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Hepatic microsomal mono-oxygenase activity and P450IA mRNA in North Sea dab *Limanda limanda* from contaminated sites

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ABSTRACT: Mature pre-spawning female dab *Limanda limanda* were sampled during the Bremerhaven Workshop from stations in the southern North Sea along a transect heading approximately NW from the Elbe estuary (the 'German Bight Transect') and around an abandoned drilling site. Hepatic microsomal mono-oxygenase activity as shown by ethoxyresorufin O-deethylase (EROD) and cyanoethoxycoumarin O-deethylase (CN-ECOD) declined with increasing distance offshore, but rose slightly at the outermost station on the Dogger Bank. Microsomal P4501A1 mRNA, detected with a new synthetic oligonucleotide probe, followed the same trend. Activities of EROD and CN-ECOD and concentrations of P4501A1 mRNA were well correlated with the major chlorobiphenyl residues measured in dab and with hydrocarbon concentrations in sediments at these stations. Dab sampled from the drilling site had hepatic EROD and CN-ECOD activity and P4501A1 mRNA contents similar to those in fish from the offshore German Bight stations.

INTRODUCTION

Hepatic mono-oxygenase (mixed function oxidase: MFO) induction in fish is being used increasingly to show the presence and effects of certain organic contaminants. Addison & Payne (1986) have reviewed the earlier literature on the subject, and recent marine or estuarine studies are described by Addison & Edwards (1988), Stegeman et al. (1988, 1990) and Sulaiman et al. (1991). The rationale for measuring MFO induction is the well-defined cause-effect relationship established in laboratory and field studies: environmental contaminants accumulated by the fish are transported to the liver, where they interact with a cytosolic receptor; this leads to production of a mRNA which is translated to cytochrome P4501A1 which in turn (with appropriate co-factors) catalyses the MFO reactions.

The usual approach to measuring MFO activity is to measure catalytic activity, most commonly ethoxyresorufin O-deethylase (EROD) which is catalysed quite specifically by P4501A1. Measurement of P450s with immunochemical probes is becoming increasingly popular (e.g. Stegeman et al. 1988, Goksøyr et al. 1992) although only a few appropriate probes are available commercially. Cytochrome P4501A1 mRNA has been measured (in both experimental and field studies) using naturally derived cDNA probes (Haasch et al. 1989, Wirgin et al. 1991). In this paper, we describe hepatic MFO activity in dab *Limanda limanda* from the German Bight and drilling site transects described elsewhere (Stebbing & Dethlefsen 1992). Using the conventional catalytic measurement of EROD as a reference, we have examined the use of a synthetic oligonucleotide cDNA probe for P4501A1 mRNA, and also a potentially sensitive catalytic index, that of cyanoethoxycoumarin O-deethylase (CN-ECOD: White 1988).

MATERIALS AND METHODS

Dab *Limanda limanda* were sampled from German Bight Stns 3 to 9 during short cruises in March 1990 by the RV 'Viktor Hensen' and were brought back live to Bremerhaven, usually within 2 d of capture. Weights, length and sex were recorded and livers were removed for analysis. Microsomes were prepared essentially as described elsewhere (Addison & Edwards 1988) but using a bench-top ultracentrifuge (Beckman Instruments). Activity of EROD (Burke & Mayer 1974) was determined as described by Addison & Edwards (1988), and CN-ECOD activity (White 1988) as described by Addison et al. (1991). Total cytochrome P450 was estimated from the CO difference spectrum of dithionite-reduced microsomes (Omura & Sato 1964). Protein was determined using the Pierce BCA reagent (Anon. 1989).

Dab were sampled at Stns A, G & R on the 'Drilling Site Transect' on a cruise of the RV 'Holland'. Activities of EROD and CN-ECOD were estimated aboard ship, on 10000 × *g* supernatants (post-mitochondrial or 'PMS') prepared from liver homogenates. Aliquots were frozen for subsequent protein determinations and P4501A1 mRNA determinations.

A cDNA oligonucleotide probe was used to measure P4501A mRNA. The probe was synthesised by the Regional DNA Synthesis Laboratory, University of Calgary, and had the sequence 5'-d(GTGCTGTGG-GGGATGGTGAAGGG)-3' corresponding to nucleotides 705-727 in rat P450d, as listed in Genebank. This sequence differs by 3 nucleotides from the corresponding segment of trout P4501A1 (Heilmann et al. 1988). The probe was labelled with (γ^{32}P)-ATP by the method of Maxam & Gilbert (1980).

Total RNA was extracted from liver as described by Sambrook et al. (1989) and denatured by heating to 60 °C for 15 min in the presence of formaldehyde. Two-fold serial dilutions of RNA were made with diethylpyrocarbonate-treated water and loaded on to nylon filters (Genescreen*Plus*) using a Bio-Rad BIO-DOT slot blot apparatus. The RNA was immobilised by baking at 80 °C for 2 h. The final slot blot loads (0.2 ml slot^{-1}) ranged from 20 to 0.16 µg total RNA.

Slot blots were pre-hybridised in 4.5 ml of 6× SSPE (20× SSPE contains 3M NaCl, 0.2M NaH_2PO_4 and 0.02M EDTA at pH 7.4), 1 % sodium dodecyl sulphate, 0.1 % Ficoll, 0.1% bovine serum albumin, 0.1 % polyvinylpyrrolidone and 0.1 mg ml^{-1} sheared salmon sperm DNA at 42 °C for 4 h in a hybridisation water bath. Hybridisation was carried out with 5 pmol of the ^{32}P-labelled probe for 18 h at 48 °C in the same buffer but without salmon sperm DNA. Blots were washed in 2× SSC (10× SSC contains 1.5M NaCl and 0.15M sodium citrate) at room temperature for 10 min followed by four 10 min washes at 60 °C. Blots were subjected to autoradiography and intensities of the bands were quantified by densitometry of the developed film. Quantitation of mRNA per sample by hybridisation with an oligo(dT)$_{18}$ probe was used to normalise the data (Harle 1987).

Statistical analyses were carried out with BMDP software (Dixon et al. 1983) usually on ln-transformed data.

RESULTS AND DISCUSSION

Table 1 shows indices of MFO activity in dab from the German Bight. Only data from mature females weighing from 80 to 160 g are presented, since MFO activity varies with maturity (Förlin 1980) and with body size (Addison & Willis 1982) in other fish species. Mean fish weights were around 100 g at all stations, except for Stns 3 & 9 which yielded larger fish; discarding data from the larger fish (> 160 g) from Stn 9 did not reduce the mean weight appreciably, and only reduced the sample size. All fish were gonadally mature but had not spawned. Liver weight expressed as a percentage of body weight varied quite widely, with Stn 9 having the highest values (possibly related to larger body size); microsomal protein also varied but showed no clear trend. Activities of EROD were over 300 pmol min^{-1} mg^{-1} microsomal protein at Stns 3 & 5, declined along the transect to Stn 8, and rose again slightly at Stn 9. The data from the 'offshore' stations (7 to 9) were usually lower than those from the inshore stations (3 & 5). Activities of CN-ECOD followed the same trend as those of EROD; activities at Stn 3 were over 2 nmol min^{-1} mg^{-1} microsomal protein, declining to less than one-tenth of that at Stn 8. Total P450 showed a roughly similar trend with higher values at the inshore, and lower values at offshore stations; this was surprising as total P450 usually does not respond dramatically to either field or experimental induction. The general conclusion from the EROD, CN-ECOD and total P450 data is that MFO activity was induced in inshore samples (possibly falling slightly between Stns 3 & 5) and declined offshore to a minimum at Stn 8, rising slightly again at Stn 9. P4501A1 mRNA analysis supported these general conclusions. No samples were available from Stn 5, but samples from Stn 6 (which was further offshore than Stn 5) and Stns 7 to 9 all had lower P450IA mRNA levels than did samples from Stn 3 (Table 2).

These trends are consistent with preliminary chemical data. Chlorobiphenyl (CB) concentrations have been reported in dab liver from the German Bight Stns 3, 5, 6, 7, 8 & 9 (Cofino et al. 1992). The major CBs (IUPAC # 118, 138 and 153) accumulated in dab liver tissue from these sites were present in mean con-

Table 1. *Limanda limanda*. Indices of hepatic MFO activity in mature female dab weighing 80 to 160 g from the German Bight. Data are as means ± SD. EROD: ethoxyresorufin O-deethylase; CN-ECOD: cyanoethoxycoumarin O-deethylase. Data in the same column with the same suffixes do not differ significantly ($p > 0.05$ by t-test of ln-transformed data)

Station (no. of samples)	Fish wt (g)	Liver % body wt	Microsomal protein (mg g^{-1} liver)	EROD (pmol min^{-1} mg^{-1} microsomal protein)	CN-ECOD (pmol min^{-1} mg^{-1} microsomal protein)	P450 (nmol g^{-1} liver)
3 (n = 8)	119^{a} ± 26.2	1.72^{ab} ± 1.09	29.4^{a} ± 6.76	354^{a} ± 210	2414^{a} ± 2016	0.420^{a} ± 0.206
5 (n = 8)	109^{a} ± 13.1	1.67^{a} ± 0.55	35.3^{ab} ± 10.1	319^{ab} ± 382	857^{ab} ± 1074	0.220^{bc} ± 0.106
7 (n = 6)	97.1^{a} ± 28.9	1.78^{a} ± 0.30	25.5^{a} ± 4.60	61.9^{b} ± 19.8	440^{b} ± 190	0.246^{ac} ± 0.043
8 (n = 7)	107^{a} ± 18.3	1.53^{a} ± 0.31	43.4^{bc} ± 9.66	38.8^{c} ± 21.7	159^{c} ± 68.6	0.160^{b} ± 0.036
9 (n = 4)	155^{b} ± 6.69	2.93^{b} ± 0.57	29.7^{ac} ± 9.50	136^{ab} ± 85.5	494^{b} ± 143	0.196^{bc} ± 0.032

centrations of 1148, 719, 727, 292, 566 and 335 µg kg^{-1} lipid along this transect. Correlation coefficients between EROD or CN-ECOD activities and these CB concentrations (for Stns 3, 5, 7, 8 & 9) were 0.82 and 0.89, respectively ($p < 0.05$ in each case). The correlation coefficient between mRNA data (Stns 3, 6, 7, 8 & 9) and these CB concentrations was 0.90 ($p < 0.05$). PAH concentrations in total sediment along the transect declined similarly to those of CBs in dab liver. Activities of EROD and CN-ECOD were also well correlated with total naphthalenes in whole sediment ($r = 0.77$ and 0.94, respectively) and with total PAH containing >2 fused rings ($r = 0.84$ and 0.98, respectively). These correlations do not necessarily mean that any of these compounds were responsible for the induced MFO activity, but if they were not, they covaried with the inducers.

Table 2. *Limanda limanda*. P4501A1 mRNA content of liver from dab sampled from the North Sea as described in text. Data are presented in optical absorbance units as mean ± SE of determinations

Station	P4501A1 mRNA (absorbance units)
German Bight	
3	1.102 ± 0.151
6	0.449 ± 0.042
7	0.412 ± 0.036
8	0.520 ± 0.089
9	0.372 ± 0.016
Drilling Site Transect	
A	0.503 ± 0.05
G	0.700 ± 0.099
R	0.920 ± 0.101

Table 3 shows the CN-ECOD data from samples from the drilling site transect. Since these fish were analysed at sea it was not possible to determine total body or liver weights, nor to prepare microsomes. Fish from the reference station (R) were slightly longer ($p < 0.05$ by t-test) than those from the 2 other stations (A & G). However, MFO activity, as shown by CN-ECOD expressed in terms of PMS protein did not differ between any of the 3 sites sampled. If microsomal protein represents about 25 % of PMS protein (as it does in trout: Addison et al. 1977) then these CN-ECOD activities are similar to those observed at Stn 7 in the German Bight. However, the variance in the drilling site samples was so high that it is impossible to infer any differences in contamination between the German Bight and the southern North Sea. Analysis of the P4501A1 mRNA content of microsomes prepared from drilling site samples after the cruise supported

Table 3. *Limanda limanda*. Cyanoethoxycoumarin O-deethylase activity (CN-ECOD: pmol mg^{-1} PMS protein min^{-1}) in female dab from the drilling site gradient. Data as mean ± SD. Data in the same column with the same suffix do not differ significantly by t-test ($p > 0.05$)

Site (no. of samples)	Length (cm)	CN-ECOD activity
A (n = 10)	18.4 ± 1.3^{a}	271 ± 300^{a}
G (n = 8)	20.6 ± 1.4^{b}	133 ± 173^{a}
R (n = 8)	20.2 ± 1.8^{b}	245 ± 213^{a}

these conclusions: there was no difference in mRNA content between dab from Stns A, G & R (Table 2) and mRNA content from these stations were usually at the lower end of the range of data obtained from the German Bight samples. Again, the chemical data available support this conclusion: the major CBs (IUPAC 118, 138 and 153) were present in dab lipid at concentrations of 256 and 411 µg kg from Stns R & O (the position of the drilling site itself) respectively. These levels are similar to those in dab from the 'offshore' stations in the German Bight. Thus, these data suggest that surface sediments from the drilling site transect have 'recovered' from any impact of past hydrocarbon exploration activity.

These data illustrate a number of points. First, MFO measurements have been shown once again to be a reliable indicator of spatial trends in environmental contamination. In one sense, this was surprising. The Bremerhaven Workshop was timed to coincide with spawning (and high disease incidence) among North Sea dab, and this was expected to cause problems in interpreting biochemical measurements, as the 'noise' in such measurements attributable to reproduction (and possibly to migration: Rijnsdorp et al. 1992) seemed likely to mask any 'signal' due to variation in contamination. The fact that trends were observed in the MFO response which were consistent with the pattern of chemical distribution indicates the robustness of the approach.

Second, the results illustrate the value of 2 indicators of MFO activity which have not been extensively evaluated: CN-ECOD and P450IA mRNA measurements. CN-ECOD has previously been reported only in experimental studies (White 1988, Addison et al. 1991) but in those it emerged as being a somewhat more sensitive indicator than EROD of induction, e.g. by β-naphthoflavone treatment. In the present work, CN-ECOD also appeared to be slightly more sensitive than EROD: the difference between activities at Stns 3 & 8 in the German Bight (maximum and minimum, respectively) were about 9-fold for EROD and about 15-fold for CN-ECOD. The specificity of CN-ECOD as an indicator of P4501A induction is not known, though White (1988) has suggested that CN-ECOD may be catalysed by other P450 isozymes. This raises the possibility that fish may contain inducible P450s other than P4501A1.

Induction of P4501A1 mRNA has previously been measured only with natural cDNA probes extracted from experimentally induced fish. The synthetic oligonucleotide probe described here proved to be a specific and sensitive indicator of MFO induction, whose results were consistent with those from the more classical techniques. It is worth emphasising that one reason that MFO induction has proved to be a reliable indicator of contamination by a suite of organic compounds is that the mechanism of the induction process is well established. With the development of the probe described here, it is now possible to measure routinely a number of key aspects of the induction process: the transcription of P4501A1 mRNA, P4501A1 itself (using immunochemical probes: Stegeman et al. 1988, Goksøyr et al. 1992) and finally, the catalytic activity of the P4501A1 as indicated by EROD and related measurements. The internal consistency of these measurements increases their reliability.

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