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**Biological effects of contaminants:
Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA)
techniques for the measurement of marine fish vitellogenins**

A.P. SCOTT

Centre for Environment, Fisheries and Aquaculture Science
Fisheries Laboratory
Pakefield Road
Lowestoft, Suffolk NR33 0HT
United Kingdom

and

K. HYLLAND

Norwegian Institute for Water Research
P. O. Box 173
Kjelsas, N-0411 Oslo
Norway

International Council for the Exploration of the Sea
Conseil International pour l'Exploration de la Mer

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Abstract

This document describes immunochemical methods to quantify the egg-yolk precursor protein vitellogenin in fish plasma. Vitellogenin is normally produced by the liver of mature female fish in response to 17β -oestradiol (E_2) in the blood. If male or reproductively immature fish are exposed to oestrogenic substances, either in the water or the diet, their livers will also be stimulated to produce vitellogenin. Concentrations of vitellogenin in the plasma of induced and uninduced fish can differ by a factor of between 10^6 and 10^7 . This makes vitellogenin induction in male and immature fish a very good biomarker for environmental oestrogens. All necessary steps in the development of both RIA (radioimmunoassay) and ELISA (enzyme-linked immunosorbent assay) are described, as are special precautions that need to be considered during the analysis of this protein.

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Key words: vitellogenin, 17β -oestradiol, radioimmunoassay, ELISA

1 INTRODUCTION

1.1 Reasons for Measuring Vitellogenin

It is now well established that oestrogenic substances of both natural and xenobiotic origin are entering the aquatic environment and disrupting the reproduction of fish and other animals (Toppari *et al.*, 1996). One of the clearest effects of these oestrogenic substances is the induction of vitellogenin (VTG) production in male and immature fish. VTG is the egg yolk precursor. It is normally only produced by the liver of mature female fish in response to 17β -oestradiol (E_2) in the blood; the E_2 , in its turn, is normally only produced by developing ovaries in response to stimulation by pituitary gonadotrophins. However, if male or reproductively immature fish are exposed to oestrogenic substances, either in the water or the diet, their livers too will be stimulated to produce VTG. The degree to which this happens can be so large in some species that concentrations of VTG in the blood plasma of induced and uninduced fish can differ by a factor of between 10^6 and 10^7 . This makes VTG induction in male and immature fish an exceptionally good biomarker for environmental oestrogens (Purdom *et al.*, 1994; Sumpter and Jobling, 1995).

There are other good reasons for measuring vitellogenin. It is a useful way of monitoring the progress of oogenesis in natural and captive broodstocks (Bon *et al.*, 1997; Methven *et al.*, 1992). It provides useful information on the duration and extent of vitellogenesis in fish fed on different diets (Cerda *et al.*, 1994; Navas *et al.*, 1998) or induced to spawn at different times of the year by photoperiod manipulation (Blythe *et al.*, 1994). It can be used to distinguish males and females at an early stage of reproductive development (Gordon *et al.*, 1984). It is also a valuable tool for fundamental studies on the mechanism of induction of vitellogenesis by the liver *in vitro* or *in vivo* (Peyon *et al.*, 1997).

The simplest methods of detecting VTG induction involve measuring plasma concentrations of either phosphoprotein phosphorus or calcium (with which the VTG is loosely complexed). However, these methods are very insensitive (Tyler and Sumpter, 1990). The degree of sensitivity which is required for environmental studies (where all levels of induction are likely to be found) can only be achieved with immunoassays. There are two main types: radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs). This paper describes how to establish a VTG RIA which has sufficient sensitivity to carry out studies in areas with very low levels of oestrogenic contamination (Matthiessen *et al.*, 1998). A robust ELISA is also described (cf. Hylland and Haux, 1997).

In order to set up an immunoassay, it is necessary to have a more or less pure VTG preparation and an anti-VTG serum. The first step in developing a RIA or an ELISA is the purification of VTG from a particular species.

1.2 Inducing VTG Production

Prior to purification, the production of VTG must be induced in captive fish (preferably males). This can be achieved by maintaining high concentrations of E_2 ($> 20 \text{ ng ml}^{-1}$) in the blood of the fish for a period of two to three weeks. Numerous ways have been devised to do this. Oestradiol (generally at concentrations between 1 mg kg^{-1} and 10 mg kg^{-1}) has been suspended in either saline solution (Mananos *et al.*, 1994b; Mourot and Le Bail, 1995; Silversand *et al.*, 1993; Yao and Crim, 1996), corn oil (Norberg, 1995), peanut oil (Hylland and Haux, 1997; Kishida and Specker, 1993), polypropylene glycol (Hara *et al.*, 1993; Inaba *et al.*, 1997), or coconut oil (Roubal *et al.*, 1997), and injected repeatedly into fish (at two- to three-day intervals) either intraperitoneally or intramuscularly. Following injection of 2 mg of E_2 per kg of fish at two-day

intervals, it took only ten days for VTG concentrations in sea bass to reach 60 mg ml⁻¹ (Mananos *et al.*, 1994b). Repeated injections are unnecessary if the E₂ is applied to the fish in a form which can sustain its release for two to three weeks. This can most easily be done by suspending the steroid in cocoa butter according to the method described by Pickering *et al.* (1987).

1.3 Purification of VTG

Because VTG is normally abundant in E₂-primed fish (between 5 mg ml⁻¹ and 100 mg ml⁻¹), its purification does not present a major problem, apart from in very small fish, where there is the problem of obtaining enough blood. There are also a few species that do not contain very large amounts of VTG. In carp, for example, even after E₂ treatment, concentrations of VTG rarely rise above 1 mg ml⁻¹ (Tyler and Sumpter, 1990). When concentrations are below 5 mg ml⁻¹, it appears to be very difficult to precipitate VTG from plasma (Hara *et al.*, 1993).

The procedure for precipitating fish VTGs has been described in detail by Silversand *et al.* (1993), who used it on plasma from cod (*Gadus morhua*), turbot (*Scophthalmus maximus*), and wolffish (*Anarhichas lupus*). It involves mixing plasma with solutions of EDTA (ethylene diamino tetra-acetic acid) and MgCl₂ (in molar ratios which need to be determined for each species). The VTG comes out of solution, is collected by centrifugation, redissolved in 1 M NaCl, and then reprecipitated by the addition of distilled water. Norberg (1995) found it difficult to precipitate halibut (*Hippoglossus hippoglossus*) VTG from plasma using MgCl₂ and EDTA alone, but overcame the problem by adding distilled water concomitantly.

Nunez Rodriguez *et al.* (1989) used precipitation as the only step in the purification of sole (*Solea vulgaris*) VTG. It is more common, however, for a further chromatographic step to be carried out. Silversand *et al.* (1993) for cod, turbot, and wolffish VTG, Norberg and Haux (1988) for brown trout (*Salmo trutta*) VTG, and Norberg (1995) for halibut VTG, all followed up the precipitation step with anion-exchange chromatography on DEAE-Sephacel. Copeland and Thomas (1988) for spotted sea trout (*Cynoscion nebulosus*) VTG, followed up with gel filtration on Sepharose 6B. Idler *et al.* (1979) for Atlantic salmon (*Salmo salar*) VTG, followed up with anion-exchange chromatography on TEAE-cellulose and then gel filtration on Bio-gel A-0.5M.

One advantage of the precipitation step is speed. The other is that it provides very strong evidence of the identity of the VTG. If plasma is subjected directly to chromatography, it is necessary to perform one or more tests on the fractions to determine which ones contain the VTG peak. This can be done by comparing the UV-absorption profiles of E₂-primed and untreated fish (Bon *et al.*, 1997; Sumpter, 1985), by incorporating radioactive isotopes into the VTG during the E₂-induction process (Chan *et al.*, 1991; Tyler and Sumpter, 1990), measuring phosphate concentrations (Borzawa-Gerard and Dumas-Vidal, 1991; Tao *et al.*, 1993), or establishing the degree of immunological cross-reaction with antisera (Matsubara *et al.*, 1994).

Studies in which VTG has been purified without the use of precipitation include Sumpter (1985) and Zanuy *et al.* (1987), who applied the plasma of rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*), respectively, directly to Sepharose 6B, followed by affinity chromatography on Con A-Sepharose. Tyler and Sumpter (1990) and Mananos *et al.* (1994b) used gel filtration on Sepharose 6B, followed by DEAE-cellulose, for carp (*Cyprinus carpio*) and sea bass VTGs, respectively. Yao and Crim (1996) used gel filtration on Sephacryl S300 followed by DEAE-cellulose for purification of ocean pout (*Macrozoarces americanus* L.), lumpfish (*Cyclopterus lumpus*), and Atlantic cod (*Gadus morhua*) VTGs. Tao *et al.* (1993) and Roubal *et al.* (1997) used DEAE-cellulose followed by Sepharose 6B for purification of English sole (*Pleuronectes vetulus*) and striped bass (*Morone saxatilis*) VTGs, respectively. Tao *et al.*

(1993) initially tried hydroxylapatite chromatography, but abandoned this method when they found that the striped bass VTG which had been prepared in this way was difficult to redissolve. Matsubara *et al.* (1994) used immunosorbent column chromatography to purify VTG from Japanese sardine (*Sardinops melanostictus*).

1.4 Use of Enzyme Inhibitors

Fish VTGs have been found to be very susceptible to proteolytic damage at all stages of collection, purification, and storage. This is a major concern in VTG assays. To prevent the fragmentation of VTG during its preparation, it is essential to use enzyme inhibitors and keep temperatures below 4 °C. The most commonly used inhibitors are aprotinin, phenyl methyl sulphonyl fluoride (PMSF), and 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF). Aprotinin has been shown to be very effective. PMSF is considerably cheaper and has been used by several research groups. However, there is doubt as to its effectiveness. Specker and Anderson (1994) found that PMSF did not prevent damage during the purification of striped bass VTG. Also, Inaba *et al.* (1997) identified a 170 kDa trypsin-like protein in tilapia (*Oreochromis niloticus*) plasma which degraded VTG and showed that this enzyme could be inhibited by aprotinin but not by PMSF. AEBSF is a more easily soluble form of PMSF.

1.5 What Form does Proteolytic Damage Take?

There are many instances in the literature where authors have reported the isolation of homogeneous peaks of VTG by gel filtration and anion-exchange chromatography but, following treatment with 2-mercaptoethanol and separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the VTG dissociates into two, and sometimes three, protein bands with molecular weights of approximately 170 kDa, 110 kDa, and 90 kDa (Mananos *et al.*, 1994b; Norberg, 1995; Silversand *et al.*, 1993). It is generally accepted, though it has never been proven, that the 170 kDa band (which is usually the most abundant) represents intact VTG and that the other bands represent proteolytic fragments. What has not been clearly explained is why these fragments are not separated from the intact protein during the process of gel filtration. Norberg (1995) and Silversand *et al.* (1993) suggest that the fragments form by rapid proteolysis at the time the VTG is added to the SDS-PAGE sample buffer. However, this seems doubtful. A more likely explanation is provided by the fact that fish VTGs contain the amino acid cysteine (Yao and Crim, 1996). Pairs of cysteine units form covalent disulphide bridges which join different parts of the amino acid chain to each other. The presence of these bridges means that a protein can be broken (“nicked”) in several places (by proteolytic enzymes) but still maintain its structural integrity (as recently shown for lipovitellin by Hartling *et al.* (1997)). These “nicks” occur during the preparation and storage of VTG but are not readily noticeable until the VTG is treated with 2-mercaptoethanol (which occurs just prior to SDS-PAGE). The 2-mercaptoethanol splits the disulphide bridges, which releases the broken fragments.

Silversand *et al.* (1993) found that VTGs from marine fish appear to be more liable to proteolytic damage than those from freshwater fish. The fact that these damaged VTG preparations resolve into only a small number of bands on SDS-PAGE indicates that only a few positions on the molecule are prone to “nicking”. A main concern for setting up an immunoassay for VTG then becomes whether any of these positions fall within the antibody recognition site. If one does, then there are likely to be major problems with assay stability (as “nicking” is not prevented by frozen storage, see Section 1.6, below).

1.6 Coping with Unstable VTG Standards

With the majority of published RIAs and ELISAs for VTG, standards have been prepared by pooling the peak fractions from the final chromatographic purification stage, estimating the protein concentration (by comparing with standards made up from bovine serum albumin, ovalbumin or freeze-dried VTG), and storing aliquots frozen at -20°C . However, Norberg (1995), Silversand *et al.* (1993), and Sumpter (1985) have all found that purified VTG is unstable in frozen form. Norberg (1995), for example, pooled the peak fractions from anion-exchange chromatography of halibut VTG and dialysed them for 3 hours at 4°C against a mixture of 0.05 M Tris-HCl buffer (pH 8.0) and glycerol (1:1) and stored the solution at -20°C . However, after one month of storage, the VTG was found to have been extensively “nicked”. Similar damage occurred within three days in the preparations made by Silversand *et al.* (1993). This damage may not be a problem if the antibody binding sites are unaffected. However, with evidence of such active degradation of standards during freezing and thawing, the long-term immunological stability of VTG solutions cannot be guaranteed and it is therefore wise to take steps to minimize or circumvent this problem. Methods which have been used include:

- a) storing VTG at -20°C in a freeze-dried (presumed stable) form and weighing out and dissolving a fresh batch each time an assay is carried out (as for the RIA described in this paper);
- b) using a pool of plasma from E_2 -treated fish as a standard (as for the ELISA described in this paper); this is based on the observation by Silversand *et al.* (1993) that VTG is far more stable in plasma than in its purified form;
- c) using lipovitellin, which is the main yolk protein in the oocyte and is formed by the cleavage of VTG; thus, the two proteins should show good immunological cross-reaction; lipovitellin appears to be intrinsically more stable than VTG and is, furthermore, resistant to heat treatment (which can be used to destroy residual enzyme activity (Hartling *et al.*, 1997)). This method is only of any use, however, if lipovitellin and VTG yield parallel dilution curves in the immunoassay.

2 PROCEDURE FOR PURIFYING VTG

2.1 Induction of VTG Production

Powdered E_2 should be added to melted cocoa butter at a concentration of 50 mg ml^{-1} . The powder should then be evenly dispersed in the butter by ultrasonication (in a bath containing warm water) and injected intramuscularly into males at the rate of $400\text{ }\mu\text{l}$ (20 mg) per kg of fish. Blood should be collected after an interval of two weeks.

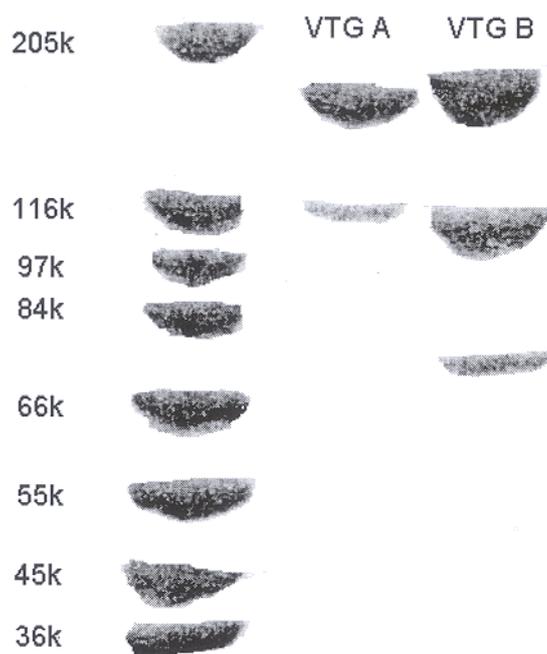
2.2 Collection of Blood

Prior to the collection of blood samples, 2 ml syringes should be rinsed with a saline solution containing $8\text{ Trypsin Inhibitor Units (TIU) ml}^{-1}$ of aprotinin and 500 IU ml^{-1} sodium heparin (to prevent blood clotting). Blood should be transferred to collection tubes, on ice, which contain $50\text{ }\mu\text{l}$ of heparin solution, with AEBSF and aprotinin at 1 mg ml^{-1} and 8 TIU ml^{-1} , respectively. A maximum of 2.5 ml blood is added to each tube, which is spun at 2000 rpm and 4°C for 15 minutes, and the plasma is thereafter removed, frozen, and stored in liquid nitrogen.

In flounder (*Platichthys flesus*), the authors have found that this and the other procedures described below are still not good enough to yield totally damage-free VTG. About 5 % of the

VTG shows evidence of proteolytic “nicking” (Figure 1; preparation A). This is a problem in many other species (especially marine fish) and Silversand *et al.* (1993) suggest that it can be overcome by injecting fish with aprotinin 30 minutes before the blood is taken. A certain amount of proteolytic damage need not necessarily be a problem for immunoassays, however. In an effort to establish whether “nicking” affected the immunological properties of flounder VTG (on which the RIA described below is based), the authors prepared some VTG without the use of protease inhibitors. This preparation ran as a single peak on gel filtration and anion-exchange chromatography (i.e., it maintained its structural identity) but showed extensive evidence of proteolysis (Figure 1; preparation B), i.e., three prominent bands, similar to halibut VTG prepared at room temperature by Norberg (1995) on SDS-PAGE. However, the immunological cross-reactivity of this preparation (Figure 2) was identical to that of relatively undamaged VTG, indicating that the antibody-binding site probably does not span any of the positions where damage occurs. This may not apply to antibodies raised to VTGs in all species, however. For example, a rainbow trout VTG antiserum which the authors employ is strongly affected by the integrity of the VTG standard (own unpublished observations).

Figure 1. Separation on 4–15 % gradient SDS-Polyacrylamide Gel Electrophoresis of flounder VTG which was purified either with (VTG A) or without (VTG B) the use of AEBSF and aprotinin. The preparations were denatured with 2-mercaptoethanol prior to separation and run alongside a range of protein markers of known molecular weights (which are shown in Daltons).



2.3 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. The authors have successfully used it to prepare VTGs from flounder, rainbow trout, and Atlantic salmon.

Beforehand, the following solutions should be made up and chilled on ice:

- 1) 20 ml of 20 mM EDTA, adjusted to pH 7.6 with 2 M NaOH, containing 20 mg AEBSF;

- 2) 2 ml of 0.5 M MgCl₂, containing 20 mg AEBSF;
- 3) 100 ml of distilled water, containing 36 mg AEBSF;
- 4) 4 ml of 1 M NaCl in 50 mM Tris-HCl, pH 8.0, containing 20 mg AEBSF and 0.8 TIU aprotinin;
- 5) 20 ml of 50 mM Tris-HCl, pH 8.0, containing 20 mg AEBSF and 3.2 TIU aprotinin.

Plasma samples from two E₂-injected male fish are thawed and dispensed as 1 ml aliquots into 150 mm × 16 mm glass tubes. Each tube then receives 150 µl of the MgCl₂ solution, 3 ml of the EDTA solution, and 15 ml of distilled water. This produces a heavy white precipitate. The precipitate is compacted by centrifugation, and the supernatant discarded; the precipitate is washed with 3 ml of distilled water and compacted again.

2.4 Chromatographic Purification

The precipitates from all tubes are redissolved, pooled in a maximum of 600 µl of the Tris-HCl/NaCl solution, and then slowly made up to 20 ml with the Tris-HCl buffer. This solution is then injected through a 43 µm filter to remove particulate matter and loaded onto a prepared DEAE-Sephacel column (1 cm i.d. × 26 cm) at a rate of 0.5 ml min⁻¹, and at a temperature of 4 °C. The column is developed with a gradient formed by two pumps. Pump A reservoir contains 50 mM Tris-HCl, pH 8.0, and pump B reservoir contains 50 mM Tris-HCl, 1 M NaCl, pH 8.0. Both buffers also contain 0.16 TIU ml⁻¹ of aprotinin. The flow rate is 0.5 ml per minute. After the sample is loaded, the column is run with 5% B for 40 minutes and then a gradient of 5 % B to 35 % B over 200 minutes. Four-minute fractions are collected. The effluent is monitored with a UV detector. Material which appears in the wash is discarded. A major UV-absorbing peak should elute at about 150 minutes (corresponding to about 200 mM NaCl, although this will vary between species). The fractions around the peak are pooled, sealed in dialysis tubing, and dialysed overnight against 5 litres of distilled water. The liquid is then rapidly frozen in a flask of liquid nitrogen and freeze-dried. In flounder, this yields approximately 25 mg of VTG per ml of plasma.

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

Despite the highly successful application of the method described above to the preparation of VTG from the blood plasma of flounder, rainbow trout, and salmon, the authors have failed to get it to work well with North Sea plaice (*Pleuronectes platessa*) or cod (*Gadus morhua*). In these species, VTG precipitation is more difficult to obtain. Also, after ion-exchange chromatography, although the main peak separates into the same three bands on PAGE as described above, the band corresponding to “intact” VTG is a minor component (despite the use of enzyme inhibitors). In order to overcome this problem, the authors have resorted to a “single-step” gel filtration procedure, as described by Hylland and Haux (1997), the details of which are laid out in Section 4.1. A minor disadvantage of this latter procedure is that, because of the recommended loading limit for the gel filtration column, it is not so suitable for preparing large amounts of VTG standards in a powdered form.

2.5 Antiserum Production

Antibodies can be 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 (Harlow and Lane, 1988). The rabbits are injected six times at two- to three-week intervals, and bled at least four times, at approximately monthly intervals.

3 RADIOIMMUNOASSAY PROCEDURE

3.1 Iodination

Fish VTG should be labelled with Na^{125}I using Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril) as the oxidizing agent (Salacinski *et al.*, 1981; Tyler and Sumpter, 1990). The Iodogen is dissolved in dichloromethane at a concentration of $100 \mu\text{g ml}^{-1}$, and $20 \mu\text{l}$ is allowed to dry in the bottom of a 1.5 ml Eppendorf vial. The vial is placed on its side. Freshly weighed VTG is made up in distilled water at a concentration of 1 mg ml^{-1} , and $10 \mu\text{l}$ is mixed with $20 \mu\text{l}$ of 0.5 M sodium phosphate buffer (pH 7.4) and pipetted onto the side of the vial. Ten μl of Na^{125}I (3.7 MBq of radioactivity) is also pipetted onto the side of the vial, which is 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 is allowed to proceed for 10 minutes and then terminated by the addition of 1 ml of 0.05 M sodium phosphate buffer (pH 7.4). This is added to 1.5 ml of assay buffer (see below), and applied to a prepared PD10 column (which contains Sephadex G25). The eluate from the column (2.5 ml) is allowed to go to waste. The radiolabelled protein fraction is then eluted by the addition of a further 3.5 ml of assay buffer and collected in a glass vial containing 3.5 ml of glycerol (to prevent the mixture freezing) and stored at $-20 \text{ }^\circ\text{C}$. Unattached Na^{125}I remains on the column. The level of incorporation of ^{125}I into VTG (which can be crudely assessed by comparing the radioactivity in the PD10 column and that in the glass vial with a hand-held monitor) should be at least 40 % (but preferably > 60 %). The authors have found no problems with using radiolabelled flounder VTG for at least 21 days, apart from a gradual diminution in the proportion that binds to antibody. Copeland and Thomas (1988) also reported that radiolabelled sea trout VTG was usable for about 3 weeks. However, Tyler and Sumpter (1990) reported that radiolabelled carp VTG could be used for up to 60 days if it was repurified on a PD10 column prior to each assay. Several other methods of radioiodination of VTG have been tried, but without success (Idler *et al.*, 1979; So *et al.*, 1985).

3.2 Standard

About 1 mg VTG powder is weighed out and dissolved in distilled water at a concentration of 1 mg ml^{-1} . This is diluted 100-fold with assay buffer to form a solution with a concentration of $10 \mu\text{g ml}^{-1}$.

3.3 Basic Assay Procedure

Plastic tubes are set up in trays—those which will receive the plasma samples (the “unknowns”) are labelled 1 to 60 in duplicate; those which will receive the standards are labelled S1 to S11 in duplicate. Three pairs of tubes are labelled M, B, and T. Aliquots of $50 \mu\text{l}$ assay buffer are dispensed into all the sample tubes and $100 \mu\text{l}$ into S1 to S11, M and B. The volume in the sample tubes is made up to $100 \mu\text{l}$ by adding $50 \mu\text{l}$ of plasma in a neat or pre-diluted form (see below). A vial containing diluted standard ($10 \mu\text{g ml}^{-1}$) is thawed and $100 \mu\text{l}$ is added to each of the S1 tubes. The tubes are mixed and $100 \mu\text{l}$ is transferred to each of the S2 tubes. This

procedure is repeated up to S11, when the final 100 μl is discarded. This yields a standard curve with concentrations ranging from 5000 ng ml^{-1} to 5 ng ml^{-1} . Anti-VTG serum is diluted 1:100,000 with assay buffer, and 100 μl is added to all tubes except those labelled B and T. The tubes labelled B receive 100 μl of assay buffer. After mixing, the tubes are centrifuged briefly to remove any drops on the sides of the tubes. They are returned to the rack, covered with aluminium foil, and placed in the refrigerator overnight. The next morning, ^{125}I -VTG is diluted with assay buffer and 50 μl (20,000 cpm) is added to all tubes. After another brief centrifugation, the tubes are incubated at room temperature for a further 6 hours. To separate the bound from the free radiolabel, 100 μl of Sac-Cel (second antibody covalently linked to cellulose) is added to all tubes except those labelled T. After a further 30 minutes, 1 ml of distilled water is added to all tubes except those labelled T. The tubes are then centrifuged for 10 minutes to compact the cellulose; the aqueous phase is removed by aspiration, and the radioactivity adhering to the pellets is measured with an automatic gamma counter. The T tubes represent the “total” radiolabel added to each tube. The B tubes represent the amount of radiolabel bound to Sac-Cel in the absence of any anti-VTG antibody (i.e., non-specific binding). The M tubes represent the amount of radiolabel bound to anti-VTG antibody in the absence of any unlabelled VTG (i.e., maximum binding).

When the percent of label bound is plotted against the logarithm of the VTG concentration, it should form a sigmoid curve (Figure 2). Unknowns should be calculated from the steep middle portion of the curve. The only exception is samples with very low concentrations. Where possible, these should be reassayed under more sensitive conditions (see Section 3.4, below). However, some plasma samples will still need to be quantified from the upper, less accurate, part of the curve. Generally, this is of minor concern, as the problem only occurs within a range of VTG concentrations (approximately 5–20 ng ml^{-1}) which is miniscule in comparison to the total range encountered within most species.

As a measure of quality control, one or two pools of plasma should be made up and included in every assay. This will give an indication of any major problems associated with degradation of the standard, as well as yielding a measure of the inter-assay coefficient of variation (for which an acceptable value would be 10 %). Where possible, all samples from a single study should be assayed at the same time, using an identical batch of standard.

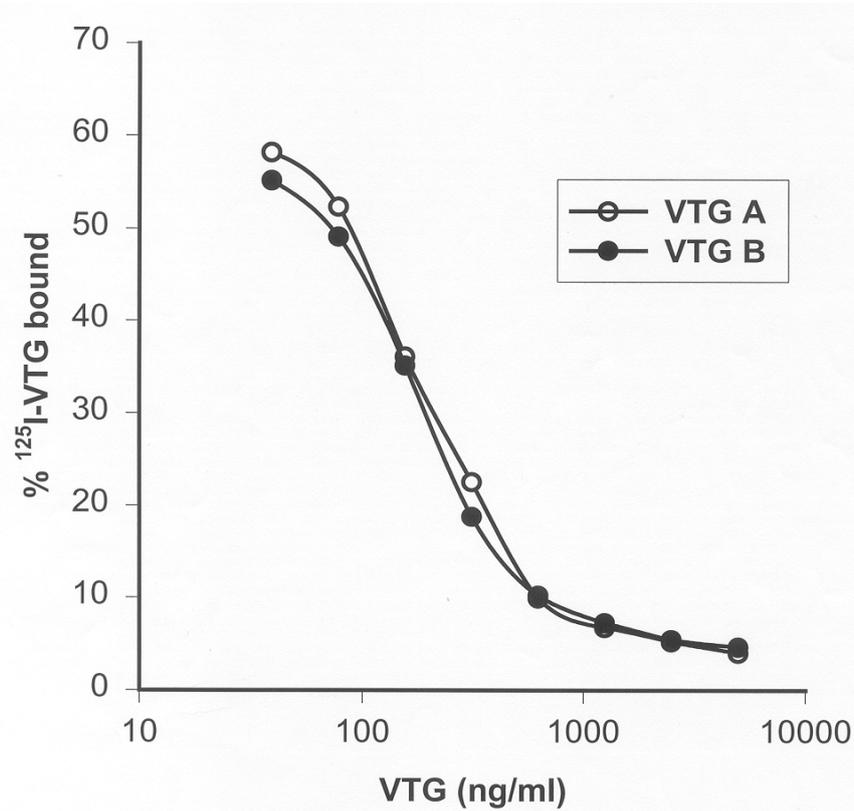
3.4 Plasma Dilutions

Depending on species, VTG is found at concentrations ranging from $< 1 \text{ ng ml}^{-1}$ to 200 mg ml^{-1} . This poses a major problem, as the accurate (i.e., steep) part of the standard curve only covers a small range (in the case of the flounder RIA, from 80 ng ml^{-1} to 1250 ng ml^{-1}). This means that most plasma will either need to be diluted or to be measured more sensitively. One strategy is to assay all male and immature female plasmas at a dilution of 1:2 (i.e., 50 μl of undiluted plasma is added to the assay tube and made up to 100 μl with assay buffer), and plasma from mature females at two dilutions of 1:5,000 and 1:50,000. A better strategy is to screen the plasmas with a simple direct ELISA method prior to carrying out the RIA.

A value should only be accepted if it falls within the steep middle range of the standard curve, where both male and female plasmas should show good parallelism with the standard VTG. If the value is greater than 1250 ng ml^{-1} , then the plasma is retested at a range of dilutions up to as much as 1:200,000. If the value is less than 80 ng ml^{-1} , then the plasma is retested using a sensitive assay procedure which, in the case of the flounder, involves incubating standard and unknowns for two days at 4 °C, with antiserum diluted 1:200,000, and for a further one day with 50 μl of 10,000 cpm of ^{125}I -VTG. Sensitivity can be improved still further by using even less of

both reagents (e.g., antiserum at 1:1,000,000 and radiolabel at 10,000 cpm). In the case of the flounder VTG RIA, no problem has been experienced in using undiluted plasma.

Figure 2. RIA of two flounder VTG preparations which were prepared either with (VTG A) or without (VTG B) the use of protease inhibitors; this shows that despite large differences in their degree of proteolytic “nicking” (Figure 1), the differences in their immunoreactive potency are negligible.



3.5 Establishing an Initial Antibody Dilution

As a first step in the development of the RIA, serial dilutions of the antiserum should be made between 1:10,000 and 1:2,000,000 and incubated for the appropriate time interval with 20,000 dpm of radiolabel prior to separation. The percentage of radio-iodinated VTG that binds to an excess of antibody should be at least 75 %. The antibody dilution corresponding to 50 % of bound radiolabel should be used in the RIA.

4 ELISA PROCEDURE

4.1 Preparation of Standard

VTG synthesis is induced in fish by treatment with E₂ (see Section 1.2, above). Plasma from 5 to 10 individuals is thoroughly mixed, divided into a large number of 0.5 ml aliquots, and frozen in liquid nitrogen.

Prior to each assay, an aliquot is thawed and passed through a 0.22 µm filter. Approximately 150 µl is then applied to a HR 10/30 Superdex 200 column coupled to a Fast Performance Liquid Chromatography (FPLC) single-pump system. Elution is carried out with 100 mM Tris-

HCl, pH 7.8, at 4 °C at a rate of 0.1 ml min⁻¹. For cod, salmon, flounder, wrasse, and brown trout, this results in a major UV-absorbing peak, in which all the immunoreactivity resides and which corresponds to a molecular weight of 550–580 kDa, eluting at about 100 minutes (Hylland and Haux, 1997). A general protein assay, using bovine IgG as a standard, is carried out on this peak in order to establish the concentration of VTG in the original aliquot. The aliquots are then used as standards and for coating the plates.

4.2 Basic Assay Procedure

1) *Coating*: The ELISA is performed using 96-well microtitre plates designed for high protein adsorption. 100 µl of antigen solution (100 ng VTG ml⁻¹ in carbonate buffer, pH 9.6) is pipetted into all wells except A1 to B1 (blanks) and C1 to D1 (non-specific binding (NSB)), which receive carbonate buffer only. The plates are incubated in a moist chamber overnight at 4 °C. The plates are then washed three times with 350 µl of TTBS (Tris-buffered saline with 0.5 % Tween-20) (see below) and blocked by incubating them for 30 minutes at room temperature with a further 350 µl of TTBS. Following aspiration of the TTBS, the plates can be used immediately or sealed and frozen at –80 °C for future use.

2) *Primary antibody incubation*: 100 µl of TTBS is pipetted into the blank wells, 50 µl into the NSB wells, and 50 µl of standard or appropriately diluted sample into the remaining wells. 50 µl of anti-cod VTG serum (diluted 1:80,000 in TTBS) is added to all wells except the blanks. The plates are sealed and incubated overnight at 4 °C.

3) *Secondary antibody incubation*: The plates are washed three times with TTBS. 100 µl of Horseradish Peroxidase-conjugated goat anti-rabbit IgG (at a dilution of 1:10,000 in TTBS) is added to all wells except the blanks. 100 µl of TTBS is pipetted into blank wells. The plates are sealed and incubated for 6 hours at 4 °C.

4) *Colour development*: The plates are washed five times in TTBS. 100 µl of substrate solution (0.04 % O-phenylene-diamine in 150 mM phosphate, 50 mM citrate buffer, pH 5.7, with 0.012 % hydrogen peroxide) is added to all wells. The substrate solution should be prepared immediately prior to use. Plates are incubated in the dark at room temperature for 20 minutes to 40 minutes. The reaction is stopped by the addition of 50 µl of 2 N HCl to all wells. Absorbance is measured at 490 nm. VTG concentrations are calculated using a sigmoid (4-parameter) curve-fit to standards.

4.3 Establishing Optimum VTG Coating, Antibody and Standard Dilutions

VTG is dissolved in carbonate buffer at dilutions of 50 ng ml⁻¹, 100 ng ml⁻¹, 200 ng ml⁻¹, and 400 ng ml⁻¹. These solutions are added, respectively, to rows 1 to 3, 4 to 6, 7 to 9, and 10 to 12 of a single microtitre plate (100 µl per well). The plate is sealed and incubated overnight at 4 °C. After washing three times with TTBS, doubling dilutions of primary antiserum (starting at 1:1,000) are added to rows A to H (50 µl per well). The plates are resealed and incubated overnight at 4 °C. After washing three times with TTBS, secondary antiserum (100 µl per well) is added to: columns 1, 4, 7, and 10 at a dilution of 1:3,000; columns 2, 5, 8, and 11 at a dilution of 1:9,000; and columns 3, 6, 9, and 12 at a dilution of 1:27,000. The plate is then resealed, incubated for 6 hours at 4 °C, washed and incubated with the colour reagent. The appropriate concentrations of coating, primary, and secondary antisera can then be determined by visual inspection of the plate or measurement on a plate reader.

5 FURTHER CONSIDERATIONS

5.1 Collection, Handling, and Storage of Plasma Samples

Blood should be collected into plastic syringes that have been flushed with heparinized saline (500 units ml⁻¹), centrifuged to compact the blood cells and the plasma frozen at -20 °C. Several authors, among them Tyler *et al.* (1996) and Mananos *et al.* (1994a), add aprotinin (2 T.I.U.) or PMSF (1 mM) to the heparin solution. However, not all authors do this—and there is not enough experimental evidence to suggest that this is a necessary step. The authors are also not aware of any experimental data on the effects of either time (between collection and centrifugation), temperature or haemolysis on the stability of VTG immunoreactivity in blood samples. Clearly, more research is needed in this area.

Norberg and Haux (1988) stated that, when brown trout plasma samples were repeatedly thawed and frozen, the apparent VTG concentration increased—sometimes as much as one hundred-fold. This finding causes concern and suggests that one needs to store several aliquots of every plasma sample. However, it possibly does not apply to every species. For example, in flounder and cod, the authors have found no evidence for any change in VTG concentrations in repeatedly thawed and frozen plasma samples.

Another problem is that the concentrations of VTG found in males from some contaminated areas may be so high (Matthiessen *et al.*, 1998) that, after freezing and thawing, the plasma forms a dense white precipitate. In the authors' experience, such precipitates readily redissolve in assay buffer.

Owing to the very large range of VTG concentrations found in many species, cross-contamination of plasma samples must be assiduously avoided.

5.2 Homologous versus Heterologous Reagents

Although VTGs from different species have a common genetic origin, there are appreciable differences in their amino acid sequences. This means that an antiserum raised to a VTG isolated from one species will not necessarily cross-react with a VTG from another. The best solution (with the fewest problems likely to be encountered with specificity and parallelism) is to use “homologous” reagents (i.e., both VTG and antiserum raised within a species). “Second best” is an immunoassay with a homologous VTG and a heterologous antiserum. The least desirable combination (although it may be necessary when working with small fish species (Tyler *et al.*, 1996)) is an immunoassay with both reagents being derived from a different species. Assays that employ one or more heterologous reagents need to be particularly well evaluated for specificity and parallelism. Interestingly, an attempt has recently been made to develop an antiserum to a sequence of amino acids which is heavily conserved in fish VTGs (Folmar *et al.*, 1995; Heppell *et al.*, 1995). This approach offers the possibility of a “universal” assay for fish VTG.

5.3 Parallelism

It is very important that plasma samples produce radiolabel displacement curves which are parallel with the standard (otherwise VTG concentrations cannot be estimated accurately). This is normally done by testing doubling dilutions of a relatively few pools of plasma. However, an alternative way is to assay every single plasma at two dilutions (5- or 10-fold apart). In many cases, both dilutions will fall on the steep accurate part of the curve. When many paired readings have been collected, they can be compared with each other using regression analysis to

give a broader indication of parallelism. This procedure will also detect whether there are problems with samples from particular areas or with samples that have been handled in different ways.

5.4 Sensitivity

Sensitivity is most commonly defined as “the least detectable sample concentration that is significantly different from zero standard” (Specker and Anderson, 1994). The various steps that can be taken to improve the sensitivity of an assay include:

- 1) reducing the total volume of buffer;
- 2) pre-incubating the antibody with the standards and unknowns;
- 3) using smaller amounts of antibody and antigen (in combination with longer incubation periods);
- 4) adding as much plasma sample as possible to tubes or wells (within the constraints set by non-specific interference, see below);
- 5) improving the avidity of the antibody (by prolonging the course of injections of VTG into rabbits (Harlow and Lane, 1988)).

At present, it is very difficult, if not impossible, to make meaningful comparisons between published VTG assays in regard to sensitivity. The main reason for this is the lack of cross-laboratory (and cross-species) standardization. Clearly, such problems will eventually need to be resolved (through the use of interlaboratory calibration studies). Fortunately, for the majority of applications, it is not essential to know the exact mass of VTG in a plasma sample. It is entirely adequate that, within each study, the **relative** differences in VTG concentrations should be accurately recorded.

5.5 Establishing Baseline Concentrations

Plasma samples from immature fish and males should be collected in areas that are known to have little or no environmental contamination. These fish will hopefully yield “baseline” VTG concentrations against which all other sites can be compared. However, even if environmental and dietary sources of oestrogens can be eliminated, there are at least two other ways in which males might receive E₂ stimulation. In some species, there appears to be substantial endogenous E₂ production by males. Plasma of male brown bullheads (*Ictalurus nebulosus*) contains E₂ up to 800 pg ml⁻¹ (Rosenblum *et al.*, 1987). The plasma of the closely related male channel catfish (*Ictalurus punctatus*) contains VTG at concentrations of between 5 µg ml⁻¹ and 5,000 µg ml⁻¹ (Goodwin *et al.*, 1992). In male North Sea plaice, plasma E₂ concentrations rise as high as 3 ng ml⁻¹ at the height of the spawning season (Wingfield and Grimm, 1977). Preliminary results suggest that these elevated E₂ concentrations are also associated with elevated VTG concentrations (authors’ own unpublished observations). Elevated plasma concentrations of VTG are also found in the males of some species (e.g., goldsinny wrasse (*Ctenolabrus rupestris*) (authors’ own unpublished observations)).

In captive fish, another potential source of E₂ in males is females. Although E₂ itself has not been specifically examined, it has been shown that fish can readily release sex steroids into the water via their gills and just as readily reabsorb them (Vermeirssen and Scott, 1996). This means that in crowded conditions and with low water flow rates, there might be sufficient transfer of E₂ from females to be able to induce VTG production in males.

5.6 Comparison between RIA and ELISA

RIA has several obvious disadvantages. The radioiodination process is unpleasant and potentially hazardous. Radioactive handling and disposal facilities are normally confined to only a few laboratories; the radiolabel has a very limited lifespan (3 to 6 weeks). One advantage is that it is easier and quicker to establish optimum assay conditions than for ELISA (as there are fewer combinations of reagents to be tested). Another advantage is that RIAs do not appear to be as prone to non-specific interference from plasma (“matrix effects”) as ELISAs. There are several published ELISA procedures in which it is reported that plasmas have to be diluted at least 1:16 (and sometimes as much as 1:100) before being added to wells—otherwise they generate “false positive” signals (Bon *et al.*, 1997; Christiansen *et al.*, 1998; Goodwin *et al.*, 1992; Mananos *et al.*, 1994a; Mourot and Le Bail, 1995). The need to dilute samples clearly reduces the working sensitivity of an assay. However, matrix effects, the causes of which are still not clear, do not affect all ELISAs. They have not presented a problem with the ELISA for cod VTG which has been described above. Okumura *et al.* (1995) have also reported an exquisitely sensitive sandwich ELISA method for Japanese eel (*Anguilla japonica*) VTG which appears to be free from problems of non-specificity.

Readers are referred to the papers by Specker and Anderson (1994) and Denslow *et al.* (1999) for discussion of different ELISA techniques and further general advice on the development and validation of VTG ELISAs.

5.7 Intercalibration

An intercalibration on VTG determination has been performed among seven European laboratories involved with the EU project COMPREHEND (contract ENV4-CT98-0798). Twenty-four plasma samples from rainbow trout with varying levels of VTG were distributed by Brunel University for analysis. The participating laboratories used western blot, RIA, and ELISA. Both homologous (i.e., rainbow trout) and heterologous (e.g., salmon) antisera were used in the assays. All assays used rainbow trout VTG as the standard. All assays were able to separate high (mg ml^{-1}), intermediate ($\mu\text{g ml}^{-1}$), and low (ng ml^{-1}) concentrations of plasma VTG. Assays using homologous antisera gave a better separation and lower detection limit than assays using heterologous antisera. In this intercalibration, RIA generally gave a lower detection limit than ELISA.

6 LIST OF REAGENTS AND EQUIPMENT REQUIRED FOR RIA

The reagents and equipment needed for RIA are listed below; the sources for footnoted items are listed on p. 15.

For purification of VTG:

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

Disposables: glass tubes (150 mm × 16 mm; carrying out precipitations); Acrocap filter (0.45 μm)²; HiTrap Q (1 ml) column¹; PD-10 column (containing Sephadex G-25 M)¹; dialysis tubing.

Chemicals: 17 β -oestradiol; cocoa butter; aprotinin; 4-(2-aminoethyl)-benzene-sulphonyl fluoride; ethylenediaminetetraacetic acid; sodium hydroxide; magnesium chloride; 0.05 M Tris-

HCl buffer (made from 0.97 g Trizma base and 6.61 g Trizma hydrochloride dissolved in 1 litre of distilled water [pH 8.0 at 5 °C]); sodium chloride; DEAE-Sephacel; Sepharose 6B.

For iodination:

Equipment: radioactivity monitor.

Disposables: eppendorf tube; PD-10 column¹; glass scintillation vial (20 ml) for storing radioactive label; lead pots; pipettes; pipette tips; gloves.

Chemicals: ¹²⁵Iodine³; 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 litres of 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 α ,6 α -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 μ l to 5 ml); multiple pipettor (for repetitive dispensing of reagents).

Disposables: polystyrene tubes (12 mm \times 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 μ l Tween-20, and 1 g bovine serum albumin dissolved in 1 litre of distilled water; pH 7.2); Sac-Cel (second antibody covalently coupled to cellulose)⁴; 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).

7 LIST OF REAGENTS AND EQUIPMENT REQUIRED FOR ELISA

The reagents and equipment needed for ELISA are listed below; the sources for footnoted items are listed on p. 15.

Equipment: plate-reader fitted with a 490 nm filter; plate washer; multichannel pipettes; FPLC (or HPLC) equipment (to quantify VTG in the plasma which is used as a standard); 0.22 μ m syringe filter; Superdex 200 HR 10/30 column¹; liquid nitrogen container.

Disposables⁵: microtitre plates; pipetting reservoirs; pipette tips; sealing tape.

Chemicals: coating buffer (1.70 g sodium carbonate and 2.86 g sodium hydrogen carbonate dissolved in 1 litre of distilled water; pH 9.6); assay buffer (1.7 g Trizma base, 8.8 g Trizma hydrochloride, 29.2 g sodium chloride, and 500 μ l Tween-20 dissolved in 1 litre of distilled water; pH 8.5); secondary antibody (Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G); substrate solution (0.04 % O-phenylene-diamine in 150 mM phosphate, 50 mM citrate buffer, pH 5.7 with 0.012 % hydrogen peroxide); 2 N hydrochloric acid.

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

- ¹Amersham Pharmacia Biotech, 23, Grosvenor Road, St. Albans, Herts, AL1 3AW, UK;
²Gelman Sciences Ltd, Brackmills Business Park, Caswell Road, Northampton NN4 7EZ, UK;
³Amersham Life Science Ltd, Amersham Place, Little Chalfont, Bucks, HP7 9NA, UK;
⁴Immunodiagnostic Systems Ltd, Boldon Business Park, Tyne & Wear, NE35 9PD, UK;
⁵Nunc, Roskilde, Denmark.

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ABBREVIATIONS

AEBSF	4-(2-aminoethyl)-benzenesulphonyl fluoride
DEAE	diethylaminoethyl
E ₂	17 β -oestradiol
EDTA	ethylene diamino tetra-acetic acid
ELISA	enzyme-linked immunosorbent assays
FPLC	fast performance liquid chromatography
HPLC	high performance liquid chromatography
NSB	non-specific binding
PMSF	phenyl methyl sulphonyl fluoride
RIA	radioimmunoassay
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEAE	triethylaminoethyl
TTBS	Tris-buffered saline with 0.5 % Tween-20
VTG	vitellogenin

ICES Techniques in Marine Environmental Sciences

- No. 1 Cadmium and lead: Determination in organic matrices with electrothermal furnace atomic absorption spectrophotometry
- No. 2 Trace metals in sea water: Sampling and storage methods
- No. 3 Cadmium in marine sediments: Determination by graphite furnace atomic absorption spectroscopy
- No. 4 Lipophilic organic material: An apparatus for extracting solids used for their concentration from sea water
- No. 5 Primary production: Guidelines for measurement by ^{14}C incorporation
- No. 6 Control procedures: Good laboratory practice and quality assurance
- No. 7 Suspended particulate matter: Collection methods for gravimetric and trace metal analysis
- No. 8 Soft bottom macrofauna: Collection and treatment of samples
- No. 9 Sediments and suspended particulate matter: Total and partial methods of digestion (*videotape available*)
- No. 10 Organic halogens: Determination in marine media of adsorbable, volatile, or extractable compound totals
- No. 11 Biological effects of contaminants: Oyster (*Crassostrea gigas*) embryo bioassay
- No. 12 Hydrocarbons: Review of methods for analysis in sea water, biota, and sediments
- No. 13 Biological effects of contaminants: Microplate method for measurement of ethoxyresorufin-O-deethylase (EROD) in fish
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- No. 22 Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds
- No. 23 Biological effects of contaminants: Determination of CYP1A-dependent mono-oxygenase activity in dab by fluorimetric measurement of EROD activity
- No. 24 Biological effects of contaminants: Use of imposex in the dogwhelk (*Nucella lapillus*) as a bioindicator of tributyltin pollution
- No. 25 Biological effects of contaminants: Measurement of DNA adducts in fish by ^{32}P -postlabelling
- No. 26 Biological effects of contaminants: Quantification of metallothionein (MT) in fish liver tissue
- No. 27 Soft bottom macrofauna: Collection, treatment, and quality assurance of samples
- No. 28 Biological effects of contaminants: *Corophium* sp. sediment bioassay and toxicity test
- No. 29 Biological effects of contaminants: Sediment bioassay using the polychaete *Arenicola marina*
- No. 30 Chlorophyll *a*: Determination by spectroscopic methods
- No. 31 Biological effects of contaminants: Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques for the measurement of marine fish vitellogenins