SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 17, No. 11, pp. 2347–2353, 1998

Printed in the USA
0730-7268/98 $6.00 + .00

CHRONIC RETENE EXPOSURE CAUSES SUSTAINED INDUCTION OF CYP1A ACTIVITY AND PROTEIN IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

NUNO M. FRAGOSO,† JOANNE L. PARROTT,‡ MARK E. HAHN,§ and PETER V. HODSON*†
†School of Environmental Studies, Queen's University, Kingston, Ontario K7L 3N6, Canada
‡National Water Research Institute, Canada Centre for Inland Waters, Burlington, Ontario L7R 4A6, Canada
§Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

(*Received* 25 *November* 1997; *Accepted* 14 *April* 1998)

Abstract—The removal of persistent chlorinated organic compounds from pulp mill effluents has not eliminated mixed function oxygenase (MFO) induction by these effluents. Therefore, continuous MFO induction downstream of pulp mills may be due to exposure to more labile compounds, such as retene (an alkyl-substituted phenanthrene), which typically cause transient induction after a single brief exposure. Because fish are exposed continuously to pulp mill effluents, we have tested, and rejected, the null hypothesis that continuous exposure of fish to retene does not cause sustained MFO induction. Rainbow trout exposed continuously to retene, a component of some pulp mill effluents and sediments downstream of pulp mills, showed concentration-dependent increases in hepatic ethoxyresorufin-*O*-deethylase (EROD) activity. The increase in EROD activity was sustained over 32 d of continuous exposure, but it diminished to background levels within 4 d after transfer to clean water. The enzymatic response was confirmed by measuring changes in the content of immunodetectable cytochrome P4501A (CYP1A) protein. These data support a role for labile, nonhalogenated compounds in chronic effects of pulp mill effluents on fish.

Keywords—Retene Cytochrome P4501A Ethoxyresorufin-*O*-deethylase Induction Pulp mill effluent

INTRODUCTION

Pulp and paper mill effluents have many deleterious effects on fish including histopathologic changes, deformities, physiologic stress and toxicity, delayed sexual maturation, impaired reproduction, changes in growth rate, and changes in population structure [1–3]. Elevated activity of mixed function oxygenase (MFO) enzymes is a common biochemical response observed in fish downstream of many pulp and paper mills [2] and is consistently observed throughout the year [4]. Mixed function oxygenase induction and the toxicologic effects of bleached-kraft mill effluents (BKME) have been attributed to the presence of halogenated compounds, such as dioxins and furans [1,5,6]. However, many sublethal effects, including elevated MFO activity, have been seen in fish exposed to effluent from mills not using chlorine bleaching, or from mills using ClO_2 bleaching and secondary treatment to eliminate chlorinated dioxins and furans [2,7].

Munkittrick et al. [8] showed that 1 year after the installation of secondary treatment at a mill in Jackfish Bay, Ontario, Canada, treated BKME was still a potent inducer, suggesting that inducing agents had not been removed by secondary treatment or that persistent inducers were still present in contaminated sediments. However, within 2 weeks of a mill shutdown, MFO induction was reduced in all three fish species that inhabited the waters near the mill. Hence, it appears that induction is not related to the presence of persistent compounds in the receiving water ecosystem, including its sediments. Transfer of wild, caged, or laboratory-exposed fish to clean water after BKME exposure results in a rapid loss of induction, with an estimated half-life of less than 8 d for induction [2]. In contrast, laboratory experiments have shown that induction with dioxins and furans is sustained for much longer periods (weeks), even after a brief exposure [9,10].

These data suggest that labile compounds are present that may mimic the induction of persistent dioxinlike compounds. Burnison et al. [11] used bioassay-driven fractionation techniques to confirm that inducers in secondary-treated BKME included multi-ring polycyclic aromatic hydrocarbons (PAHs) with alkyl substitutions instead of chlorine substitution. This raises the possibility that prolonged exposure to labile compounds may sustain MFO induction, as does dioxin exposure.

We examined whether BKME-derived PAHs can cause sustained induction of MFO activity in rainbow trout (*Oncorhynchus mykiss*), using continuous exposure to retene (7-isopropyl-1-methylphenanthrene) as a model. Retene is thought to be formed from the in situ anaerobic metabolism of abietic or dehydroabietic acid, two resin acids found in some pulp mill effluents [12]. Resin acids are natural plant products and are major contributors to toxicity with other wood extractives in spent cooking liquors [13,14]. Retene has been found in unbleached kraft pulp and paper mill effluent [15] and is believed to be formed from its precursors in anoxic pockets within aeration treatment ponds [16,17]. Retene is used as an indicator of anthropogenic sources of PAHs in sediments, and concentrations range from 10 to 800 ng/g dry weight in uncontaminated lakes of forest watersheds [12]. In sediments downstream of pulp mills, retene concentrations can be much higher, 2,000 μg/g dry weight (M. Fox, personal communication). This indicates that indigenous fish could be exposed to retene in effluent or to retene in sediments. Preliminary work by Parrott et al. [18] has shown that retene induces MFO activity, whereas abietic acid and phenanthrene do not. Thus, we examined the ability of chronic retene exposure to cause sustained MFO induction in juvenile rainbow trout and as-

* To whom correspondence may be addressed (hodsonp@biology.queensu.ca).

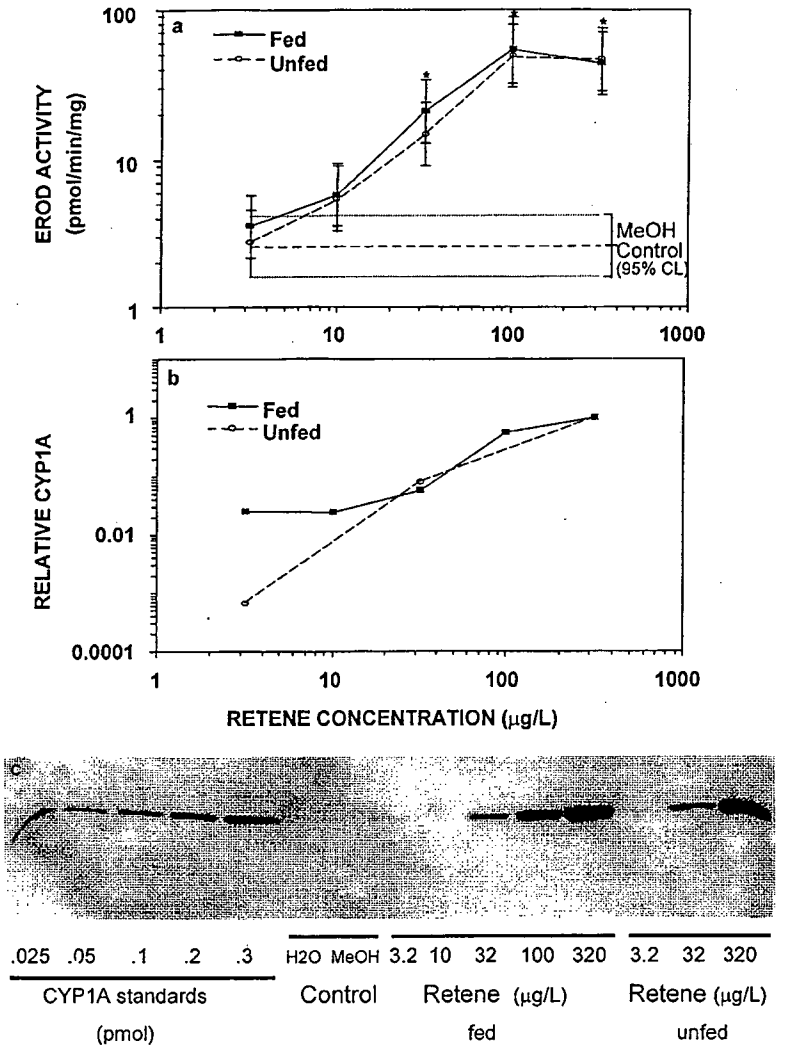

Fig. 1. Concentration dependence of rainbow trout hepatic (a) ethoxyresorufin-*O*-deethylase (EROD) activity, (b) cytochrome P4501A (CYP1A) protein during continuous exposure to waterborne retene over 4 d, and (c) western blot of CYP1A. An asterisk (*) denotes that both fed and unfed retene treatments are significantly different from control at a probability level of 0.05.

sessed the loss of MFO induction upon transfer to clean water. We have tested, and rejected, the null hypotheses that continuous exposure of fish to retene does not cause sustained induction and that no change in ethoxyresorufin-*O*-deethylase (EROD) activity would be observed during depuration of retene.

MATERIALS AND METHODS

Experimental design

To measure the potency of retene for inducing MFO enzymes, trout were exposed for 4 and 32 d to five concentrations of retene and two controls. Because of the extended exposure, the effect of feeding on EROD induction was assessed with fed and unfed treatments. The ability of retene to sustain induction was tested by continuous exposure of fish to 100 µg/L of retene for 4 and 32 d. The persistence of MFO induction was assessed by exposing trout to retene for 2 d, followed by transfer to clean water for up to 4 d, when activity approached control levels.

Fish holding

Experiments were carried out in a 15°C cold room during the summer of 1996 at the National Water Research Institute, Canada Centre for Inland Waters, Burlington, Ontario, Canada. Rainbow trout (~2 g) were obtained from Rainbow Springs Hatchery (Thamesford, ON, Canada) or from Spring Valley Trout Farm (Petersburg, ON, Canada). Fish were held under a constant 16h light: 8 h dark cycle, in dechlorinated Burlington water with a hardness of 131 ± 3 mg $CaCO_3$/L and a water flow above 1 L/g of fish/d.

Fish were fed Trout Starter (1.5 GR; Martin Mills Profishnet Aquaculture Feed, Tavistock, ON, Canada) at a rate of 1.5% body weight/d. During exposure–response experiments, fish were divided into fed and unfed treatments. Feeding was stopped in the unfed treatments at least 48 h before exposures and fish remained unfed throughout 4- and 32-d exposures. Fed fish were given food once daily throughout the exposure period, up to 48 h before sampling. Fish in the time series and depuration experiments were fed by a similar regime.

A loading density of ≤1 g of fish per liter of water was

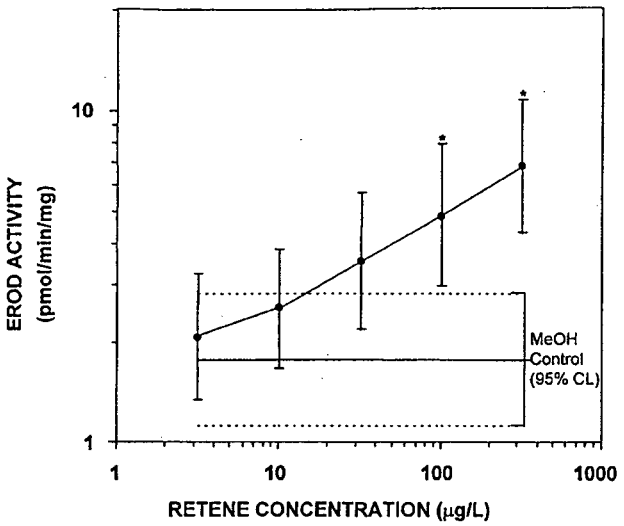

Fig. 2. Concentration dependence of rainbow trout hepatic ethoxyresorufin-*O*-deethylase (EROD) activity during continuous exposure to waterborne retene over 32 d. An asterisk (*) denotes that value is significantly different from control at a probability level of 0.05.

achieved by placing five 2-g fish in 10 L of water or five 4-g fish in 20 L of water. Aquaria were 20-L, black, high-density polyethylene plastic buckets, lined with clear food-grade polyethylene bags (3 mil; Apache Plastic, Burlington, ON, Canada). Because of slime build up, bags were replaced every 8 d during 32-d exposures. Tanks were unaerated throughout the experiments. All exposures were waterborne (nominal concentrations reported) with static daily renewal of water and solutions. Water quality was monitored daily, and mean (±SD) temperature was 14.6 ± 0.5°C, pH was 7.3 ± 0.5, and dissolved oxygen was 7.0 ± 0.2 mg/L.

Exposure regimes

Exposure–response experiments consisted of groups of five fish exposed to 3.2, 10, 32, 100, and 320 µg retene/L, and to solvent (methanol [MeOH]) and negative (water) controls. The total volume of MeOH in treated and solvent tanks was 0.2 ml/L of water. Fish were sampled after 4 or 32 d of continuous exposure.

In time-series and depuration experiments, fish were exposed to 100 µg/L of retene (the concentration causing maximum induction in exposure–response experiments), 10 µg/L

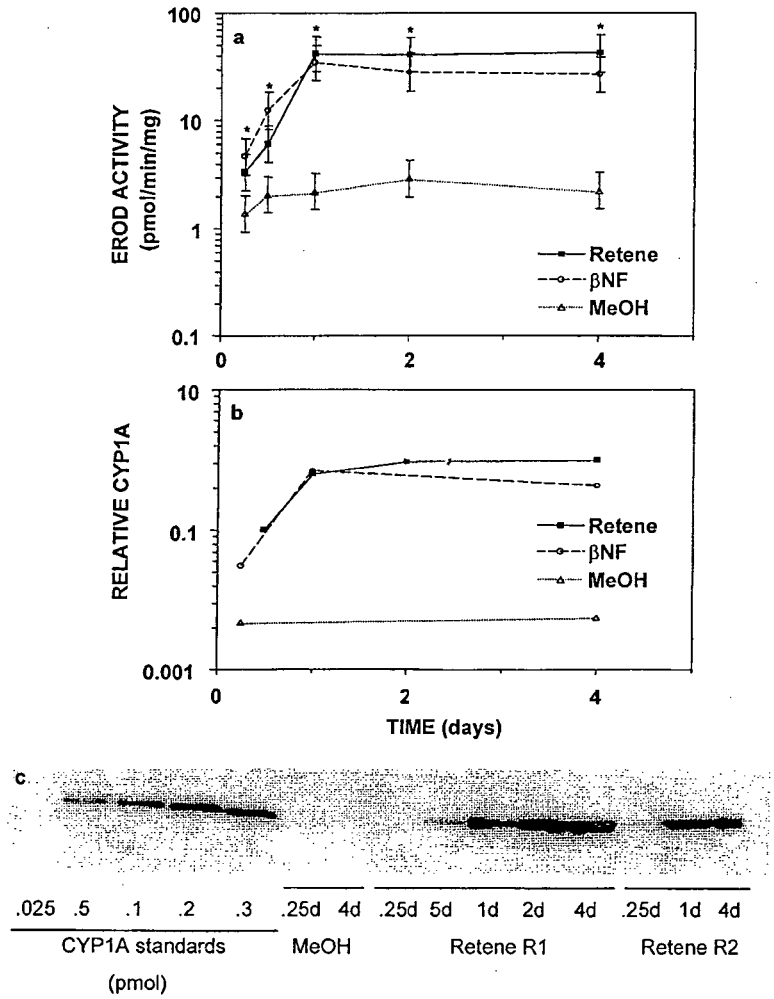

Fig. 3. Time course of induction of rainbow trout hepatic (**a**) ethoxyresorufin-*O*-deethylase (EROD) activity, (**b**) cytochrome P4501A (CYP1A) protein during 4 d of continuous exposure to waterborne retene (100 µg/L), beta-naphthoflavone (βNF) (10 µg/L; positive control), or methanol (MeOH) (200 µl/L; negative control), and (**c**) western blot of CYP1A protein. An asterisk (*) denotes that both retene and βNF treatments are significantly different from the MeOH control at a probability level of 0.05.

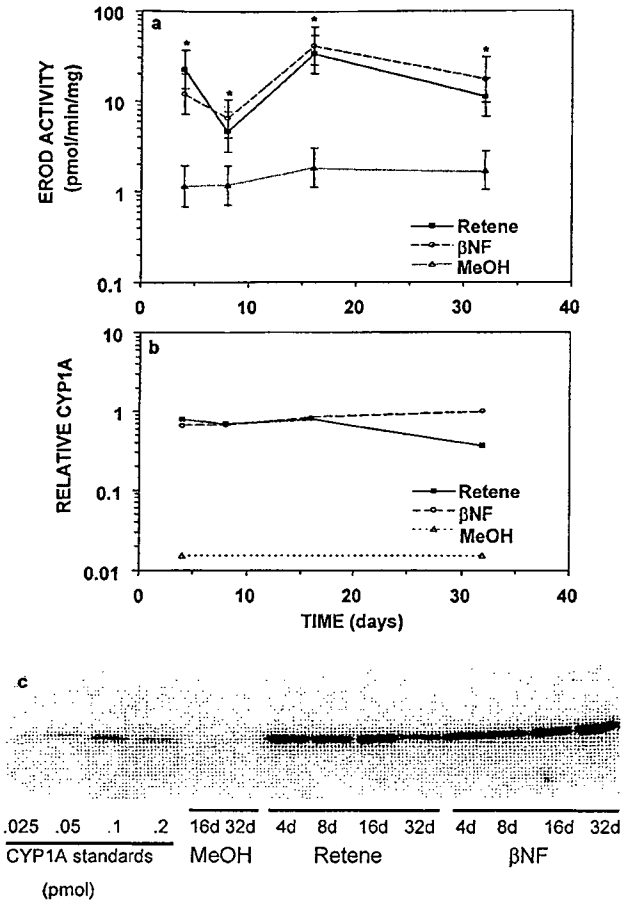

Fig. 4. Time course of induction of rainbow trout hepatic (a) ethoxyresorufin-*O*-deethylase (EROD) activity, (b) cytochrome P4501A (CYP1A) protein during continuous exposure to waterborne retene (100 μg/L), beta-naphthoflavone (βNF) (10 μg/L; positive control), or methanol (MeOH) (200 μl/L; negative control), and western blot of CYP1A protein. An asterisk (*) denotes that both retene and βNF treatments are significantly different from the MeOH control at a probability level of 0.05.

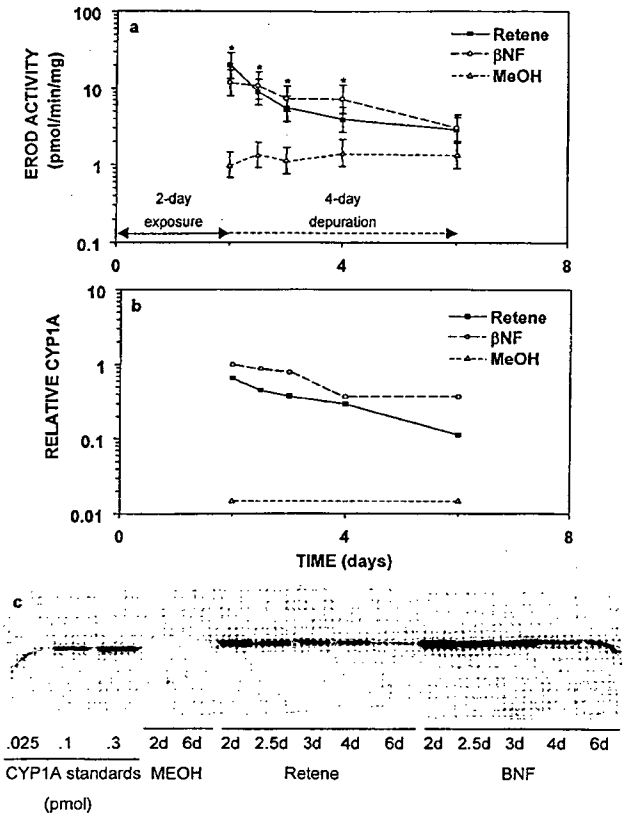

Fig. 5. Hepatic (a) ethoxyresorufin-*O*-deethylase (EROD) activity, (b) cytochrome P4501A (CYP1A) protein in rainbow trout exposed to waterborne retene (100 μg/L), beta-naphthoflavone (βNF) (10 μg/L; positive control), or methanol (MeOH) (200 ml/L; negative control) for 2 d followed by a 4-d depuration period, and (c) western blot of CYP1A protein. An asterisk (*) denotes that both retene and βNF treatments are significantly different from the MeOH control at a probability level of 0.05.

of beta-naphthoflavone (βNF) (positive control), 2 ml of MeOH (solvent control), or water (negative control). Time-series experiments lasted 4 or 32 d and fish were sampled on days 0.25, 0.5, 1, 2, and 4, for 4-d exposures, or on days 4, 8, 16, and 32 for 32-d exposures. Fish in depuration experiments were transferred to clean water after 2 d of exposure to retene (100 μg/L) or βNF (10 μg/L). They were sampled 2 d after exposure started (0 d of depuration) and 0.5, 1, 2, and 4 d after transfer to clean water.

At the end of the exposure period, fish were given a blow to the head, their spinal cords were severed, they were weighed, and their livers were removed and weighed. Livers less then 30 mg were homogenized in microcentrifuge tubes in 500 μl of HEPES grinding buffer (11.184 g KCl, 5.206 g HEPES sodium salt buffer, 1 L water, pH 7.5). Another 250 μl of buffer was added for every 10 mg of liver over 30 mg. Homogenates were centrifuged at 9,000 *g* for 20 min at 2°C. The supernatant (S9 fraction) was removed using a Pasteur pipette, placed in a cryovial, quick frozen in liquid nitrogen, and stored at −80°C.

Assay for EROD activity

All samples were assayed in triplicate for EROD activity and total protein. An aliquot of 50 μl of S9 fraction was added to each well of a Falcon or Costar 96-well tissue culture treated microplate (Fisher Scientific, Ottawa, ON, Canada). Ten microliters of resorufin standards in dimethylsulfoxide (DMSO) were added to one row of wells, consisting of six concentra-

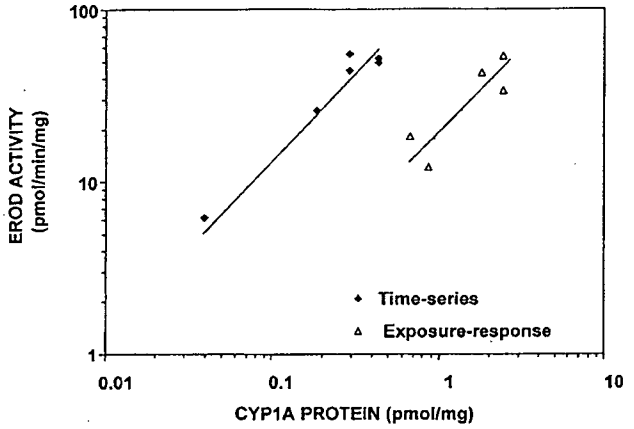

Fig. 6. Regression analysis of hepatic ethoxyresorufin-*O*-deethylase (EROD) activity versus cytochrome P4501A (CYP1A) protein in rainbow trout exposed continuously to waterborne retene during a 4-d time series (100 μg/L) and a 4-d exposure–response experiment (3.2–320 μg/L).

tions in duplicate, ranging from 0 to 5.0 μg/ml. Fifty microliters of 7-ethoxyresorufin (22 μM) in DMSO diluted 10-fold in HEPES sodium salt buffer (100 mM, pH 7.8) was added to each well and the plates were incubated in the dark for 10 min. Enzyme reactions were started by the addition of 10 μl of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), tetrasodium salt (cofactor; 24 mM) to each well. Changes in fluorescence were measured once per minute for 12 min at room temperature with a CYTOFLUOR® 2300 Plate Reader (PerSeptive Biosystems, Burlington, ON, Canada) equipped with an excitation filter of 530 nm, 30 nm bandwidth and emission filter of 590 nm, 35 nm band width.

Micro Bio-Rad protein assay

Fifty microliters of S9 fraction was diluted into 500 μl of water and 10 μl of diluted sample was added to each well, in triplicate. Similarly, 10 μl of protein standards (bovine serum album, 0–50 mg/ml) was added to an empty row of wells with 200 μl of Micro Bio-Rad Protein Dye (Bio-Rad, Mississauga, ON, Canada). The plate was incubated for 5 min at room temperature and the absorbence read at 600 nm.

Rates of specific enzyme activity were calculated from the slopes of fluorescence versus time, standard curves of resorufin, and measured protein concentrations. Final values were expressed in pmole/min/mg protein.

Data analysis

Data were checked for homogeneity of variance using Bartlett's test, and then log transformed [19]. Log-transformed data were used in multifactorial analysis of variance (ANOVA) at a 0.05 probability level. The actual p values from the ANOVAs are reported in the text. Means and 95% confidence limits were calculated according to Hodson et al. [19].

Western analysis

The S9 fraction from livers of selected fish from each experiment were assayed for cytochrome P4501A (CYP1A) by immunoblot analysis [20], with modifications as described in Hahn et al. [21]. Representative samples from each treatment group (i.e., samples with an EROD activity close to treatment mean values) were analyzed.

Total protein, measured in the Micro Bio-Rad assay, was used to calculate the volume of sample required to load 100 μg of total protein into each well. Samples were diluted in a sample treatment buffer solution (0.25 M Tris HCl, 40% glycerol, 4% sodium dodecyl sulfate [SDS], 0.008% bromophenol blue, 5% β-mercaptoethanol). Three or five CYP1A standards (microsomes from scup [*Stenotomus chrysops*] injected with βNF, containing known amounts of CYP1A) were diluted in the same manner, for a range of concentrations (0.025–0.3 pmol CYP1A/lane). Samples and standards were loaded onto gels using a 100-μl Hamilton syringe.

Proteins were run on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel over a 16-h period (initial I = 35 mA; V = 95 V), after which gels were soaked in Tris-buffered saline solution (100 mM Tris, 2.5 M NaCl, pH 7.5) for 15 min. Proteins were transferred to a nitrocellulose membrane (Nytran®) using a Semi-Dry Transfer Apparatus (Bio-Rad, Hercules, CA, USA) at 15 V, 0.9 A for 40 min. Reversible staining with Ponceau Stain (Fisher Scientific, Pittsburgh, PA, USA) for 5 min confirmed protein transfer. The membrane was preincubated in Rad-Free blocking buffer (Schleicher and Schuell, Keene, NH, USA) for 1 h, after which fresh buffer containing the anti-scup CYP1A monoclonal antibody (MAb), 1-12-3 (10 μg/ml) [20] was added. After 1 h, the membrane was washed with deionized water and rinsed in Tris-buffered saline solution three times. The membrane was incubated for 1 h in the buffer containing a second antibody, alkaline phosphatase-conjugated goat anti-mouse antibody (GAMAP; Schleicher and Schuell) and washed as before.

Monoclonal antibody 1-12-3-reactive protein (CYP1A) on the membrane was visualized using Tropix ready-to-use solution (Tropix, Bedford, MA, USA) or chemiluminescent substrate sheets (Schleicher and Schuell) and x-ray film (Kodak X-OMAT, Sigma Chemical, St. Louis, MO, USA). Exposed film was photographed using a Kodak DCS 2000 digital camera with micro 55-mm lens (Eastman Kodak, Rochester, NY, USA). Images were captured in Adobe Photoshop [22]. Relative band density was quantified using NIH Image software [23]. Because most samples were outside the range of the standard curve, all bands were normalized relative to the darkest band on the gel. Thus, CYP1A protein values represent relative, rather than absolute, amounts.

RESULTS

Exposure–response experiments

Four- and 32-d exposures to retene caused MFO induction in trout liver (Figs. 1a and 2). As retene exposure concentration increased, EROD activity increased above basal levels, relative to clean water- and MeOH-exposed fish ($p < 0.001$). The EROD activity reached a maximum in fish exposed to 100 and 320 μg/L of retene, in 4- and 32-d experiments, respectively, but fish exposed for 4 d were significantly more induced (13-fold relative to controls) than those exposed for 32 d (two-fold) ($p < 0.001$). The CYP1A protein showed a similar response to retene exposure and a strong correlation occurred between EROD activity and CYP1A protein content of the individual fish in the 4-d experiment (Fig. 1b and c). The CYP1A content was not measured in 32-d fish, as no samples were available.

Feeding did not have a significant effect on EROD activity, in either 4- or 32-d experiments ($p = 0.071$ and $p = 0.338$, respectively), and no differences occurred among replicates ($p = 0.585$). However, some mortality of unfed fish (30 of 70 fish died) occurred in all treatments (water, MeOH, retene) near the completion of the 32-d experiment, but none occurred in fed treatments.

Time series

Both retene- and βNF-exposed fish showed elevated EROD activity compared to MeOH-exposed fish within 12 h of exposure. Activity reached a maximum within 24 h and stayed elevated over 4 d of continuous exposure (Fig. 3a). The EROD activity was also significantly elevated in retene- and βNF-exposed fish over 32 d ($p < 0.001$) (Fig. 4a). Changes in EROD activity were paralleled by changes in CYP1A protein (Fig. 3b and 4b).

Depuration

After a 2-d exposure, the EROD activity of fish exposed to retene and βNF was significantly higher than for fish exposed to MeOH ($p < 0.001$). The EROD activity of retene- and βNF-exposed fish started to decline within hours of transferring fish to clean water (Fig. 5a) and approached control (MeOH) values within 4 d postexposure. A replicate effect was observed ($p = 0.008$), that is, the EROD activity of rep-

licate two fish was higher than that of replicate one fish for both retene- and βNF-treated fish. Analysis of EROD data using fish and liver weight as covariables by analysis of covariance eliminated the difference between replicates (p = 0.584). The CYP1A protein content was highest in retene and βNF exposures at day 2 (beginning of depuration) and decreased rapidly during depuration (Fig. 5b). The CYP1A protein content of MeOH-exposed fish was barely visible on gels (<0.025 pmol/lane) and did not change during the 4 d of depuration (Fig. 5c).

DISCUSSION

Induction of EROD was a sensitive indicator of retene and βNF exposure. Induction of the MFO system was evident with retene concentrations as low as 10 μg/L and it was sustained in both time-series and exposure–response experiments over 32 d. The importance of a continuous exposure in sustaining EROD induction was demonstrated in the depuration experiment: a rapid loss of EROD activity suggests that retene is labile and rapidly eliminated by fish. Parallel changes in EROD and CYP1A protein content show that increased EROD activity is due to increased levels of catalytic protein (CYP1A) that can likely be attributed to ligand binding to the aryl hydrocarbon receptor (AhR) and subsequent gene activation. The strong correlation also demonstrated that enzyme activity was a good measure of protein concentration and induction.

The EROD activity increased with retene exposure, confirming the earlier results of Parrott et al. [18], and the lack of induction in negative and solvent controls (water and MeOH) demonstrated that retene was causing the effect independent of other experimental parameters, such as pH, dissolved oxygen, and temperature. The benefit of feeding fish during experiments was a lower incidence of mortality relative to unfed fish. The lack of a feeding effect on MFO induction allowed us to feed fish during time-course experiments and to avoid the bias caused by mortality in unfed treatments in 32-d exposures. Death was likely a result of starvation, as the frequency of mortality was similar in all unfed treatments (water, MeOH, and retene).

The parallel increase in EROD activity and CYP1A protein (Fig. 6) supports the involvement of the AhR and the *CYP1A* gene in retene-induced changes in catalytic activity. However, a difference was found among experiments in the amount of CYP1A protein associated with a given amount of catalytic activity (Fig. 6: time-series versus exposure–response experiments), perhaps because protein concentrations were outside the range of standards (Figs. 1c and 3c) and we measured relative and not absolute concentrations. Direct studies of AhR binding will be needed to demonstrate retene binding and activation of the AhR by retene and to determine AhR binding affinity relative to tetrachlorodibenzo-*p*-dioxin (TCDD) or βNF. This model is further supported by the similar responses of fish exposed to βNF, a model CYP1A inducer.

Continuous exposure of fish to retene (100 μg/L) and to βNF (10 μg/L) caused sustained MFO induction over 32 d. Beta-naphthoflavone was tested at 10 μg/L because it inhibits microsomal EROD activity at higher concentrations (50 ppb βNF in 1 μl of dimethylformamide) [24], and retene was tested at 100 μg/L, the concentration giving maximum induction in 4-d exposures (Fig. 1). Trout hepatic CYP1A responded identically to both compounds. After 32 d, mean EROD activities for retene- and βNF-exposed fish were 6- and 10-fold greater than for MeOH-exposed fish, an induction similar to that of fish exposed to BKME in the wild and in the laboratory [19]. However, retene-induced EROD activity (5–50 pmol/min/mg) was much lower than activities of fish exposed to dioxins. Dioxin-treated rainbow trout commonly have EROD values ranging from 100 to 1,000 pmol/min/mg [25], presumably because of the persistence of dioxins and their high affinities for the AhR. Prolonged activation of the *CYP1A* gene in rainbow trout, perch, and trout hepatocytes in culture is attributed to continuous binding of dioxin to the receptor complex [10,26–29]. The lower EROD values caused by retene suggest that its efficacy for activation of the AhR is lower than that of dioxin and/or that it is more readily metabolized and excreted. Retene behaves more like βNF, which is known to cause transient induction of CYP1A relative to sustained induction by polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans [26,30]. Thus, the strength and duration of a retene–receptor complex would be less than for dioxin, allowing gene transcription and protein translation to decline sooner than with dioxin.

The apparently rapid depuration of retene (and βNF) can be balanced by continual exposure, as demonstrated by the continuously elevated catalytic activity measured during the 32-d experiments (Fig. 4). Therefore, prolonged exposure of fish to labile PAHs found in pulp mill effluent might be one explanation for the continuous elevation of MFO activity observed downstream of pulp mills. Retene precursors (abietic acid and dehydroabietic acid) are among the most abundant and persistent resin acids present in some bleached and unbleached pulp mill effluents [31]. If anaerobic conditions exist in aeration ponds or sediments, transformation of these compounds to retene may occur, as proposed by Wakeham et al. [12]. Fish inhabiting these waters would be exposed to retene and other labile PAHs throughout the year, resulting in continuously elevated MFO activity in these fish relative to fish from reference sites.

A major limitation in the present experiments is that EROD activity and CYP1A protein content were related to nominal concentrations of retene added to each tank; the actual exposure concentration is unknown. A synchronous fluorometric scanning method has recently been developed to measure retene in water [32]. Measurements independent of this experiment demonstrated a 60% reduction in nominal concentrations of retene within 5 h of adding retene to exposure tanks (S. Billiard, personal communication). Adsorption of retene to the walls of the aquaria, air stones, and air tubing was shown by recovery of retene with MeOH rinses. The rapid loss of MFO induction between days 4 and 8 of the 32-d exposure may be due to an augmented adsorption associated with slime accumulation on tank walls. Therefore, the actual effective concentrations of retene are about 40% of those added to exposure tanks.

In conclusion, the results presented here show that sustained MFO induction in fish exposed to retene is not due to the persistence of the compound in the organism but to the persistence of the exposure. Thus, labile PAHs may cause the same prolonged induction as persistent chlorinated compounds such as TCDD, and might cause toxic effects typically associated with these more persistent compounds.

Acknowledgement—We thank Mark Baker, Joe Mendoza, and Diana Franks for providing valuable research assistance and John Stegeman for providing the CYP1A antibodies. We thank the National Water Research Institute for providing support to carry out the fish exposures. Funding for this research was provided by an Advisory Re-

search Council Grant and Research Initiation Grant from Queen's University. Financial support was also provided by the U.S. National Oceanic and Atmospheric Administration National Sea Grant College Program Office grant NA46RG0470, Woods Hole Oceanographic Institution Sea Grant project R/B-124 to M.E. Hahn. This is contribution 9567 from the Woods Hole Oceanographic Institution.

REFERENCES

1. Hodson PV, et al. 1992. Effects of bleached kraft mill effluent on fish in the St. Maurice River, Quebec. *Environ Toxicol Chem* 11:1635–1651.
2. Munkittrick KR, Van Der Kraak GJ, McMaster ME, Portt CB, van den Huevel MR, Servos MR. 1994. Survey of receiving-water environmental impacts associated with discharges from pulp mills. 2. Gonad size, liver size, hepatic EROD activity and plasma sex steroid levels in white sucker. *Environ Toxicol Chem* 13:1089–1101.
3. Kloepper-Sams PJ, Swanson SM, Marchant T, Schryer R, Owens JW. 1994. Exposure of fish to biologically treated bleached-kraft effluent. 1. Biochemical, physiological and pathological assessment of Rocky Mountain whitefish (*Prosopium williamsoni*) and longnose sucker (*Catostomus catostomus*). *Environ Toxicol Chem* 13:1469–1482.
4. Gagnon MM, Dodson JJ, Hodson PV, Van Der Kraak GJ, Carey JH. 1994. Seasonal effects of bleached-kraft mill effluent on reproductive parameters of white sucker (*Catostomus commersoni*) populations of the St. Maurice River, Quebec, Canada. *Can J Fish Aquat Sci* 51:337–347.
5. Servizi JA, Gordon RW, Martens DW, Lockhart WL, Metner DA, Rogers IH, McBride JR, Norstrom RJ. 1993. Effects of biotreated bleached kraft mill effluent on fingerling Chinook salmon (*Oncorhynchus tshawytscha*). *Can J Fish Aquat Sci* 50:846–857.
6. Kloepper-Sams PJ, Benton E. 1994. Exposure of fish to biologically treated bleached-kraft effluent. 2. Induction of hepatic cytochrome P4501A in mountain whitefish (*Prosopium williamsoni*) and other species. *Environ Toxicol Chem* 13:1483–1497.
7. Martel PH, Kovacs TG, O'Connor BI, Voss RH. 1994. A survey of pulp and paper mill effluents for their potential to induce mixed function oxidase enzyme activity in fish. *Water Res* 28:1835–1844.
8. Munkittrick KR, Van Der Kraak GJ, McMaster ME, Portt CB. 1992. Response of hepatic activity and plasma sex steroids to secondary treatment of bleached kraft pulp mill effluent and mill shutdown. *Environ Toxicol Chem* 11:1427–1439.
9. Muir DCG, Yarechewski AL, Metner DA, Lockhart WL, Webster GRB, Friesen KJ. 1990. Dietary accumulation and sustained hepatic mixed function oxidase enzyme induction by 2,3,4,7,8-pentachlorodibenzofuran in rainbow trout. *Environ Toxicol Chem* 9: 1463–1472.
10. Hahn ME, Stegeman JJ. 1994. Regulation of cytochrome P4501A1 in teleosts: Sustained induction of CYP1A1 mRNA, protein, and catalytic activity by 2,3,7,8-tetrachlorodibenzofuran in the marine fish *Stenotomus chrysops*. *Toxicol Appl Pharmacol* 127:187–198.
11. Burnison BK, Hodson PV, Nuttley DJ, Efler S. 1996. A bleached-kraft mill effluent fraction causing induction of a fish mixed-function oxygenase enzyme. *Environ Toxicol Chem* 15:1524–1531.
12. Wakeham SG, Schaffner C, Giger W. 1980. Polycyclic aromatic hydrocarbons in recent lake sediments—II. Compounds derived from biogenic precursors during early diagenesis. *Geochim Cosmochim Acta* 44:415–429.
13. Kovacs TG. 1986. Effects of bleached-kraft mill effluents on freshwater fish: A Canadian perspective. *Pollut Res J Can* 21: 91–118.
14. Hodson PV, Maj MK, Efler S, Burnison BK, van Heiningen ARP, Girard R, Carey JH. 1997. MFO induction in fish by spent cooking liquors from kraft pulp mills. *Environ Toxicol Chem* 16:908–916.
15. Buelow CR. 1982. Organics in the Lower Fox River system: Selected neutral compounds. MS thesis. University of Wisconsin, Madison, WI, USA.
16. Stuthridge TR, Tavendale MH. 1996. Biotransformation and partitioning of resin acids within an aeration stabilisation basin. *Proceedings*, Technical Association of Pulp and Paper Mill Industry Environmental Conference, Orlando, FL, USA, May 5–8, pp 17–30.
17. Zender JA, Stuthridge TR, Langdon AG, Wilkins AL, Mackie KL, McFarlane PN. 1994. Removal and transformation of resin acids during secondary treatment at a New Zealand bleached kraft pulp and paper mill. *Water Sci Technol* 29:105–121.
18. Parrott JL, Burnison BK, Hodson PV, Comba ME, Fox ME. 1995. Retene induces hepatic mixed function oxygenase in rainbow trout. *Proceedings*, 2nd World Congress, Society of Environmental Toxicology and Chemistry, Vancouver, BC, Canada, November 4–9, p 182.
19. Hodson PV, Efler S, Wilson JY, El-Shaarawi A, Maj M, Williams TG. 1996. Measuring the potency of pulp mill effluents for induction of hepatic mixed function oxygenase activity in fish. *J Toxicol Environ Health* 49:101–128.
20. Park SS, Miller H, Klotz AV, Kloepper-Sams PJ, Stegeman JJ, Gelboin HV. 1986. Monoclonal antibodies to liver microsomal cytochrome P-450E of the marine fish *Stenotomus chrysops* (scup): Cross reactivity with 3-methylcholanthrene induced rat cytochrome P-450. *Arch Biochem Biophys* 249:339–350.
21. Hahn ME, Woodward BL, Stegeman JJ, Kennedy SW. 1996. Rapid assessment of induced cytochrome P4501A protein and catalytic activity in fish hepatoma cells grown in multiwell plates: Response to TCDD, TCDF, and two planar PCBs. *Environ Toxicol Chem* 15:582–591.
22. Adobe Systems. 1995. *Adobe Photoshop®, Version 3.0.5*. San Jose, CA, USA.
23. Rasband W. 1995. *NIH Image, Version* 1.60. National Institute of Health, Washington, DC, USA.
24. Haasch ML, Quardokus EM, Sutherland LA, Goodrich MS, Lech JJ. 1993. Hepatic CYP1A1 induction in rainbow trout by continuous flow through exposure to β-napthaflavone. *Fundam Appl Toxicol* 20:72–82.
25. Parrott JL, Hodson PV, Dixon DG. 1995. Rainbow trout hepatic mixed-function oxygenase induction by polychlorinated dibenzo-*p*-dioxins as a function of time and tissue concentration. *J Toxicol Environ Health* 46:301–316.
26. Pesonen M, Goksoyr A, Andersson T. 1992. Expression of P4501A1 in a primary culture of rainbow trout hepatocytes exposed to β-naphthoflavone or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Arch Biochem Biophys* 292:228–233.
27. Branson DR, Takahashi IT, Parker WM, Blau GE. 1985. Bioconcentration kinetics of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rainbow trout. *Environ Toxicol Chem* 4:779–788.
28. Kleeman JM, Olson JR, Chen SM, Peterson RE. 1986. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin metabolism and disposition in yellow perch. *Toxicol Appl Pharmacol* 83:402–411.
29. Kleeman JM, Olson JR, Chen SM, Peterson RE. 1986. Metabolism and disposition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rainbow trout. *Toxicol Appl Pharmacol* 83:391–401.
30. Parrott JL, Hodson PV, Servos MR, Huestis SL, Dixon DG. 1995. Relative potency of polychlorinated dibenzo-*p*-dioxins and dibenzofurans for inducing mixed-function oxygenase activity in rainbow trout. *Environ Toxicol Chem* 14:1041–1050.
31. Pesonen M, Andersson T. 1992. Toxic effects of bleached and unbleached paper mill effluents in primary cultures of rainbow trout hepatocytes. *Ecotoxicol Environ Saf* 24:63–71.
32. Fragoso NM. 1998. Mixed function oxygenase activity of rainbow trout (*Oncorhynchus mykiss*) in relation to exposure, accumulation and elimination of retene. MS thesis. Queen's University, Kingston, ON, Canada.