Hepatic CYP1A induction in rainbow trout (*Oncorhynchus mykiss*) after exposure to benzo[*a*]pyrene in water

Morten Sandvik, Tor Einar Horsberg, Janneche Utne Skaare and Kristian Ingebrigtsen

Juvenile rainbow trout were exposed to unlabelled benzo[*a*]pyrene (BaP) and ^{3}H-benzo[*a*]pyrene (^{3}H-BaP), in a static exposure system for 2 days. The initial concentration was 30 μg l^{-1} and 0.625 μCi l^{-1}, corresponding to 6 mg kg^{-1} body weight and 125 μCi kg^{-1} body weight. Hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity was measured during the exposure and depuration periods, elucidating the time-course pattern of CYP1A induction. Maximum induction (11-fold) of EROD activity was observed on day 2 after addition of BaP to the water. Tissue distribution of ^{3}H-BaP was studied by liquid scintillation counting and whole-body autoradiography. The concentration of ^{3}H-BaP-derived radioactivity was highest in the bile at all sampling times. High levels of radiolabelled compound were also present in the gills, liver and the olfactory organ. There was an overall decrease in all tissues during the depuration period. The elimination of ^{3}H-BaP-derived radioactivity from the gills, however, was slow compared with liver and blood (6.2 days vs 2.7 and 2.9 days, respectively).

Keywords: *Benzo[a]pyrene, rainbow trout, CYP1A induction, whole-body autoradiography.*

Introduction

Polyaromatic hydrocarbons (PAHs) are widespread environmental contaminants of concern, mainly because of the mutagenicity and carcinogenicity of the 4- to 7-ring PAHs (Grimmer *et al.* 1981). The main sources are petroleum spillage and dry fallout from the atmosphere or rainfall. Most of the PAHs entering the aquatic environment remain close to sites of deposition, so that lakes, rivers, and coastal marine environments near centres of human population are primary repositories. There have been several reports of an increased incidence of cancer-like lesions in aquatic animals living in areas with PAH-contaminated sediments. These findings provide strong evidence of a link between mutagenic PAHs in sediments and tissue lesions in bottom-living fish (Hodgins *et al.* 1977, Varanasi *et al.* 1986).

The cytochrome P450-dependent monooxygenase system (CYP) plays a key role in the toxicity of PAHs. Even though many CYPs, including CYP1A, are involved in metabolic detoxification pathways (Gonzalez 1990), highly reactive electrophilic intermediates are produced during metabolism, and these may, unless further metabolism occurs, react with DNA and other cellular macromolecules (Conney 1982). CYP1A is probably solely responsible for 7-ethoxyresorufin-*O*-deethylase (EROD) activity and is highly inducible upon exposure to PAHs, some polychlorinated biphenols (PCBs) and polychlorinated dibenzofuranes and dibenzodioxins (Skaare *et al.* 1991, Stegeman and Lech 1991, Bernhoft *et al.* 1994, Hektoen *et al.* 1994, Goksøyr 1995). The induction of the CYP1A system has therefore been used as a biochemical indicator of pollution in the aquatic environment (Payne *et al.* 1987). Biomarkers are defined as sub-lethal biological measures of responses to, and effects of, pollutants in living organisms (Peakall 1994). Generally, biomarkers are selected for their responses to particular classes of environmental contaminants rather than to specific individual chemicals. PAHs, some PCBs, and polychlorinated dibenzofuranes and dibenzodioxins are potent inducers of the CYP1A family of the cytochrome P450-dependent monooxygenase system (CYP), and CYP1A activity had therefore been used as a biochemical indicator of pollution in the aquatic environment (Payne *et al.* 1987). The interpretation of such data requires comprehensive knowledge of dose–response relationships after various routes of exposure to CYP1A-inducers. The aims of the present work were to investigate the time-course pattern of hepatic CYP1A induction in rainbow trout after exposure to BaP in water, and to study the tissue distribution and elimination of the inducer.

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MATERIALS AND METHODS

Chemicals

BaP was obtained from British Greyhound Chromatography and Allied Chemicals (Birkenhead, Merseyside, UK), ^{3}H-BaP (specific activity, 57 Ci mmol^{-1}; radiochemical purity, 98.9%) from Amersham Life Science (Amersham, UK), ethoxyresorufin and NADPH from Sigma Chemical Co. (St Louis, MO, USA), while Bio-Rad protein assay was obtained from Bio-Rad Laboratories GmbH (Munchen, Germany). All other chemicals were of analytical grade.

Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes, weighing 85 ± 15 g, were obtained from a commercial hatchery in late May. The fish were kept in two 600 l fibre-glass tanks with aerated, filtered (biological filters) and recirculating fresh water at 9–10 °C. Lighting was artificially adjusted to a 12-h light (7 am to 7 pm) – 12-h dark cycle. The fish were acclimatized in the fibre-glass tanks for 2 weeks before treatment. They were fed fresh fillets of green pollack (*Pollachius virens*) once daily. Commercially pelleted fish feed was avoided, as it may contain etoxyquin, an inductor of CYP1A-activities.

Treatment

One week before dosing, 48 fish were transferred to four 200 l glass tanks with the same holding conditions as the fibre-glass tanks. The biomass in each tank was 6 g l^{-1} (12 fish). The fish were starved 3 days before exposure. After removal of pumps and filters, 600 μl of a solution of unlabelled BaP and ^{3}H-BaP in acetone was added to the water in two tanks, to give a final concentration of 30 μg l^{-1} and 0.625 μCi l^{-1}, corresponding to 6 mg kg^{-1} fish and 125 μCi kg^{-1} fish. Acetone only

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was added to the other two tanks. The exposure period was 2 days in aerated water. The water was then renewed, and thereafter renewed every second day during the depuration period. Pumps and filters containing charcoal were reconnected to remove possible radiolabelled materials excreted from the fish. Four BaP-exposed fish and four corresponding controls were sacrificed by a blow on the head 1, 2, 4, 6, 8, and 10 days after the start of exposure. In order to study tissue distribution by means of whole body autoradiography, eight fish were exposed to Bap and ^{3}H-BaP in the water in a separate tank. The holding conditions and treatment were the same as for the other BaP-exposed fish, except that the concentration of ^{3}H-BaP in the water was 7.5 μCi l^{-1} (1.5 mCi kg^{-1} fish). Two fish were euthanized with an overdose of benzocaine in water 2, 4, 6 and 10 days after dosing. They were immediately frozen in liquid nitrogen and prepared for whole-body autoradiography.

Preparation of microsomes and enzyme assays

The livers were removed carefully without disrupting the gall bladder and the microsomes were prepared as described by Beyer *et al.* (1996). Enzyme activities (EROD) were recorded immediately by the method described by Klotz *et al.* (1984), with the exception that the assay was carried out in 0.1 M Na-phosphate buffer (pH 7.4), and at an assay temperature of 20°C. The protein content was determined by the method of Bradford (1976) using lyophilized bovine serum albumin as standard.

Liquid scintillation counting

Samples (10–200 mg) of gill, muscle, skin, bile, liver, blood, trunk kidney and head kidney were digested in Soluene-350 (Packard) (1 ml per sample) for 24 h. Hionic fluor (Packard) scintillation fluid (10 ml per sample) was then added. All samples were equilibrated at room temperature for 24 h before counting in a Packard Tri-Carb 1900 CA liquid scintillation analyser. The counting efficacy was controlled by a Packard automatic ^{3}H-quenching standard. Tissue concentrations of BaP or its metabolites were calculated from counts (dpm mg^{-1}) in each tissue, and the specific radioactivity of ^{3}H-BaP. The results are presented as BaP equivalents g^{-1} (BaP equivalents ml^{-1} for blood and bile).

Whole-body autoradiography

The fish were embedded in a 1% (w/v) carboxymethylcellulose gel and immersed in a bath of hexane and solid carbon dioxide (–75°C). Sagittal whole-body sections (30 μm) from different levels of the body were collected at –20°C on adhesive tape (No. 821, 3M Co., St Paul, MN, USA) in a PMV cryomicrotome (PMV 450 MP, Palmstierna Mekaniska Verkstad, Stockholm, Sweden) as described by Ullberg (1954, 1977). After freeze-drying at –20°C, the sections were exposed to Hyperfilm-^{3}H (Amersham, UK). The photographic exposure period was 3 months.

Statistical methods

The datasets were examined for significant difference ($p<0.05$) according to Student's *t*-test. Log transformations of the sample data were employed in order to allow the use of parametric statistical methods. Parametric tests were preceded by Bartlett's test for homogeneity of variance (JMP® software, version 3.0.2, SAS Institute Inc., Cary, NC, USA). The data from the depletion study were summarized using the statistical software program Excel Analysis Toolpack (version 1 from GreyMatter International Inc., Cambridge, MA, USA). Tissue concentrations of BaP-derived radioactivity in the liver were correlated with hepatic CYP1A levels. Log-transformed tissue concentration of BaP was regressed against sampling time. Regression lines were fitted for each organ and 95% confidence intervals for the slopes obtained. Elimination rates were regarded as being significantly different in two compartments when there was no overlap of 95% confidence intervals for their respective slopes (Sokal and Rohlf 1981). ND (not detected) values at time points where both ND and positive values were found, were assigned a random value between the limit of detection and zero (Bhattacharyya and Johnson 1977).

Results

CYP1A induction

The time-course patter of hepatic EROD activity following exposure to BaP in water is shown in Figure 1. A significant induction of EROD activity was observed 1 day after the start of the exposure, the activity on day 2 being 11-fold higher than in the corresponding controls. When the BaP-exposed fish were transferred to clean water after 2 days of exposure, the enzyme activity decreased towards control levels by day 10. The EROD activity, nevertheless, was still significantly higher than the corresponding controls at 4, 6 and 8 days after exposure. The multiple regression analysis showed that the hepatic CYP1A levels were positively correlated (r^2=0.98) to the levels of BaP-derived radioactivity in the liver during the depuration phase (day 2 to day 10).

Tissue distribution and elimination of BaP-derived radioactivity

^{3}H-BaP-derived radioactivity was present in all tissues analysed 1 day after the start of the exposure (Table 1). The bile contained the highest concentration of BaP-derived radioactivity at all time points, peak levels being reached 4 days after dosing. Peak levels in liver, muscle, gill and blood were observed after 1 day of exposure, while the highest concentrations in skin, trunk kidney and head kidney were reached after 2 days. The concentration decreased in all these tissues during the depuration period. The estimated half-lives of BaP-derived radioactivity in liver, gills and blood were 2.9, 6.2 and 2.7 days, respectively (Figure 2).

The results from the autoradiographic study were in good agreement with those from the liquid scintillation counting. The highest concentrations of radiolabelled compound were

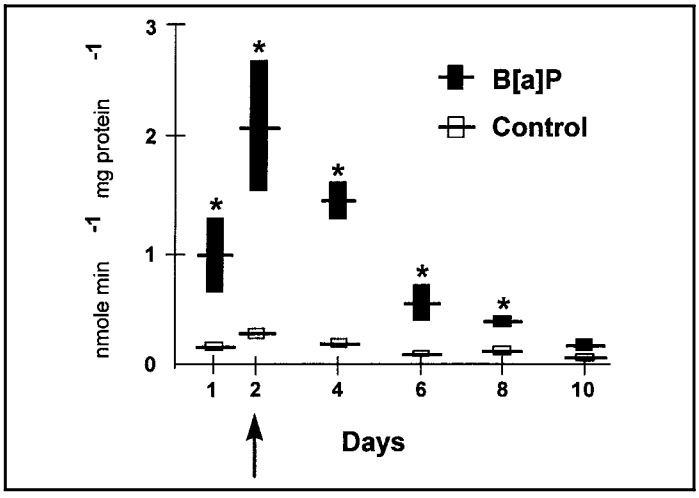

Figure 1. Hepatic CYP1A activity (EROD) in rainbow trout after 2 days of exposure to BaP in water (30 μl l^{-1}). The fish were transferred to clean water after day 2 (↑). The results are given as means±SE (n=4). *: Significantly different from corresponding controls.

Days after dosing	Liver	Muscle	Skin	Gill	Head kidney	Trunk kidney	Bile[a]	Blood[a]
1	4.054±0.813	0.011±0.001	0.156±0.024	0.968±0.120	0.065±0.009	0.119±0.031	30.880±4.422	0.062±0.007
2	3.724±0.789	0.009±0.002	0.272±0.044	0.914±0.165	0.187±0.106	0.294±0.095	71.520±7.831	0.043±0.012
4	2.845±0.571	0.004±0.001	0.075±0.003	0.560±0.071	0.081±0.029	0.192±0.053	92.591±14.873	0.022±0.009
6	1.447±0.490	0.004±0.001	0.052±0.009	0.548±0.046	0.157±0.078	0.099±0.010	66.736±19.431	0.009±0.005
8	1.003±0.107	0.002±0.001	0.045±0.006	0.478±0.058	0.090±0.006	0.170±0.071	34.205±9.666	0.014±0.005
10	0.505±0.050	0.004±0.001	0.030±0.005	0.309±0.020	0.096±0.023	0.018±0.008	41.705±14.171	0.001±0.001

Table 1. BaP equivalents in tissues (μg g^{-1}) of rainbow trout after administration of BaP in water (30 μg l^{-1}). The fish were transferred to clean water after day 2. The values are expressed as BaP or metabolically transformed equivalents, calculated from counts (dpm g^{-1}) in each tissue, and the specific radioactivity of ^{3}H-BaP.

[a]μg ml^{-1}.

Values are means±SE (n=4).

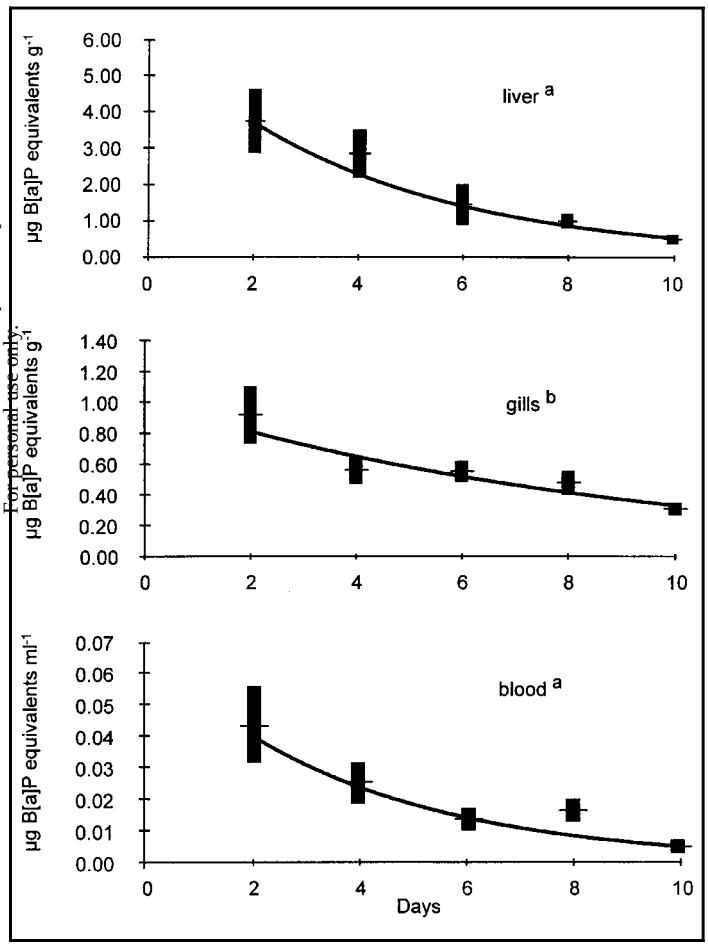

Figure 2. Predicted elimination rates (—) and tissue levels in rainbow trout during depuration following 2 days of exposure to BaP (30 μg l^{-1}) in water. The values are expressed as BaP equivalents or metabolically transformed equivalents, calculated from counts (dpm g^{-1}) in each tissue, and the specific radioactivity of ^{3}H-BaP. The results are given as means±SE (n=4). Half-lives of BaP-equivalents in liver, gills and blood were 2.9, 6.2 and 2.7 days, respectively. Elimination rates in organs with the same letter were not significantly different.

found in the gills, liver, bile, intestinal contents, intestinal mucosa and the olfactory organ (Figures 3 and 4). The level of radiolabelled compound in the intestinal mucosa increased during the experimental period, whereas those in the other tissues decreased. At day 10, however, radioactivity was still present in the gills, liver, intestinal mucosa and the olfactory organ.

Discussion

Extensive field studies and experimental work have been carried out in order to establish the relationship between exposure to CYP1A-inducers and the responses in terms of CYP1A activity in various species (Stegeman 1993, Goksøyr 1995). The majority of dose–response and time-course studies have involved oral and intraperitoneal administration. Experimental studies where fish have been exposed to CYP1A-inducers in the surrounding water, however, are scarce (Gerhart and Carlson 1978, Smolowitz *et al.* 1992, Levine *et al.* 1994).

In our study, an 11-fold induction of EROD activity was observed in the liver following 2 days of exposure to 30 μg l^{-1} BaP in water. The pattern observed, with an initial rapid increase in EROD activity followed by a slow decrease during depuration, is in accordance with previous studies in which rainbow trout and other fish species have been administered single doses of PAH-type inducers (Collier and Varanasi 1991, Upshall *et al.* 1993, Eggens and Boon 1996). The dose of aqueous BaP corresponded to a body burden of 6 mg kg^{-1} b.w. This is within the range of reported doses used in earlier studies with different routes of administration of BaP in fish (Upshall *et al.* 1993, Beyer *et al.* 1996). The dose level of aqueous BaP was exceeding its solubility in water. However, levels up to 1 mg l^{-1} with waterborne BaP have previously been reported (Smolowitz *et al.* 1992). The positive correlation (r^2=0.98) between the hepatic CYP1A activities and the levels of BaP-derived radioactivity in the liver during the depuration phase strongly suggests that the induction response reflects the kinetics of the inducer. This is supported by the longer lasting induction response of CYP1A in studies where more persistent compounds such as 2,3,7,8-TCDD and certain PCBs have been employed as inducers (Hektoen *et al.* 1994, Beyer *et al.* 1996).

Liquid scintillation counting and tape section autoradiography were employed in order to obtain information on the tissue distribution and elimination of BaP and its metabolites. When using these techniques it should be

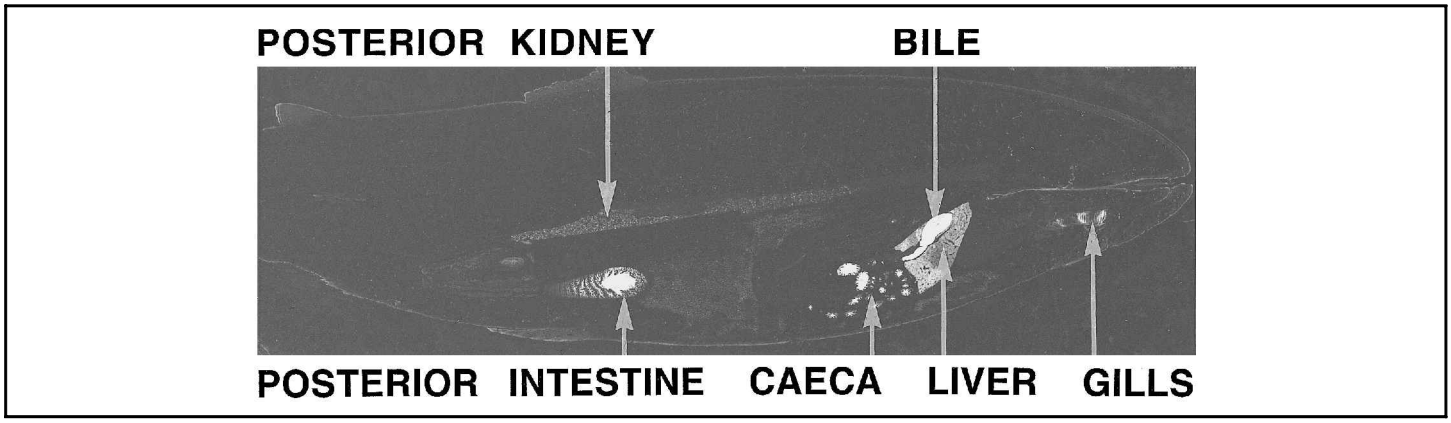

Figure 3. Autoradiogram of rainbow trout 2 days after exposure to ^{3}H-BaP in water (30 μg l^{-1} and 7.5 μCi l^{-1}: 6 mg kg body weight and 1.5 mCi kg^{-1}). White areas correspond to high concentrations of radiolabelled compound. Note radioactivity in the liver, bile and intestinal tract.

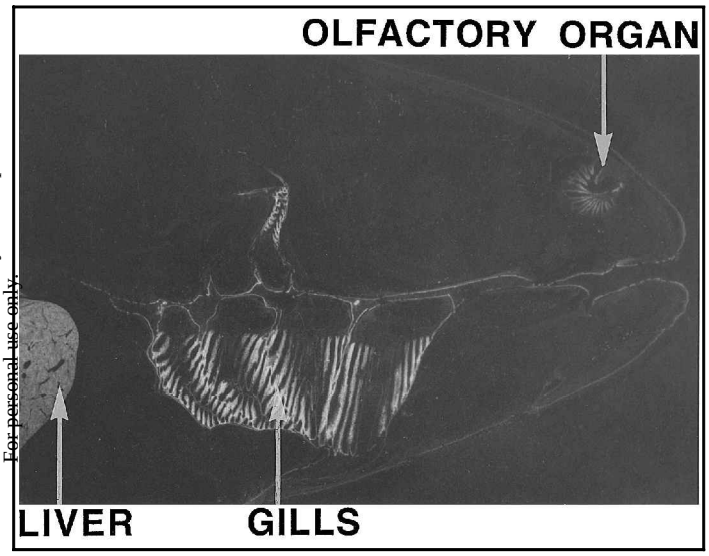

Figure 4. Autoradiogram of the head region of rainbow trout 2 days after exposure to ^{3}H-BaP in water (30 μg l^{-1} and 7.5 μCi l^{-1}: 6 mg kg body weight and 1.5 mCi kg^{-1}). White areas correspond to high concentrations of radiolabelled compound. Note radioactivity in the gills and olfactory organ.

borne in mind that the observed radiolabelling reflects the sum of radioactivity of the parent compound and its metabolites. In the present study no further attempts were done to characterize or quantify the parent compound or its metabolites. However, studies on metabolism of BaP in rainbow trout and other fish species indicate that BaP is readily metabolized and that a large portion is converted to water-soluble metabolites, mainly glucuronic acid conjugates (Ahokas *et al.* 1977, Plakunov *et al.* 1987).

The high concentration of radiolabelled compound in the gills obviously reflects the role of this organ as the main absorption site for aqueous BaP. Xenobiotics absorbed through the gills are distributed to various organs via the dorsal aorta. The fish kidney receives a major fraction of the arterial blood, whereas the liver is mainly supplied with venous blood. Accordingly, it has been suggested that the kidney may contribute significantly to the removal of many xenobiotics by urinary excretion (Klaassen 1975). In the present study, however, the concentration of BaP-derived radioactivity in the liver was 40- and 10-fold higher than in the trunk kidney at days 1 and 2, respectively. This observation, together with the high and increasing levels of radioactivity in the bile, suggest that BaP and metabolites are mainly excreted by the hepatobiliary route in rainbow trout, and that renal excretion plays a minor role only.

The autoradiographic study revealed a high amount of radiolabelled compound in the olfactory organ, implicating that this tissue may be a susceptible target organ for PAHs in the water. This is supported by Smolowitz *et al.* (1992), who reported a strong CYP1A induction in the olfactory epithelium of topminnows (*Poeciliopsis lucida*) following exposure to BaP in water. On the other hand, no induction occurred when BaP was injected intraperitoneally, indicating that the olfactory epithelium is more heavily exposed to the inducer when this is administered via water. Accordingly, the authors suggested that the olfactory organ may represent a specific marker tissue for waterborne exposure to CYP1A inducers.

The observed half-lives of BaP-derived radioactivity in the liver and blood were 2.7 and 2.9 days, respectively. This is in accordance with previous studies of BaP depuration in rainbow trout and other fish species (Niimi 1987, Lemaire *et al.* 1990). Elimination of BaP-derived radioactivity was, however, significantly slower in the gills ($t_{\frac{1}{2}}$=6.2 days). PAH-type compounds are capable of eliciting strong induction of CYP1A proteins in many organs of fish (Miller *et al.* 1989, Stegeman *et al.* 1991), including the gills (Smolowitz *et al.* 1992, Husøy *et al.* 1996). Furthermore, Steward *et al.* (1990) found significant amounts of BaP-derived radioactivity irreversibly bound to macromolecules in tissues of brown bullhead (*Ictalurus nebulosus*) following intraperitoneal administration. This radioactivity was most likely due to reactive BaP intermediates formed by CYP1A-mediated metabolism. Finally, Andersson and Pärt (1989), employing a perfused gill preparation, found that BaP was extensively metabolized in the gills of rainbow trout. They concluded that the gills express the enzyme systems needed for formation of reactive intermediates which may bind covalently to essential macromolecules and thereby injure cell function. Whether BaP metabolites covalently bound to

tissue macromolecules partly contribute to levels of radiolabelled compound observed in the gills of rainbow trout in the present study, is not known at present. Nevertheless, the high concentration of BaP-derived radioactivity in the gills throughout the experimental period renders this tissue of particular interest as a potential target organ for waterborne exposure to PAHs. The possibility of using the gills as a specific marker tissue for waterborne exposure to PAHs should be explored further.

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