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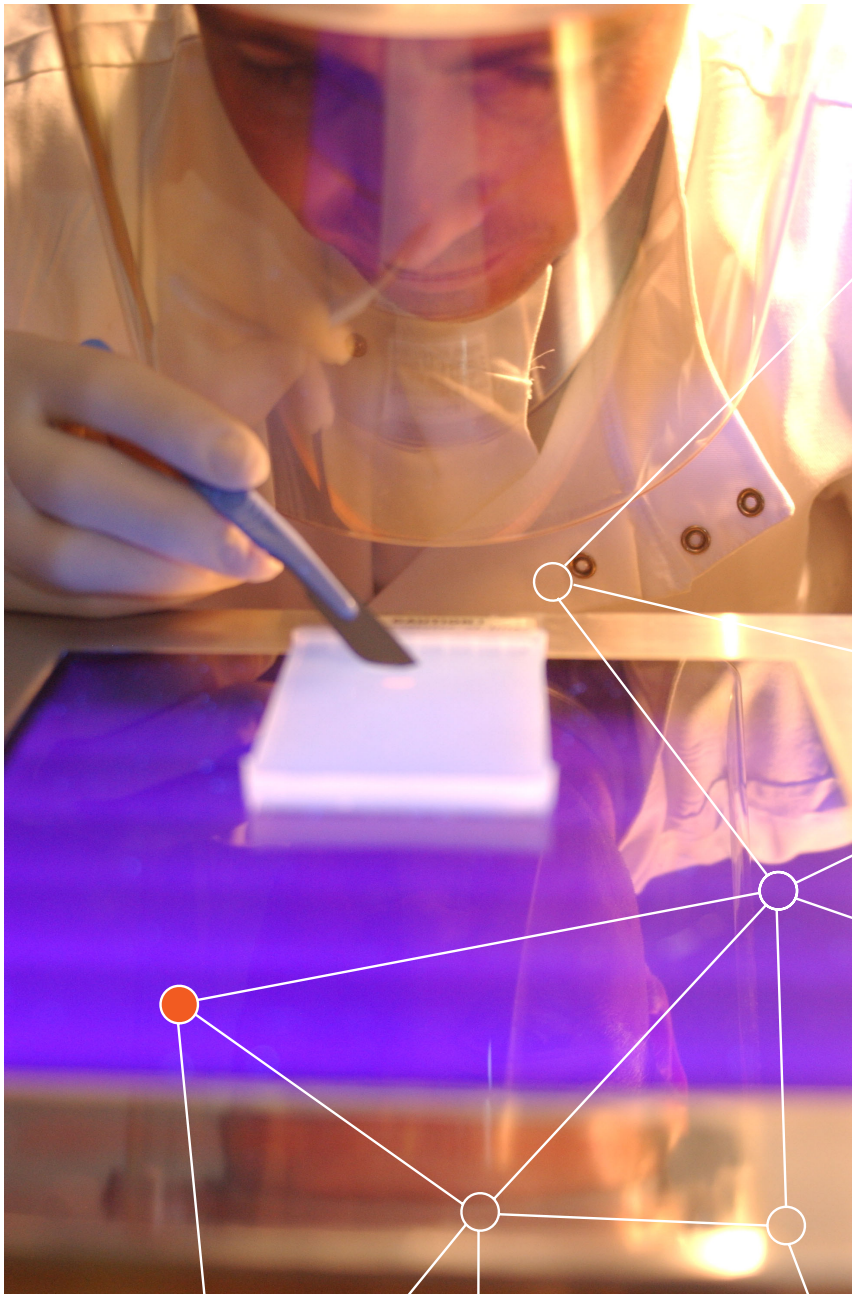

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Determination of CYP1A-dependent mono-oxygenase activity in dab by fluorimetric measurement of EROD activity in S9 or microsomal liver fractions

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This series presents detailed descriptions of methods and procedures relating to chemical and biological measurements in the marine environment. Most techniques described have been selected for documentation based on performance in ICES or other intercalibration or intercomparison exercises: they have been carefully evaluated and shown to yield good results when correctly applied. They have also been subject to review by relevant ICES working groups, but this is not to be construed as constituting official recommendation by the Council.

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Contents

Abstract

This paper describes a method for the determination of cytochrome P4501A (CYP1A) in fish liver fractions by the measurement of 7-ethoxyresorufin *O*-deethylase (EROD) activity. The proposed method is by fluorescent assay of resorufin using internal standardization. The method is specifically for measurements made in dab (*Limanda limanda* L.), but is suitable for adaptation to other species. The principle of the method, the sampling requirements, the assay procedures and the reporting of the results are described. Sources of error and quality control procedures are also specified. The document is a modification of a 1998 publication to allow measurement of EROD activity from both S9 and microsomal fractions to be undertaken.

Key words: CYP1A, EROD, fish, dab (*Limanda limanda*), biological effects of contaminants

1 Introduction

This document is a revision of a 1998 publication by Stagg and McIntosh (1998). The major revision is the inclusion of the analysis of microsomal fractions of the liver in addition to the S9 fraction. There are also numerous significant revisions to incorporate the results of research and monitoring since 1998.

Cytochrome P450-dependent mono-oxygenases (CYP P450) are membrane-bound enzymes which metabolize a range of exogenous (organic contaminants) and endogenous (steroids, fatty acids) substrates. They are ubiquitous throughout the animal kingdom, but in fish are often present at higher concentrations in tissues such as the liver. CYP P450s are Phase I enzymes which catalyse the insertion of an oxygen atom into a xenobiotic (foreign) molecule to facilitate conjugation to an endogenous substrate. The terminal oxygenase of this enzyme system is an iron-containing haemoprotein, namely, cytochrome-P450. The system is called P450 because the wavelength absorption maximum, after reduction with carbon monoxide, is at 450 nm. The conjugation reactions are carried out by a second group of enzymes (Phase II) such as uridinediphosphate (UDP)-glucuronyl transferase (UDP-GT) and glutathione *S*-transferase (GST). These combined reactions have the effect of making neutral, lipid-soluble xenobiotic compounds more water soluble, thus facilitating their elimination from the body. Although this sequence of events is generally a detoxification mechanism, it can result in the activation of relatively inert chemicals to highly reactive and damaging intermediates.

In fish, one form of CYP P450 termed CYP1A is induced by environmental exposure to a diverse range of planar molecules, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins and dibenzofurans (Spies *et al*., 1982; Luxon *et al*., 1987; Addison and Edwards, 1988; Jimenez *et al.,* 1988; Payne *et al*., 1988; Stegeman *et al*., 1988; Van Veld *et al*., 1990; Stein *et al*., 1992; Stagg *et al*., 1995). The enzymes responsible for this activity belong to the 3-methylcholanthrene inducible family of cytochrome P450 and in fish there appears to be only a single gene (CYP1A1) in this family (Nebert and Gonzalez, 1987; Nelson *et al*., 1993).

The basis for the use of CYP1A in monitoring is the phenomenon of induction, whereby levels of the enzyme are increased by the synthesis of new protein following exposure and the levels of enzyme activity are measurably increased. The induction of CYP1A can therefore be used to monitor exposure to bioavailable planar contaminants and, for example, is of value in determining exposure to PAHs, such as are found in hydrocarbon discharges associated with the oil industry (Davies *et al*., 1984; Stagg *et al*., 1995).

Stegeman (1993a, 1993b) and Stegeman and Hahn (1994) provide recent reviews of fish P450s, and Goksøyr and Förlin (1992), Stegeman *et al*. (1992), and Livingstone (1993) have reviewed the application of CYP1A as a monitoring tool.

Induction of CYP1A has also been proposed to be a measure of deleterious biological effects in the sense that compounds such as PAHs can be activated to compounds which cause DNA damage (Stegeman, 1987). There is considerable support from field observations correlating CYP1A induction to contaminant levels, DNA damage, hepatic carcinogenesis, and other pathological conditions (see reviews by Livingstone, 1993, and Livingstone *et al*., 1994). Many steps in this cascade, for example, the effects of PAHs on CYP1A induction, DNA damage, and carcinogenesis, have also been substantiated by experimental studies. Induction of CYP1A activity can therefore be seen as an essential process involved in the aetiology of tumour initiation and carcinogenesis by the activation of compounds such as PAHs into pre-carcinogens. Whether or not the reactive intermediates produced in Phase I reactions will cause damage depends in part on the

capacity of Phase II enzymes to conjugate and detoxify the compounds formed. However, there are considerable differences between fish species in this regard, which partly explains species susceptibility to carcinogenesis (Collier *et al*., 1992). Therefore, although the mechanistic link between elevated CYP1A activity and deleterious biological effects is well established, there is still a need for considerable care in interpreting CYP1A induction.

CYP1A mono-oxygenase activity is most conveniently measured as 7-ethoxyresorufin *O*-deethylase (EROD) activity and the method described here is that originally developed by Burke and Mayer (1974). 7-Ethoxyresorufin is an artificial substrate but is used because the assay is simple, sensitive, highly specific for CYP1A, and also presents a low hazard to the operator. Aryl hydrocarbon hydroxylase (AHH) was used in many earlier studies, but it is catalysed by a broader spectrum of P450s. A previous TIMES leaflet (Galgani and Payne, 1991) gives details of a microplate method using a fluorescent plate reader. Although advantageous from the point of view of being able to process many samples very quickly, it suffers from high levels of variability and the lack of internal standardization, and therefore is not widely used.

The present document describes a method for use with conventional fluorimeters, employing internal standardization. Interlaboratory comparisons of EROD activity measurements (Munkittrick *et al*., 1993; Stagg and Addison, 1994; Minchin et al., 2002) have shown the importance of developing a standard methodology to allow comparable data to be obtained from different laboratories, both for the absolute levels of EROD activity reported and for the ability to detect differences between induced and non-induced groups of fish.

1.1 Principle of the method

Microsomal mono-oxygenase activity is the overall activity of a multi-enzyme system residing in the sarcoplasmic reticulum which comprises an NADPH reductase and multiple cytochrome P450s. Other components involved in the reaction are membrane lipids, cytochrome b_5 reductase and cytochrome b_5. NADPH reductase is a flavoprotein which accepts electrons from NADPH and passes them on to P450. The cytochrome P450 component provides the system with its substrate specificity, which in the case of CYP1A is selective for planar aromatic molecules. 7- Ethoxyresorufin *O*-deethylase (EROD) reacts exclusively with CYP1A and is converted from 7-ethoxyresorufin to resorufin by the deethylation of the hydroxyl group on the *para* position (Figure 1).

To measure enzyme activity, the liver is dissected from the fish, a subsample is homogenized in an appropriate buffer and then centrifuged usually to yield a 10,000 × **g** supernatant (the 'S9 fraction'). Many investigators prefer to work with a more refined sample in which the S9 fraction is further centrifuged to yield a 100,000 × **g** pellet which is then resuspended in buffer ('microsomal preparation'). For research purposes, microsomal fractions are generally preferred as there are fewer interferences from cytosolic and lysosomal components of S9 fractions. However, the long centrifugation times associated with fraction preparation, the requirement for an ultra-centrifuge, and the slower sample throughput means that S9 fractions are often used in monitoring programmes. The fraction analysed should always be reported with the data.

EROD is measured in S9 and microsomal samples in the presence of NADPH using 7-ethoxyresorufin (ethoxyphenoxazone) as substrate; the product, resorufin, is determined fluorimetrically (Burke and Mayer, 1974). The reaction can be followed in the reaction cuvette because the characteristic emission spectra of 7-ethoxyresorufin (λEx 470 nm; λEm 560 nm) and resorufin (λEx 535 nm; λEm 585 nm) show not only good

separation of the maxima, but also the relative fluorescence intensity for resorufin compared to that of 7-ethoxyresorufin is at least one order of magnitude greater (Prough *et al.*, 1978). An important source of variability of EROD measurements (Munkittrick *et al.*, 1993; Stagg and Addison, 1994) is the purity of the resorufin used to calibrate the assays. Therefore, resorufin standards should be made up taking into account the purity of the resorufin stocks used. This is most conveniently done by reference to the extinction coefficient of resorufin (Klotz *et al.*, 1984) and preparing the standard to account for the purity measured.

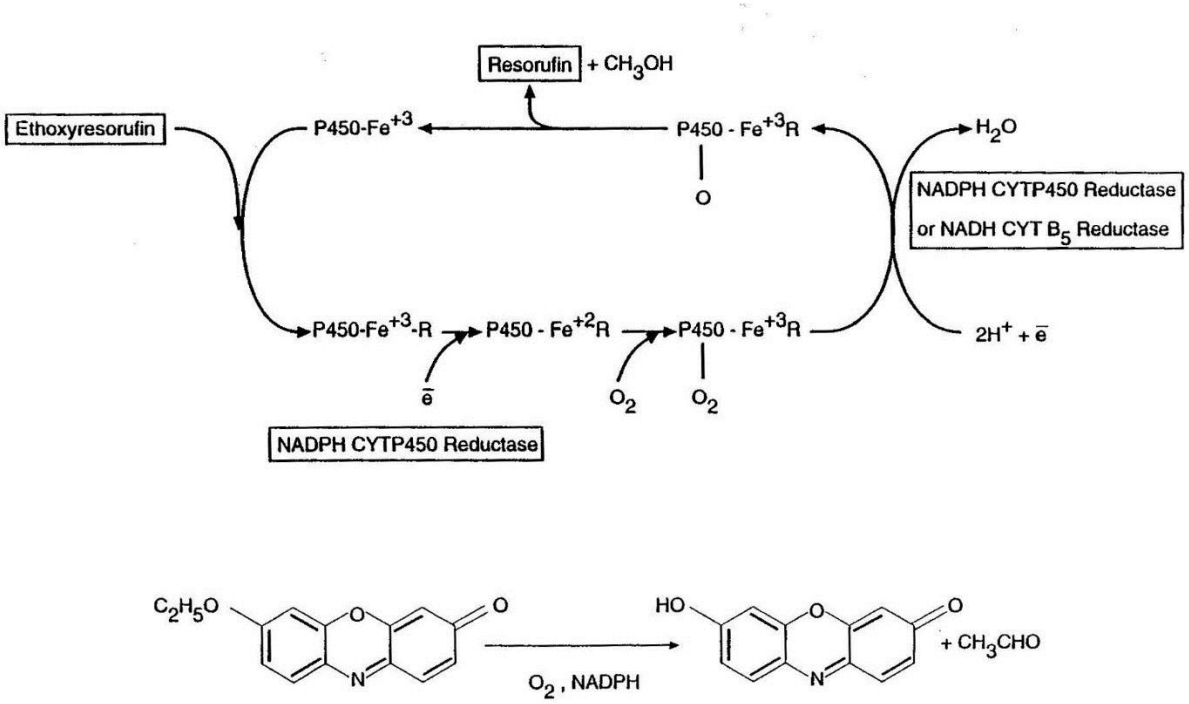

Figure 1. The microsomal 7-ethoxyresorufin O-deethylase (EROD) reaction

2 Protocols

2.1 General applicability of the method

This method has been written specifically for the dab (*Limanda limanda* L.). This species has been selected because it is the preferred species for monitoring offshore under the Oslo and Paris Commissions' Joint Assessment and Monitoring Programme (JAMP). The method is equally appropriate for other flatfish species such as the flounder (*Platichthys flesus* L.) or plaice (*Pleuronectes platessa* L.) and is used for gadoids in monitoring by some ICES Member States. Reaction conditions for these species should be checked before the method is applied universally since it has been reported (Munkittrick *et al*., 1993) that suboptimal conditions (e.g. inadequate 7-ethoxyresorufin concentrations) have been used by some workers when methods have been transposed from one species to another without validating the conditions. Within any particular national or international programme, identical methodology should be used. However, this does not preclude studies on other species, in different geographical regions with slightly different methods. Variations in method are tolerable with regard to the buffers used, homogenization, normalization processes, method of freezing, and type of assay as long as they are standardized in any particular programme.

2.2 Fish sampling

Many intrinsic and extrinsic factors affect EROD activity in the liver of fish. Differences between sexes and the effect of seasonal variation due to differences in the state of sexual maturation are well documented (Edwards *et al*., 1988; Förlin and Haux, 1990; Goksøyr *et al*., 1992; Larsen *et al*., 1992). EROD is a temperature-dependent reaction which is carried out at a specified temperature (usually 20 °C) but because of the adaptation of fish to ambient temperatures, strong correlations between EROD activity and environmental temperature, independent of the assay temperature, have been observed (e.g. off the Dutch coast (Sleiderink *et al*., 1995)). Therefore, the water temperature at the time of sampling must be recorded and the sampling programme designed to either avoid or account for the temperature effect. Disease and associated liver damage may suppress EROD activity; for example, fish with overt signs of lesions can have a reduced level of EROD activity (see Figure 2).

Inhibition or inactivation of EROD activity has been reported at high concentrations of some contaminants, particularly heavy metals and some PCB congeners (e.g. George, 1989; Skaare *et al*., 1991). In some cases, there have also been reports of enhanced activity following exposure to certain combinations of contaminants (e.g. aromatic hydrocarbons and resin acids (Croce *et al*., 1995)).

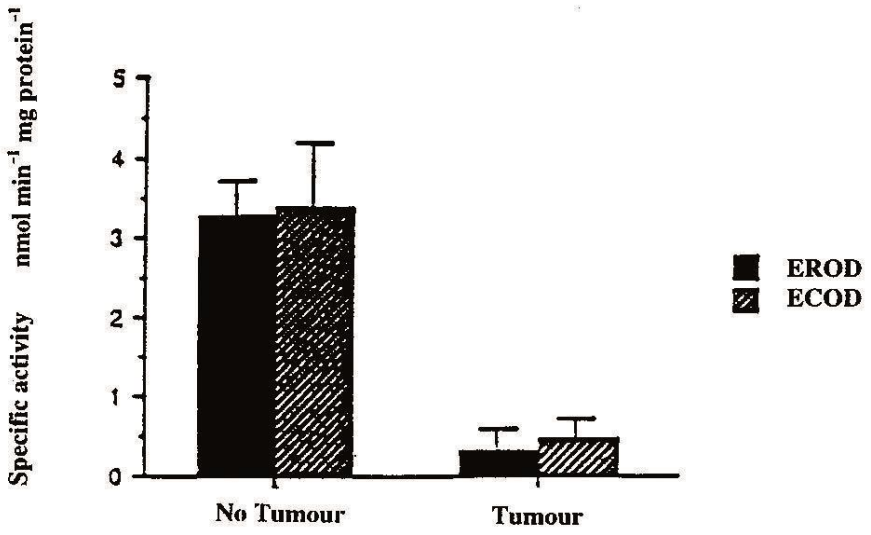

Figure 2. Specific activity (nmol min^{-1} mg protein^{-1}) of 7-ethoxyresorufin *O*-deethylase (EROD) and 7-ethoxycoumarin *O*-deethylase (ECOD) in livers of female dab (*Limanda limanda*) with and without overt hepatic tumours. (From Stagg *et al.*, 1995)

Sampling strategies should attempt to take account of these factors in any monitoring programme, and the following guidelines should be applied to the sampling:

1. Induction is usually higher in male fish and therefore separate analyses should be reported for males and females, or only males analysed.
2. Gonad-somatic index (GSI, 100 × weight of gonad/weight of fish) should be calculated and reported as a measure of the state of maturity.
3. Animals should not be collected during or immediately before or after the spawning or migration periods.
4. The bottom temperature (for flatfish) should be measured at the time of capture of the fish and reported for each group of fish analysed.
5. Healthy fish with no external signs of disease should be sampled.
6. The sample size should be selected to give a coefficient of variation in EROD activity that is less than 10 % of the mean from the log-transformed data. Normally this would require a minimum of ten individuals of each sex at every station.
7. The length of the fish should be within the size range 20–24 cm for dab, 25–29 cm for flounder, if possible.
8. A reference population from a pristine, uncontaminated environment should be used.
9. If negative results are found, the use of western blots or ELISA techniques is recommended to check that the lack of response is not due to de-activation of the enzyme.

2.3 Dissection

Samples of live fish should be obtained and each fish should be processed, within 5 minutes of killing, up to the point where the sample is cryopreserved or homogenized. The liver is removed by dissection following opening of the peritoneal cavity (most conveniently from the blind side of flatfish species). There is some evidence that biliary metabolites may inhibit EROD activity and, therefore, the liver should be dissected free without contaminating the tissue with bile (Hodson *et al.*, 1991). Since the liver is a

heterogeneous tissue, it is also prudent to standardize on the lobe or portion of the lobe removed for assay. Care should be taken to ensure that the tissue is kept cold at all times during dissection. Cryopreserved samples should be freeze-clamped in liquid nitrogen and either kept in liquid nitrogen or, more conveniently, transferred to a –70 °C freezer.

2.4 Homogenization and fraction preparation

All procedures should be carried out at 0–4 °C. A small piece (250+/-20 mg) of fresh or cryopreserved liver is homogenized on ice in 1 ml homogenizing buffer (Table 1) using a Potter-Elvehjem homogenizer at 700 rpm. Dithiothreitol (DTT) is unstable once in solution, meaning that homogenizing buffer should be made up daily or frozen in batches and defrosted on the day of use.

The homogenates should be transferred to polyethylene microfuge tubes and centrifuged at 10,000 × **g** for 20 minutes at 4 °C. An aliquot of the supernatant is then aspirated and transferred to a fresh Eppendorf tube, kept on ice and either assayed immediately or after storage at below –70 °C. Care should be taken not to aspirate the fat layer overlying the supernatant, which is seen in some samples. These S9 supernatants should contain approximately 5–20 mg protein ml^{-1}.

Table 1. Homogenizing buffer for the determination of EROD activity.

Reagent	Concentration
K_2HPO_4/KH_2PO_4 (pH 7.4)	100 mM
EDTA	1 mM
Dithiothreitol (DTT)	1 mM
KCl	150 mM

Should analysis on microsomal fractions be required, S9 fractions are decanted into balanced ultracentrifuge tubes and further centrifuged at 100,000 x g for 90 minutes at 4 °C. The cytosolic fraction is decanted and discarded and the remaining microsomal pellet is resuspended in cold fresh resuspension buffer (Table 2) using gentle working of a small hand-held Teflon pestle in the tube on ice until a homogeneous suspension is obtained. Microsomal fractions can then be analysed or aliquoted into cryovials for snap freezing in liquid nitrogen and storage at below -70 °C prior to analysis.

Table 2. Resuspension buffer for the determination of EROD activity.

Reagent	Concentration
K_2HPO_4/KH_2PO_4 (pH 7.4)	100 mM
EDTA	1 mM
Dithiothreitol (DTT)	1 mM
KCl	150 mM
Glycerol	20% (v/v)

2.5 Assay conditions

The reaction rate will be determined by the conditions under which the assays are carried out and will be affected by factors such as: pH, ionic strength and type of buffer; the concentrations of enzyme, substrate and co-factors; and the temperature of the assay. For most temperate fish species, including dab, the temperature optima are usually between 20°C and 25 C and the pH optimum is 7.4. The effects of changes in the concentrations of NADPH and 7-ethoxyresorufin on EROD activity in liver from the dab are shown in Figure 3. The data show that in both induced and non-induced fish, concentrations of NADPH and 7-ethoxyresorufin of 0.25 mmol l^{-1} and 2.0 µmol l^{-1}, respectively, give maximal activity.

A temperature-controlled (20 C) cuvette should be used with continuous stirring using a small magnetic flea. The assays should be performed in a 2 ml reaction volume comprising: 1.96 ml of buffer to give a final concentration of 100 mM K_2HPO_4/KH_2PO_4 (pH 7.4) and 150 mM KCl, 10 µl of 7-ethoxyresorufin in DMSO (dimethylsulphoxide) or water free methanol to give a final concentration of 2 µM, and 20 µl of S9 supernatant or microsomes (see Table 2). NADPH (10 µl) in distilled water, to give a final concentration of 0.25 mM, is added to start the reaction and the rate of resorufin production is measured fluorimetrically over the linear part of the response curve. NADPH is prepared fresh daily.

To measure true reaction rates, the instrument sensitivity should be set to give a full-scale deflection of approximately 1.0–1.5 nmol resorufin and reaction rates measured corresponding to resorufin production up to a maximum of 0.25–0.5 nmol min^{-1}. Higher reaction rates regularly exhibit non-linear kinetics and/or result in underestimation of the true reaction rate, especially in induced animals (see below). In such cases, it is necessary to dilute the S9 supernatant in a homogenizing buffer until an appropriate reaction rate is achieved. Due to the quenching effect of variable amounts of protein, the assay is calibrated internally by the addition of an appropriate amount of resorufin in 10 µl of phosphate buffer. Typically, this would be 0.125 nmol but may vary depending on the instrument settings required for any particular sample. Fluorescence of resorufin should be measured by excitation at 535 nm and recording the emission at 580 nm.

Stock solutions of both 7-ethoxyresorufin and resorufin should be made by dissolving weighed amounts of crystalline solid in DMSO or water free methanol and then stored at 4 C in the dark. Working solutions of these stocks should be prepared fresh daily by serial dilution into either DMSO or 0.1 M phosphate buffer (pH 8.0) for 7-ethoxyresorufin and resorufin, respectively. Both resorufin and 7-ethoxyresorufin degrade rapidly on exposure to light. These compounds should therefore be stored in the dark and protected from strong light during use. The preparation of standards and solutions should be carried out in subdued light.

Table 3. Reagent volumes and concentrations used in the determination of 7-ethoxyresorufin *O*-deethylase (EROD) activity in dab (*Limanda limanda*) liver.

Reagent	Reagent concentration	Volume	Final concentration
Buffer	102.0 mM	1.96 ml	100 mM
7-ethoxyresorufin*	0.4 mM	10 µl	2.0 µM
NADPH	100.0 mM	10 µl	0.25 mM
S9 / microsomal fraction	5–10 mg protein ml^{-1}	20 µl	50–100 µg ml^{-1}

*Dissolved in DMSO

2.6 Purity of resorufin standards

The purity of commercially obtained resorufin varies widely depending on source, batch and storage conditions (Munkittrick *et al*., 1993; Stagg and Addison, 1994). Resorufin is also unstable and since the resorufin solution is the primary standard in the assay the concentration of the standard must be verified before use. This is preferably done each day by preparing the standard to a concentration that is determined using the extinction co-efficient of resorufin and then preparing the working calibration standard(s) from this. Pure resorufin has an absorption maximum of 572 nm at pH 8.0 in 0.1 M phosphate buffer with a molar absorbance of 73.2 mM^{-1} cm^{-1} (Klotz *et al*., 1984).

The following equation should be used:

$$A = \varepsilon CL$$

where A = the absorbance at 572 nm

ε = the extinction coefficient (0.0732 μM^{-1} cm^{-1} in 0.1M phosphate buffer, pH = 8.0)

C = the concentration (μM)

L = the path length (cm).

For example, for a 25 μM working solution of resorufin, the absorbance A_{572} in a 1 cm cuvette should be 1.83 ± 0.04. The absorbance of the actual stock solutions (in 0.1 M phosphate buffer at pH 8.0) used to calibrate the assays should therefore be measured each day at 572 nm, and the working calibration standard solution(s) prepared fresh taking this in to account. The molar absorbance in mM^{-1} cm^{-1} in 0.1M phosphate buffer (pH 8.0) should also be reported with the EROD measurements.

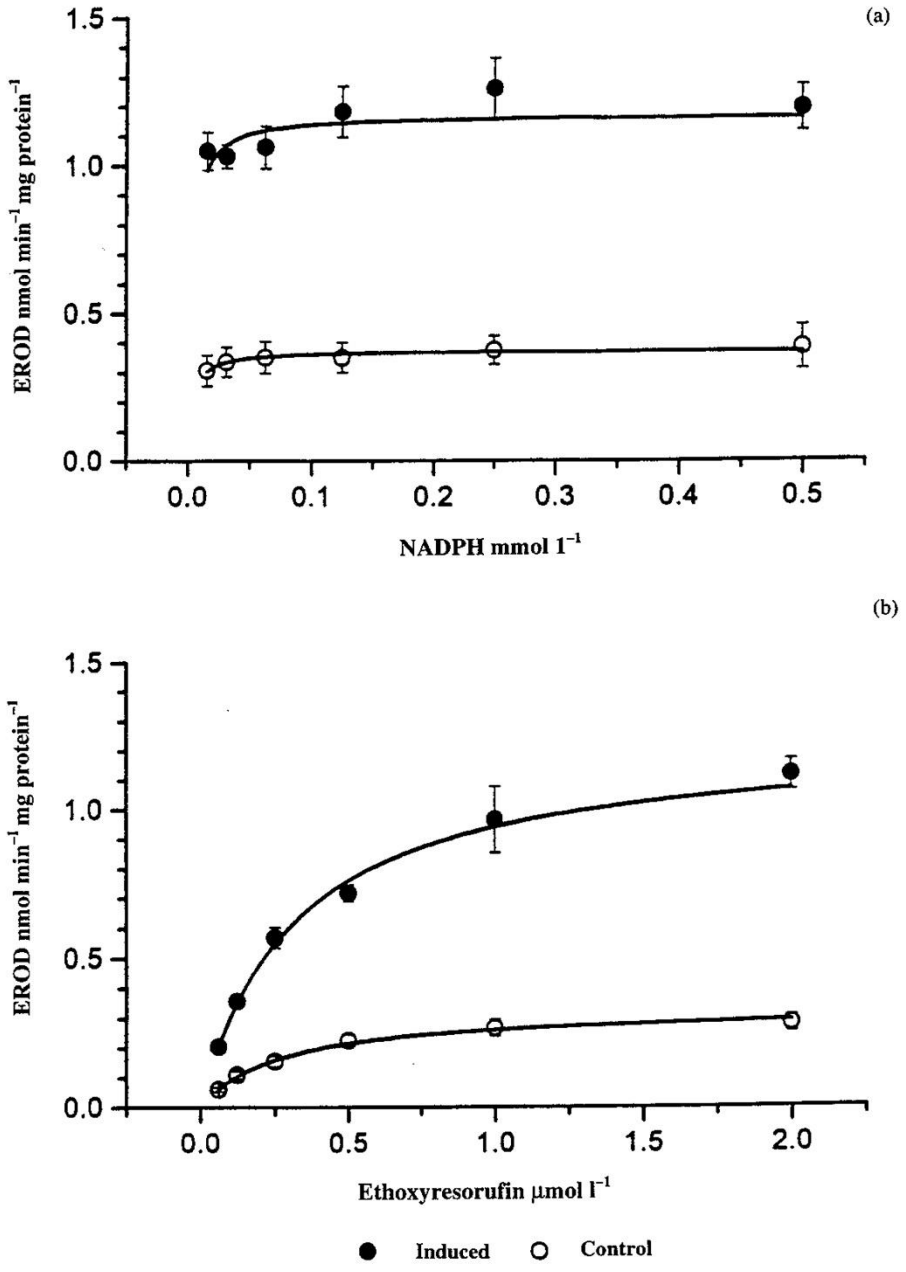

Figure 3. The dependence of hepatic 7-ethoxyresorufin *O*-deethylase (EROD) activity in induced (100 mg kg^{-1} β-naphthoflavone, intraperitoneal injection) and control dab (*Limanda limanda*) on the concentration of (a) NADPH and (b) 7-ethoxyresorufin in the reaction medium.

2.7 Normalization of enzyme activity to protein and wet weight of tissue

Enzyme activity can be expressed relative either to the protein content of the S9 / microsomal fraction or to the wet weight of the tissue used in the reaction (i.e. per mg protein or per gramme wet weight tissue). The latter has advantages in that it eliminates the possibility of a change in EROD activity due to changes in unrelated background protein. However, the argument for using protein is that in some monitoring situations, particularly when carrying out EROD measurements at sea, it is not possible to weigh accurately small pieces of tissue. Both Munkittrick *et al*. (1993) and Stagg and Addison (1994) demonstrated that protein determination was a major source of differences in the EROD values reported from different laboratories. Variation can be due to differences in the method or standards used and it is, therefore, imperative that a standard protein assay is used. The most widely used traditional protein determination is the Lowry assay (Lowry *et al*., 1951), but many workers now use the Coomassie blue dye-binding method (Bradford, 1976). However, the latter method has poor linearity, is prone to interference, and is also markedly affected by the standards used. Modified protein assays, based on the method by Lowry *et al*. (1951), are available (e.g. BioRad DC assay, BioRad Laboratories). These assays are fast, have much improved linearity compared to the Bradford method, and are suitable for use with 96-well microplates. These methods should be used with bovine serum albumin (BSA) as a standard.

S9 supernatants or microsomal fractions are diluted in fresh homogenizing or resuspension buffer respectively to give an expected protein concentration in the range 0.5 to 1.0 mg ml^{-1} protein. Ten µl of samples or standards prepared in fresh homogenizing or resuspension buffer (0.2–1.6 mg ml^{-1} BSA) are pipetted into a microtitre plate, 25 µl of alkaline copper tartrate reagent is added to each, followed by 200 µl Folin reagent (BioRad Laboratories). The plate is mixed and the absorbance read at 650 nm after 20-25 minutes incubation.

It has been noted at the authors' institute that because DTT is unstable in solution and affects the modified Lowry protein assay (matrix matching the samples and standards accounts for this effect), homogenizing and resuspension buffers should always be used fresh or freshly defrosted when preparing protein samples and standards for assay.

2.8 Expression of results

The output of a typical EROD assay is shown in Figure 4A and the method of determining the rate of resorufin production is presented in Figure 4B. In an active sample, the amount of fluorescence increases after the addition of NADPH, but often the rate decreases with time. The rate measured should be as close as possible to the initial linear rate (a) shown in Figure 4B. The amount of resorufin produced over time is computed from the rate of increase in fluorescence divided by the increase in fluorescence produced by the addition of a known standard amount of resorufin (b) in Figure 4B. Occasionally, traces are produced in which the fluorescence dips immediately after the addition of NADPH (Figure 4C). In such cases, the trace should be read on the linear part of the curve. In measurements with S9 supernatants, a rate may also be observed prior to the addition of NADPH (Figure 4D) which is most likely due to the presence of endogenous NADPH in the sample.

Results should be presented normalized to the protein content of the S9 fraction and reported as nanomoles or picomoles of resorufin liberated for one minute per mg of protein (nmol min^{-1} mg $protein^{-1}$).

2.9 Sources of error

The following sources of error have been identified and should be taken into account:

1) Sample integrity

- contamination of the sample by bile salts during dissection;
- deterioration during storage or processing owing to an increase in temperature;
- deterioration due to long-term storage.

2) Sample preparation

- changes in the speed and duration of centrifugation;
- variation in type of homogenizer and the speed and duration of homogenization;
- preparation of fractions other than an S9 supernatant.

3) Assay conditions

- deviation from assay conditions (temperature, pH and substrate concentrations);
- deterioration of substrate and co-factors;
- deterioration of resorufin standards;
- differences in the purity of the resorufin;
- protein determination (method, standards used, etc.);
- graphical errors in the interpretation of fluorescence data.

A) Typical EROD trace showing the increase in fluorescence with time and the addition (τ) of an aliquot of resorufin to calibrate the assay.

B) The same trace as in (A) to show the rate measurement (a) and the calibrant spike (b) used to compute the rate of resorufin production.

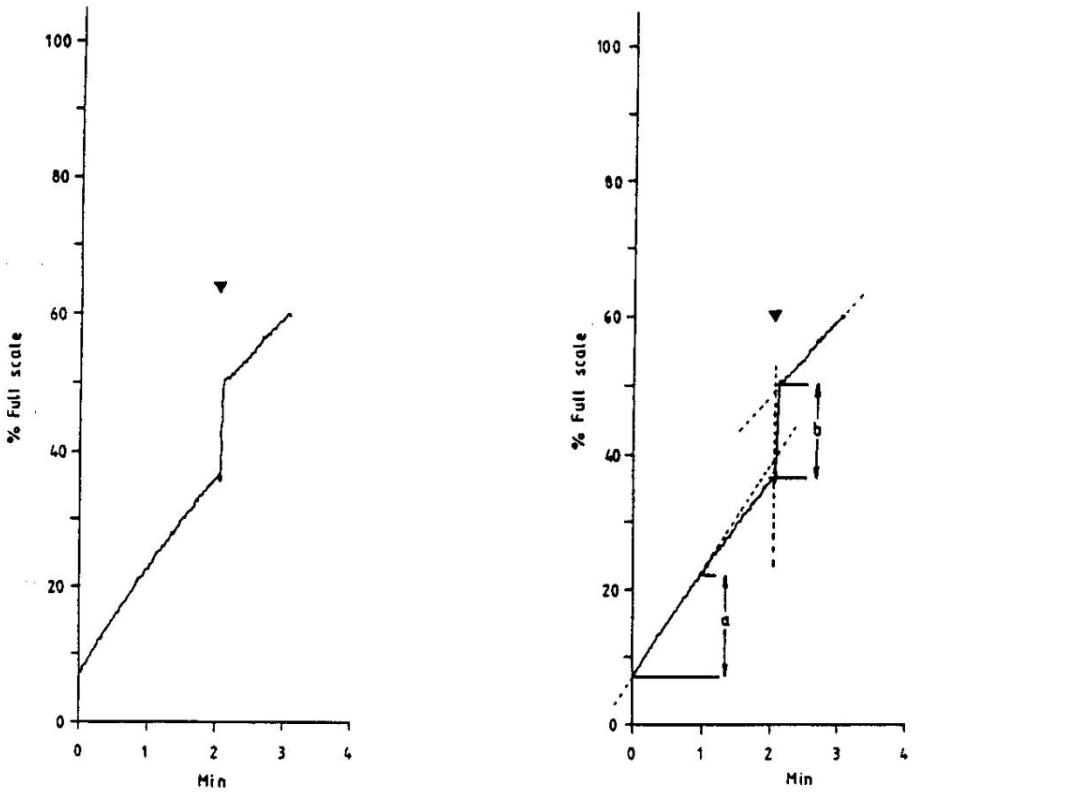

Figure 4. Fluorescent output during hepatic 7-ethoxyresorufin *O*-deethylase EROD assays from the dab (*Limanda limanda*).

C) A typical trace demonstrating an initial decline in fluorescence values after the addition of NADPH to start the reaction. Resorufin is added at the arrow (τ).

D) Trace to show the effects of endogenous NADPH after the addition of 7-ethoxyresorufin at arrow (a) and resorufin at arrow (b).

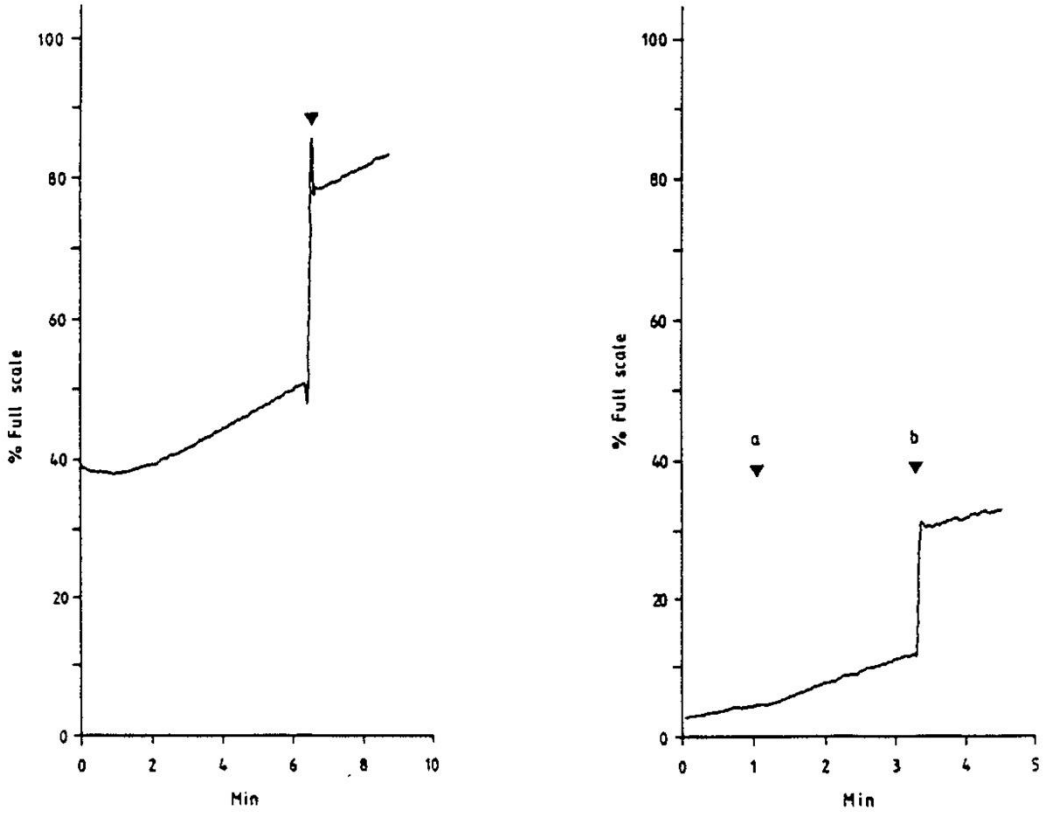

Figure 4 continued.

2.10 Quality control

Laboratories should build appropriate quality control into their measurements; the use of laboratory reference materials and the maintenance of quality control charts for both protein and EROD assays is recommended. Accreditation and participation in external ring-trials and proficiency testing is encouraged. The following sources of error are particularly relevant to the measurement of EROD activity and appropriate steps to control these errors should be taken.

1. Instrument variation

   Equipment (especially balance, pipettes, fluorometer, absorbance spectrometer), should be serviced and calibrated at least annually and performance checked daily.

2. Resorufin standards

   The molar absorbance of the working solutions used to make up the standards should be reported with each batch of assays performed.

3. Laboratory reference materials

   An internal laboratory reference material should be analysed at least once per day. This should consist of homogenous aliquots that are thawed and assayed once only. They may be a stock of microsomal or S9 fractions from one or more induced fish (e.g. one β-naphthoflavone-induced salmon, or several induced dab that are homogenized together and aliquoted).

3 Acknowledgements

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