory based experiments (Kirby *et al.*, 2000; Reynolds *et al.*,

2003), was readily available from a farm supplier and its reproductive cycle and behaviour are well known (Janssen *et al.,* 1995; Janssen *et al.,* 1996). Most significantly, however, Flounder have been used extensively in the monitoring of a wide range of biomarkers in the UK and, in particular, for studies of both hepatic EROD (Kirby *et al.,* 1999; Kirby *et al,.* 2004) and male plasma VTG (Allen *et al.,* 1999; Kirby *et al.,* 2004). This meant that there could be a rapid transfer of the findings from this study to both current field studies and existing datasets.

Of equal importance to the choice of species was the selection of the chemicals to be studied. In achieving this, an extensive literature search was conducted to ascertain those of most relevance to the UK environment. For MFO inducers a range of polycyclic aromatic hydrocarbons (PAH) and other planar organics (e.g. certain poly chlorinated biphenyls (PCBs)) were investigated and selected on the basis of potency and environmental relevance. The most appropriate oestrogenic chemicals for study were selected on the basis of potency and their presence in domestic and industrial effluents that discharge to estuarine environments (Matthiessen *et al.,* 1998) and included natural and synthetic oestrogens and breakdown products of alkylphenol-derived surfactants which are persistent in the environment.

The general approach adopted for the study is outlined below but the overarching aim was to enable us to establish known responses to environmentally relevant contaminants and to investigate how these responses were affected by means of basic binary mixture experiments. Furthermore, an opportunity was taken to utilise emerging gene expression techniques by collaborating with colleagues from Birmingham University. The ultimate aim was to enable us to have more confidence in our interpretation of biomarker monitoring data and to provide added value to the datasets that we have already collected.

2. GENERAL APPROACH, AIMS AND OBJECTIVES

As a general approach the project was broken into four distinct elements;

i.) Establish standard EROD responses to specific MFO inducers
ii.) Establish standard VTG induction responses to oestrogenic chemicals
iii.) Investigate up or down regulation of these standard responses when fish are exposed to a combination of two chemicals
iv.) Interpret findings in terms of potential relevance to and interpretation of existing and future EROD and VTG monitoring data.

The detail of the experimental approach and set-ups are described below but, briefly, these aims were achieved by establishing a standard dosing and exposure regime in which all easily controllable factors that might affect the results (e.g. temperature, feeding regime, water quality, water flow, exposure duration) were set and maintained. Under these standard and reproducible conditions standard responses were first established and then compared to the experimental responses observed when the test species were co-exposed to binary mixtures of chemicals. This approach would not only allow the determination of whether up or down regulation (crosstalk) of biomarker responses could occur but, by varying the concentration/ dose regimes, thresholds for both response and crosstalk influence could be investigated and related to environmentally relevant concentrations. For the majority of experiments fish were exposed to the chemicals via constant flow, water-borne introduction, with the use of appropriate solvent carriers if necessary. Where resources allowed, chemical determination of the exposure concentrations were made. Where certain responses or crosstalk relationships were apparent additional experiments using a second, and less resource intensive, exposure method, intraperitoneal injection in a benign oil carrier, were conducted to provide further evidence.

The core scientific project objectives were established at the outset as:

1. Develop a priority list of known MFO inducers and endocrine disruptors that are of greatest concern in the estuarine and nearshore environment.

2. Characterise the responses which the main MFO inducers and endocrine disruptors induce in the flounder, *Platichthys flesus,* on an individual basis with respect to dose and exposure time.

3. Investigate levels of interaction with respect to measured responses of MFO inducers and endocrine disruptors (EROD activity and plasma VTG respectively) when fish are exposed to simple combinations of the two.

4. Apply results of system interaction experiments to the interpretation of field collected data.

In order to address these objectives 25 experiments were conducted throughout the project duration. A summary of all experiments is shown in Table 1.

3. MATERIALS AND METHODS

Experimental Set Up
All dosing studies were carried out in a constant temperature facility using temperature controlled seawater at 12°C (±1°C). Experimental rigs were set up enabling a maximum of 18 tanks to be used in any given test. Seawater was supplied to each 50 litre tank via an individually controlled flow-through set-up. Continuous aeration was provided to each tank using 50mm airstones.

Chemicals
Dibenz [a,h] anthracene, (DbA) 97%,
Benzo[k]fluoranthene (BkF) 98%,
β-Naphthoflavone, (BNF),
Benzo[a]pyrene, (BaP) 97%,
17-α Ethynylestradiol, (EE2) 98% and
β-Estradiol (E2) 97%, from Sigma-Aldrich

Aroclor 1254, (Aroclor), Monsanto.
4-Nonylphenol technical mix (NP) 85%, Fluka
Tetrabromobisphenol A (TBBP-A) as Saytex CP-2500, and
Hexabromocyclododecane (HBCD) as Saytex HP-900 from Abermarle

Acetone, Glass Distilled Grade, Rathburns
Methanol, Glass Distilled Grade, Rathburns
Ethanol, HPLC grade, Rathburns
Corn Oil

Fish
At the start of the project, the flounder used were mainly wild fish trawled from the River Alde in Suffolk, UK which is known to be free from significant contamination. Early in the study (Test A1140-6) captive bred flounder from the Port Erin Marine Hatchery on the Isle of Man were used. Before tests were initiated, each batch of fish was acclimated for several weeks in 1200 litre holding tanks with a flow through supply of filtered seawater and continuous aeration. They were fed Nutramarine or Europa pellet food from Skrettings, formerly Trouw Aquaculture (Wincham, Northwich, Cheshire, UK)

Water Borne Exposures
Dosing stocks of chemicals were prepared, dispensed into amber glass bottles and placed on magnetic stirrer plates on the testing rig. Peristaltic pumps (series 200, Watson Marlow Bredel, Falmouth, Cornwall, UK) were used to transfer the dosing stocks at a controlled rate into the incoming seawater via ptfe tubing. The stocks mixed with the inflowing seawater before reaching the tank.

Stocks were dissolved in de-ionised water, methanol or acetone as appropriate. The stock flow rate and seawater flow rate were adjusted such that the any solvent concentration in the tanks was at a concentration of 0.1 ml / litre. Dosing was checked daily by weight change (twice daily for the less soluble compounds) and adjusted where necessary to maintain the appropriate dose.

Chemical analysis of the tank contents was carried out for each 10 day test. 1 litre samples of water were taken from each tank and liquid/liquid extracted with dichloromethane (DCM) using silanised glass vessels throughout the procedure so as to prevent or minimise adsorption of the test chemicals to the glass surfaces.

Fish were exposed for 10 days and were not fed during that time. This was to allow bile volume to increase and to minimise loss of dosed chemicals via adsorption to organic particulates in the water.

Intraperitoneal (IP) Injection Exposures
Dosing stocks were made up in corn oil. Stocks were thoroughly mixed using a combination of ultrasonic and vortex mixing. Concentrations were calculated to use 2 ml of corn oil per kilogram of fish weight.

Fish were weighed and the volume of dosing stock was calculated. This was then injected into the peritoneal cavity using a syringe and hypodermic needle.

Fish were placed into 50 litre tanks and kept for 5 days under flow through conditions with continual aeration.

Sampling
Fish were taken from their tank and a blood sample was taken from the caudal vein using a heparinised syringe. Samples were transferred to a 1.5 ml Eppendorf vial and centrifuged using a refrigerated centrifuge (Hettich Mikro 22R) at 10,000 rpm and 4°C for 5 minutes. The plasma was drawn off for VTG analysis using a fine-tip pipette and stored at –80°C.

Fish were killed by a cephalic blow according to UK Home Office guidelines and the length and weight were recorded.

The liver was excised and weighed for determination of the somatic index. Samples of liver tissue were taken for EROD analysis and frozen immediately at –80°C.

Bile samples, or complete gall bladders were taken for metabolite analysis and frozen at –80°C.

Gonads were removed and weighed for determination of the somatic index.

Radioimmunoassay procedure for the determination of oestradiol (E_2)and Ethinyloestradiol (EE_2)
Make a 0.5M; pH 7.6 sodium phosphate buffer by dissolving 115g Na_2HPO_4 (anhydric) and 29.6g $NaH_2PO_4.2H_2O$ in 2 litres of distilled water.

Assay buffer is made up when needed and consists of:

50 ml 0.5M buffer
450 ml water
1 g bovine albumin (Sigma A-4503)
4 g Sodium chloride
0.15 g EDTA acid
0.05 g Sodium azide.

Antisera are frozen, without dilution, in 100µl aliquots. A stock solution is made by diluting 100 µl of antiserum with 10 ml of 0.05M phosphate buffer and 0.05g of sodium azide.

The antiserum to E_2 (510/5) was a gift from Dr Barry Furr of ICI (1977).

The EE_2 antiserum (292/2) was raised in-house.

Radiolabels
[2,4,6,7,16,17-3H]-oestradiol; TRK587 from Amersham BioSciences or
[17α-[6,7-3H(N)]-Ethynylestradiol; NET-462 from Perkin Elmer Life Sciences Inc
Stored at –20 C. The stock solution is made by diluting 50 µCi in 3 ml ethanol

Standard
Standards are stored at a concentration of 500 µg per ml in ethanol.

Assay
The assay involves addition of 100 µl of each sample to duplicate tubes.
Serial dilutions of standard are made using assay buffer in a range of 9 standard concentrations from 500 pg per 100µl to 2 pg per 100 µl.

The reagent is then made by addition of:

- 18 ml assay buffer
- Radioactive stock label (the volume being chosen to produce a working dilution of 5000 dpm/100 µl).
- Antiserum stock (the volume being chosen to give a maximum binding of 50%).

E_2 assay: Buffer, 18 ml; radiolabel, 25 µl; antiserum, 80 µl.
EE_2 assay: Buffer, 18 ml; radiolabel, 40 µl; antiserum, 50 µl.

After addition of the antiserum, 100 µl of the working solution is added to all tubes. The rack of tubes is covered with foil and placed in the fridge overnight.

A charcoal suspension is made by dissolving 0.5 g gelatin powder in warm distilled water. Add 50ml 0.5M phosphate buffer and make up to 500 ml with distilled water. Add 2.5 g of Norit charcoal and 0.5 g of Dextran T70. Shake, then refrigerate overnight.

The next morning, embed the rack of tubes in crushed ice and dispense 1 ml of ice-cold charcoal suspension to all tubes except those designated as 'totals'. The total tubes are given 1 ml of assay buffer. After 25-30 minutes, load the tubes into centrifuge racks and spin at 1000 g for 12 minutes. Pour the contents into a polyethylene scintillation vial and add 7 ml of Optiphase 'Hisafe II' from Fisher Scientific. The vials are then counted.

The same procedure is used for all steroid radioimmunoassays.

Yeast Assay for oestrogen receptor agonist potency
Some extracted tank-water samples were tested for oestrogenic activity using a yeast-based oestrogen screen (YES) using the method of Routledge and Sumpter (1996).

Samples were added to the 96 well microtitration test plate at a range of concentrations and then allowed to evaporate to dryness at room temperature. An assay medium consisting of the chromogenic substrate chlorophenol red β-galactopyranoside (CPRG) and a growth medium inoculated with yeast cells, was added to the plate. The plate was incubated for 3 days at 32 °C and was shaken vigorously for 2 minutes each day. On the third day any change in the colour of the CPRG was read colourimetrically using a UV/ VIS plate-reader (Bio-Tek instruments, Inc.) at an absorbtion wavelength of 540 nm for colour and 620 nm for turbidity. A dilution series of 17β-oestradiol (E2) (as a positive control and oestrogenic standard) together with a solvent blank, were assayed alongside each batch of samples.

The oestrogenic activity of each sample was determined by comparing responses with that of the E2 standard.

PCB Analysis
Tank extracts for the Aroclor 1254 experiment were analysed using the methods described by Allchin *et el* (1989).

GC/MS Determination for PAHs
PAHs in tank extracts were analysed according the the methods specified in Kelly *et al*., 2000. Aliquots of the extracts (1 ìl) were analysed using a column coated with 5% phenyl methyl silicone fluid (0.25ìm film thickness). The carrier gas was helium. Injection was made via a split/splitless injector operated in splitless mode. The GC column was directly coupled to the ion source of a GCQ ion trap mass spectrometer. This was interfaced to and controlled by a data system using XCalibur software running under Windows NT. The mass spectrometer was operated in full scan electron-impact (EI) ionisation mode, and scans from 35 to 325 Daltons. A cycle time of 1 s was used to ensure an adequate number of data points across fast eluting capillary GC peaks.

Quantification of PAH was carried out using deuterated analogues of the parent PAH as internal standards, and experimentally determined response factors.

EROD Activity Analysis
Homogenate preparation
Liver samples were stored in an -80^{o}C freezer. A 200mg (± 10) sliced sample of liver was homogenised with 1 ml of ice cold homogenising buffer (100mM K_2HPO_4/KH_2PO_4 pH 7.5, 1mM EDTA, 1mM dithiothrietol, 150mM KCL) using 6 strokes of a Potter-Elvehjem automatic homogeniser set at 4000rpm. The homogenate was transferred to a 1.5 ml polyethane Eppendorf vial and centrifuged at 10,000g for 20 minutes at 4^{o}C. Supernatants were removed and stored on ice.

EROD activity determination
EROD activity was determined using the standard ICES method (Stagg *et al*., 1995). A Perkin Elmer LS50B fluorescence spectrometer set at 535 nm excitation and 580 nm emission wavelengths with a cuvette stirring function was used. Assay reagents were kept at 20^{o}C (±1) in a water bath to maintain assay temperature. The final reaction mixture (2ml volume) consisted of 1.96ml assay buffer (100mM K_2HPO_4/ KH_2PO_4 pH 7.5, 100mM KCL), 20ul liver supernatant, 10ul ethoxyresorufin substrate (0.2nM in dimethylsulphoxide) and 10ul resorufin internal standard 3.125ìM, adjusted by absorbance to allow for variation in the percentage purity of resorufin stock, in pH 8.0 phosphate buffer.

The reaction was initiated by the addition of 10ul NADPH (0.25mM), fluorescence emission readings were taken at 0 and 60 seconds. EROD activity was normalised to protein content, which was determined using a plate reader modification of the Bradford method (1976) using a bovine serum albumin standard.

VTG Analysis
VTG was determined by an ELISA method as described by Scott and Hylland (2002). Polystyrene plates were coated with 100ìl of diluted VTG coating buffer (VTG standard with carbonate buffer at 1:1000). These plates were then stored in wet paper at 4^{o}C overnight.

Plasma samples and standards (high standard: 1:20 and Low standard 1:100, VTG standard in assay buffer) were incubated with primary antiserum. This was prepared by the formation of a set of ten fold dilutions of all samples and standards, followed by the addition of 135ìl of assay buffer to each well of the polypropylene plates. 65ìl of primary antiserum (diluted 1:10,000 in assay buffer) was added to each well containing sample and standard dilutions. The plates were then shaken for 4 minutes on a plate shaker, wrapped in wet paper and incubated over night at 4^{o}C.

On day 2 the incubations were transferred to the polystyrene plates which had been washed with washing buffer using an automated plate-washer. Samples and standards (150ìl) were transferred from the polypropylene plates to the corresponding well of the polystyrene plates. Plates were then incubated in wet paper for 4-6hr at room temperature. Following incubation the cells were rinsed with washing buffer and secondary antiserum (150ìl, diluted 1:15,000 in assay buffer) added to each of the wells. The plates were incubated at 4^{o}C overnight in wet paper.

On day 3 the plates were washed with distilled water and 150ìl p-NPP added to each well and colour allowed to develop, the plates were read using a microplate reader set at an absorption wavelength of 405nm.

VTG concentrations were calculated from the dilution of each plasma sample that gave a reading closest to the most accurate part of the standard curve (the mid range). Once the concetrations had been determined, all samples which gave a reading below 0.5 ìg/ml were reassayed using a more sensitive ELISA procedure. This was the same as described above but using a coating buffer one fifth the concentration, antiserum one seventh the concentration and the starting concentration of the two standards at 10 and 2ìg/ml. The limit of quantification for the assay was established as 0.2ìg/ml, equivalent to 1:100 dilution of plasma.

cDNA Microarray Analysis

Preliminary gene expression studies of a subset of the BREEDIF flounder liver samples were carried out using a European flounder cDNA microarray developed for the EU-funded GENIPOL project.

The clone set used to construct this array was derived from a variety of sources –

1) Approx. 12,000 clones isolated from a normalised "induced" European flounder liver cDNA library constructed in lambda TriplEx2 vector (BD Bioscience UK). "Induced" libraries were produced by pooling RNA samples extracted from fish tissues 48 hours and 72 hours post-treatment with 3-MC, Arochlor 1254, E2, 3-methyltestosterone, PFOA, Cd , PCN and tBHP. Although normalized, some redundancy is still present amongst this clone set.

2) 333 distinct clones isolated by PCR-Select (BD Bioscience UK) Suppressive, Subtractive, Hybridisation (SSH) from cadmium, benzo[*a*]pyrene or environmentally exposed *P.flesus* as described previously by Sheader *et al.*, (2004).

3) 133 distinct clones from *P. flesus* corresponding to toxicologically relevant genes isolated by means of degenerate PCR (Williams *et al.,* 2003).

4) 14 clones from the plaice (*P. platessa*) and 17 clones from the Winter flounder (*P. americanus*) were obtained as the kind gifts of Dr S. George (University of Stirling, UK) and Dr W. Baldwin (University of Texas, USA) respectively.

PCR products derived from the above clones were purified and printed in duplicate on GAPSII (Corning) slides at The University of Birmingham Functional Genomics Laboratory according to the protocol described by Williams *et al.,* (2003).

All array experiments employed the reference cDNA technique. This has advantages in comparison to standard two colour experiments, which are described in detail by Dudley *et al.*, (2002). The reference cDNA was made by combining PCR amplicons (1:1:1 by concentration) amplified from no-insert pTriplEx2, pCR2.1 and pBluescript plasmids.

Messenger RNA was prepared from *P.flesus* liver samples using the Poly-Attract 1000 system (Promega). SuperscriptII reverse transcriptase (Invitrogen) and random primers (Alta Bioscience) were used to synthesise cDNA. Reference and *P.flesus* derived cDNA was labelled with the fluorophores Cy3-dCTP and Cy5-dCTP (Amersham Bioscience) respectively using the Bioprime random priming kit (Invitrogen) with non-biotinylated dNTPs. Labelled cDNA was purified with a QIA-quick spin column (Qiagen); the amount of dye incorporated into each cDNA sample was determined by spectrophotometry at 550nm (Cy3) and 650nm (Cy5). Fifteen picomoles Cy3 and eighty picomoles Cy5 labelled cDNA were mixed for use in each hybridisation. All slide hybridisations, wash steps and scanning were conducted as described by Williams *et al.,* (2003). One sample from each animal was arrayed separately against a reference sample, with three animals of each sex per exposure group being analysed. Fish were selected from experiment A1140-11 and represented control, DbA only, EE2 only and a DbA/EE2 mix treatments.

All data normalisation and analysis was carried out using the Genespring software package (Silicon Genetics). The signal intensity (determined at a wavelength of 635nm) of each gene was divided by its control channel value (determined at 532nm) in each sample. Any feature with signal intensities below the threshold calculated by the cross-gene error model component of the Genespring software was discarded. The resulting ratio was divided by the mean ratio for all features for a given gene on all arrays, including replicates (per-gene normalisation). The ratio for each gene was then divided by the mean ratio for all features on that array (per-chip normalisation). Median polishing was performed to bring the medians for each normalisation step into concordance (Silicon Genetics). The result of this procedure was that each normalised spot ratio represented an expression level relative to a value of one.

Each data set was put through successive filter steps in order to remove low-trust, and non-differentially expressed genes. The first filter removed all data points for which the recorded raw fluorescence intensities were small and therefore of low trust. The cut-off fluorescence intensity value used in this filter was calculated using the cross-gene error model component of the Genespring software package. A second filter was then performed on normalised data to remove non-differentially expressed genes from the data-set. Any gene for which all treatments showed a fold change in expression less than two standard deviations from the mean expression value for that gene for entire experiment was removed.

Statistically significant differences were determined using a parametric Welch *t*-test using global error model variances derived by combining measurement variation and inter-sample variation for array data from the replicates, grouped by treatment (Silicon Genetics). A multiple testing correction (Benjamini and Hochberg, 1995) was used with a *P*-value cut-off of 0.05.

4. RESULTS

Significance
Where significant differences are referred to in the text this means that a comparison of the sets of data using ANOVA found a value for p to be <0.05.

Data Correlations
Several parameters were recorded for each individual fish used in the tests (length, weight, sex, liver weight, gonad weight, GSI, HSI, EROD and VTG). Each parameter was tested for strong correlations with any of the other parameters from fish in the same tank / test regime. Most tanks of fish showed strong correlations, as expected, between the following sets of data:

Length / Weight,
Weight / Liver Weight,
Gonad Weight / GSI,
Liver Weight/ HSI and
GSI / Sex.

Several other strong correlations were noted, but these were isolated cases which were not found across replicates, or in fish exposed to the same chemical in a different test. No detectable trends could be uncovered.

EROD inducers
A number of experiments were run using both known and suspected EROD inducers in order to characterise both the concentration at which, and the time period within which, they affected EROD activity in flounder. The two initial experiments examined the responses of flounder from two different sources. These were wild fish from the River Alde (Suffolk, UK), and captive bred fish from a fish farm (Port Erin, Isle of Man, UK), to establish which would be the most suitable as a test population. These tests were run using a concentration range of benzo[*a*]pyrene (BaP), a PAH known to exert a strong effect on EROD activity in several fish species (Maria *et al* 2002, Sandvik *et al*., 1998, Telli-karakoc *et al.*, 2002). Tests were run over 21 days and samples were taken at day 21 only. The EROD responses noted in these tests were not particularly

high but it was possible to establish that the farmed fish exhibited a higher response than the wild population. While the results from the first two tests were being analysed, a third test was run to establish the change in EROD activity over time using a nominal concentration range of BaP from 0.01 to 10 µg/l. The response from this test was compared with a similar test of VTG induction over time (A1140-4). From the results of these two tests, an optimum test time of ten days was established for assessing both responses in a binary exposure experiment.

The induction of EROD by BaP in these tests was fairly low, even at a nominal concentration of 10 µg/l.

In test 5 we tested three further PAHs using the established 10 day test protocol. One of these substances was β-Naphthoflavone (BNF). This was chosen as a positive control because it is a powerful EROD inducer although it has little environmental relevance. BNF was tested at a nominal concentration of 10 µg/l on fish from both sources and produced very high EROD activity particularly in the Port Erin fish. Samples taken at day six and day ten of test 5 showed a peak response at day 6, with a lower but still significantly elevated activity by day 10 (Figure 1).

This test also investigated two further PAHs, dibenz[*a*,*h*]anthracene (DbA) (Figure 2) and benzo[*k*]fluoranthene (BkF) (Figure 3) which were suspected to be significant contributors to PAH activity in environmental samples with high EROD inducing properties. These compounds were dosed at nominal concentrations of 10 and 50 µg/l and fish were sampled on days 6 and 9.

At day 6, BkF dosed fish showed a rise in EROD activity when compared with the solvent control fish although this rise was only significant in the 50 µg/l concentration tank. The magnitude of the response was reduced after day 6, but it was significantly higher in both concentrations at day 9 than that observed in the solvent control tank.

DbA also produced a significant elevation in EROD activity at 10 µg/l by day 9, and in the 50 µg/l concentration on both sampling days. The rise in EROD activity was higher than that shown for BkF and was maintained until the end of the test.

Test 7 investigated two flame retardant chemicals for their potential as EROD inducers, hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBP-A). The range-finding study was carried out with concentrations reaching up to the mg/l range. No significant effects were observed for either chemical on EROD levels, although at very high concentrations indications of an inhibitory and inducing potential were evident for HBCD and TBBP-A respectively (Figure 4).

The last individual EROD inducer that we examined was the commercial PCB formulation Aroclor 1254. PCBs are well known persistent organic pollutants (POPs) with an established scientific history of EROD inducing effects (Yawetz *et al* 1998, Brumley *et al* 1995, Straus *et al* 2000). In test 13, we dosed Aroclor 1254 in a nominal concentration range from 3.3 µg/l to 333 µg/l dissolved in methanol. Two tanks were dosed with 100 µg/l, one dissolved in methanol as a carrier solvent and the other in acetone. Semi-quantitative chemical analysis carried out on samples taken during the study showed that Aroclor was present in all tanks and that there was a concentration range across the tanks up to a maximum concentration of 227 µg/l. The highest Aroclor concentration was recorded in the tank where acetone was used as the carrier solvent. However, none of the concentrations induced any elevated EROD activity in the fish by day 10 of the test.

A summary of the EROD activity found for the chemicals tested can be seen in Table 2. From this series of tests the PAH compound DbA was selected for use as a model environmental EROD inducer in binary mixture exposures. DbA is a xenobiotic contaminant commonly detected in environmental samples and for this reason it was preferred over the more potent, but less environmentally relevant β-naphthoflavone.

VTG induceing studies
Early experiments focussed on the need to establish a suitable time period and EE2 concentration for the first mixture studies, so a time series experiment for EE2 in methanol was conducted. Wild flounder from the River Alde were exposed to 1, 10 and 100 ng/l EE2 for 14 days. Blood was taken on days 9 and 14. At 1 ng/l E2 exposure there was no significant increase in VTG induction compared to the control on either day 9 or 14. At 10 ng/l EE2 there was a significant induction of VTG, and this was also true for 100 ng/l EE2 at day 9 and 14. (Figure 5). A standard concentration for use in mixture exposures was selected as 20 ng/l EE2, and 10 days as a suitable exposure period.

This experiment was repeated using farmed fish. Blood samples were taken at 6 and 10 days, and EE2 concentrations used were 20 and 50 ng/l EE2. There was a significant induction of VTG at both 6 and 10 days for both concentrations. 50 ng/l EE2 gave significantly higher VTG response than 20 ng/l EE2. Therefore a concentration of 20 ng/l EE2 over a 10 day exposure would also be suitable for farmed fish used in future experiments.

In experiment 16 flounder were exposed to 10, 33, 100, 330 and 1000 ng/l E2. No significant VTG induction was observed at concentrations of 100 ng/l or below. A significant induction of VTG occurred at both 333 ng/l and 1000 ng/l E2. At 10 days, a slightly higher mean VTG concentration was observed compared to day 6 but the difference was not significant (Figure 6). From these results the E2 concentration selected for use in future mixture experiments was 200 ng/l.

In experiment 8 flounder were exposed to a technical mixture of nonylphenol isomers in order to establish a suitable time period and dose for exposure. In this experiment, fish were exposed to 33, 100 and 330 µg/l NP. There was no significant response at 33 µg/l NP. The 330 µg/l NP exposure showed a significantly higher response compared to the control, however there was also some induction at 100 µg/l. Therefore a 100 µg/l NP over a 10 day exposure was used as a standard VTG response concentration. (Figure 7).

In order to establish suitable concentrations of VTG inducing chemicals by intraperitoneal injection, flounder were injected with a range of concentrations of E2, EE2 and NP. Over a range of 0.01 - 0.03 mg/kg E2 there was an increase in VTG induction (Figure 8). Although very low VTG was observed at 1 mg/kg E2, this result was from only one fish and it was considered that this was not representative of the true degree of VTG induction. A concentration of 1mg/kg E2 was therefore chosen as the standard concentration for VTG response as at this concentration the response was still increasing which gave the greatest scope for demonstrating both up and down regulation.

IP dosed EE2 also yielded an increase in VTG induction over the concentration range 0.0033 mg/kg – 1 mg/kg EE2. A concentration of 0.33 mg/kg EE2 was selected as the standard concentration for VTG response in the following binary dose experiment, as this concentration allowed for the observation of both up and down regulation (Figure 9).

NP gave a significantly lower VTG response than either E2 or EE2, however an increase was still observed over the dose range 1-100 mg/kg NP (Figure 10). Therefore it was decided that 100 mg/kg NP would be used as the standard VTG inducing response for use in mixture experiments.

A summary of the VTG activity observed for these chemicals can be seen in Table 3.

Binary Mixtures

DbA and EE2 Mixtures
The first simple binary mixture exposure was conducted as test 6 and involved BNF, DbA and EE2. The test involved single contaminant tanks of BNF (1 µg/l), DbA (40 µg/l) and EE2 (20 ng/l) and mixture tanks dosed with BNF / EE2 and DbA / EE2.

Although DbA and BNF were detected in water samples from the tanks, the EROD response was low across all tanks. There were significant differences in the mixture tanks compared to the single dose PAH tanks. These showed EROD response to DbA to be up-regulated by EE2, whilst the response to BNF was down regulated by EE2 (Figure 11). Although significant, the overall range of the results was too low to allow any conclusions to be drawn.

The expected VTG response to EE2 was also not seen in any of the tanks, and further tests were carried out to establish the cause. Test 11 was a repeat of test 6 and produced very good VTG induction in both the EE2 and the mixture tanks (Figure 12).

Although dosed at nominal concentrations that should have produced a response, the DbA and BNF failed to stimulate the EROD activity to levels which allowed up or down regulation to be measured. These substances caused elevation of EROD activity in subsequent tests which suggests that the dose delivery system was at fault.

Test 14 was a binary mixture test using DbA and EE2. There were several dosing problems encountered during this test that led to variable EROD and VTG induction between replicate treatments. Interpretation was, therefore, impossible so the test was repeated as test 15. The tank dosing went according to protocol in this test, with chemical analysis showing near nominal concentrations of DbA in all the appropriate tanks. All the EE2 dosed tanks in this test showed a high VTG induction with no obvious variations between tanks (Figure 13). The EROD activity showed a significant rise in all the tanks containing DbA, but the response in tanks dosed with the mixture of DbA and EE2 was significantly down regulated when compared to the DbA alone (Figure 14).

Test 17 was designed to study the effects of varying the cocnentration of EE2 in the mixture whilst maintaining the same concentration of DbA. DbA was again dosed at a nominal 40µg/l concentration in all mixture tanks and when dosed alone. EE2 was dosed at six concentrations ranging from 0.2 ng/l to 66 ng/l.

VTG was induced at concentrations of 20 ng/l and 66 ng/l EE2 with no response apparent at lower concentrations (Figure 15). EROD was induced in all tanks dosed with DbA, to activity levels significantly higher than those of the solvent control tanks. This elevated EROD response was significantly down-regulated in the 20ng/l and 66ng/l EE2 mixtures when compared to tanks where DbA was dosed on its own (Figure 16).

IP Injection of DbA and EE2 Mixtures

The same mixtures were tested using IP injection techniques to find out whether the water-borne tank test results were repeatable using this technique. An initial range finding test suggested that a DbA loading of 30 mg/kg would give a similar response to the waterborne exposure of 40 µg/l. The same test investigated VTG response to IP injection of EE2 and indicated that VTG induction similar to the 20 ng/l water exposure would be achieved by a loading of between 0.1 and 0.33 mg/kg.

In test 24, the fish were dosed via IP injection either with DbA on its own, or with a mixture of DbA and EE2. Earlier water-borne dosing tests undertaken within this project had shown a potential for other steroids or steroid mimics to affect the EROD activity at concentrations below those that affected VTG induction. To investigate this using IP dosing, EE2 was injected at six concentrations ranging from 0.0033 mg/kg to 1 mg/kg. This covered a range from definite VTG induction at the highest dose, down to two orders of magnitude below the dose at which VTG should be induced.

The results showed that VTG induction in the mixtures began at an EE2 dose of 0.01 mg/kg, although similar levels to the 20 ng/l water dose weren't reached until the 0.1 mg/kg IP dose. The 1 mg/kg dose had a minimal effect on VTG induction, possibly due to a problem with the IP injection process. The results from this dose were not considered subsequently.

The EROD results were more difficult to interpret, but showed a high level of induction with the 30 mg/kg dose of DbA. This was down regulated in mixtures with the three highest EE2 doses (0.033, 0.1 and 3.3 mg/kg). The results were less clear in mixtures with lower EE2 doses. The 0.01mg/kg dose showed no significant effect on EROD induction, whilst the lowest dose of 0.0033 mg/kg appeared to cause a significant down-regulation compared to the DbA alone (Figure 17). It is not possible from these data to pinpoint the lowest concentration of EE2 that causes a down regulation of the EROD response.

DbA and E2 Mixtures

Flounder were exposed to DBA and E2 in a waterborne experiment to investigate whether E2 causes a down regulation in EROD when exposed to E2 with DBA compared to DBA alone. It was discovered that when flounder were exposed to DbA alone, the EROD activity was raised significantly compared to all controls. When exposed to DbA and E2 the EROD activity was significantly reduced compared to that of DbA alone (Figure 18). VTG levels were also analysed in this test, and it was found that there was no significant difference between flounder exposed to E2 alone and those exposed to DbA and E2 together (Figure 19). Chemical analysis of water taken from each of the tanks showed some variation in DbA concentrations. However, dosing in these tanks was considered to be good and it is thought that the variation may be due to the extraction process (Figure 20). No correlations between EROD, VTG and somatic indices were observed in any of the treatments.

As a result of this experiment a further test was conducted to identify the threshold concentration at which E2 down regulated EROD activity. Flounder were exposed to DbA at 40 µg/l and E2 concentrations ranging from 2 – 660 ng/l. The DbA alone treatment significantly raised the EROD activity above that of the control. Over the DbA/E2 treatment range, a significant reduction in EROD activity occurred at the lowest E2 concentration (2 ng/l) (Figure 21). VTG analysis showed expected values for VTG induction over the E2 concentration range. However, a significant difference (compared to the control) is not observed until a concentration of 200 ng/l E2. This indicates that E2 causes a significant decrease in EROD levels when combined with DbA at concentrations 100 times lower than those at which significant VTG induction occurs.

In order to confirm the above result a repeat experiment was run using lower concentrations of E2 than in the earlier test. Flounder were exposed to DbA at 40 µg/l and E2 at concentrations ranging from 0.5 ng/l – 150 ng/l E2. Again, the DbA treatment alone significantly raised the EROD activity above that of the control. EROD analysis showed that there was no significant decrease in EROD activity when flounder were exposed to 0.5 ng/l E2 combined with DbA. However, as seen in the previous experiment, a significant decrease in EROD activity compared to DbA alone was seen when fish were exposed to 2 ng/l E2 and DbA together (Figure 22). VTG analysis showed no significant increase in VTG response at concentrations of 20 ng/l or lower, but a significant response at 150 ng/l E2, again demonstrating that E2 causes a significant decrease in EROD activity at concentrations considerably lower than those at which VTG induction occurs. In this experiment there were no correlations observed between VTG and EROD and somatic indices in any of the treatments.

Once this result had been confirmed in water borne experiments, Intraperitoneal injection experiments were used to investigate whether the same outcome would be observed. Flounder were injected with DbA and E2 at a range concentrations (0.0033-1 mg/kg). When injected with DbA alone, EROD levels were significantly higher than in the control tank. Injecting the flounder with 0.0033 mg/kg E2 and DbA did not significantly reduce the EROD activity compared to DbA alone (Figure 23). A significant decrease was observed at and above a concentration of 0.01 mg/kg E2 mixed with DbA compared to DbA alone (Figure 23). VTG analysis showed significant VTG induction at 0.01 mg/kg, the same concentration at which a reduction in EROD activity was observed (Figure 24). This is in contrast to the waterborne experiments which showed a down regulation of EROD at considerably lower levels than those which induce VTG.

DbA and Nonlyphenol mixtures

An experiment was run in order to investigate the potential of NP to down regulate EROD induction in flounder. 100 µg/l NP was used as the standard concentration for tests of VTG induction (Figure 7). Flounder were exposed to 40 µg/l DbA alone, 100 µg/l NP alone, and a mixture of both chemicals. Controls (seawater, acetone only, methanol only and acetone/methanol mix) were run to confirm that neither solvent affected the VTG or EROD response. It was found that there were no significant differences between seawater and solvent controls. When exposed to DbA alone, flounder showed a significantly higher response than those in the acetone control tank. When exposed to 100 µg/l NP and DbA there was no significant difference between the treatment and DbA alone (Figure 25) suggesting that NP does not down regulate EROD at concentrations similar to those that induce VTG.

To further establish whether NP has the ability to down regulate EROD a second experiment was conducted. This time flounder were exposed to a standard DbA concentration and a range of NP concentrations (10, 33, 66, 100, 200 and 400 µg/l). Fish exposed to DbA alone had EROD levels that were significantly higher than those in the control. When exposed to a mixture of NP and DbA a significant decrease in EROD activity was observed at 100 µg/l NP (Figure 26). VTG analysis for fish from this experiment showed that significant induction of VTG occurred at 200 µg/l NP, indicating that down regulation of EROD in the presence of NP occurs at concentrations lower than those at which VTG is induced. VTG analysis showed no induction of VTG at 100 µg/l NP, in contrast to previous tests which showed VTG induction at 100 µg/l NP (Figure 7). Therefore it may be possible that a concentration of 100 µg/l is the threshold at which VTG induction occurs and so small variations in the actual dosing concentration could have a large affect on VTG induction. However, chemical analysis has shown that the NP concentrations achieved in the tanks were much lower than the nominal concentrations. This may also explain the variation in results and the non-induction of VTG at 100 µg/l. From these experiments, it is unclear whether down regulation of EROD occurs prior to the induction of VTG, but it has been shown that down regulation of EROD by NP occurs in the concentration range 66-100 µg/l and that VTG induction occurs between 100-200 µg/l.

An IP experiment was used to test whether flounder EROD levels responded in the same way as they did in the waterborne experiment. Flounder were injected with DbA alone and with DbA mixed with a range of NP concentrations (10, 100 and 500 mg/kg). Injection with DbA produced an EROD response significantly higher than the control. When injected with DbA/NP mixtures both a significant down regulation of EROD and a significant induction of VTG occurred at 100 mg/kg (Figure 27).

E2 with varying DbA Mixtures

In a further investigative test using IP injections, flounder were dosed with a standard concentration of E2 (1 mg/kg) and a range of DbA concentrations (1, 30 and 100 mg/kg). VTG analysis showed that significant induction of VTG occurred at 1 mg/kg E2, and that there was no significant difference between the induction found for 1 mg/kg E2 alone and that found for any of the mixtures.

As expected 30 mg/kg DbA caused a significant increase in EROD activity compared to that observed in the control. The 1 mg/kg DbA concentration in combination with 1 mg/kg E2, produced no significant increase in EROD activity compared to the control. When injected with 1 mg/kg E2 and 30 mg/kg DbA the flounders showed a significant increase in EROD compared to the control and a significant decrease in EROD compared to the 30 mg/kg DbA alone. This pattern was repeated for the 1 mg/kg E2 and 100 mg/kg DbA mixture (Figure 28).

cDNA Microarray Results

There were 2,967 clones which showed a differential expression of more than 2-fold when groups of the same sex and treatment were compared with the relevant controls. However, these are not likely all to be statistically significant. After using a Welch t-test with a multiple-testing correction, the expression of 703 clones were found to be significantly different when data were grouped by treatment and compared with all untreated controls. Of these 703 clones, 125 were then sequenced, or had already been sequenced. The rest remain to be identified and will provide further insights into the effects of these treatments.

The 125 sequenced clones were clustered, by gene expression profile, into four main groups-

A) Induced with DbA, not EE2
B) Repressed with treatment
C) Induced with EE2, not DbA
D) Induced with all treatments

These are illustrated in Figure 29.

The induction of recognized biomarker genes was apparent, CYP1A (cytochrome P450 1A) during DbA treatment, vitellogenin and choriogenin (ZPP / ZRP / VEP) during EE2 treatment. The transcript expression levels correlated well with EROD values for CYP1A and with plasma vitellogenin values for vitellogenin mRNA and choriogenin mRNA (Figures 30 and 31).

Group A , those genes generally induced by DbA but not by EE2, including (as well as CYP1A), FABP (Fatty-acid binding protein), BHMT (Betaine-homocysteine methyltransferase), MHC1r (major histocompatibility class 1 receptor), e1B-AP5 (a putative nucleocytoplasmic mRNA transporter) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Group B, those genes generally repressed in comparison with the untreated controls. His group includes cathepsins (lysosomal proteases), transferrin, complement component C3, apolipoprotein H and hepcidin (acute phase proteins), siah-interacting protein and a proteosome component (involved in intracellular protein degradation), a methytransferase, microsomal stress 70 ATPase core precursor, NADH-ubiquinone oxidoreductase (a respiratory enzyme), peroxiredoxin (a thiol-specific antioxidant), a chemotaxin, and cystatin (a protease inhibitor).

Group C, those genes generally induced by EE2 but not DbA, included vitellogenin, choriogenin and TFIIA P12, a transcription initiation factor.

Group D, those genes generally induced by all treatments, containing mostly digestive proteases, such as trypsins, elastases and carboxypeptidases. A transcript similar to high-choriolytic enzyme 1 precursor was also induced, this is another protease, but is more usually associated with involvement in egg development. Additionally induced were; beta-microglobulin, a component of the major histocompatibility complex and beta-globin, ie haemoglobin.

Other transcripts of interest were induced, but not to the level of statistical significance used for this analysis. These included reticulon 1 (an ER membrane protein previously implicated in signalling in neuroendocrine cells) which was induced during EE2 treatment. Induced in response to DbA treatment were a MAP-kinase-associated serine threonine kinase (potentially involved in stress-associated protein kinase cascade), glutathione peroxidase (involved in the response to oxidative stress) and metallothionein (generally associated with heavy metal stress but also seen to respond to various other stressors).

Review of selected field data

CEFAS has generated field monitoring data for hepatic EROD activity in target fish species as part of the National Marine Monitoring Programme (NMMP) for many years. These data have formed an integral part of datasets that have been used both to target further surveys and to inform overview documents such as the State of the Seas Report (currently in preparation). Much of the EROD monitoring data is reported regularly in the CEFAS Aquatic Environment Monitoring Reports (CEFAS, AEMR's Nos.. 51-54).

More recently CEFAS has undertaken extensive surveys of hepatic EROD and male plasma vitellogenin in flounder (*P. flesus*) from UK estuaries (Kirby *et al.,* 2004a; Kirby *et al.,* 2004b). These survey results, from areas of known multi-contaminant inputs, clearly show that many flounder exhibit elevated levels of both EROD activity and plasma VTG. Consequently, it is probable that the fish which make up the samples are experiencing up or down regulation as a result of co-exposure to both EROD inducers and oestrogen mimics.

As part of this study we have made a basic review of these datasets in light of the results gained in this study.

4. DISCUSSION

EROD Inducers: Relative Potency

Several EROD inducers and potential inducers were tested during the course of these experiments and table 2 summarises the concentrations at which they were found to influence EROD activity. BaP and Aroclor 1254, contrary to expectations, both failed to produce any elevation in EROD activity at the concentrations used in these tests. Flounder EROD has been found to be relatively unresponsive to BaP in previous tests at CEFAS, although it was expected that a concentration as high as 10 µg/l would show some increased activity. It was decided to conclude the BaP testing when no response could be determined after testing with concentrations up to 10 µg/l over 21 days. At this concentration the two other PAHs tested, DbA and BkF, were showing significant effects on the EROD over a shorter 10 day test.

Aroclor 1254 is an acknowledged MFO system inducer and has been used in this way in a variety of studies (Brumley *et al*., 1995, Straus et *al*., 2000, Yawetz *et al.,* 1998). The lack of any apparent effect on EROD activity in test 13 appears to indicate either that the flounder are not sensitive to this range of concentrations, or that the test was compromised in some way. Semi-quantitative chemical analysis showed that concentrations of Aroclor 1254 in the tanks ranged up to 227 µg/l. This concentration is towards the higher range of reported environmental levels and it was expected to yield an obvious EROD response. It may be that Aroclor 1254 is not an effective EROD inducer in flounder. Several IP based studies on other species have found an EROD response only at doses of Aroclor 1254 which were significantly higher than the 3.3 mg/kg required for induction by DbA in this study. (Brumley *et al.*, 1995, Hewitt *et al*., 1998, Troxil *et al*., 1997). This may suggest that Aroclor 1254 is a far less potent inducer of EROD activity than we supposed previously.

The brominated flame retardants, HBCD and TBBP-A were included as relatively new chemicals of concern. HBCD showed no up-regulation of EROD activity when the water was dosed up to a nominal concentration of 1 mg/l.

TBBP-A showed a very small but significant rise in EROD activity at 1 mg/l nominal concentration (figure 4). This may well have indicated the lowest concentration of an EROD inducing potential, but at a concentration unlikely to be found in the environment anywhere other than at a point-source pollution incident.

BNF performed as expected (figure 1) and strongly induced EROD activity at 10 µg/l, the sole concentration tested.

DbA and BkF (figures 2 and 3) were found to induce EROD activity at the 10 µg/l concentration but DbA had a much higher response than BkF at the 50 µg/l concentration, which led to its selection as the model EROD inducer for mixtures testing.

From the results found in this study, relative potencies to elevate EROD activity appear to be:

BNF > DbA > BkF > TBBP-A > HBCD
{BaP}
{Aroclor 1254}

After BkF it is not possible to complete the ranking of these chemicals due to the lack of induction found when testing Aroclor 1254 and BaP. We know that TBBP had a response at 1mg/l which was not found in HBCD at the same concentration. BaP and Aroclor 1254 were not tested at this concetration, so we have no data to show whether they are more or less potent than TBBP.

A study by Bols *et al*., (1999) using a rainbow trout liver cell line found BkF > DbA > BaP. A similar study by Fent and Batscher (2002) demonstrated that DbA was more potent than BkF, agreeing with the results from this study.

Oestrogens (VTG Inducers): Relative Potency

There is an acknowledged disparity in the scientific literature between the efficacy of various oestrogens and oestrogen mimics when tested either *in vivo* or *in vitro*. Various studies have shown that *in vitro* tests underestimate the effects found *in vivo* (Hugget *et al*., 2003, Leroy *et al*., 2002, Van den belt *et al*., 2004) whilst other studies show the opposite trend (Segner *et al*., 2003)). In this study we used an *in vivo* assessment of VTG production to assess the concentration of each chemical needed to cause significant elevations in the plasma VTG concentrations. Our water borne tests showed the order of potency to be EE2 > E2 > NP, with EE2 approximately 10 times more potent than E2, whilst NP was >100 times less potent than E2 (figures 5, 6 and 7). This order corresponds to the generally accepted order of oestrogenic potency of these chemicals (Folmar *et al.*, 2002, Petit *et al*., 1999, Segner *et al*., 2003, Van den Belt *et al.*, 2003). The relative potencies that were found in the IP tests were in the same rank order, but EE2 was closer to E2, requiring approximately one third of the dose to stimulate a similar amount of VTG production. NP was at least 100 times less potent than E2, with strong indications that, compared to E2, it may be 2-3 times less potent in the IP tests than it was in the water borne tests. This would place its IP potency against EE2 on a par with the waterborne tests, suggesting that perhaps the E2 exercises a different potency depending on the route of exposure.

Water Borne and Interperitoneal Route

Most of the experiments conducted in this study focused upon the water borne exposure of fish to test chemicals. This was to allow any results obtained to be of greater environmental relevance and to draw some conclusions about how water borne mediated effects in the environment might be modulated in multi-contaminant areas. However, water borne exposures are significantly more expensive and are more prone to technical failure than, for example, intraperitoneal (IP) exposures which are often used to investigate contaminant effects in fish. In light of the apparent modulating effects seen in water borne, binary exposures of MFO inducers and oestrogenic chemicals it was pertinent to investigate these same effects using an intraperitoneal exposure route to confirm the results and to assist in our interpretation.

Exposure to the three oestrogenic chemicals (E2, EE2 and NP) via the IP route confirmed the VTG induction potency ranking established with water borne exposures to flounder (figures 8, 9,and 10). EE2 is the most potent inducer in water borne exposures, being approximately 5-10 times more potent than E2 and ~1000 times more potent than NP. For IP based exposure in a corn oil carrier, EE2 is also the most potent VTG inducer being 2-30 times more potent than E2 and ~1000 times more than NP.

The similarity in potency ranking for water borne and IP exposure routes was not unexpected and suggests that IP is an appropriate method to deploy when screening for possible effects and/or potency of oestrogens in order to direct and design more environmentally realistic studies. Interestingly, the IP exposure route was also able to differentiate between the different oestrogens on the basis of their ability to inhibit DbA induced EROD activity in flounder. In water borne experiments, E2 was found to be approximately 10 times more potent than EE2 and 50,000 times more potent than NP in its ability to inhibit EROD activity. Again, the IP route reflected this, showing E2 to be approximately 15 times more active that EE2 whilst 4-NP was 10,000 times more active. Due to the small number of IP experiments conducted, these results need to be treated with caution but it is apparent that, for this relatively small selection of chemicals, IP exposure generally reflects the effects seen under waterborne exposure conditions. Furthermore, the use of the second exposure route confirms the down regulating ability of oestrogenic chemicals on the activity of the MFO system and bears out the discovery that E2 (figure 23), unlike EE2 (figure 17) and NP(figure 27), is significantly more potent in reducing MFO activity than in inducing VTG production. The IP approach did not suggest that EROD inhibition would occur at substantially lower concentrations than VTG induction. Via the IP route the down regulation of EROD occurred at doses between 1 and 33 times lower than VTG induction. In water borne exposures EROD inhibition was apparent at concentrations up to 75 times lower than those causing VTG induction. Therefore, for greater confidence in the results and their ability to be applied to environmentally relevant scenarios it is recommended that IP exposure is only used as a screening test.

Mixture Experiments: Suppression Levels

Each of the oestrogenic chemicals studied has demonstrated the ability to downregulate DbA mediated EROD activity. Furthermore, and especially for E2 and EE2, the down regulation threshold concentration is similar to the concentrations currently found in some sewage effluents. Concentrations of oestrogenic chemicals in water bodies receiving oestrogenically contaminated effluents are likely to be much lower than these thresholds, due to subsequent dilution. However, monitoring data suggest that high plasma VTG concentrations occur in fish inhabiting waters with low water-borne contaminant levels (Kirby *et al*. 2004b). The responses are probably due to cumulative exposure from all routes including sediment contact and diet and, therefore, we can expect the same cumulative exposure mechanism to result in concentrations which can impact the induction of the MFO system.

Significantly, the established oestrogenic potency of EE2 > E2 >NP, reconfirmed in this study, was not reflected in their ability to inhibit the MFO system. In terms of DbA mediated EROD activity suppression the potency ranking was E2 > EE2 > NP. In general the EROD inhibition threshold for EE2 and NP occurred at around the same concentration as that required to elicit VTG production in male flounder. However, for E2, EROD activity was suppressed at water borne concentration much lower than those required for VTG induction. This suggests that, in terms of MFO system crosstalk, the natural oestrogen E2 works in a different manner to that of its man made counterparts (oestrogen mimics).

For EE2, 20 ng/1 exposure over 10 days resulted in a 50% reduction in EROD activity, with a concentration of over 66 ng/l required to inhibit activity completely. For E2 the results from a range of experiments showed some variation. An early experiment (Figure 18) suggested that concentrations as high as 200 ng/l E2 caused about a 50% reduction in EROD activity. However, this experiment used only one concentration and later studies (Figures 21 and 22) suggested that EROD inhibition was occurring at concentrations as low as 2 ng/l. In experiment A1140-19 (figure 21) complete inhibition of EROD was apparent at 2 ng/l and in experiment A1140-21 (Figure 22) the lowest concentrations of 0.5 and 2 ng/l caused approximately 30% inhibition of EROD, with almost complete inhibition by a concentration of 20 ng/l. The inhibitory effect of E2 on EROD or CYP1A confirms that found in previous studies (Arukwe *et al.,* 1997; Navas and Segner, 2000; Stegeman *et al.,* 1982; Forlin *et al.,* 1984; Snowberger *et al.,* 1991). However, these other studies used either *in vitro* cell cultures or IP injection, rather than *in vivo* exposure as we have done. We have demonstrated in this study that the effect occurs, perhaps to a greater extent, via water borne exposure. Furthermore, we have also demonstrated the EROD inhibiting effects of the synthetic steroid EE2.

It is more difficult to fully explain the differences we have observed between the potency of E2 and EE2. Fundamentally their modes of action at the oestrogen receptor are similar and therefore it may be the lower solubility, higher persistence and greater resistance to biodegradation (Desbrow *et al.,* 1998) that contribute to the greater potency of EE2 in terms of VTG induction. However, these characteristics do not explain why E2 demonstrates a greater potency in terms of EROD inhibition. This may be related to the greater solubility of E2, indirect effects on other systems or the activity of its breakdown products on the MFO system. The mechanisms involved are probably complex and will require further study to elucidate. It also has to be remembered that the suppression of EROD activity is mediated by unrealistically high water borne concentrations of DbA. However, we have also noted an EE2 suppression of β-naphthoflavone (BNF) mediated EROD induction which contradicts that reported for rainbow trout hepatocytes by Navas and Segner (2001). At this stage we are unable to state that the EROD inhibitory effects of E2 and EE2 are not affected by the chemical(s) used to induce the MFO response.

Suppression of DbA mediated EROD activity by water borne nonylphenol (NP) occurs at much higher concentrations than for E2 and EE2. From figure 26 it is possible that inhibition of the EROD response may occur at concentrations as low as 33$\mu g\ l^{-1}$ but it is apparently about 50% by a concentration of 100 $\mu g\ l^{-1}$. At no time, even at NP concentrations as high as 400 $\mu g\ l^{-1}$ was the EROD activity completely suppressed to baseline activity levels. The ability of NP to inhibit EROD activity has also been noted in IP injected juvenile salmon (Arukwe *et al.,* 1997) at doses as low as 1 mg/kg. However, Arukwe *et al.* (1997) looked only at the suppression of baseline EROD activity and not at its ability to down regulate the activity induced by PAHs. Data from this study have shown that DbA mediated EROD activity was not significantly reduced at 10 mg/kg, was reduced by approximately 40% at 100 mg/kg and was almost completely inhibited at 500 mg/kg (Figure 27).

What is notable as an outcome from this study is that there is very little evidence for effects on VTG induction in mixes of oestrogens with MFO inducers when compared to the effects noted with the oestrogens alone. This was unexpected as it is well known that certain PAH and other MFO inducers can exhibit both oestrogenic and anti-oestrogenic properties. BNF has been shown to suppress estradiol dependent VTG synthesis in rainbow trout liver cell cultures (Anderson *et al.,* 1996). Moreover, other studies have demonstrated that certain PAH can significantly reduce circulating levels of endogenous E2 in flounder (Monteiro *et al.,* 2000) which could ultimately lead to reduced VTG expression. Further examples of the oestrogenic/ antioestrogenic potential of PAH are given in a review by Nicolas (1999).

This study has routinely used a nominal water-borne concentration of 40 µg/l DbA in the co-exposure experiments with oestrogenic chemicals. There has been no evidence that this level of PAH has lead to any up or down regulation of either E2 or EE2 mediated VTG synthesis under the experimental conditions used in this study. However, there is some tentative evidence that this level of DbA may be able to suppress VTG induction by NP. In experiment A1140-12 (Figure 32) a very low level of VTG induction is seen in the 100 µg/l NP exposure, but in combination with DbA no VTG induction was apparent. Again in experiment A1140-20, no VTG induction was seen at the 100 µg/l NP exposure when DbA was present. This is very scant data on which to base conclusions and, as 100 µg/l represents a concentration close to the threshold for VTG response for NP, it could be caused simply by fluctuations of actual NP concentrations in the experiments. However, as mentioned above, the potential for VTG suppressing effects of PAH is well known so these results seem reasonable. The fact that we have seen some evidence for suppression of NP mediated VTG synthesis and yet not for E2 or EE2 also suggests that, in the environment, the contaminants responsible for VTG production at different sites might also impact how the VTG response is affected by the presence of other co-contaminants such as PAH.

Possible Modes of Action for Cross-Talk Activity

This study has produced significant evidence that estrogens and estrogen-like compounds down-regulate PAH-induced EROD activity. There is also some tentative indication that there may be a reciprocal response with β-naphthoflavone possibly reducing the VTG production expected from NP. Although evidence for this is sparse, similar effects, including reduction of plasma levels of E2 in the presence of PAHs, have been noted in previous studies (Casillas *et al*., 1991; Forlin *et al*., 1984; Stegeman *et al*., 1982). BNF has been shown to reduce synthesis of VTG mRNA (Chen and Sonstegard, 1984) and Spies *et al* (1984; 1985) demonstrated an inverse relationship between MFO inducers (PCBs in this case) and fertilisation success in Starry flounder (*Platichthys stellatus).*

The down-regulation of the EROD response may have significant survival implications for fish populations in contaminated waters. The reduction in their capacity to biotransform and excrete PAHs could lead to higher levels of DNA adduct formation and consequently initiation of neoplasias in the liver and possibly other organs which will affect the long term health of the fish (Stegeman and Lech, 1991). There is also some evidence that the presence of MFO inducers and estrogenic chemicals together cause a different stress response in fish than either compound on its own (Teles *et al*., 2004). This supports the evidence that the effect of mixtures is not always simple additivity.

A number of studies have investigated the link between the two endpoints, EROD activity and VTG induction, used in this study, (Arukwe *et al.,* 1997, Elskus *et al.*, 1991, Forlin *et al.,* 1984, Navas and Segner, 2000, Solé *et al.,* 2000). The potential links are numerous due to the nature of the assays used, which measure the endpoints of complex hormone, enzyme and gene mediated pathways.

EROD activity is associated with the expression of a particular cytochrome P450 enzyme family referred to as CYP1A. The function of CYP1A enzymes is to oxidise and hydroxylate compounds, making them more polar so as to aid conjugation and excretion. The same mechanisms are also used in the transformation of natural steroids eg progesterone to 17á-hydroxyprogesterone and testosterone to 11-ketotestosterone (Lee, 1988). The production of VTG is controlled by hormones found in the pituitary gonadal axis, and the enzymes involved in the production of these hormones are all dependent on cytochrome P450 expression. (Lee, 1988).

The potential for cross talk between the two linked systems is obvious, but the way in which this is manifested seems to be dependent on the specific compounds involved and their concentrations. This makes a definitive understanding of the mechanism hard to establish.

It is possible that E2 may exert some effect on CYP1A through mediation of the E2 receptor (ER) and estrogen responsive elements (Navas and Segner, 2000). Effects on gene transcription (Stegeman *et al.*, 1993) or on estrogen-responsive genes / receptors have also been suggested (Arukwe *et al.*, 1997). Certainly the presence of E2, EE2 or other estrogens can cause a decrease in MFO activity and P450 levels (Forlin *et al.*, 1984; Elskus *et al.*, 1991; Arukwe *et al.*, 1997; Navas and Segner, 2000; Solé *et al.*, 2000). This is not entirely true of NP, which may need to be considered separately as it appears to affect the P450 system in a different way. Presence of NP seems to increase P450 levels at all concentrations. At low concentrations CYP1A activity is increased, up-regulating EROD. This switches over to activity in another cytochrome, CYP3A, at higher concentrations whilst CYP1A is suppressed, down-regulating EROD activity. NP seems to be a substrate for the CYP1A enzyme, competitively inhibiting its expression, whilst up-regulating CYP3A (Teles *et al.*, 2004).

NP may also impact on a different part of the VTG synthesis process than that affected by E2 and EE2 and has been suggested to directly affect the E2 feedback system or pituitary gland rather than acting at the oestrogen receptor (Jobling *et al*, 1996). Conversely to the action of NP on the MFO system, β-naphthoflavone seems to elevate EROD activity but not P450 levels, and when combined with NP it prevents the associated elevation of P450 activity (Teles *et al.*, 2004).

When MFO inducers and steroids are acting in combination, the suppressing or down-regulating effect of the steroids is more prominent on CYP1A protein levels which catalyse the EROD activity (Arukwe *et al.*, 1997) than on the EROD activity itself. This indicates that the crosstalk actually takes place at a profound level in the MFO system, which is then expressed as a reduction in EROD activity in the presence of steroids.

cDNA Gene Array

The data provided from the flounder microarray analysis of fish from experiment 11 is semi-quantitative and subject to experimental variability. Due to these factors, future replication is essential in order to allow robust analysis of the data. We have found, in other studies with flounder, that five or more biological replicates for each condition are preferable. In this preliminary study, only three replicate fish of each sex for each treatment were used and therefore the data does require cautious interpretation. Therefore only gene expression changes of a very high magnitude have been considered for discussion here. However, even though the microarray technique requires further verification to provide greater confidence in the data it is clear that certain effects were occurring.

It can be wrong to assume that gene expression differences as shown on the array will always translate into ultimate differences in protein levels or enzyme activities. There are other important processes, such as translational regulation and protein construction, that might also be disrupted before gene expression ultimately results in these higher level changes. However, it is clear that for CYP1A expression and EROD activity (Figure 30) and vitellogenin/choriogenin mRNA expression and plasma VTG concentrations (Figure 31) a very close relationship was obvious. Microarray results for these fish, although still preliminary, can begin to highlight the biochemical pathways which are disrupted during toxicant exposure. It is worth recognizing that the gene expression changes identified here represent sustained responses over ten days of exposure, rather than the acute transcriptional changes represented by, for example, heat-shock proteins, which often return to basal (pre-exposure) transcriptional levels within a day.

DbA treatment clearly induced CYP1A, and this is reflected in the elevated EROD activities observed. More unusually, fatty acid binding protein was also induced. This protein has previously been observed to bind metabolites of hydrophobic xenobiotics (Larsen *et al.*, 1991) and therefore its presence is understandable, but it is usually more closely associated with dietary changes. It is, perhaps, conceivable that the induction of FABP was necessary to facilitate transport of fatty acids in response to oxidation of the membrane lipids due to production of reactive metabolites of DbA by CYP1A. Other genes induced by DbA included betaine-

homocysteine methyltransferase; metallothionein; GAPDH; glutathione peroxidase and a MAP-kinase related kinase. These responses were generally characteristic of DbA treatment and were not seen with EE2 treatment.

Marked inductions of vitellogenin and choriogenin, the egg proteins, were seen in both male and female EE2-treated fish. As with CYP1A, these responses provide a validation of the experiment, especially when combined with the vitellogenin protein measurements. TFII P12, a transcription initiation factor, could conceivably be induced to facilitate the dramatic increases in transcription of vitellogenin and choriogenin. The discovery of reticulon induction is interesting as it has previously only been found in neuroendocrine cells. This may provide a clue to signalling in response to the presence of xenoestrogens in the liver.

One of the main objectives of this experiment was to investigate the effects of a co-treatment with a PAH and an endocrine disruptor. Levels of vitellogenin and choriogenin expression in fish from the DbA/EE2 mixture exposures are generally similar to those gained with oestrogen exposure alone (Figure 31). There does appear to be a slight reduction of the vitellogenin and choriogenin response in the co-treated females compared with those treated with EE2 alone. Assessment of the same response in males was not possible due to the lack of response from one of the fish. It was evident that the females expressed more of these transcripts than the males, as might be expected if, for example, the oestrogen receptor-mediated pathway components were more common in cells of the females.

With CYP1A, however, a more complex interaction was seen. EE2 caused an apparent reduction of CYP1A levels in comparison with those seen in the controls. It has previously been reported that endocrine disrupters can repress CYP1A transcription, and it has also been suggested that oestradiol might have a protective effect against oxidative stress, which may explain the apparent reduction of FABP expression with EE2 (Figure 30). With the co-treatment, FABP remained at low expression, consistent, perhaps, with this protective effect. However, CYP1A displayed a sex-specific response. In females, CYP1A expression remained low, but in males, an induction of CYP1A was seen. This may be related to the magnitude of vitellogenesis, which was far higher in the females than the males, and hints at an oestrogen-receptor mediated effect, as the dose of EE2 was identical.

Correlations

As well as searching for evidence of up or down regulation in the EROD or VTG responses the data were assessed for patterns of co-dependency between the two biomarkers. There is evidence in the literature of a relationship between hepatic EROD activity and plasma VTG in male flounder caught in estuaries (Kirby *et al.* 2004b). However, these correlations are not consistent and can be both positive and negative on different occasions. The positive correlations in the study reported by Kirby *et al.*, were probably due to the strong seasonal influences associated with the reproductive cycle, within which MFO activity reaches a peak in the spring which corresponds to a similar trend discovered in male plasma VTG concentrations (Kirby *et al.*, 2004b). Certain estuaries showed negative correlations where apparent suppression of EROD occurred when plasma VTG concentrations were high. This is more consistent with what would be expected if we assume that exogenous oetrogenic exposure has the ability to suppress MFO system activity. The data from these field studies must be treated with caution, however, because there are only very few data points and a plethora of other factors, both internal and external to the fish, that could be involved.

Correlation analysis of the EROD and VTG data in this study reveal no consistent relationship between the two biomarkers from any of the treated or non-treated groups. Both biomarkers show high levels of individual variability, which was also noted at the gene expression level, from fish receiving the same treatments but these, also, could not be attributed to the activation of either the MFO or vitellogenic system. This was not unexpected as all the fish used in these studies were reproductively immature and some of the co-dependent effects seen in the field data are certainly related to the breeding cycle. Furthermore, most of the effects of, for example, the ability for estradiol to down regulate EROD from the literature have measured these effects in baseline, non-induced, EROD activities. The fact that we have studied co-dependent responses in situations where both systems are stimulated may represent a more complex relationship.

It is also well know that somatic indices such as the hepato- and gonado-somatic indices (HSI and GSI) can also be related to biomarker responses (Kirby *et al.*, 1999). For example, GSI is generally positively related to EROD in reproductively mature North Sea flounder as EROD in both sexes increases as gametogenesis begins in the autumn to reach a peak at spawning time during the winter after which it crashes again to

baseline levels by the spring (Janssen *et al.,* 1995; 1996a). An increase in HSI can also be related to EROD and VTG. For example, Everaarts *et al.* (1993) have demonstrated increased HSI's in fish exposed to contaminated sediments and Theodorakis *et al.* (1992) have shown an increase in HSI to mirror a trend in elevated EROD activity in bluegill sunfish. Janssen *et al.* (1996b) have shown that HSI increases in female flounder at the onset of, and during, vitellogenesis, and Allen *et al.* (1997) have shown that plasma vitellogenin levels in flounder are elevated in UK estuaries where mean HSI is high.

In this study, however, no consistent correlation was found in any experiment for VTG or EROD with GSI or HSI. Prolonged exposure to contaminants would no doubt result in changes, but after only 10 days exposure gross somatic effects were not observed.

Other potential influences on EROD Activity

There are a number of factors which can cause variability of EROD levels, these include season, temperature, size and age.

Seasonal factors such as annual temperature trend, the body fat content due to the availability of food and the sexual maturity cycle will all affect the EROD response. EROD activity is known to be highest in reproductively active males, inactive males and inactive females and lowest in reproductively active females. (Stegeman and Hahn, 1994). This suppression in reproductively active females is due to the production of oestradiol. Females show a decrease in EROD activity pre spawning and a rise during the post spawning period. Seasonal cycles have also been related to water temperature variations and studies have shown that in the European plaice, EROD activities were low in the summer, rose gradually in the autumn and winter and peaked in the spring (George *et al.,* 1990). A similar pattern was also seen in this investigation using flounder. This pattern is inverse to the change in water temperature and suggests a temperature dependant effect. Higher EROD levels at lower ambient temperatures may be due to an increase in enzymatic activity so as to compensate for lower reaction rates (Ankley *et al.,* 1985).

The size of the fish is another EROD influencing factor, for example EROD levels have been found to decrease with increasing fish mass and this is thought to be because increasing mass leads to dilution of contaminants. However smaller fish may have lower EROD activities because of increased metabolism which allows them to excrete more contaminants. (Hodson *et al.*,1996). To be confident in the EROD values obtained, consistency of fish size was maintained in the same range for each experiment.

Diet and nutrition can have a significant effect on the level of the EROD response, and this was the reason that flounder were not fed either on day 0 or throughout the experiment. If fish from the wild were used it would not have been possible to control their diet and so using farmed fish is an easier means of controlling dietary influences. In the wild, flounder will feed intensely from March to October followed by a period of sporadic feeding or starvation until after spawning. During this feeding time their livers are in much better condition. This kind of feeding was observed in the farmed fish used in our experiments and could explain the variation in results that are observed in day 0 results.

To ensure flounder were exhibiting the highest EROD levels and lowest levels of variation immature fish were used in all experiments. When deciding whether to use wild flounder from a clean site or farmed fish, an initial experiment was run to measure EROD response. This showed significantly higher EROD response in farmed fish, although activity levels were no higher than 100 pm EROD /mg protein/min in wild fish compared to farmed fish, which reached in excess of 800 EROD pm/mg protein/min. These differences between farmed and wild fish may have resulted from a number of influences. For example the female Alde fish even though there was no significant difference between weight of fish compared to Port Erin fish, had a GSI significantly higher than the farmed fish (control female fish compared). This would mean that wild female fish had significantly larger gonads and were therefore more sexually mature and are more likely to be affected by seasonal changes in EROD. A negative correlation was also observed between GSI and EROD in the wild female fish that was not observed in the farmed fish. This indicated that more mature, larger gonads resulted in lower EROD levels. Even if wild fish were caught at a standard size (therefore using immature fish) they are still exposed to internal and external factors which are less controllable than with farmed fish in a controlled environment.

All of the above factors were taken into account when designing experiments in order to ensure confidence in the EROD values obtained and minimise variation. For example immature farmed fish were used, they were kept at a constant temperature during the experiment and fish of a standard size were used. However individual variation in Flounder EROD levels due to genetic differences is unavoidable and may explain some of the variation seen in the experiments.

Other Potential Influences on VTG Production

Female flounder are known to have four phases in their normal VTG plasma concentration cycle these are; a build up of VTG concentrations between October and December (increase), a peak in January - February before the spawning migration (peak), decrease in VTG between March and June (decrease), and July-September sexual inactivity (rest). (Kleinkauf *et al*., 2004). Male VTG levels have also been found to vary throughout the year in wild fish even at clean sites (Kirby *et al*., 2004b). It was found that male flounder caught in September had lower VTG than those caught in later months. It is thought that this seasonal variation in VTG is due to the migratory behaviour of wild flounder. Variation between fish is thought to occur due to differences in age, health, nutrition, temperature, exposure to pollutants, time of migration in to an estuary and whether the fish is a first time or repeat spawner. (Kleinkauf *et al*., 2004). In this investigation no VTG trend was found though out the year in farmed fish although there were variations between months. This was expected because there is no migration in farmed fish. However, the variation between months may have been due to a number of factors including temperature, feeding, genetics and gonad size. It is better to use farmed fish to show oestrogenic disruption because of the lack of control over parameters such as migratory behaviour, time of year and food availability which are inherent in the use of wild flounder.

Implications for Monitoring Data

This study has clearly demonstrated the potential for 'crosstalk' between the regulation of individual biomarker responses when binary mixtures of MFO inducers and oestrogens occur. Surprisingly, we have not noted routine effects of PAH exposure on the production of oestrogen induced plasma VTG in male flounder. However, significant inhibition of EROD activity and appropriate effect threshold concentrations have been established for three important environmental oestrogens, E2, EE2 and nonylphenol.

Translation of these effects to the environment cannot be considered straight forward. It is known, however, that concentrations of oestrogens and MFO inducers likely to cause activity are present together in, for example, certain UK estuaries (Kirby *et al.*, 2004a &b). Furthermore, the crosstalking effects have been studied in flounder which is the primary fish species for biological effects monitoring in UK (and across Europe) estuarine environments. Therefore, it is highly likely that crosstalking, and the associated up or down regulation of responses, is occurring in wild fish populations. New techniques such as the DNA microarray may be able to help to indicate where this crosstalk is happening in future.

The realisation that certain responses are being modified in the presence of other contaminants is particularly significant for biological effects monitoring in multi-contaminant areas, such as estuaries and coastal waters. In general, individual biomarkers, such as EROD or plasma VTG, have been determined and the data interpreted in isolation with no consideration of how crosstalk might be affecting the data. Table 4 shows the low concentrations of E2 and EE2 required in this study to moderate the EROD response. If, as the evidence presented here suggests, EROD levels are being suppressed in areas of oestrogenic contamination it is likely that EROD monitoring data are being interpreted incorrectly.

Figure 33 shows mean hepatic EROD activity data of male flounder from eight estuarine monitoring sites taken during the Autumns of 1999 and 2001. These fish were also assessed for their plasma VTG levels as a measure of oestrogenic contaminant exposure. If it is assumed that higher mean plasma VTG concentrations indicate higher levels of oestrogenic contamination in these areas it is also reasonable to suggest that the down regulation of EROD is potentially greatest in the areas showing the highest plasma VTG in males flounder. On this basis a basic assessment of the likely level of EROD suppression can be applied depending on the mean VTG concentration in the fish. To this end % suppressions of EROD activity were assumed for the following VTG concentrations: $<0.2\ \mu g\ ml^{-1}$ = 0%; 0.2-10 = 10%; 10-100 = 20%; 100-500 = 40% and >500 = 60%. These figures are somewhat arbitrary but are based on the data gathered within this project. Figure 33 shows the same two sets of EROD monitoring data adjusted on the basis of their VTG levels.

It is clear from the adjusted EROD figures that, if crosstalk is occurring in the environment, monitoring data may be significantly underestimating impacts to fish as measured by the stimulation of the MFO system. The adjusted data suggests that most sites could be showing significantly more EROD activity, compared to the Alde reference site, if possible oestrogenic down-regulation is accounted for. This is especially evident in the data from the Dabholm Gut site on the River Tees, where oestrogenic suppression of the MFO system could well be masking a much more significant effect on EROD activity.
It is acknowledged that this may be a simplistic approach and that, in reality, the mechanisms involved and other potential influences may result in a much more complex web of interactions. Nevertheless, the potential for crosstalk to occur between these systems in the environment is high and it is recommended that further research is required to ensure that our interpretation of monitoring data is sound. This is certainly true for the impacts of oestrogens on the MFO system and, while not as certain from this study, the scientific literature strongly suggests that organic contaminants, such as PAH, may also be interfering with plasma steroid levels and associated VTG concentrations.

Achievement of Project Aims and Objectives
This project was initiated with four core aims and objectives (see Introduction). A number of oestrogenic chemicals and MFO inducers that are also important environmental contaminants were identified and selected. A standardised experimental set up was established utilising farmed, immature flounder and a 10 day exposure regime. Under these conditions, standard, concentration-related responses in terms of EROD activity and plasma VTG production were established. Up or down regulation in these standard responses were then investigated under the same conditions with binary combinations of oestrogens and MFO inducers. The potential for up or down regulation was established and the concentration thresholds of effect established. This information has then been applied to real field data as an example of how crosstalk in multi-contaminant environments may be affecting the biomarker monitoring data. To this end all of the core objectives set out at the onset of the project have been achieved.

6. CONCLUSIONS

- The hepatic EROD inducing potential of several organic chemicals were established in flounder after a 10 day water borne exposure under specific test conditions. The classic model inducer, βNF, was found to be the most potent inducer, with high EROD induction occurring at a concentration of 10 μg/l. Of the PAH tested DbA and BkF were found to be more potent than BaP. DbA and BkF showed low levels of induction at concentrations of 10 μg/l.
- The potency of several oestrogenic chemicals under standard conditions of water borne exposure were established. EE2 was found to be the most potent inducer, with a VTG induction threshold between 1 and 10 ng/l after 10 days water borne exposure . The VTG induction threshold under the same conditions were between 100 to 200 ng/l and 100 to 330 μg ng/l for estradiol, and technical nonylphenol respectively.
- The ability of EE2, E2 and NP to down-regulate PAH induced hepatic EROD activity in flounder was established. The water borne concentrations at which EE2 and NP demonstrated the ability to down-regulate the EROD were approximately those at which they were also able to induce plasma VTG. However, E2 was able to significantly downregulate EROD at concentrations over 50 times lower than those at which VTG was produced. It has been shown that under standard test conditions concentrations as low as 2 ng/l were able to inhibit PAH induced EROD activity.
- No conclusive evidence of up or down regulation of VTG production in male flounder as a result of oestrogenic exposure was discovered. It is possible that nominal concentrations of 40 μg/l DbA suppressed the ability of threshold concentrations of NP (~100 μg/l) to induce VTG.
- The exposures conducted via the intraperitoneal route confirmed the potency rankings of the oestrogens as gained via water borne exposure. This route also confirmed the ability of E2 to suppress EROD activity at significantly lower doses as that required to induce VTG production. The use of IP exposures can therefore be used as a useful screen to forecast potential water borne effects. However, because the IP route did not predict the extent to which E2 could suppress EROD in terms of the ratio of this effect to its VTG inducing threshold IP should only be used as a screen when investigating possible environmental effects.

- The use of a cDNA microarray proved to be a useful tool in aiding with mechanism interpretation. The results suggest that the inhibiting effect of oestrogens on the MFO system are also occurring at the gene expression level and not just at the enzyme activity level.
- Using the findings from this study it is concluded that data gained as a result of biomarker monitoring in samples from multi contaminant areas (e.g. estuaries) are probably being influenced by crosstalk system interactions. It has been demonstrated that EROD, for example, may be significantly underestimated in estuaries where oestrogenic chemicals are present. Furthermore, this could be occurring even where these chemicals are not at active levels in terms of their ability to induce VTG production in males.

7. RECOMMENDATIONS AND FUTURE RESEARCH

- Biomarker monitoring data is almost certainly being influenced by cross system interactions. In order to more confidently interpret these data a greater understanding of the nature and extent to which crosstalk is occurring in the wild is required.
- Further research is recommended in order to further understand theses interactions and the mechanisms by which they occur. In particular there is a need to focus on the effects caused by known active chemicals in standard monitoring species. The use of cDNA microarray technology will be useful in elucidating the nature of the crosstalk mechanisms.
- The high variability of response for EROD, VTG induction and level of EROD suppression gained in groups of fish under the same regimes of exposure suggest that individual genetic makeup may be highly influential in the determination of response level and therefore potential sensitivity to deleterious effects of pollution. It is recommended that a greater understanding of how and why different individuals and whole populations respond differentially to pollutants. This will allow monitoring data from geographically distinct areas to be compared with greater confidence and will enable a more accurate assessment of risk to individual populations.

8. ACKNOWLEDGEMENTS

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Table 1. Summary of test dates and chemical exposure

Test	Start date	End date	Fish Source	Chemical
1	29/06/01	20/07/01	Port Erin	BaP range finding test
2	10/07/01	31/07/01	Alde	BaP range finding test
3	30/11/01	21/12/01	Alde	BaP dose-response 21 day test
4	22/01/02	11/02/02	Alde	EE2 dose-response 21 day test
5	09/04/02	25/04/02	Alde	DbA, BkF, BNF EROD response test
6	05/07/02	16/07/02	Port Erin	Dba, BNF alone, EE2 mixtures
7	16/07/02	19/08/02	Port Erin	HBCD and TBBP-A range finding test
8	01/11/02	12/11/02	Port Erin	NP range finding test
9	06/12/02	12/16/02	Port Erin	EE2 VTG response finding test
10	28/01/03	07/02/03	Port Erin	EE2 VTG response finding test
11	14/03/03	25/03/03	Port Erin	Dba, BNF alone, EE2 mixtures
12	23/05/03	02/06/03	Port Erin	Dba and NP alone and mixtures
13	10/06/03	20/06/03	Port Erin	Aroclor range finding test
14	27/06/03	06/07/03	Port Erin	Dba and EE2 alone and mixtures
15	12/08/03	21/08/03	Port Erin	Dba and EE2 alone and mixtures
16	17/10/03	27/10/03	Port Erin	E2 range finding test
17	07/11/03	18/11/03	Port Erin	DbA in mixtures with varying EE2
18	01/12/03	11/12/03	Port Erin	Dba and E2 alone and mixtures
19	06/02/04	17/02/04	Port Erin	DbA in mixtures with varying E2
20	05/03/04	16/03/04	Port Erin	DbA in mixtures with varying NP
21	07/06/04	17/06/04	Port Erin	DbA in mixtures with varying E2
22	19/05/04	24/05/04	Port Erin	IP range finding: E2, EE2, NP, Dba
23	07/07/04	12/07/04	Port Erin	IP test with DbA and E2
24	16/07/04	21/07/04	Port Erin	IP test with DbA and EE2
25	29/07/04	03/08/04	Port Erin	IP tests: E2 vary Dba, DbA vary NP

Table 2. Results of EROD inducers range test to find EROD inducing concentration

Chemical	Exposure	NOEC	Approximate LOEC
Benzo[a]pyrene	Waterborne	10 µg/l	Not found
B-Napthoflavone	Waterborne	1µg/l	10 µg/l
Benzo[k]fluoranthene	Waterborne	<10µg/l	10 µg/l
Dibenz[a,h]anthracene	Waterborne	<10µg/l	10 µg/l
	Intraperitoneal	1 mg/kg	3.3 mg/kg
Hexabromocyclododecane	Waterborne	1 mg/l	Not found
Tetrabromobisphenol A	Waterborne	1 µg/l	1 mg/l
Aroclor 1254	Waterborne	330 µg/l	Not found

Table 3. Results of oestrogen ranging tests to find VTG inducing concentration

Chemical	Exposure	NOEC	Approximate LOEC
Estradiol	Waterborne	33ng/l	100-200ng/l
	Intraperitoneal	0.1mg/kg	0.33mg/kg
Ethynylestradiol	Waterborne	1ng/l	10ng/l
	Intraperitoneal	0.033mg/kg	0.1 - 0.33mg/kg
Nonylphenol	Waterborne	33µg/l	100-330µg/l
	Intraperitoneal	33mg/kg	100mg/kg

Table 4. Concentrations of oestrogen's mixed with DbA at which VTG induction and down-regulation of EROD occurs

Chemical	Exposure	VTG induction	Down regulation of EROD
Estradiol	Waterborne	150ng/l	2ng/l
	Intraperitoneal	0.01mg/kg	0.01mg/kg
Nonylphenol	Waterborne	100 - 200µg/l	66 -100µg/l
	Intraperitoneal	100mg/kg	100mg/kg
Ethynylestradiol	Waterborne	20ng/l	20ng/l
	Intraperitoneal	0.01mg/kg	0.033mg/kg

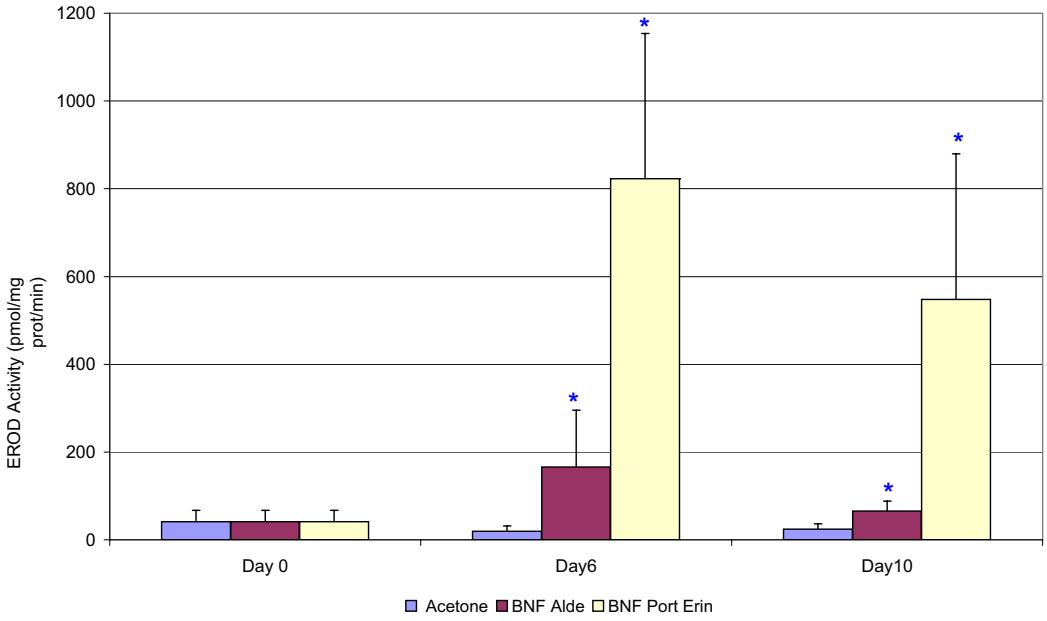


Figure 1. EROD Results from Flounder from Port Erin Hatchery, and the River Alde, after dosing with BNF at 10ng/l for 10 days

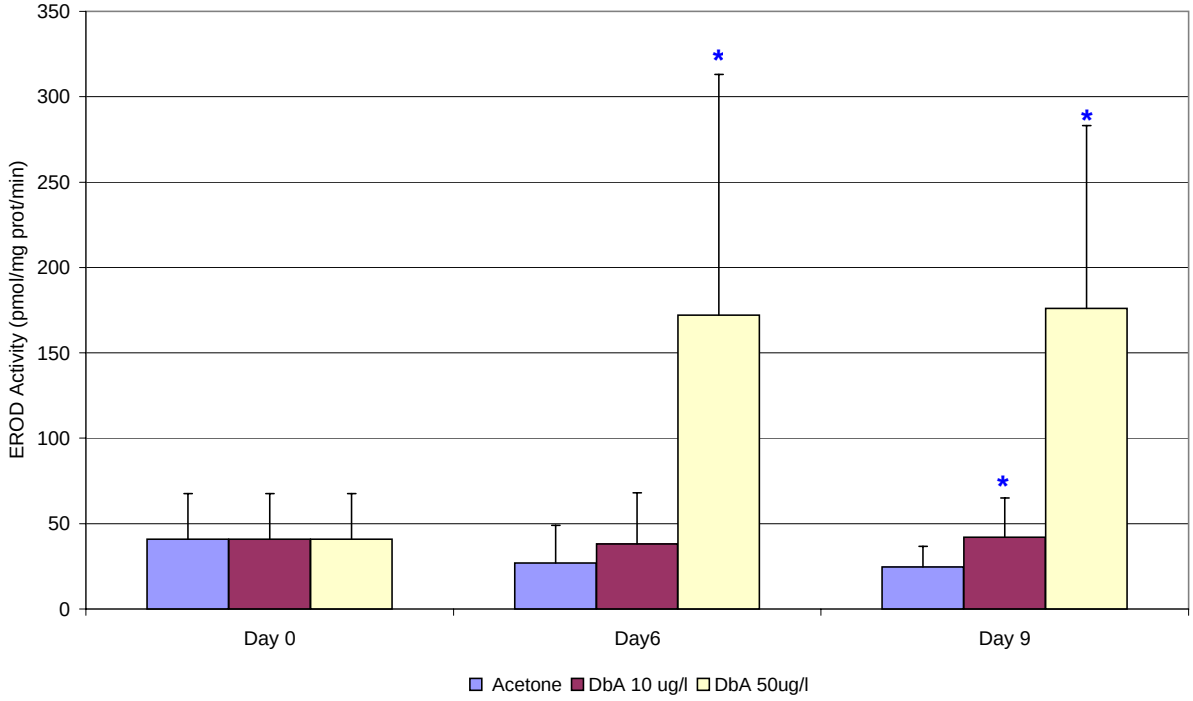


Figure 2. EROD Results from flounder dosed with DbA

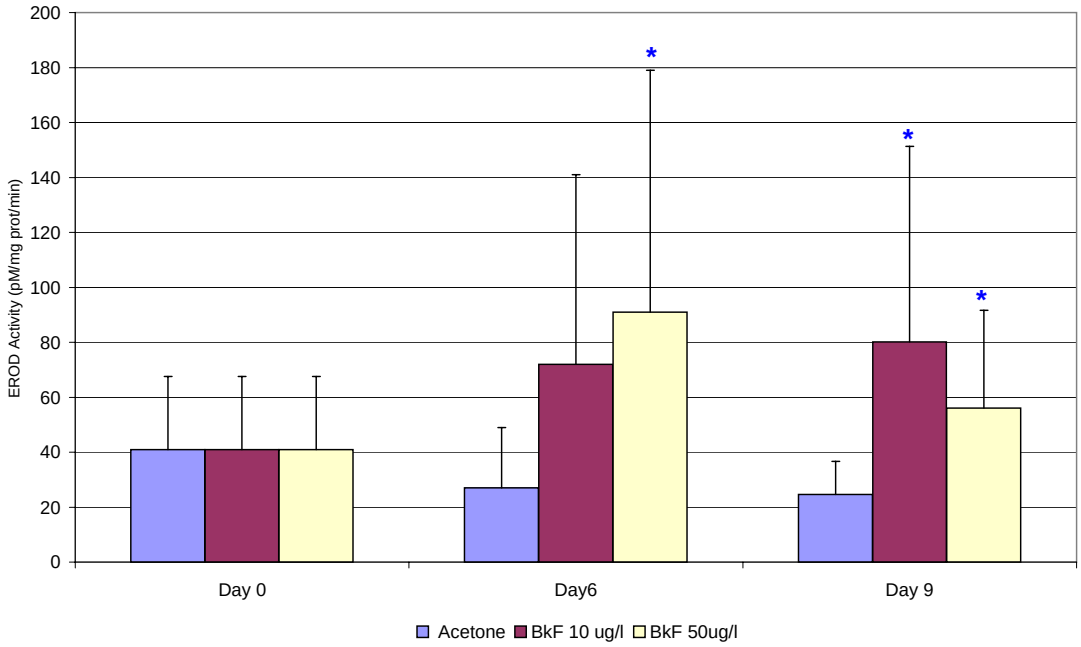


Figure 3. EROD Results from flounder dosed with BkF

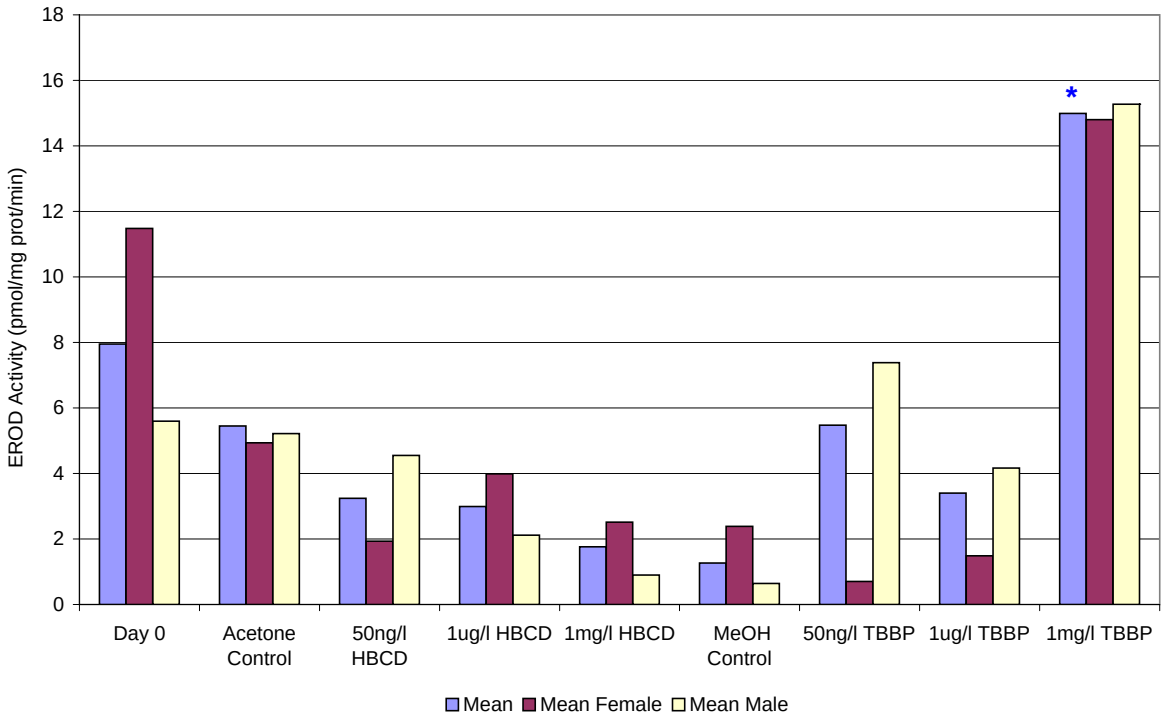


Figure 4. EROD Results from flounder dosed with HBCD or TBBP for 10 days.
**** denotes significant difference compared to control.***

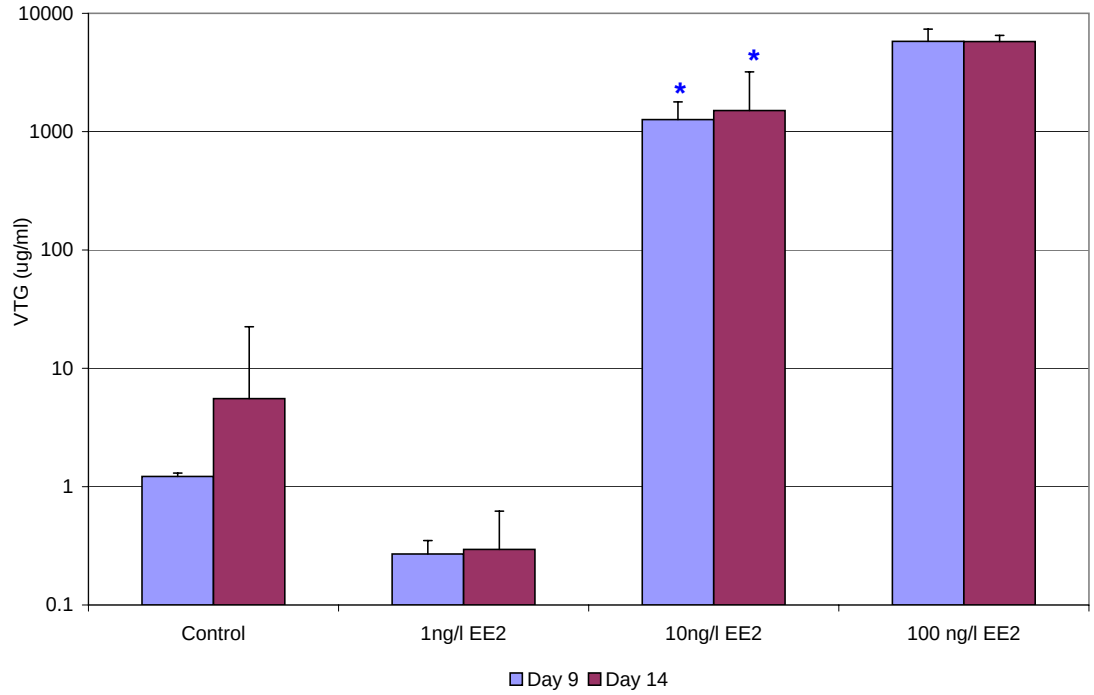


Figure 5. Plasma VTG Results from flounder dosed with EE2. * denotes significant difference from control

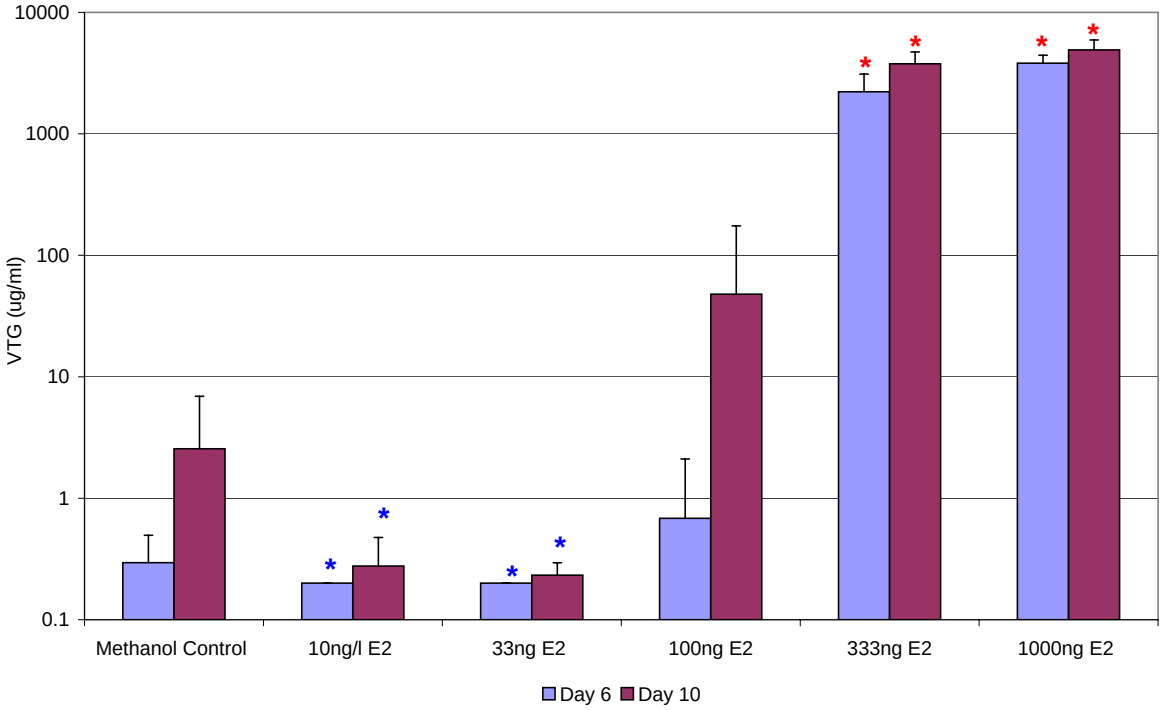


Figure 6. Plasma VTG Results from flounder dosed with E2. * denotes significantly lower than control. * denotes significantly higher than control

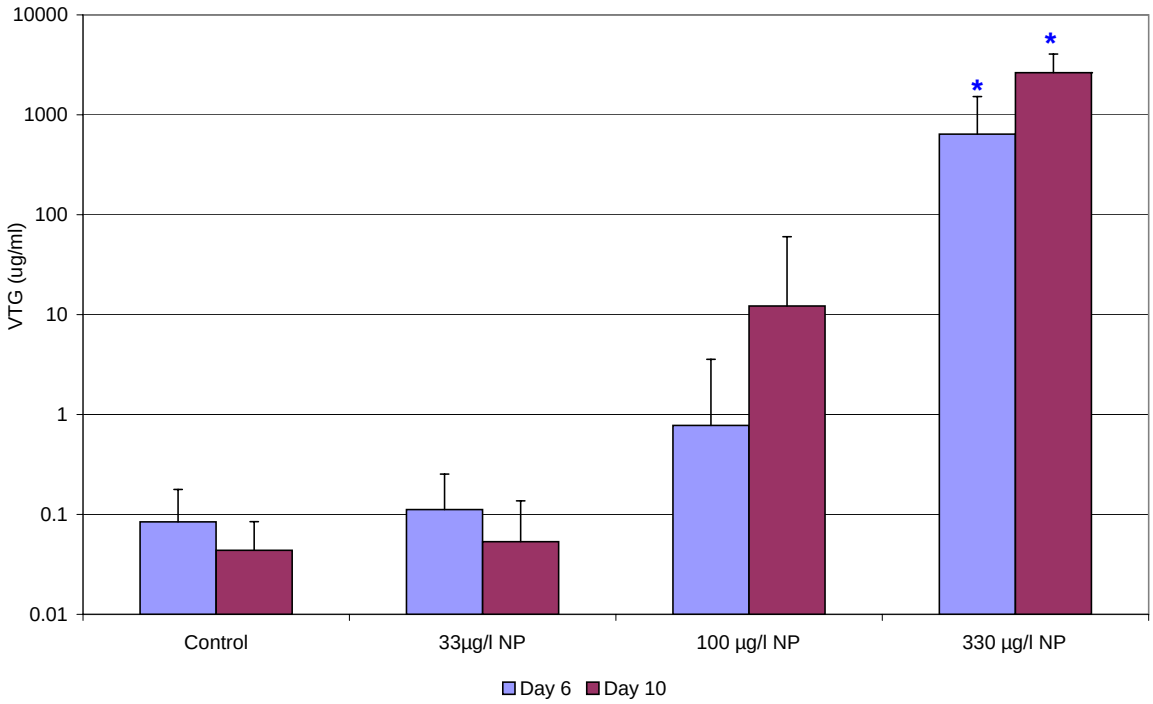


Figure 7. Plasma VTG Results from flounder dosed with NP. * denotes significant difference compared to the control

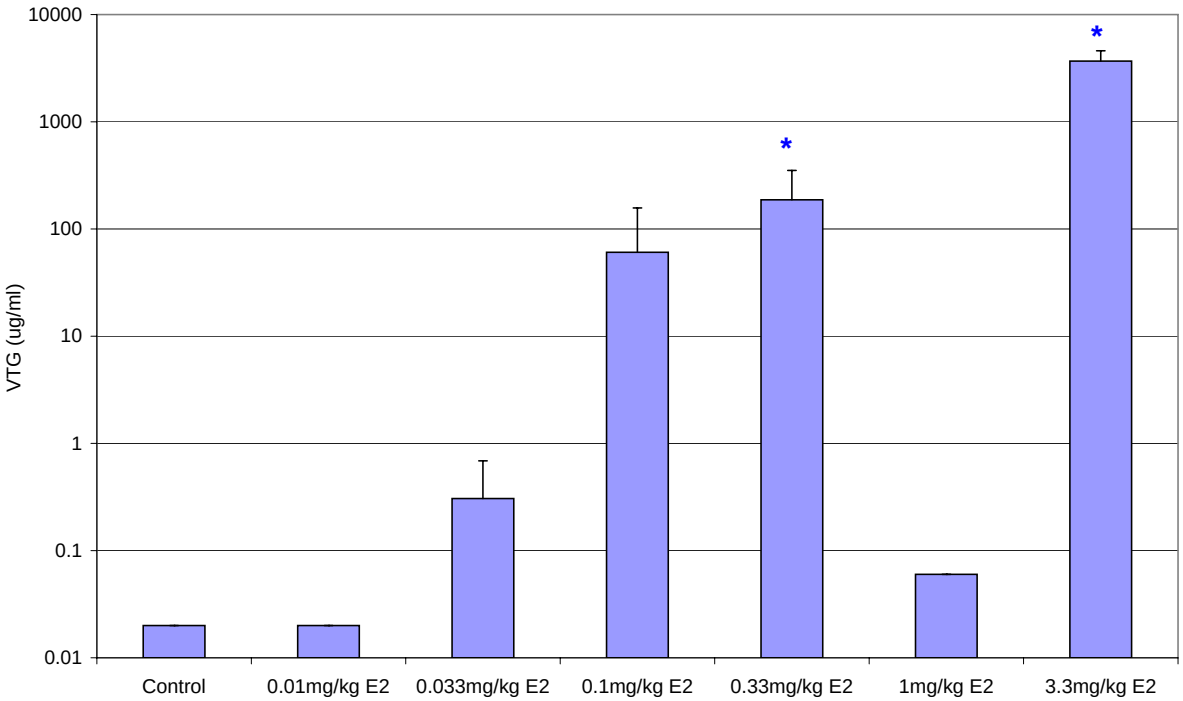


Figure 8. Plasma VTG Results from flounder dosed with E2 via IP injection. * denotes significant difference compared to the control

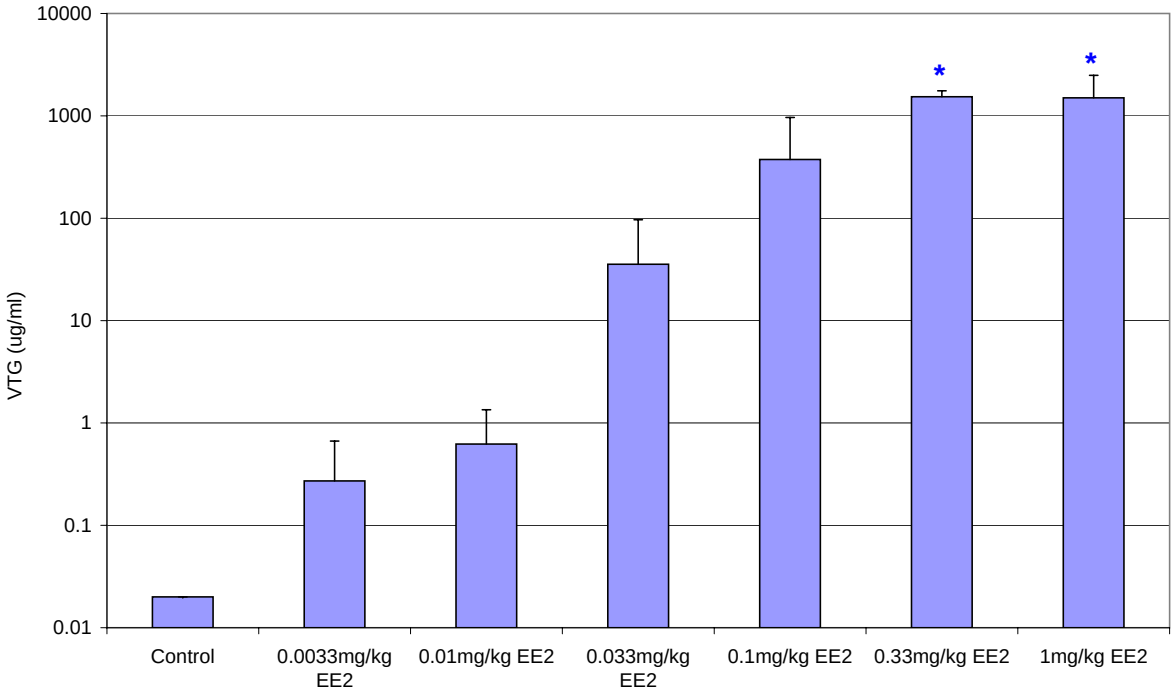


Figure 9. Plasma VTG Results from flounder dosed with EE2 via IP injection. * denotes significant difference compared to the control

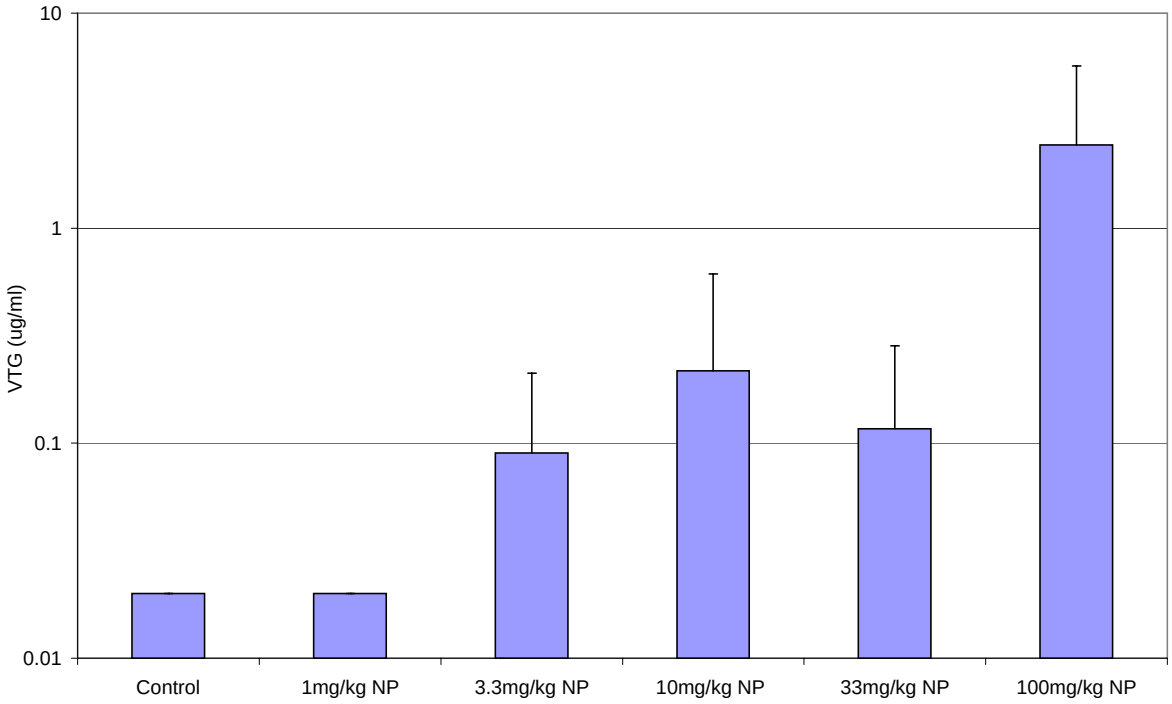


Figure 10. Plasma VTG Results from flounder dosed with NP via IP injection

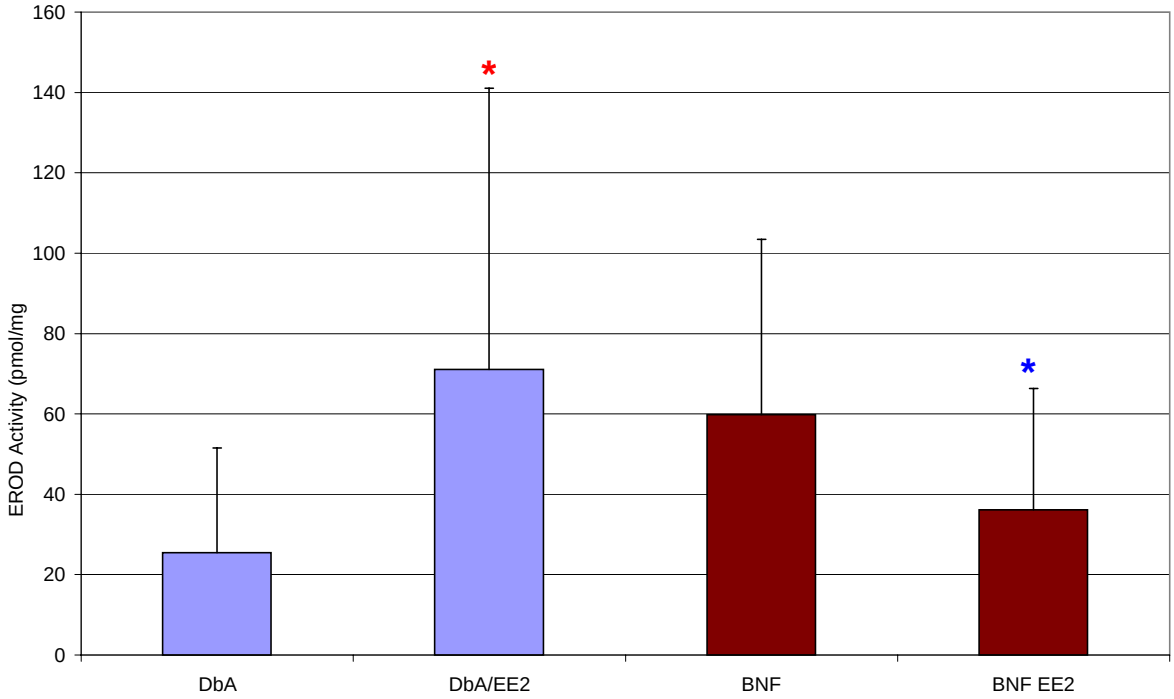


Figure 11. EROD activity in flounder dosed with DbA, BNF, DbA/EE2 mixture or BNF/EE2 mixture. * denotes significant difference from BNF. * denotes significant difference from DbA

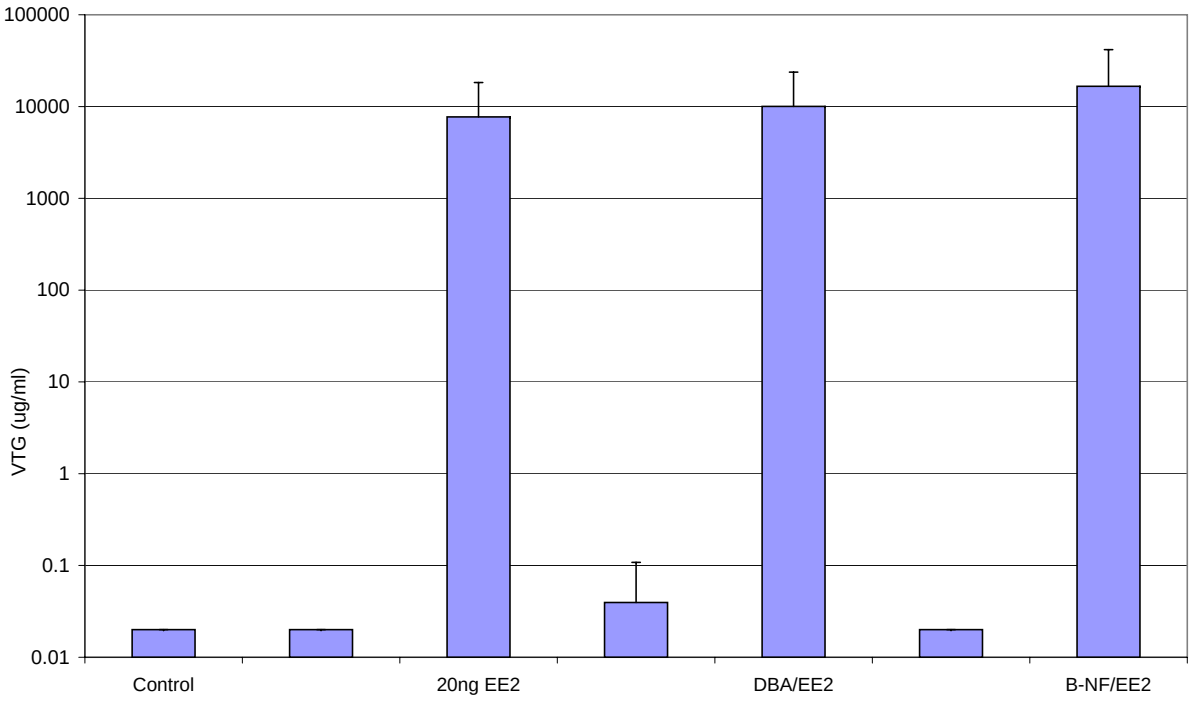


Figure 12. Plasma VTG concentrations in flounder dosed with DbA, BNF, EE2, DbA/EE2 mixture or BNF/EE2 mixture

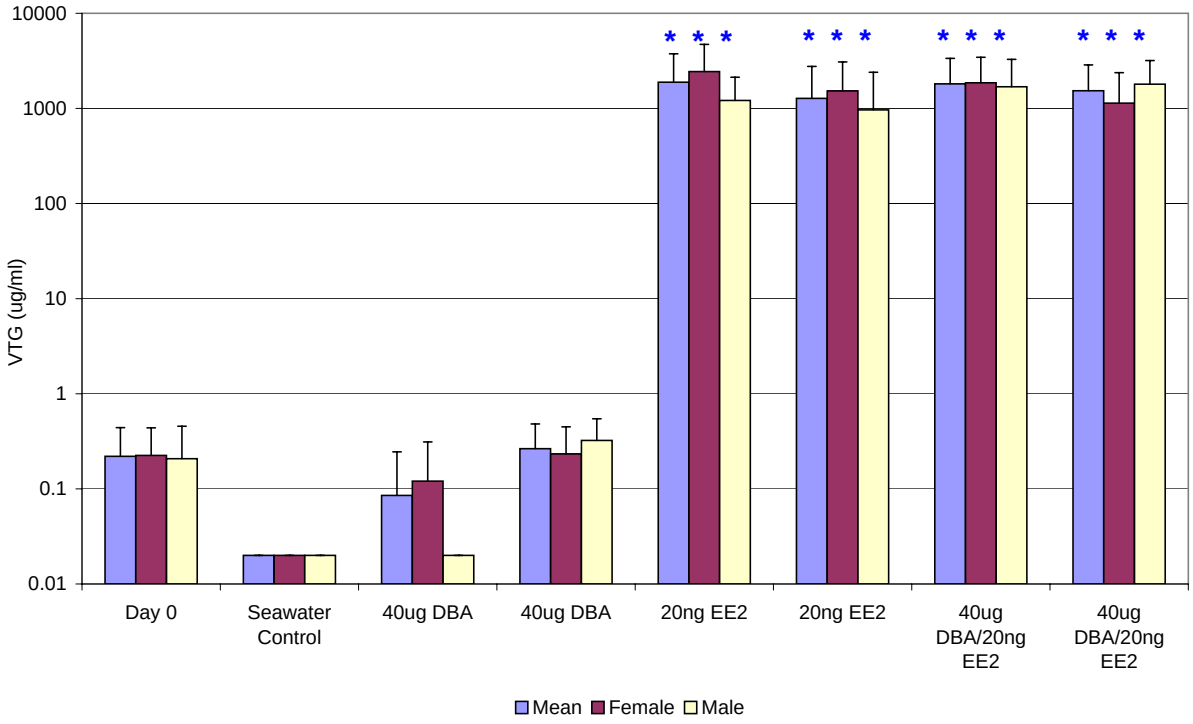


Figure 13. Plasma VTG concentrations in flounder dosed with DbA, EE2, or a mixture of DbA and EE2. * denotes significant difference compared to the control

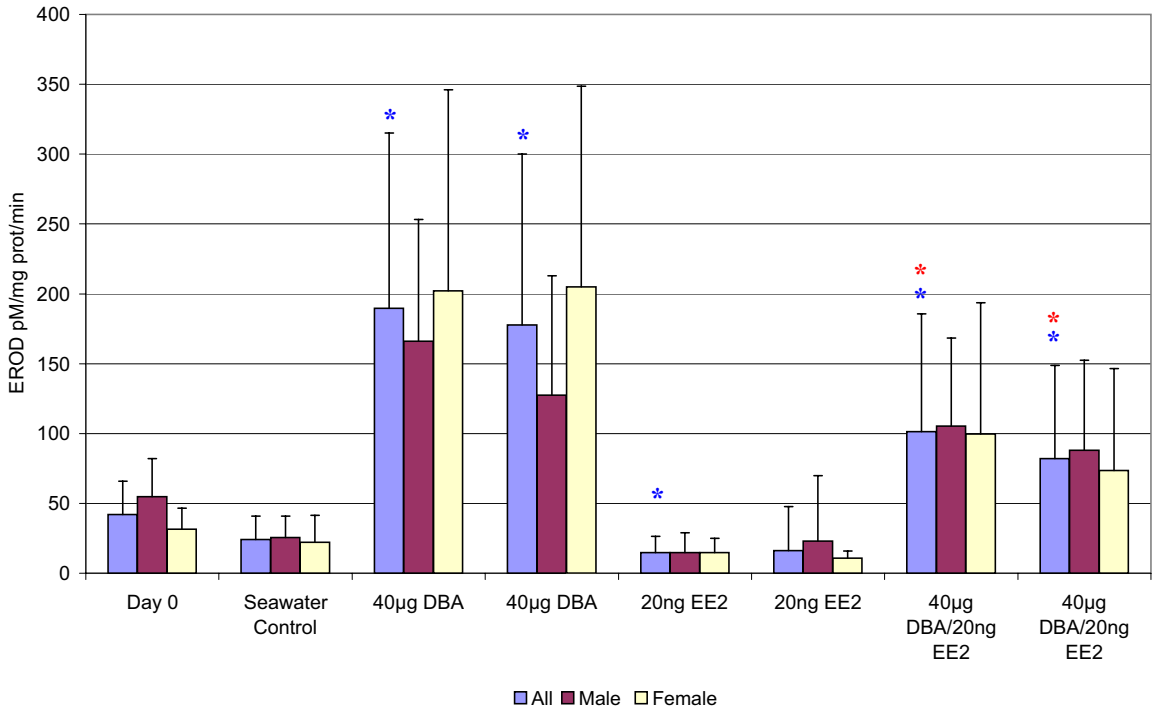


Figure 14. EROD activity in flounder dosed with DbA, EE2, or a mixture of DbA and EE2. * denotes significant difference compared to the control. * denotes significant difference compared to the DbA alone. ANOVA only carried out on means data

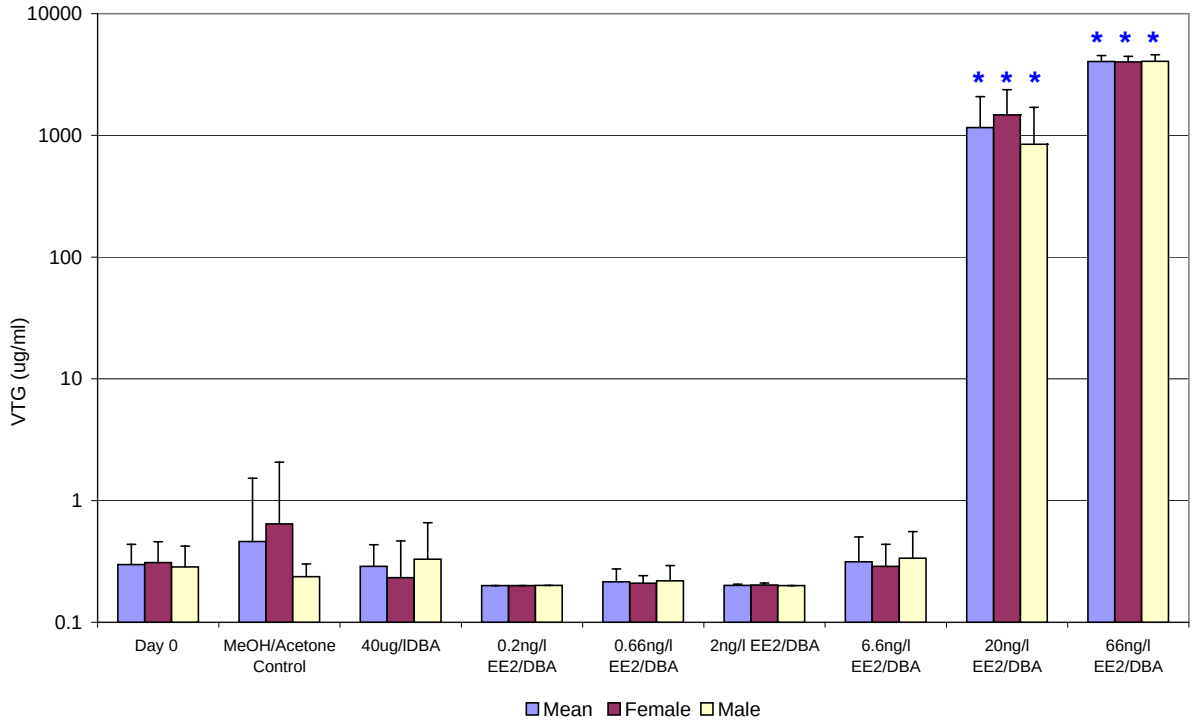


Figure 15. Plasma VTG concentrations in flounder dosed with DbA or a mixture of DbA and a concentration range of EE2. * denotes significant difference compared to the control

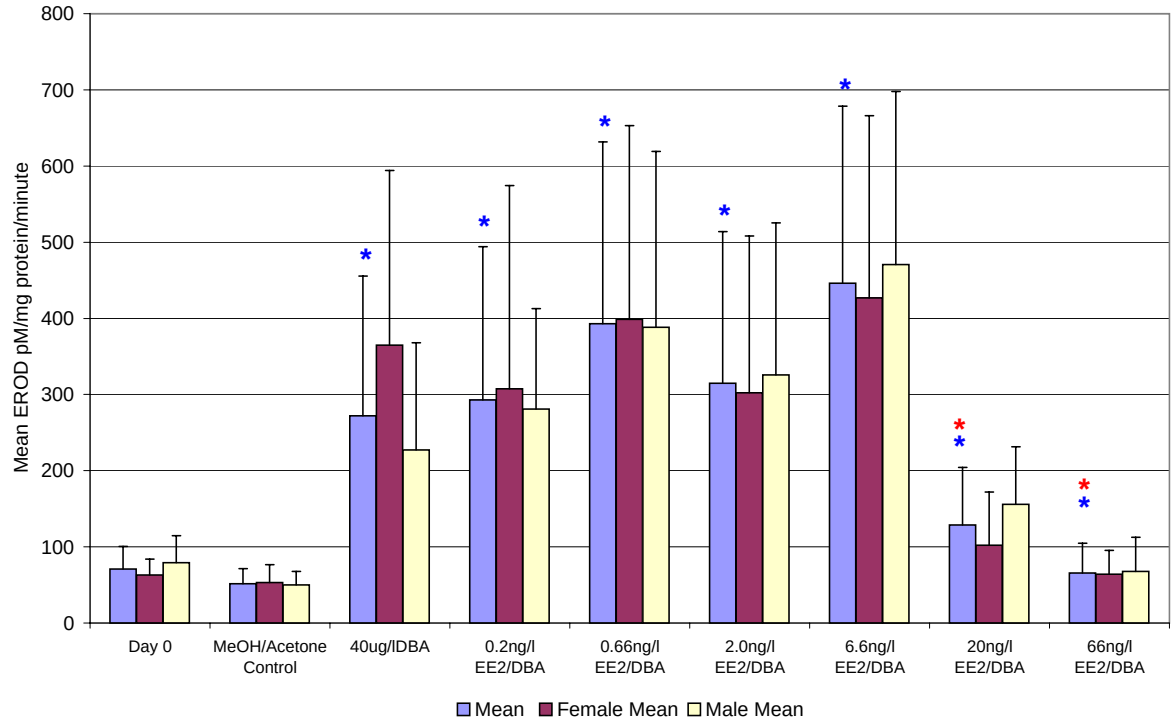


Figure 16. EROD activity in flounder dosed with DbA or a mixture of DbA and a concentration range of EE2. * denotes significant difference compared to the control. * denotes significant difference from DbA

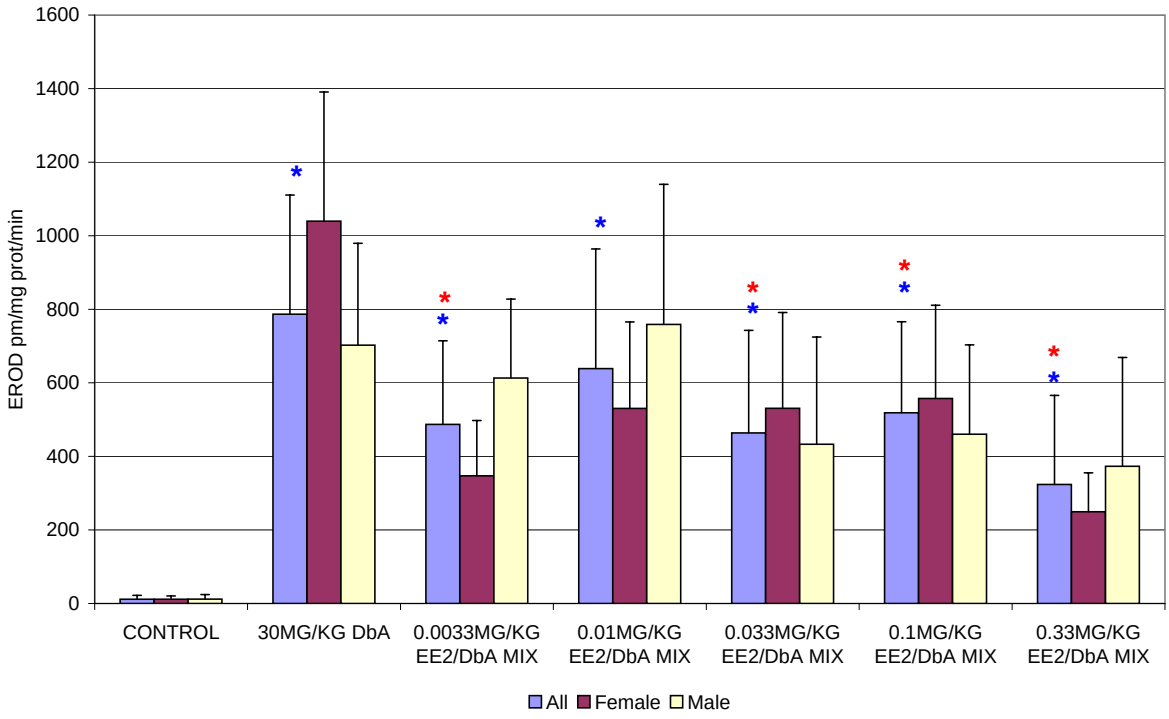


Figure 17. EROD activity in flounder dosed via IP injection with DbA or a mixture of DbA and a concentration range of EE2. * denotes significant difference compared to the control. * denotes significant difference from DbA

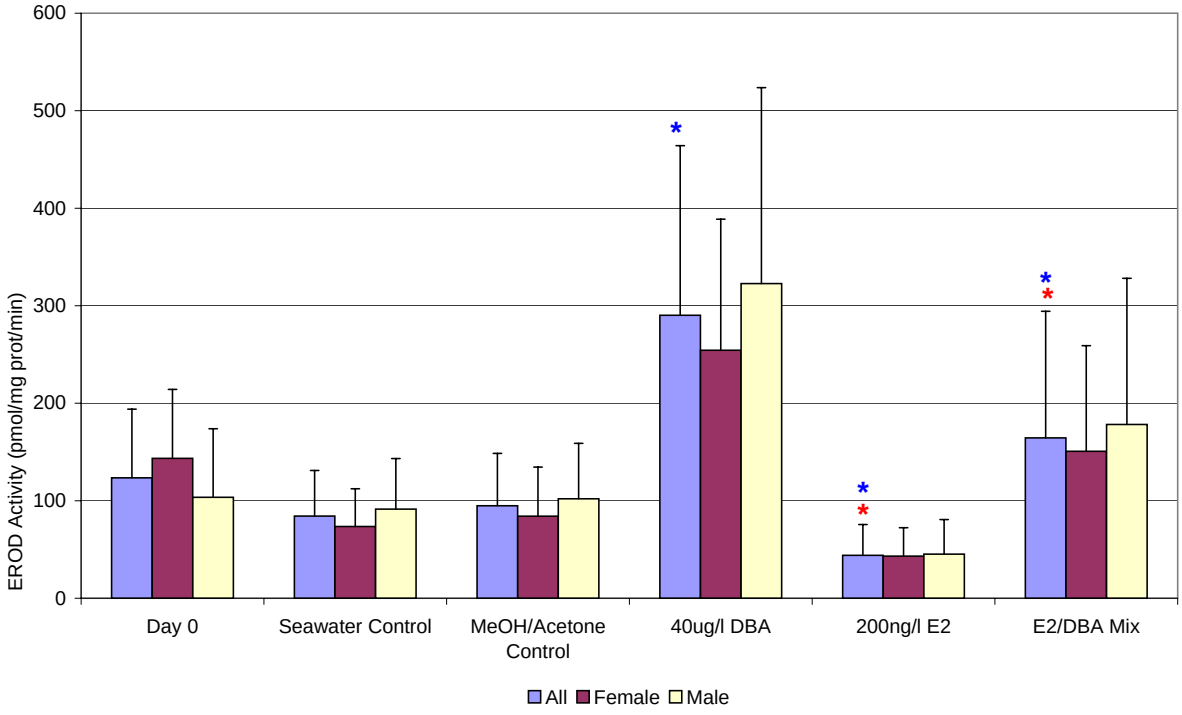


Figure 18. EROD activity in flounder dosed with DbA, E2, or a mixture of DbA and E2. * denotes significant difference compared to the control. * denotes significant difference from DbA

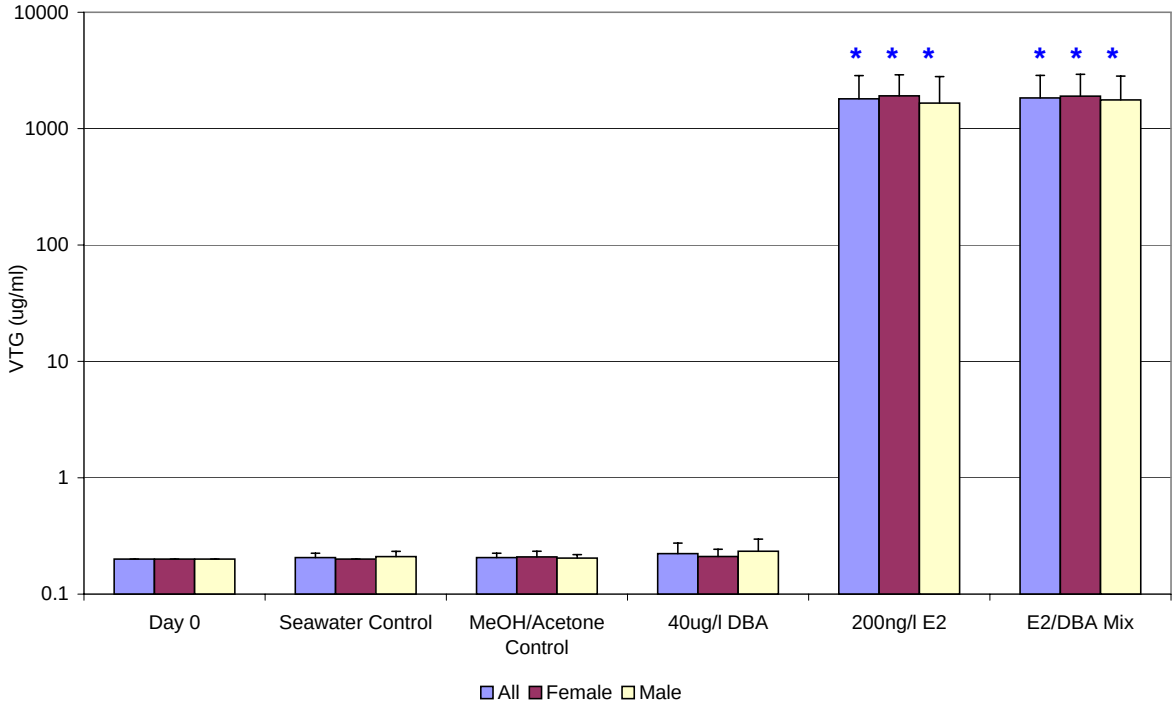


Figure 19. Plasma VTG concentrations in flounder dosed with DbA, E2, or a mixture of DbA and E2. * denotes significant difference compared to the control

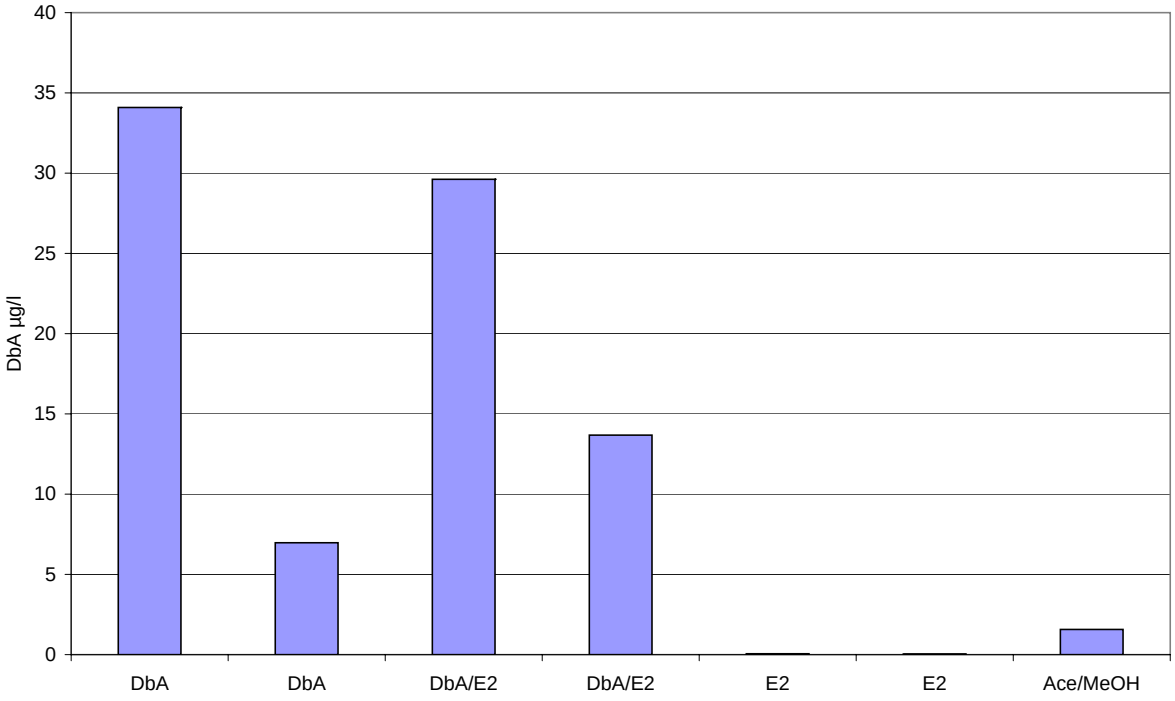


Figure 20. Chemical analysis of tank extracts from test using DbA, E2 or a mixture of DbA and E2

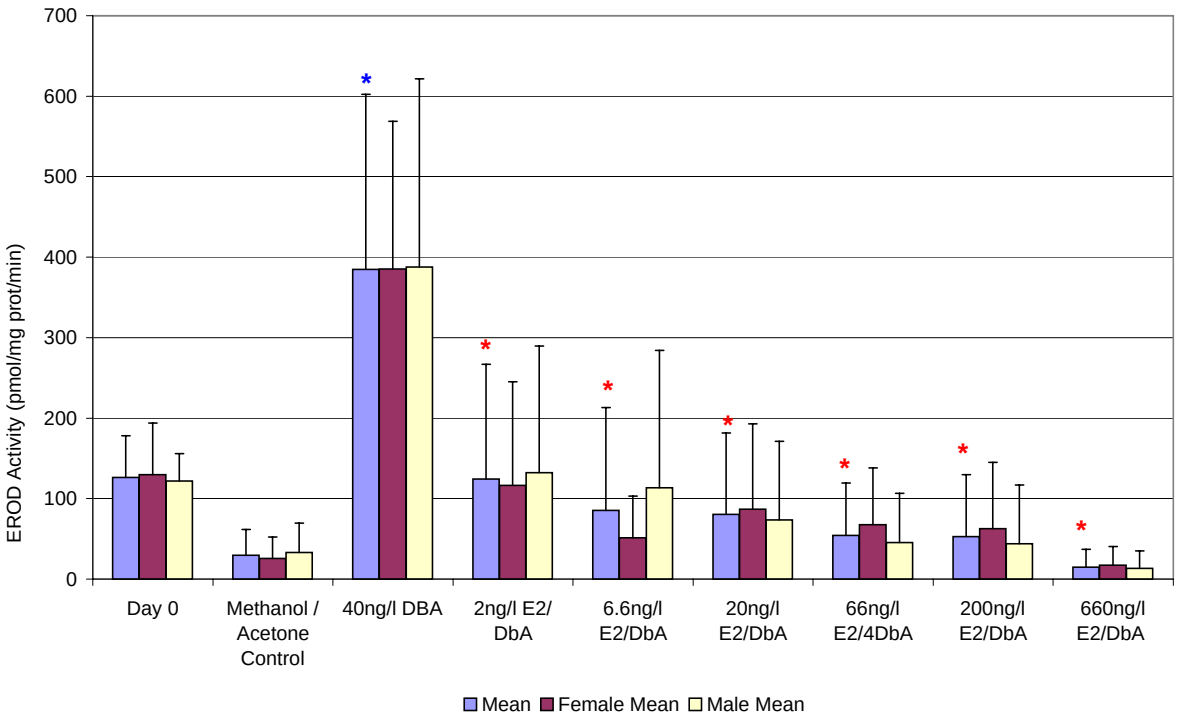


Figure 21. EROD activity in flounder dosed with DbA or a mixture of DbA and a concentration range of E2 from 2 ng/l to 660 ng/l. * denotes significant difference compared to the control. * denotes significant difference from DbA

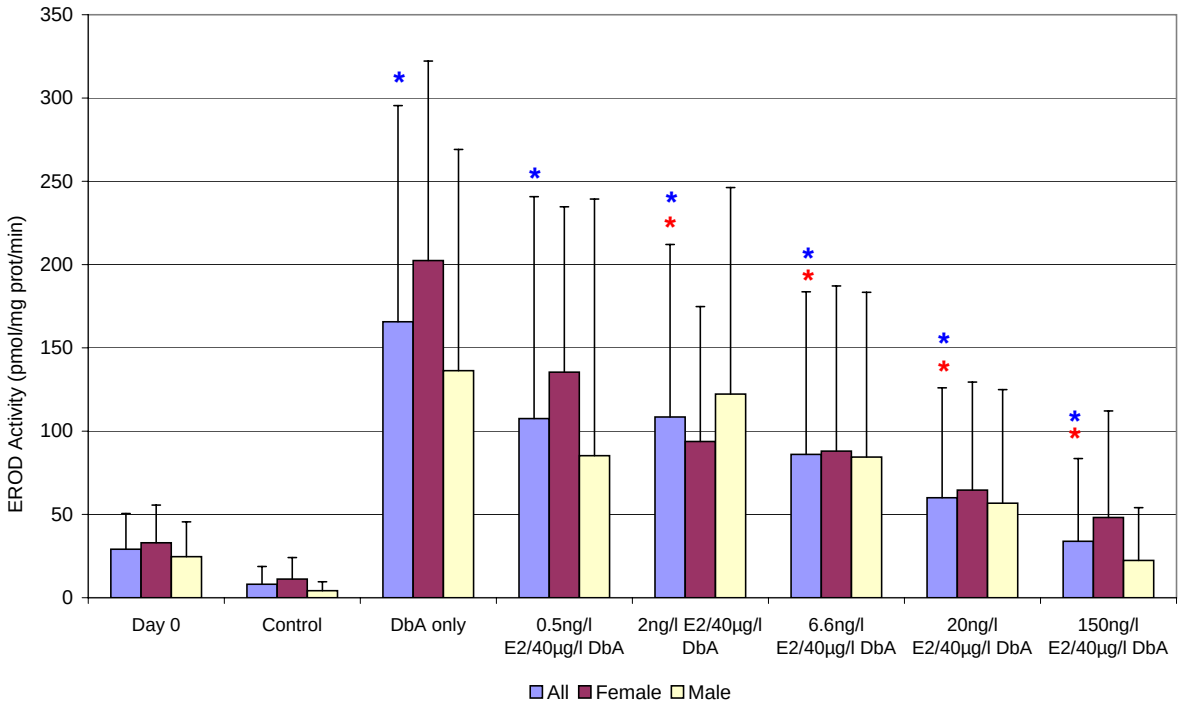


Figure 22. EROD activity in flounder dosed with DbA or a mixture of DbA and a concentration range of E2 from 0.5 ng/l to 150 ng/l. * denotes significant difference compared to the control. * denotes significant difference compared to the DbA alone. ANOVA only carried out on means data

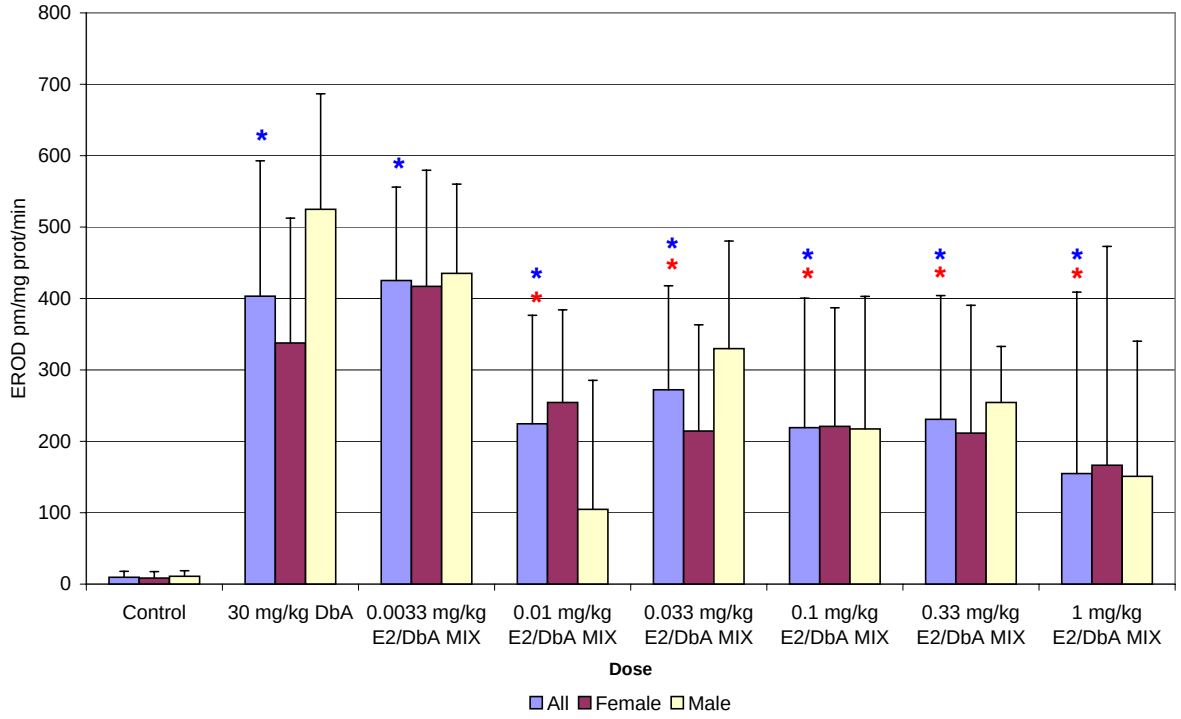


Figure 23. EROD activity in flounder dosed via IP injection with DbA or a mixture of DbA and a concentration range of E2. * denotes significant difference compared to the control. * denotes significant difference compared to the DbA alone. ANOVA only carried out on means data

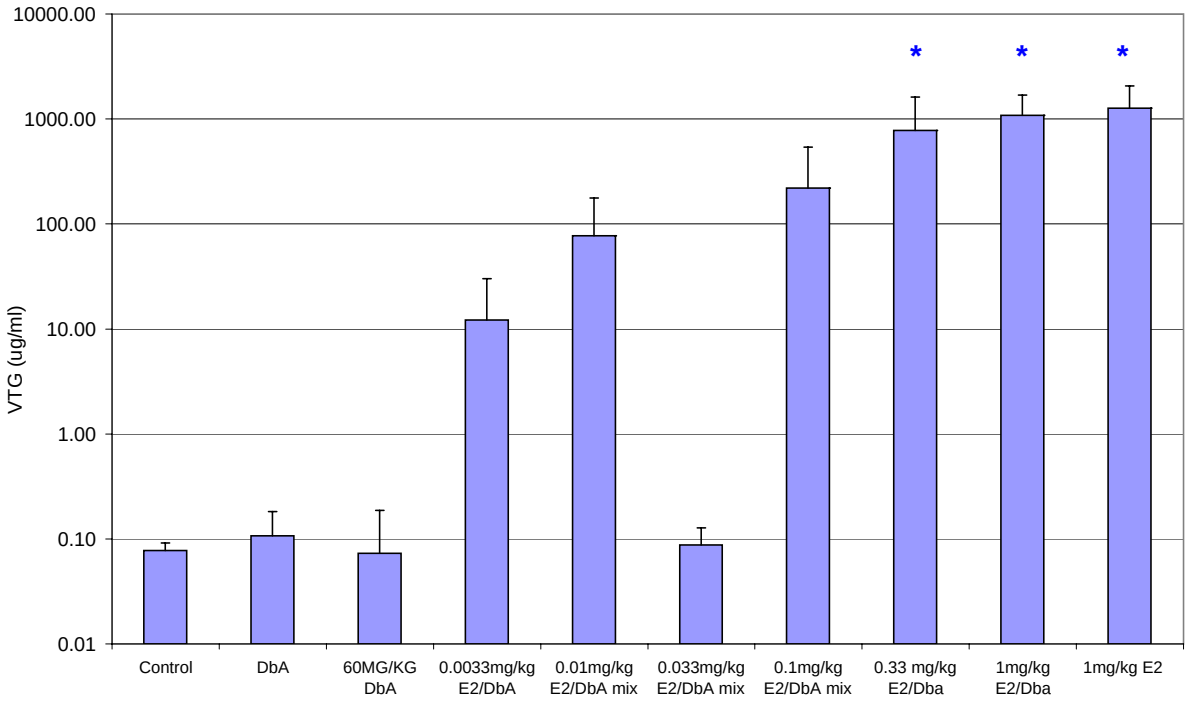


Figure 24. Plasma VTG concentration in flounder dosed via IP injection with DbA or a mixture of DbA and a concentration range of E2. * denotes significant difference compared to the control

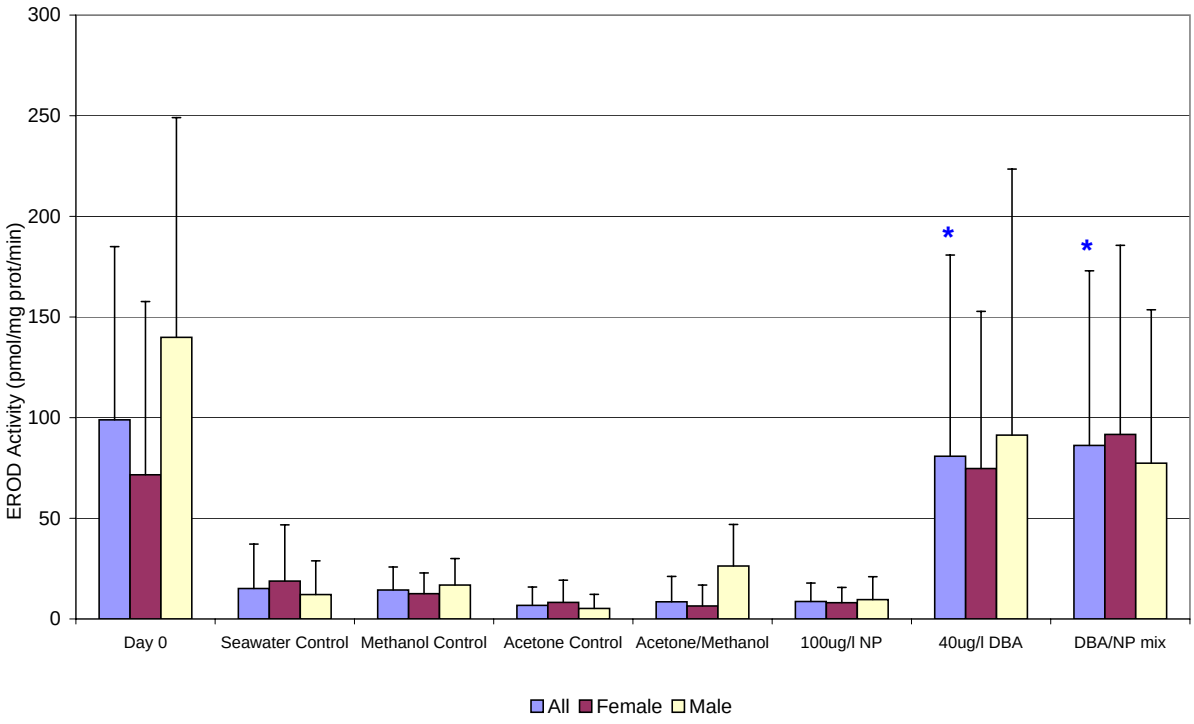


Figure 25. EROD activity in flounder dosed with DbA, NP, or a mixture of DbA and NP. * denotes significant difference compared to the control. ANOVA only carried out on means data

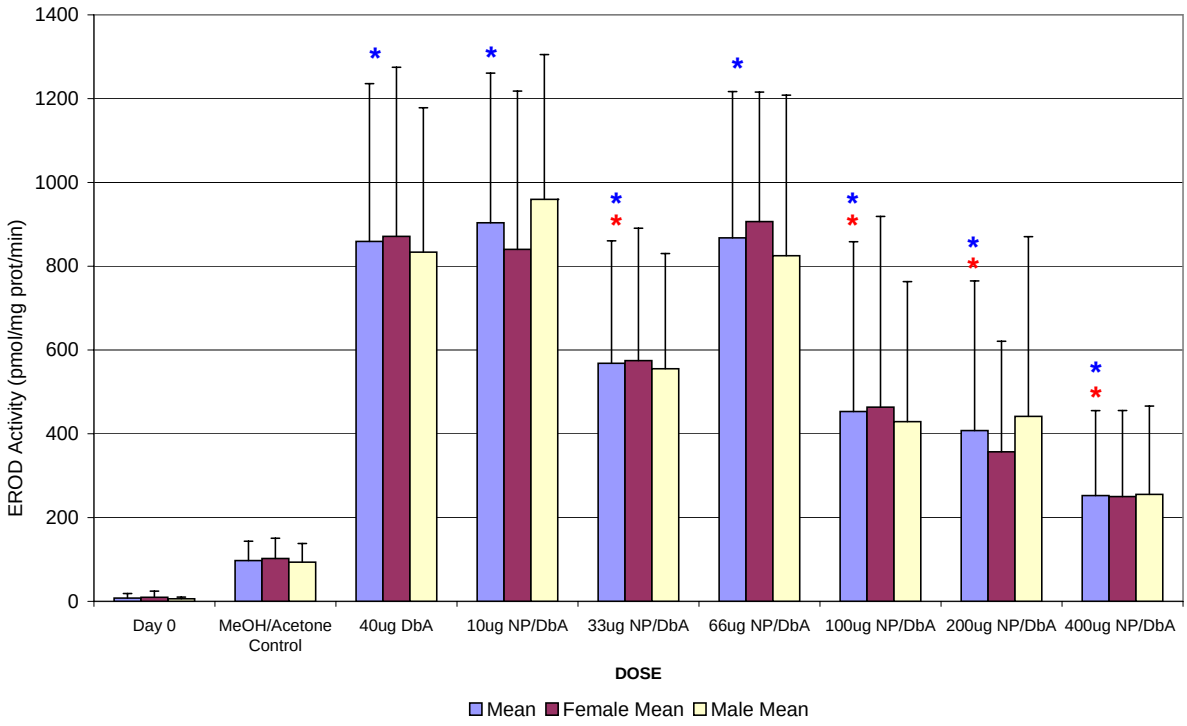


Figure 26. EROD activity in flounder dosed with DbA or a mixture of DbA and a concentration range of NP. * denotes significant difference compared to the control. * denotes significant difference compared to the DbA alone. ANOVA only carried out on means data

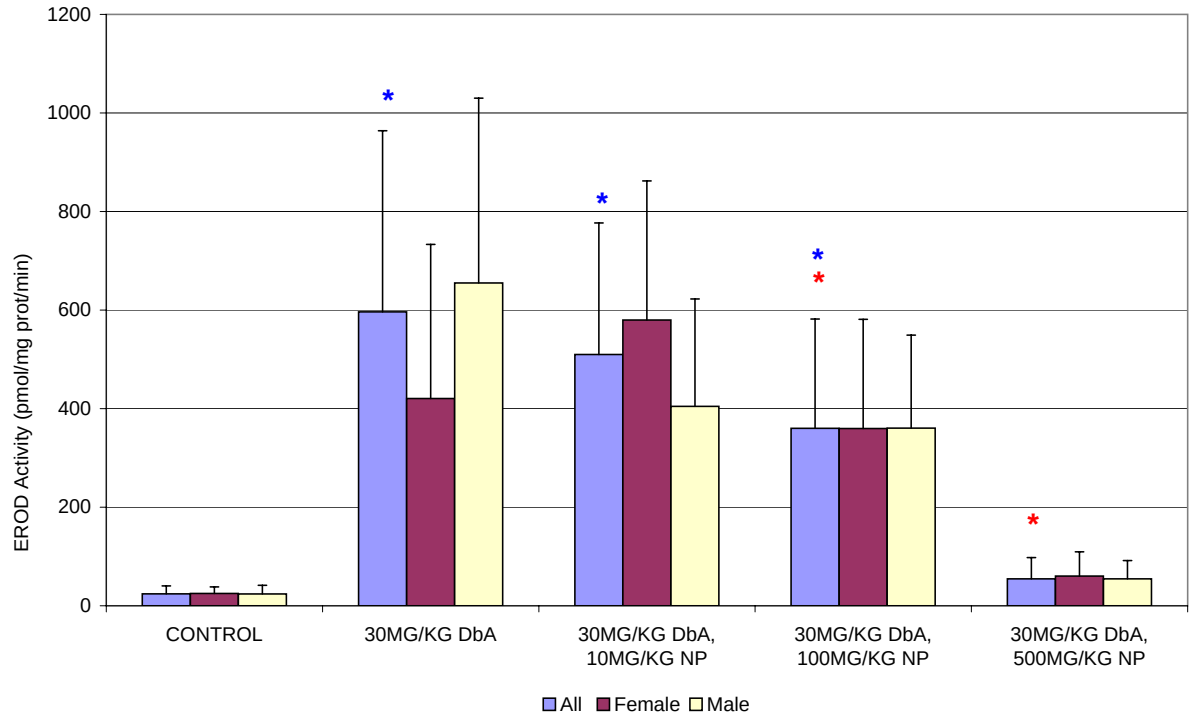


Figure 27. EROD activity in flounder dosed via IP injection with DbA or a mixture of DbA and a concentration range of NP. * denotes significant difference compared to the control. * denotes significant difference compared to the DbA alone. ANOVA only carried out on means data

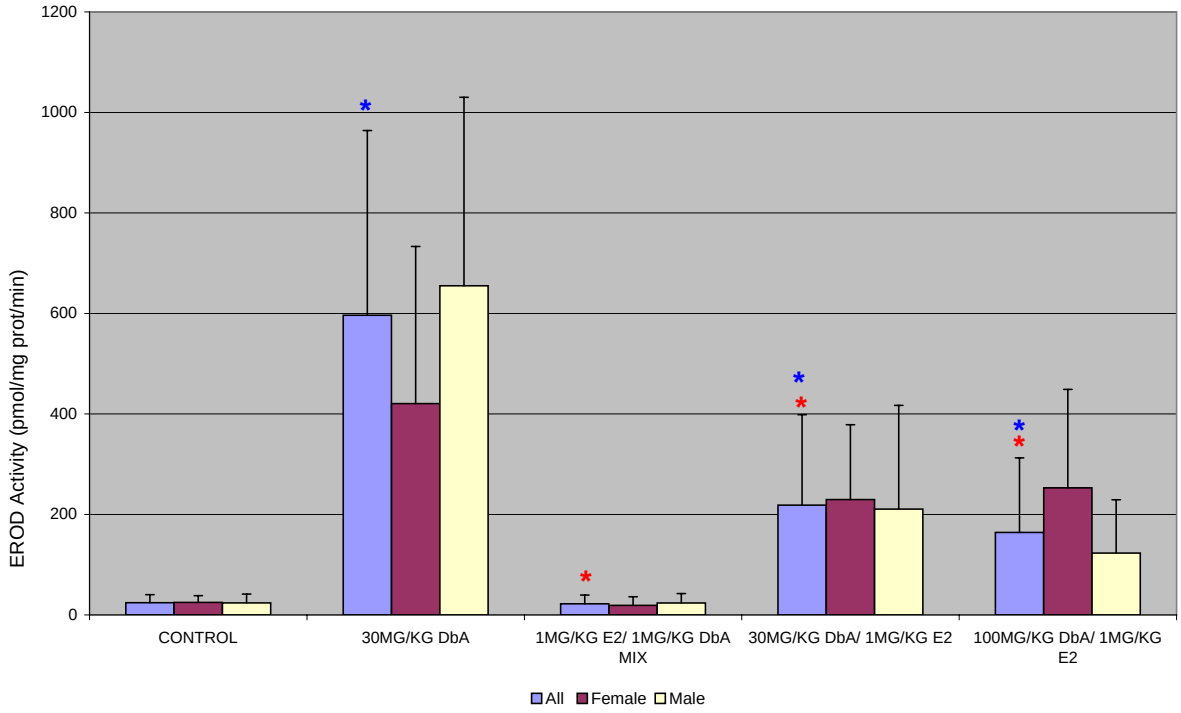
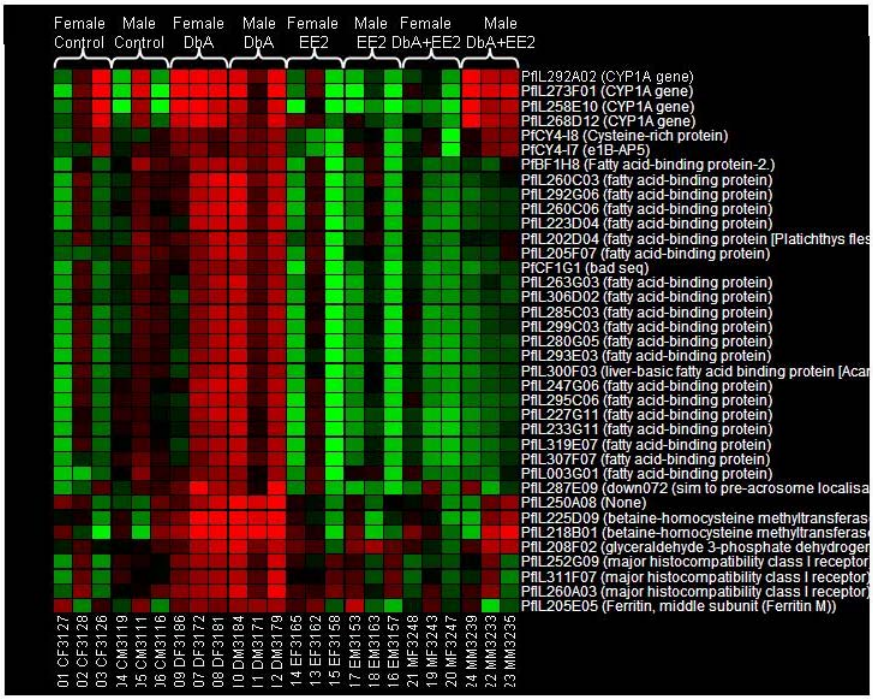


Figure 28. EROD activity in flounder dosed via IP injection with DbA on its own, or varying DbA doses with 1mg/kg E2. * denotes significant difference compared to the control. * denotes significant difference compared to the DbA alone. ANOVA only carried out on means data

A



B

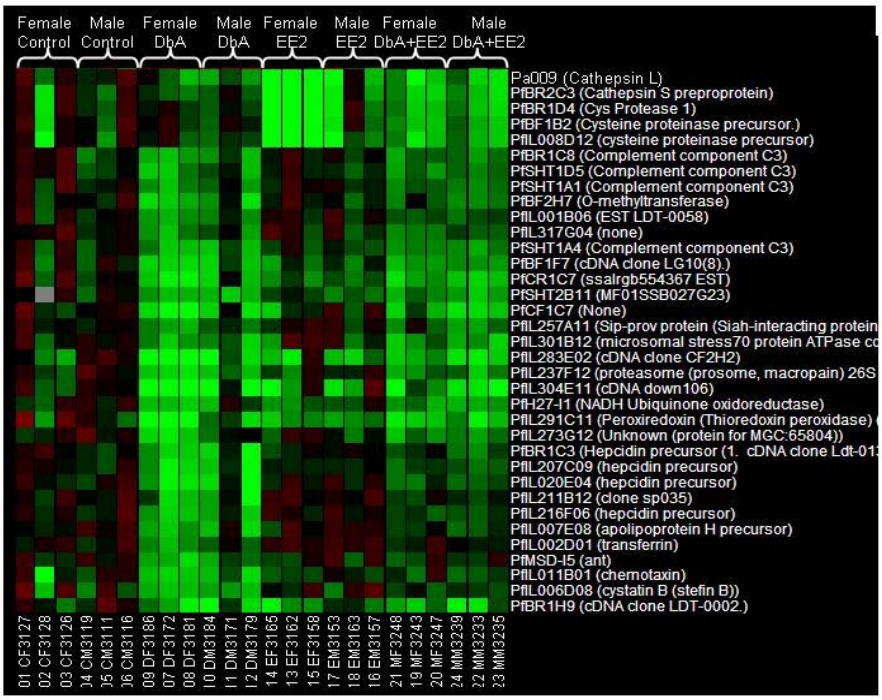
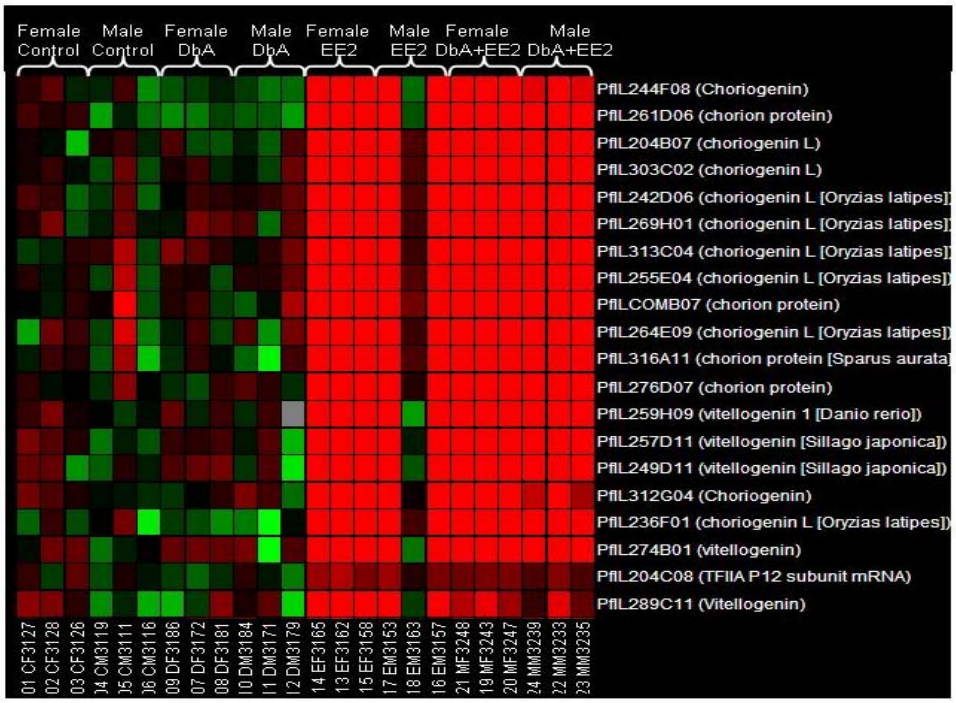


Figure 29. cDNA microarray results showing 4 groups clustered by gene expression profile. See text for details.Figures represent illustrations of genes differentially regulated (Red indicates induction, green repression, with respect to the mean of the control values, colour intensity indicates the magnitude of response, to a maximum intensity at 5-fold)

C



D

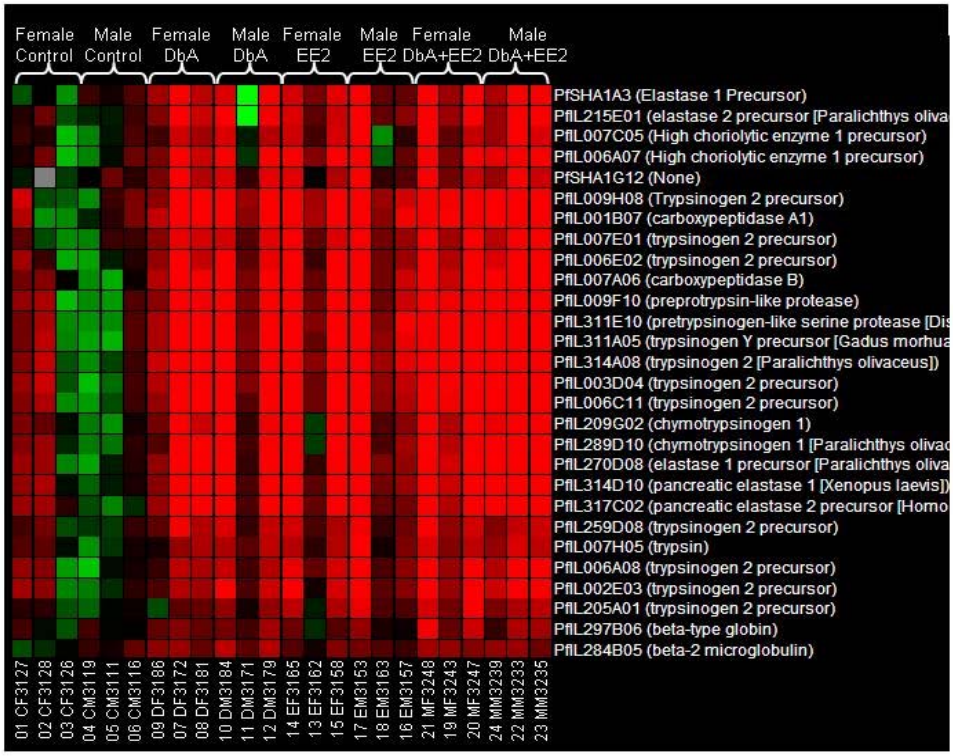
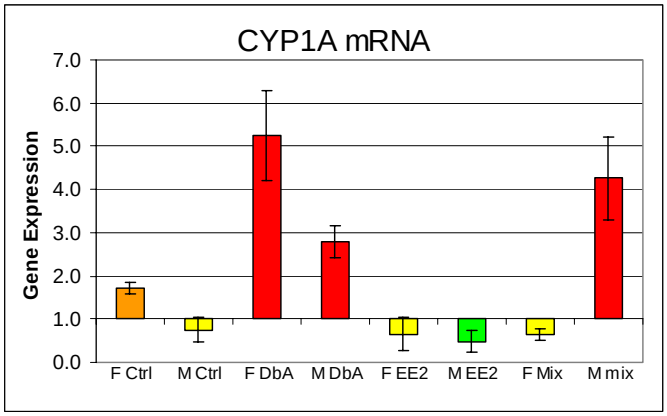
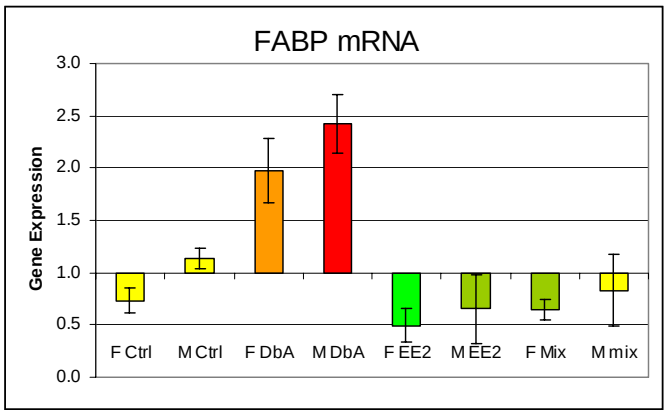


Figure 29. continued - cDNA microarray results showing 4 groups clustered by gene expression profile. See text for details.Figures represent illustrations of genes differentially regulated (Red indicates induction, green repression, with respect to the mean of the control values, colour intensity indicates the magnitude of response, to a maximum intensity at 5-fold)





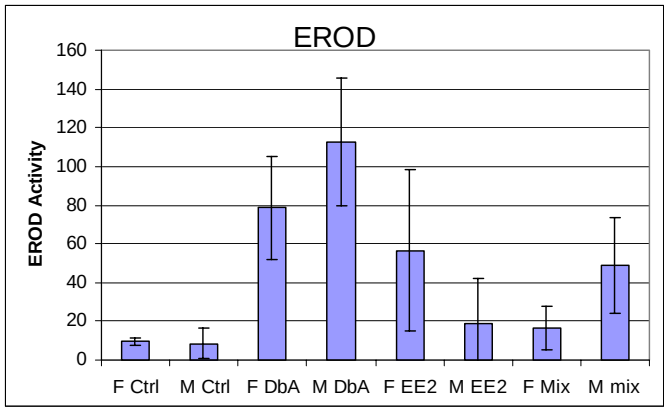
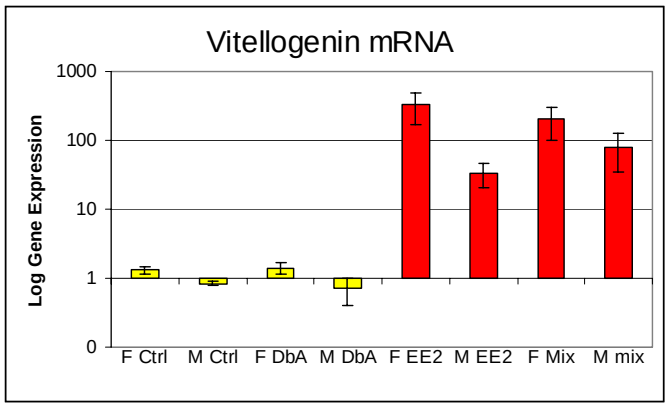
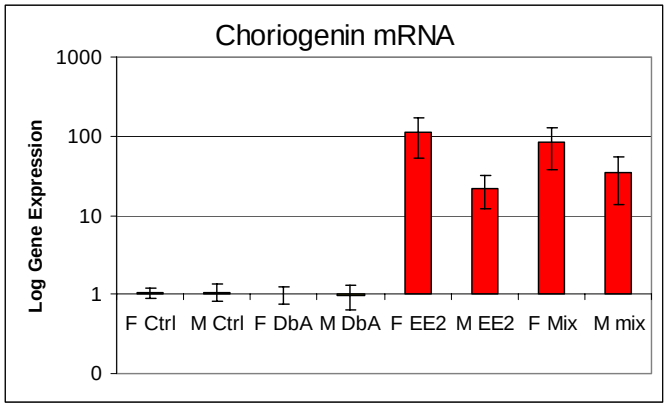


Figure 30. CYP1A mRNA, Fatty-acid binding protein mRNA and EROD activity for the flounder liver samples tested by microarray. Gene expression was calculated as the mean fold change over the mean for all control samples. Error bars indicate standard deviation. Note that a linear scale is used





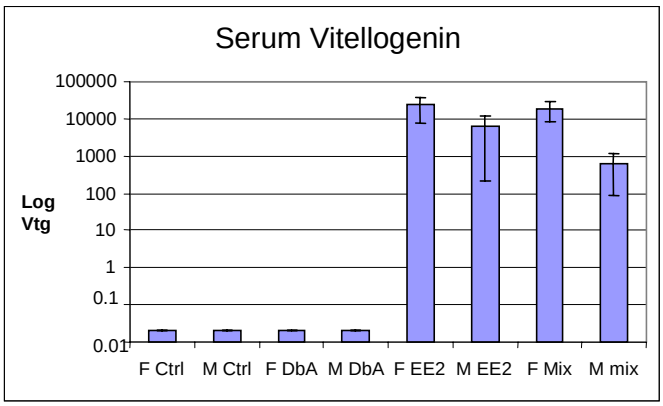


Figure 31. Vitellogenin mRNA, Choriogenin mRNA and serum Vitellogenin protein measurement for the flounder liver samples tested by microarray. Gene expression was calculated as the mean fold change over the mean for all control samples. Error bars indicate standard deviation. Note that a logarithmic scale is used

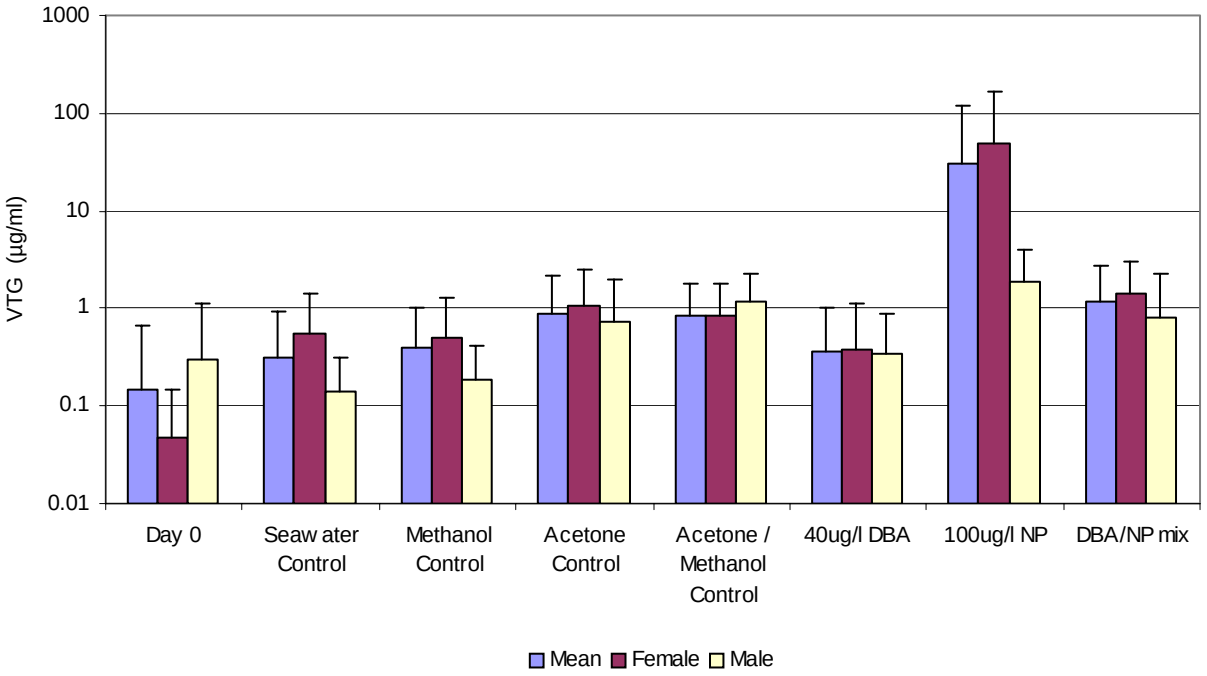


Figure 32. Plasma VTG concentrations in flounder dosed with DbA, NP, or a mixture of DbA and NP

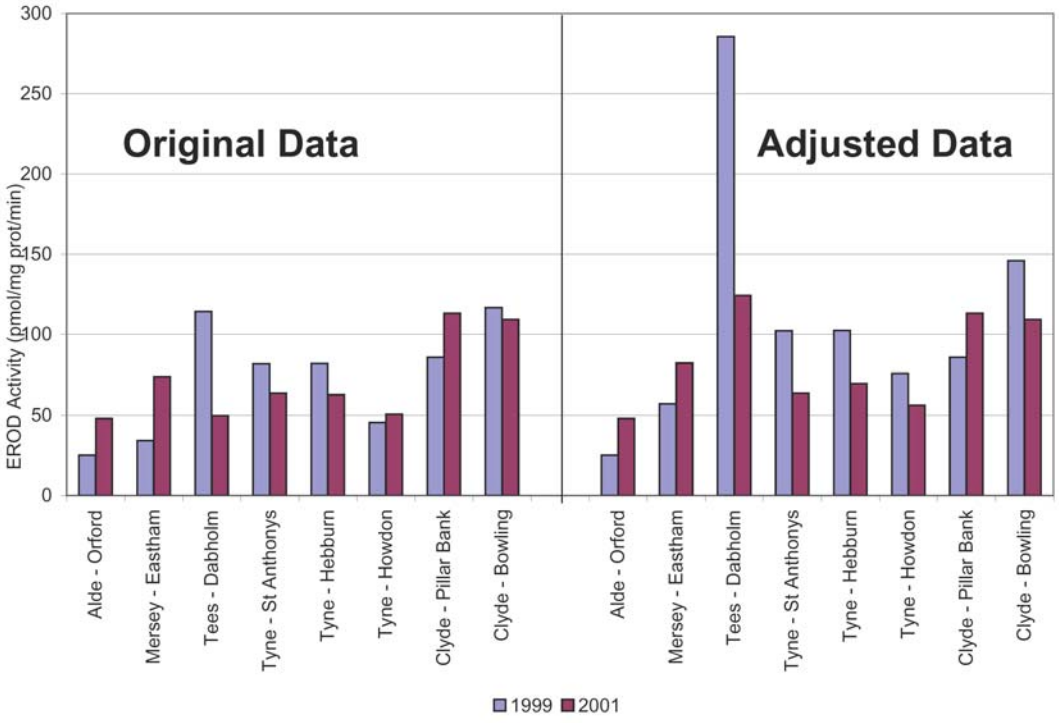


Figure 33. Hepatic EROD monitoring data in UK estuaries: Implications of level of plasma VTG adjustment

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