



BIOACCUMULATION OF DEHYDROABIETIC ACID IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): ROLE OF BIOTRANSFORMATION ENZYMES

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ABSTRACT

The role of Phase I and Phase II metabolism in rainbow trout on the bioaccumulation of dehydroabietic acid, an abundant and persistent compound found in pulp and paper mill effluents, was investigated using known inducer/inhibitor compounds for cytochromes P450 (P450s), ethoxyresorufin *O*-deethylase (EROD) and uridine diphosphoglucuronosyltransferase (UDPGT). The inducer/inhibitor compounds consisted of piperonyl butoxide (PBO), salicylamide (SAL) and β -naphthoflavone (BNF). PBO induced EROD, SAL had no effect on the measured biotransformation enzyme activities and BNF induced EROD and UDPGT. Treatment of trout with these compounds altered the bioaccumulation and distribution of dehydroabietic acid (DHAA) between bile, liver, and muscle in trout exposed to both the pure compound and an untreated mill effluent indicating that biotransformation enzyme activities may directly influence these bioconcentration processes in recipient biota. © 1997 IAWQ. Published by Elsevier Science Ltd.

KEYWORDS

Bioaccumulation; biotransformation enzymes; bleached pulp and paper effluent; Rainbow trout; resin acids; toxicity.

INTRODUCTION

Biotransformation enzymes have been widely used to assess the potential biological impacts of pulp and paper mill effluent discharges. A recent review by Hodson (1996) considers the relationships between mixed function oxidase (MFO) induction and other toxic effects, the fate and distribution of MFO inducing compounds in the environment or effluent treatment systems and the nature and identity of MFO inducers in pulping effluents. The use of conjugating enzymes for assessing the impacts of pulp and paper effluent has been discussed by Andersson *et al.* (1988) and Hodson *et al.* (1991). Although it is now commonly recognised that MFO induction in fish is an indicator of exposure to pulping effluents, there are no defined mechanisms linking induction to specific adverse biological effects at either the individual or population level.

The effects that MFO induction and biotransformation reactions have on the toxicity and bioaccumulation of xenobiotics in fish have been reviewed by Kleinow *et al.* (1987). The extent of chemical biotransformation

may appreciably affect the concentration and persistence of xenobiotics. Furthermore, changes in chemical structure and physical properties due to transformations may markedly alter the toxicological properties of chemicals and may affect the disposition of these compounds (Kleinow *et al.*, 1987). Such alterations will affect the elimination, bioaccumulation and compartmentalisation of potentially toxic components in fish (Kleinow *et al.*, 1987).

Effluents from pulp mills (kraft and mechanical) contain many compounds which are toxic to aquatic organisms and that readily bioaccumulate. Resin acids are an important group of chemicals acutely toxic to fish and dehydroabietic acid (DHAA) is one of the most persistent and abundant (Oikari *et al.*, 1983). The acute toxicity or LC50 (median lethal concentration) of DHAA has been previously determined in rainbow trout, *Oncorhynchus mykiss*, (Leach and Thakore, 1976; Chung *et al.*, 1979; Davis and Hoos, 1975). The principal detoxification route for DHAA is glucuronidation (Oikari *et al.*, 1983). However, the mode-of-action of DHAA toxicity and the effect of biotransformation enzyme induction or inhibition on the accumulation of DHAA within fish remains unknown.

Previous research has incorporated piperonyl butoxide (PBO) as an inhibitor/inducer of MFOs (Phase I metabolism) in rainbow trout (Glickman *et al.*, 1977; Melancon *et al.*, 1977; Erickson *et al.*, 1988; Erickson *et al.*, 1992). Lech (1974) and Lech and Statham (1975) used salicylamide (SAL) as an inhibitor of glucuronosyltransferase (Phase II metabolism) in rainbow trout and sea lamprey. A recent study of Podowski *et al.* (1991) utilised both of these inhibitors to study the biotransformation and disposition of hexachlorocyclopentadiene in goldfish (*Carassius auratus*). Another compound, β -naphthoflavone (BNF), is a known inducer compound for cytochromes P450. Currently no information exists on the relationships between biomarker activities of fish exposed to PBO, SAL and BNF and how these affect the chemical disposition of pulp and paper mill effluent derived contaminants in aquatic biota.

The present study was undertaken to ascertain the role of Phase I and Phase II metabolism within rainbow trout on the bioaccumulation of DHAA by incorporating PBO, SAL, and BNF into the bioassays. Cytochromes P450 quantification (P450s), ethoxyresorufin *O*-deethylase (EROD) and uridine diphosphoglucuronosyltransferase (UDPGT) enzyme biomarker activities were measured to observe the *in vivo* response from PBO, SAL, and BNF and how these effects were related to the DHAA concentration in trout bile, liver and muscle.

MATERIALS AND METHODS

Animals and Treatments. Rainbow trout, *Oncorhynchus mykiss*, were obtained from a local fish hatchery and held for several weeks at $12 \pm 1.0^\circ\text{C}$. Fish were fed a commercial, 35% protein, crumbled feed every other day to maintain body weight; they were not fed for 48 hr prior to or during the studies.

Exposure experiment 1. Static exposure tests to pure DHAA were conducted for 96 hr in 90 L glass aquaria. Water temperatures were maintained at $12 \pm 1.0^\circ\text{C}$ with a 12 hr light/dark cycle. Aquaria were filled with 80 L of water and seven fish were placed in each aquarium. Dissolved oxygen concentrations were maintained at greater than 90% saturation. Fish were added to aquaria containing 1 mg/L PBO or 25 mg/L SAL and pre-treated for 4 hr prior to the addition of DHAA to a concentration of 1.4 mg/L. Acetone was used as the carrier for the chemicals and there were 3 replications per treatment. Average fish weight and total length were 19.2 g and 126.6 mm respectively.

Exposure experiment 2. In order to assess the effect of Phase I and II enzyme manipulation on DHAA bioaccumulation from mill effluents, a similar exposure experiment to those outlined above was undertaken but diluted (1% v/v) untreated BKME replaced the 1.4 mg/L solution of DHAA. Whole mill effluent was obtained from an integrated, bleached kraft pulp and paper mill processing *Pinus radiata* softwood. The undiluted effluent contained 1970 $\mu\text{g/L}$ DHAA to give a fish exposure concentration of 20 $\mu\text{g/L}$. Treatments consisted of acetone (control), PBO and SAL (as above) and BNF. The BNF treatment fish were injected at 50 mg BNF/kg body weight into the intraperitoneal cavity. Corn oil was used as the vehicle at 10 ml/kg of

fish. There were 3 fish per treatment. Average fish weight and total length was 110 g and 226 mm respectively.

Tissue Samples and biotransformation enzyme analysis. At the termination of each 96 hr assay, fish from the control and treatments were sacrificed and the recovered liver was rinsed with saline and quickly chilled to decrease enzyme activity. All subsequent steps were carried out at 0-4°C. Microsomal fractions were isolated as described by Forsyth and Chambers (1989).

EROD activity was quantified using a modification of the method of Pohl and Fouts (1980) and Munkittrick *et al.* (1993). UDPGT activity was quantified by a modification of the method of Castrén and Oikari (1983) which used p-nitrophenol as a substrate. Protein concentrations were quantified by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Tissue analysis for DHAA. Bile and liver samples were analysed for total DHAA content (conjugated and free) using the ethanolic hydrolysis method outlined by Johnsen *et al.* (1995). Free resin acid content (unconjugated) in the liver and muscle samples was determined by blending the tissues with anhydrous sodium sulphate containing sodium metabisulphite (0.5% w/w) and sulphuric acid (0.5% w/w) prior to extraction with dichloromethane/methyl-t-butyl (1:1). Lipid and fatty acid constituents were removed from the concentrated extracts using selective methylation with methanol/toluenesulphonic acid (Mahood and Rogers, 1975) and acid/base partitioning. O-methylpodocarpic acid was used as a surrogate recovery standard. The resin acid fraction was silylated and analysed by GC/MS.

Table 1. Hepatic biomarkers in rainbow trout exposed to potential detoxification enzyme inducer/inhibitor compounds

Treatment	P450 nmol · mg protein ⁻¹	EROD pmol · min ⁻¹ · mg protein ⁻¹	UDPGT nmol · min ⁻¹ · mg protein ⁻¹
DHAA expt			
control	0.31 (0.05) A ^a	30.96 (13.38) A	0.91 (0.29) A
PBO	0.26 (0.02) A	106.49 (32.57) B	0.64 (0.20) A
SAL	0.36 (0.08) A	15.95 (7.50) A	0.77 (0.30) A
effluent expt			
control 1 ^b	0.24 (0.03) A	7.01 (7.11) A	0.53 (0.20) A
control 2	0.30 (0.09) A	14.02 (4.42) A	0.57 (0.17) A
PBO	0.25 (0.02) A	24.10 (11.61) B	0.44 (0.03) A
SAL	0.36 (0.08) A	6.46 (7.37) A	0.37 (0.09) A
BNF	0.35 (0.20) A	196.02 (36.04) B	0.84 (0.12) B

a: Mean (95% C.I.) S-N-K index: Means within a column not followed by the same capital letter are significantly different ($p \leq 0.05$) from control fish by the Student-Neuman-Keuls means comparison test on log transformed data.

b: Control 1 fish exposed to dilutant only; others exposed to 1% effluent and treatment.

RESULTS AND DISCUSSION

Biotransformation enzymes

The biotransformation enzyme results for the acute bioaccumulation study showed that PBO significantly induced EROD whereas no effect was observed in P450 and UDPGT (Table 1). BNF treatment gave a significant induction of EROD and UDPGT. The effluent itself had no effect on hepatic biomarkers.

SAL has been used as an inhibitor of glucuronosyltransferase in several previous studies (Lech, 1974; Lech and Statham, 1975; Podowski *et al.*, 1991), UDPGT responses in those studies were not quantified *in vivo*. Unexpectedly, SAL had no significant effect on P450, UDPGT or EROD (Table 1). Clarke *et al.* (1991) reported that several isoforms of UDPGT are found in fish, each having different substrate specificity. Results of the present study suggest that, if SAL inhibited UDPGT, it effected an isoform other than that which is specific for *p*-nitrophenol. This is further supported by the significantly higher percentage of unconjugated DHAA found in liver from SAL-treated trout, suggesting that SAL, and to a lesser extent PBO, had inhibited glucuronide formation relative to the control (Fig. 1).

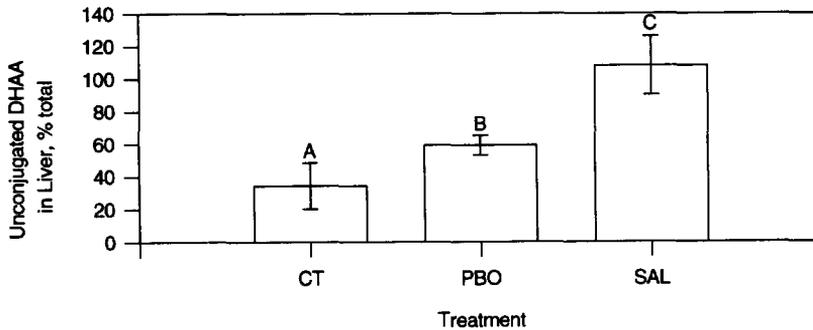


Figure 1. Unconjugated DHAA in liver of trout exposed to DHAA in the water column. Means within a column not followed by the same capital letter are significantly different ($p \leq 0.05$) from control fish (CT) by the Student-Neuman-Keuls means comparison test.

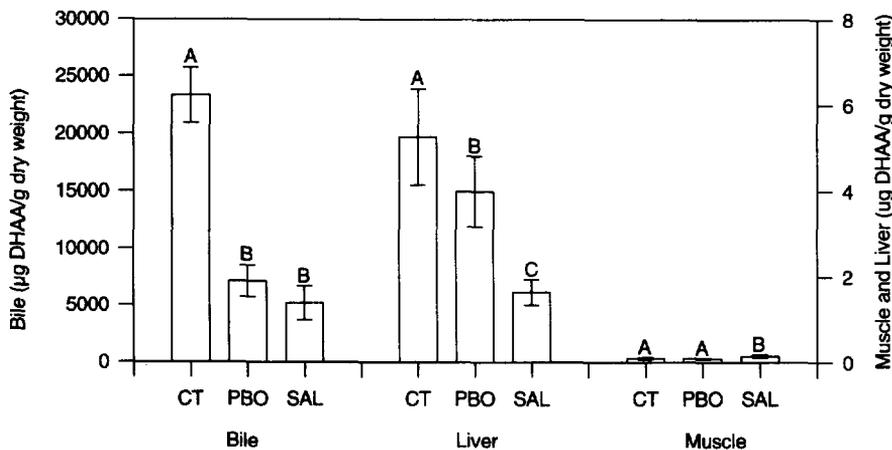


Figure 2. Mean DHAA concentrations in bile, liver, and muscle of trout exposed to 1.4 mg/L DHAA. Means within a column not followed by the same capital letter are significantly different ($p \leq 0.05$) from control fish (CT) by the Student-Neuman-Keuls means comparison test.

Exposure to pure DHAA

Tissue Bioaccumulation. Tissue concentrations of dehydroabiatic acid were determined in fish exposed to pure DHAA. Bile DHAA concentrations were decreased relative to the control for both the PBO and SAL treatments in fish exposed to 1.4 mg/L DHAA (Fig. 2). Liver concentrations of DHAA were decreased and muscle concentrations increased in SAL-treated fish. Thus, the observed changes in SAL-treated fish only partially correlated to the conventional model that inhibited glucuronidation activity would cause a decreased bile concentration, and increased liver and muscle concentrations due to decreased biliary and tissue excretion (Förlin and Wachmeister, 1989). Similarly, the lowered concentrations of DHAA in bile and

liver tissues from PBO-treated fish may be a consequence of the apparent decrease in conjugation activity implicated in Fig. 1.

Exposure to untreated BKME

Tissue Bioaccumulation. DHAA accumulation results for bile, liver and muscle tissues from effluent-exposed fish were not consistent with those observed for the pure compound exposure (Fig. 3). Significant differences in DHAA concentrations relative to the control were only found in the livers of fish treated with SAL. Decreased bile DHAA concentrations were not observed for either the PBO or SAL treatments. The significance and contribution of the effluent matrix to these differences is currently being investigated.

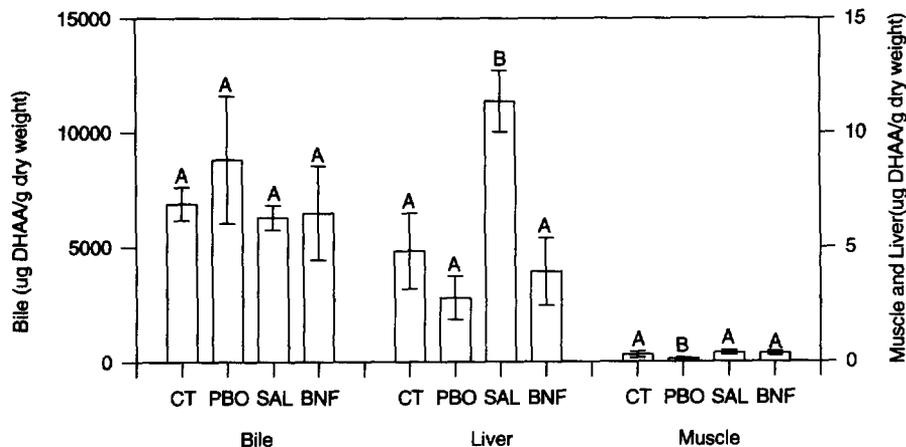


Figure 3. Mean DHAA concentrations in bile, liver, and muscle of trout exposed to 1% untreated effluent. Means within a column not followed by the same capital letter are significantly different ($p \leq 0.05$) from control fish (CT) by the Student-Neuman-Keuls means comparison test.

For the effluent exposure experiment, trout were also treated with BNF, a known inducer of both EROD and UDPGT (Andersson *et al.*, 1985; Förlin and Haux, 1985; Kleinow *et al.*, 1987; Williams *et al.*, 1996). The BNF treatment had no effect on the disposition of DHAA in the fish relative to the control. Förlin and Wachtmeister (1989), found that induction of UDPGT resulted in higher concentrations of conjugated chlorophenolic metabolites in the bile.

Tissue distribution. The relative distributions of DHAA in tissues taken from control and treated fish for the effluent exposure experiment are given in Fig. 4. A significant increase in bile DHAA concentrations relative to those in liver and muscle tissues was observed in PBO-treated fish, possibly indicating that PBO treatment increased the relative excretion rate of DHAA from muscle and liver tissues. Treatment with salicylamide lowered TDF (bile:liver) and increased TDF (liver:muscle) suggesting that some inhibition of resin acid conjugation enzymes had occurred in these fish. Whilst the measured resin acid tissue distribution factors and bile bioconcentration factors (BCF) were comparable in all treatments to those previously reported in effluent exposed trout, liver and muscle BCF values were somewhat lower than expected (Table 2; Stuthridge *et al.* (1995)).

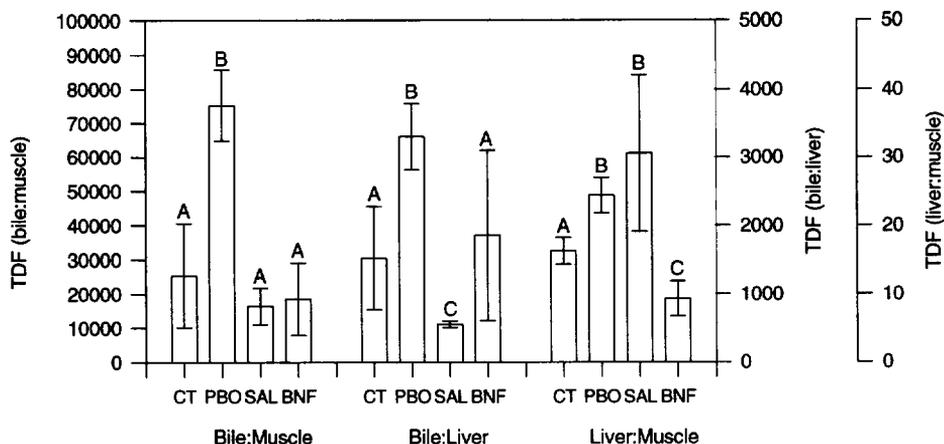


Figure 4. Mean Tissue Distribution Factors (TDF: [tissue 1, $\mu\text{g/g dw}$]:[tissue 2, $\mu\text{g/g dw}$]) in trout exposed to 1% untreated effluent. Means within a column not followed by the same capital letter are significantly different ($p \leq 0.05$) from control fish (CT) by the Student-Neuman-Keuls means comparison test.

Table 2. Tissue bioconcentration factors in trout exposed to 1% untreated effluent

Treatment	log BCF - bile ^{a,b}	log BCF - liver	log BCF - muscle
control	5.55 (0.07) A	2.38 (0.15) A	1.17 (0.20) A
PBO	5.66 (0.22) A	2.14 (0.15) A	0.75 (0.20) B
SAL	5.50 (0.04) A	2.75 (0.09) B	1.28 (0.12) A
BNF	5.51 (0.14) A	2.21 (0.16) A	1.25 (0.01)
Stuthridge <i>et al.</i> (1995)	5.8	3.3	2.2

^a: bioconcentration factor: $\log([\text{tissue}, \mu\text{g/g dw}]/[\text{effluent}, \mu\text{g/mL}])$

^b: Mean (95% C.I.) S-N-K index: Means within a column not followed by the same capital letter are significantly different ($p \leq 0.05$) from control fish by the Student-Neuman-Keuls means comparison test on log transformed data.

CONCLUSIONS

In this study, trout exposure experiments utilising pure DHAA and DHAA in an effluent matrix have shown that potential biotransformation enzyme inducer/inhibitor compounds can affect the bioaccumulation and tissue distribution of DHAA in the fish. The observed differences in the behaviour of DHAA between the two experiments indicate that the relationships between biotransformation enzymes and the bioaccumulation and disposition of such compounds are complex.

Numerous studies have shown that bile analysis in fish may be a useful tool for assessing the environmental distribution and biota exposure of polar water-borne xenobiotics derived from pulp and paper mill effluent discharges (Oikari and Holmbom, 1986; Förlin and Wachtmeister, 1989; Oikari and Lindström-Seppä, 1990). Pulp and paper mill effluents are known to contain compounds that may either induce or inhibit detoxification enzyme activities. Therefore, caution may be required when interpreting data related to the bioaccumulation of xenobiotics into specific tissues of fish.

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