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Development of cytochrome P450 as a biomarker of organic pollution in *Mytilus* sp.: field studies in United Kingdom ('*Sea Empress*' oil spill) and the Mediterranean Sea

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Literature on the properties, forms and regulation of cytochrome P450 (CYP) in digestive gland of *Mytilus* sp., including studies indicating the existence of an organic contaminant-inducible CYP1A-like protein, are briefly reviewed. Laboratory and field studies show increases in digestive gland microsomal CYP1A-immunopositive protein levels and/or benzo[*a*]pyrene hydroxylase (BPH) activity (i.e. metabolism of benzo[*a*]pyrene to phenols) with exposure of *Mytilus* sp. to certain polycyclic aromatic hydrocarbons (PAHs) and polychlorobiphenyls. In order to examine further the relationship between these two parameters, *M. edulis* were collected 25 and 130 days after the release of oil following the grounding of the tanker '*Sea Empress*' in South Wales, UK (15 February 1996); and *M. galloprovincialis* were sampled from sites in south-western France and south-eastern Spain during a cruise aboard the IFREMER Research Vessel '*L'Europe*' (2–18 August 1996). In both studies, sites with higher levels of CYP1A-immunopositive protein showed higher levels of BPH activities. Positive correlations were observed between the two measurements—$R = 0.65$ (*M. edulis*) and 0.68 (*M. galloprovincialis*), and both fitted linear regression models ($P < 0.05$). The CYP1A-immunopositive protein levels and BPH activities tended to be highest at sites with greatest PAH body burden for the Mediterranean study. It is concluded that development of the CYP1A-like protein into a robust biomarker of exposure to organic contaminants will depend upon sequencing of the gene/protein and the subsequent production of *Mytilus*-specific cDNA and antibody probes. Such probes will then allow a full characterization of the enzyme's properties and gene regulation.

Keywords: molecular markers, benzo[*a*]pyrene hydroxylase, CYP1A, immunopositive.

Abbreviations: BaP, benzo[*a*]pyrene; BPH, benzo[*a*]pyrene hydroxylase; CYP, cytochrome P450; CYP1A, cytochrome P4501A; DMF, dimethylformamide; EROD, 7-ethoxyresorufin *O*-deethylase; IFREMER, Institut Français de Recherche pour l'Exploitation de la Mer; MFO, mixed-function oxygenase; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorobiphenyl; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulphate.

Introduction

An increasing complexity of organic, metal and other contaminants is entering aquatic ecosystems and being taken up into the tissues of biota, posing a threat both to the resident organisms and to man (Walker and Livingstone 1992, Rand 1995, Hughes 1996). Effective monitoring of environmental pollution requires integrated measurement of selected chemical contaminants and biological responses/effects in the biota (biomarkers). The application, advantages and limitations of molecular, cellular/tissue and whole-animal biomarkers have been discussed extensively in

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detail elsewhere (McCarthy and Shugart 1990, Huggett *et al*. 1992, Livingstone 1993, Depledge and Fossi 1994, Schlenk 1996, Walker 1996). Such biomarkers may be obtained from sentinel organisms, or any organisms of particular interest. Mytilids and many other molluscan bivalve species have been used extensively as sentinels in pollution monitoring around the world (so-called 'Mussel Watch' programmes) because of their ability to bioaccumulate high tissue concentrations of contaminants, sessile nature and widespread distribution (Livingstone and Pipe 1992, Livingstone 1996, Livingstone and Goldfarb 1998).

Cytochrome P450 (CYP) is the terminal component of the mixed-function oxygenase (MFO) system, which catalyses the first step in the biotransformation of many organic foreign compounds (xenobiotics), including certain polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs) and dioxins. The MFO system occurs in the endoplasmic reticulum of the cell, and is often found in highest levels in the liver (or equivalent tissue) and tissues involved in the processing of food (Livingstone 1991a). Induction of the CYP isoform cytochrome P4501A (CYP1A; NB the term 'CYP1A1' is only used when the full sequence of the gene or protein is known—see Stegeman (1992) and Nelson *et al*. (1996)) by PAHs, PCBs, dioxins and other compounds has been used extensively, worldwide as a biomarker of exposure to organic pollution in the liver of wild rodents (Qualls *et al*. 1998), birds (Walker 1998), reptiles (Ertl and Winston 1998) and, in particular, fish (Livingstone 1993, 1996, Stegeman and Hahn 1994, Bucheli and Fent 1995, Goksøyr 1995, Livingstone and Goldfarb 1998). Induction of CYP1A in fish has been measured at the levels of mRNA, protein and the catalytic activities 7-ethoxyresorufin *O*-deethylase (EROD; NB CYP1A1 is the sole or major catalyst of EROD activity) and, to a lesser extent, benzo[*a*]pyrene hydroxylase (BPH; NB CYP1A1 is the major catalyst of BPH activity). Induction following exposure to planar aromatic compounds is indicated to occur in all post-embryo lifestages (Peters and Livingstone 1995, Peters *et al*. 1997).

Although the MFO system and its CYP isoforms are much less well characterized in aquatic invertebrate taxa, recent studies have indicated the existence of a CYP1A-like protein, which is inducible by organic contaminants such as PAHs and PCBs, in digestive gland of *Mytilus* sp. and other molluscan species (Livingstone 1996, Herron and Falckh 1998, Livingstone and Goldfarb 1998, Peters *et al*. 1998a, b). Early laboratory and field studies on *Mytilus* sp. exposed to PAHs, PCBs and other organic contaminants demonstrated increases in digestive gland microsomal total CYP content, '418-peak' content (putative denatured CYP), or benzo[*a*]pyrene (BaP) metabolism (i.e. total metabolites—see later) (Livingstone 1991b, Narbonne *et al*. 1991, Michel *et al*. 1993), whereas more recent studies showed increases in CYP1A-immunopositive protein and/or BaP metabolism or BPH activity (i.e. phenols only—see later) (Michel *et al*. 1994, Livingstone *et al*. 1995, 1997, Solé *et al*. 1996, 1998, Peters *et al*. 1998a). However, although there is indirect evidence for the CYP1A-like enzyme being involved in the microsomal metabolism of BaP (Porte *et al*. 1995, Wootton *et al*. 1996), there have been inconsistencies in the responses of CYP1A-immunopositive protein and BPH activity with exposure to organic contaminants, with increases in the former not being accompanied by increases in the latter (Solé *et al*. 1996) and vice versa (Solé *et al*. 1998). The present paper reviews the state of knowledge on the existence of a molluscan inducible CYP1A-like enzyme, and also presents new data from two field studies ('*Sea Empress*' oil spill in South West Wales, UK and the Mediterranean Sea) designed to examine the relationship between

CYP1A-immunopositive protein and BPH activity in digestive gland microsomes of the mussels *Mytilus edulis* and *Mytilus galloprovincialis*. Finally, the overall data are assessed in the context of the current status and potential of a CYP1A-like enzyme as a biomarker of organic pollution in *Mytilus* sp..

Characteristics of the molluscan MFO system

The MFO system or its components have been identified in 21 species of mollusc (Livingstone 1991a, Cheah *et al*. 1995) and at least 13 MFO activities have been described in tissues of *Mytilus* sp. (Peters and Livingstone 1998). Molluscan CYP content has been determined in subcellular digestive gland preparations of molluscs, either as sodium dithionite-difference spectra of the carbon monoxide-liganded samples (Stegeman 1985), or as the carbon monoxide-difference spectra of sodium dithionite-reduced samples (Ade *et al*. 1982, Livingstone *et al*. 1989, Cheah *et al*. 1995). Both methods report a wavelength peak (λmax) between 447 and 452 nm and a second peak between 416 and 424 nm. Levels of mytilid microsomal CYP were determined to be highest in digestive gland compared with other tissues, due in part to higher levels of associated MFO components and MFO activities (Livingstone and Farrar 1984). Relatively little is known of the endogenous functions of molluscan CYP. However, *in vitro* enzyme activities indicate roles in both xenobiotic and steroid metabolism (Livingstone 1991a). To date, three molluscan CYP families have been sequenced, two have been identified as unique, CYP10 from the pond snail *Lymnaea stagnalis* (Teunissen *et al*. 1992) and CYP30 from the clam *Mercenaria mercenaria* (Brown *et al*. 1998) whereas the third CYP4 has been sequenced in abalone (CYP4C17, *Haliotis rufescens*) and *M. galloprovincialis* (CYP4Y1) (Snyder 1998).

Mytilus sp. digestive gland microsomes metabolize BaP, in the presence of NADPH, to the 7,8-, 9,10- and 4,5-dihydrodiols, 3- and 9-phenols and putative tetrols (Stegeman 1985, Michel *et al*. 1992, Livingstone *et al*. 1997). However the major microsomal metabolites (47–65% of the total polar metabolites) are the 1,6-, 6,12- and 3,6-quinones (Michel *et al*. 1992, Lemaire and Livingstone 1993, Lemaire *et al*. 1993, Porte *et al*. 1995, Livingstone *et al*. 1997). *In vitro* metabolism of BaP leading to the formation of quinones, dihydrodiols and phenols has also been observed in the absence of added NADPH (Livingstone *et al*. 1989, Michel *et al*. 1992, Lemaire *et al*. 1993). Other apparent NADPH-independent MFO activities, e.g. 7-ethoxycoumarin *O*-deethylase, testosterone hydroxylase, *N*,*N*-dimethylaniline demethylase and benzphetamine *N*-demethylase have been described in *M. edulis* digestive gland microsomes (Kirchin 1988, Livingstone *et al*. 1989). CYP-inhibitor studies revealed sensitivity to α-napthoflavone and SKF525A (Livingstone and Farrar 1984, Moore *et al*. 1989, Michel *et al*. 1992) for both NADPH-dependent and NADPH-independent BaP metabolism, indicating that the *in vitro* metabolism of BaP involves more than one metabolic pathway (Stegeman 1985, Livingstone *et al*. 1989, 1997). More recently, the differential effect of the CYP-inhibitors, α-naphthoflavone and clotrimazole on digestive gland microsomal NADPH-dependent BaP metabolism (respectively inhibited dihydrodiol/phenol and quinone formation) has also indicated the involvement of more than one CYP isoform in BaP metabolism (Livingstone *et al*. 1997).

The existence and expression of molluscan CYP forms have been examined using Northern, Southern and Western blot techniques with vertebrate probes, and

reverse transcriptase polymerase chain reaction (RT-PCR) methods (Wootton *et al*. 1995, 1996, Falchk and Herron 1998, Herron and Falchk 1998, Peters *et al*. 1998a, b). Northern blot analysis demonstrated hybridization of nucleic acid probes with CYP1A1-, CYP3A-, CYP4A-, and CYP11A-like mRNA from *Mytilus* sp. tissue (Wootton *et al*. 1995, 1996). Southern blot analysis of genomic DNA presented further evidence of *CYP3A* and *CYP11A*-like genes (Wootton 1995). Seasonal variation of CYP1A1-, CYP3A- and CYP4A-like mRNA profiles through the year were different for different CYPs, indicating potential differential regulation of CYP genes (Wootton *et al*. 1995, 1996). Immunorecognition studies using Western blots have also indicated the expression of multiple CYPs using polyclonal anti-CYP1A,-CYP2B, -CYP2E, -CYP3A and -CYP4A antibodies to fish or mammalian CYPs (Peters *et al*. 1998a, b). A polyclonal antibody raised against perch (*Perca fluvialitis*) hepatic CYP1A immunoreacted with a CYP1A-like epitope of a partially purified preparation of *M. edulis* digestive gland CYP (Porte *et al*. 1995). A similar epitope was also immunoidentified in digestive gland microsomal preparations from *M. edulis* and *M. galloprovincialis* (Livingstone *et al*. 1997, Peters *et al*. 1998a,b, Solé *et al*. 1996, 1998). Levels of the CYP1A-immunopositive protein may be elevated by laboratory (Livingstone *et al*. 1997) and field exposure to organic xenobiotics (Peters *et al*. 1998a), although little is known of the mechanisms involved. Induction of CYP1A-like mRNA has been detected in digestive glands from PCB-treated *M. edulis* using degenerate primers to fish CYP1A1 in RT-PCR studies (Herron and Falchk 1998). Similar methods did not indicate the expression of a constitutive CYP1A-like mRNA in the cephlapod mollusc *Octopus pallidus* (Falchk and Herron 1998).

Changes in the molluscan MFO system following xenobiotic exposure

Elevation of molluscan CYP levels or related catalytic activities, has been observed both in laboratory and field studies: specifically following laboratory exposure to fuel oil (Livingstone 1987), 3-methylcholanthrene (Livingstone *et al*. 1988, Michel *et al*. 1994) and PCBs (Michel *et al*. 1993, Livingstone *et al*. 1997), whereas field studies have demonstrated positive correlations between microsomal BPH activity, or other CYP-related measurements, and environmental exposure to PAHs (Livingstone 1988, Michel *et al*. 1994, Solé *et al*. 1995a, 1996). Recent studies have indicated higher levels of digestive gland microsomal CYP1A-immunopositive protein in indigenous populations of *Mytilus* sp. sampled from PAH- and PCB-impacted compared with reference sites (Solé *et al*. 1996, Peters *et al*. 1998a). An apparent elevation of CYP1A-immunopositive protein was also observed following transplantation of *M. galloprovincialis* from a reference to a PCB-impacted site in the Venice Lagoon, Italy (Peters *et al*. 1998a).

The apparent induction of *M. galloprovincialis* digestive gland CYP1A-immunopositive protein has been investigated following water-borne exposure to 20 ppb PCB mixture Arochlor 1254 for 4 and 10 days, or 4 days after a single injection (2.5 μg g^{-1} wet weight) into the mantle cavity of PCB congener CB-138 (2,2´,3,4,4´5´-hexachlorobiphenyl) (Livingstone *et al*. 1977). Levels of the digestive gland microsomal 48 kDa (apparent molecular weight) CYP1A-immunopositive protein increased 72 % and 59 % respectively, 10 days after exposure to Arochlor 1254 and 4 days after exposure to CB-138. No changes, however, were seen in the levels of digestive gland CYP1A-like mRNA 4 days after

injection of CB-138 (as determined by Northern blot analysis using cDNA probe to hepatic CYP1A of *Oncorhynchus mykiss*). This apparent absence of induction may be due to a number of causes such as poor hybridization between the *O. mykiss* cDNA probe and the *M. galloprovincialis* mRNA, the up-regulation of a different CYP gene as well as CYP protein modifications, e.g. post-translational stabilization leading to an elevation of CYP1A-immunopositive protein and the induced levels of CYP1A mRNA being much more short-lived than the CYP1A-protein and catalytic activity—as seen for fish species (Kloepper-Sams and Stegeman 1989). Elevation of CYP1A-immunopositive protein was also determined in digestive gland tissue (13 400 *g* supernatants) of *M. galloprovincialis* exposed to water-borne BaP (Canova *et al.* 1998). In this study, a 2- to 11-fold elevation of CYP1A-immunopositive protein levels was detected following exposure to BaP, although the levels of immunorecognition were lower than those observed in microsomal fractions. Livingstone *et al.* (1997) observed that concurrent with elevation of CYP1A-immunopositive protein, there was a shift in NADPH-dependent BaP metabolism towards phenol and dihydrodiol and away from quinone production, the phenols and diols increasing from 32 % to 85 % of total free metabolites.

Both fluorometric and radiometric methods have been used to examine the formation of the oxygenated metabolites of BaP (Livingstone and Farrar 1984, Stegeman 1985, Livingstone 1987, Narbonne *et al.* 1991, Lemaire *et al.* 1993, Michel *et al.* 1993, 1994). Chromatographic separation has enabled the resolution of specific metabolites from both microsomal incubations (Lemaire *et al.* 1993) and partially purified CYP incubations in reconstituted systems (Porte *et al.* 1995). Elevation of *in vitro* BaP metabolism by *Mytilus* sp. digestive gland sub-cellular fractions has been demonstrated following *in vivo* exposure to mammalian CYP inducers/substrates such as 3,3′,4,4′-tetrachlorobiphenyl (Michel *et al.* 1993) and 3-methylcholanthrene (Livingstone *et al.* 1988, Michel *et al.* 1994). Non-planar compounds, e.g. 2,2′,4,4′,5,5′-hexachlorobiphenyl (Michel *et al.* 1993), phenobarbital or clofibrate (Livingstone *et al.* 1988, Michel *et al.* 1994) failed to elevate BaP biotransformation. Molluscan MFO parameters therefore have the potential to be applied in field studies as biomarkers of organic contaminants, e.g. PAHs and some PCBs (Narbonne *et al.* 1991, Michel *et al.* 1994, Livingstone *et al.* 1995, Solé *et al.* 1996, 1998, Butty and Holdway 1997).

'*Sea Empress*' and Mediterranean Sea field studies

This section describes the results of two field studies undertaken along the coast of South Wales, UK and the Mediterranean Sea. The UK study was prompted by the release of approximately 72 000 tonnes of 'Forties Blend' crude oil and 360 tonnes of heavy fuel oil when the *Sea Empress* oil-tanker ran aground at the entrance to Milford Haven, South Wales on the 15 February 1996. Most of the oil moved south and eastwards parallel to the coast affecting approximately 200 km of shoreline. *M. edulis* were sampled 25 and 130 days following the release of oil, from sites at different distances from the location of the accident. The study in the Mediterranean Sea was undertaken as a joint initiative involving collaboration between laboratories in France, Italy, Spain, The Netherlands and Germany. *M. galloprovincialis* were collected from sites in south-western France and south-eastern Spain (Catalonia) during a research cruise aboard the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER) Research Vessel *L'Europe*.

In both studies, digestive gland microsomal BPH activities (fluorometric assay—measures mostly phenols only) and levels of CYP1A-immunopositive protein were determined to examine the interrelationship between the two MFO parameters and evaluate them as potential biomarkers of organic contaminants.

Materials and methods

Chemicals. All biochemicals including β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). BaP and lauryl sulphate (sodium dodecyl sulphate—SDS), were from Sigma-Aldrich Chemical Company Ltd (Poole, UK). Glycerol (AnalaR grade) and methanol (technical grade) were ordered from Merck Ltd (Leicestershire, UK). Nitrocellulose (Hybond-C super) and 'Rainbow' pre-stained molecular weight markers were from Amersham International plc (Buckinghamshire, UK). 3-HydroxyBaP was from NCI Chemical Carcinogen Repository, Kansas City, USA. Rabbit anti-perch (*P. fluviatilis*) CYP1A antibody was a gift from Dr L. Förlin (University of Göteborg, Sweden) and the sheep anti-rabbit antibody–alkaline phosphatase conjugate was from Sigma-Aldrich Chemical Company Ltd (Poole, UK). Partially purified *M. edulis* digestive gland CYP (octyl sepharose fraction) was prepared according to Porte *et al.* (1995).

Collection of M. edulis *following the* 'Sea Empress' *oil spill, South Wales, UK. M. edulis* were collected from the inter-tidal zone 25 days and 130 days after the initial release of oil from the tanker. The sites for the first sampling were (number in brackets describe size range); Tenby (1.5–3.0 cm), St Ishmael's (2.0–6.0 cm), Burry Port (2.0–6.0 cm), and Goodwick (4.0–6.0 cm), whereas Dale (4.7–6.0 cm), Manorbier (3.3–4.3 cm), St Ishmael's (3.5–5.3 cm), Burry Port (5.0–6.2 cm), and Goodwick (5.8–6.6 cm), were the locations for the second sampling (figure 1). Digestive gland tissue was dissected out, the style removed, damp-dried, subsequently plunged into liquid nitrogen and stored at –70 °C. Extensive external and internal oiling of the shell was observed from samples of *M. edulis* collected from Tenby during the first sampling time point.

Collection of M. galloprovincialis *in the south-western Mediterranean Sea. M. galloprovincialis* were collected sub-tidally from 3–18 August 1996 during a scientific cruise aboard the IFREMER Research Vessel *L'Europe*. Samples were collected from nine sites in south-western France and south-eastern Spain at the following locations (number in brackets describe size range); Le Planier (5.2–7.5 cm), Carteau (4.5–9.5 cm), Fort Brescou (4.6–7.5 cm), Port Leucate (5.2–7.5 cm), Port Vendres outside

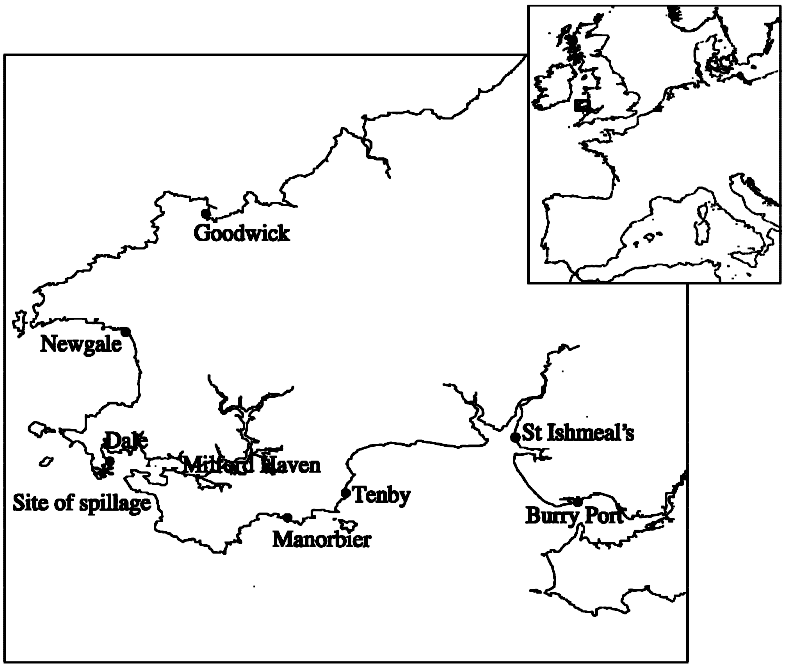

Figure 1. Location of *Mytilus edulis* sampling sites in South Wales, UK following the release of oil from the *Sea Empress* oil tanker on 15 February 1996. * Indicates site of spillage.

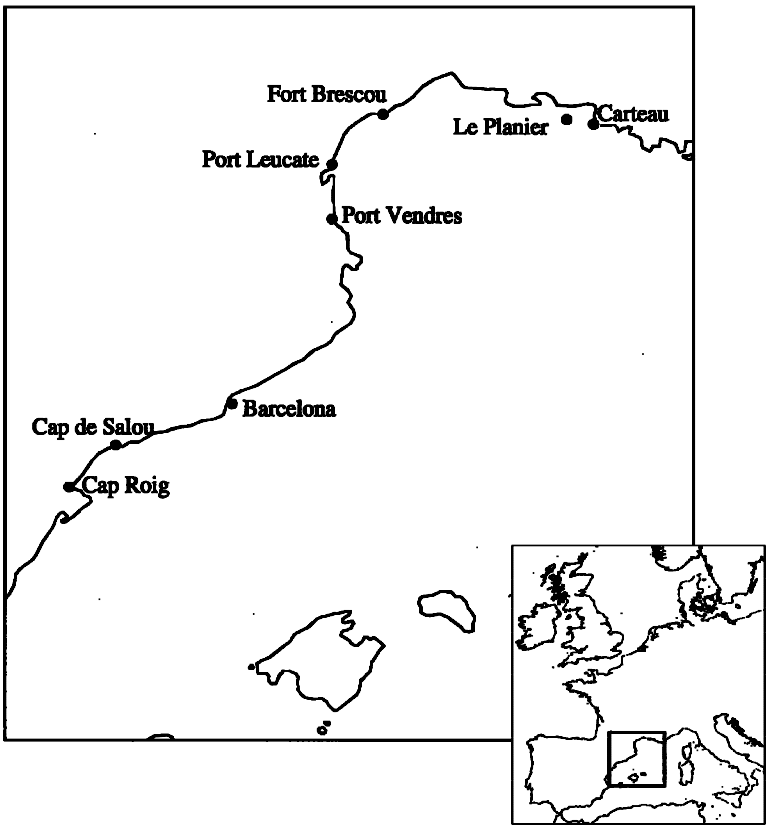

Figure 2. Location of *Mytilus galloprovincialis* sampling sites in the Mediterranean Sea, south-western France and south-eastern Spain (Catalonia). Samples were collected during a cruise of the IFREMER Research Vessel *L'Europe*, 2–18th August 1996.

harbour (4.8–6.8 cm), Port Vendres inside harbour (6.9–8.6 cm), Barcelona inside port (5.8–6.8 cm), Cap de Salou (3.4–4.8 cm) and Cap Roig (6.2–8.4). Digestive gland tissue was dissected on board, the style removed, damp dried and immediately plunged into liquid nitrogen. Samples were stored under liquid nitrogen for the duration of the cruise but for the journey to the UK, frozen samples were maintained in dry ice until transfer to –70 °C in Plymouth (UK).

Preparation of Mytilus *sp. digestive gland microsomes.* Microsomes were prepared at 4 °C according to the method of Livingstone (1988). Aliquots of frozen digestive glands were weighed (*c.* 2.5 *g* total wet weight) and homogenized on ice in 10 ml of 20 mM Tris–HCl, pH 7.6, 0.5 M sucrose, 0.15 M KCl. Homogenates were centrifuged at 500 *g* for 15 min and the resultant supernatants were transferred and centrifuged at 10 000 *g* for 45 min. Occasionally, a viscous lipid layer formed on the resulting supernatants and was discarded before the supernatants were transferred. The supernatants were then centrifuged at 100 000 g for 90 min and the microsomal pellets once formed, were re-suspended in homogenization buffer and then centrifuged for 30 min at 100 000 g. The washed microsomal pellet was re-suspended in 1 ml of microsomal buffer containing 20 mM Tris–HCl, pH 7.6, 20% glycerol (v/v) and stored at –70 °C before Western blot and BPH activity determinations. An aliquot of each microsomal preparation was frozen separately for total protein measurements. Total proteins were determined according to the method of Lowry *et al*. (1951).

Determination of Mytilus *sp. digestive gland microsomal BPH activity.* Digestive gland microsomal BPH activity was determined fluorometrically, essentially following the method of Dehnen *et al*. (1973). The assay volume was 1 ml and contained 50 mM triethanolamine–HCl pH 7.5, 0.74 mM NADPH, 0.8–1.6 mg microsomal protein and 60 µM BaP dissolved in dimethylformamide and water. Assay incubations were undertaken in a shaking water-bath under yellow light at 25 °C for 12 min. Buffer, water and NADPH were pre-incubated for 2–3 min at 25 °C. Microsomal protein was added 1 min before the assay was initiated by the addition of BaP. After 12 min, the reaction was stopped using 1 ml

of cold acetone. Time-zero controls were performed for each sample under the same conditions as above, except acetone was added prior to the addition of BaP. The contents of the tubes were centrifuged at approximately 1000 *g* (to pellet precipitated protein) for 10 min. Resulting supernatants (0.9 ml) were transferred to tubes containing 2.08 ml of 8 % triethylamine (v/v). The contents were vortex-mixed and centrifuged at approximately 1000 *g* for 5 min. The fluorescence of the supernatants was determined using a Perkin-Elmer 3000 fluorometer (excitation wavelength 467 mm, emission wavelength 525 nm) against a quinine solution where 2.43 μg ml^{-1} quinine sulphate was equivalent to 0.38 pmol ml^{-1} solution of 3-hydroxy BaP. For the analysis of the samples collected in the Mediterranean Sea, the concentration of the microsomal protein in the assay was maintained at 0.75 mg by diluting the protein samples with water, as described by Michel *et al*. (1994).

Determination of Mytilus *sp. CYP1A-like immunopositive protein.* The Western blot, immuno-identification and quantification procedures were undertaken according to the method of Peters *et al*. (1998b). Microsomal protein prepared from tissues sampled from South Wales and the Mediterranean Sea were diluted with water to protein concentrations of 7.8 μg μl^{-1} 6.0 μg μl^{-1} respectively. Samples were then boiled for 2–3 min with an equal volume of 0.125 M Tris–HCl pH 6.8, 4 % SDS (w/v), 20 % glycerol (v/v), 10 % 2-mercaptoethanol (v/v) and 0.001 % bromophenyl blue (w/v). Denatured microsomal preparations corresponding to 39 μg protein (South Wales samples) or 30 μg protein (Mediterranean Sea samples) were loaded on 10 % SDS gels with pre-stained molecular weight markers and 40 μg partially purified *M. edulis* CYP protein, and electrophoresis was performed according to the method of Laemmli (1970). Proteins were then semi-dry blotted onto nitrocellulose following the method of Town *et al*. (1979). Blots were washed with 'GT-TBS' comprising 0.5 % gelatin (w/v), 0.2 % Tween 20 (v/v), 10 mM Tris–HCl pH 8.0, 0.15 M NaCl for 30 min. They were then incubated for 1 hr with rabbit anti-perch (*P. fluviatilis*) CYP1A antibody diluted 1:1000 (w/v) in GTB-TBS containing 0.1 % sodium azide (w/v). After washing two times for 15 min with a 'T-TBS' solution of 0.2 % Tween 20 (v/v), 10 mM Tris–HCl pH 8.0, 0.15 M NaCl, the blots were incubated for 1 h with anti-rabbit antibody–alkaline phosphatase conjugate diluted 1:3000 (v/v) in GT-TBS. Blots were subsequently washed twice with T-TBS for 15 min and 100 mM Tris–HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ for 5 min. Protein bands were visualized by incubation with a solution containing 0.48 mM nitroblue tetrazolium, 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate, 59.3 mM $MgCl_2$, and 10 mM Tris–HCl pH 9.2. The visualization reaction was stopped by the addition of water and blots were quantified using a Bio-Rad model GS-690 imaging densitometer.

Statistical analysis. Inter-site comparisons between BPH activities and CYP1A-immunopositive protein levels were analysed using analysis of variance (Statview statistical package); significance set at $P < 0.05$.

Results and discussion

Digestive gland microsomal BPH activities and CYP1A-immunopositive protein levels were determined in *Mytilus* sp. collected (a) following an oil spill in South West Wales, UK, and (b) sites in the Mediterranean Sea. The UK study had two components; firstly, samples were collected from sites impacted or potentially affected by the spill 25 days after the release of oil from the tanker *Sea Empress* and secondly, samples were collected 130 days after the release of oil. The two time points corresponded to a spring and summer season sampling and as such, the study contained a temporal component in the evaluation of the responses of the MFO parameters. Since the release of oil occurred near to the commercial port of Milford Haven (see figure 1), Godwick was selected as a reference site because it was a nearby port with commercial shipping activity, but was not affected by the oil spill. Due to tidal and wind influences, the oil tended to disperse offshore. However significant oiling of the shoreline occurred along the southern coast of Wales in an easterly direction (SEEEC 1996). Table 1 reports the observed levels of *M. edulis* digestive gland microsomal BPH activity and CYP1A-immunopositive protein in samples collected 25 days (spring sampling) after the release of oil. There were no differences in BPH activities between sampling sites, and activities were observed to show considerable biological variability ($P > 0.05$). The specific activities in spring were low compared with later in the year (see figure 3), and a number of sites had BPH activities below the detection limits of the assay (minimum detection

Table 1. *M. edulis* digestive gland microsomal benzo[*a*]pyrene hydroxylase (BPH) activity (mean ± SEM, $n = 4–6$) and levels of cytochrome P4501A (CYP1A)-immunopositive protein (mean ± SEM, $n = 4–5$), sampled 25 days after the release of oil from the *Sea Empress* oil tanker on 15 February 1996.

Site	BPH activity (pmol min^{-1} mg^{-1} protein)	CYP1A-immunopositive protein (arbitrary units)
Tenby	0.0009 ± 0.0009	0.837 ± 0.081
St Ishmael's	0.0007 ± 0.0007	0.811 ± 0.040
Burry Port	0.0024 ± 0.0006	0.486 ± 0.043*
Goodwick	0.0014 ± 0.0010	0.867 ± 0.080

* $P < 0.05$ vs all other sites (analysis of variance).

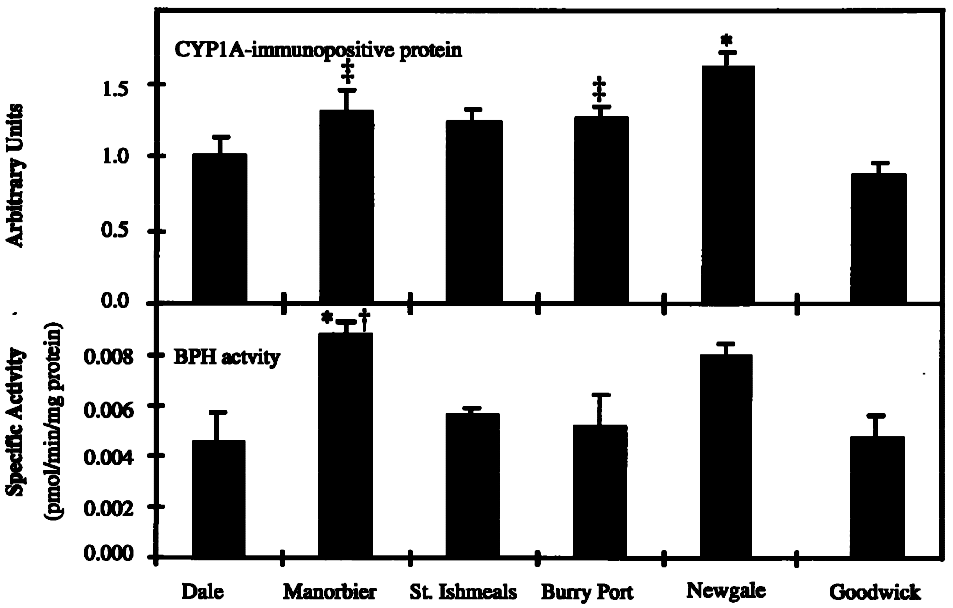

Figure 3. Digestive gland microsomal cytochrome P4501A (CYP1A)-immunopositive protein levels and benzo[*a*]pyrene hydroxylase (BPH) activity determined in *Mytilus edulis* sampled along the coast of South Wales, 130 days (summer sampling) after the release of oil from the the *Sea Empress* oil tanker. Mean ± standard error; $n = 3–6$ (BPH activities) or $n = 3–5$ (CYP1A-immunopositive protein levels); * $P < 0.05$ vs Dale and Goodwick; † $P < 0.05$ vs Bury Port; ‡ $P < 0.05$ vs Goodwick (analysis of variance).

limit estimated to be 0.003 pmol min^{-1} mg^{-1} protein). These results concur with other studies reporting lower levels of mytilid MFO parameters in spring compared with other seasons (Livingstone *et al.* 1989, Kirchin *et al.* 1992, Solé *et al.* 1995b, Wootton *et al.* 1996). In contrast, the CYP1A-immunopositive protein level was measured in all microsomal preparations from the spring samples. Burry Port, the site furthest from the spill, showed lower levels of CYP1A-immunopositive protein than in samples from either the reference site Goodwick or the other sites closer to the oil spill (table 1).

BPH activity and CYP1A-immunopositive levels for the summer sampling are shown in figure 3. Samples collected from Burry Port during the summer (130 days

after the release of oil) reported 1.5-fold higher levels of CYP1A-immunopositive protein compared with levels determined from tissue sampled from the reference site, Goodwick (figure 3). Levels of the CYP1A-immunopositive protein were also observed to be 1.8- and 1.5-fold higher at two other sites, respectively Newgale and Manorbier compared with Goodwick. From figure 1, it may be observed that Dale was the sampling site closest to the release of oil, but tissue from Dale showed CYP1A-immunopositive protein levels and BPH activities similar to those determined in Goodwick, St Ishmael's and Burry Port (figure 3). This may reflect the effectiveness of the remedial action undertaken following the spill and Dyrynda *et al*. (1997) reported a rapid decline of PAH content in *M. edulis* sampled from Dale, 77 days after the oil spill. In contrast Manorbier, reported 1.8-fold higher BPH activity compared with the reference site Goodwick samples. BPH activities in the summer sampling were also variable, but only a single sample was below the detection limit of the assay, compared with 13 samples from the spring collection (data not shown). Thus, seasonal variation in *Mytilus* sp. microsomal BPH activity may limit its application as a biomarker when the specific activities are near the detection limits of the fluorometric assay.

From figure 3, it may be observed that sites with higher mean CYP1A-immunopositive protein levels also demonstrated higher levels of mean BPH activity. Figure 4 describes a positive correlation ($R = 0.65$) between CYP1A-immunopositive protein and BPH activity for digestive gland microsomes of *M. edulis* collected during both sampling times. The data supported a linear regression model ($P < 0.05$). In previous field studies, there have been no observed correlations between mytilid digestive gland microsomal CYP1A-immunopositive protein levels and BPH activities; either following the release of oil from the tanker '*Aegean Sea*' off the Galician coast, Spain (Solé *et al*. 1996) or after the transplantation of animals from a clean site in the Faroe Islands, to contaminated sites in the Skagerrak and Kattegat (Solé *et al*. 1998). In the former study, CYP1A-immunopositive protein levels (but not BPH activities) were indicated to increase along a pollution gradient originating from the source of the oil-spill (Solé *et al*. 1996), whereas BPH activities (but not CYP1A-immunopositive protein levels)

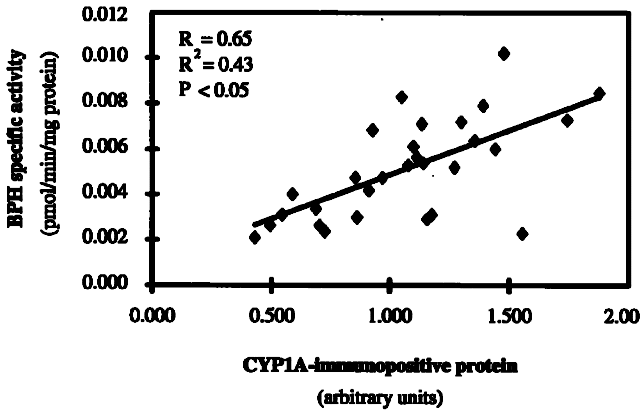

Figure 4. Correlation and regression of *Mytilus edulis* digestive gland microsomal benzo[*a*]pyrene hydroxylase (BPH) activity vs cytochrome P4501A (CYP1A)-immunopositive protein levels. Data presented as the combination of spring and summer samples with the exclusion of BPH data below the detection limits of the assay, $n = 29$.

were elevated following transplantation from the Faroe Islands to the Skagerrak and Kattegat (Solé *et al*. 1998).

M. galloprovincialis digestive gland microsomal BPH activities and CYP1A-immunopositive protein levels were found to vary between sites in the Mediterranean Sea (figure 5). In general there was a good agreement between CYP1A-immunopositive protein levels and BPH activity, i.e. sites with high mean levels of CYP1A-immunopositive protein showed high mean levels of BPH activity, e.g. Port Vendres and Barcelona, whereas low mean immunopositive protein levels corresponded to low mean enzyme specific activities, e.g. Port Leucate. The PAH body burdens (ng g^{-1} dry weight) were measured in pooled whole animal samples collected at each site and were reported in Baumard (1997). It may be observed that *M. galloprovincialis* sampled from sites with higher PAH body burdens (ports and harbours) also demonstrated higher levels of digestive

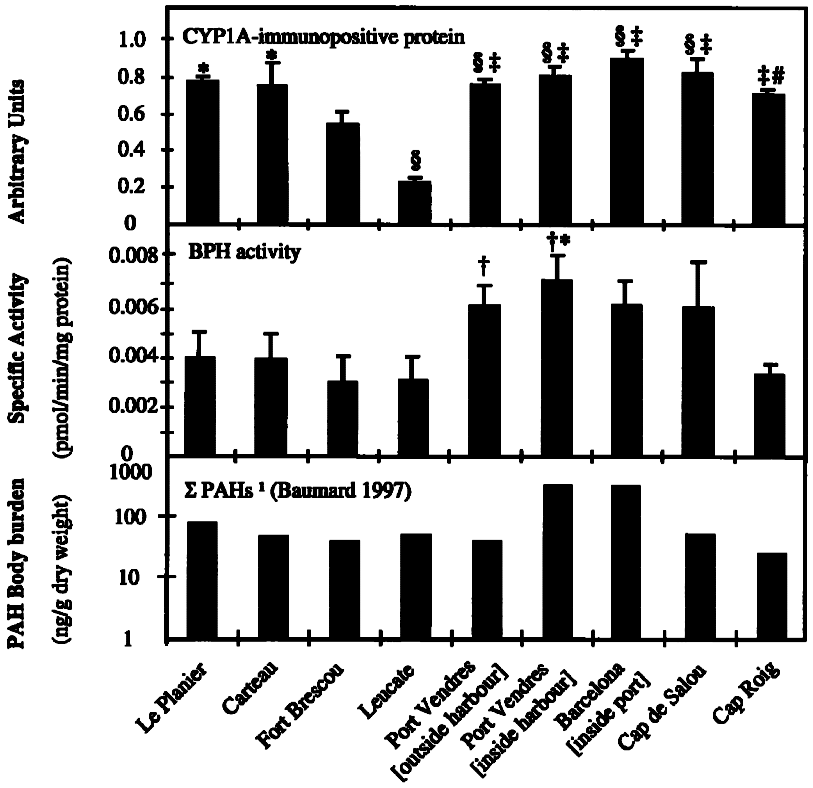

Figure 5. Digestive gland cytochrome P4501A (CYP1A)-immunopositive protein levels and benzo[*a*]pyrene hydroxylase (BPH) activity determined in *Mytilus galloprovincialis* sampled along the coast of south-western France and south-eastern Spain (Catalonia) during a cruise of the IFREMER Research Vessel *L'Europe*, 2–18 August 1996. Mean ± standard error; n = 4–6 (BPH activities) or n = 4–5 (CYP1A-immunopositive protein levels); * $P < 0.05$ vs Brescou and Leucate; † $P < 0.05$ vs Cap Roig; § $P < 0.05$ vs Brescou; # $P < 0.05$ vs Barcelona (inside port); ‡ $P < 0.05$ vs Leucate (analysis of variance). [1] Sum of phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene/triphenylene), benzo[*b*]fluoranthene/benzo[*j*]fluoranthene/benzo[*k*]fluoranthene), benzo[*a*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, perylene, indeno[1,2,3-*cd*]pyrene, benzo[*ghi*]perylene, dibenzo[*a*,*h*]anthracene/dibenzo[*a*,*c*]anthracene) *M. galloprovincialis* body burdens determined by Baumard (1997) from organisms sampled during the same research cruise.

gland microsomal CYP1A-immunopositive protein levels and BPH activities, e.g. Port Vendres (inside harbour) vs Port Leucate. Levels of CYP1A-immunopositive protein varied four-fold between sites and 15 inter-site comparisons were determined to be significantly different by the Fisher PLSD test (analysis of variance). This is contrasted by a 2.3-fold variation in BPH activity between sites and only three inter-site comparisons calculated to be different by the Fisher PLSD test.

Considering the results from the two studies using *M. edulis* and *M. galloprovincialis*, digestive gland CYP1A-immunopositive protein levels were determined in all microsomal preparations. In contrast, BPH activities in 13, 1 and 6 microsomal preparations (corresponding to the spring and summer UK studies and the summer Mediterranean study respectively) were below the detection limits of the assay. These 'zero' results have the potential to bias the estimated mean BPH activities for a specific site. In accordance with Michel *et al.* (1994), *M. galloprovincialis* BPH activities in the Mediterranean Sea study were determined with a constant digestive gland microsomal protein concentration in the assay. This methodological standardization and/or the low BPH activities detected, tended to shift the calculated microsomal specific activities towards an apparent discontinuous data set. As a consequence, a correlation determined in the same way as for the *M. edulis* study could not be undertaken. However a limited correlation could be made between mean BPH specific activities and mean CYP1A-immunopositive protein levels for each site (figure 6). Although there were only nine data points, a positive correlation ($R = 0.68$) was determined that fitted ($P = 0.05$) a linear regression model.

Status and potential of CYP1A-like protein/enzyme as a biomarker of organic pollution

Levels of hepatic CYP1A protein or EROD activity have been used world-wide in numerous fish studies as biomarkers of exposure to organic pollutants such as certain PAHs, PCBs, and related compounds (Livingstone 1996). Recent advances have demonstrated a CYP1A-like protein in certain mytilid species with the

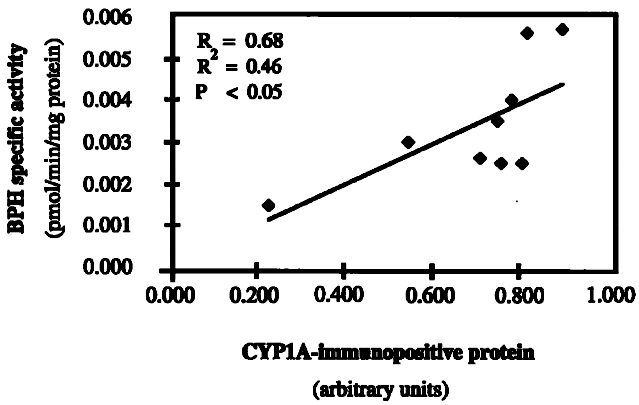

Figure 6. Correlation and regression of *Mytilus galloprovincialis* digestive gland microsomal mean benzo[*a*]pyrene hydroxylase (BPH) activity vs mean cytochrome P4501A (CYP1A)-immunopositive protein levels at each site sampled in the Mediterranean Sea.

potential for similar field applications (Peters *et al*. 1998a), although the sequence identity of this protein is unknown (i.e. whether it would be classified in the CYP1A or in a new unique CYP gene family—see Nelson *et al*. (1996) Livingstone and Goldfarb 1998, Peters and Livingstone 1998). Indirect evidence indicates the CYP1A-like protein is involved in microsomal BaP metabolism to phenols (Livingstone *et al*. 1997). Previous studies have demonstrated changes in mytilid digestive gland MFO parameters in response to exposures to PAHs and PCBs, indicating the selectivity of the MFO response (Livingstone 1985, 1987, Narbonne *et al*., 1991, Michel *et al*. 1993, 1994, Solé *et al*. 1996, Livingstone *et al*. 1997, Solé *et al*. 1998). However, despite these advances, a robust molluscan MFO biomarker for exposure to organic contaminants has not, to date, been applied world-wide in routine pollution monitoring.

Total BaP metabolites (determined radiometrically) were up to 2.5-fold higher in soft-tissue microsomes prepared from *M. galloprovincialis* collected at heavily PAH-contaminated compared with less contaminated sites (Narbonne *et al*. 1991). In the same study, a positive correlation between microsomal BaP metabolism and PAH levels in the sediment was observed, but only after three data points, representing 13 % of the samples, were rejected. Radiometric methods also showed a similar two-fold increase in digestive gland microsomal BaP metabolism following exposure to 3,3´,4,4´-tetrachlorobiphenyl (Michel *et al*. 1993). Using the fluorometric method to assay BPH activity, similar increases in MFO activities were observed in both field studies and experimental exposure to 3-methylcholanthrene (Michel *et al*. 1994). Digestive gland microsomal BPH activities were also elevated up to 2.4-fold in *M. edulis* transplanted from a clean reference to contaminated sites in the North Sea (Solé *et al*. 1998). However, in contrast, no changes in BPH activity (but increases in CYP1A-immunopositive protein), were observed in samples collected along a PAH-gradient following an oil spill (Solé *et al*. 1996). Although the above studies applied both radiometric and fluorometric methods for assessing BaP metabolism, it may be summarized that elevation of mytilid BPH activity, or BaP metabolism (total metabolites) did not occur in every study; and when the activity increased, the magnitude of the response was of the order of 2–3-fold. Such levels of response (i.e. differences between putative clean and contaminated sites) were also observed in digestive gland microsomal preparations following the *Sea Empress* oil spill and from field samples around the Mediterranean Sea (see previous section).

Partially purified *M. edulis* CYP showed catalytic properties similar to microsomal MFO activities (NADPH-dependent and NADPH-independent BaP metabolism) and a protein epitope recognized by an anti-perch (*P. fluviatilis*) CYP1A antibody (Porte *et al*. 1995). This partially purified CYP containing CYP1A-immunopositive protein subsequently added detection of a CYP1A-like protein in digestive gland microsomes of field *Mytilus* sp. (Solé *et al*. 1996, 1998, Peters *et al*. 1998a,b). The current *Sea Empress* and Mediterranean Sea studies (see previous section) showing positive correlations between CYP1A-immunopositive protein levels and BPH activities in digestive gland microsomes of *Mytilus* sp. from a wide range of geographical sites and exposure conditions, provide further indirect evidence that the CYP1A-like protein is involved in the metabolism of BaP to phenols. The positive correlations cannot of course, provide any direct insight into the functional relationship between the CYP1A-like protein and BPH activity, nor can it explain the lack of correlation between the two measurements in the studies

of Solé *et al*. (1996, 1998). However possible explanations for the elevation of CYP1A-like protein but not BPH activity could be inhibition of the latter by high PAH levels (Solé *et al*. 1996), whereas the reverse situation could be related to limited specificity of the anti-fish CYP1A antibody at low levels of induction of mussel CYP1A-like protein (Solé *et al*. 1998). Another factor in the latter discrepancy could be the time sequence of events in the induction process, in the synthesis of apoprotein and the appearance of catalytic activity, of which nothing is known in *Mytilus* sp. (Livingstone *et al*. 1997).

The previous and present studies provide strong evidence for the existence and biomarker potential of a CYP1A-like protein in digestive gland of *Mytilus* sp. The route to the development of a robust biomarker lies in the sequencing and identification of the protein, which is already underway (Herron and Falchk 1998). This in turn will lead to cDNA and antibody probes for gene regulation studies and field biomarker application; and to the characterization of catalytic properties through heterologous gene expression of CYP1A-like proteins.

Finally, in order to fully evaluate the CYP1A-like protein as a robust field biomarker, processes such as seasonal variation, nutritional status, the effects of sub-tidal vs intertidal sampling, and species polymorphism should be addressed (Livingstone 1996). Current studies in our laboratory are examining seasonal variation of *M. edulis* digestive gland CYP-immunopositive protein forms at different UK field sites, with other parameters such as 'scope for growth', DNA damage (strand breakage and adduct formation), contaminant body burden, reproductive status, growth and population structure.

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