ORIGINAL ARTICLE

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Sources of measurement error in assays of EROD activity of fish for biological effects monitoring

Abstract The paper describes the technical, procedural and biological aspects of 7-ethoxyresorufin O-deethylase (EROD) activity measurements in dab, *Limanda limanda*, from the North Sea. Several sources of measurement error in assays of EROD activity measurements for biological effects monitoring exercises could be defined, particularly for assay conditions, sample storage and sampling. In addition to variability caused by technical errors, biological features inherent in the surveyed fish may lead to even more severe errors in EROD activity measurements. This is particularly true of the choice of sampling time. Disregarding the species-specific spawning periods may lead to a loss of activity by a factor of as much as 90 in females and 40 in males, thus making it necessary to design sampling surveys appropriately.

Key words EROD activity · Dab · Measurement error · Biological effects monitoring

Introduction

Ever since pollution was recognised as a major threat to the marine environment, national and international programmes have been initiated to monitor its development, with the objective of eventually controlling and reduce the input of xenobiotic substances into marine ecosystems (Akkerman et al. 1990). Starting with the chemical and the biological monitoring in the 1970s, which determined pollutants in water, sediments and biological matrices, the introduction of the concept of 'biological effects monitoring' opened up a new dimension in pollution abatement thinking (see Stebbing et al. 1992a). Aside from the mere analytical investigation of xenobiotics in conventional monitoring, biological effects monitoring is concerned with the effects of these xenobiotics

on the biota. These effects may be manifested at different organisational levels of the ecosystem, such as on a subcellular level, at the level of organs, individuals, populations or even at the ecosystem level.

In the framework of the approach to protect the marine environment, chemical analysis is but one option to demonstrate pollution. Assessing the ecological significance of the multitude of xenobiotics on marine biota is another possible way to demonstrate the deleterious effects of pollutants to the public and to decision-makers. It is for this reason that the biological effects monitoring approach in the future will take a central position in the concept of pollution abatement and environmental monitoring.

In order to be able to describe structure and function of an ecosystem adequately (Schaeffer et al. 1988; Stebbing et al. 1992b), or only one aspect thereof such as biodiversity (Noss 1990), a suite of indicators would have to be used. However, for pragmatic purposes in regional monitoring only a few but effective indicators are employed (Johnson 1988; Hunsaker and Carpenter 1990), as was the case in the execution of the Monitoring Master Plan for the North Sea, when establishing the 1993 Quality Status Report of the North Sea. Thus, aside from fish diseases, benthos studies and the oyster-embryo essay, changes in the liver-microsomal enzymes (7-ethoxyresorufin-O-deethylase; EROD) has been proposed as a biomarker since it has frequently been used to describe the exposure of fish to organic pollutants (e.g. Payne et al. 1987; Achazi 1989; Eggens and Galgani 1992; Goksøyr and Förlin 1992; Sleiderink and Boon 1995). The pollutant-induced activity increase in the enzymatic biotransformation system is thought to be particularly well suited to an assessment of organic pollutants such as the co-planar polychlorinated biphenyls (PCB), polyaromatic hydrocarbons (PAH), chlorinated dibenzodioxines (CDD) and dibenzofurans (CDF).

Today, a large body of information on the reaction of mixed function oxygenases in fish towards organic pollutants is available. Because of their application to environmental monitoring, protocols and interlaboratory comparisons have been established to reduce sources of

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variability (Hodson et al. 1991; Munkittrick et al. 1993; Pluta 1995; Stagg and Addison 1995; Stagg and McIntosh 1998). Despite existing protocols in which common laboratory procedures were agreed upon, other important sources of variability remain.

Quality control is of particular importance for the validation of results that may find themselves used by decision-makers for management purposes in environmental issues. An important issue to be considered in this respect is the fact that in most cases biological material sampled in the field has to be stored for different periods of time before EROD activity can be measured in the laboratory. As the hemoprotein cytochrome P-450 (the terminal oxidase enzyme responsible for the biochemical reaction) is a membrane-bound enzyme, the conservation procedure may drastically affect its activity (Monod and Vindimian 1991; Stagg and McIntosh 1998). Thus, in the following, while applying the protocol outlined by Hodson et al. (1991), some procedural aspects of the method have been scrutinised under various hypothetical aspects of differing laboratory performances. In particular, errors caused by storage of chemicals and biological material have been identified. These will be outlined in the following, and their degree of deviation from an optimal procedure discussed. Any procedure that caused a weakening of the analytical signal was considered to be a methodical disadvantage.

Material and methods

All measurements were conducted with liver material obtained from male and female dab, *Limanda limanda,* and flounder, *Platichthys flesus,* caught in the southern North Sea during the years 1991–1994, as well as some laboratory-reared turbot. Female dab were generally larger than 22.5±2.6 cm mean total length. Male fish had a mean total length of at least 20.3±1.5 cm. Flounders measured between 18.0 and 25.0 cm total length in both sexes (for detailed information on biological variables see Krüner et al. 1996). The fish were caught on a 100-nautical-mile transect leaving the Elbe estuary in north-west direction, where fish were sampled using a bottom trawl. Duration of trawling was 15 min. Immediately after capture fish were sacrificed by a blow to the head and livers were removed by careful dissection without damaging the gallbladder. Samples were immediately shock frozen in 2-ml cryo-tubes in liquid nitrogen and stored for up to 100 days for later determination of the EROD activity.

EROD activity was measured after the method described by Burke and Mayer (1974), altered after Andersson et al. (1985). Between 0.3 and 0.75 g of liver tissue was homogenised in four volumes of 0.1 M sodium phosphate buffer including 0.15 M potassium chloride (pH 7.4). Homogenisation was carried out using a Potter-Elvehjem glass and Teflon homogeniser. The homogenate was centrifuged at 10000 g for 20 min at 4°C. The supernatant was decanted completely and its volume determined. For total protein determination it was necessary to take 200 µl from the supernatant.

The rest of the supernatant was then centrifuged at 105000 g for 60 min in a cooled (4°C) ultracentrifuge. After this procedure, the second supernatant was decanted and the resulting microsomal pellet was suspended in 1.0 ml 20% glycerine phosphate buffer (0.1 M) where the total volume of the pellet was determined. MFO determination was conducted within 3 h. The same sample also provided material for the determination of microsomal protein. During the handling before measuring mixed-function-oxygenase (MFO) activity, storage on ice is necessary. If the determination of

MFO activity was not possible within the 2 h time span, samples were stored in 1 ml cryo-tubes in liquid nitrogen.

Artificial substrates such as 7-ethoxyresorufin were metabolised in a biochemical reaction to the first intermediate product resorufin. Resorufin was measured with a spectrofluorometer (Shimadzu RF 5001 PC; excitation at 530 nm, recording at 586 nm) with a temperature-controlled stirrer cuvette (Burke and Mayer 1974). The measured fluorescence corresponded to the amount of resorufin produced by the enzymatic reaction. This was equivalent to the crude MFO activity of the sample measured as production of resorufin per minute. Total and microsomal protein content were determined according to Lowry et al. (1951) with an autoanalyser (Technicon, TRAACS 800) using bovine serum albumin as a standard.

The EROD assay was conducted in a 1-cm-quartz-glass cuvette, using the following amounts of reagents: 1840 µl phosphate buffer at pH 7.4; 100 µl substrate; 50 µl sample; 10 µl NADPH solution.

For the preparation of the 7-ethoxyresorufin stock solution, 1.0 mg substrate was dissolved in 83 ml DMSO (dimethylsulfoxide), to obtain a 50-µM solution. This stock solution may be stored at room temperature in the dark for more than 12 weeks. For measurement, 1-ml units were diluted with 4 ml buffer and stored before use at 4°C, protected from direct light. A freshly prepared mix of stock solution and buffer was needed every day.

For the preparation of the NADPH solution, 8 mg NADPH was dissolved in 1.0 ml buffer to yield a 1.0-mM solution and stored at 4°C, protected from direct light. A freshly prepared solution was needed for every day of measurements.

Results and discussion

For organisational reasons, measurements could not be conducted from identical livers throughout. Thus, at times, absolute values between experimental regimes appear different due to choice of different total numbers for the particular assays. In some species, i.e. turbot, livers are relatively large, so there was a choice of sample location. In preliminary experiments it could be confirmed that the expression of EROD activity in turbot liver was not organ location-specific.

Assay conditions and handling

According to the methodological procedures found in the literature (Burke and Mayer 1974; Andersson et al., 1985; Pluta 1995), the NADPH solution may be stored frozen (–20°C) for up to 1 month. Yet Stagg and McIntosh (1998) suggested that the NADPH solution be prepared fresh daily. We conducted experimental storage series with an NADPH solution and found that after storage for 24 h at 4°C no differences in activity could be detected. With increasing storage time of the NADPH solution the measured EROD activity decreased significantly. After 20 days of storage the measured activity approached zero. Thus, it is advisable to renew the NADPH solution every 24 h, in order to keep the measuring error low. Hodson et al. (1991) even suggested preparing NADPH solutions fresh immediately before use.

For the choice of substrate solvent two substances are generally used (Achazi et al. 1995), methanol and
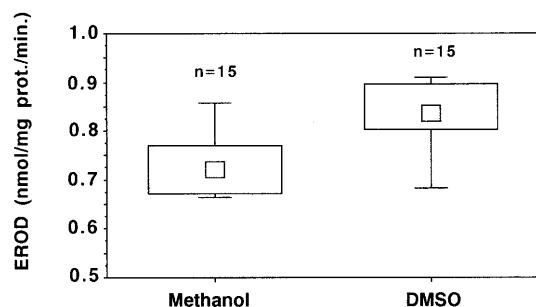
Fig. 1 EROD activity (medians) in livers of North Sea dab using two different substrate solvents. *Vertical bars* 95% confidence limits; *boxes* quartiles (25%, 75%) and median values; *n* number of samples

Fig. 2 a,b EROD activity (medians) in dab liver depending on substrate solvent and substrate amount added. **a** substrate dissolved in methanol; **b** substrate dissolved in DMSO. *Difference significant (*P*<0.05) compared to preceding measurement. *Vertical bars* 95% confidence limits; *boxes* quartiles (25%, 75%) and median values. Number of samples for each measurement was 13. Scales in **a** and **b** are not identical

DMSO, and only they were compared in the present study. We used one half of the same dab liver for each comparative measurement (*n*=15). The results indicate a significant (*P*<0.05) depression in EROD activity when the substrate was dissolved in methanol as compared to DMSO (Fig. 1). The median value from measurements made in DMSO was 34% higher than the corresponding value from the methanol measurements.

Since in some species the liver is relatively small and it is at times required to split livers (for instance, for residue analysis in combination with EROD expression), it was tested whether the amount of liver tissue used for analysis could possibly influence the measured EROD activity. This aspect was tested with livers from turbot, since the species has a sufficiently large liver to conduct repetitive experiments. For this purpose juvenile turbot (200 g) were injected intraperitoneally with β-naphtho-flavon (25 mg kg^{-1} wet wt.). In an initial test it was assured that there was no regional within-variation in EROD activity in individual turbot livers when analysing a proximal, middle and distal section of three different livers. Thus, different size pieces of liver tissue were stored shock-frozen in liquid nitrogen. In assays based on 0.3–0.7 g of liver tissue, no effects of the amount of tissue used were detectable in EROD activity. Whenever smaller pieces of tissue were used, activity dropped as low as 40% of the value measured in 0.7 g tissue. Thus, the minimum tissue amount should not be <0.3 g.

A similar effect is apparent when different amounts of dissolved substrate are used. Since the amount may vary between 50 and 300 µl, the signal is strongly influenced depending on the nature and amount of the substrate added. Figure 2 shows the kinetic of the EROD activity at variable substrate amounts, which shows an increase up to 75 µl and then, following the stabilisation in the sense of a Michaelis-Menten equation, a descent due to the increasing toxicity of the solvents. For both assays a maximum reaction is achieved at 75 µl. Above this, the toxic nature of the solvent is particularly noticeable in the methanol-assay (Fig. 2a), which was commented upon by Achazi et al. (1995) who said that the concentrations of either of the two solvents should not exceed 0.5–0.8% of the assay volume. At 300 µl the activity de-
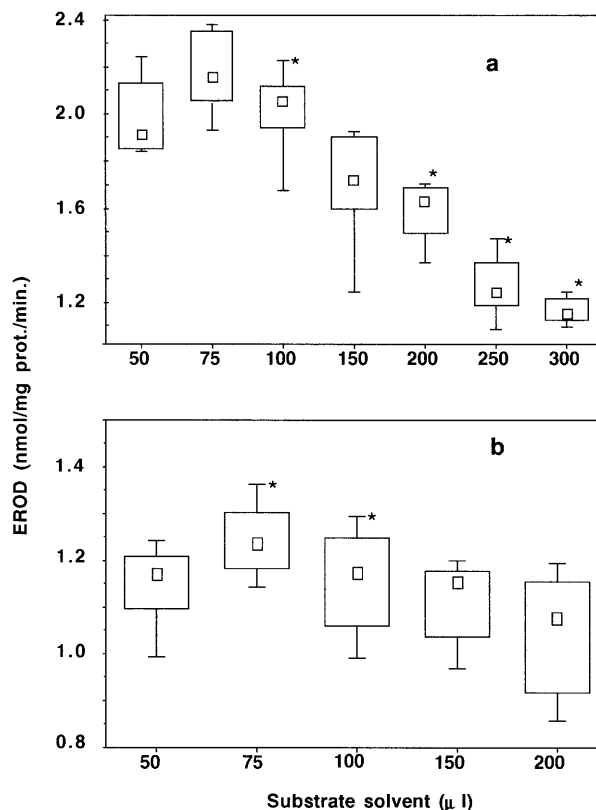
creased by almost 50%. In the DMSO assay solvent-dependent inhibition is less pronounced, but nevertheless, the activity at 200 µl is only 88% of that obtained when 75 µl substrate solvent is used.

Also, Lange (1996) reported a 20% higher EROD activity when DMSO was used as substrate solvent instead of methanol. Although generally suggested as the preferred solvent (see also Recommendations of the ICES/IOC intercalibration and preliminary protocol workshop on the determination of EROD activity in fish liver; Pluta 1995), other solvents such as acetone may even yield better results than DMSO, depending on the species analysed (M. Eggens, personal communication).

Optimum pH conditions for the expression of EROD activity in the two potential substrates were found between 7.5 and 7.6 in the methanol assay and fell off rapidly at pH >7.7 and <7.4. In DMSO, optimum conditions were located between 7.2 and 7.5 pH while activity declined at pH <7.6, with only 79% of the optimum reaction at pH 7.9 (Fig. 3).

In former calibration exercises a major source of variability resulted from the estimates of protein content (Munkittrick et al., 1993; Stagg and McIntosh 1998) when different techniques were used. In our own protein
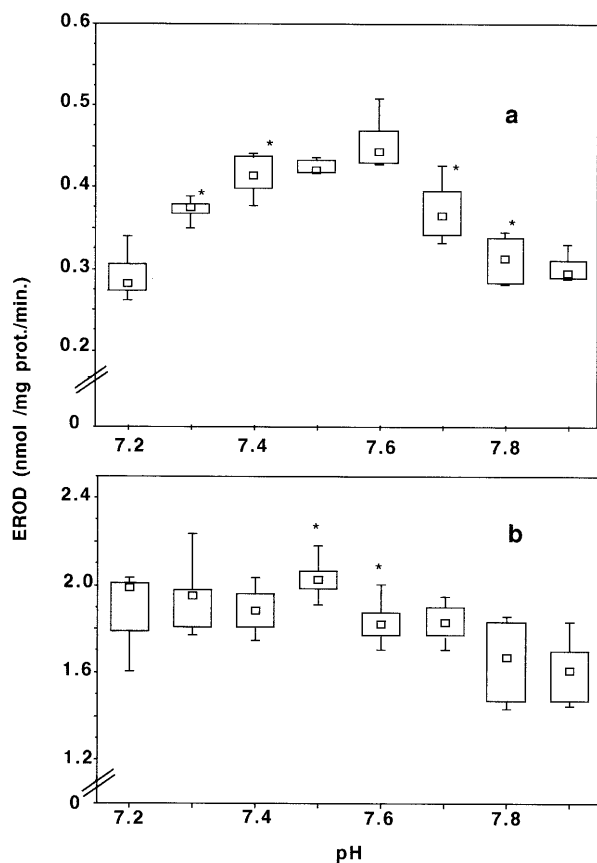
Fig. 3 a, b EROD activity (medians) in dab liver depending on pH of phosphate buffer. **a** Substrate dissolved in methanol; **b** substrate dissolved in DMSO. *Difference significant ($P<0.05$) compared to preceding measurement. *Vertical bars* 95% confidence limits; *boxes* quartiles (25%, 75%) and median values. Number of samples for each measurement was 8
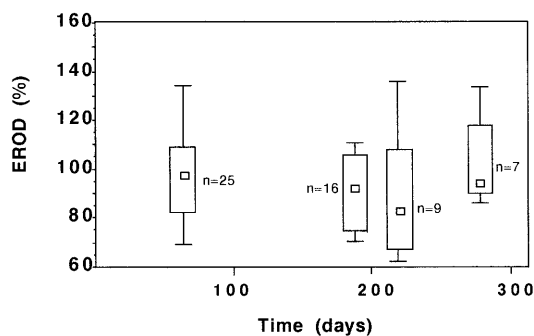

Fig. 4 EROD activity (%) in dab liver kept for extended periods of time in liquid nitrogen. Values from field-frozen measurements were considered 100%. *Vertical bars* 95% confidence limits; *boxes* quartiles (25%, 75%) and median values; *n* number of samples per period.
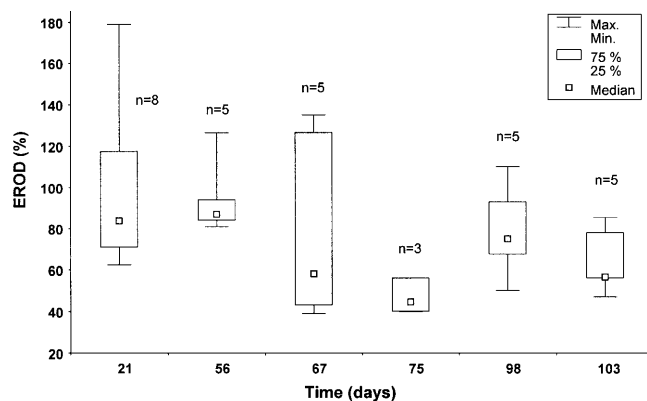

Fig. 5 Decrease in EROD activity (pmol resorufin s^{-1} in turbot liver during storage in liquid nitrogen and repetitive thawing for subsampling. Values from field-frozen first measurements were considered 100%. *n* Number of samples per period

determinations deviations from the mean were usually low (for $n=50$, 2.2%). Therefore, we do not consider protein determination as particularly crucial.

The 7-ethoxyresorufin stock solution did not lose activity compared to a freshly prepared solution even after storage of up to 12 weeks at room temperature. Newly prepared stock solutions when compared with 12-week-old ones yielded the same activities under assay conditions using identical substrate.

Storage of samples

In theory it is considered advantageous to use fresh livers for EROD activity measurement (Monod and Vindimian, 1991). In practice, this is not practicable since ultracentrifugation and spectrophotometry are not always possible on a research vessel. Given that during cruises large numbers of liver tissue samples may be obtained, storage of fresh livers in liquid nitrogen for extended periods of time becomes inevitable. In storage experiments, when pieces of liver tissue were shock-frozen in 2-ml cryo-tubes under liquid nitrogen for a period of

up to 283 days, no significant differences between the measured EROD activity of livers from the same individuals were detected, when samples had been kept deep frozen throughout the whole period (Fig. 4). This finding is in good agreement with what is known from other workers, who stored liver of white sucker (*Catostomus commersoni*) at –80°C for 6 months without losing EROD activity (Munkittrick et al. 1993).

In contrast, turbot liver tissue that had been frozen in liquid nitrogen as a whole and was slightly thawed each time a sample had to be sliced off for preparation lost activity continuously over a period of 100 days (Fig. 5), maintaining only 70% of its initial activity. It is for this reason that this kind of procedure (refreezing samples) should not be adopted (see also Hodson et al. 1991).

In view of the necessity of sample storage for extended periods of time also the microsomal fraction was submitted to a storage test in two different temperature regimes; at –80 and at –196°C in a glycerol/phosphate buffer mixture. Storage up to a period of 36 days did not cause any loss of activity under either temperature regime (Fig. 6). Yet, after 70 days at –196°C a significant loss of activity occurred, falling below 50% of the initial activity. The
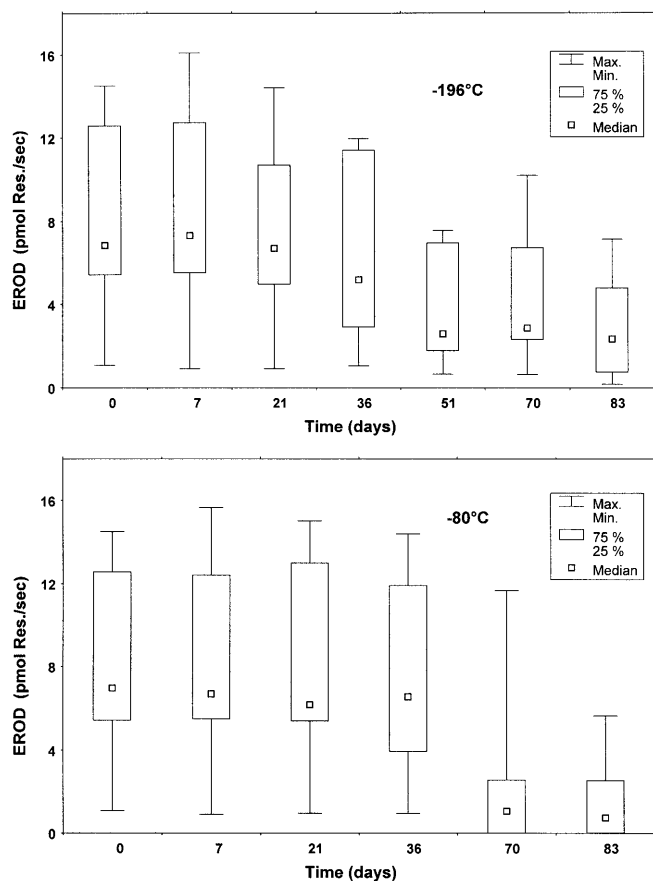
Fig. 6 Course of EROD activity (pmol resorufin s^{-1}) in microsomal fraction of dab livers stored at –80 and –196°C for different temperatures for different periods of time. EROD activity was not calculated on protein basis due to limited material for protein determination. Number of samples per period was 6

Fig. 7 EROD activity in mature dab from the Elbe estuary. *Vertical bars* Standard deviation; *n* number of sample

same trend was visible at –80°C, although the material was not sufficiently large to allow statistical analysis. The results are in accordance with the findings of Förlin and Andersson (1985) and Hodson et al. (1991) who were able to store liver microsomes of rainbow trout for extended periods of time without substantial loss of activity. Similarly, Danner-Rabovsky and Groseclose (1982) were able to store rat liver microsomes at –70°C for up to 2 weeks without major activity losses.

Effects of biological parameters

Compared to the technical errors, possible deviations from the real situation due to faulty biological sampling can be much more severe. As reported by Saborowski (1996) and Saborowski et al. (1995), sampling at the wrong time, for instance during the spawning period, may suppress the EROD signal by a factor of 90 in the case of female fish (sampled during the reproductive season) or 40 in the case of male fish (when sampled at or just before the onset of gonadal activity). A similar relationship between EROD activity and spawning period
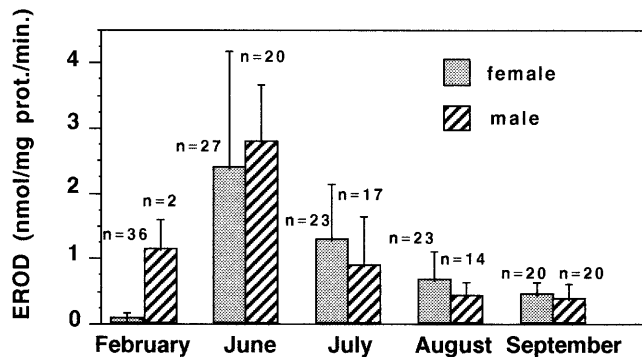
has been reported for various other fish species as well (Edwards et al. 1988; Galgani et al. 1991; Mathieu et al. 1991; Larsen et al. 1992). Another biological feature that may possibly influence the expression of EROD activity is the length of the fish, which ultimately works via the state of maturity and may thus be only a covariable.

The most abundant data as far as a North Sea species is concerned have been collected for dab, *Limanda limanda* (Lange et al. 1992; Sleiderink et al. 1993; Krüner and Westernhagen 1995; Sleiderink and Boon 1995; Sleiderink et al. 1995a,b; Lange 1996; Saborowski 1996; and others). Another North Sea species that has been relatively well investigated for its EROD activity is flounder, *Platichthys flesus* (see Eggens 1996); thus, a direct comparison as far as EROD activity expression is concerned may be best established for these species.

In dab, natural variation in EROD activity is large (male: factor 40; female: factor 90) and is predominantly sex-specific. Although the expression of EROD activity in both sexes follows a common pattern, male fish may at times display an EROD activity up to ten times higher than females under the same conditions (environment, season and physiological state) (Fig. 7). When comparing both species a different timing of the seasonally determined EROD activity is apparent. In dab, highest activities for both sexes are measured in June, while a strong suppression of activity in females is apparent in February during the reproductive season, when activities are as low as 0.05 nmol resorufin mg^{-1} microsomal protein min^{-1}. In male dab, lowest activity is expressed during August/September, with lowest values of 0.1 (nmol resorufin mg^{-1} microsomal protein min^{-1}). In flounder, the situation is different, following information provided by Eggens et al. (1995a) for flounder from the Dutch Wadden Sea. The data indicate that mean EROD activity in flounder is relatively stable over the year except for a clear peak occurring in January–February, after the spawning period and declining thereafter. In principle this behaviour parallels the situation in dab from the German Bight, which shows highest EROD activity in June. Given that the spawning period in dab is not as narrowly defined as it is for flounder and extends until May–June (see Cameron et al. 1997) the peak in EROD

activity in dab occurs also after the spawning period, declining thereafter and reaching particularly low values for females in spawning condition.

As already pointed out and as may be expected, there are also considerable species-specific differences in the height of the EROD activity (Krüner et al. 1996). Again, some of the better investigated species in this respect are dab and flounder. It is generally the case that whenever both species are sampled under the same conditions or at the same sites the expression of EROD activity in dab (males) is three to four times higher than in flounder (Westernhagen et al., this Vol.; Eggens et al. 1995b, 1996) and resembles that of plaice from the same sites. Therefore a direct comparison of absolute EROD activity in different species is not always meaningful, although general patterns should be the same, as evident from the seasonal expression of EROD activity in both species (Westernhagen et al. this Vol.). In some species (mullet, dab) though, ranges may be very similar (Beliaeff and Burgeot 1997).

Discussion

As laid out above, several independent factors could influence the generation of EROD activity data in a measuring programme, even when an agreed-upon protocol (Hodson et al., 1991) was applied. Most factors were merely technical, that is, they depended on the measuring laboratory. However, variability still reached the factor seven. In addition, the more important causes of variability were biologically defined, causing a considerable difference in the expression of EROD activity, which was rather independent of contamination (up to the factor 90 in females and 40 in males), and depended on the sex and the sampling season. Since both types of variability act independently of each other, their influence may be either additive or diminutive, causing potentially considerable error. Therefore we consider it of rather great importance for a measuring programme to keep both sources of variability in mind in the design and conduction of a monitoring programme that employs EROD activity measurements. This observation was also confirmed by Lange (1996) who noticed, that fish used during his studies and by Saborowski (1996), although from the same origin (same haul), yielded different EROD activity values when determination was conducted independently. From the present investigation it appeared that for both species, dab and flounder, June was the best month to sample for EROD activity determination in the framework of a monitoring programme.

Due to the higher expression of EROD activity in dab, this species lends itself better to marine monitoring purposes than flounder and, given its wide distribution and high abundance, is also better suited than plaice or turbot. For inner estuarine conditions, flounder will be the obligatory target species since neither dab nor plaice occur at such low salinity, as stated already by Eggens (1996).

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Communicated by A. Diamant