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APPLICATION OF CYTOCHROME P4501A INDUCTION AS A BIOMARKER FOR IMPACT BY ORGANIC POLLUTION IN GOBY (*Zosterisessor ophiocephalus*) AND MUSSEL (*Mytilus galloprovincialis*) IN VENICE LAGOON, ITALY

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ABSTRACT

The use of cytochrome P4501A (CYP1A) as a biomarker of organic pollution was investigated in liver of goby (*Z. ophiocephalus*) and digestive gland of mussel (*M. galloprovincialis*) from sites in and near to Venice as part of the UNESCO-MURST Venice Lagoon Ecosystem Project. Tissue contaminants (PAHs, PCBs) and biochemical measurements varied seasonally. Elevated 7-ethoxyresorufin *O*-deethylase (EROD) activity and CYP1A-protein levels in goby were correlated with high tissue contaminant levels at the industrial Porto Marghera site. Indications were obtained of elevated levels of *CYP1A*-like mRNA, CYP1A-like protein and microsomal metabolism of benzo[a]pyrene to free metabolites in mussels from the industrial CVE site in Venice compared to a site in the Adriatic Sea. The studies demonstrate the usefulness of CYP1A as a biomarker for organic pollution in fish, and indicate some potential for its application in molluscs.

INTRODUCTION

Lipophilic organic contaminants, such as polynuclear aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs) and dioxins, are readily taken up into the tissues of marine organisms, both from the water and sediment interfaces, and through the food chain (Livingstone, 1991a; Walker and Livingstone, 1992). The integrated use of biomarkers and chemical contaminant levels has been advocated as an effective means of

monitoring the impact of such pollution (McCarthy and Shugart, 1990; Stegeman *et al.*, 1992, Livingstone, 1993). This approach was applied to a study of organic pollution in Venice Lagoon as part of the UNESCO-MURST Venice Lagoon Ecosystem Project.

Induction of the biotransformation enzyme cytochrome P4501A (CYP1A) in liver of fish by PAHs, PCBs, dioxins and other chemicals has been used extensively worldwide as a biomarker for impact by organic pollution. Responses have been measured at the level of enzyme activity (7-ethoxyresorufin *O*-deethylase [EROD] and benzo[a]pyrene (BaP) hydroxylase activities), enzyme amount or mRNA. To date some 20 or more successful field studies, involving over 25 species of fish, have been carried out in Europe and the USA (Livingstone, 1993). Use of the biomarker in fish is based on a fundamental understanding of the properties and regulation of the enzyme (Goksøyr and Förlin, 1992). Much less is known of the enzyme in molluscs such as sessile bivalves, and the success of field results using various measurements of cytochrome P450, or the mixed-function oxygenase (MFO) system, has been limited (Livingstone, 1991a, b). However, recent studies indicate the existence of a CYP1A-like enzyme in *Mytilus* sp. (Porte *et al.*, 1993; Wootton *et al.*, 1993) with possible potential for biomonitoring. Measurements of CYP1A were made in liver of goby (*Zosterisessor ophiocephalus*) and digestive gland of mussel (*Mytilus galloprovincialis*) from industrial and other sites in and near Venice Lagoon.

MATERIALS AND METHODS

Male and female gobies and mussels were collected for chemical and biochemical determinations at several times of the year from putative clean and polluted sites. These included Lio Grande and Crevan (N. Venice lagoon) and Porto Marghera (central lagoon - industrial) for goby, and Platform site (Adriatic Sea) and CVE (central lagoon - industrial) for mussel (Fig. 1). Either individual goby livers or pooled mussel digestive glands were used. The numbers of samples taken are given in the text. Tissues were immediately dissected out, frozen in liquid nitrogen, and stored at -70 ^{o}C before use.

Chemicals and biochemicals were of AnalaR grade or equivalent. Following alkaline saponification, hexane extraction and alumina/silica gel chromatography, hydrocarbons and PCBs were measured by fluorometry and gas chromatography (Fossato *et al.*, 1989). Cytosolic (9,000g) and microsomal (100,000g resuspended pellet) fractions were prepared by standard procedures in 0.15 M KCl/1 mM EDTA pH 7.5 (goby) or 10 mM Tris-HCl/0.5 M sucrose/0.15 M KCl pH 7.6 (mussel) (20 % w/v glycerol was included in microsomal buffer) (Livingstone, 1988; Livingstone *et al.*, 1992). EROD activity was measured fluorometrically (Burke and Mayer, 1974) in hepatic 9000g or microsomal fractions of goby. Metabolism of BaP to free metabolites (dihydrodiols, phenols, diones) was measured radiometrically, in the presence of NADPH using ^{3}H-BaP, in digestive gland microsomes of mussel (Lemaire *et al.* (1993). Free polar metabolites were resolved by reverse phase HPLC and quantified by on-line radiometric counting. Western blotting for CYP1A was carried out on goby and mussel microsomes as described by Towbin *et al.* (1979) using polyclonal anti-CYP1A from perch (*Perca fluviatilis*) (alkaline phosphatase visualization, quantified by image analysis). Analysis of *CYP1A1*-like mRNA in mussel digestive gland was

carried out by RNAzol B extraction of RNA and Northern blotting using ^{32}P-radiolabelled cDNA probe for *CYP1A1* (pfP_1450-3' from rainbow trout - *Oncorhynchus mykiss*) as described in Wootton *et al.* (1993). Values are given as mean ± SEM and were compared by one-way analysis of variance ($P < 0.05$).

RESULTS AND DISCUSSION

Preliminary results for goby liver and mussel digestive gland are presented in respectively Tables 1 and 2. Total PCB levels are given for goby, and PCBs, PAHs and

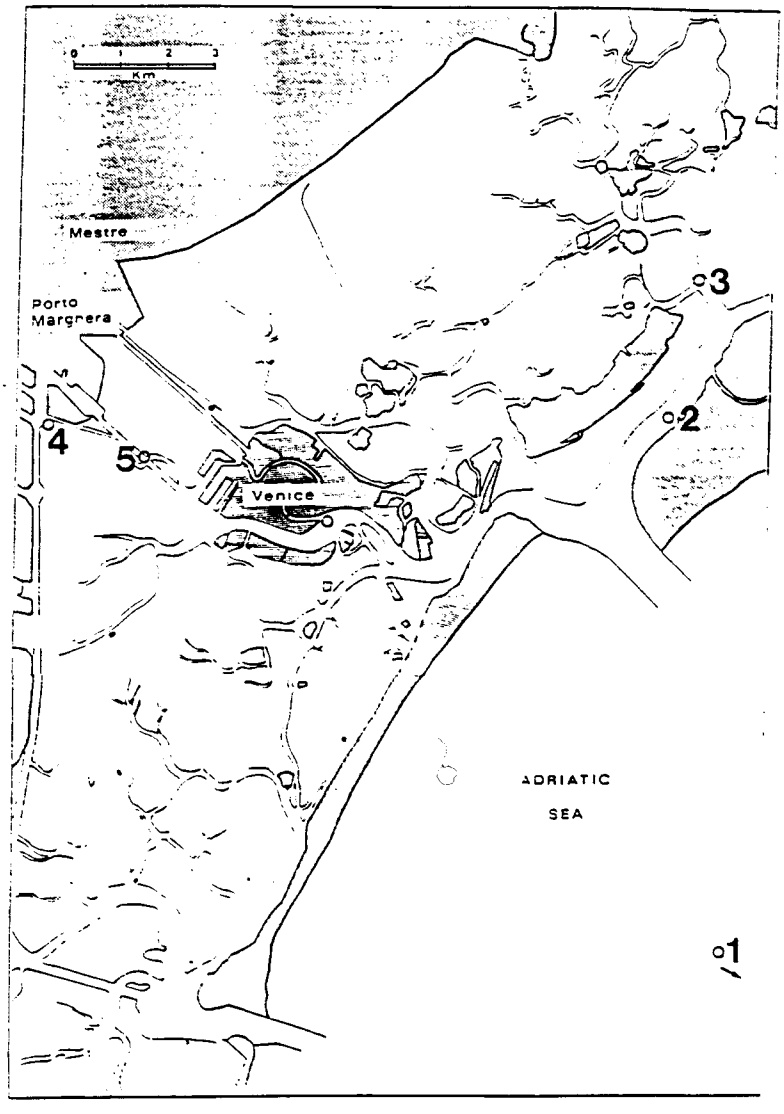

Figure 1. Animal collection sites in Venice Lagoon and the Adriatic Sea. Goby (*Zosterisessor ophiocephalus*) : Lio Grande (2), Crevan (3) and Porto Marghera (4 - industrial site); mussel (*Mytilus galloprovincialis*) : Platform site (1) and CVE (5 - industrial site).

aliphatic hydrocarbons for mussel. Tissue contaminant levels changed seasonally in both goby and mussel (seasonal data shown only for goby - Table 1), possibly reflecting variation in discharge and metabolism of contaminants, but most likely due to changes in tissue lipid levels (Walker and Livingstone, 1992). Contaminants were generally highest in both goby and mussel at the industrial sites of respectively Porto Marghera (Table 1) and CVE (Table 2). Maximal PCB levels were higher in goby than mussel (18,110 compared to 1,461 ng g^{-1} dry weight), consistent with the low level of metabolism of PCB congeners by marine organisms and their bioaccumulation to highest levels at the top of food chains (Walker and Livingstone, 1992). In contrast, PAHs are metabolised and eliminated much faster by fish than molluscs (Walker and Livingstone, 1992), and tissue levels of these contaminants were generally lower in goby liver than mussel digestive gland (data not shown).

Hepatic EROD activity was generally similar in male and female goby, but changed considerably with season (Table 1), consistent with observations for other fish species (Goksøyr and Förlin, 1992; Livingstone, 1993). However, EROD activity in both male and female goby was higher at Porto Marghera than at Crevan on all sampling occasions (x 3 - x 57 higher), and, to a lesser extent, was also higher at Lio Grande than at Crevan (up to x 5). A similar pattern was observed for levels of CYP4501A protein, *viz.* for selected samples from June in arbitrary units 2.76 ± 0.71

Table 1. Hepatic 7-ethoxyresorufin *O*-deethylase (EROD) activity and total polychlorobiphenyl (PCB) levels in goby (*Z. ophiocephalus*) at different times of the year from Venice Lagoon. Male EROD (upper values), female EROD (lower values) and total PCBs in unsexed single livers (square parenthesis).

Collection time	Lio Grande	Crevan	Porto Marghera
April 1992	14.1 ± 3.4 *	3.0 ± 0.7 #	39.6 ± 0.2 x
	13.5 ± 2.8 *	3.7 ± 0.5 #	20.3 ± 3.6 *
	[2727]	[1993]	[8863]
June 1992	5.2 ± 1.1 *	4.6 ± 0.6 *	142 ± 15 #
	2.3 ± 0.3 *	2.9 ± 0.7 *	164 ± 46 #
	[1240]	[2250]	[18110]
September 1992	13.8 ± 4.8 *	2.9 ± 0.8 *	17.8 ± 7.2 *
	22.1 ± 7.0 *	4.6 ± 1.1 #	11.2 ± 4.2 #
	[1740]	[1360]	[7200]

EROD in pmol min^{-1} mg^{-1} protein; total PCBs in ng g^{-1} dry weight; data in the same row sharing the same symbol (* # x) do not differ significantly ($P > 0.05$); values are mean ± SEM (n = 3 to 6).

(Porto Marghera), 0.64 ± 0.22 (Lio Grande) and 0.01 ± 0.01 (Crevan) ($P < 0.05$; n=3). Thus, on most occasions site-specific differences in EROD activity paralleled those of PCB levels. The results support the usefulness of hepatic CYP1A induction as a biomarker for organic pollution in fish (Goksøyr and Förlin, 1992; Livingstone, 1993).

The existence of a CYP1A-like enzyme in marine invertebrates, which is readily inducible by organic pollutants such as particular PCBs and PAHs, is as yet unclear (Livingstone, 1991a, 1991b). Hepatic EROD activity is catalysed solely by CYP1A in fish (Stegeman, 1989; Goksøyr and Förlin, 1992) and mammals, but is either not detectable or only present in low activity in invertebrates (Livingstone, 1990, 1991a). BaP hydroxylase activity is mainly catalysed by CYP1A, plus some other CYP isoenzymes, in vertebrates (Åstrom and DePierre, 1986; Stegeman, 1989), and is widely detectable in marine invertebrates (Livingstone, 1991a). Apparent induction of the MFO system has been indicated for some marine invertebrate species, but responses in most have been absent or low, and certainly much lower than for vertebrates, e.g 3-fold increases in BaP hydroxylase in the starfish *Asterias rubens* (Den Besten *et al.*, 1993), compared to up to several hundred-fold increases for hepatic EROD in fish. In field studies with mussels some success has been achieved with digestive gland BaP hydroxylase activity (Narbonne *et al.*, 1991), the "418-peak" (putative denatured CYP450) (Livingstone, 1988) and other biochemical measurements (Livingstone, 1991b), but as yet no single parameter has emerged as a widely used biomarker for organic pollution in molluscs.

Table 2. Levels of chemical contaminants, *CYP1A*-like mRNA, CYP1A-like protein and microsomal benzo[a]pyrene (BaP) metabolism (to free polar metabolites) in digestive gland of mussels (*M. galloprovincialis*) from Venice Lagoon and the Adriatic Sea (collected June 1992).

Measurement	Platform site (Adriatic Sea)	CVE (Venice)
UCM [a]	339	814
PAHs [b]	149	443
PCBs [b]	185	1461
CYP1A-like mRNA [c]	10.5 ± 5.6 *	39.5 ± 0.9
CYP1A-like protein [c]	6.0 ± 1.3	10.9 ± 3.8
BaP metabolism [d]	1.25 ± 0.61	3.02 ± 0.93

[a] Unresolved complex mixture of aliphatic hydrocarbons in $\mu g\ g^{-1}$ dry weight; [b] total polynuclear aromatic hydrocarbons (PAHs) and polychlorobiphenyls (PCBS) in $ng\ g^{-1}$ dry weight; [c] arbitrary units; [d] pmol polar metabolites (sum of dihydrodiols, diones and phenols) $min^{-1}\ mg^{-1}$ protein; * $P < 0.05$ comparing sites; values are mean ± SEM (n = 3 to 6).

More recently, additional evidence has been obtained of the existence of a CYP1A-like enzyme in digestive gland of mussel. Western blotting of partially purified CYP450 with perch polyclonal anti-CYP1A gave a single band of 54 kD (Porte *et al.*, 1993). Similarly, Northern blotting with rainbow trout *CYP1A1*-cDNA probe gave a single mRNA band (Wootton *et al.*, 1993). These measurements of CYP1A-like protein and *CYP1A*-like mRNA, plus enzyme activity (metabolism of BaP to free metabolites), were applied to the study of mussels in the Venice area. All three were either higher, or indicated to be higher, in mussels at the industrial CVE site compared to the site in the Adriatic Sea (Table 2). Correlation was therefore seen with the higher levels of hydrocarbons and PCBs at the CVE site (Table 2). The results are considered indicative of the existence of an inducible CYP4501A-like enzyme in mussel and argue for further study to investigate its biomarker potential.

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