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Apparent cytochrome P-450 induction as an indication of exposure to environmental chemicals in the flounder *Platichthys flesus*

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ABSTRACT: Flounder *Platichthys flesus* from a pollution gradient in Langesundfjord, Norway, and from experimental exposures to diesel oil in mesocosm basins, were analyzed during the GEEP Workshop for evidence of specific cytochrome P-450 induction. Ethoxyresorufin O-de-ethylase (EROD) activity and the content of microsomal cytochrome P-450 in liver were positively correlated with the field pollution gradient, as indicated by residues of PAH and PCB in mussels at the 4 sites. Monoclonal antibody 1-12-3 to the PAH- and PCB-inducible scup P-450 isozyme (P-450E) recognized a single protein band in *P. flesus* liver microsomes. The amount of this protein correlated positively with levels of EROD activity and microsomal P-450 content in the field-sampled fish. By contrast, fish from control and treated mesocosm basins all possessed relatively low levels of EROD activity and immunodetected protein. Levels of high molecular weight PAH known to induce teleost P-450 were likewise low in these basins, although there was a high content of other aromatics. We conclude that both EROD activity and levels of the *P. flesus* counterpart to P-450E indicate induction by environmental chemicals in *P. flesus* from Langesundfjord.

INTRODUCTION

Oxidative metabolism, usually carried out by enzymes termed mono-oxygenases, is the initial step in transformation of most lipophilic foreign compounds. Cytochrome P-450 comprises a family of haemo-proteins that are the dominant catalysts for mono-oxygenase reactions with a large number of drugs, carcinogens and pollutants, including aquatic pollutants. Understanding the functions and nature of cytochrome P-450 in aquatic species is essential to understanding the toxicology of aquatic pollutants. Several reviews treat these topics (e.g. Bend & James 1978, Stegeman 1981).

In addition to catalysing transformation of organic pollutants, many forms of cytochrome P-450 are regulated by these compounds. Regulation is evident in an induction, or increased level of mono-oxygenase activity, following treatment of animals with specific xenobiotic compounds. Aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin O-de-ethylase (EROD) activities catalysed by cytochrome P-450 are known to be induced in various fish species by aromatic hydrocarbons and polychlorinated biphenyls (Stegeman 1981). Levels of mono-oxygenase activity have thus been suggested in some cases to indicate environmental exposure of animals to pollutant mixtures that include such inducers (e.g. Payne 1976).

Induction of AHH and EROD activities in fish has now been linked to specific forms of cytochrome P-450 which have been purified from several species. Among marine teleosts, this inducible form of P-450 has been purified from scup (cytochrome P-450E, Klotz et al. 1983) and cod (cytochrome P-450c, Goksoyr 1985). The activity of these proteins, or more explicitly the levels of the proteins themselves, could indicate the presence of biochemically significant levels of specific classes of pollutants. Recently, polyclonal and monoclonal antibodies have been developed against cytochrome P-450E from scup. These antibodies have been used to evaluate the presence of environmentally-induced cytochrome P-450E in scup (Kloepper-Sams et al. 1987), and the apparent induction of a counterpart to cytochrome P-450E by environmental chemicals in

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other species (e.g. Stegeman et al. 1987). Here we describe the analysis of flounder *Platichthys flesus* from field and experimental conditions in Norway, employing both EROD activity and heterologous antibodies to induced teleost cytochrome P-450 to characterize the fish. An objective is to further validate the use of monooxygenase activity or cytochrome P-450 induction in analysis of the biological effects of aquatic pollutants.

METHODS

Bakke et al. (1988) and Follum & Moe (1988) describe the sampling and transport of *Platichthys flesus* from 4 field sites (1 to 4) in Langesundfjord, Norway, and from 4 experimental basins at the Solbergstrand mesocosms, where controlled exposures of *P. flesus* to differing concentrations of a diesel oil and copper mixture were carried out. Data on length, weight and sex of the fish appear in the companion paper by Addison & Edwards (1988). Within hours of capture, fish were examined for external lesions, then killed by a blow to the head, and livers excised and placed in ice-cold buffer. Hepatic microsomes were prepared in collaboration with Addison & Edwards (1988), and frozen in liquid N_2. Protein was analyzed by the method of Lowry et al. (1951).

Ethoxyresorufin O-de-ethylase (EROD) activity was measured by the spectrophotometric method described by Klotz et al. (1984). It directly measures product formation, like the fluorometric analysis originally described and most recently detailed by Burke et al. (1985), and employed in the companion studies by Addison & Edwards (1988). However, in our studies resorufin is detected by absorbance rather than fluorescence. Conditions of assay were as follows. The reaction mixture contained 0.1 *M* Tris-HCl, pH 8.0, with 0.1 *M* NaCl, 2 μM 7-ethoxyresorufin and ca 100 µg of microsomal protein in a final volume of 1 ml. The reaction was initiated by the addition of 0.5 m*M* NADPH and run at 26 °C. Formation of resorufin (extinction coefficient 72 mM^{-1} cm^{-1}) was followed at 572 nm on a Pye-Unicam SP8-200 recording spectrophotometer. All assays were done in duplicate.

Cytochrome P-450 levels were determined by dithionite difference spectra of microsomes which had been bubbled with CO, and treated with NADH, according to methods previously described (Stegeman 1987) using the spectrophotometer as above. Calculation of specific content of cytochrome P-450 in microsomes was based on an extinction coefficient of 91.5 $nmol^{-1}$ cm^{-1}, and cytochrome P-420 on an extinction coefficient of 110 $nmol^{-1}$ cm^{-1}

Monoclonal antibody designated MAb 1-12-3 to cytochrome P-450E was obtained as previously described (Parks et al. 1986) in ascites fluid. This antibody was used in immunoblotting according to methods described by Kloepper-Sams et al. (1987). Immunoblot analysis was carried out on liver microsomes of *Platichthys flesus* that had been shipped on dry ice from Oslo to the Woods Hole Oceanographic Institution USA. EROD activity and cytochrome P-450 levels were determined on samples without prior knowledge of their origin. The immunoblot analyses were likewise done in the laboratory by personnel who had no prior knowledge of the identity or nature of these samples, except for the levels of protein to be applied to the gel. Immunoblotting was carried out repeatedly to verify results.

Statistical analysis consisted of 1-way analysis of variance (ANOVA) and *t*-tests.

RESULTS AND DISCUSSION

Samples from Langesundfjord

Microsomal cytochrome P-450

Hepatic microsomal cytochrome P-450 content differed significantly between field sites. Mean cytochrome P-450 content ranged from 0.18 nmol mg^{-1} in *Platichthys flesus* from Site 1 to 0.53 nmol mg^{-1} at Site 4 (Table 1), with content increasing in the order 1, 2, 3, 4. None of the field samples showed absorbance at 420 nm, indicating no degradation of cytochrome P-450.

Table 1 *Platichthys flesus*. Liver microsomal cytochrome P-450 content (nmol mg microsomal $protein^{-1}$), EROD activity (pmol min^{-1} mg $protein^{-1}$) and EROD per unit P-450 (nmol min^{-1} nmol $P\text{-}450^{-1}$), for the 4 field sites and 4 experimental treatments (C: control, L: low, M: medium, H: high exposure to diesel oil and Cu). Means ± SD (n = 11 or 12, but mesocosm flounder with more than 25 % cytochrome P-420 excluded)

Source	n	Cyt. P-450	EROD	EROD/P-450
Site				
1	11	0.18 ± 0.05	39 ± 19	0.52 ± 0.47
2	11	0.30 ± 0.11**	269 ± 291**	0.84 ± 0.74
3	12	0.36 ± 0.08**	372 ± 285**	1.25 ± 0.83*
4	12	0.53 ± 0.11**	547 ± 236**	1.05 ± 0.44
Basin				
C	7	0.10 ± 0.02	129 ± 71	1.28 ± 0.53
L	12	0.14 ± 0.05	107 ± 49	0.80 ± 0.45
M	12	0.12 ± 0.05	59 ± 42	0.57 ± 0.37
H	10	0.12 ± 0.07	80 ± 46	0.64 ± 0.37

For field sites, significant differences from the reference site (1) indicated by $^{*}p<0.05$, $^{**}p<0.01$ (P-450 and EROD from Site 4 also differed from Sites 2 and 3, which were not separable)

There was no apparent association between the levels of cytochrome P-450 and the sex of individuals within any group. This was not unexpected, given that gonads were regressed in the animals at the time of sampling.

EROD activity

There was nearly a 15-fold difference in mean levels of EROD activity per mg protein between the groups with lowest and highest activity (Sites 1 and 4, Table 1). At Sites 2 and 3 mean levels of EROD activity were intermediate (though individual values varied considerably, with the range at Site 3 being nearly 12-fold and that at Site 2 nearly 40-fold). Fewer differences between sites were seen for the estimated turnover number for EROD activity (activity per nmol of microsomal cytochrome P-450) though levels were significantly lower for fish from Site 1 than elsewhere, indicating that the P-450 form responsible for EROD activity comprised a lower percentage of the total complement of microsomal P-450 in this group.

Immunoblot analysis

Total microsomal cytochrome P-450 in fish includes many different forms, only some of which are inducible by foreign compounds (Stegeman & Kloepper-Sams 1987). Microsomal cytochrome P-450 content and levels of EROD activity in Table 1 suggest higher levels of some cytochrome P-450 in flounder from Sites 2, 3 and particularly 4, relative to Site 1. Analysis of these fish with monoclonal antibody 1-12-3 to scup cytochrome P-450E confirmed increasing levels of a specific form of cytochrome P-450 across Sites 2, 3 and 4. An example of the immunoblotting results (Fig. 1) shows the substantially greater amount of staining by MAb 1-12-3 in flounder from Sites 2, 3 and 4 relative to 1. Here, and in all other cases, there was staining of only a single protein band in liver microsomes. Based on the specificity of the monoclonal antibody 1-12-3 established in other studies (Kloepper-Sams et al. 1987), we conclude that the epitope recognised by MAb 1-12-3 represents a *Platichthys flesus* counterpart to scup cytochrome P-450E, a teleost P-450 form proven to be inducible by PAH and PCB, and shown to be the major EROD catalyst. Immunoblot analysis of all individuals again showed that there was a steady increase

Table 2. *Platichthys flesus*. Immunoblot analysis of liver microsomes of individuals from the 4 field sites and 4 experimental conditions: anti-scup P-450E from staining with MAb 1-12-3 to scup P-450E (pmol of P-450E equivalent per mg microsomal protein, assuming the same affinity of MAb 1-12-3 for P-450E and the epitope in *P. flesus*); anti-cod P-450c from staining with polyclonal Ab to cod P-450c (area under the curve in densitometric analysis of Samples 1 to 4 in each group-samples were loaded onto gels with identical amounts of microsomal protein). Means ± SD

Source	*n*	Anti-scup P-450E	Anti-cod P-450c
Site			
1	10	3.5 ± 1.6	73 ± 62
2	11	28.8 ± 22.9**	151 ± 92
3	12	33.6 ± 15.6**	198 ± 40**
4	12	47.9 ± 18.7**	195 ± 42**
Basin			
C	10	5.2 ± 6.0	–
L	12	6.0 ± 7.3	–
M	12	8.3 ± 9.4	–
H	12	2.8 ± 3.8	–

For field sites, significant differences from reference site 1 indicated by ** $p<0.01$

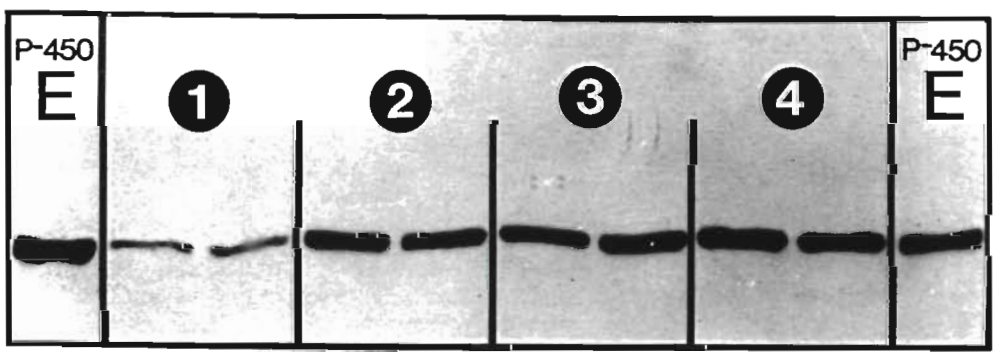

Fig. 1. *Platichthys flesus*. Immunoblot of liver microsomes. Two samples from each of the 4 field sites (1 to 4). Samples blotted had values for EROD activity and microsomal P-450 content near the means for each site. Each lane had 100 µg microsomal protein applied and the blot was stained as in Material and Methods

in the amounts of the apparent cytochrome P-450E counterpart found across Sites 1 to 4 (Table 2). The values obtained with MAb 1-12-3 in Table 2 correspond to about 2 % of total P-450 present as 'P-450E' in Site 1 fish, and 9 to 10 % in fish from the other sites. However, the percentage that was 'P-450E' ranged as high as 22 % in some individuals.

A similar immunoblot analysis of a subset of samples from each of the 4 sites was done using polyclonal antibodies to BNF-inducible cytochrome P-450c from cod (Goksoyr 1985), a homologue of scup P-450E (Goksoyr et al. unpubl.). This analysis gave results broadly similar to those seen with the monoclonal antibody 1-12-3, with a much lower amount of staining in fish from Site 1 than from the other sites. This supports the interpretation that the cross-reacting protein in *Platichthys flesus* is the PAH or PCB-inducible P-450.

Correlation with environmental chemicals

Levels of EROD activity, total microsomal cytochrome P-450, and immunodetected cytochrome P-450 each showed a strong positive correlation with the levels of certain types of compounds detected at the Langesundfjord sites. These include aromatic hydrocarbons and PCBs detected in mussels by Klungsøyr et al. (1988), with steady increases across Sites 1 to 4 in the ratios 1 : 2.6 : 5.2 : 6.9 for aromatic hydrocarbons, and 1 : 2.3 : 2.9 : 3.6 for total PCBs. It is generally agreed that the mussel *Mytilus edulis* has limited capacity for metabolism of higher molecular weight hydrocarbons or chlorobiphenyls, and thus that the body burden in mussels largely reflects the levels of bioavailable compounds present in their environment. Uptake of hydrocarbons and chlorobiphenyls by the flounder at these sites would most likely occur in a similar rank order. The levels of PCBs measured in livers of the flounder (Addison & Edwards 1988) are fully consistent with this conclusion. However, the exact compounds responsible for the induction of cytochrome P-450E homologue in these samples are not known.

Samples from the mesocosm experiment

Microsomal cytochrome P-450

In contrast to the results obtained with flounder from the field, there were few differences in results for differing mesocosm treatments (C: control, L: low, M: medium, H: high dose of a diesel oil and Cu mixture, Bakke et al. 1988). Levels of cytochrome P-450 ranged from 0.08 nmol mg^{-1} in Basin C, to 0.14 nmol mg^{-1} in Basin L, with M and H flounder being intermediate; none of these means were significantly different. Thus, the levels of cytochrome P-450 in all groups from Solbergstrand were on average lower than levels from Site 1 in Langesundfjord. In some flounder from the mesocosm there was a substantial peak of absorbance at 420 nm. This presumably reflects conversion of cytochrome P-450 to the inactive form cytochrome P-420, evidence of degradation of cytochrome P-450. This was most pronounced in C flounder. The data presented in Table 1 therefore exclude individuals with putative cytochrome P-420, but all mesocosm groups still showed lower average values for microsomal P-450 content than was seen at Field site 1.

EROD activity

Mean levels of EROD activity were also not significantly different between treatments (Table 1), and the variances were high (as for field samples). Five of the C flounder had little or no detectable EROD activity, and these were mainly the individuals with high levels of putative cytochrome P-420 (the latter are excluded from Table 1). This correlation would be expected if there had been inactivation of cytochrome P-450 in those fish, with a loss of active EROD catalyst.

Immunoblot analysis

Levels of cytochrome P-450 and EROD activity were low enough in each of the mesocosm flounder to suggest either (1) that there was little induction of cytochrome P-450 by MC-type inducers, or (2) that any cytochrome P-450 that might have been induced had been somehow inactivated. The immunoblot analyses of these samples showed very similar levels of immunodetected P-450 in mesocosm control flounder to fish from Field site 1. The levels in other groups did not differ significantly from control values (Table 2), so that there was much less induction of a counterpart to cytochrome P-450E in the mesocosm flounder than in most of the fish from the fjord. This could be interpreted as indicating that active inducing compounds were either less abundant or less bioavailable in the mesocosm systems.

Chemical residues

The conclusion that there was little induction is consistent with the levels of certain types of hydrocarbons detected in mussels held in the mesocosm basins. Total content of aromatic hydrocarbons in whole mussel tissues differed substantially across the basins, in line with the dosing. However, there was little difference in

the content of aromatic hydrocarbons from fluoranthene to perylene, reflecting the nature of the diesel oil used for these exposures (Klungsøyr et al. 1988). Induction of cytochrome P-450 in fish by aromatic hydrocarbons has generally been associated with higher molecular weight compounds, such as methylcholanthrene, benzo[a]pyrene, benzanthracene and other multi-ring aromatics. Lower molecular weight compounds have been little studied for inductive capacity in fish, but based on results with mammalian systems, we would expect them to be less active at induction of cytochrome P-450E. The content of higher molecular weight compounds in mussels from all 4 basins were lower than at Site 1 in Langesundfjord. Thus, on the basis of estimated bioavailable hydrocarbon content, one could predict that the fish in the mesocosm would generally show a lower degree of induction than at the field sites.

External lesions

Some *Platichthys flesus* from the mesocosm basins had obvious external lesions, appearing as fin erosion, reddish patches or gross ulcerative lesions. Serious lesions were seen only in H flounder, the high-exposure group. Eight of 12 flounder had evident lesions, and 4 of these had serious ulcerative disease, with lesions 2 to 5 mm deep. Examples of these are shown in Fig. 2. The nature of the lesions was not determined.

CONCLUSIONS

Several general conclusions can be drawn from these results. Firstly, the levels of EROD activity and the immunodetected cytochrome P-450 in *Platichthys flesus* from the environment correlate well with the levels of environmental contaminants known to induce the related form of P-450 in other fish species. The similar correlation between environmental contaminants and microsomal cytochrome P-450 suggests that the majority of cytochrome P-450 detected spectrally in fish from contaminated sites was induced. However, whether the apparent cytochrome P-450E counterpart accounts for all of this increased microsomal cytochrome P-450 is yet to be established; levels of other forms also might have been increased.

Secondly, the low levels of hydrocarbons having multi-ring aromatic structures in the mesocosm dosing are consistent with lower levels of cytochrome P-450 and EROD activity in these flounder. However, the inductive capacity of the lower molecular weight aromatics or the heterocyclic compounds like those

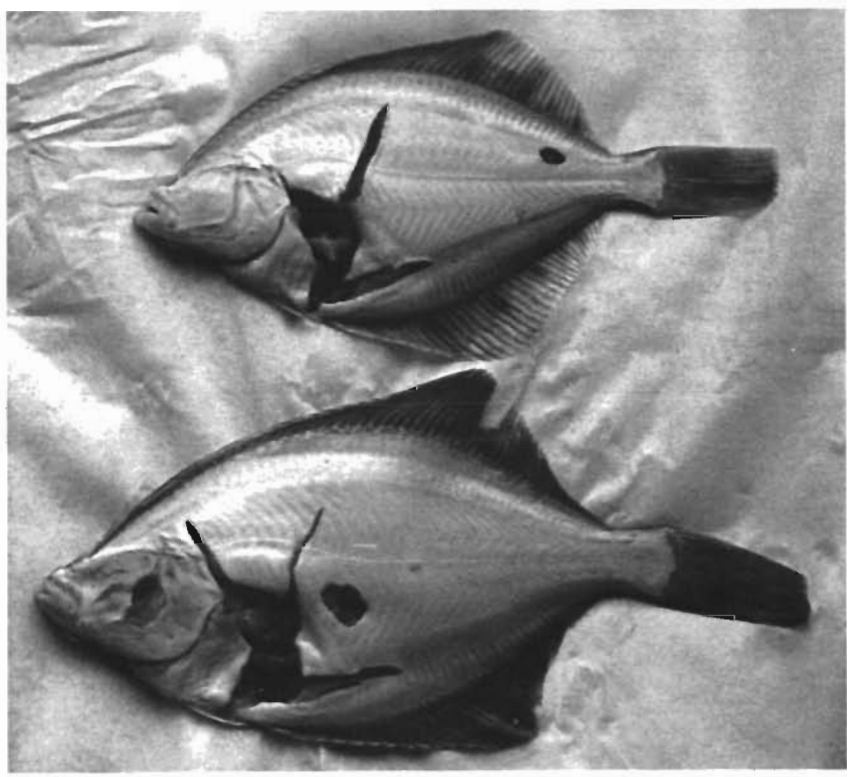

Fig. 2. *Platichthys flesus*. External lesions in individuals from the high-dose basin of the mesocosm experiment. (Photograph by Allison Edwards)

abundant in the mesocosm basins is not known in fish and needs to be established. Such lower molecular weight aromatics might produce toxicity in the absence of induction of P-450.

Thirdly, our results with the spectrophotometric method for analysis of EROD activity showed the same pattern as did the fluorometric analysis employed in the companion paper by Addison & Edwards (1988). Note also that the EROD activity here correlated with the AHH activity in the analysis by Addison & Edwards (1988). In previous studies of individual *Platichthys flesus* we saw a similar correlation between EROD and AHH activity (Stegeman 1987).

In summary, this study demonstrates the potential for use of mono-oxygenase catalytic function (EROD activity) and the levels of specific cytochrome P-450 forms detected by antibodies to scup cytochrome P-450E, or its teleost counterparts, to indicate contamination by biochemically significant levels of certain classes of organic pollutants. The results fully support previous studies demonstrating use of EROD activity and/or antibodies to cytochrome P-450E or its homologues to indicate environmental induction (Stegeman et al. 1986, 1987, Varanasi et al. 1986, Kloepper-Sams et al. 1987). Optimisation of assays and accounting for variables that might influence levels and turnover of the specific inducible forms of cytochrome P-450 should enhance this potential.

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