



# **Oestrogenic endocrine disruption in flounder (*Platichthys flesus* L.) from United Kingdom estuarine and marine waters**

**P. Matthiessen, Y. T. Allen, C. R. Allchin, S. W. Feist, M. F. Kirby, R. J. Law, A. P. Scott, J. E. Thain and K. V. Thomas**

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FLOUNDER (*PLATICHTHYS FLESUS* L.) FROM  
UNITED KINGDOM ESTUARINE AND MARINE WATERS**

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# 1. EXECUTIVE SUMMARY

- 1.1** This report describes one aspect of a phenomenon known as endocrine disruption (ED) which concerns the interference of environmental chemicals with the normal functioning of the endocrine (hormone) system. The aspect considered is that of substances in the UK marine environment which are able to mimic female vertebrate hormones (oestrogens) such as 17 $\beta$ -oestradiol, and thereby cause inappropriate feminisation of male fish (and other organisms).
- 1.2** It is already known that natural and synthetic oestrogenic hormones, and at least one industrial chemical, in sewage treatment works (STW) discharges are causing widespread feminisation of male river fish in the UK. This feminisation involves the unnatural synthesis of the yolk-precursor protein vitellogenin (VTG), and the induction of a condition known as ovotestis, in which egg cells develop in otherwise normal testes. Fish with this condition are described as 'intersex' although they remain genetically male. It is thought that this process may be damaging the ability of some freshwater fish to breed, but this has not yet been unequivocally established.
- 1.3** The present investigation set out to look for similar effects in the sea, and has focused on a common estuarine flatfish, the flounder (*Platichthys flesus* L.). This species, although not the basis of a significant fishery in the UK, was considered suitable for this survey because it is heavily exposed to contaminants associated with the muddy sediments in which it lives. Furthermore, it spends most of its life in its home river and estuary, only venturing offshore to breed. This means that studying feminisation in flounder will reveal something about the geographical location of the oestrogenic discharges which are contributing to any effects.
- 1.4** The primary techniques used in this survey included the measurement of VTG in male and female flounder, a search for feminised testes and other intersex conditions, and measurement of gonadal weights and sex ratios. Supplementary information was provided by chemical analysis for a range of contaminants in flounder liver tissue, measurement of certain steroid hormones in flounder, and measurement of the degree of induction of the cytochrome P450 mixed-function oxidase enzymes, a major system by means of which fish are able to degrade and excrete unwanted substances.
- 1.5** Vitellogenin levels in male blood plasma in the period autumn 1996 to spring 1998 were found to

be significantly elevated (in comparison with a clean reference site on the Alde estuary) in at least one sample from most of the 11 estuaries investigated. The exceptions were the Tamar and the Dee where fish appeared entirely normal. In broad terms, the degree of oestrogenic contamination as measured by male vitellogenesis in the various estuaries can be ranked in the following descending order: Tees > Mersey > Tyne > Wear = Humber = Clyde = Southampton Water = Thames > Crouch > Dee = Tamar. VTG concentrations in Tees, Mersey and Tyne fish were extremely high (>100,000 ng ml<sup>-1</sup>), and often exceeded those normally found in sexually mature females. There were no major differences between VTG levels measured in 1996/97 and 1997/98.

- 1.6** Flounder were also sampled from 5 coastal sites, and from several sites in the central southern North Sea. In all cases, VTG was significantly induced, and in the case of fish from Liverpool Bay and Red Wharf Bay (Anglesey), the degree of induction was large (although not as large as in the Mersey). Laboratory experiments indicated that VTG in male fish disappears rather slowly, with a half-life of about 2 weeks, and it is therefore suggested that the high VTG in fish at these two sites could have been induced in an estuary (probably the Mersey) before they migrated to sea to breed. However, local oestrogenic contamination at coastal and offshore sites cannot be entirely ruled out.
- 1.7** At most locations, ovotestis conditions in male flounder were entirely absent. However, in 1996, 17% of Mersey fish showed this abnormality, with large numbers of primary and secondary egg cells (oocytes) in the testes. In 1997, 9% of male Mersey fish and 7% of male Tyne fish contained ovotestis. In a few cases, eggs were fully developed with yolk granules. Most testes did not show gross morphological abnormalities related to oestrogenic exposure, although one testis from the Mersey appeared to be almost entirely composed of eggs. Unexpectedly, in view of the high levels of VTG induction, no intersex fish were seen in the Tees, but this is probably due to the fact that the sample size from this estuary was very small. Broadly speaking, ovotestis only seems to occur in flounder populations when mean plasma VTG levels in male adults exceed 100,000 ng ml<sup>-1</sup>.
- 1.8** Abnormal sex ratios were not seen in any estuary, although this is based on histological and not genetic data; i.e. it is possible that some of the overt females may have been genetic males. There was no consistent pattern to the distribution of testis:body weight ratios (i.e. gonadosomatic

ratio), but hepatosomatic ratios (i.e. liver:body weight) were increased in males which had elevated VTG. This shows that oestrogenic exposure had caused abnormal liver growth (hypertrophy) in order to synthesise VTG, thus placing strain on the metabolism of these fish.

**1.9** Due to resource limitations, only female Dee estuary flounder were sampled for natural steroid hormones (in September 1997). Male hormones (the androgens 11-ketotestosterone and testosterone) were not detectable, while the oestrogen 17 $\beta$ -oestradiol was only present at a low level in one fish. This was probably due to the season of sampling, and it would be desirable to conduct more analyses of this type in order to establish whether hormone metabolism is altered in the more oestrogen-contaminated fish.

**1.10** The pattern of induction of the P450 mixed function oxidase system in flounder liver (as measured by the activity of the enzyme ethoxyresorufin-*o*-deethylase, or EROD) showed considerable similarities with the pattern of VTG induction, with highest EROD activity in the Mersey, Tees and Tyne, intermediate activity in the Humber, Wear and Southampton Water, and low activity in the Alde reference site. It is possible that the EROD was being induced by the same substances responsible for oestrogenicity, but even if not, there may still be reproductive implications because the P450 system is responsible for metabolising steroids. The main EROD inducers are the planar polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins and furans (PCDDs and PCDFs), and polycyclic aromatic hydrocarbons (PAHs), several of which are known to have endocrine disrupting properties.

**1.11** In broad terms, the flounder from the Mersey and Tees were the most contaminated with organochlorines and PAHs, although appreciable amounts of PCBs were also present in Thames and Tamar fish. However, the levels of these contaminants in liver were rather low (max. median  $\Sigma$ PCB = 1.3 mg kg<sup>-1</sup> wet wt.; max. median  $\Sigma$ DDT = 0.5 mg kg<sup>-1</sup> wet wt.; max.  $\Sigma$ PAH = 0.4 mg kg<sup>-1</sup> wet wt.). It is doubtful whether these residues made a major positive or negative contribution to the observed oestrogenic effects, but they at least indicate that the fish which were most contaminated with common manmade chemicals were also those most impacted by oestrogenic hormones and/or their mimics. It is clear that more investigative chemistry is required to identify the causative compounds.

**1.12** It seems unlikely that the major causative substances will prove to be oestrogenic hormones derived from domestic sewage, although these

materials are almost certainly contributing to the observed effects. There is little correlation between VTG induction and the volumes of domestic sewage discharged to each estuary, but there is a much clearer relationship with the volume of industrial effluent. Again, this does not prove a causal link with industrial chemicals, but it suggests that non-hormonal substances are major players.

**1.13** The full biological implications of these results are not yet understood, but it is a possibility that flounder in the most contaminated estuaries (Tees, Mersey, Tyne) have impaired reproductive output and reduced overall fitness. Whether this is likely to have long-term implications for the survival of these flounder populations is unknown, but it seems unlikely that flounder in the UK as a whole are seriously endangered from oestrogenic exposure. However, it should be noted that the most oestrogen-sensitive part of the flounder's life cycle (the larva undergoing gonad formation) occurs at sea where contaminant levels are relatively low. It is therefore likely that species which breed in estuaries will be experiencing more serious effects.

**1.14** It is recommended that further research is conducted in order to clarify the following:-

- What are the major oestrogenic substances in UK estuaries and what are the main sources?
- What are the implications for the reproductive output (and hence for populations) of fish species which breed in the more contaminated situations?
- What impacts are oestrogens having on the reproductive output of other estuarine organisms (e.g. crustaceans and molluscs)?
- Are UK estuaries significantly contaminated with other endocrine disrupters such as androgen and thyroid hormone mimics, and their antagonists?

Many of these questions are being addressed by a new 4-year research programme (**Endocrine Disruption in the Marine Environment, or EDMAR**) funded mainly by the British Government (Ministry of Agriculture, Fisheries and Food – MAFF; Department of Environment, Transport and the Regions – DETR; Environment Agency – EA; Scotland and Northern Ireland Forum for Environmental Research – SNIFFER), but also by the European Chemical Industry Association (CEFIC). The programme is being managed by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS), but other collaborating organisations include Fisheries Research Services (FRS) Marine Laboratory, Aberdeen, Zeneca Brixham Environmental Laboratory, and the Universities of Liverpool and Plymouth.



## 2. INTRODUCTION AND BACKGROUND

### 2.1 Introduction to endocrine disruption

The endocrine system is the primary means by which multicellular organisms control their biochemical, reproductive, physiological and developmental functions. This very complex system is orchestrated by the brain via the hypothalamus and pituitary, using substances known as hormones which act as so-called chemical messengers. These are extremely potent, and generally have physiological activity in concentrations of parts per trillion or lower. Put simply, most hormones are transported by the blood and 'dock' with cellular receptor protein molecules uniquely sensitive to them, the resulting hormone-receptor complex then interacting with specific DNA sequences in the cell nucleus. This in turn leads to the transcription of particular genes into proteins which go on to cause a cascade of changes resulting in the ultimate effect for which the hormone is the trigger (e.g. yolk production). Clearly, any external interference with this system could have far-reaching and potentially damaging consequences.

This report concerns one aspect of a pollution phenomenon now known as endocrine disruption which has probably been in existence since man-made organic chemicals were first released into the environment. Indeed, a process which could be termed endocrine disruption has a much longer history because plants have almost certainly been chemically disrupting the endocrine systems of animals for defensive purposes since herbivores first evolved, although this can scarcely be described as pollution. However, it has only been in the last decade that a variety of disparate environmental chemicals has been recognised as having common modes of action on the endocrine system, and consequent harmful impacts on organisms.

An endocrine disrupter (ED) is now defined (European Commission, 1996) as **“an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function”**. However, there is debate about whether *any* effect on the endocrine system (adverse or otherwise) should be described as disruption. This is akin to the wider argument about whether small changes in biochemical or cellular markers of pollution, because they may predict long-term damage, can rightfully be described as adverse in themselves when they may lie entirely within an organism's normal homeostatic range. Clearly, modulations of the endocrine system are occurring all the time due to entirely natural environmental factors, so small perturbations caused by anthropogenic chemicals may not push the system into the pathological range. The key questions are therefore whether biochemical systems, and the cells which

contain them, can adapt successfully to chemical exposure, and if not, to what extent there will be knock-on consequences for individuals and populations.

Since the early 1990s, there has been growing interest in the phenomenon of endocrine disruption (ED). Theo Colborn drew popular attention to this type of pollution in a book entitled *Our Stolen Future* (Colborn *et al.*, 1996), and it is the subject of numerous research programmes, reviews, workshops and conferences (e.g. Colborn *et al.*, 1993; IEH, 1995; Toppari *et al.*, 1995; European Commission, 1996; Kavlock *et al.*, 1996; Tattersfield *et al.*, 1997; Kendall *et al.*, 1998). In Britain, ED effects are now a high priority for evaluation by the Environment Agency which has recently issued a document setting out its policy for tackling the issue (EA, 1998(a)).

ED embraces a multitude of mechanisms of action, including effects on growth, behaviour, reproduction and immune function. The best-known form of ED involves substances which mimic or block the action of hormones at their receptor molecules (i.e. agonistic or antagonistic action), but it is also possible for xenobiotic substances to interfere with receptor protein synthesis itself, to affect the synthesis, metabolism, transport or excretion of hormones, to interfere with the feedback mechanisms operating through the hypothalamus and pituitary gland, or to damage endocrine organs directly. By far the most intensely studied type of ED concerns substances which mimic or antagonise the steroid hormones (e.g. oestrogens), thereby potentially producing unwanted effects on *inter alia* the vertebrate reproductive system. However, environmental examples do exist of several other possible ED effects in several invertebrate taxonomic groups, including insects, crustaceans and molluscs, as well as in vertebrates like fish, reptiles, birds and mammals.

Ironically, the most widely-publicised type of putative ED concerns certain effects in humans, including the declining sperm count in some populations (Swan *et al.*, 1997) and the rising incidence of testicular and breast cancer (Hakulinen *et al.*, 1986), but none of these have yet been firmly identified as being caused or potentiated by EDs (specifically, oestrogens). There is no doubt, however, that humans like other vertebrates are potentially at risk of ED effects, all vertebrates sharing essentially the same system of steroid hormones. This is evidenced by the tragic consequences for some children during the 1950s to 1970s of prescribing the synthetic steroid diethylstilbestrol (DES) to their pregnant mothers to prevent spontaneous abortion (Linn *et al.*, 1988). Effects observed in vertebrate wildlife can therefore be considered as early warnings of possible impacts on humans.

It is clear that some of the effects now labelled as ED have been known for a long time (e.g. Dodds *et al.*, 1938), but the recent interest stems largely from the fact

that these substances have the potential to act at often very low concentrations, and that some effects (e.g. intersex or hermaphrodite conditions) can be triggered during embryonic or larval development, but can only be expressed during adulthood or in subsequent offspring.

Furthermore, particular agonists and their mimics, for example, are able to act additively at their receptors (synergistic action has not been clearly demonstrated). Many apparently unrelated but nevertheless agonistic or antagonistic substances, at individually negligible concentrations, can therefore potentially contribute to adverse effects in organisms exposed to complex mixtures. Such exposure is the norm for wildlife, particularly aquatic organisms in rivers and estuaries, and raises the possibility that current chemical-specific risk assessment procedures may be inadequate for quantifying the full range of environmental effects of new chemicals. Indeed, the recent EMWAT workshop (Tattersfield *et al.*, 1997) made it clear that present chemical hazard assessment procedures for wildlife are almost entirely unsuitable for detecting ED, although some types of reproductive toxicity can, of course, be picked up in standard tests for pesticides.

## 2.2 Background to research in United Kingdom estuaries

This report describes an investigation into the occurrence and effects of oestrogenic contaminants in United Kingdom estuaries, but it grows out of previous research in both the freshwater and marine environments. Indeed, the majority of ED effects reported to date in the UK and elsewhere are connected with aquatic systems (Matthiessen and Sumpter, 1998).

The earliest known example of endocrine disruption in UK wildlife concerns the effects of the anti-fouling compound tributyltin (TBT) on marine molluscs (Champ and Seligman, 1996; Matthiessen and Gibbs, 1998) which were first observed in the UK in the early 1970s. This substance inhibits the normal metabolism of the androgenic hormone testosterone to the oestrogen 17 $\beta$ -oestradiol, and probably also interferes with testosterone excretion, thus causing testosterone to accumulate and produce masculinisation in female gastropods and bivalves. The best known of these effects is the phenomenon of imposex in dogwhelks which causes females to grow a penis and become sterile. This has resulted in the elimination of many UK dogwhelk populations and is also having effects on edible whelks in the vicinity of shipping lanes. In addition, TBT has caused more subtle, but nevertheless serious, effects on the sexuality of other molluscs (periwinkles and bivalves), all of which led to its withdrawal in 1987 from use on small vessels. However, continuing environmental effects are still being caused by the use of TBT on larger vessels, indicating the desirability of a total international ban on its use.

Other reliable examples of ED in UK marine waters are scarce, although Moore and Stevenson (1991) have reported intersexuality in harpacticoid copepods from the Firth of Forth which appears to be associated with certain sewage discharges, and Waring *et al.* (1996) reported that a 1% suspension of sewage sludge impairs reproduction in sand gobies. However, a range of effects (reproductive precocity or inhibition, developmental abnormalities, immunosuppression, cancers) in crustaceans, fish and mammals from other marine areas have also been attributed to endocrine disruption caused by a number of lipophilic organochlorine contaminants, although cause-effect data are rare. Perhaps the best experimental data were obtained by Reijnders (1986) who conducted a large reproduction experiment with common seals, and showed that females fed on PCB-contaminated fish from the Wadden Sea failed to become pregnant, while those fed on relatively uncontaminated fish bred normally. It is probable that this effect was caused by PCB-induced vitamin A and thyroid hormone deficiency in which a PCB metabolite displaced retinol and thyroid hormone from their plasma protein transport complex (Brouwer *et al.*, 1989). Similar work by Ross *et al.* (1995) showed that contaminated Baltic herring caused immunosuppression in seals, although it is not certain whether this was due to ED. It is possible, perhaps even probable, that effects of this type have been responsible for declines in various marine mammal populations, and for certain disease outbreaks in seals and cetaceans, but proof is lacking. Other experimental data with flounder held in mesocosms containing contaminated harbour dredgings (Janssen *et al.*, 1997) show that certain unidentified contaminants are responsible for precocious female maturation, and field observations with flatfish in industrialised North American waterways (Johnson *et al.*, 1997) have shown similar associations. Subtle related observations have been reported from certain North Sea and Baltic Sea fish populations (Lang *et al.*, 1995; Rijnsdorp and Vethaak, 1997; Petersen *et al.*, 1997), but biomarkers of endocrine disruption were not measured, so alternative explanations are possible.

Treated domestic sewage discharges have been identified as a major cause of oestrogenic effects on fish in the United Kingdom and United States freshwater environments (e.g. Purdom *et al.*, 1994; Folmar *et al.*, 1996). In the first instance, this was detected by measuring the biomarker vitellogenin (VTG) in the blood plasma of male fish. VTG is the protein precursor of yolk and is synthesised by the liver exclusively in response to oestrogens. In males there is almost no natural oestradiol (normally produced by the ovaries in response to gonadotropin hormone originating in the pituitary), so VTG induction in these fish is an excellent marker of exposure to exogenous oestrogenic materials. Using caged rainbow trout, Harries *et al.* (1995, 1996, 1997) have shown that some UK rivers downstream of sewage treatment works (STW) discharges are oestrogenic for several kilometres, although the effect

usually declines rapidly with distance due to dilution and other processes. Furthermore, testicular growth can be retarded in the caged animals, and this effect has been replicated in laboratory experiments with alkylphenols (Jobling *et al.*, 1996). Recent results by Jobling and co-workers at Brunel University (Jobling *et al.*, 1998) have now shown that this oestrogenic exposure is accompanied in most instances by the presence of intersex conditions in wild roach. In some cases (e.g. Rivers Aire and Nene), 100% of the male fish contain oocytes in their testes (ovotestis), and effects are more marked below STW discharges in comparison with upstream stretches partially isolated by means of weirs. No UK roach populations appear to be totally free of ovotestis, but it is not known whether background levels of this condition are natural or due to the absence of completely pristine surface waters.

Many of the effects in rivers can be attributed to natural and synthetic oestrogenic hormones derived from glucuronide-conjugated material excreted by women and livestock (Desbrow *et al.*, 1998). De-conjugation occurs in sewage treatment works and in rivers, leading to the reappearance of fully potent hormone. However, other substances certainly contribute to the effects – for example, much of the response in the River Aire has been due to nonylphenol originating from wool scouring processes (Harries *et al.*, 1995), and it is probable that other substances (perhaps including phyto-oestrogens derived from plants) make significant contributions in some places.

Sewage and industrial waste enters estuaries and the sea as well as rivers (both from riverine discharges, and directly), so the question arose whether marine fish were also being affected by oestrogenic materials. As it had already been established (Harries *et al.*, 1995) that very little dilution was required to completely nullify the oestrogenic effects of very potent treated sewage in which the major contaminants were oestrogenic hormones, it was expected that effects would be minimal in the sea due to the generally good dilution available in most coastal waters. However, an initial investigation of wild flounder (*Platichthys flesus*) in the Tyne estuary by Lye *et al.* (1997) at Newcastle University showed that VTG was indeed present in some males, although the amounts were not quantified. It was therefore decided by CEFAS, with financial support from MAFF and logistic support from the Environment Agency, to carry out a wider survey of wild flounder, after having developed a radio-immunoassay with which to quantify flounder VTG.

Flounder (Plate 1) seemed to be a good choice for this work, for several reasons. Although the UK fishery is small, this flatfish (which grows up to about 50 cm in length) is reasonably easy to catch by beam trawling and is widespread in muddy estuaries throughout the British Isles. It is the only European flatfish which also makes migrations into freshwater, but it spends most of its life

in the low-salinity waters of its home estuary, and only migrates into the open sea to breed. These breeding migrations in the March-June period can exceed 300 km but are generally less than 30 km, down to depths of about 55 m. After the floating eggs hatch, the larvae gradually sink and metamorphose into young flatfish at a length of 15-30 mm and return to estuarine waters, thereafter residing on the bottom. The young fish feed on copepods and diatoms, but as they grow, they graduate to eating a large variety of other invertebrates including amphipods, mysids, decapod shrimps, young crabs, bivalves and polychaetes. The males reach adulthood at 11 cm, and the females at 17 cm.

It was therefore expected that flounder would be more heavily exposed to sediment-associated lipophilic contaminants than the midwater fish studied in rivers, both through direct exposure when they bury themselves in sediment, and through their heavy consumption of benthic invertebrates. Furthermore, their confinement to a home estuary for at least 8 months of the year ensures that any contaminant-related effects will probably be due to local inputs. A possible disadvantage is that the most oestrogen-sensitive part of the life cycle, that of sexual differentiation in the larvae, probably occurs at sea under relatively uncontaminated conditions, although the precise period of greatest sexual lability is unknown.

The major objectives of the project described in this report (MAFF project code A1107, 1995-1998) were therefore to establish whether oestrogenic materials are present in UK estuarine and marine waters at biologically significant concentrations, and to investigate some of the possible effects which these may have in the flounder. Some of the early results have already been published by Allen *et al.* (1997, 1998).

### 3. METHODS

#### 3.1 Development and operation of a radioimmunoassay for flounder vitellogenin

##### 3.1.1 Introduction

The radioimmunoassay (RIA) for flounder vitellogenin VTG, required the following steps:

- Male flounders were injected with oestradiol (E<sub>2</sub>) in order to induce VTG production
- After 2 weeks, the fish were bled and the plasma was collected
- VTG was precipitated from the plasma by addition of salts and distilled water
- VTG was further purified by ion-exchange chromatography and then freeze-dried
- Some of the VTG was injected repeatedly into rabbits in order to induce antibody production

- Once the antibodies were formed (3 to 4 months), VTG was radiolabelled with Iodine-125, a standard was made up and a set of validation experiments were carried out to establish the optimum concentrations of reagents and incubation times for RIA.

### 3.1.2 Purification of flounder vitellogenin

#### *Induction of VTG production*

Powdered E<sub>2</sub> was added to melted cocoa butter at a concentration of 50 mg ml<sup>-1</sup>. The powder was then evenly dispersed in the butter by ultrasonication (in a bath containing warm water) and injected intramuscularly into 10 male flounders (200-400 g) at the rate of 400 µl (20 mg) per kg of fish. Blood was collected after an interval of 2 weeks.

#### *Collection of blood*

Prior to collection of the blood, 2 ml syringes were rinsed with a saline solution containing 8 Trypsin Inhibitor Units (TIU) ml<sup>-1</sup> of aprotinin and 500 IU ml<sup>-1</sup> sodium heparin (to prevent blood clotting). Blood was transferred to 4 ml collection tubes, on ice, which contained 50 µl of heparin solution, with AEBSF and aprotinin at 1 mg ml<sup>-1</sup> and 8 TIU ml<sup>-1</sup>, respectively. A maximum of 2.5 ml blood was added to each tube, spun at 2000 rpm and 4°C for 30 minutes and the plasma removed, frozen and stored in liquid nitrogen.

#### *Precipitation of VTG from plasma*

The protocol which is described here is closely based on that described by Norberg (1995) for the preparation of Atlantic halibut (*Hippoglossus hippoglossus*) VTG. Beforehand, the following solutions were made up and chilled on ice: 20 ml 20mM ethylenediaminetetraacetic acid (EDTA; adjusted to pH7.6 with 2M NaOH), containing 20 mg AEBSF; 2 ml 0.5M MgCl<sub>2</sub>, containing 20 mg AEBSF; 100 ml distilled water, containing 36 mg AEBSF; 4 ml 1M NaCl in 50 mM Tris(hydroxymethyl)aminomethane (Tris) pH 8.0, containing 20 mg AEBSF and 0.8 TIU aprotinin; 20 ml 50 mM Tris pH 8.0, containing 20 mg AEBSF and 3.2 TIU aprotinin.

Plasma samples from two E<sub>2</sub>-injected male fish were thawed and dispensed as 1 ml aliquots into 150 mm x 16 mm glass tubes. Each tube then received 150 µl of the MgCl<sub>2</sub> solution, 3 ml of the EDTA solution and 15 ml distilled water. This produced a heavy white precipitate. The precipitate was compacted by centrifugation, the supernatant discarded, washed with 3 ml distilled water and compacted again.

#### *Chromatographic purification*

The precipitates from all tubes were redissolved, pooled in a maximum of 600 µl of the Tris/NaCl solution and then slowly made up to c. 20 ml with the Tris buffer.

This solution was then injected through a 43 micron filter to remove particulate matter and loaded on to a prepared DEAE-Sephacel column (1 cm i.d. x 26 cm) at a rate of 0.5 ml min<sup>-1</sup>, and at a temperature of 4°C. The column was developed with a gradient formed by two pumps. Pump A reservoir contained 50 mM Tris pH8.0 and pump B reservoir contained 50 mM Tris, 1 M NaCl pH 8.0. Both buffers also contain 16 TIU ml<sup>-1</sup> of aprotinin. The flow rate was 0.5 ml per minute. After the sample was loaded, the column was run with 5% B for 40 minutes and then a gradient of 5% B to 35% B over 200 minutes. Fractions were collected at four minute intervals. The effluent was monitored with a UV detector. Material which appeared in the wash was discarded. A major UV-absorbing peak eluted at about 150 minutes. The fractions around the peak were pooled, sealed in dialysis tubing and dialysed overnight against 5 litres of distilled water. The liquid was then rapidly frozen in a flask of liquid nitrogen and freeze-dried. This yielded ca. 25 mg of VTG per ml of plasma.

The VTG was further purified by size-exclusion chromatography on Sepharose 6B with a buffer consisting of 50 mM Tris (pH 8.0) and 50 mM NaCl at a flow rate of 0.25 ml min<sup>-1</sup>. There was a single symmetrical UV-absorbing peak. The VTG was then concentrated from these fractions by passing them slowly through a 1 ml HiTrap Q column (Pharmacia Biotech). The VTG became absorbed to the column matrix and was eluted with 2.5 ml 50 mM Tris (pH 8.0) and 400 mM NaCl. The buffer was then exchanged with distilled water using a desalting column (PD-10; Pharmacia Biotech) and the VTG solution frozen and freeze-dried.

#### *Antiserum production*

Antibodies were produced in rabbits by giving intramuscular injections of VTG (3 mg per injection per rabbit) dissolved in saline and emulsified with Freund's complete adjuvant. The rabbits were injected six times at two to three week intervals, and bled at least four times, at approximately monthly intervals.

### 3.1.3 Radioimmunoassay procedure

#### *Iodination*

Flounder VTG was labelled with Na<sup>125</sup>I using Iodogen (1,3,4,6-tetrachloro-3',6'-diphenylglycouril) as the oxidising agent (Tyler and Sumpter, 1990). The Iodogen was dissolved in dichloromethane at a concentration of 100 µg ml<sup>-1</sup>; and 20 µl was allowed to dry in the bottom of a 1.5 ml Eppendorf vial. The vial was placed on its side. Freshly weighed VTG was made up in distilled water at a concentration of 1 mg ml<sup>-1</sup>; and 10 µl was mixed with 20 µl of 0.5 M sodium phosphate buffer (pH 7.4) and pipetted onto the side of the vial. Ten µl of Na<sup>125</sup>I (3.7 MBq of radioactivity) was also pipetted onto the side of vial, which was then placed upright - allowing both solutions to run down into the base of the vial, where

they come into contact with the Iodogen. The reaction was allowed to proceed for 10 min and then terminated by the addition of 1 ml 0.05M sodium phosphate buffer (pH7.4). The labelled VTG was separated from free  $N^{125}I$  by rapid gel-filtration on a PD10 column containing Sephadex G25 and eluted with assay buffer (see below). The labelled protein fraction was collected in a glass vial, mixed with 3.5 ml glycerol (to prevent the mixture freezing) and stored at  $-20^{\circ}C$ . The level of incorporation of  $^{125}I$  into VTG (which was crudely assessed by counting the radioactivity in the PD10 column and the glass vial with a hand-held monitor) was between 40 and 60%. The radiolabelled flounder VTG was usable for at least 21 days - apart from a gradual diminution in the proportion that bound to antibody.

### ***Standard***

About 1 mg VTG powder was weighed out and dissolved in distilled water at a concentration of  $1\text{ mg ml}^{-1}$ . This was diluted 100-fold with assay buffer to form a solution with a concentration of  $10\text{ }\mu\text{g ml}^{-1}$ , which was then frozen in 1 ml aliquots. A fresh batch of standard was prepared each time that a fresh batch of radiolabel was prepared.

### ***Basic assay procedure***

Plastic tubes were set up in trays - those which received the plasma samples (the 'unknowns') were labelled 1 to 60 in duplicate; those which received the standards were labelled S1 to S11 in duplicate. Three pairs of tubes were labelled M, B and T. Aliquots of  $50\text{ }\mu\text{l}$  assay buffer were dispensed into each sample tube and  $100\text{ }\mu\text{l}$  into S1 to S11, M and B. The volume in the sample tubes was made up to  $100\text{ }\mu\text{l}$  by adding  $50\text{ }\mu\text{l}$  of plasma in a neat or pre-diluted form (see below). A vial containing diluted standard ( $10\text{ }\mu\text{g ml}^{-1}$ ) was thawed and  $100\text{ }\mu\text{l}$  was added to each of the S1 tubes. The tubes were mixed and  $100\text{ }\mu\text{l}$  transferred to each of the S2 tubes. This procedure was repeated up till S11, when the final  $100\text{ }\mu\text{l}$  was discarded. This yielded a standard curve with concentrations ranging from 5000 to  $5\text{ ng ml}^{-1}$ . Anti-VTG serum was diluted 1:100,000 with assay buffer; and  $100\text{ }\mu\text{l}$  was added to all tubes except for those labelled B and T. The tubes labelled B received  $100\text{ }\mu\text{l}$  assay buffer. After mixing, the tubes were centrifuged briefly to remove any drops on the sides of the tubes. They were returned to the rack, covered with aluminium foil and placed in the fridge overnight. The next morning,  $^{125}I$ -VTG was diluted with assay buffer and  $50\text{ }\mu\text{l}$  ( $20,000\text{ cpm}$ ) added to all tubes. After another brief centrifugation, the tubes were incubated at room temperature for a further 6 h. To separate the bound from the free radiolabel,  $100\text{ }\mu\text{l}$  of Sac-Cel (second antibody covalently linked to cellulose) was added to all tubes except for those labelled T. After a further 30 min, 1 ml distilled water was added to all tubes except those labelled T. The tubes were then centrifuged for 10 min to compact the cellulose, the aqueous phase removed by aspiration and the radioactivity adhering to the pellets measured with an automatic gamma counter. The T tubes represented the

'total' radiolabel added to each tube. The B tubes represented the amount of radiolabel bound to Sac-Cel in the absence of any anti-VTG antibody (i.e. non-specific binding). The M tubes represented the amount of radiolabel bound to anti-VTG antibody in the absence of any unlabelled VTG (i.e. maximum binding). Using a spreadsheet, the activities in the standard and sample tubes were converted to 'percent bound' by dividing them by the total counts. When the percent of label bound was plotted against the logarithm of the VTG concentration, it formed a sigmoid curve. Unknowns were calculated from the steep middle portion of the curve. The only exception was for samples with very low concentrations. Where possible, these were reassayed under more sensitive conditions (see below). However, some plasma samples still needed to be quantified from the upper, less accurate, part of the curve. This was considered of minor concern, as the problem only occurred within a range of VTG concentrations (ca.  $5\text{ to }20\text{ ng ml}^{-1}$ ) which was minuscule in comparison to the total range encountered within flounder (which went as high as  $200\text{ ng ml}^{-1}$ ).

As a measure of quality control, a pool of plasma was made up and included in every assay. Where possible, all samples from a single study were assayed at the same time - using an identical batch of standard. The interassay coefficient of variation was ca. 11%.

### ***Plasma dilutions***

All male and immature female plasmas were initially assayed at a dilution of 1:2 (i.e.  $50\text{ }\mu\text{l}$  of undiluted plasma is added to the assay tube and made up to  $100\text{ }\mu\text{l}$  with assay buffer); and those from mature females at two dilutions of 1:5,000 and 1:50,000. A value was only accepted if it fell within the steep middle range of the standard curve where both male and female plasmas showed good parallelism with the standard VTG. If the value was greater than  $1250\text{ ng ml}^{-1}$ , then the plasma was retested at a range of dilutions up to as much as 1:200,000. If the value was less than  $80\text{ ng ml}^{-1}$ , then the plasma was retested using a sensitive assay procedure which, in the case of the flounder, involved incubating standard and unknowns for two days at  $4^{\circ}C$ , with antiserum diluted 1:200,000, and for a further one day with  $50\text{ }\mu\text{l}$  of  $10,000\text{ cpm}$  of  $^{125}I$ -VTG. Sensitivity could be improved still further by using even less of both reagents (e.g. antiserum at 1:1,000,000 and radiolabel at  $5,000\text{ cpm}$ ). No problems were experienced in using undiluted plasma.

### ***Establishing an initial antibody dilution***

As a first step in the development of the RIA, serial dilutions of antiserum were made between 1:10,000 to 1:2,000,000 and incubated for the appropriate time interval with  $20,000\text{ dpm}$  of radiolabel prior to separation. The percent of radio-iodinated VTG which bound to an excess of antibody was 75%. The antibody dilution corresponding to 50% of bound radiolabel was used in the RIA.

### 3.1.4 List of reagents and equipment used in the radioimmunoassay

#### **For purification of VTG:**

**Equipment:** ultrasonic bath; FPLC dual pump chromatography system (with fraction collector and UV monitor)<sup>1</sup>; liquid nitrogen container; freeze drier; centrifuge.

**Disposables:** glass tubes (150 mm x 16 mm; carrying out precipitations); Acrocap filter (0.45 µm)<sup>2</sup>; HiTrap Q (1 ml) column<sup>1</sup>; PD-10 column (containing Sephadex G-25 M)<sup>1</sup>; dialysis tubing.

**Chemicals:** 17β-oestradiol; cocoa butter; aprotinin; 4-(2-aminoethyl)-benzene-sulphonyl fluoride; ethylenediaminetetraacetic acid; sodium hydroxide; magnesium chloride; 0.05 M Tris buffer (made from 0.97 g Trizma base and 6.61 g Trizma hydrochloride dissolved in 1 l distilled water [pH 8.0 at 5°C]); sodium chloride; DEAE-Sephacel; Sepharose 6B.

#### **For iodination:**

**Equipment:** radioactivity monitor.

**Disposables:** eppendorf tube; PD-10 column<sup>1</sup>; glass scintillation vial (20 ml) for storing label; lead pots; pipettes; pipette tips; gloves.

**Chemicals:** <sup>125</sup>Iodine<sup>3</sup>; 0.5 M sodium phosphate buffer (made from 115 g di-sodium hydrogen phosphate [anhydrous] and 29.6 g sodium di-hydrogen phosphate [dihydrated] dissolved in 2 l distilled water and stored frozen in 50 ml aliquots; pH 7.4); 0.05 M sodium phosphate buffer; dichloromethane; Iodogen (1,3,4,6-tetrachloro-3',6'-diphenylglycouril); glycerol; assay buffer (see below).

#### **For radioimmunoassay:**

**Equipment:** standard laboratory centrifuge with buckets which can hold up to at least 144 assay tubes; automatic gamma counter; water suction pump (i.e one which is attached to a tap) for the aspiration of the 'free' radiolabel fraction following the second antibody separation; tube racks; pipettes (covering the range from 5 µl to 5 ml); multiple pipettor (for repetitive dispensing of reagents).

**Disposables:** polystyrene tubes (12 mm x 75 mm); pipette tips; polystyrene pots (for mixing up reagents).

**Chemicals:** assay buffer (10.2 g di-sodium hydrogen phosphate [anhydrous], 3.87 g sodium di-hydrogen phosphate [monohydrate], 8.18 g sodium chloride, 2.01 g potassium chloride, 500 µl Tween-20 and 1 g bovine serum albumin dissolved in 1 l distilled water; pH 7.2); Sac-Cel (second antibody covalently coupled to cellulose)<sup>4</sup>; radioiodinated VTG (stored at -20°C; anti-VTG serum (stored at 4°C at a dilution of 1:100 in 0.05 M sodium phosphate buffer, plus a few grains of sodium azide to prevent bacterial growth).

All chemicals and disposables were normally obtained from Sigma Chemical, Fancy Road, Poole, Dorset, BH12 4XA, UK except for:

- <sup>1</sup> - Amersham Pharmacia Biotech, 23, Grosvenor Road, St. Albans, Herts, AL1 3AW, UK;
- <sup>2</sup> - Gelman Sciences Ltd, Brackmills Business Park, Caswell Road, Northampton NN4 7EZ, UK;
- <sup>3</sup> - Amersham Life Science Ltd, Amersham Place, Little Chalfont, Bucks, HP7 9NA, UK;
- <sup>4</sup> - Immunodiagnostic Systems Ltd, Boldon Business Park, Tyne & Wear, NE35 9PD, UK.

## 3.2 Laboratory studies with flounder

### 3.2.1 Vitellogenin induction studies

Laboratory trials with flounder caught by beam trawling from the clean control estuary (the Alde in Suffolk - see below) were conducted in November and December 1996, in order to assess the sensitivity of this species in comparison with rainbow trout (which had been used for monitoring oestrogens in freshwater). Two separate trials were carried out using the synthetic oestrogen ethynylestradiol (EE2) and the oestrogen mimic *p*-nonylphenol (NP). Fish were dosed continually for three weeks with either 1 and 10 ng l<sup>-1</sup> EE2 or 10 and 30 µg l<sup>-1</sup> NP in 1500 l capacity circular tanks. Stock solutions of the test chemicals were introduced into mixing containers via peristaltic pumps. Diluent water flowed into the tanks continuously at a rate of 1 l min<sup>-1</sup> from the mixing chambers which were supplied with a predetermined amount of the test substance.

The estuary of the River Alde on the east coast of Suffolk (Figure 1) was chosen as the clean control site. Only 3 small STWs (total discharge = 145 m<sup>3</sup> day<sup>-1</sup> of secondary treated effluent) directly enter the estuary (Figure 2(a)) and male flounder caught locally were expected to have low background concentrations of vitellogenin in their blood plasma. As well as using Alde fish for the laboratory trials, comparisons of plasma vitellogenin concentrations could be made with fish from other estuaries and tested for significant differences.

The flounder (adults and nearly-adult juveniles) collected from the River Alde estuary near Orford were brought back to the laboratory where they were randomly allocated to tanks and acclimated to experimental conditions (temperature 10°C ± 1°C; dissolved oxygen (%ASV) > 80%; salinity 34 ± 2; photoperiod 12:12 light:dark) for a period of 48 hours. At test initiation, fish were randomly sampled from the acclimation tanks and anaesthetised with 2-phenoxyethanol (0.5 ml l<sup>-1</sup>). Each fish was measured for length and wet weight, and a 0.5-1 ml blood sample was removed from the caudal vein on the ventral side via heparinised syringe. A marking technique, whereby the fins were injected with different combinations of acrylic paints, was used to identify individual fish. This technique has been found by CEFAS to be harmless to flatfish. A total of 25 fish was added to each treatment

tank and the tests were run for a period of three weeks. Fish were fed chopped mussel every other day. Any dead fish observed in the tanks were removed and identified. At test termination each fish was length-measured, weighed, blood sampled and then sacrificed by lethal dose of anaesthetic. The gonads and livers were removed and weighed for calculation of gonado- and hepatosomatic indices (GSI, HSI) respectively:

$$\text{GSI} = (\text{total gonad weight} / \text{total body weight}) \times 100$$

$$\text{HSI} = (\text{liver weight} / \text{total body weight}) \times 100$$

A section of gonad was preserved for histopathological assessment (see below). The blood samples were centrifuged at 12,000 rpm for 5 minutes, the plasma pipetted off and stored frozen (-20°C) in cryovials. The concentration of vitellogenin in the plasma was measured using the newly developed specific radioimmunoassay (RIA - see section 3.1 above). Samples of water in each of the tanks and in the stock bottles were analysed at least once weekly for EE2 or NP for the duration of the trials. EE2 and NP were analysed by gas chromatography-mass spectrometry (GC/MS) following solid phase extraction.

### 3.2.2 Vitellogenin elimination study

In March 1998 another laboratory study was initiated to investigate the process of VTG elimination from the bloodstream of flounder after transferral to 'clean' conditions. Adult and nearly adult fish were collected from the Alde estuary and acclimated in tanks as described above. At test initiation, fish were randomly sampled from the acclimation tanks and anaesthetised with 2-phenoxyethanol (0.5 ml l<sup>-1</sup>). Each fish was measured for length and wet weight, and a 0.5 ml blood sample was removed from the caudal vein on the ventral side via heparinised syringe. A marking technique, whereby the fins were injected with different combinations of acrylic paints, was used to identify individual fish. Two circular 1500 l capacity tanks, each containing 30 fish, were dosed continuously for three weeks with 10 ng l<sup>-1</sup> EE2, using the same dosing system described for the VTG induction studies. Two tanks, each containing 20 fish, were set up as controls (dosed only with seawater and solvent at an equivalent concentration to the treatment tanks). Stock solutions of the test chemicals/solvent controls were introduced into mixing containers via peristaltic pumps. Diluent water flowed into the tanks continuously at a rate of 1 l min<sup>-1</sup> from the mixing chambers, which were supplied with a predetermined amount of the test substance. Fish were fed live brown shrimp every other day. After three weeks of dosing, the fish were lightly anaesthetised and another 0.5 ml blood sample taken from each. The tanks were thoroughly cleaned out and filled with seawater. The fish were returned to the tanks and the test continued for a further three weeks in seawater only (flow rate 1 l min<sup>-1</sup>). Blood samples were taken at weekly intervals. Any dead fish observed in the tanks

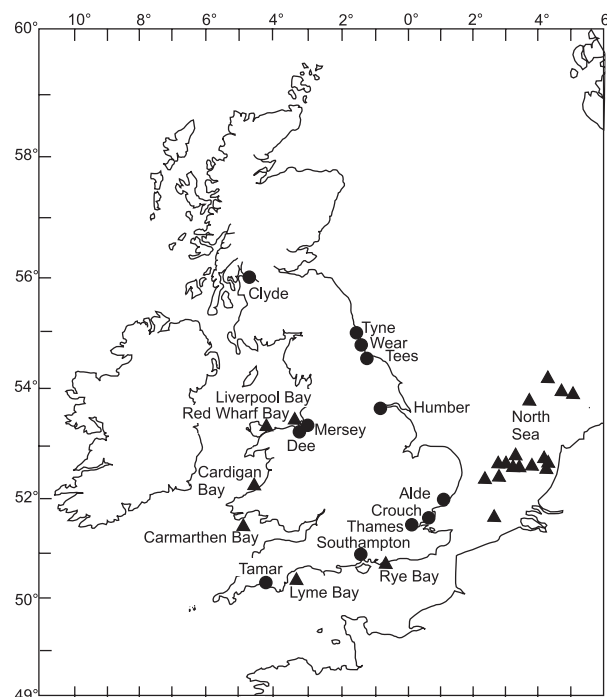
during the six week period were removed, identified and the sex determined.

All blood samples were centrifuged at 12,000 rpm for 5 minutes, the plasma pipetted off and stored frozen (-20°C) in cryovials. The concentration of vitellogenin in the plasma was measured using an ELISA (Enzyme-Linked Immunosorbent Assay) technique. Samples of water in each of the tanks were analysed at least once weekly for EE2 for the duration of the trial.

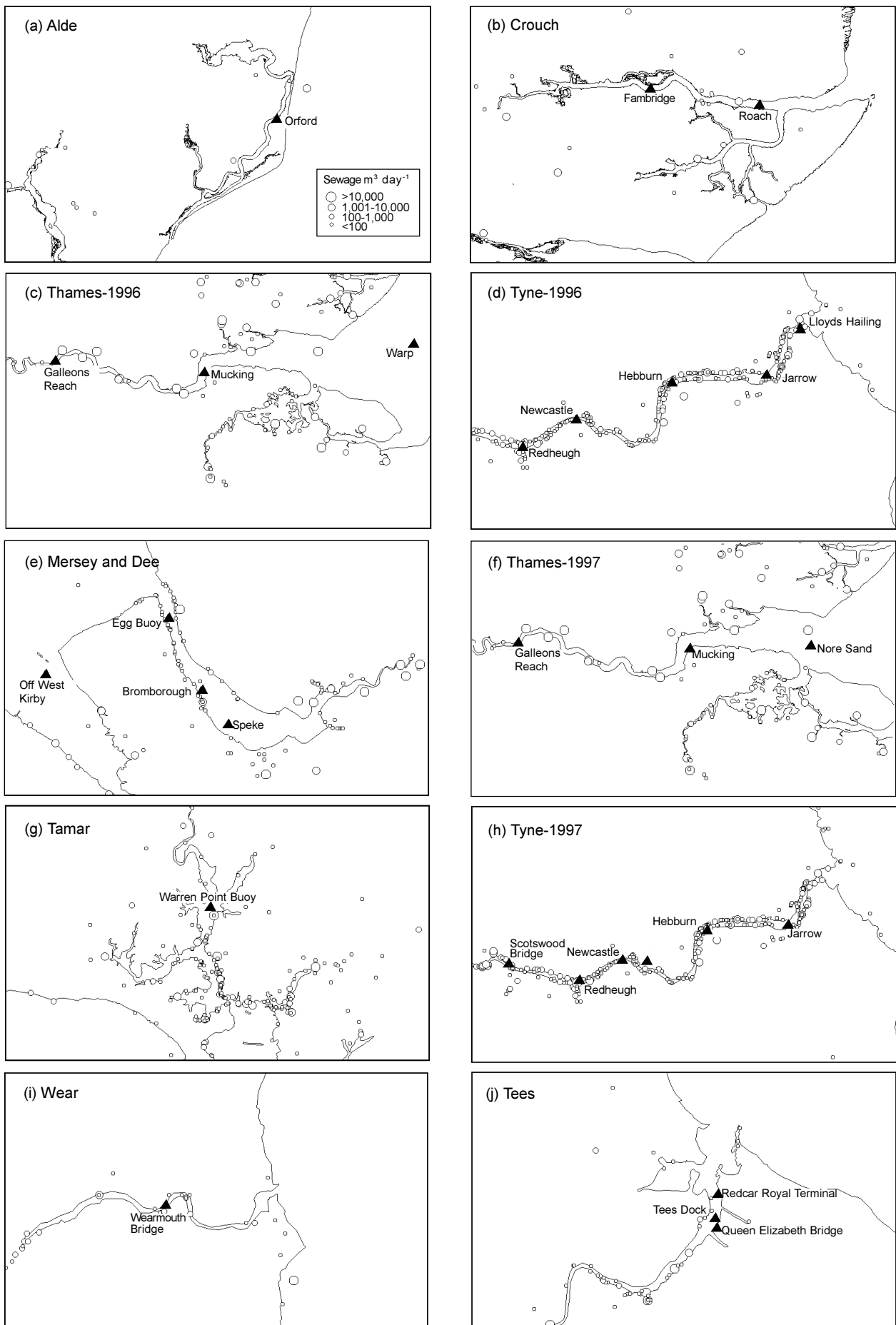
### 3.3 Field studies with flounder

Between September and December 1996, a total of five English estuaries (including the Alde) were sampled for flounder using the U.K. Environment Agency research vessels *COASTAL GUARDIAN* and *WATER GUARDIAN*, and also private charter vessels. During January and February 1997, flounder were sampled from a number of coastal and offshore sites in the North Sea and Irish Sea using the CEFAS research vessels *RV CORYSTES* and *RV CIROLANA*. Between May and December 1997 another series of surveys was carried out, sampling a total of eleven estuaries.

The estuaries surveyed in 1996 in addition to the Alde were the Tyne, Thames, Crouch and Mersey (Figures 1 and 2). In 1997 the Alde, Mersey, Tyne and Thames were re-surveyed and, in addition, samples were obtained from the Tees, Wear, Dee, Tamar, Clyde, Humber and Southampton Water (Figure 1). Between two and five sites on each estuary were chosen *a priori*.

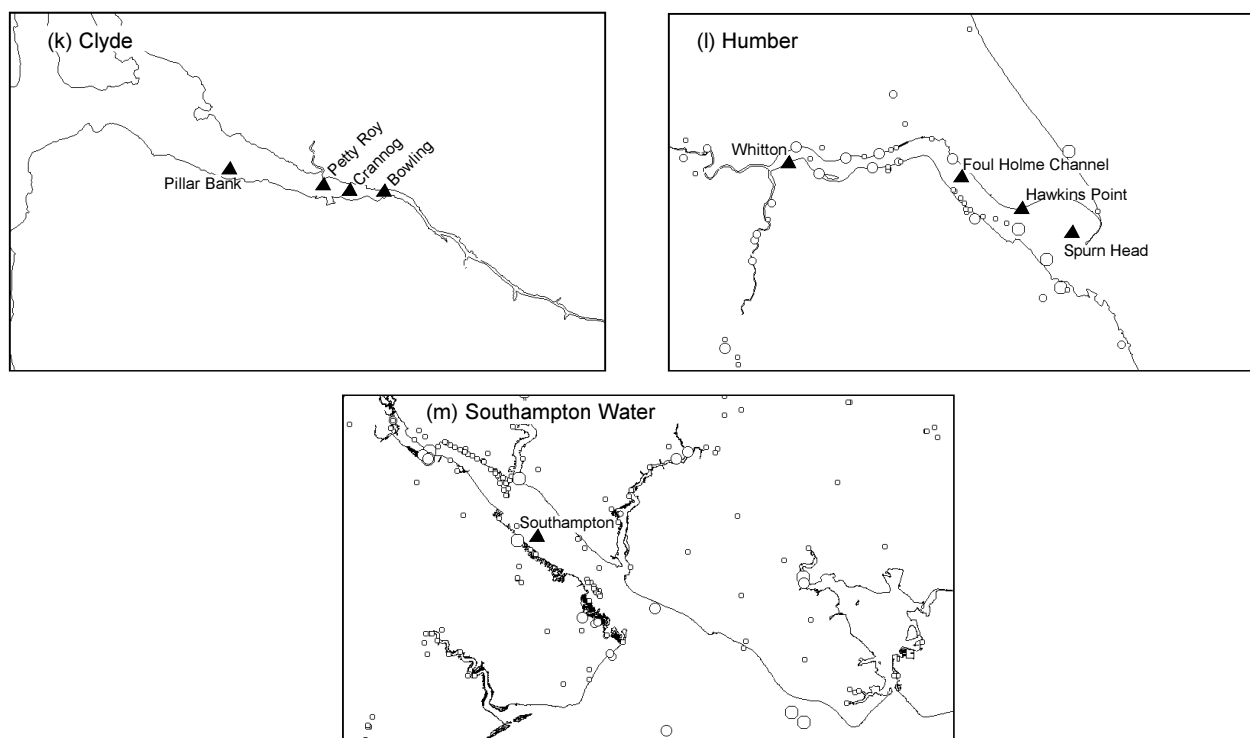


**Figure 1. Location of the estuaries (●) and offshore sites (▲) sampled for flounder between September 1996 and December 1997**



**Figure 2.** Location of the sampling sites on each estuary, denoted by ▲, along with location and size of STW discharges





**Figure 2. continued**

If flounder were not present at a chosen site then trawling was carried out at alternative sites until fish were found. A one mile stretch of water was trawled several times until at least ten males had been caught. A blood sample was taken from both male and female fish, centrifuged immediately and the plasma stored frozen (-20°C) for analysis for VTG using the specific RIA. Liver, gonad and whole fish weights were recorded for calculation of HSI and GSI. Gonad samples were also preserved for histopathological assessment (see below).

In the 1997 survey, liver samples from male and female flounder were collected from some estuaries for EROD induction measurements (see below). The liver was excised immediately the fish was killed, placed in a vial and then immediately transferred to liquid nitrogen for cryogenic storage.

Several sites in the south-eastern North Sea (Figure 1) were trawled but yielded only a few male flounder at each. For the purposes of statistical comparison of VTG concentrations with estuarine fish, the males were pooled to obtain an overall value for the southern North Sea.

In 1997, a total of six sites in the Irish Sea and English Channel (Figure 1) were trawled - Liverpool Bay, Red Wharf Bay, Cardigan Bay, Carmarthen Bay, Lyme Bay and Rye Bay. While at Carmarthen Bay only one male flounder was caught, five or more males were sampled from the others.

### 3.4 Caged flounder trials

In the spring of 1998, a preliminary trial to assess the suitability of using caged flounder for localising oestrogenic activity was carried out in the Crouch estuary adjacent to the CEFAS Burnham Laboratory. A heavy-duty galvanised steel cage (1.24 x 0.5 x 0.5 m) was placed below the mean low water mark and attached securely to a permanent wooden jetty with lengths of rope. The cage, because of its weight, sank into the soft mud a few inches, which provided the flounder with its natural habitat while confined. Initially, twenty small (10-16 cm) flounder were added to the cage. In a second trial fourteen large flounder (>20 cm) were added. Survival was monitored at low tide every few days by pulling the cage from the mud and counting the number of live fish.

### 3.5 Histopathology

Flounder gonads from laboratory VTG induction trials and field studies were removed and placed in neutral buffered formalin (NBF) without delay. Following fixation for a minimum of 48 hours, gonads were trimmed with a razor blade and placed in cassettes in 70% alcohol. Specimens were then processed to paraffin wax blocks using conventional techniques and a vacuum infiltration processor. 5 micron sections were stained with haematoxylin and eosin (H&E) and examined using a Nikon Eclipse E800 microscope. One section from each gonad was examined. Representative

specimens were photographed conventionally and additionally were stored for comparative purposes using Lucia Image Archiving software (Nikon UK Ltd).

At a later date, selected blocks previously found to contain intersex gonads (i.e. ovotestis), as well as normal gonads from fish collected from the Mersey, were sectioned at 200  $\mu\text{m}$  intervals to investigate the distribution pattern of oocytes within intersex testes and to test the sensitivity of histology for the detection of the condition, respectively. Representative specimens were photographed conventionally and in addition, images were stored for comparative purposes using Lucia Image Archiving software.

### 3.6 Statistical analyses

Values for VTG concentrations which were below the minimum detection limit were assigned the value of the minimum detection limit for that assay batch. All data were log transformed and checked for normality and homogeneity of variances using Chi Square and Bartlett's Test respectively. In the laboratory induction studies, the log-ratios of each of the treatment groups (i.e. dosing levels) were compared for significance using a one-way Analysis of Variance (ANOVA). This was followed by *ad hoc* assessments of any detected differences, using the Fisher Protected Difference Test. For the field studies, mean male VTG concentrations from each of the sites were compared to the mean male VTG concentration in the Alde reference estuary (for the 1996 survey, this was equivalent to the pooled initial values from the two laboratory tests) using one-way ANOVA with the Tukey multiple range test or the non-parametric Kruskal-Wallis test. All tests were conducted at an  $\alpha = 0.05$ .

### 3.7 Analysis of polycyclic aromatic hydrocarbons (PAH) in flounder liver

Homogenised samples of flounder liver (collected in 1996-8) were bulked by site in groups of 5 for PAH analysis. In brief, the samples were digested under reflux with methanolic potassium hydroxide, the PAH and non-saponifiable lipids extracted into pentane, and the extract cleaned up by passage through an alumina column prior to determination of PAH by means of gas chromatography/mass spectrometry. Quantification of individual compounds and groups was made relative to known amounts of a range of fully deuterated PAH analogues added as internal (surrogate) standards before sample digestion, and both blanks and reference samples were run alongside each batch of flounder livers to ensure that the methods were under control. Fuller details of methods can be found elsewhere (Law *et al.*, 1988; Kelly and Law, in press).

### 3.8 Analysis of organochlorines in flounder liver

A range of organochlorine compounds including a suite of twenty-five individual chlorobiphenyl congeners was determined in samples of pooled flounder liver (each from 5 individuals). The samples were collected in 1996-8 and analysed using standard procedures (Allchin *et al.*, 1989) with some modifications. Organochlorine residues were determined in the final prepared extracts by gas chromatography with electron capture detection using a 50 m x 0.2 mm i.d. column coated with crosslinked 5% phenyl methylsilicone with a film thickness of 0.33  $\mu\text{m}$ . Quantification was by multilevel internal standard calibration. Data quality was assured by adherence to rigorous in house procedures and cross checked by the use of certified reference materials and participation in external collaborative exercises.

### 3.9 Measurement of EROD induction in flounder liver

Measurement of EROD activity in fish liver is a convenient way of revealing the degree of induction of the cytochrome P4501A1 enzyme system responsible for the degradation of a number of environmental substances as well as endogenous materials such as steroids. It is generally used as a marker of exposure to planar compounds such as PCBs and PAHs, but may also indicate potential for the induction of liver carcinomas.

#### 3.9.1 Homogenate preparation

Flounder liver samples collected in Sept/Dec 1997 were processed within 3 months of collection, after having been stored in liquid nitrogen. A 200 mg ( $\pm 10$ ) slice of liver was homogenised with 1 ml of ice cold homogenising buffer (50 mM TRIS pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 150 nM NaCl) using six strokes of a Potter-Elvehjem automatic homogeniser set at 4000 rpm. The homogenate was transferred to a lidded polyethylene Eppendorf tube and stored on ice. The homogenates were then centrifuged at 10,000 g for 20 minutes in a refrigerated unit set at 4°C. Supernatants were removed, stored on ice and assayed within 30 minutes.

#### 3.9.2 EROD activity determination

EROD activity determinations were conducted using a modification of the method described in Stagg *et al.* (1995). A Perkin Elmer LS50B fluorescence spectrometer set at wavelengths of 535 nm excitation and 580 nm emission with a cuvette stirring function was used. All assay reagents were kept at 20°C ( $\pm 1$ ) in a water bath in order to control the assay temperature.

The reaction mixture, final volume 2 ml, consisted of 1.96 ml assay buffer (100 mM pH 7.5 TRIS, 100 mM NaCl), 20 µl liver homogenate supernatant, 10 µl ethoxyresorufin substrate (0.4 µM in dimethylsulphoxide, DMSO). The assay was calibrated by the addition of 10 µl of resorufin standard (25 µM in DMSO) which equates to the addition of 250 pM of resorufin. To ensure consistency and quality control of the standard and substrate, batches were made and individual aliquots of 1-2 ml were frozen and defrosted prior to use in the assay. The reaction was initiated by the addition of 10 µl NADPH (0.25 mM) and emission readings were recorded at 0 and 60 seconds post addition.

EROD activity was normalised to protein content and expressed as pM resorufin/minute/mg of protein (pM/min/mg pro). Protein analyses were carried out on the same liver homogenate supernatant as the EROD activity using a plate reader modification of the Bradford (1976) method with a bovine serum albumin standard.

### **3.10 Measurement of oestrogenic and androgenic steroid hormones in flounder blood plasma**

It is possible to determine the effects of endocrine disrupting contaminants on oestrogen and androgen concentrations in blood plasma by measuring the ratio of 17β-oestradiol to the androgens testosterone and 11-ketotestosterone. One analytical technique which is particularly suited for such measurements is high performance liquid chromatography coupled to mass spectrometry (LC-MS). Unlike other techniques, such as gas chromatography-mass spectrometry (GC-MS), both free steroids and conjugates (e.g. glucuronide or sulphate) can be determined without derivatisation whilst offering high sensitivity and specificity. A comprehensive review of steroid analysis, including the application of various LC-MS techniques, is available (Makin *et al.*, 1995).

The method described uses solid phase extraction (SPE) to extract the analytes from the blood serum, followed by analysis using HPLC atmospheric pressure chemical ionisation (APCI) mass spectrometry, operated in the single ion monitoring (SIM) mode. Atmospheric pressure chemical ionisation is particularly suited for this form of analysis since it is considered a 'soft' ionisation technique which often results in protonated [M+H]<sup>+</sup> or deprotonated [M-H]<sup>-</sup> ions being formed. Operation of the mass spectrometer in the SIM mode allows each ion to be measured and detected individually which results in increased sensitivity when compared to acquiring all the ions generated.

#### **3.10.1 Materials**

HPLC grade methanol and water were obtained from Rathburns (Walkeburn, UK) whilst 17β-oestradiol, 11-ketotestosterone, testosterone, progesterone and bovine serum were obtained from Sigma (Gillingham, Dorset, UK). All authentic reference compounds were certified to be of a purity > 95%.

#### **3.10.2 Preparation of standards**

Standards were prepared at concentrations of 0.05, 0.1, 0.5 and 1 ng µl<sup>-1</sup> in methanol. Reference material was prepared by adding 20 ng of 17β-oestradiol, 11-ketotestosterone and testosterone to bovine serum (10 ml).

#### **3.10.3 Solid phase extraction**

Plasma samples (*ca.* 2 ml) were pre-treated by adding 8 ml of saline solution (0.9% NaCl) and 100 µl of internal standard solution (40 ng ml<sup>-1</sup> progesterone in methanol). The sample was then heated (90°C, 15 min) before being passed through a solvated (2 ml methanol followed by 2 ml water) C18 SPE column (500 mg monofunctional; International Sorbent Technology, Hengoed, UK) at a flow rate of ~ 5 ml min<sup>-1</sup>. Once extraction was complete the column was dried thoroughly using vacuum aspiration for 20 minutes. The analytes were then recovered by eluting the column with methanol (500 µl) which was allowed to soak for 5 minutes. This was then repeated.

#### **3.10.4 High performance liquid chromatography**

HPLC was carried out using a Hewlett Packard 1050 system fitted with a quaternary pump and an ODS2 column (5 µm, 15 cm x 3.4 mm; Phenomenex, Macclesfield, UK). The mobile phase was acetonitrile/water run over a gradient (10:90%, acetonitrile:water linear to 100% acetonitrile for 20 min and held for 5 min). Column temperature was maintained at 30°C. 10 µl injections were made onto the column following a 5 min post-run equilibration period.

#### **3.10.5 Mass spectrometry**

Mass spectra were obtained on a VG Platform bench-top mass spectrometer (VG, Altrincham, UK). The mass spectrometer was initially tuned on background solvent ions (CH<sub>3</sub>CNH<sup>+</sup>, *m/z* 42). Tuning was then optimised on the 17β-oestradiol deprotonated molecular ion [M-H]<sup>-</sup> ion (*m/z* 272) and mass calibrated in the positive ion mode on a mixture of PEG 200, 600 and 1000 as per manufacturer's instructions. The instrument was used in both the positive and negative modes as summarised in Table 1. Peak areas of the respective ions were used for quantitation (Table 2).

**Table 1. Typical mass spectrometer operating conditions**

Mass spectrometer function	Mode	
	Positive	Negative
Corona (kV)	3.2	2
High voltage lens (kV)	0	0
Ion energy (V)	2	2
Source temperature (°C)	150	150
Probe Temperature (°C)	400	400
High mass resolution	12.5	12.5
Low mass resolution	12.5	12.5
Multiplier (V)	650	650

**Table 2. Single ion monitoring conditions**

SIM function	Analyte			
	17 $\beta$ -oestradiol	Testosterone	11-ketotestosterone	progesterone
Mode	(-)ve	(+)ve	(+)ve	(+)ve
SIM ion ( $m/z$ )	272	289	303	316
Monitoring time (min)	10.5-13	12.5-25	0-10.5	12.5-25
Cone voltage (V)	45	20	20	20

## 4. RESULTS

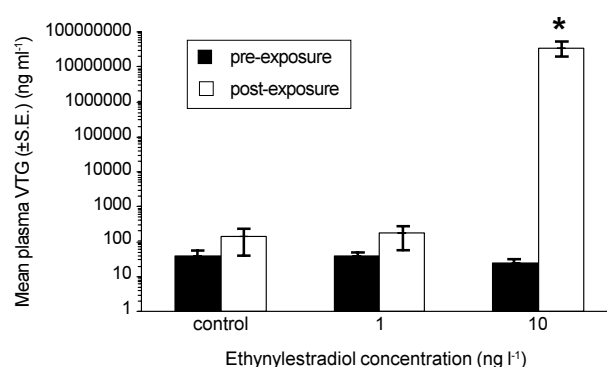
### 4.1 Laboratory studies with flounder

#### 4.1.1 VTG induction studies

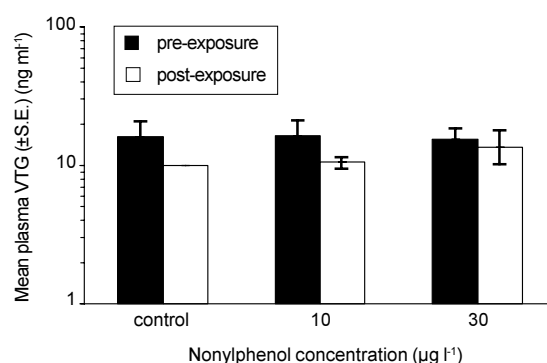
Male flounder caught in the control estuary had low concentrations of VTG at the start of both exposure studies. The mean concentration in males was 29.5 ng ml<sup>-1</sup> at the start of the EE2 study and 16.0 ng ml<sup>-1</sup> for the NP study. Exposure for three weeks to 1 ng l<sup>-1</sup> EE2 did not induce vitellogenin production in male (or female) fish but at a concentration of 10 ng EE2 l<sup>-1</sup> VTG was significantly ( $p < 0.001$ ) increased in males (Figure 3). Female flounder exposed to 10 ng l<sup>-1</sup> EE2 also had significantly increased concentrations of VTG; the majority of females in this treatment were immature with low (<200 ng ml<sup>-1</sup>) initial values of VTG. Two maturing females which had moderate concentrations at the beginning of the experiment increased their plasma VTG concentrations 10 and 100-fold.

Exposure to NP did not produce a response (Figure 4) at either 10 or 30  $\mu$ g l<sup>-1</sup> ( $p = 0.86$ ). A repeat experiment using NP concentrations of 30 and 100  $\mu$ g l<sup>-1</sup> confirmed that 30  $\mu$ g l<sup>-1</sup> does not produce a response, but 100  $\mu$ g l<sup>-1</sup> proved to be a lethal concentration. HSI increased significantly after exposure to 10 ng l<sup>-1</sup> EE2 and 30  $\mu$ g l<sup>-1</sup> NP (Figure 5). There was no effect of either EE2 or NP on GSI (Figure 6). Overall, these results showed that VTG induction in male flounder is about 10 times less sensitive to exogenous oestrogens than in rainbow trout (Sheahan *et al.*, 1994; Jobling *et al.*, 1996).

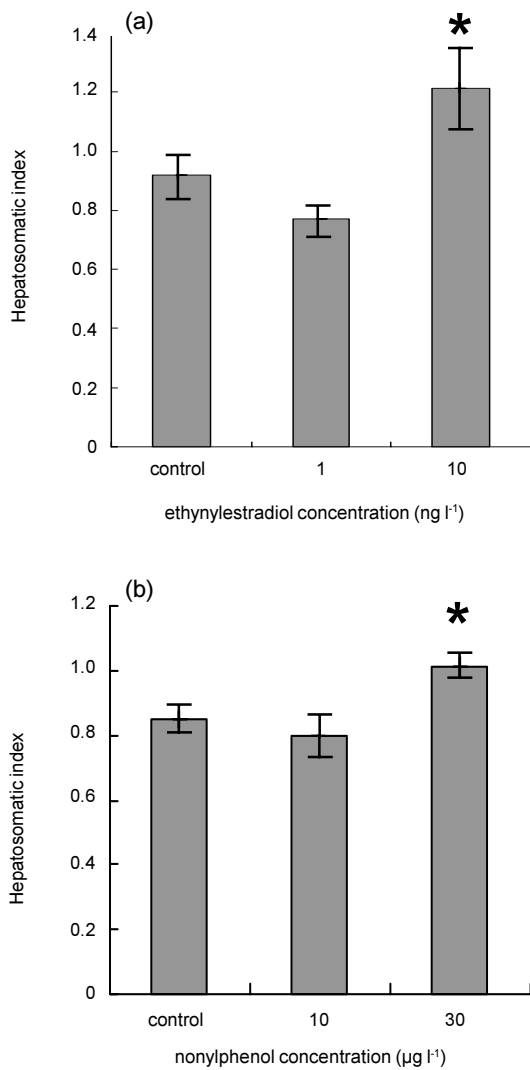
Histopathological examination of both male and female gonads showed no significant abnormalities.



**Figure 3. Mean plasma vitellogenin induction in male flounder exposed to ethynylestradiol (EE2) for 3 weeks. \* denotes significant differences from the control ( $p < 0.001$ ). Error bars represent standard error of the mean**

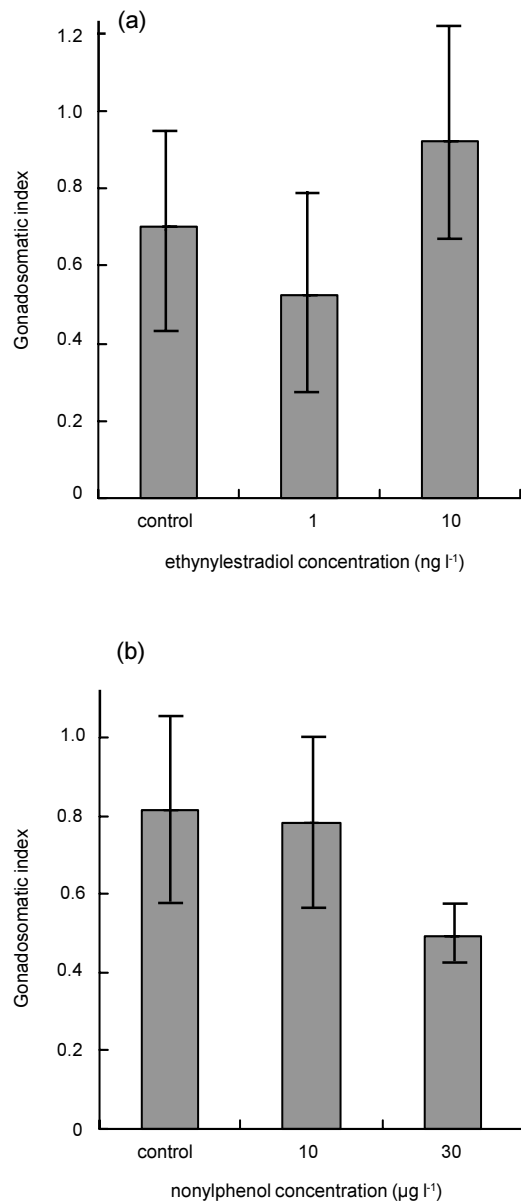


**Figure 4. Mean plasma vitellogenin concentrations in male flounder exposed to nonylphenol for 3 weeks. Error bars represent standard error of the mean**



**Figure 5.** Mean Hepatosomatic Indices (HSI) in male flounder exposed to EE2 and NP for 3 weeks. \* denotes significantly different from control ( $p < 0.05$ ). Error bars represent standard error of the mean

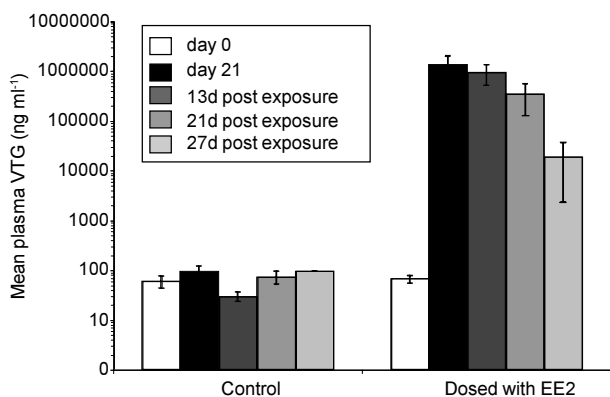
Chemical analysis of tank water showed that the mean EE2 concentration in the control, 1 and 10 ng l<sup>-1</sup> treatments was <1.5, <6.0 and 14.5 ng l<sup>-1</sup> respectively. The stock solutions were also measured; nominal stock concentrations of 2 and 20 µg l<sup>-1</sup> had measured concentrations of 1.4 and 22 µg l<sup>-1</sup> respectively. In the NP tank test, mean measured concentrations of <0.25, 7.2 and 24.5 µg l<sup>-1</sup> were recorded for the control, 10 and 30 µg l<sup>-1</sup> treatments. A nominal stock concentration of 120 mg l<sup>-1</sup> NP, used to dose the 30 µg l<sup>-1</sup> tank, was measured to be 121 mg l<sup>-1</sup>. The less concentrated stock used to dose the 10 µg l<sup>-1</sup> tank could not be accurately measured due to a build up of bacterial slime which interfered with the extraction procedure.



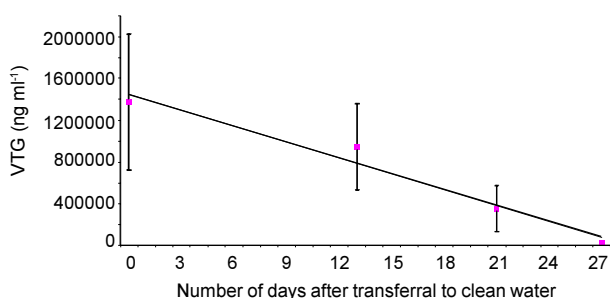
**Figure 6.** Mean Gonadosomatic Indices (GSI) in male flounder exposed to EE2 and NP for 3 weeks. Error bars represent standard error of the mean

#### 4.1.2 VTG elimination study

Data from the two treatment and two control tanks were pooled. The mean VTG concentration on Day 0 (i.e. at the start of the experiment) was 61.2 (S.E. ± 16.1) ng ml<sup>-1</sup> in control male flounder and 68.8 (S.E. ± 12.3) ng ml<sup>-1</sup> in males placed in the treatment tanks. After three weeks exposure to 10 ng l<sup>-1</sup> EE2, the mean plasma VTG concentration in males had risen to 1.37 mg ml<sup>-1</sup>. Control male mean VTG concentration was 98.1 ng ml<sup>-1</sup>. Subsequent measurements made over the next three weeks, after EE2-exposed fish were transferred back to clean water, showed a decline in the VTG concentration (Figure 7) while VTG concentrations in control males



**Figure 7.** Mean plasma vitellogenin concentrations in male flounder after exposure to EE2 for 3 weeks and at various intervals after transferral to clean conditions



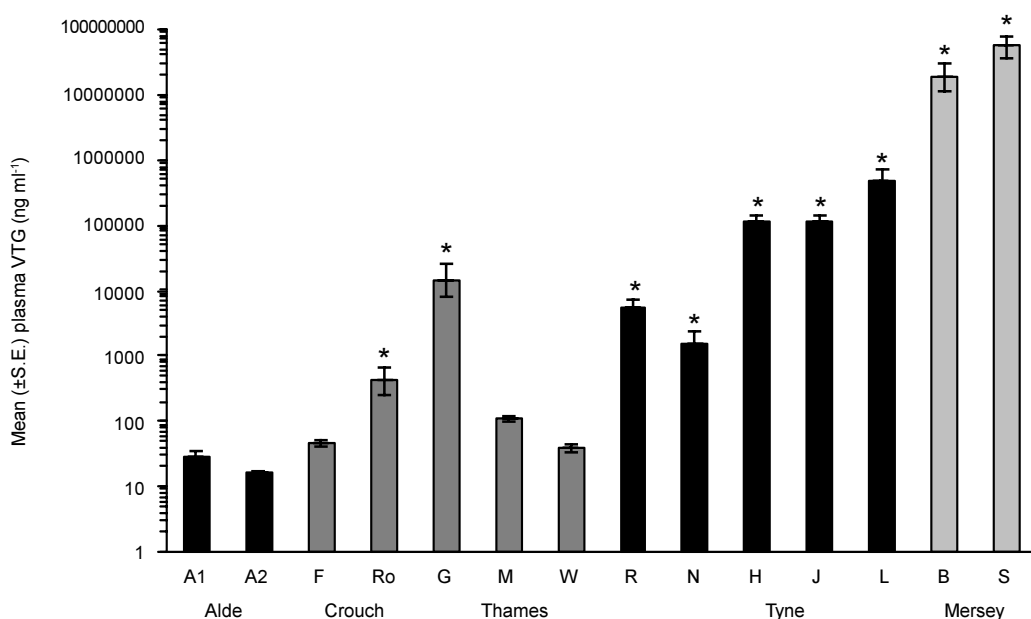
**Figure 8.** Elimination of VTG from blood plasma after 3 weeks continuous exposure to 10 ng l<sup>-1</sup> EE2

remained low. This elimination of VTG from the blood was linear (Figure 8), with a calculated (linear regression) half-life of 13.5 days. Approximately three weeks after transferral to clean conditions, mean plasma VTG concentrations had dropped to 19,424 ng ml<sup>-1</sup>.

## 4.2 Field studies

### 4.2.1 1996 estuarine survey

On the Tyne estuary, male flounder were sampled at Redheugh, Newcastle, Hebburn, Jarrow and Lloyds Hailing Station (Figure 2). Plasma VTG concentrations at all sites were significantly elevated ( $p < 0.001$ ) compared to those in flounder from the control site (Figure 9). Fish at the three outermost stations had the highest mean concentrations of 0.113, 0.116 and 0.484 mg ml<sup>-1</sup> VTG. Three sites on the Thames estuary were sampled: Galleons Reach, Mucking and Warp (Figure 2(c)). Male fish from the innermost site alone, Galleons Reach, had significantly elevated concentrations (Figure 9) of VTG (mean = 14,568 ng ml<sup>-1</sup>). Two sites on the Crouch estuary, Fambridge and Roach, were surveyed (Figure 2(d)). The mean VTG concentration was significantly increased at the latter site (Figure 9) although was much less (443 ng ml<sup>-1</sup>) than at most sites in other estuaries which had significantly increased VTG. Male fish from both sites sampled on the Mersey estuary, Bromborough Port and Speke (Figure 2(d)), had very significantly elevated concentrations of VTG and the highest recorded concentrations of all surveyed sites

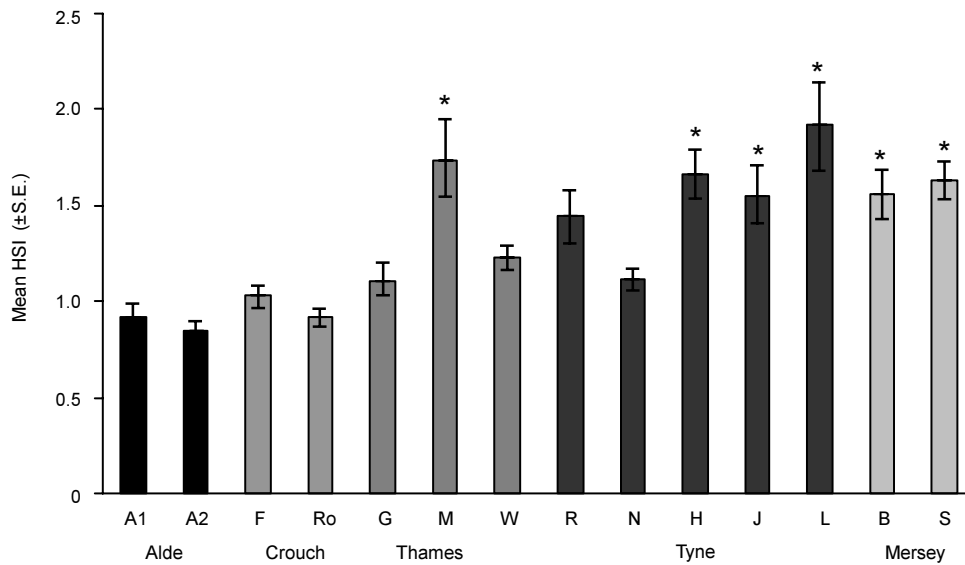


**Figure 9.** Mean plasma vitellogenin induction in male flounder from various estuaries around England. Alde: A1=November sample, A2=December sample; Crouch: F=Fambridge, Ro=Roach; Thames: G=Galleons Reach, M=Mucking, W=Warp; Tyne: R=Redheugh, N=Newcastle, H=Hebburn, J=Jarrow, L=Lloyds Hailing Station; Mersey: B=Bromborough Port, S=Speke. \* denotes significantly different from pooled control estuary (Alde) fish ( $p < 0.001$ ). Error bars represent standard error of the mean

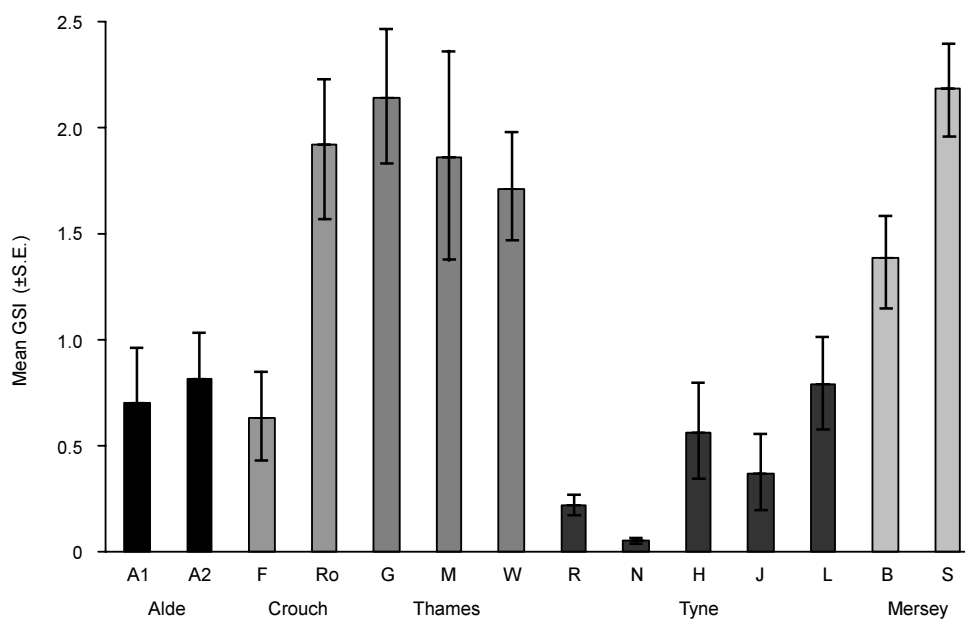
(Figure 9). Mean plasma VTG was 19 and 59 mg ml<sup>-1</sup> respectively, six orders of magnitude greater than the reference VTG concentrations. As expected, male Hepatosomatic Indices were elevated in fish with high VTG titres (Figure 10), but no consistent effects on Gonadosomatic Index were seen (Figure 11).

Male and female fish from the estuaries surveyed in 1996 were classed as either immature or mature, according to gonad weight, (with arbitrary cut off values

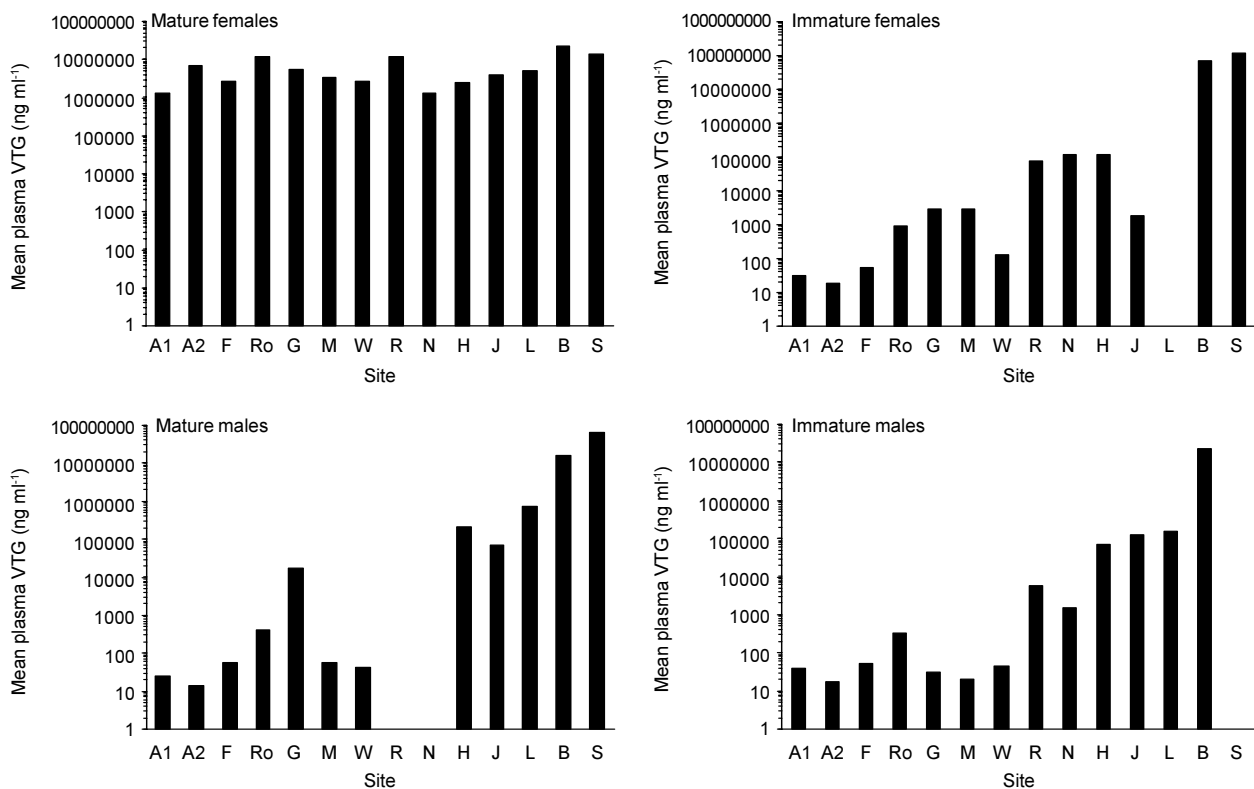
of 3g for females and 1g for males) and the concentrations of VTG in each of the four groups were then compared between sites (Figure 12). It is interesting to note that mature females from the Tyne, Mersey, Crouch and Thames did not have plasma vitellogenin concentrations which were any more elevated than mature females caught in the Alde. Males and immature females from the Mersey, however, had vitellogenin concentrations which were much higher than those found in mature females.



**Figure 10. Mean Hepatosomatic Indices (HSI) in male flounder from various estuaries around England. Site abbreviations as Figure 9. \* denotes significantly different from pooled control estuary (Alde) fish ( $p < 0.001$ ). Error bars represent standard error of the mean**



**Figure 11. Mean Gonadosomatic Indices (GSI) of male flounder from various estuaries around England. Site abbreviations as Figure 9. Error bars represent standard error of the mean**



**Figure 12. Mean plasma vitellogenin concentrations in mature females, immature females, mature males and immature males from sampled estuaries around England. Site abbreviations as Figure 9**

A gross abnormality in the testes of some male Tyne fish was noted - this consisted of an elongation of the normal triangular shaped testicular lobe, which was then bent round into a U-shape (Plate 2). No serious histological abnormalities were seen in the Tyne, Alde, Thames or Crouch fish but 17% of male Mersey fish (i.e. 5 out of a total of 30 males examined) displayed large numbers of primary and secondary oocytes in the testis tissue i.e. they were apparently hermaphrodite or intersex. In severe cases, cords of oocytes with densely staining cytoplasm were distributed along the septae of connective tissue separating the testicular lobules (Plates 3 and 4). Numerous spermatocytes and spermatids could still be clearly differentiated (Plate 4) together with early developmental stages of oocytes. In one fish, a few more mature oocytes containing conspicuous yolk droplets were observed (Plate 5). In all cases the basic lobular architecture of the testis (Plate 7) was retained. No pathological changes were observed in flounder ovaries (Plate 8).

#### 4.2.2 1997 North Sea and Irish Sea surveys

North Sea male flounder had a mean plasma VTG concentration of 77 (S.E.  $\pm 4$ ) ng ml<sup>-1</sup>, which, although small, was significantly greater than fish from Thames Warp, Crouch Fambridge and the Alde ( $p < 0.001$ ). In the Irish Sea and English Channel surveys, VTG in male fish from all sites, with the exception of Carmarthen Bay where only one male fish was caught, was significantly

( $p < 0.01$ ) elevated compared with the Alde (Figure 13). None of the male fish from these samples contained the ovotestis condition, and none of the females contained abnormal ovaries.

To confirm that the significant differences which were found between male flounder vitellogenin concentrations in the North Sea and the three other areas which had apparently low oestrogenic contamination (the Thames Warp, Crouch Fambridge and the Alde) were not due to interassay variability and/or to reduced accuracy engendered by reading values close to the top of the standard curve, 15 plasma samples (including males and immature females) from each area were measured with a single sensitive assay. The mean  $\pm$  S.E. concentrations (in ng ml<sup>-1</sup>) were: North Sea, 61  $\pm 6$ ; Thames Warp, 26  $\pm 3$ ; Crouch Fambridge, 33  $\pm 3$ ; Alde, 23  $\pm 7$ . The results confirmed that males caught in the North Sea had significantly higher vitellogenin concentrations (at the 5% confidence level) than males and immature fish in the other three areas.

A single plasma sample (from a male flounder caught in the Tyne) was run in each assay as a control to establish inter-assay variability. In sixteen assays, the mean ( $\pm$  S.E.) concentration of this sample remained close to 45.0 ( $\pm 2.1$ )  $\mu$ g ml<sup>-1</sup>. The coefficient of variation was 19%. There was no evidence that the concentration of vitellogenin in this sample was affected by repeated thawing and freezing.

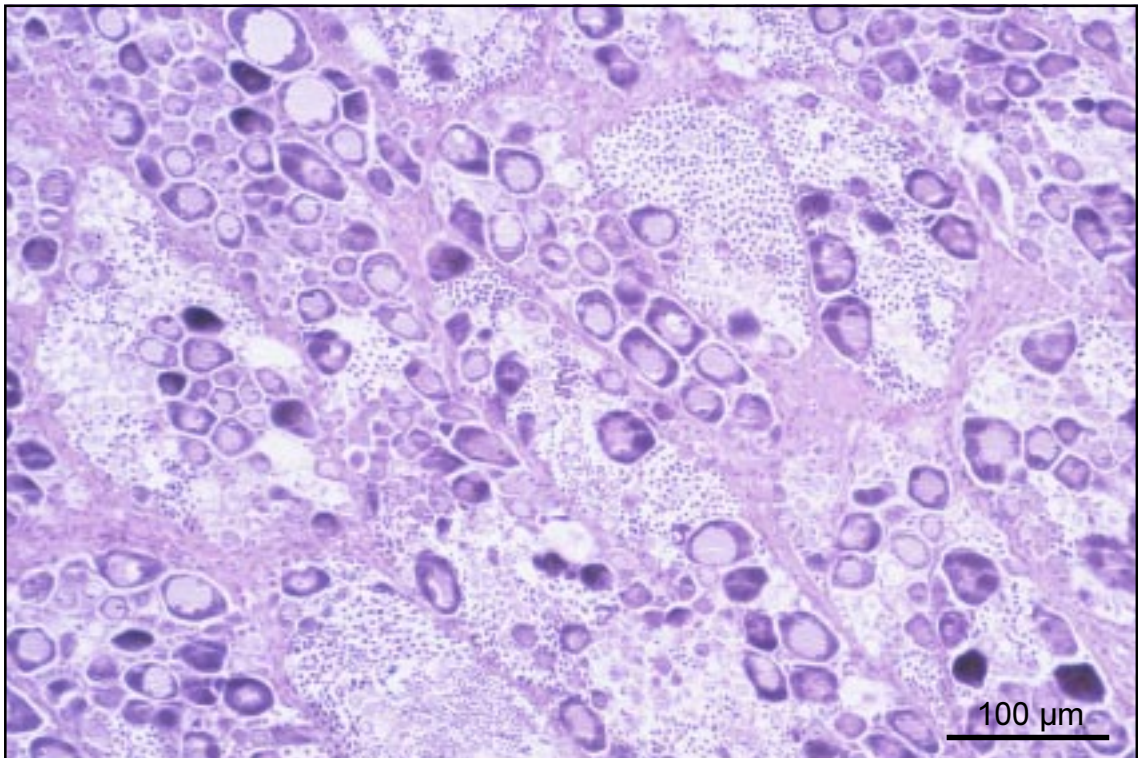




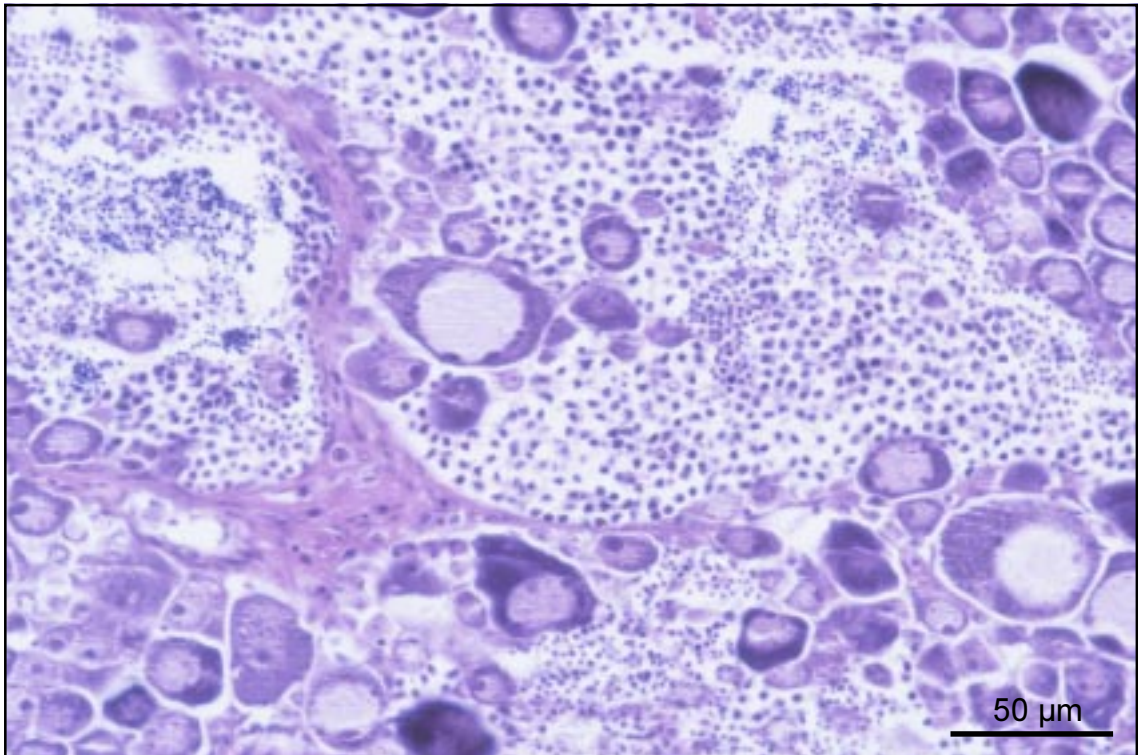
*Plate 1. A specimen of the flounder Platichthys flesus (L.)*



*Plate 2. Two male flounder from the Tyne estuary. The larger one has a normal triangular-shaped testis, but the testis of the smaller fish is grossly misshapen although histologically normal*

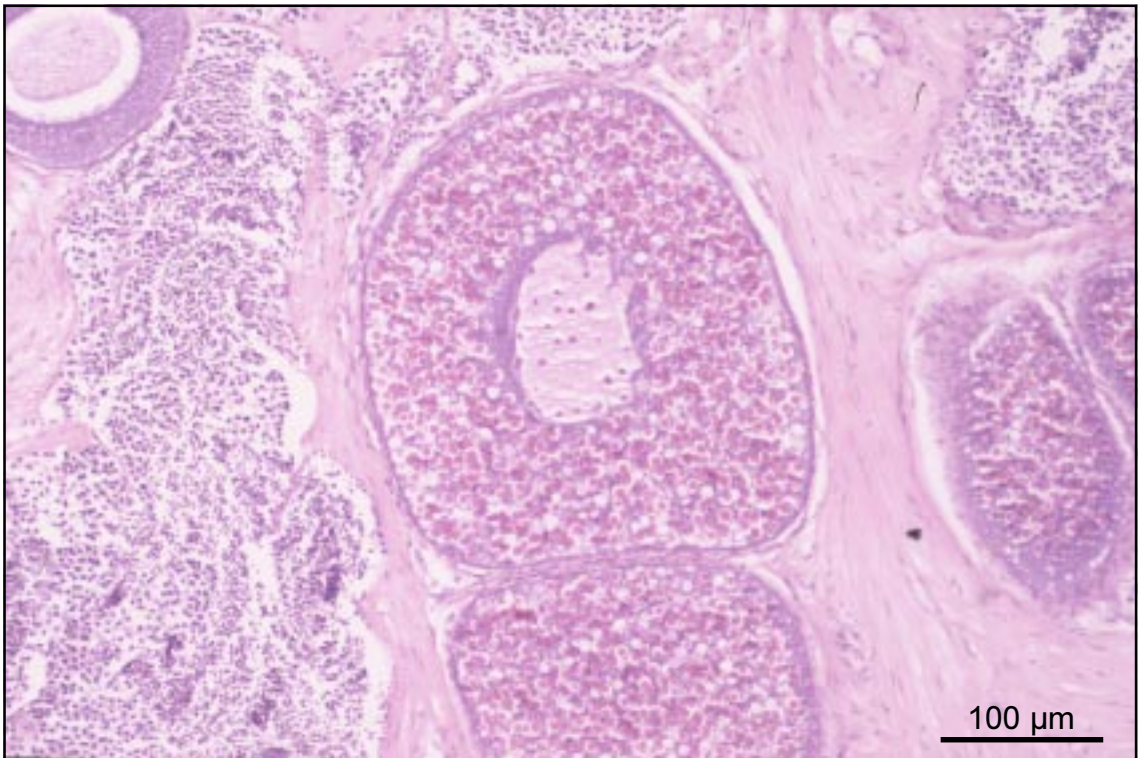


**Plate 3.** *An example of testis histology from an intersex male flounder, showing developing primary and secondary oocytes among the testicular tissue*

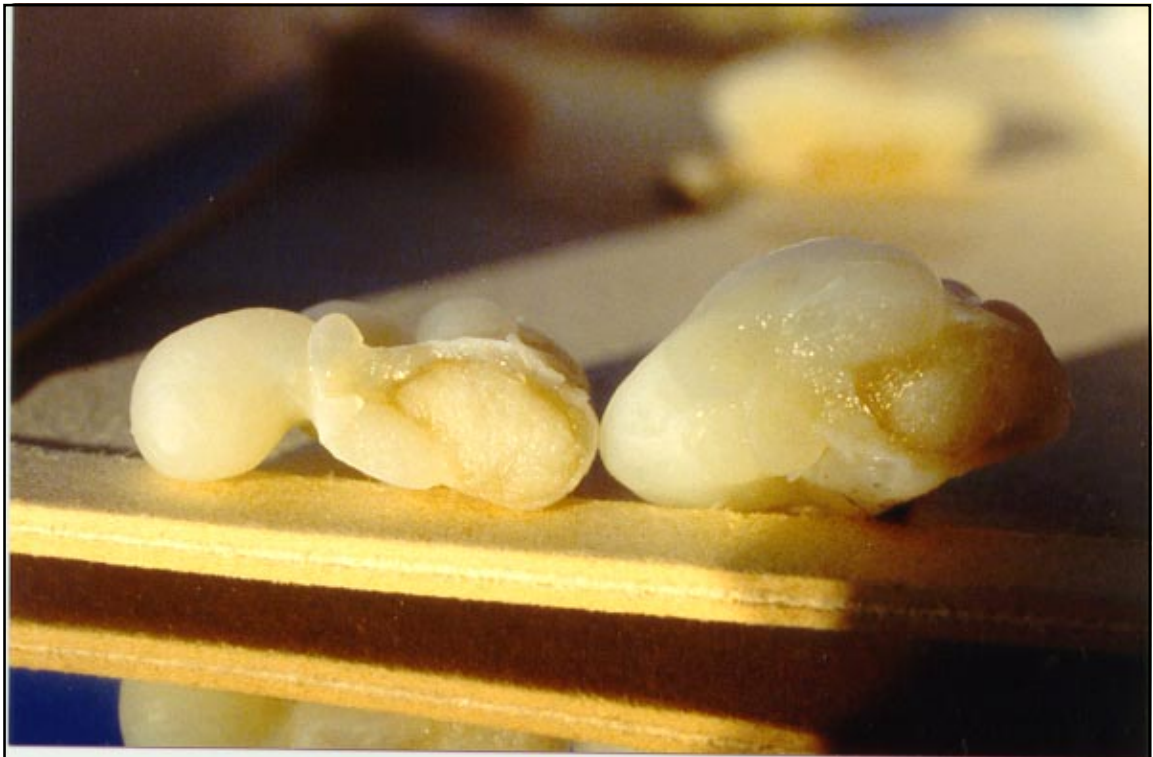


**Plate 4.** *A higher magnification of testicular tissue from an intersex male flounder*

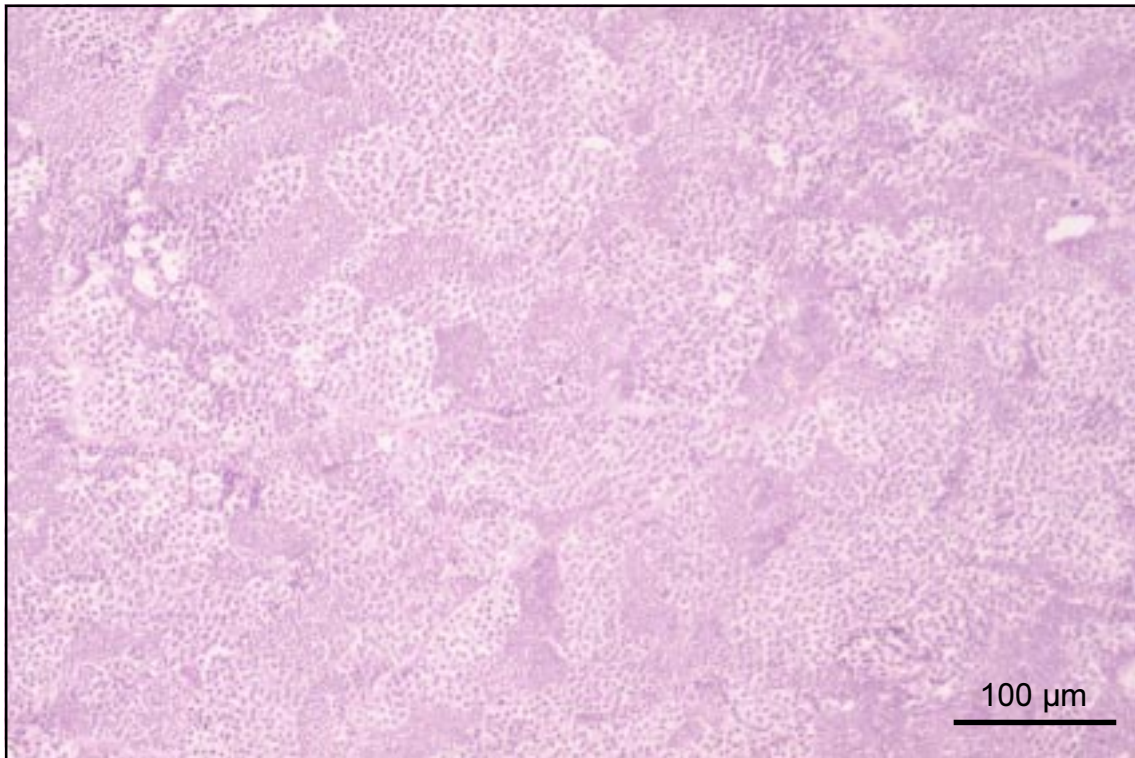




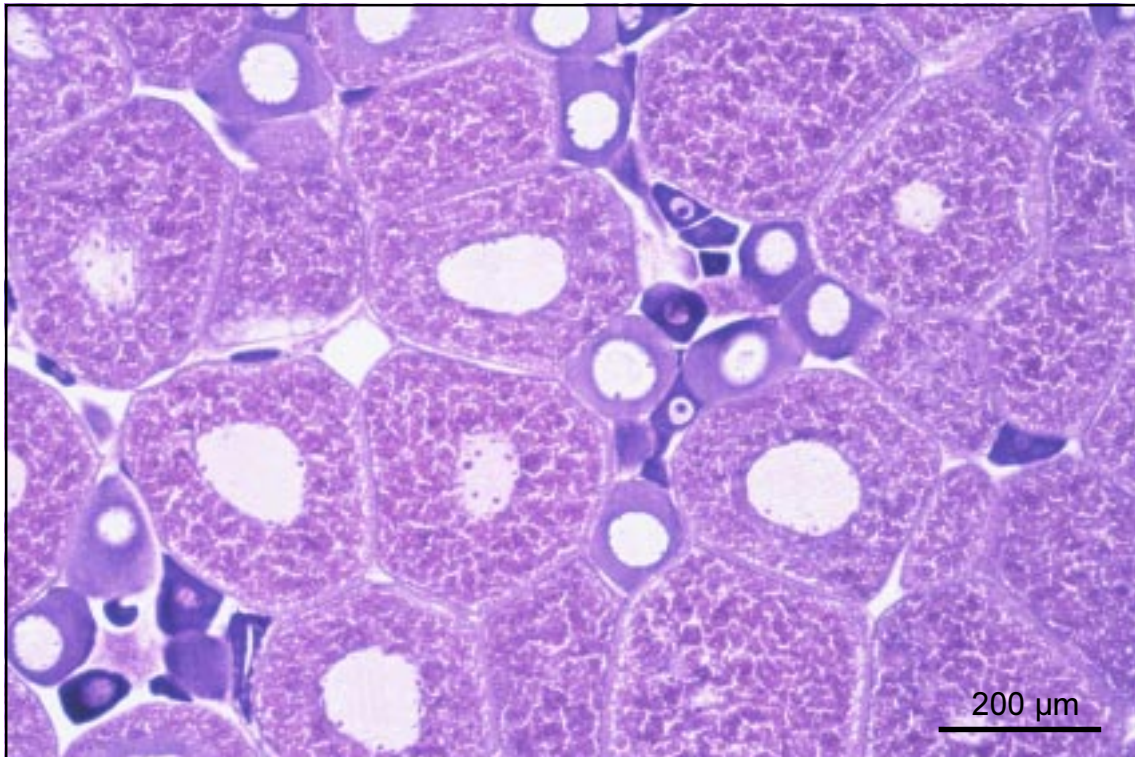
**Plate 5.** *Vitellogenic oocytes in the testis of an intersex male flounder*



**Plate 6.** *A testis from an intersex male flounder showing complete separation of testicular and ovarian tissue*

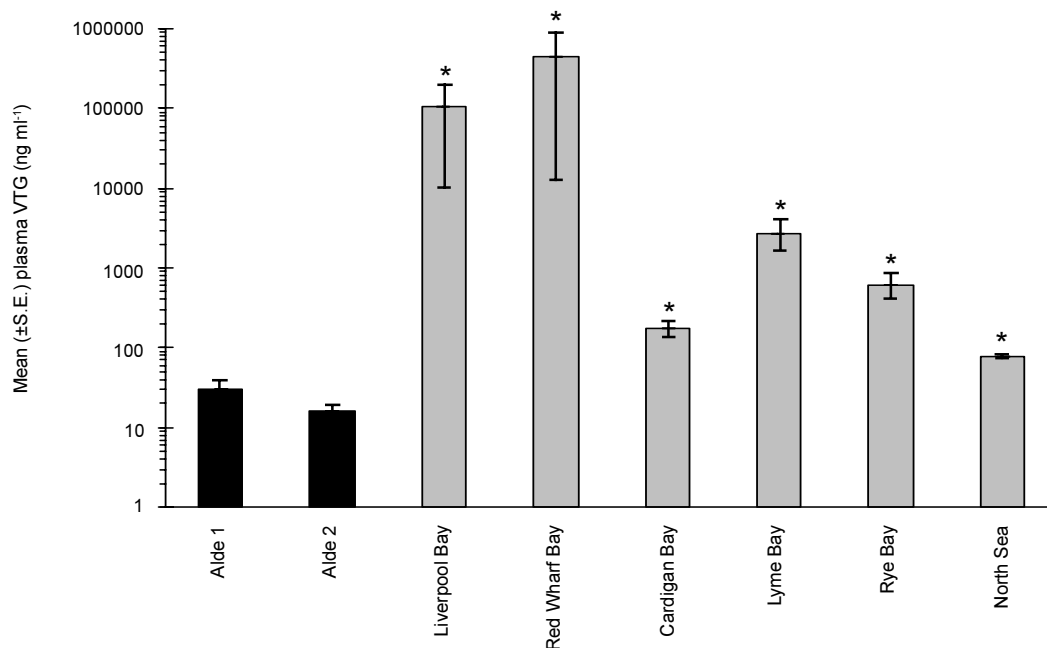


**Plate 7.** *Histology of testicular tissue from a normal male flounder*



**Plate 8.** *Histology of ovarian tissue from a normal female flounder, showing several stages of oocyte development*





**Figure 13.** Mean plasma vitellogenin induction in male flounder from offshore sites around the coast of England. \* denotes significantly different from pooled control estuary (Alde) fish ( $p < 0.001$ ). Error bars represent

#### 4.2.3 1997 estuarine survey

Mean plasma VTG concentrations in male flounder caught in the Alde in 1997 were again low and were very similar to concentrations in 1996 (23.8 versus 22.7 ng ml<sup>-1</sup> for 1997 and 1996 respectively). As in the previous year, flounder from the Tyne and Mersey had very high concentrations of VTG in their plasma (averaging around 1 mg ml<sup>-1</sup>), although Mersey concentrations were lower than in 1996 and similar to those of the Tyne (Figure 14). Although the number of males caught in the Tees and Wear was small, all showed elevated VTG concentrations which were on a par with the Tyne and Mersey. Flounder caught in the Dee and Tamar had the lowest concentrations of VTG and were not significantly elevated when compared with those of Alde fish. VTG levels in fish from sites on the Thames were slightly elevated, but not significantly so. VTG concentrations in Clyde fish were generally low with the exception of the fish from Crannog which had a mean concentration of 27,000 ng ml<sup>-1</sup>. Flounder from Southampton Water and one of the four sites sampled on the Humber (Hawkins Point) had significantly elevated VTG concentrations but these were two or more orders of magnitude lower than the Tyne and Mersey.

Male flounder HSI and GSI values from all estuaries are presented in Figures 15 and 16 respectively. Generally, fish in which VTG concentrations were found to be moderately or highly elevated had correspondingly high HSI values. However, flounder from the Tamar and the Dee, which had low concentrations of VTG, had HSI values which were comparable to fish from contaminated estuaries such as the Mersey,

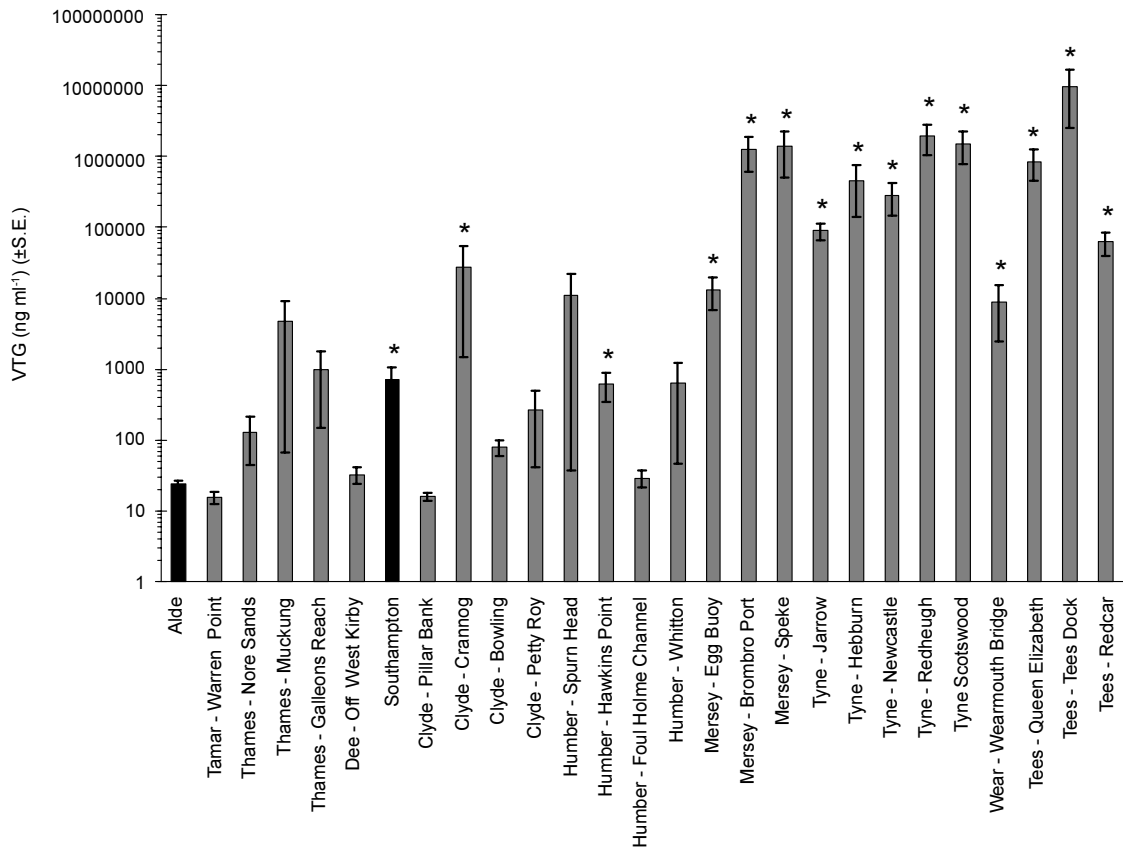
Tees and Tyne, possibly indicating exposure to non-oestrogenic contaminants that cause liver hypertrophy. As in 1996, there were no consistent effects on GSIs.

Histopathological examination of gonad tissue showed that only males from the Tyne and Mersey displayed ovotestis, and no pathological changes were observed in flounder ovaries. On the Mersey, a total of 6 males out of a total of 65 examined (i.e. 9.2%) contained oocytes; on the Tyne, 5 males out of 65 (7.5%) contained oocytes. The majority of these were primary and secondary stage oocytes, but some individuals contained fully developed yolky eggs. Generally, the basic lobular architecture of the testis was retained, although in extreme cases the majority of the testis was replaced by vitellogenic oocytes. One fish from the Mersey was affected so severely that separate testicular and ovarian tissue was visible by the naked eye (Plate 6) and it was difficult to discern whether the true sex of the fish was male or female.

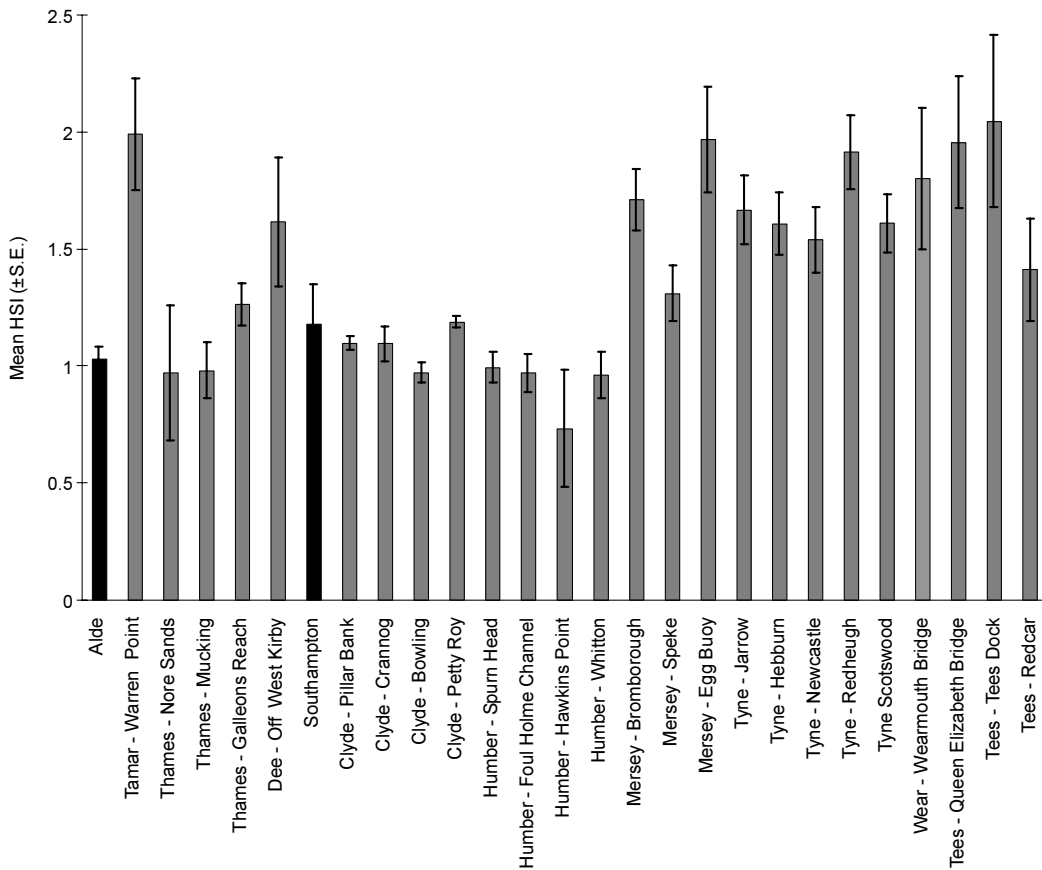
There was no correlation between the concentration of plasma VTG and the occurrence of ovotestis in

**Table 3.** VTG concentrations (ng ml<sup>-1</sup>) in individual intersex male flounder from the Mersey and Tyne in the 1997 survey

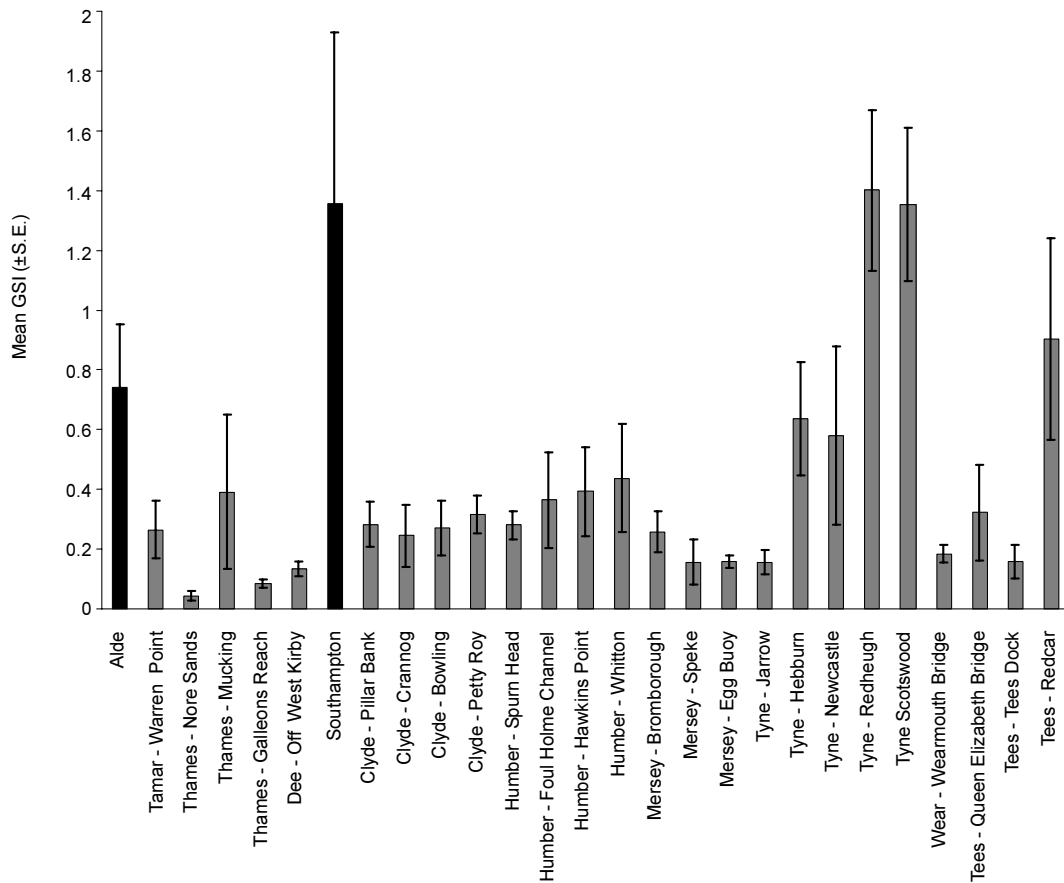
Mersey	Tyne
34.9	33.9
56.6	406.4
200.6	2149.5
2300.2	4184.4
17003.3	5794.9
23697.0	-



**Figure 14. Mean plasma vitellogenin induction in male flounder from estuaries sampled in 1997. \* denotes significantly different from control estuary (Alde) fish ( $p < 0.001$ ). Error bars represent standard error of the mean**



**Figure 15. Mean Hepatosomatic Indices (HSI) in male flounder from various estuaries around England and Scotland sampled in 1997. Error bars represent standard error of the mean**



**Figure 16. Mean Gonadosomatic Indices (GSI) of male flounder from various estuaries around England and Scotland sampled in 1997. Error bars represent standard error of the mean**

individual fish (Table 3). In both estuaries, males with VTG concentrations ranging from low to medium displayed ovotestis, which confirms that ovotestis induction is not necessarily associated with VTG induction in adults. Consideration of sex ratios (Table 4) was somewhat hampered by the fact that sample sizes from some estuaries were too small to draw reliable

conclusions. However, enough fish from the Alde, Mersey, Clyde, Tyne and Humber were obtained to show that sex ratios did not depart markedly from unity in these estuaries. Furthermore, the male:female ratio for all sites pooled together was 1:1.07. It should be noted, however, that these data are based on phenotypic sex characteristics and not on genotypic sex.

**Table 4. Flounder sex ratios in each of the surveyed estuaries in 1997**

Estuary / Coastal Site	Male : Female sex ratios in different size classes					Classes combined
	≤15.9 cm	16.0–20.9 cm	21.0-25.9 cm	26.0-30.9 cm	≥31 cm	
Alde	0:0	0:0	11:17	11:10	1:0	23:27
Cardigan Bay	0:0	0:0	0:0	2:2	1:0	3:2
Clyde	0:0	28:27	35:30	1:2	1:2	65:61
Dee	0:0	4:1	5:10	5:7	1:10	15:28
Humber	0:1	8:12	36:28	9:19	2:1	55:61
Liverpool Bay	0:0	0:0	1:1	4:1	0:4	5:6
Lyme Bay	0:0	0:0	4:0	6:0	12:14	22:14
Mersey	1:1	15:18	14:13	21:26	12:24	63:82
Red Wharf Bay	0:0	0:0	1:0	7:1	5:1	13:2
Rye Bay	0:0	0:0	5:0	2:3	2:4	9:7
Southampton	0:0	1:2	1:1	1:3	2:1	5:7
Tamar	0:0	0:0	2:1	4:2	3:4	9:7
Tees	0:0	4:1	4:5	0:3	2:0	10:9
Thames	0:0	4:4	8:10	2:5	1:0	15:19
Tyne	1:0	19:22	16:19	18:19	16:16	70:76
Wear	0:0	0:1	1:1	0:0	0:0	1:2

Overall ratio = 383 : 410 = 1 : 1.07

**Table 5.** Concentrations of PAH in flounder liver ( $\mu\text{g kg}^{-1}$  wet weight).  $C_1\text{-N}$ ,  $C_2\text{-N}$ , and  $C_3\text{-N}$  represent alkyl naphthalenes of increasing substitution.  $\Sigma\text{PAH}$  is the sum of the concentrations of PAH determined. In addition to the compounds shown,  $C_1$ -phenanthrenes, fluoranthene, pyrene, benz[a]anthracene, chrysene/triphenylene, 2,3-benzanthracene, benzo[b+j+k] fluoranthenes, benzo[e]pyrene, benzo[a]pyrene, perylene, benzo[ghi]perylene and indeno[123-cd]pyrene were determined, but found to be below the limit of detection (ca.  $0.1 \mu\text{g kg}^{-1}$  wet weight per component) in all samples. P/A represents the ratio of the concentrations of phenanthrene and anthracene

Location	Naphthalene	$C_1\text{-N}$	$C_2\text{-N}$	$C_3\text{-N}$	Phenanthrene	Anthracene	$\Sigma\text{PAH}$	P/A
Alde	30	29	14	9.4	27	0.3	109.7	90
Dee	17	26	24	21	4.6	5.8	98.4	0.8
Humber	5.2	15	20	15	13	3.0	71.2	4.3
Humber	5.5	18	31	41	3.5	1.6	100.6	2.2
Mersey	17	64	43	133	69	37	363	1.9
Tamar	8.1	11	13	11	2.3	2.3	47.7	1.0
Tees	28	99	107	107	14	10	365	1.4
Thames	12	17	33	53	38	4.7	157.7	8.1
offshore Tyne/Tees	6.4	23	43	11	2.0	2.1	87.5	1.0

Serial sections through affected testes revealed that oocytes tended to be distributed evenly throughout the organ, although occasionally in small clusters. Serial sections of testes from ten fish which did not show evidence of ovotestis in the single section made originally confirmed the absence of the condition in these samples.

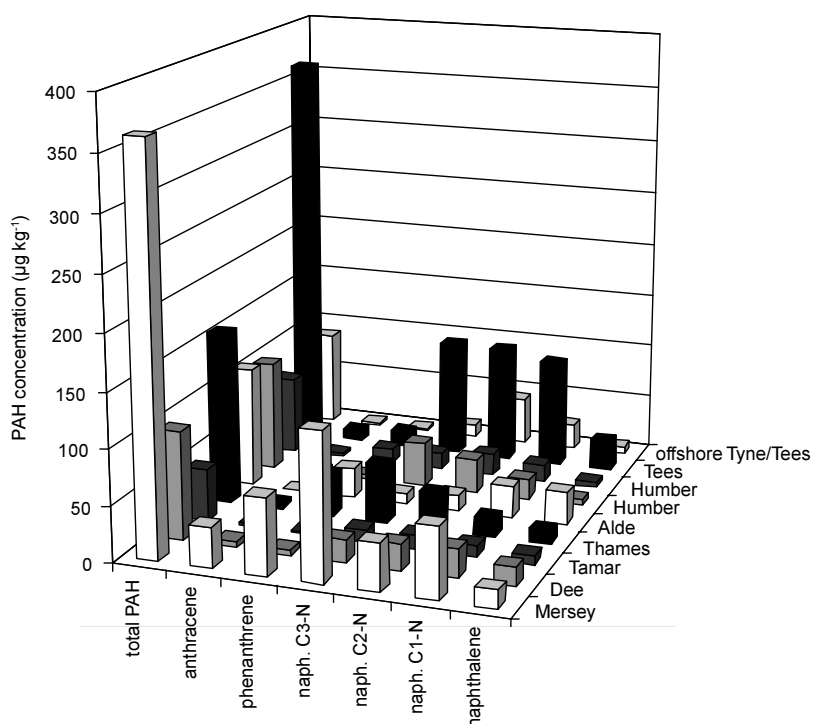
### 4.3 Caged flounder trials

Small flounder placed in the cage did not survive for more than 5 days. With the larger sized fish, there was 100% survival after 2 weeks confinement and after five weeks 50% remained alive. The success of flounder

caging studies in estuaries as part of future research programmes will therefore be dependent on using larger individuals, in order to achieve a sufficient survival rate over a three week exposure period.

### 4.4 PAH residues in flounder liver

$\Sigma\text{PAH}$  concentrations in bulked samples of flounder liver ranged from 48 to  $365 \mu\text{g kg}^{-1}$  wet weight (Table 5; Figure 17). The highest concentrations (363 and  $365 \mu\text{g kg}^{-1}$  wet weight respectively) were seen in samples from the Mersey and Tees. In all cases naphthalene and its



**Figure 17.** PAH concentrations ( $\mu\text{g kg}^{-1}$  wet wt.) in pooled flounder liver



alkyl derivatives predominated, accounting for 71 to 95% of the ΣPAH concentration. PAH larger than 3-ring compounds (including the majority of combustion origin, and those with carcinogenic potential) were not detected in any of the samples. This is in accord with the studies undertaken following the *SEA EMPRESS* oil spill in Wales in 1996 (Law *et al.*, in press). The low molecular weight PAH are relatively water soluble, and so are taken up by fish across gill surfaces. Fish also possess an effective mixed-function oxygenase enzyme system, and PAH are rapidly metabolised thereby minimising accumulation. The phenanthrene/anthracene ratios range from 0.8 to 8.1 at all sites, except the River Alde control estuary where the value is 90 (Figure 18). Phenanthrene is much more abundant than anthracene in oil, whereas when the major source is combustion processes values closer to unity are observed. In the Alde the ratio suggests an oil source, possibly small boat traffic. With the larger human populations around the other estuaries the main or dominant source seems to be combustion.

#### 4.5 Organochlorines in flounder liver

Dieldrin, alpha and gamma hexachlorocyclohexane, DDT group compounds, hexachlorobenzene and chlorobiphenyl residues were commonly found in all samples (Table 6; Appendices 1 and 2; Figure 19).

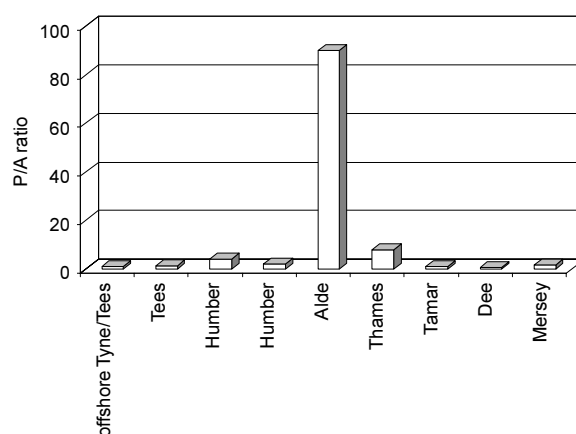
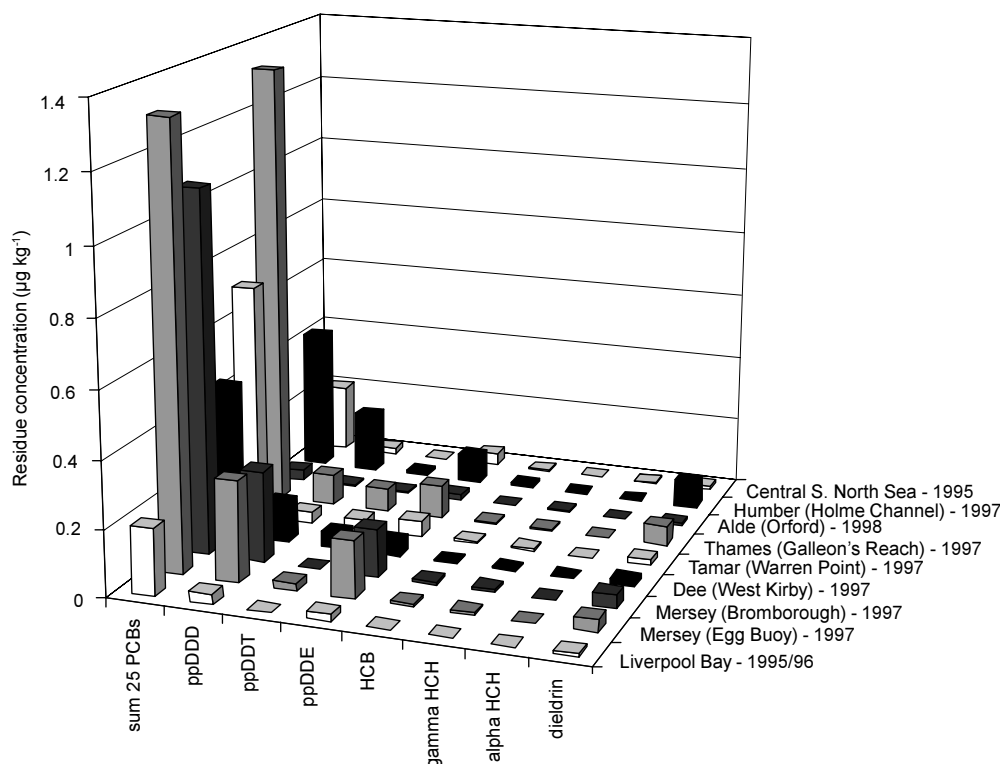


Figure 18. Phenanthrene / anthracene ratios in pooled flounder liver

There are no standards for these compounds in fish from England and Wales but the values presented are similar to those found in previous surveys which concentrated on the Liverpool and Morecambe Bay areas. These surveys indicate that the PCB values for flounder are in the medium and upper category of the guidelines issued by the Joint Monitoring Programme (JMP) of the Oslo and Paris Commissions (CEFAS, 1997).

Table 6. Median organochlorine residue concentrations (mg kg<sup>-1</sup> wet wt.) in pooled flounder liver (5 livers per pool). Number of pools = 1-7

Location	dieldrin	alpha HCH	gamma HCH	HCB	pp'-DDE	pp'-DDT	pp'-DDD	sum 25 PCBs	% lipid
Alde (Orford) - 1998	0.008	0	0.004	0	0.014	0.005	0.005	0.032	9
Central S. North Sea - 1995	0.011	0.002	0.003	0.002	0.037	0.001	0.017	0.203	9
Dee (West Kirby) - 1997	0.017	0.002	0.004	0.005	0.044	0.047	0.12	0.453	21
Humber (Holme Channel) - 1997	0.082	0	0.002	0.011	0.085	0.009	0.18	0.424	10
Liverpool Bay - 1995/96	0.008	0.001	0.002	0.001	0.021	0	0.03	0.2	9
Mersey (Bromborough) - 1997	0.041	0.002	0.01	0.008	0.14	0	0.27	1.082	-
Mersey (Egg Buoy) - 1997	0.037	0	0.007	0.009	0.17	0.021	0.3	1.315	22
Tamar (Warren Point) - 1997	0.019	0	0.005	0.007	0.048	0.03	0.033	0.708	19
Thames (Galleon's Reach) - 1997	0.059	0.002	0.006	0.005	0.1	0.068	0.093	1.33	13



**Figure 19. Organochlorine residue concentrations in pooled flounder liver ( $\text{mg kg}^{-1}$  wet wt.) (median values for 1-7 groups of pooled livers – 5 livers per pool)**

#### 4.6 EROD induction in flounder liver

The span of EROD activities from all the sites sampled is represented in Figure 20. Significantly ( $p < 0.05$ ) elevated activity, compared to the Alde reference site, was evident in ten out of the 16 stations sampled. The sites from the Mersey and Tees showed the greatest induction, reaching a maximum of 161.6 pM/min/mg protein, a four fold increase over the reference level.

It is clear from this survey that the mixed function oxygenase (MFO) enzyme system in flounder is significantly induced in several of the estuaries where VTG is also induced in males. These results can be considered as a marker for the relatively high general contamination of estuaries such as the Mersey, Tees and Tyne and suggest that the flounder, and probably other fish, populations in these areas are undergoing sub-lethal contaminant stress.

#### 4.7 Steroid hormone levels in flounder blood

Both positive and negative modes of ionisation were evaluated using a  $1 \text{ ng } \mu\text{l}^{-1}$  solution of each steroid which was injected directly into the mass spectrometer and data acquired in full scan mode (*ca.* 100-350 AMU).

Positive ionisation produced strong protonated molecular ions  $[\text{M}+\text{H}]^+$  for testosterone ( $m/z$  289), 11-ketotestosterone ( $m/z$  303) and progesterone ( $m/z$  316) whilst  $17\beta$ -oestradiol was poorly ionised and produced a fragmented mass spectrum which was unsuitable for SIM. Negative ionisation of  $17\beta$ -oestradiol produced a deprotonated molecular ion  $[\text{M}-\text{H}]^-$  ( $m/z$  272) which was suitable for SIM. As is common with APCI, the cone voltage and probe temperature were then optimised to minimise fragmentation and maximise instrument sensitivity (Doerge and Bajic, 1992). Good chromatographic separation of each analyte prior to introduction into the mass spectrometer allows the instrument to be used in the positive mode to determine androgen and progesterone concentrations whilst then being switched to the negative mode to determine the concentration of  $17\beta$ -oestradiol. A chromatogram of the respective steroids spiked into bovine serum is shown in Figure 21. Each peak is resolved to baseline with good peak shape. Analyte recovery data are presented in Table 7. Repeat injections ( $n=8$ ) at  $1 \text{ ng ml}^{-1}$  concentrations demonstrated that the detection method shows good precision whilst acceptable recoveries were obtained for all analytes. The limits of detection obtained are sufficient to enable the use of HPLC-APCI-MS for the determination of E/A ratios in blood plasma.

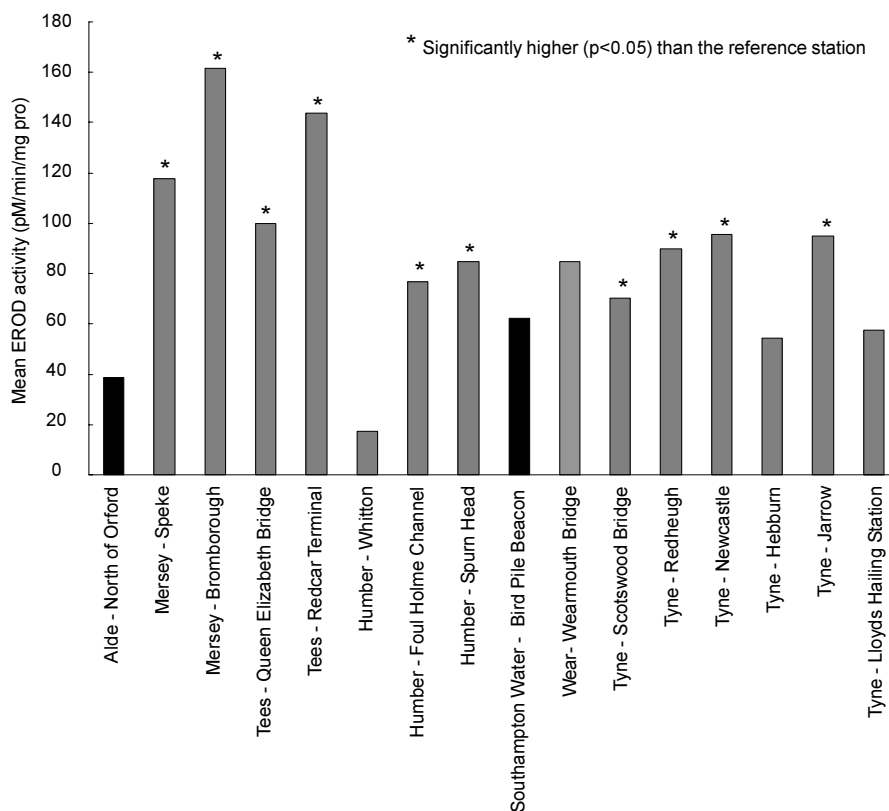


Figure 20. Mean EROD activities in English estuaries (pM/min/mg protein)

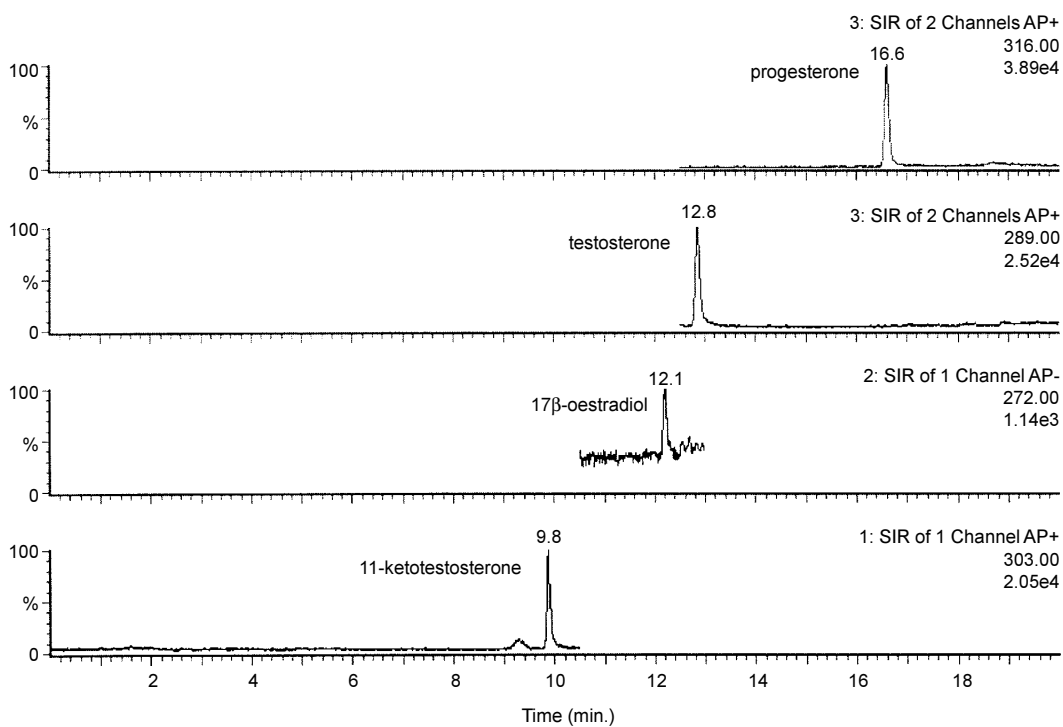


Figure 21. Chromatograms showing the analysis of steroid standards extracted from bovine serum. Retention times (min): 11-ketotestosterone (9.8), 17 $\beta$ -oestradiol (12.1), testosterone (12.8) and progesterone (16.6)

**Table 7. Recovery of selected steroids from spiked serum**

Analyte	Mean Recovery (%) <sup>†</sup>	Std.Dev. (n=8)	RSD %	L.O.D. <sup>‡</sup> (ng ml <sup>-1</sup> )
11-ketotestosterone	106	0.25	12	0.1
17β-oestradiol	61	0.20	16	0.1
testosterone	68	0.18	13	0.1

<sup>†</sup> Calculated using:  $Recovery = 100(X_S - X_U) / K$ , where  $X_S$  = concentration measured in spiked sample,  $X_U$  = concentration measured in unspiked sample and  $K$  = known value of the spike in the sample. 4 ng spiked into each sample (2 ml)

<sup>‡</sup> Calculated with a signal to noise ratio of 10

#### 4.7.1 Analysis of flounder blood plasma samples

Blood plasma samples were obtained from female flounder caught in the Dee estuary during September 1997. The serum samples were extracted using the procedure outlined above. The data are presented in Table 8. However, many of the samples contain steroid concentrations that are below the limits of detection for this method. This is possibly due to seasonal changes in blood steroid concentrations linked to the flounder's reproductive cycle. Fish caught later in the year would provide samples with elevated steroid concentrations which would allow an assessment of the impact of environmental endocrine disrupters on blood plasma oestrogen:androgen ratios to be made.

#### 4.7.2 Conclusion of investigation of steroid analysis using HPLC

A simple method has been developed for the measurement of oestrogen/androgen ratios in blood plasma using HPLC followed by mass spectrometric detection using APCI. The method has been shown to have both good precision and accuracy whilst also giving limits of detection which are sufficient for the detection of steroidal compounds in blood plasma at certain periods during the reproductive cycle. The method should be a valuable addition to existing tools that are available for determining the effects of environmental endocrine disrupters.

**Table 8. Oestrogen/androgen concentrations for flounder blood plasma obtained from the River Dee estuary, September 1997**

Sample	Sex	Length (cm)	17β-oestradiol (ng ml <sup>-1</sup> )	11-ketotestosterone (ng ml <sup>-1</sup> )	Testosterone (ng ml <sup>-1</sup> )
Dee 1	F	35.4	<0.1	<0.1	<0.1
Dee 2	F	33.7	<0.1	<0.1	<0.1
Dee 12	F	32.6	0.24	<0.1	<0.1
Dee 15	F	34.9	0.1	<0.1	<0.1
Blank	n/a	n/a	<0.1	<0.1	<0.1
Reference plasma*	n/a	n/a	0.86	1.17	1.09

\* Bovine plasma spiked with steroids

## 5. DISCUSSION

### 5.1 Apparent differences in oestrogenic activity between fresh and salt water

The work of Harries, *et al.* (1995) had led to the discovery that a mere 1:3 dilution of a strongly oestrogenic sewage treatment works (STW) effluent at Harpenden completely removed its ability to produce vitellogenesis in male trout. Furthermore, oestrogenic activity in rivers downstream of STW effluents also declined very rapidly with distance in almost all cases, reducing the activity to a number of more or less localised peaks as one moved past a series of STW inputs. The exception to this rule was the River Aire downstream of Marley STW (Harries *et al.*, 1997) which remained at maximum oestrogenic potency for a distance of at least 5 km. This discharge was dominated by alkylphenol ethoxylate (APE) surfactants and their degradation products originating mainly from local textile mills, whereas at other locations studied, the major oestrogenic inputs consisted of natural and synthetic steroid hormones derived from domestic sewage (Desbrow *et al.*, 1998).

The details of why the effects of an APE-dominated discharge should not significantly diminish with distance downstream have not been fully explored, but they may be associated with the fact that alkylphenols become adsorbed to, and persist in, bottom sediments. The alkylphenol-contaminated mud in the Aire therefore probably acted as a slow-release source of oestrogenic activity over long stretches of river, whereas the more water-soluble steroids tend to dissipate and degrade fairly rapidly. In addition, the rather modest increases in dilution of STW effluents which occur during winter due to heavier rainfall and hence increased riverine discharge, also produced reductions in the oestrogenic potency of water in the River Lea (where steroid hormones predominate). These observations all led to the expectation that the considerably greater dilutional capacity in most UK estuaries compared with UK rivers would minimise the impact of oestrogenic discharges to their waters, especially if those discharges were once more found to be steroid dominated. It was certainly not unreasonable to expect that STWs discharging to estuarine waters would be just as contaminated with steroids as their freshwater counterparts; indeed, this was confirmed by Desbrow *et al.* (1998) for Southend STW which discharges to the Thames estuary.

Many of the results presented in this report have therefore confounded our early expectations. With the exceptions of the Tamar and Dee, all nine other estuaries studied in this survey contained at least one station where male flounder were expressing statistically significant amounts of vitellogenin (in comparison with our reference sites on the Alde). This is despite their

lower sensitivity than trout to oestrogens as determined in our laboratory vitellogenesis experiments. Due to the fairly sedentary nature of flounder for much of the year, which incidentally was evidenced by the huge difference in the VTG responses of Dee and Mersey fish which are separated by less than 20 km of water, one can be reasonably confident that the effects seen were due primarily to local inputs. A probable exception to this is the strong VTG induction of male fish caught in Liverpool Bay and Red Wharf Bay, at increasing distances from the Mersey. Especially in the latter case, local sources of oestrogenic materials are probably negligible. The same applies to the much more weakly induced fish caught in Cardigan Bay, Lyme Bay, Rye Bay and the central North Sea.

Although our data cannot rule out the possibility that the greater or lesser VTG induction seen in coastal and offshore fish was partly due to local contamination, our experimental results with male fish allowed to recover from an exposure to ethynylestradiol show that reduction of plasma VTG levels is fairly slow, with a half-life of approximately 2 weeks. This is not unexpected given that the metabolism of male fish is unlikely to be organised to degrade or excrete this protein with any rapidity. This means that VTG-induced males migrating offshore to breed would still be experiencing significant plasma VTG levels after several months – a sort of VTG ‘memory’ effect. For example, if one assumes that the Mersey fish sampled at the end of 1996 (containing about 40 mg ml<sup>-1</sup> plasma VTG) migrated offshore soon thereafter and ceased to receive any oestrogenic stimulation, then their VTG content could have taken 12-14 weeks to reach the levels seen in the fish in Red Wharf Bay (0.4 mg ml<sup>-1</sup>). This is ample time to explain the VTG levels seen in the Red Wharf Bay fish caught about 2 months after those in the Mersey, which if they had migrated from that estuary (a plausible hypothesis), would only have had to travel a straight-line distance of 80 km. A flatfish could easily manage this in 3 months, although not all flounder migrate this far to breed. Even the mildly induced fish in the central southern North Sea were only about 100-300 km from the nearest large estuaries (Thames, Rhine, Ems, Weser, Elbe, Humber), which again is a feasible distance for a flounder to travel on its breeding migration. Of course, these deductions cannot be taken as proof that the coastal and offshore fish received their oestrogenic exposure in contaminated estuaries, but a ‘memory’ effect of this type certainly appears plausible.

### 5.2 Routes and types of contaminant exposure

Part of the reason that UK estuaries have shown an apparently stronger oestrogenic signal than expected may be connected with the habits of the fish species used for monitoring. The fish that have been used to survey UK rivers (mainly trout and roach) are essentially

mid-water species which feed on surface and planktonic food as well as on benthos. Furthermore, they do not like to bury themselves in the mud like flounder, and so are not exposed to contaminants primarily associated with particulate matter and interstitial water. This means that flounder will tend to be exposed to a different range of contaminants than midwater riverine fish, both directly from the sediments in which they live, and indirectly via the sediment-dwelling invertebrates on which they feed. Such contaminants will tend to be the more adsorptive and lipophilic substances which preferentially accumulate in sediments, and are less likely to be the relatively water-soluble oestrogenic steroids which dominate the oestrogenic load in the water column of some UK rivers. This does not mean that estuarine flounder are not exposed to exogenous steroids, but it may well shift the balance of oestrogenic exposure more towards lipophilic substances such as the organochlorines (OC), phthalates and alkylphenols. For example, it is known (EA, 1998(b)) that several DDT isomers and metabolites (*o,p'*-DDT; *p,p'*-DDT; *p,p'*-DDE; *o,p'*-DDD), as well as lindane, dieldrin, endosulfan, chlordecone, camphechlor, methoxychlor, some phthalates, and some hydroxylated polychlorinated biphenyl (PCB) metabolites all show oestrogenic activity *in vitro* or *in vivo* (or both). Furthermore, *p,p'*-DDT and *p,p'*-DDE (as well as at least one phthalate) also show anti-androgenic activity which could contribute towards phenotypic feminisation of males. On the other hand, other lipophilic materials (e.g. several PCBs, tetra- and penta-chlorodibenzodioxin, pentachlorodibenzofuran, and several polycyclic aromatic hydrocarbons (PAH) including the common benzo[*a*]pyrene) are anti-oestrogenic, so the situation in estuaries which experience multiple contaminants is a very complex one.

As we have shown, flounder from the more industrialised estuaries indeed contain measurable amounts of various oestrogenic and anti-androgenic OCs in their livers, and also contain PAHs (although not the known anti-oestrogenic ones – these are likely to be metabolised too fast to bioaccumulate to measurable levels, even if they are having an effect). In broad terms, the concentrations of OCs (up to 1.3 mg kg<sup>-1</sup> wet wt. of ΣPCB and 0.5 mg kg<sup>-1</sup> wet wt. of ΣDDT, in flounder liver) do not seem to be extremely high or primarily responsible for observed male vitellogenesis. For example, the liver tissue concentrations associated with reproductive endocrine effects in kelp bass from a contaminated site on the coast of southern California were 0.9 mg kg<sup>-1</sup> wet wt. ΣPCB and 3.4 mg kg<sup>-1</sup> wet wt. ΣDDT, and there were no abnormal increases in plasma VTG (as measured by plasma protein phosphorus - Spies and Thomas, 1997). Furthermore, abnormal sex ratios in freshwater bloaters from Lake Michigan only seem to have occurred at *p,p'*-DDE concentrations (whole fish) above approximately 2.5 mg kg<sup>-1</sup> wet wt. (Brown *et al.* and Hesselberg *et al.* reported in Monosson *et al.*, 1997). Perhaps the most thorough

studies of the relationship of OC residues with adverse reproductive effects in fish have been made in lake trout from the Great Lakes which had bred poorly or not at all for many years (Giesy and Snyder, 1998). In many of these waters, lake trout are now breeding again, and by 1993, mean whole-body residue concentrations had already dropped to 2-12 mg kg<sup>-1</sup> wet wt. ΣPCB and 1-8 mg kg<sup>-1</sup> wet wt. ΣDDT. On the basis of a huge amount of observational and experimental data, it is thought that the main responsible contaminants were non- and mono-*ortho* substituted PCBs, PCDD and PCDF. Flounder data are not available for the latter two substances, but the highest PCB levels we have observed in flounder are low by comparison with the ~20 mg kg<sup>-1</sup> wet wt. ΣPCB seen in lake trout from Lakes Huron and Michigan in the mid-1970s when the reproductive effects were at their height.

It is impossible to make an assessment of the overall contribution (or antagonism) of the substances we measured to the observed oestrogenic effects without conducting much more detailed investigatory chemistry of inputs and sediments, and at this stage it seems unlikely that the compounds we detected are major components of the integral effect. Nevertheless, the present residue data show that some of the estuaries causing oestrogenic exposure are also contaminated with industrial chemicals and pesticides, while some sites with only weakly responding fish appear to be only slightly contaminated with such substances. Whether these relationships are causal remains to be seen.

There is no doubt that the industrialised estuaries where the biggest effects in flounder have been observed contain large numbers of synthetic chemicals. For example, Matthiessen *et al.* (1993) found 71 industrially-derived organic chemicals in the Tyne, Humber, Mersey and Tees. Rogers *et al.* (1990) found 64 in the Mersey alone, and it seems likely that this estuary and the adjacent Liverpool Bay are the marine areas most heavily contaminated with OCs in the UK (Leah *et al.*, 1994; MAFF, 1994). Among these are probably several substances (OCs and others) which could be contributing to oestrogenic effects, including the methyl lindane group of compounds which is present at concentrations of up to 0.31 mg kg<sup>-1</sup> wet wt. in flatfish muscle tissue from the Mersey (McNeish *et al.*, 1997).

There are, however, additional circumstantial data which suggest that synthetic chemicals may be major contributors to the oestrogenic effects we have measured. Table 9 shows the total daily volumes of domestic and industrial effluents recorded by the Environment Agency or the Scottish Environment Protection Agency as directly entering the estuaries in question (discharges to the rivers upstream have not been considered in this exercise because of the known rapid decline of oestrogenicity with distance below effluent inputs to these systems). These data show that although the volumes of domestic sewage (in which the

**Table 9. Domestic and industrial effluent discharged direct to each of the surveyed estuaries**

Estuary	Domestic discharges maximum dry weather flow (m <sup>3</sup> day <sup>-1</sup> )					Flushing time (days)	Industrial discharges (m <sup>3</sup> day <sup>-1</sup> ) <sup>d</sup>
	Crude	Primary	Secondary	Tertiary	Total		
Alde	0	0	145	0	145 <sup>a</sup>	-	0
Clyde	-	-	-	-	672,421 <sup>c</sup>	-	209,745 <sup>c</sup>
Crouch	10	7	16,218	900	17,135 <sup>a</sup>	-	0
Dee	0	1901	46,384	2,562	50,847 <sup>a</sup>	-	62,455
Humber	-	-	-	-	207,642 <sup>b</sup>	5	944,201
Mersey	19,954	966	59,181	0	80,102 <sup>b</sup>	30.4	671,158
Southampton	14,621	34,286	195,854	120	244,881 <sup>a</sup>	-	671,336
Tamar	19,674	40,586	33,144	2,252	95,656 <sup>a</sup>	-	19,634
Tees	2,727	93,508	197,192	0	293,427 <sup>b</sup>	-	959,483
Thames	0	0	2,408,400	0	2,408,400 <sup>b</sup>	-	45,045 <sup>e</sup>
Tyne	18,604	250,010	38	0	268,652 <sup>b</sup>	0.77	1,162,931
Wear	15,608	168	15,800	0	31,576 <sup>b</sup>	0.69	216,745

<sup>a</sup> Domestic sewage data obtained from the CEFAS FEPA licensing database, details correct at December 1997

<sup>b</sup> Domestic sewage data obtained from Environment Agency regional databases

<sup>c</sup> Data obtained from SEPA West and DETR

<sup>d</sup> Industrial discharge data obtained from CEFAS licensing database, details correct at December 1997. Note that the domestic discharges also contain some industrial effluent – on average, about 7% of total flow

<sup>e</sup> Industrial discharge data obtained from Environment Agency

main oestrogenic materials are probably steroids) entering the Thames, Mersey, Tamar, Humber, Tyne, Tees, Southampton Water and Clyde all exceed 80,000 m<sup>3</sup>/day, generally only those with very high inputs of industrial effluent (Mersey, Tyne, Wear, Tees) also show high levels of oestrogenic activity. One exception to this trend is the Humber, where the inputs of industrial effluents are relatively high, but oestrogenic responses are rather moderate. The reason for this is unknown, but may be connected with the fact that the flushing time of the Humber is relatively rapid compared with a more enclosed estuary like the Mersey. Southampton Water, which also only produces a mild oestrogenic response in flounder, also receives a relatively large amount of industrial effluent, although over 0.5 million m<sup>3</sup>/day of this emanates from a single oil refinery near the mouth of the estuary and therefore also receives good dilution.

A contributory reason for the relatively slight oestrogenicity in the Thames may be that the high proportion of secondary treated domestic effluent which it receives in comparison with the other estuaries has been preferentially purged of steroids. However, there is no firm evidence for this idea, and it could be argued that secondary treatment would result in enhanced deconjugation of the conjugated steroids derived from human and animal excretion, thereby increasing oestrogenicity. The influence of STW processes on the oestrogenicity of sewage is a question in need of urgent attention.

### 5.3 EROD induction and associated factors

The apparent correlation between EROD and VTG induction in the flounder caught in 1997/98 is interesting, but as with the relationship with residue levels, the two may not be directly related. Most of the residues measured in flounder are known EROD inducers, but it of course does not follow that these substances were also the main causes of oestrogenicity. The following paragraphs indicate the complexity of influences on the cytochrome P450 system.

Gonado-somatic index (GSI) is a measure of gonadal maturity and has been shown to be well correlated with EROD activity in dab during the spawning season (Kirby *et al.*, 1998). It has been shown that GSIs in North Sea flounder start to increase in September for both males and females in preparation for winter spawning, dropping back to baseline levels in February and March for females and males respectively (Janssen *et al.*, 1995; and Janssen *et al.*, 1996).

In the present study in 1997/98, the mean female GSI at the Alde reference site was 0.72 which is at or below baseline levels, also suggesting that these fish were in their resting/pre-spawning phase. Those females sampled at the two Mersey sites also had low mean GSI values of 0.82 and 0.98, which were not significantly different from the Alde. However, at all other sites mean

female GSIs were elevated, significantly so ( $p < 0.05$ ) in all except one case. No clear explanation exists as to why enlarged female gonads were present in the Tyne and Humber samples. If it were contaminant induced, one might expect those in the Mersey to be enlarged also. It is possible that the later sampling date of the Tyne/Humber fish (a month after the Mersey) meant that they had entered an early reproductive phase. However, this early maturation was not mirrored at the reference site which was sampled later than the Tyne/Humber but still demonstrated relatively low female GSIs. The high female mean GSI (2.52) in the Southampton Water samples would suggest that these fish had definitely entered the phase of gonadal maturation as would be expected for the December sampling date.

Mean male GSI values were calculated for fewer sites and only included in statistical analyses when sample numbers exceeded three. The value of 0.76 from the Alde was relatively high and suggests that the males at this site may have begun to enter their gonadal maturation phase as would be expected at this time of year (Janssen *et al.*, 1996). This also explains the high figure (1.36) for the December sampled fish from Southampton Water. However, of the sites where mean male GSI was statistically compared to the reference site, only the value of 0.16 at Jarrow in the Tyne was significantly ( $p < 0.05$ ) different. This low value was probably associated with the fact that the fish from this site were significantly ( $p < 0.05$ ) smaller. These GSI figures correspond well to those measured in 1996.

EROD activity in flounder has already been shown to exhibit high levels of seasonal fluctuation but these influences appear to be at a minimum during June to October (Eggens *et al.*, 1996). The influence of reproduction may significantly obscure the EROD response to contaminants (Goksøyr *et al.*, 1996) but the samples taken in this study in 1997/98 were timed to coincide with a period when reproductive influences were thought to be minimal. We are therefore confident that the inter-estuary differences shown are contaminant-induced. However, correlation analysis of mean GSI with mean EROD does suggest that even in the optimal sampling period there may be a residual effect of the reproductive cycle on the mixed function oxygenase (MFO) system in some estuaries. For example, the female samples from the Tyne sites showed a high negative correlation ( $r = -0.84$ ) of EROD with GSI which reflects a trend that is more evident during the reproductive season of several flatfish species (Eggens *et al.*, 1996; Kirby *et al.*, 1998; Elskus *et al.*, 1992).

Livingstone *et al.* (1997) mention several instances where increased EROD activity in fish has been linked to higher order effects on processes such as disease and reproduction. This study has unequivocally shown that the MFO system in flounder is significantly elevated above baseline levels in several English estuaries.

Whilst the MFO system is essential for detoxification of certain xenobiotics and the breakdown of some endogenous compounds (for example steroid hormones and vitamins), its induction may also produce deleterious side effects. First, whilst many xenobiotics are rendered less harmful, others form carcinogenic or genotoxic compounds after transformation by the MFO system (e.g. formation of benzo[*a*]pyrene diol-epoxide from the parent polycyclic aromatic hydrocarbon (PAH)). Secondly, since the metabolism of essential endogenous substances such as steroid hormones is regulated by the MFO system, its elevated activity could have serious repercussions for an organism's normal reproductive development. Furthermore, it has been shown in liver tissue culture (Anderson *et al.*, 1996) that while some EROD inducers (e.g. dioxins and dibenzofurans) inhibit the ability of hepatocytes to synthesise VTG in response to oestrogen, others (e.g. PCB 114) actually potentiate this effect.

Further research is clearly required to clarify the interactions between exogenous oestrogens and the MFO system, and the potentially damaging feedback on steroid metabolism and reproductive success. It seems, however, that the EROD induction seen in flounder from the more industrialised estuaries is a warning that such effects are probably occurring.

#### **5.4 Relationship of male vitellogenesis with ovotestis pathology**

There appears to be a high vitellogenesis threshold (approximately 100,000 ng VTG ml<sup>-1</sup> of plasma) in adult male flounder which is associated with ovotestis pathology in the population, although it must be noted that oestrogenic exposure of adults is not thought capable of producing ovotestis (see below). The majority of estuaries surveyed therefore appear to contain no intersex fish, and sex ratios are apparently normal everywhere. The exception to this is the Tees where male VTG levels were the highest on record, but no ovotestis was seen. The reason for this is unknown, but is probably connected with the fact that only 10 male fish could be caught in this estuary. The sample size was thus so small that it would not have been expected to detect a condition which was only seen in a maximum of 18% of the fish in the Mersey and Tyne.

However, another important factor is that ovotestis is almost certainly induced through exposure of flounder larvae at the stage of gonadal development, and not through exposure of juveniles or adults. Supporting evidence for this is that intersex adults show no consistent pattern of VTG induction, suggesting that their condition was induced much earlier in their life history. The precise age of gonadal development in flounder larvae is unknown, but it seems likely that it occurs while the majority of larvae are still at sea. This



would tend to minimise the appearance of intersex in this species, but implies that species which breed in estuaries are likely to show a higher prevalence of this condition. It is also possible that flounder larvae are predominantly exposed to (lipophilic) oestrogenic contaminants passed on to them in the egg from their mothers, a mechanism which would tend to overshadow effects potentially caused by the more water-soluble materials.

## 5.5 Implications of abnormal vitellogenesis and induction of intersex for reproductive success in flounder

The prevalence of ovotestis in male flounder in even the most contaminated estuaries is evidently relatively low in comparison with that found in roach (up to 100%) from many UK rivers (Jobling *et al.*, 1998). Furthermore, nothing is known about the ability of intersex males (or apparently normal oestrogen-exposed animals) to produce qualitatively and quantitatively normal sperm. Observations were not made of the vas deferens in the flounder, so it is not known whether there was any feminisation of this duct. Such an effect has been shown in male laboratory carp (Gimeno *et al.*, 1996) and wild male roach (Jobling *et al.*, 1998), and could potentially prevent any viable gametes from reaching the exterior. In addition, no abnormalities were seen in any female gonads, perhaps because oestrogens do not stimulate the oocytes to absorb VTG, they only stimulate VTG synthesis which in female flounder at the times sampled would probably have been proceeding maximally (Campbell and Idler, 1976). Also, there was no clear evidence for delayed testicular development or precocious ovarian maturation. There is no doubt that even the most oestrogen-contaminated estuarine flounder populations are still recruiting at least some young fish, so it could be argued that all is well. However, it should be remembered that most UK estuaries with large human populations in their catchment were almost devoid of fish until inputs of grossly polluting organic matter with high biochemical oxygen demand were better controlled in the 1950s and 1960s (Rivers (Prevention of Pollution) Act 1951; Clean Rivers (Estuaries and Tidal Waters) Act 1960; Rivers (Prevention of Pollution) Act 1961), so we may be witnessing a situation which has not yet stabilised.

Furthermore, it seems likely that excessive production of VTG in males will damage their general fitness to compete for resources and fight disease. It is known that vitellogenesis in males and immature females can cause serious metabolic stress due to the drain on energy reserves, and can also lead to kidney and liver damage and necrosis (Herman and Kincaid, 1988). Furthermore, vital amino acids and lipids can be diverted from their target tissues, and calcium may be withdrawn from the scales and skeleton in order to support VTG synthesis

(whose molecule contains a calcium atom) (Carragher and Sumpter, 1991).

The full implications of the present observations cannot therefore be predicted without further research, although it seems likely that flounder in the most contaminated estuaries (Tyne, Tees, Wear and Mersey) are experiencing some adverse effects as a result of oestrogenic exposure. The implications for the reproductive success of organisms which complete their whole life cycles in contaminated estuaries are potentially more grave because their most sensitive stages will probably be experiencing much higher exposure than flounder eggs and/or larvae.

## 6. RECOMMENDATIONS

The following research is recommended to clarify the full biological and chemical implications of the findings reported here:-

- 6.1 A programme of investigative analytical chemistry is required to identify the main compounds responsible for the oestrogenic effects observed in UK estuaries. This should take the form of toxicity-directed fractionation of water, sediment and tissue extracts using a suitable bioassay such as the yeast (o)estrogen screen (YES), i.e. the deployment of Toxicity Identification and Evaluation (TIE) procedures.
- 6.2 Surveys to localise the major sources of these oestrogenic materials.
- 6.3 A research programme to develop improved biomarkers of oestrogenic and androgenic effects in fish, crustaceans and molluscs.
- 6.4 Additional surveys of UK marine waters using these new biomarkers, in order to obtain a more rounded picture of the extent of endocrine disruption which may be occurring.
- 6.5 Investigations into the reproductive output of organisms in the most ED-contaminated areas, focusing on those which reproduce in estuaries.
- 6.6 Computerised modelling of the population-level implications of any reductions in reproductive output.

The majority of these recommendations (with the exception of work on molluscs) have already been taken on board by a new 4-year research programme entitled **Endocrine Disruption in the Marine Environment, or EDMAR**, funded mainly by the British Government (Ministry of Agriculture, Fisheries and Food – MAFF; Department of Environment, Transport and the Regions

– DETR; Environment Agency – EA; Scotland and Northern Ireland Forum for Environmental Research – SNIFFER), but also by the European Chemical Industry Association (CEFIC). The programme is being managed by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS), but other collaborating organisations include Fisheries Research Services (FRS) Marine Laboratory, Aberdeen, Zeneca Brixham Environmental Laboratory, and the Universities of Liverpool and Plymouth.

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## 8. REFERENCES

- ALLCHIN, C. R., KELLY, C. A. AND PORTMANN, J. E., 1989. Methods of analysis for chlorinated hydrocarbons in marine and other samples. *Aquat. Environ. Prot.: Analyt. Meth.*, MAFF Direct. Fish. Res., Lowestoft, **6**: 25pp.
- ALLEN, Y., THAIN, J., MATTHIESSEN, P., SCOTT, A. P., HAWORTH, S. AND FEIST, S., 1997. A survey of oestrogenic activity in UK estuaries and its effects on gonadal development of the flounder *Platichthys flesus*. ICES, C.M. 1997/U:01, 13 pp + figs.
- ALLEN, Y., SCOTT, A.P., MATTHIESSEN, P., HAWORTH, S., THAIN, J.E. AND FEIST, S., 1998. A survey of estrogenic activity in UK estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. *Environ. Tox. Chem.*, (in press).
- ANDERSON, M. J., MILLER, M. R. AND HINTON, D. E., 1996. In vitro modulation of 17 $\beta$ -estradiol-induced vitellogenin synthesis: effects of cytochrome P4501A1 inducing compounds on rainbow trout (*Oncorhynchus mykiss*) liver cells. *Aquat. Toxicol.*, **34**: 327-350.
- BRADFORD, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.*, **72**: 248-254.
- BROUWER, A., REIJNDERS, P. J. H. AND KOEMAN, J. H., 1989. Polychlorinated biphenyl (PCB) – contaminated fish induces vitamin A and thyroid hormone deficiency in the common seal (*Phoca vitulina*). *Aquat. Toxicol.*, **15**: 99-105.
- CAMPBELL, C. M. AND IDLER, D. B. 1976. Hormonal control of vitellogenesis in hypophysectomised winter flounder (*Pseudopleuronectes americanus* Walbaum). *Gen. Comp. Endocrinol.*, **28**: 143-150.
- CARRAGHER, J. F. AND SUMPTER, J. P., 1991. The mobilisation of calcium from calcified tissues of rainbow trout (*Oncorhynchus mykiss*) induced to synthesise vitellogenin. *Comp. Biochem. Physiol.*, **99A**: 169-172.
- CEFAS, 1997. Monitoring and surveillance of non-radioactive contaminants in the aquatic environment and activities regulating the disposal of wastes at sea, 1994. *Sci Ser., Aquat. Environ. Monit. Rep. CEFAS, Lowestoft*, **47**: 59pp.
- CHAMP, M. A. AND SELIGMAN, P. F. (EDS.), 1996. *Organotin: Environmental Fate and Effects*. Chapman and Hall, London, 623pp.
- COLBORN, T., DUMANOSKI, D. AND MYERS, J. P., 1996. *Our Stolen Future*. Penguin Books USA Inc., New York, 306pp.
- COLBORN, T., VOM SAAL, F. S. AND SOTO, A. M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.*, **101**: 378-384.
- DESBROW, C., ROUTLEDGE, E., BRIGHTY, G. C., SUMPTER, J. P. AND WALDOCK, M., 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. *Environ. Sci. Technol.*, **32**: 1549-1558.
- DODDS, E. C., GOLDBERG, L., LAWSON, W. AND ROBINSON, R., 1938. Oestrogenic activity of certain synthetic compounds. *Nature*, **141**: 247-248.
- DOERGE, D. R. AND BAJIC, S., 1992. Analysis of pesticides using liquid chromatography atmospheric pressure chemical ionisation mass-spectrometry. *Rapid Comm. Mass Spectrom.*, **6**: 663-666.
- EA, 1998(a). Endocrine-disrupting substances in the environment: what should be done? Environmental Issues Series, Environment Agency, Bristol, UK, 13pp.
- EA, 1998(b). Endocrine-disrupting substances in wildlife. A review of the scientific evidence and strategic response. Environment Agency, Bristol, 80pp.
- EGGENS, M. L., OPPERHUIZEN, A. AND BOON, J. P., 1996. Temporal variations of CYP1A indices, PCB and 1-OH pyrene concentration in flounder, *Platichthys flesus*, from the Dutch Wadden Sea. *Chemosphere*, **33**: 1579-1596.
- ELSKUS, A. A., PRUELL, R. J. AND STEGEMAN, J. J., 1992. Endogenously-mediated, pretranslational suppression of cytochrome P4501A in PCB-contaminated flounder. *Mar. Environ. Res.*, **34**: 97-101.

- EUROPEAN COMMISSION, 1996. European Workshop on the Impact of Endocrine Disrupters on Human Health and Wildlife. Report of the Proceedings 2-4 December 1996, Weybridge, UK, Report EUR 17549, 125pp.
- FOLMAR, L. C., DENSLow, N. D., RAO, V., CHOW, M., CRAIN, D. A., ENBLom, J., MARCINO, J. AND GUILLETTE, L. J., 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environ. Health Perspect.*, **104**: 1096-1101.
- GIESY, J. P. AND SNYDER, E. M., 1998. Xenobiotic modulation of endocrine function in fishes. pp. 155-237, *In*: (Kendall R J; Dickerson R L; Giesy J P; Suk W A (eds.)) Principles and Processes for Evaluating Endocrine Disruption in Wildlife. SETAC Technical Publication, SETAC Press, Pensacola.
- GIMENO, S., GERRITSEN, A., BOWMER, T. AND KOMEN, H., 1996. Feminization of male carp. *Nature*, **384**: 221-222.
- GOKSØYR, A., BEYER, J., EGAAS, E., GRØSVIK, B. E., HYLLAND, K., SANDVIK, M. AND SKAARE, J. U., 1996. Biomarker responses in flounder (*Platichthys flesus*) and their use in pollution monitoring. *Mar. Pollut. Bull.*, **33**: 36-45.
- HAKULINEN, T., ANDERSEN, A. A., MALKER, B., RIKKALA, E., SHOU, G. AND TULINIUS, H., 1986. Trends in cancer incidence in the Nordic countries. A collaborative study of the five Nordic cancer registries. *Acta Pathologica et Microbiologica Immunologica Scandinavica*, **94A, Suppl. 288**: 1-151.
- HARRIES, J. E., JOBLING, S., MATTHIESSEN, P., SHEAHAN, D. A. AND SUMPTER, J. P., 1995. Effects of Trace Organics on Fish - Phase 2. Report to the Department of the Environment, Foundation for Water Research, Marlow, Report no. FR/D 0022, 90pp.
- HARRIES, J. E., SHEAHAN, D. A., JOBLING, S., MATTHIESSEN, P., NEALL, P., ROUTLEDGE, E., RYCROFT, R., SUMPTER, J. P. AND TYLOR, T., 1996. A survey of estrogenic activity in United Kingdom inland waters. *Environ. Tox. Chem.*, **15**: 1993-2002.
- HARRIES, J. E., SHEAHAN, D. A., JOBLING, S., MATTHIESSEN, P., NEALL, P., SUMPTER, J. P., TYLOR, T. AND ZAMAN, N., 1997. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ. Tox. Chem.*, **16**: 534-542.
- HERMAN, R. L. AND KINCAID, H. L., 1988. Pathological effects of orally administered oestradiol to rainbow trout. *Aquaculture*, **72**: 165-172.
- IEH, 1995. Environmental Oestrogens: Consequences to Human Health and Wildlife. Institute for Environment and Health, Leicester, UK, 107pp.
- JANSEN, P. A. H., LAMBERT, J. G. D. AND GOOS, H. J. T., 1995. The annual ovarian cycle and the influence of pollution on vitellogenesis in the flounder, *Platichthys flesus* (L.). *J. Fish Biol.*, **47**: 509-523.
- JANSEN, P. A. H., MONTEIRO, P. M. R. R., LAMBERT, J. G. D. AND GOOS, H. J. T., 1996. Spermatogenesis, testicular steroidogenesis and plasma steroid profiles in the male flounder, *Platichthys flesus* (L.), during the annual reproductive cycle and after long-term exposure to polluted harbour sediment. *Can. J. Fish. Aquat. Sci.*, in press.
- JANSEN, P. A. H., LAMBERT, J. G. D., VETHAAK, A. D., GOOS, H. J. T., 1997. Environmental pollution caused elevated concentrations of oestradiol and vitellogenin in the female flounder, *Platichthys flesus* (L.). *Aquat. Toxicol.*, **39**: 195-214.
- JOBLING, S., SHEAHAN, D. A., OSBORNE, J. A., MATTHIESSEN, P. AND SUMPTER, J. P., 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ. Tox. Chem.*, **15**: 194-202.
- JOBLING, S., TYLER, C. R., NOLAN, M. AND SUMPTER, J. P., 1998. The identification of oestrogenic effects in wild fish. R&D Technical Report W119, Environment Agency, Bristol, UK.
- JOHNSON, L.L., SOL, S.Y., YLITALO, G.M., HOM, T., FRENCH, B., OLSON, O.P. AND COLLIER, T.K., 1997. Precocious sexual maturation and other reproductive anomalies in English sole from an urban waterway. International Council for the Exploration of the Sea, Copenhagen, ICES C.M. 1997/U:07, 15 pp + figs.
- KAVLOCK, R. J., DASTON, G. P., DEROSA, C., FENNER-CRISP, P., GRAY, L. E., KAATTARI, S., LUCIER, G., LUSTER, M., MAC, M. J., MACZKA, C., MILLER, R., MOORE, J., ROLLAND, R., SCOTT, G., SHEEHAN, D. M., SINKS, T. AND TILSON, H. A., 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. *Environ. Health Perspect.*, **104**: 715-740.
- KELLY, C. A. AND LAW, R. J., (IN PRESS). Monitoring of PAH in fish and shellfish following the *Sea Empress* oil spill. Proceedings of the *Sea Empress* Conference, Cardiff, Wales, 11-13 February 1998.
- KENDALL, R. J., DICKERSON, R. L., GIESY, J. P. AND SUK, W. A. (EDS), 1998. Principles and Processes for Evaluating Endocrine Disruption in Wildlife. SETAC Technical Publication, SETAC Press, Pensacola, Florida, 491pp.
- KIRBY, M. F., NEALL, P. AND TYLOR, T., 1998. EROD activity measured in flatfish from the area of the *Sea Empress* oil spill. *Chemosphere*, In press.

- LANG, T., DAMM, U. AND DETHLEFSEN, V., 1995. Changes in the sex ratio of North Sea dab (*Limanda limanda*) in the period 1981-1995. ICES C.M. 1995/G:25 Ref E, 11pp.
- LAW, R. J., FILEMAN, T. W. AND PORTMANN, J. E., 1988. Methods of analysis for hydrocarbons in marine samples. Aquat. Environ. Protect.: Analyt. Meth., MAFF Direct. Fish. Res., Lowestoft, 2: 25pp.
- LAW, R.J., THAIN, J.E., KIRBY, M.F., ALLEN, Y.T., LYONS, B.P., KELLY, C.A., HAWORTH, S., DYRYNDA, E.A., DYRYNDA, P.E.J., HARVEY, J.S., PAGE, S., NICHOLSON, M.D. AND LEONARD, D.R.P., (IN PRESS). The impact of the *Sea Empress* oil spill on fish and shellfish. Proceedings of the Sea Empress Conference. Cardiff, Wales, 11-13 February 1998.
- LEAH, R. T., CONNOR, L. AND MCNEISH, A. S., 1994. Persistent organic compounds within the Mersey estuary. R&D Note no. 328, National Rivers Authority (now Environment Agency), Bristol, 117pp.
- LINN, S., LIEBERMAN, E., SCHOENBAUM, S. C., MONSON, R. R., STUBBLEFIELD, P. G. AND RYAN, K. J., 1988. Adverse outcomes of pregnancy in women exposed to diethylstilbestrol *in utero*. J. Repro. Med., 33: 3-7.
- LIVINGSTONE, D. R., FÖRLIN, L. AND GEORGE, S. G., 1997. Molecular biomarkers and toxic consequences of impact by organic pollution in aquatic organisms. pp154-171, *In*: (Sutcliffe, D. W. (ed.)), Water quality and stress indicators in marine and freshwater ecosystems. Linking levels of organisation (individuals, populations, communities). ISBN 0-980386-54-1.
- LYE, C. M., FRID, C. J. J., GILL, M. E. AND MCCORMICK, D., 1997. Abnormalities in the reproductive health of flounder *Platichthys flesus* exposed to effluent from a sewage treatment works. Mar. Pollut. Bull., 34: 34-41.
- MAFF, 1994. Monitoring and surveillance of non-radioactive contaminants in the aquatic environment and activities regulating the disposal of wastes at sea, 1992. Aquat. Environ. Monit. Rep., MAFF Direct. of Fish. Res. (now CEFAS), Lowestoft, 40: 83pp.
- MAKIN, H.L.J., GOWER, D.B. AND KIRK, D. (EDS), 1995. Steroid Analysis, Chapman and Hall, 736pp.
- MATTHIESSEN, P. AND GIBBS, P. E., 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ. Tox. Chem., 17: 37-43.
- MATTHIESSEN, P. AND SUMPTER, J. P., 1998. Effects of estrogenic substances in the aquatic environment. pp. 319-335, *In*: (Braunbeck, T., Hinton, D.E. and Streit, B. (eds)) Fish Ecotoxicology. Birkhäuser Verlag, Basel, Switzerland.
- MATTHIESSEN, P., THAIN, J. E., LAW, R. J. AND FILEMAN, T. W., 1993. Attempts to assess the environmental hazard posed by complex mixtures of organic chemicals in UK estuaries. Mar. Pollut. Bull., 26: 90-95.
- MCNEISH, A. S., JOHNSON, M. S. AND LEAH, R. T., 1997. Methyl lindane and other analogues of hexachlorocyclohexane in dab and plaice from the Mersey estuary. Aquat. Toxicol., 40: 11-22.
- MONOSSON, E., KELCE, W. R., MAC, M., AND EARL GRAY, L., 1997. Environmental antiandrogens: potential effects on fish reproduction and development. pp. 53-60, *In*: (Rolland, R. M., Gilbertson, M. and Peterson, R. E (eds)), Chemically induced alterations in functional development and reproduction of fishes. SETAC Press, Pensacola.
- MOORE, C. G. AND STEVENSON, J. M., 1991. The occurrence of intersexuality in harpacticoid copepods and its relation with pollution. Mar. Pollut. Bull., 22: 72-74.
- NORBERG, B., 1995. Atlantic halibut (*Hippoglossus hippoglossus*) vitellogenin: induction, isolation and partial characterization. Fish Physiol. Biochem., 14: 1-13.
- PETERSEN, G. I., GERUP, J., NILSSON, L., LARSEN, J. R. AND SCHNEIDER, R., 1997. Body burdens of lipophilic xenobiotics and reproductive success in Baltic cod (*Gadus morhua* L.). ICES C.M. 1997/U:10, 22pp.
- PURDOM, C. E., HARDIMAN, P. A., BYE, V. J., ENO, N. C., TYLER, C. R. AND SUMPTER, J. P., 1994. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol., 8: 275-285.
- REIJNDERS, P. J. H., 1986. Reproductive failure in common seals feeding on fish from polluted coastal waters. Nature, 324: 456-457.
- RIJNSDORP, A. D. AND VETHAAK, A. D., 1997. Changes in reproductive parameters of North Sea plaice and sole between 1960 and 1995. ICES C.M. 1997/U:14, 14pp + figs.
- ROGERS, H. R., EADIE, G. S., JAMES, H. A. AND WATTS, C. D., 1990. Sources and fates of synthetic organics. Final report to the Department of the Environment. Water Research Centre, Medmenham, report no. FR/D 0001.
- ROSS, P. S., DE SWART, P. J. H., REIJNDERS, P. J. H., LOVEREN, H. VAN; VOS, J. G. AND OSTERHAUS, A. D. M. E., 1995. Contaminant-related suppression of delayed-type hypersensitivity and antibody responses in harbor seals fed Baltic Sea herring. Environ. Health Perspect., 103: 162-167.

- SHEAHAN, D. A., BUCKE, D., MATTHIESSEN, P., SUMPTER, J. P., KIRBY, M. F., NEALL, P. AND WALDOCK, M., 1994. The effects of low levels of 17  $\alpha$ -ethynylestradiol upon plasma vitellogenin levels in male and female rainbow trout, *Oncorhynchus mykiss* (Walbaum) held at two acclimation temperatures. pp. 99-112, *In*: (Müller, R. and Lloyd, R. (eds)), *Sublethal and Chronic Effects of Pollutants on Freshwater Fish*. Fishing News Books, Osney Mead, Oxford, by arrangement with the Food and Agriculture Organisation of the United Nations, ISBN 0-85238-207-3.
- SPIES, R. B. AND THOMAS, P., 1997. Reproductive and endocrine status of female kelp bass from a contaminated site in the Southern California Bight and estrogen receptor binding of DDTs. pp. 113-133, *In*: (Rolland R. M., Gilbertson M. and Peterson R. E. (eds)), *Chemically induced alterations in functional development and reproduction of fishes*. SETAC Press, Pensacola.
- STAGG, R. M., MCINTOSH, A. AND MACKIE, P., 1995. Elevation of hepatic monooxygenase activity in the dab (*Limanda limanda* L.) in relation to environmental contamination with petroleum hydrocarbons in the northern North Sea. *Aquat. Toxicol.*, **33**: 245-264.
- SWAN, S. H., ELKIN, E. P. AND FENSTER, L., 1997. Have sperm densities declined? A reanalysis of global trend data. *Environ. Health Perspect.*, **105**: 1228-1232.
- TATTERSFIELD, L., MATTHIESSEN, P., CAMPBELL, P., GRANDY, N. AND LÄNGE, R. (EDS), 1997. SETAC-Europe/OECD / EC Expert Workshop on Endocrine Modulators and Wildlife: Assessment and Testing. EMWAT. Veldhoven, The Netherlands, 10-13 April 1997. ISBN 90-5607-010-X, Society of Environmental Toxicology and Chemistry - Europe, Brussels, 126pp.
- TOPPARI, J., LARSEN, J. C., CHRISTIANSEN, P., GIWERCMAN, A., GRANDJEAN, P., GUILLETTE, L. J., JÉGOU, B., JENSEN, T. K., JOUANNET, P., KEIDING, N., LEFFERS, H., MCLACHLAN, J. A., MEYER, O., MÜLLER, J., RAJPERT-DE MEYTS, E., SCHEIKE, T., SHARPE, R., SUMPTER, J. AND SKAKKEBÆK, N. E., 1995. *Male Reproductive Health and Environmental Chemicals with Estrogenic Effects*. Danish Environmental Protection Agency, Copenhagen, Miljøprojekt no. 290, 166pp.
- TYLER, C. R. AND SUMPTER, J. P., 1990. The development of a radioimmunoassay for carp, *Cyprinus carpio*, vitellogenin. *Fish Physiol. Biochem.*, **8**, 129-140.
- WARING, C. P., STAGG, R. M., FRETWELL, K., MCLAY, H. A. AND COSTELLO, M. J., 1996. The impact of sewage sludge exposure on the reproduction of the sand goby, *Pomatoschistus minutus*. *Environ. Pollut.*, **93**: 17-25.



## APPENDIX 1. Polychlorinated biphenyl congeners in pooled flounder liver (mg kg<sup>-1</sup> wet wt.) – raw data

Year	Site	Location	CB 101	CB 105	CB 110	CB 118	CB 128	CB 138	CB 141	CB 149	CB 151	CB 153	CB 156	CB 158	CB 170	CB 18	CB 180	CB 183	CB 187	CB 194	CB 28	CB 31	CB 44	CB 47	CB 49	CB 52	CB 66	
1995	NMP785	Morecambe Bay	0.011	0.006	0.019	0.017	0.003	0.027	0.003	0.012	0.004	0.034	0.003	0.002	0.006	nd	0.016	0.005	0.015	0.003	0.003	0.002	nd	0.001	0.003	0.004	0.006	
	NMP785	Morecambe Bay	0.007	0.004	0.013	0.013	0.003	0.02	0.002	0.009	0.003	0.026	0.002	0.001	0.005	nd	0.013	0.004	0.011	0.002	0.002	0.001	nd	nd	nd	0.003	0.004	
	NMP785	Morecambe Bay	0.008	0.005	0.015	0.013	0.003	0.022	0.002	0.01	0.004	0.027	0.003	0.001	0.005	nd	0.013	0.004	0.013	0.002	0.002	0.001	nd	0.001	0.002	0.003	0.005	
	NMP785	Morecambe Bay	ND	0.006	0.021	0.017	0.004	0.028	0.003	0.012	0.004	0.035	0.003	0.002	0.006	nd	0.016	0.005	0.015	0.003	0.003	0.002	nd	0.002	0.003	0.004	0.006	
		Central S. North Sea	0.006	0.003	0.01	0.011	0.003	0.018	0.001	0.005	0.002	0.023	0.002	0.001	0.003	nd	0.008	0.003	0.01	0.001	0.001	nd	nd	nd	nd	0.002	0.002	0.003
		Central S. North Sea	0.007	0.003	0.011	0.012	0.003	0.022	0.001	0.007	0.002	0.028	0.002	0.001	0.004	nd	0.012	0.004	0.012	0.001	0.001	nd	nd	nd	nd	0.003	0.002	0.003
		Central S. North Sea	0.011	0.005	0.016	0.019	0.005	0.031	0.002	0.01	0.003	0.039	0.003	0.002	0.005	nd	0.014	0.005	0.017	0.002	0.002	nd	nd	nd	nd	0.004	0.003	0.005
		Central S. North Sea	0.012	0.006	0.02	0.022	0.006	0.039	0.002	0.014	0.004	0.049	0.004	0.003	0.006	nd	0.018	0.007	0.021	0.002	nd	nd	nd	nd	nd	0.004	0.003	0.006
		Central S. North Sea	0.011	0.005	0.022	0.02	0.005	0.036	0.003	0.015	0.005	0.045	0.004	0.003	0.008	nd	0.021	0.007	0.019	0.002	0.002	nd	nd	nd	nd	0.003	0.003	0.005
		Liverpool Bay	0.006	0.002	0.012	0.008	0.002	0.014	0.002	0.006	0.002	0.015	0.001	nd	0.003	nd	0.007	0.002	0.007	0.001	0.002	nd	nd	nd	nd	0.002	0.002	0.003
		Liverpool Bay	0.016	0.006	0.031	0.025	0.006	0.038	0.004	0.016	0.006	0.043	0.004	0.003	0.008	nd	0.021	0.006	0.019	0.003	0.004	0.002	0.002	0.002	0.002	0.004	0.004	0.008
		Liverpool Bay	0.01	0.004	0.018	0.018	0.004	0.03	0.003	0.009	0.005	0.037	0.003	0.002	0.006	nd	0.017	0.005	0.015	0.002	0.002	nd	0.001	0.001	nd	0.003	0.005	
		Liverpool Bay	0.019	0.007	0.033	0.028	0.006	0.039	0.005	0.018	0.007	0.045	0.004	0.003	0.008	nd	0.021	0.006	0.02	0.003	0.005	0.003	0.002	0.002	0.002	0.005	0.007	0.012
	1996		Liverpool Bay	0.007	0.003	0.013	0.009	0.001	0.012	0.001	0.007	0.002	0.015	0.001	nd	0.002	0.002	0.006	0.002	0.006	nd	0.004	0.002	0.002	0.001	0.003	0.005	0.004
NMP785		Morecambe Bay	0.016	0.007	0.027	0.024	0.008	0.044	0.004	0.019	0.006	0.057	0.004	0.002	0.009	nd	0.028	0.008	0.03	0.005	0.003	0.001	nd	nd	0.007	0.005	0.008	
NMP785		Morecambe Bay	0.016	0.007	0.026	0.024	0.007	0.041	0.004	0.018	0.005	0.054	0.004	0.002	0.009	nd	0.026	0.007	0.028	0.004	0.003	0.001	nd	nd	0.005	0.005	0.009	
NMP785		Morecambe Bay	0.012	0.005	0.021	0.017	0.005	0.031	0.003	0.014	0.004	0.041	0.004	0.001	0.006	nd	0.019	0.005	0.02	0.004	0.003	0.001	nd	nd	0.006	0.005	0.006	
NMP785		Morecambe Bay	0.011	0.005	0.018	0.02	0.007	0.035	0.003	0.009	0.004	0.044	0.004	0.002	0.007	nd	0.002	0.006	0.021	0.004	0.002	0.001	nd	nd	0.005	0.002	0.007	
NMP785		Morecambe Bay	0.013	0.005	0.022	0.02	0.006	0.038	0.004	0.016	0.005	0.052	0.004	0.002	0.008	nd	0.028	0.007	0.027	0.005	0.003	0.001	nd	nd	0.009	0.005	0.006	
1997		Humber	0.028	nd	0.041	0.02	0.007	0.056	0.011	0.031	0.016	0.08	0.007	0.006	0.017	nd	0.047	0.018	0.036	0.006	0.003	0.001	0.003	0.004	0.005	0.012	nd	
		Humber	0.035	nd	0.056	0.027	0.007	0.053	0.01	0.036	0.016	0.072	0.006	0.006	0.013	0.002	0.037	0.014	0.034	0.004	0.007	0.003	0.006	0.005	0.009	0.021	0.01	
		Humber	0.03	nd	0.051	0.022	0.006	0.045	0.01	0.034	0.015	0.062	0.006	0.006	0.012	0.001	0.034	0.013	0.031	0.004	0.004	nd	0.004	0.005	0.007	0.016	nd	
		Humber	0.027	nd	0.041	0.017	0.006	0.047	0.01	0.032	0.015	0.074	0.007	0.005	0.015	0.001	0.045	0.017	0.036	0.006	0.004	0.001	0.004	nd	nd	0.014	nd	
		Humber	0.021	nd	0.035	0.014	0.006	0.038	0.008	0.026	0.012	0.055	0.005	0.004	0.011	nd	0.035	0.013	0.028	0.005	0.003	nd	0.003	nd	nd	0.011	nd	
	NMP745	Mersey Brom. Buoy	0.08	0.04	0.12	0.13	0.026	0.11	0.016	0.049	0.027	0.14	0.029	0.011	0.031	0.017	0.083	0.022	0.058	0.009	0.067	0.036	0.018	0.022	0.046	0.07	0.083	
	NMP745	Mersey Brom. Buoy	0.094	0.047	0.14	0.14	0.028	0.12	0.018	0.065	0.027	0.15	0.026	0.012	0.028	0.019	0.074	0.021	0.058	0.008	0.082	0.04	0.027	0.028	0.055	0.083	0.098	
	NMP745	Mersey Brom. Buoy	0.059	0.03	0.085	0.11	0.022	0.09	0.014	0.037	0.022	0.11	0.026	0.008	0.029	0.013	0.078	0.018	0.051	0.009	0.056	0.03	0.017	0.016	0.036	0.054	0.062	
	NMP745	Mersey Brom. Buoy	0.061	0.034	0.088	0.11	0.024	0.092	0.013	0.036	0.02	0.11	0.023	0.009	0.023	0.015	0.061	0.017	0.046	0.006	0.058	0.032	0.016	0.019	0.036	0.058	0.072	
	NMP555	Tamar Warren Point	0.053	0.027	0.088	0.12	0.022	0.098	0.01	0.02	0.009	0.097	0.023	0.014	0.016	nd	0.035	0.009	0.019	0.006	0.011	0.009	0.004	0.006	0.017	0.01	0.023	
	NMP555	Tamar Warren Point	0.047	0.026	0.075	0.12	0.026	0.099	0.009	0.017	0.008	0.093	0.023	0.014	0.016	nd	0.035	0.009	0.015	0.005	0.01	0.008	0.003	0.005	0.014	0.009	0.022	
	NMP555	Tamar Warren Point	0.032	0.017	0.056	0.073	0.015	0.064	0.005	0.017	0.007	0.066	0.013	0.008	0.01	nd	0.024	0.007	0.015	0.004	0.008	0.006	0.003	0.007	0.012	nd	0.016	
		Eastham																										
		Mersey Brom. Buoy	0.062	0.03	0.094	0.098	0.019	0.088	0.014	0.039	0.018	0.11	0.02	0.009	0.022	0.013	0.055	0.016	0.044	0.007	0.051	0.03	0.019	0.019	0.033	0.054	0.067	
		Eastham																										
		Mersey Brom. Buoy	0.11	0.06	0.17	0.18	0.033	0.16	0.025	0.073	0.034	0.21	0.034	0.017	0.041	0.011	0.1	0.032	0.078	0.011	0.062	0.032	0.02	0.028	0.05	0.076	0.1	
	Wallasey																											
	Outer Mersey	0.057	0.019	0.084	0.067	0.014	0.079	0.01	0.037	0.018	0.097	0.01	0.007	0.017	0.008	0.046	0.014	0.042	0.005	0.032	0.015	0.011	0.014	0.027	0.042	0.043		
	Wallasey																											
	Outer Mersey	0.092	0.032	0.14	0.12	0.025	0.14	0.02	0.06	0.03	0.17	0.019	0.014	0.037	0.013	0.093	0.025	0.071	0.012	0.049	0.025	0.017	0.021	0.042	0.058	0.068		

**APPENDIX 1. continued**

Year	Site	Location	CB 101	CB 105	CB 110	CB 118	CB 128	CB 138	CB 141	CB 149	CB 151	CB 153	CB 156	CB 158	CB 170	CB 18	CB 180	CB 183	CB 187	CB 194	CB 28	CB 31	CB 44	CB 47	CB 49	CB 52	CB 66	
1997	<i>cont.</i>	Wallasey																										
		Outer Mersey	0.089	0.029	0.13	0.11	0.021	0.14	0.018	0.058	0.027	0.17	0.016	0.012	0.03	0.012	0.086	0.022	0.068	0.011	0.048	0.022	0.014	0.019	0.042	0.058	0.063	
		Wallasey																										
		Outer Mersey	0.099	0.034	0.15	0.12	0.025	0.16	0.021	0.068	0.03	0.19	0.018	0.014	0.035	0.015	0.093	0.026	0.076	0.013	0.052	0.026	0.02	0.022	0.049	0.062	0.072	
		Wallasey																										
		Outer Mersey	0.06	0.022	0.089	0.085	0.015	0.1	0.014	0.032	0.02	0.13	0.012	0.01	0.025	0.01	0.069	0.019	0.05	0.009	0.034	0.015	0.011	0.014	0.027	0.038	0.045	
		West Kirby																										
		Dee	0.017	0.01	0.033	0.031	0.008	0.041	0.005	0.016	0.006	0.048	0.008	0.003	0.01	nd	0.023	0.006	0.019	0.004	0.005	0.002	0.001	0.002	0.005	0.006	0.012	
		West Kirby																										
		Dee	0.021	0.013	0.043	0.039	0.012	0.059	0.007	0.025	0.009	0.073	0.012	0.005	0.016	nd	0.034	0.01	0.028	0.005	0.005	0.002	0.002	0.003	0.005	0.008	0.017	
		West Kirby																										
		Dee	0.02	0.013	0.038	0.04	0.009	0.045	0.006	0.017	0.007	0.048	0.01	0.004	0.013	nd	0.028	0.006	0.023	0.005	0.007	0.004	0.003	0.004	0.007	0.008	0.018	
		West Kirby																										
		Dee	0.024	0.017	0.048	0.05	0.013	0.061	0.008	0.022	0.008	0.061	0.013	0.005	0.018	0.001	0.039	0.008	0.03	0.007	0.008	0.004	0.003	0.003	0.007	0.009	0.022	
		West Kirby																										
		Dee	0.027	0.017	0.054	0.052	0.01	0.055	0.007	0.021	0.008	0.061	0.012	0.005	0.016	0.001	0.034	0.007	0.029	0.006	0.009	0.006	0.004	0.005	0.009	0.011	0.025	
		Humber	0.011	nd	0.021	0.007	0.003	0.022	0.004	0.015	0.007	0.031	0.003	0.002	0.006	nd	0.019	0.007	0.017	0.003	0.003	nd	0.002	0.002	0.003	0.007	0.003	
		Humber	0.011	nd	0.021	0.006	0.003	0.019	0.003	0.015	0.006	0.025	0.002	0.002	0.005	0.001	0.015	0.006	0.014	0.002	0.003	0.001	0.002	0.002	0.003	0.008	0.003	
		NMP435 Inner Thames	0.062	0.028	0.12	0.067	0.018	0.094	0.019	0.048	0.023	0.11	0.021	0.011	0.027	0.002	0.068	0.019	0.04	0.007	0.022	0.006	0.013	0.015	0.022	0.033	0.039	
		NMP435 Inner Thames	0.094	0.037	0.19	0.095	0.024	0.13	0.028	0.08	0.035	0.15	0.027	0.015	0.036	0.004	0.092	0.024	0.059	0.01	0.035	0.008	0.025	0.028	0.037	0.056	0.06	
		NMP435 Inner Thames	0.091	0.035	0.18	0.089	0.024	0.13	0.027	0.079	0.032	0.15	0.026	0.017	0.034	0.003	0.088	0.025	0.053	0.009	0.027	0.006	0.019	0.024	0.032	0.045	0.085	
1998	NMP325	Durham (off Tees)	0.006	0.001	0.008	0.005	0.002	0.01	nd	0.004	0.001	0.014	0.001	nd	0.002	nd	0.006	0.002	0.005	nd	nd	nd	nd	nd	0.003	nd	0.001	
		Orfordness																										
		Alde	nd	nd	0.003	0.002	0.002	0.007	nd	nd	nd	0.008	nd	nd	0.001	nd	0.003	0.001	0.002	nd	nd	nd	nd	nd	0.003	nd	nd	



## APPENDIX 2. Organochlorine residues in pooled flounder liver (mg kg<sup>-1</sup> wet wt.) (with PCBs given as totals)

Sample Year	Site	Location	Dieldrin	Alpha HCH	Gamma HCH	HCB	<i>pp'</i> -DDE	<i>pp'</i> -DDT	<i>pp'</i> -DDD	Total 25 PCBs	Total ICES-7 PCBs	% Hexane Extractable Lipid
1995	NMP785	Morecambe Bay	0.008	0.002	0.002	0.002	0.025	0.005	0.033	0.205	0.112	13
	NMP785	Morecambe Bay	0.006	0.001	0.002	0.001	0.02	0.004	0.031	0.148	0.084	9
	NMP785	Morecambe Bay	0.006	nd	0.002	0.001	0.022	0.004	0.027	0.164	0.088	10
	NMP785	Morecambe Bay	0.007	nd	0.002	0.001	0.028	0.005	0.036	0.212	0.115	11
		Central S. North Sea	0.01	0.001	0.002	0.001	0.021	0.001	0.009	0.118	0.069	7
		Central S. North Sea	0.011	0.002	0.002	0.001	0.022	0.001	0.009	0.141	0.084	9
		Central S. North Sea	0.019	0.002	0.003	0.002	0.037	nd	0.018	0.203	0.119	11
		Central S. North Sea	0.017	0.003	0.004	0.002	0.043	0.003	0.017	0.248	0.143	11
		Central S. North Sea	0.011	0.002	0.003	0.002	0.039	nd	0.025	0.244	0.138	9
		Liverpool Bay	0.005	0.001	0.002	0.001	0.01	nd	0.022	0.099	0.054	6
		Liverpool Bay	0.011	0.002	0.002	0.002	0.028	nd	0.056	0.281	0.151	9
		Liverpool Bay	0.008	0.001	0.003	0.001	0.021	nd	0.03	0.2	0.117	8
		Liverpool Bay	0.015	0.003	0.004	0.003	0.034	0.021	0.082	0.308	0.164	12
		Liverpool Bay	0.007	0.001	nd	0.001	0.015	nd	0.029	0.11	0.058	3
	1996	NMP785	Morecambe Bay	0.01	nd	nd	0.004	0.047	0.03	0.044	0.322	0.177
NMP785		Morecambe Bay	0.011	nd	nd	0.003	0.047	0.026	0.044	0.305	0.169	22
NMP785		Morecambe Bay	0.01	nd	nd	0.003	0.033	0.009	0.037	0.233	0.128	22
NMP785		Morecambe Bay	0.009	nd	nd	0.003	0.038	0.018	0.031	0.219	0.116	18
NMP785		Morecambe Bay	0.008	nd	nd	0.003	0.036	0.014	0.027	0.286	0.159	21
1997		Humber	0.07	nd	0.002	0.01	0.089	0.009	0.17	0.455	0.246	8
		Humber	0.12	nd	0.002	0.017	0.089	0.009	0.2	0.489	0.252	9
		Humber	0.082	nd	0.002	0.011	0.076	0.009	0.18	0.418	0.213	6
		Humber	0.096	nd	0.002	0.012	0.085	0.01	0.21	0.424	0.228	11
		Humber	0.067	nd	0.005	0.009	0.063	0.014	0.14	0.333	0.177	14
	NMP745	Mersey Brom. Buoy	0.045	nd	0.01	0.009	0.17	0.007	0.27	1.34	0.68	
	NMP745	Mersey Brom. Buoy	0.061	0.003	0.011	0.01	0.17	nd	0.32	1.488	0.743	
	NMP745	Mersey Brom. Buoy	0.039	nd	0.01	0.007	0.13	nd	0.2	1.082	0.557	
	NMP745	Mersey Brom. Buoy	0.041	0.002	0.011	0.008	0.14	nd	0.2	1.079	0.55	
	NMP555	Tamar Warren Point	0.023	nd	0.005	0.007	0.051	0.044	0.044	0.746	0.424	24
	NMP555	Tamar Warren Point	0.019	nd	0.005	0.007	0.048	0.03	0.033	0.708	0.413	23
	NMP555	Tamar Warren Point	0.016	nd	0.004	0.005	0.031	0.016	0.032	0.485	0.267	14
	Eastham	Mersey Brom. Buoy	0.045	0.002	0.004	0.008	0.13	0.069	0.35	1.031	0.518	21
	Eastham	Mersey Brom. Buoy	0.041	0.002	0.005	0.007	0.27	0.072	0.47	1.747	0.898	17
	Wallasey	Outer Mersey	0.03	nd	0.004	0.005	0.11	0.013	0.17	0.815	0.42	16
	Wallasey	Outer Mersey	0.037	nd	0.008	0.01	0.18	0.021	0.3	1.393	0.722	26
	Wallasey	Outer Mersey	0.042	nd	0.007	0.009	0.17	0.025	0.33	1.315	0.701	28
	Wallasey	Outer Mersey	0.049	nd	0.009	0.011	0.19	0.03	0.4	1.49	0.776	29

## APPENDIX 2. continued

Sample Year	Site	Location	Dieldrin	Alpha HCH	Gamma HCH	HCB	<i>pp'</i> -DDE	<i>pp'</i> -DDT	<i>pp'</i> -DDD	Total 25 PCBs	Total ICES-7 PCBs	% Hexane Extractable Lipid
1997	Wallasey	Outer Mersey	0.028	nd	0.006	0.006	0.13	0.018	0.23	0.955	0.516	18
<i>cont.</i>	West Kirby	Dee	0.015	nd	0.002	0.002	0.034	0.038	0.083	0.321	0.171	15
	West Kirby	Dee	0.015	0.002	0.002	0.002	0.044	0.046	0.1	0.453	0.239	17
	West Kirby	Dee	0.017	0.002	0.004	0.005	0.041	0.047	0.12	0.383	0.196	20
	West Kirby	Dee	0.02	0.002	0.005	0.006	0.053	0.076	0.15	0.489	0.252	28
	West Kirby	Dee	0.021	0.002	0.005	0.007	0.054	0.059	0.15	0.491	0.249	25
		Humber	0.087	nd	0.003	0.006	0.043	0.006	0.14	0.198	0.1	15
		Humber	0.078	nd	0.002	0.006	0.037	0.006	0.12	0.178	0.087	15
	NMP435	Inner Thames	0.059	nd	0.005	0.003	0.088	0.036	0.062	0.934	0.456	10
	NMP435	Inner Thames	0.12	0.002	0.011	0.006	0.11	0.068	0.12	1.379	0.652	17
	NMP435	Inner Thames	0.045	0.002	0.006	0.005	0.1	0.071	0.093	1.33	0.62	12
1998	NMP325	Durham (off Tees)	0.007	nd	0.002	0.004	0.012	0.008	0.005	0.071	0.041	10
	Orfordness	Alde	0.008	nd	0.004	nd	0.014	0.005	0.005	0.032	0.02	9



