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Comparison of dietary and waterborne exposure to benzo[*a*]pyrene: bioavailability, tissue disposition and CYP1A1 induction in rainbow trout (*Oncorhynchus mykiss*)

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Absorption and tissue distribution of benzo[*a*]pyrene (BaP)-derived radioactivity were studied in juvenile rainbow trout following dietary or waterborne exposure. In order to compare the bioavailability of BaP, the fish were exposed to 1·5 mCi ^{3}H-BaP kg^{-1} fish, either in the diet or in the water as a 2 days static exposure. Furthermore, tissue levels of BaP-derived radioactivity bound to macromolecules in different tissues were studied in non-induced fish, and in fish induced by additional treatment with unlabelled BaP (corresponding to 5 mg kg^{-1} fish) in the water. Absorption and tissue distribution of ^{3}H-BaP were studied by liquid scintillation counting and whole-body autoradiography. BaP-derived radioactivity bound to macromolecules in different tissues was studied by autoradiography of solvent-extracted whole-body sections. The hepatic CYP1A induction was measured as EROD activity. Exposure to unlabelled BaP resulted in a marked induction of hepatic EROD activity in rainbow trout 2 days after the start of the exposure. Significant higher concentrations of radiolabelled compound were observed in waterborne-exposed fish, in contrast to dietary-exposed fish. High concentrations of radiolabelling were observed in the gills, liver, bile, intestines, olfactory organ, kidney and the skin of the waterborne-exposed fish. In the dietary-exposed fish, high levels of radioactivity were observed in the intestines and the bile, whereas lower concentrations were present in the liver. Only traces of radioactive compound were observed in the gills. In contrast to waterborne-exposed fish, no radioactivity was detected in the olfactory organ or skin. In autoradiograms of sections extracted with a series of polar and non-polar solvents, a large fraction of radioactivity was still present in the gills, olfactory organ, liver, kidney, skin and intestinal mucosa of the waterborne-exposed fish, indicating that reactive BaP intermediates formed by CYP1A-mediated metabolism were bound to macromolecules in these tissues.

Keywords: rainbow trout, benzo[*a*]pyrene, bioavailability, tissue distribution, CYP1A induction.

Introduction

The polycyclic aromatic hydrocarbons (PAHs) include a large group of chemicals that have toxic properties and are found at elevated concentrations in some aquatic ecosystems. High frequencies of preneoplastic- and neoplastic lesions in fish living in areas with high PAH levels have increased the environmental concern about these chemicals (Nishimoto and Varanasi 1985, Malins *et al.* 1988). The mutagenicity and carcinogenicity of mainly the four to seven ring PAHs (Grimmer *et al.* 1981) are linked to their metabolism through the cytochrome P450 1A monooxygenase system (CYP1A). Fish appear to be more susceptible to DNA damage caused by BaP metabolites as the repair mechanisms in

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less efficient than in mammals (Walton *et al.* 1987). In previous studies, high amounts of BaP-derived radioactivity irreversibly bound to macromolecules in several fish tissues have been observed (Steward *et al.* 1990). Furthermore, it has been suggested that CYP1A induction in the olfactory organ may bear a direct relationship to waterborne exposure to CYP1A-inducing compounds (Smolowitz *et al.* 1992). Finally, significant amounts of radiolabelling in the gills and olfactory organ of rainbow trout following waterborne exposure to ^{3}H-BaP have recently been reported (Sandvik *et al.* 1997). In order to examine whether specific tissues could candidate to serve as markers for different routes of exposure to CYP1A inducers, the tissue disposition of BaP-derived radioactivity was studied in rainbow trout following either dietary or waterborne exposure to ^{3}H-BaP.

Materials and methods

Chemicals

BaP was obtained from British Greyhound Chromatography and Allied Chemicals (Birkenhead, Meyerside, 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 (Munich, Germany). All other chemicals were of analytical grade.

Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes, in the range of 110–154 g were obtained from a commercial hatchery . The fish were kept in fibre-glass tanks (500 l) with aerated, filtered (biological filters) and recirculating fresh water at 9–10 °C. Lighting was artificially adjusted to a 12-h light, 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. One week before exposure, the fish were transferred to glass tanks (60 or 200 l) with the same holding conditions as the fibre-glass tanks. The fish were fasted for 3 days before exposure. The biomass in each glass tank was 5 g l^{-1}. The experiments were carried out under static conditions after removal of pumps and filters. The water was renewed after 2 days, and thereafter every second day

CYP1A induction by waterborne BaP

The degree of CYP1A induction was studied in eight fish (132 ± 9g) exposed to BaP by addition of 500 µl unlabelled BaP in acetone to give a final concentration of 25 µg l^{-1}; corresponding to 5 mg kg^{-1} fish. The exposure period was 2 days in aerated water. Four fish were sampled after 2 days, and the remaining fish were sampled after 4 days. The activity was compared with a corresponding number of fish receiving acetone only.

The livers were removed carefully without disrupting the gall bladder and the microsomes were prepared as described by Beyer *et al.* (1996). Hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity was 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

Waterborne exposure with ^{3}H-BaP

Eight fish weighing 128 ± 19 g were transferred to a 200 l glass tank, and ^{3}H-BaP was added to the water to give a final concentration of 7·5 µCi l^{-1} (1·5 mCi kg^{-1} fish). The exposure period was 2 days in aerated water.

To determine the distribution pattern of ^{3}H-BaP in CYP1A-induced fish, eight fish weighing 141 ± 9 g were treated with ^{3}H-BaP and unlabelled BaP in the water as described above. Two fish from each group 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.

Dietary exposure with ^{3}H-BaP

Eight fish weighing 101 ± 16 g were given a test diet (0·5 g kg^{-1} fish) by a single intragastric administration. The diet was prepared by dissolving ^{3}H-BaP in acetone, and

syringes loaded with fine grounded trout pellets. The syringes were placed in a fume hood in order to evaporate the solvent. The fish were administered pellets containing ^{3}H-BaP (3 mCi g^{-1} diet; corresponding to 1·5 mCi kg^{-1} fish), and transferred to a 200 l glass tank. 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.

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 (40 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). From each fish sagittal whole-body sections (40 µm) were taken through the entire body. All tissues were freeze-dried. Every other tissue section was extracted successively with 5 % trichloroacetic acid for 1 min, 50 % methanol for 30 s, 100% methanol for 30 s, heptane for 10 s, 100 % methanol for 30 s, 50 % methanol for 30 s and thereafter rinsed in tap water for 5 min. The sections were pressed against X-ray films (Hyperfilm-^{3}H, Amersham, UK) for 3 months. Autoradiograms obtained from the freeze-dried sections were considered to represent unchanged BaP plus its metabolites, while autoradiograms obtained from the freeze-dried solvent-extracted sections were considered to represent irreversibly bound metabolites of BaP (Bergman 1979, Brandt and Brittebo 1989).

Liquid scintillation counting

Samples (10–200 mg) of gills and liver were prepared from the carcasses remaining after the preparation of whole-body sections. The samples 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 efficiency was controlled by a Packard automatic ^{3}H-quenching standard, and the results are presented as dpm mg tissue^{-1}.

Statistical methods

The datasets were analysed 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). In order to test for differences in tissue levels of BaP-derived activity between fish exposed to dietary or waterborne BaP, the data were compared by using Wilcoxon´s ranked sign test. A significance level of $\alpha = 0{\cdot}05$ was chosen.

Results

Tissue distribution of BaP-derived radioactivity

Significantly higher concentrations of ^{3}H-BaP-derived radioactivity were observed in the tissues from fish subjected to waterborne exposure, compared with those given the test compound intragastrically (figure 1). Furthermore, in fish exposed via water, the levels of radioactivity were significantly higher in non-induced fish compared with the induced group, as determined by the Wilcoxon test.

The results from the autoradiographic study were in good agreement with those from the liquid scintillation counting. The autoradiograms from the fish exposed to ^{3}H-BaP via water generally showed higher radiolabelling than autoradiograms from dietary-exposed fish. In the fish exposed via water (figure 2), high concentrations of radiolabelled compound were observed in the gills, olfactory organ, skin, kidney, urine, liver, bile, intestinal contents and intestinal mucosa. In the dietary-exposed fish, high concentrations of radioactivity were observed in the gastrointestinal contents, intestinal mucosa and the bile, whereas the labelling in the liver was low (figure 3). In contrast to waterborne-exposed fish, only traces of radioactive compound were observed in the gills, and no radioactivity was observed in the olfactory organ and skin.

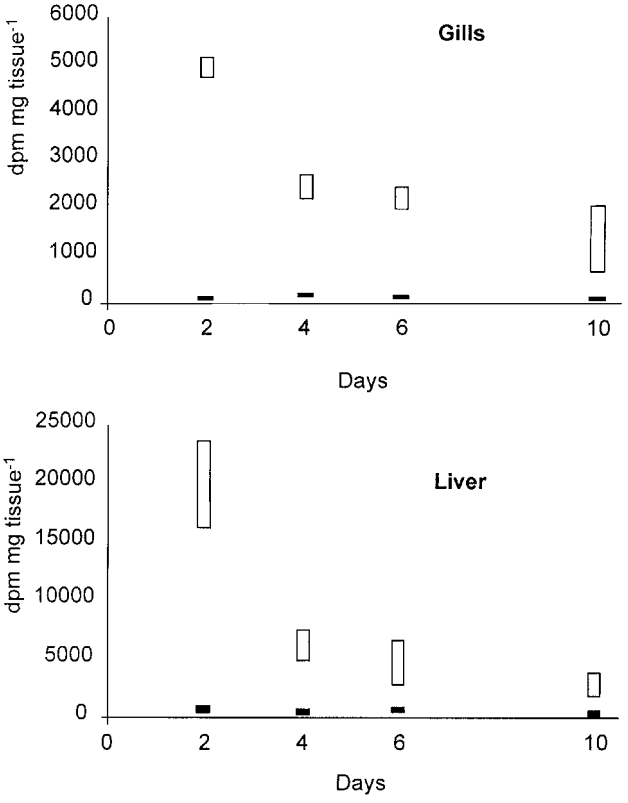

Figure 1. Concentrations of BaP-derived radioactivity (dpm mg tissue^{-1}) in gills and liver of rainbow trout following exposure to ^{3}H-BaP. The fish were exposed to 1·5 mCi kg $^{-1}$ via water (□), or 1·5 mCi kg^{-1} by intragastric administration (■). Each bar represents the range of two observations.

CYP1A induction

Table 1 shows the results of the EROD measurements. Exposure to BaP caused a marked induction of hepatic EROD activity 2 days after the start of the exposure, the activity being 10–50-fold higher than in the corresponding controls. The induction persisted for 4 days.

Tissue binding of BaP-derived radioactivity

In the solvent-extracted sections from fish exposed via water, a large fraction of radioactivity remained in the gills and olfactory organ (figure 4), the liver and intestinal mucosa (figure 5) and in the trunk kidney (figure 6). No differences in non-extractable radioactivity could be observed between non-induced and induced fish (see Table 2). In dietary-exposed fish, non-extractable radioactivity was present in the intestinal mucosa.

Discussion

The tissue levels of ^{3}H-BaP-derived radioactivity in the gills and liver after waterborne exposure was considerably higher than after dietary exposure. This

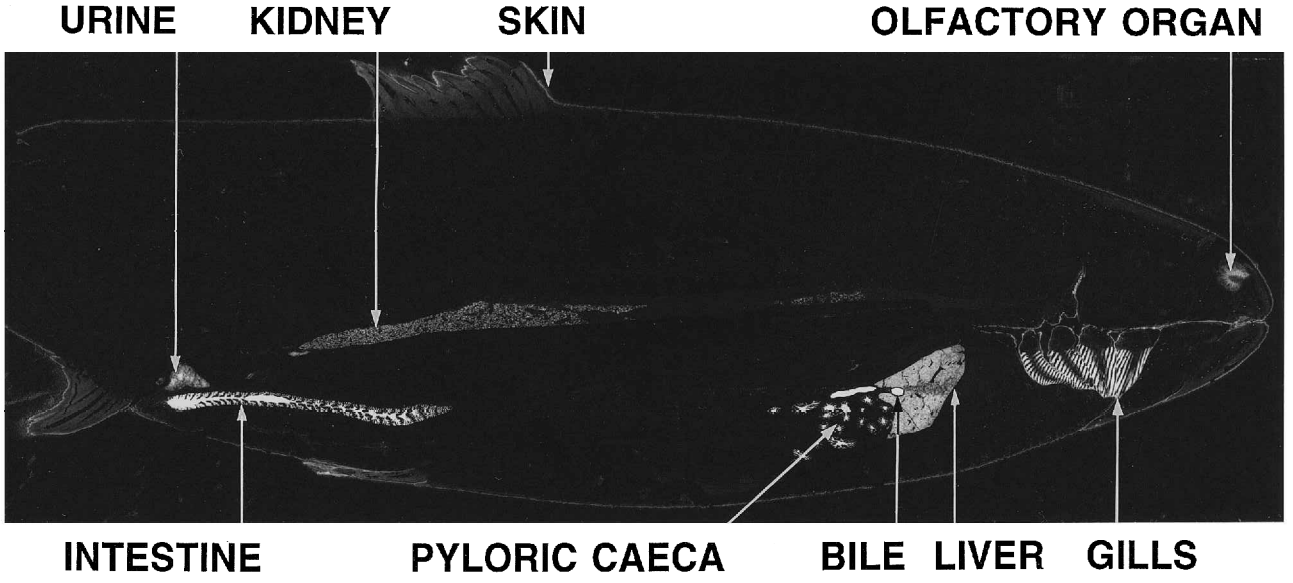

Figure 2. Autoradiogram of rainbow trout 2 days after exposure to ^{3}H-benzo[*a*]pyrene (1·5 mCi kg^{-1}) in water. White areas correspond to high concentrations of radiolabelled compound. Note radiolabelling in the olfactory organ, gills, liver, bile, kidney, urine, intestinal tract and skin.

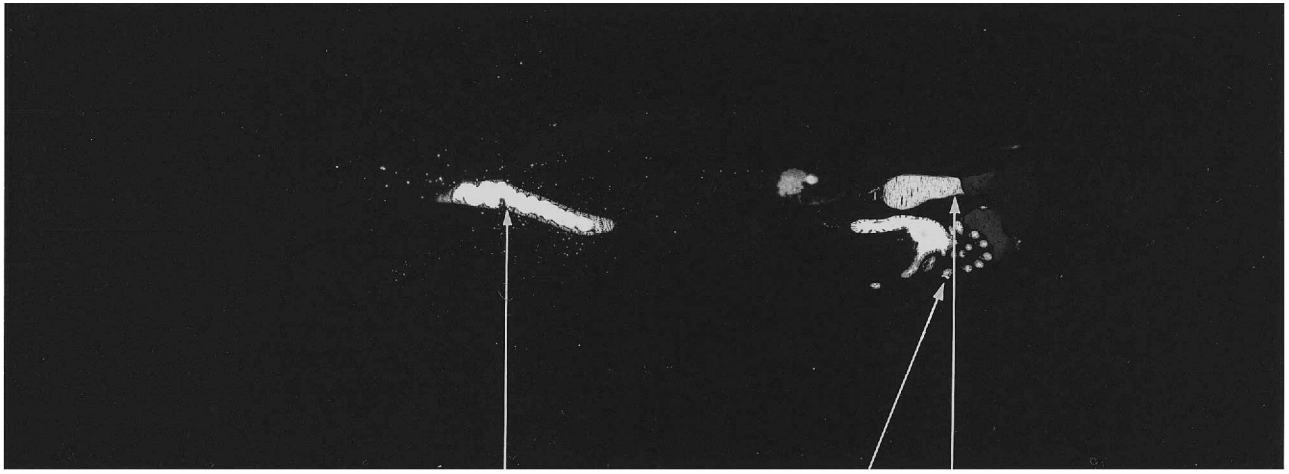

Figure 3. Autoradiogram of rainbow trout 2 days after intragastric administration of ^{3}H-benzo[*a*]pyrene (1·5 mCi kg^{-1}). White areas correspond to high concentrations of radiolabelled compound. Note that radiolabelling is present in the gastrointestinal tract and bile only.

Table 1. Hepatic EROD activities (pmoles min^{-1} mg $protein^{-1}$) in rainbow trout following waterborne exposure with BaP (25 µg l^{-1}, corresponding to 5 mg kg^{-1}).

Group	Days after exposure	EROD
Control	0	19 ± 6
Control	2	18 ± 2
Control	4	19 ± 4
B[*a*]P	2	641 ± 100*
B[*a*]P	4	341 ± 204*

Values are means ± SEM (4).
* Significantly different from corresponding controls.

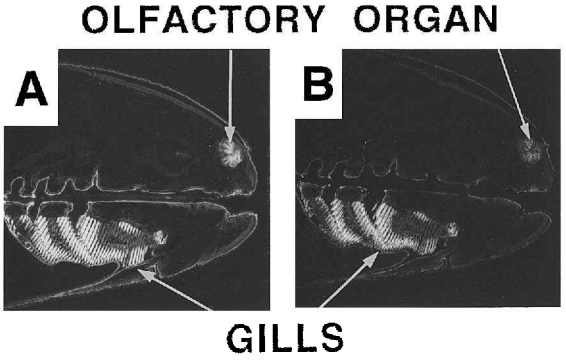

Figure 4. Autoradiograms of the head region of rainbow trout 2 days after exposure to ^{3}H-benzo[*a*]pyrene in water (1·5 mCi kg^{-1}). (A) Autoradiogram of a freeze-dried section and (B) autoradiogram of an adjacent section extracted with a series of polar and non-polar solvents prior to film exposure. White areas correspond to high concentrations of radiolabelled compound. Note tissue-bound radiolabelling in the olfactory organ and gills in B.

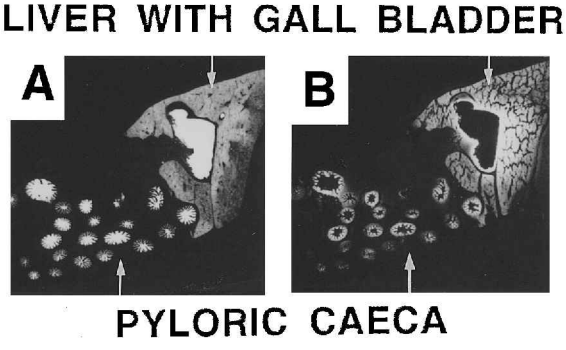

Figure 5. Autoradiograms of the intestinal region of rainbow trout 2 days after exposure to ^{3}H-benzo[*a*]pyrene in water (1·5 mCi kg^{-1}). (A) Autoradiogram of a freeze-dried section and (B) autoradiogram of an adjacent section extracted with a series of polar and non-polar solvents prior to film exposure. White areas correspond to high concentrations of radiolabelled compound. Note tissue-bound radiolabelling in the liver and intestinal mucosa in B.

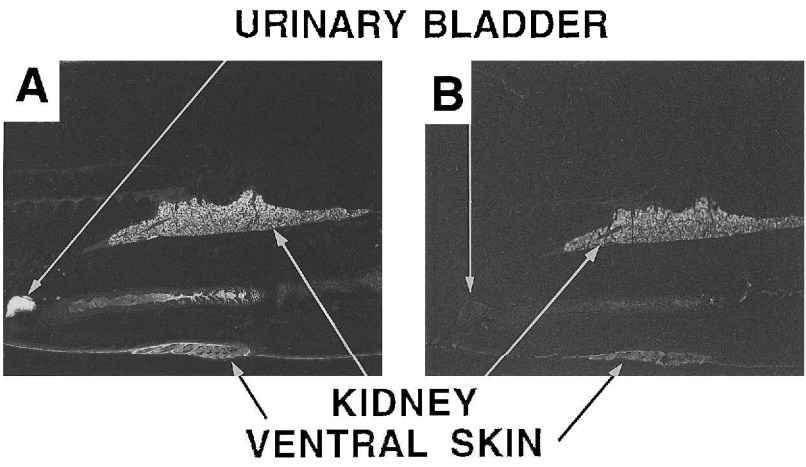

Figure 6. Autoradiograms of the trunk region of rainbow trout 2 days after exposure to ^{3}H-benzo[*a*]pyrene in water (1·5 mCi kg^{-1}). (A) Autoradiogram of a freeze-dried section and (B) autoradiogram of an adjacent section extracted with a series of polar and non-polar solvents prior to film exposure. White areas correspond to high concentrations of radiolabelled compound. Note tissue-bound radiolabelling in the kidney in B.

indicates an efficient absorption via the gills and that the systemic availability of the compound is higher after waterborne compared to dietary exposure. Fish show considerable interspecies variability in absorption of PAH from feed and more than half of an orally administered dose may remain in the digestive tract adsorbed to feed particles (Corner *et al.* 1976, Whittle *et al.* 1977). However, species differences in absorption of dietary BaP may also partly be explained by the activities of enzyme systems expressed in the digestive tract. Both phase 1 and phase 2 metabolizing enzymes are present in the intestine of rainbow trout and may contribute significantly to the biotransformation of xenobiotics occurring in the feed (Lindstrøm-Seppä *et al.* 1981). Therefore, both adsorption to feed particles as well as intestinal metabolism may have contributed to the relatively low systemic availability of ^{3}H-BaP following dietary exposure as observed in the present study.

In rainbow trout induced with unlabelled BaP, the present results demonstrate

Table 2. Amount of BaP-derived radioactivity (dpm mg^{-1} tissue) in the gills and liver 2–10 days after waterborne exposure to ^{3}H-BaP.

			Days after start of exposure			
Tissue	Group		2	4	6	10
Gills	Non-induced	1	4745	2674	1977	700
		2	5174	2200	2401	2014
	Induced[a]	1	1206	1109	617	649
		2	1843	1526	715	471
Liver	Non-induced	1	16349	5056	6513	1988
		2	23893	7362	2982	3719
	Induced[a]	1	4472	3094	480	707
		2	2716	1828	914	549

Values are individual observations (1, 2).

[a] Induction was performed by exposure to unlabelled BaP in water (25 µg l^{-1}, corresponding to 5 mg kg^{-1} fish).

that hepatic EROD activity was responsive to waterborne exposure corresponding to 5 mg BaP kg^{-1}. This is in agreement with results previously reported, where rainbow trout (Upshall *et al.* 1993, Sandvik *et al.* 1997), and other fish species (Collier and Varanasi 1991, Beyer *et al.* 1997) have been administered single doses of PAH-type inducers. Furthermore, in a study by Levine *et al.* (1994), CYP1A induction in gizzard shad (*Dorosoma cepedianum*) showed a dose dependent course in the range from 1 to 10 mg kg^{-1} after intraperitoneal injection or exposure via water.

The levels of BaP derived radioactivity in tissues in fish from the waterborne experiment were higher in non-induced fish compared with induced fish. This is in accordance with the study of Schlede *et al.* (1970), where oral treatment of rats with BaP once daily for 2 days prior to an intravenous injection of ^{3}H-BaP markedly decreased the levels of radiolabelled compound in the blood and adipose tissue compared with controls injected with ^{3}H-BaP only. Thus the present study confirms that BaP may also have a significant effect on its own metabolism in rainbow trout.

The harmful effects of PAH exposure are mainly connected to CYP1A mediated metabolism. Phase 1-metabolites containing epoxide groups are highly reactive and readily bind to macromolecules, such as proteins and nucleic acids. The formation of such adducts may in turn result in cellular damage (Hinson *et al.* 1994). Since macromolecular binding of CYP1A-mediated reactive intermediates represents an important step in BaP elicited cytotoxicity, the present autoradiographic study was designed in order to also detect tissue-bound residues of ^{3}H-BaP-derived radioactivity in various organs. Autoradiography of solvent-extracted tissue sections has formerly been successfully employed in studies on tissue-bound residues of reactive intermediates of various xenobiotics (Bergman 1979, Brandt and Brittebo 1989, Larsson *et al.* 1992).

Not unexpectedly, the results from the autoradiography of whole-body sections extracted with a series of polar and non-polar solvents showed that tissue-bound residues of ^{3}H-BaP-derived radioactivity were present in the liver and kidney. This is in accordance with the results of Steward *et al.* (1990) showing that tissue binding of BaP derivatives takes place in the liver and kidney of brown bullhead (*Ictalurus nebulosus*). In the present study however, bound residues also occurred in the intestinal mucosa, skin, olfactory organ and gills of the waterborne-exposed fish, while significant amounts of tissue-bound residues in dietary-exposed fish were only observed in the intestinal mucosa. The observed tissue distribution of bound ^{3}H-BaP-derived radiolabelling coincide with the localization of CYP1A expression which has been reported to occur in the liver and various extrahepatic tissues of several teleost species. Hepatic CYP1A is expressed in hepatocytes, biliary epithelial cells and vascular endothelial cells (reviewed by Hinton 1994, Stegeman and Hahn 1994). Localization of CYP1A in the kidney has been demonstrated in tubular cells, glomerular endothelium, vascular and sinusoidal endothelium, and nephronic and collecting ducts (Smolowitz *et al.*1991, Stegeman *et al.* 1991, Husøy *et al.* 1994) In the intestinal tract CYP1A activity is localized in the epithelial of mucosa as well as in the vascular endothelium in the lamina propria (Smolowitz *et al.* 1991,1992, Stegeman *et al.* 1991). Expression of CYP1A has also been detected in epithelial and endothelial cells of the gills (Miller *et al.* 1988, Smolowitz *et al.* 1991,1992, Stegeman *et al.* 1991, Husøy *et al.* 1994), in the olfactory organ (Smolowitz *et al.*1992) and in epithelial cells of the skin (Smolowitz

*et al.*1991, Stegeman and Hahn 1994). Therefore, macromolecular binding of reactive intermediates mediated through CYP1A-metabolism of xenobiotics might occur in these tissues, and the present results strongly indicate that this is the case for BaP.

Whether, and to what extent, such binding takes place is, however, not solely dependent on the state of CYP1A activity (i.e. induction) in the actual tissue. Since the formation of conjugated derivatives is an important step in removal of reactive intermediates, the phase II enzyme systems like glutathione S transferases (GST) and UDP-glucuronosyltransferases (UGT) may protect against tissue interactions with CYP1A-mediated epoxides. Accordingly, inhibition of both GST and UGT have been shown to increase the covalent binding to DNA of mutagenic BaP derivatives (Bock *et al.* 1981, Hesse *et al.* 1982). Therefore, the balance between the activities of oxidative and conjugative pathways determines the amount of intermediates that is available for macromolecular binding in cells. Interestingly, no quantitative differences in tissue binding of ^{3}H-BaP-derived radiolabelling could be detected between induced and non-induced fish in the present study. One explanation for this might be that the capacity of conjugation enzymes was sufficient to remove the excess of CYP1A-mediated metabolites in the induced fish. Supportive of this suggestion is that in rats pretreated with 3-methylcholanthrene or betanaphtoflavone, both activation and inactivation of BaP metabolites was much more pronounced than in untreated controls (Bock *et al.* 1981), an observation which may be explained by the coordinate induction of CYP1A and certain forms of UGT by the aryl hydrocarbon (Ah) locus. Further support to our suggestion is given by the present observation that the levels of ^{3}H-BaP-derived radioactivity in tissues of waterborne-exposed rainbow trout were higher in the non-induced compared with the induced fish. Finally it should be borne in mind that autoradiography is not a method specifically designed for quantification, and, although not presently recognized, subtle quantitative differences in tissue bound residues between the two groups might be present.

Significant amounts of tissue-bound residues in the dietary-exposed fish were present in the intestinal mucosa only, while in the waterborne-exposed fish high levels of tissue binding were also found in several other organs. This finding is obviously related to the systemic availability of the compound differing between the two routes of exposure, and should deserve attention in terms of whether or to what extent specific organs may serve as markers for various routes of exposure to environmental pollutants. Smolowitz *et al.* (1992) observed that exposure of topminnows (*Poecilliopsis lucida*) to BaP in the water elicited a strong induction of CYP1A in the olfactory epithelium, while no induction occurred in this tissue after intraperitoneal administration of the compound. Likewise, the present results showed that no radiolabelling was present in the olfactory organ after dietary exposure to ^{3}H-BaP. On the contrary, a substantial level of ^{3}H-BaP-derived radioactivity, of which a major part represented tissue-bound residues, was present after waterborne exposure. Thus the present observations strongly support the suggestion of Smolowitz *et al.*(1992) that the olfactory organ may represent a specific marker tissue for waterborne exposure to CYP1A inducers. Furthermore, the present study showed that a major fraction of the radiolabelling in the skin and gills of the waterborne-exposed fish appeared as tissue-bound residues, whereas in the dietary-exposed fish no radioactivity at all was present in the skin and only traces occurred in the gills. Consequently, these tissues may also represent potential

candidates as specific marker tissues for environmental pollutants with CYP1A inducing potential.

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