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CONFIRMATION OF IN SITU EXPOSURE OF FISH TO SECONDARY TREATED BLEACHED-KRAFT MILL EFFLUENT USING A LABORATORY SIMULATION

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Abstract—To corroborate the responses in whitefish (*Coregonus lavaretus* L.) exposed to elemental chlorine free (ECF) bleached-kraft pulp mill effluent (BKME) in situ, a 30-d laboratory exposure was carried out at concentrations simulating the field conditions. The flow-through exposures were conducted at four secondary (activated sludge) treated effluent (STE) concentrations: 1.3, 2.3, 3.5, and 7%. To evaluate the role of the secondary treatment, fish were also exposed to one concentration (3.5%) of pretreated effluent (PTE) from the mill. Compared to the control, whitefish liver 7-ethoxyresorufin-*O*-deethylase (EROD) activity was twofold in fish exposed to 3.5% STE, which was similar to monooxygenase induction in the field at the same effluent dilution. The exposure to 3.5% PTE caused a 12-fold relative induction in whitefish. The activity of pentoxyresorufin dealkylase showed a high correlation with EROD activity ($r^2 = 0.85$, $p < 0.01$). The plasma concentration of 17β-estradiol was reduced by 37% ($p < 0.05$) in fish exposed to 3.5% STE, whereas testosterone was reduced by about 40% ($p < 0.05$) in fish in both 3.5% STE and PTE groups. The accumulation of chlorophenolics (CPs) and resin acids (RAs) in the bile of the fish was negligible at the three lowest STE concentrations, reflecting the nearly nondetectable levels of CPs and RAs in secondary treated whole effluent. The measured blood parameters plasma immunoglobulin M, glucose, hemoglobin, and hematocrit were not affected by effluent exposure. The responses obtained from the laboratory simulation well accorded with the exposures in the field, although signs of reproductive impairment could be detected in the laboratory. Overall, however, it is evident that the improvements to mill processes and wastewater treatment have substantially reduced the load of harmful constituents in bleached-kraft mill effluent and biological impacts in the receiving environment.

Keywords—Bleached-kraft mill effluent Elemental chlorine-free bleaching 7-Ethoxyresorufin-*O*-deethylase activity Activated sludge treatment Reproductive steroids

INTRODUCTION

Concern about chlorinated organic substances discharged by the pulp and paper industry has been evident in worldwide governmental legislation and industrial regulations. In recent years, however, the forest industry has introduced several alterations in the manufacturing of products and treatment of wastewater. As a consequence, the chemical composition of the final effluents has changed substantially [1]. The alterations, including modified cooking, substitution of chlorine dioxide for elemental chlorine, and biological treatment, are reported to have a direct beneficial impact on effluent quality as well as on the receiving environment [2]. Pulp and paper mill effluents are complex mixtures of organic and inorganic substances, many of which have remained chemically unknown and/or unidentified, with potential harmful effects on the environment. Recently, more attention has been paid to the role of many wood-derived extracts, including phytosterols, possibly having deleterious effects on fish [3].

In fish, the induction of cytochrome P4501A (CYP1A) generally indicates exposure to a wide variety of polycyclic (PAHs) and chlorinated aromatic hydrocarbons (halogenated aromatic hydrocarbons and polychlorinated biphenyls) [4]. Consequently, several studies have attempted to relate CYP1A induction in fish to pollutants, including constituents of pulp and paper mill effluents [5]. The induction of CYP1A monooxygenases (MOs) has invoked one of the most consistent responses in fish caused by bleached- and unbleached-kraft pulp mill effluents [6,7]. Thus, MO induction has been considered as a useful biomarker of exposure to pulp and paper effluents, although the ultimate significance of the induction is largely unknown [8,9]. It has been suggested that polychlorinated dibenzo-*p*-dioxins (PCDDs) and -furans (PCDFs) are not the dominant MO inducers in bleached-kraft mill effluent (BKME) [9,10]. Compounds causing MO induction in fish are suggested to be labile, moderately hydrophobic, likely planar aromatic PAHs with low chlorine substitution [11]. One of the most meaningful responses observed in fish exposed to pulp and paper mill effluents is related to the reproductive functions of fish. Fish exposed to BKME have consistently exhibited reduced steroid levels and gonadal growth, increased age to sexual maturation, and reduced expression of secondary sexual characteristics [12,13]. However, the mechanisms and constituents responsible for these disruptive effects in fish are unknown, although some wood-derived compounds, e.g., sitosterol and resin acids (RAs), present in effluents may cause disruptive effects [3,14].

Studies conducted under field conditions may suffer from varying environmental conditions, e.g., temperature, oxygenation, etc., in addition to the varying dilution and dispersion of effluents and other site-specific factors. In laboratory simulations, however, many such factors can be controlled, enabling a more accurate evaluation of the toxicological effects of the effluent itself to be made. The objective of this labo-

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ratory study was to corroborate the results obtained in the field, where whitefish were experimentally exposed to the receiving waters of an elemental chlorine-free (ECF) pulp and paper mill [15]. Second, to estimate the role of effluent treatment in a modern activated sludge plant, we also studied the effects of both pre- (PTE) and secondary treated effluents (STE) on these fish biomarkers.

MATERIALS AND METHODS

Mill and effluent characteristics

The effluent waters used in the exposures of fish were obtained from an integrated pulp and paper mill located in Lappeenranta, Finland. For two decades, the mill and the lake area receiving effluents, southern Lake Saimaa, have been extensively studied [6,16,17]. In 1992, the mill shifted to high chlorine substitution in the bleaching and introduced an activated sludge plant for effluent treatment. At present, the mill produces 461,000 t/y (1995) ECF bleached-kraft pulp using 46% softwood (SW) and 54% hardwood (HW) with the bleaching sequence DEopDED for the SW and ODEoDEpD for the HW (D = chlorine dioxide, E = caustic extraction, Eo = caustic extraction with addition of oxygen, Ep = caustic extraction with addition of peroxide, and Eop = caustic extraction with addition of oxygen and peroxide). In addition to the bleached-kraft pulp, the mill produces 142,000 t/y mechanical pulp and low (LWC) and medium weight coated (MWC) printing paper (466,000 t/y). The effluents from the mill (flow 5,000 m^3/h) are treated with a low-loaded activated sludge process, incorporating an aerobic selector. The retention time in two primary clarifiers is about 3 h and about 12 h in three secondary clarifiers (each 65 m in diameter, volume 37,000 m^3). The percentage removal of effluent variables by the treatment system was as follows: suspended solids 97%, chemical oxygen demand 81%, biological oxygen demand 97%, total nitrogen 80%, total phosphorus 90%, adsorbable organic halogens 66%, and color 31%.

Collecting pretreated and final effluent

Four consecutive 5-d composite samples were used for the laboratory experiment. Effluents were collected by the mill in 1-m^3 polyethene containers from the primary effluent clarifier (PTE) and from the secondary clarifier of the mill (i.e., from the final flow discharged to southern Lake Saimaa). The containers were transported once a week (four times during the experiment) to the laboratory, stored at 10°C, and used for the experiments within 1 week. During the experiment, the mill was under normal process conditions. The characteristics of the PTE and the STE are given in Table 1.

Fish, experimental approach, and sampling

Hatchery-reared juvenile one-year-old whitefish (*Coregonus lavaretus* L. s.l.) from the Finnish Game and Fisheries Research Institute's Fish Culture Research Station, Laukaa, Finland, were used. Before the experiments, the fish (length 16.7 ± 1.1 cm and weight 35 ± 9 g) were acclimatized for 2 weeks. The fish were randomly distributed among six identical all-steel aquariums (590 L), with 15 fish in each. During the acclimation and the exposures, fish were fed daily with hatchery fish food (Rehuraisio, Finland) equal to about 1% of the fish biomass. The photoperiod was 12 h to 12 h (light : dark), the same as in the hatchery. All exposures were conducted in flow-through conditions (1 L/min = approx. 1 L/g of fish per day).

Table 1. Mean characteristics (n = 4) of whole elemental chlorine-free pulp and paper mill primary effluent obtained from the outlet of the primary clarifier (except those denoted by [a]) and biologically treated effluent used in the laboratory experiments

Effluent/parameter	Primary effluent	Secondary effluent
pH	7.3 (0.3)[ab]	8 (0.1)[b]
P_{total} (mg/L)	0.74 (0.20)[ab]	0.08 (0.008)[b]
N_{total} (mg/L)	3.63 (0.29)[ab]	2.1 (0.75)[b]
BOD (mg/L)[c]	411 (35)[ab]	5 (0.5)[b]
COD (mg/L)	1,355 (63)[ab]	255 (17)[b]
Suspended solids (mg/L)	50 (15)[b]	8 (1.6)[b]
AOX (μg/L)	8.7 (0.8)[ab]	2.85 (0.1)[b]
Chlorophenolics (μg/L)[d]	1.73 (1.46)	0.48 (0.31)
Resin acids (μg/L)	1,100 (990)	4.65 (2.5)

[a] Sample from primary inlet effluent (before the primary clarifier).
[b] Analyzed by the analytical laboratory of the mill.
[c] Abbreviations defined in text.
[d] Free.

The effluent waters were added to activated carbon filtered tap water (pH 7.3; conductance 14.2 msiemen/m; free chlorine <0.02 mg/L) using peristaltic pumps. The effluent concentrations were simulated on the basis of the volumes of effluent and dilution water. Fish were exposed for 30 d to nominal concentrations of 1.3, 2.3, 3.5, and 7 vol % of STE and to 3.5 vol % of PTE. The first three STE concentrations were representative of the concentrations present in the receiving area in southern Lake Saimaa and corresponded to dilutions of effluent measured at distances of 16, 11, and 3.3 km from the outfall pipe of the mill [17]. The 7% concentration, which was above the maximal concentration observed in the field experiments, was used to simulate conditions at the margin of the mixing zone of effluent. The characteristics of the experimental waters (temperature, O_2, and pH) as well as their dilution flow volumes were monitored daily.

After 30 d exposure, the fish were immobilized with a blow to the head and weighed to the nearest 0.1 g. Blood (400–800 μl) was rapidly drawn from the caudal vessels into 1-ml heparinized syringes, and subsamples were taken for hematocrit (centrifuged for 3 min, 10,000 *g*), hemoglobin (Hb), and glucose determinations. The remaining blood was immediately centrifuged for 2 min (12,000 *g*) for plasma separation. Bile (30–50 μl per fish) was aspirated from the gallbladders using a fine needle, and the whole liver was dissected. Plasma, bile, and liver tissue samples were stored in liquid nitrogen until analysis. The entire sampling procedure for one fish required approximately 6 min. The condition factor (CF) was calculated using the following formula: W(g)/L(cm^3) × 100. For the preparation of microsomes, frozen livers were thawed in ice-cold Tris–HCl buffer (0.1 M, pH 7.6), to which 1 mM ethylenediaminetetra-acetic acid dipotassium salt and 0.25 M sucrose (pH 7.6) were added. Following homogenization with a Teflon pestle in an ice bath–chilled glass cylinder (500 rpm, 10 strokes), the resultant homogenate was centrifuged (LKB 2331 Ultraspin 70) at 10,000 *g* for 20 min. The supernatant liquid was further spun at 105,000 *g* for 60 min at 4°C. The microsome pellet was resuspended in 50 mM Tris–HCl (pH 7.6) buffer containing 20% (v/v) glycerol and 1 mM ethylenediaminetetra-acetic acid. Microsomes were stored in liquid nitrogen until assayed.

Analysis of chlorophenolics and resin acids in water and bile

The free and conjugated chlorophenolics (CPs) and RAs in the bile of whitefish were analyzed using a modified method

described by Oikari and Ånäs [18]. For the free CPs, bile samples (30–100 μl) were first diluted with 0.9% NaCl solution, and an internal standard (2,6-dibromophenol) was added. After acetylation with 0.5 ml K_2CO_3 and 0.5 ml acetanhydride (shaking for 3 min), samples were extracted in 2 ml hexane and evaporated by nitrogen gas. For conjugated CPs, the aqueous phase was first hydrolyzed with 1.5 ml 37% HCl for 2 h at 70°C, neutralized with 5 M NaOH, and processed further as for the free CPs. For CPs, gas chromatography (GC) (Perkin Elmer Autosystem XL) analyses were performed using a capillary column (NB-54; 25 m, inner diameter 0.32 mm) with a temperature program of 100°C/1 min to 190°C (rate 3°C/min) and further to 260°C/5 min (rate 15°C/min) with electron capture detection. The following CPs were monitored: 2,4,6-trichlorophenol, pentachlorophenol, 4,5-dichloroguaiacol, 4,5,6-trichloroguaiacol, 3,4,5-trichloroguaiacol, tetrachloroguaiacol, 3,4,5-trichlorocathecol, tetrachlorocathecol, and 6-monochlorovanillin. For the free RAs, diluted (to 1 ml) and acidified (pH 3 with H_2SO_4) bile samples were extracted three times with 2 ml methyltributylether (MTBE); combined extracts were evaporated with nitrogen gas to 1 ml and methylated with diatsomethane. Heptadecanoic acid was used as an internal standard. For the conjugated RAs, the aqueous phase was hydrolyzed with 2 ml KOH (2 h at 70°C) after addition of the internal standard. After acidification with H_2SO_4 (pH 3), the samples were processed as for free RAs. Gas chromatographic analyses for RAs were performed with the temperature program of 150 to 250°C (rate 4°C/min) and further to 280°C/8 min (rate 10°C/min) using a flame ionization detector.

The CPs in water samples were analyzed by GC with a modified method originally described by Voss et al. [19]. For the free CPs, 50 ml of sample was first neutralized with NaOH and acetylated with 1 ml K_2CO_3 and 1 ml acetanhydride by shaking for 3 min. Samples were extracted with 5 ml hexane and evaporated with nitrogen gas. Gas chromatography was performed similarly for the bile CPs. For the RAs in water, a 250-ml sample was acidified (pH 3.5 with H_2SO_4) and then extracted three times with 20 ml + 10 ml + 10 ml MTBE. Combined extracts were evaporated with nitrogen gas to 5 ml and frozen (−20°C) for 2 to 4 h. The rethawed sample was further evaporated to 1 ml and methylated with diatsomethane. The temperature programs for the water CPs and RAs were similar to those for bile analyses.

Enzyme assays

The activity of hepatic MO was measured fluorometrically as activity of 7-ethoxyresorufin-*O*-deethylase (EROD) and pentoxyresorufin-*O*-deethylase (PROD), according to the method of Burke et al. [20], adapted for the microplate format (Ascent microplate fluorometer, Labsystems, Vantaa, Finland). Microsomes (600–800 μg protein per well) were incubated in 100 mM potassium phosphate buffer of pH 8, 2.5 μM EROD or 5 μM PROD (Sigma Chemical, St. Louis, MO, USA) and 0.5 mM of the reduced form of nicotinamide adenine dinucleotide phosphate (Sigma Chemical) to a final volume of 200 μl. Fluorescence (excitation 530, emission 584) was recorded at 30-s intervals for 4 min at 20°C. Microsomes from rainbow trout injected by β-naphthoflavone (50 mg/kg) were used as positive controls for the EROD and PROD assays. The protein concentration of microsomes was measured with a Bio-Rad DC Protein Assay Kit, using bovine serum albumin as the standard.

Table 2. The concentrations of chlorinated phenolics (free) and resin acids in the whole primary and the whole secondary effluents collected as weekly composites (4) from the primary clarifier and the secondary clarifier (final effluent) of the mill studied[a]

Compound	Primary effluent	*n*	Secondary effluent	*N*
Chlorophenolics (μg/L)				
4,5-Dichloroguaiacol	0.61	4	0.17	4
4,5,6-Trichloroguaiacol	0.03	2	<0.01	1
3,4,5-Trichloroguaiacol	ND		<0.01	2
Tetrachloroguaiacol	0.04	1	ND	
3,4,5-Trichlorocatechol	0.11	4	ND	
Tetrachlorocathecol	ND		0.02	2
2,4,6-Trichlorophenol	ND		0.01	2
Pentachlorophenol	0.01	3	<0.01	2
6-Monochlorovanillin	1.95	2	0.27	4
Resin acids (μg/L)				
Pimaric	250.18	4	1	1
Sandaracopimaric	61.03	4		
Isopimaric	308.73	4	1.4	2
Palustric	197.85	4	1.25	2
Neoabietic	196.35	4		
Dehydroabietic	343.9	1	1.95	2
Abietic	350.0		2.07	4

[a] Mean of four weekly composites; *n* = number of times found in separate composites (maximum, 4); ND = detected.

Plasma and blood parameters

The concentration of whitefish plasma immunoglobulin M (IgM) was determined with the enzyme-linked immunosorbent assay. Antibodies against trout IgM were used as trapping and detecting agents as described earlier [21]. The assay was standardized with a known concentration of purified whitefish IgM. Samples for blood glucose were immediately mixed with 0.6 M $HClO_4$ and centrifuged for 2 min at 12,000 rpm before freezing. The plasma glucose was determined using a Boehringer Mannheim test kit (GOD-Perid method 124036). Plasma testosterone and 17β-estradiol concentrations were determined using Fenzia Enzyme Immunoassay test kits (EIA, Orion Diagnostica, Espoo, Finland); samples were read with a plate reader (Labsystems EMS Reader MF, Vantaa, Finland) at 405 nm. Hematocrit (Hct) was determined using heparinized microhematocrit tubes within 3 min of sampling. Hemoglobin was measured spectrophotometrically using the cyanmethemoglobin method.

Statistics

All the data were first tested for normality and homogeneity of variance (Levene test) to meet statistical demands. The MO activities, the levels of reproductive steroids, and the bile accumulation of compounds in the different exposure groups were compared to the control group using a nonparametric Kruskal–Wallis test. The blood and blood plasma parameters were first log-transformed and compared using one-way analysis of variance followed by Tukey's HSD test. For examining correlations, linear regression analyses were performed. The significance level (denoted by an asterisk) was set to $p < 0.05$. All the statistics were performed using SPSS® software.

RESULTS

Effluent constituents

The selected CPs and RAs were analyzed from four whole effluent composites collected weekly from the primary and the secondary clarifiers of the mill (Table 2). The mean concen-

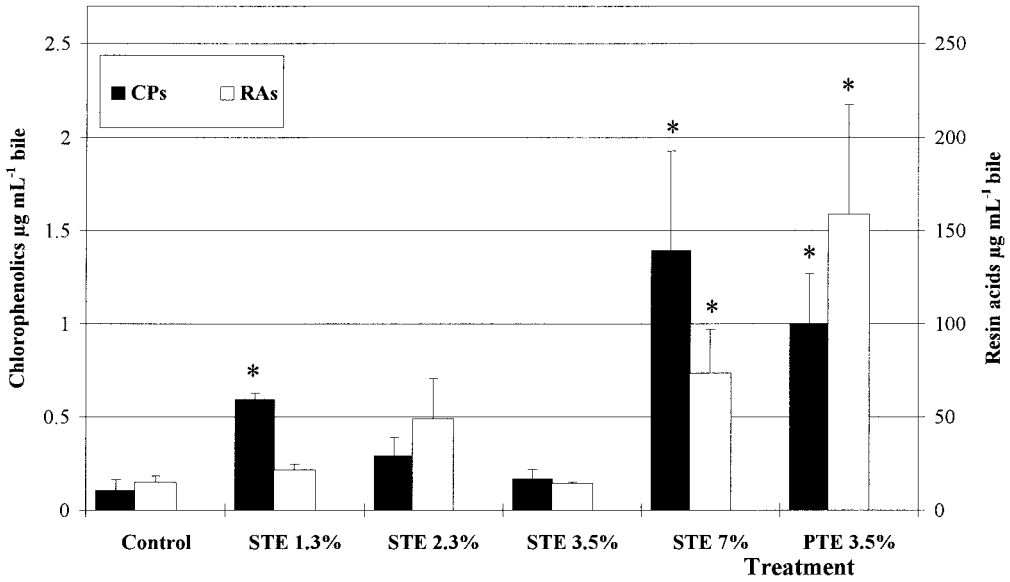

Fig. 1. The concentrations of total (conjugated and free) chlorophenolics (CPs) and resin acid (RAs) in the bile of whitefish exposed for 1 month to four concentrations (1.3, 2.3, 3.5, and 7%) of biologically treated elemental chlorine-free bleached pulp and paper mill effluent and to one concentration of primary treated effluent from the same mill. Values: mean ± standard error of the mean (n = four fish per treatment). Asterisks (*) indicate significant ($p < 0.05$) differences between control and effluent exposed groups. Significant difference ($p < 0.05$) between control and exposed group.

tration of CPs in the whole secondary effluent was 0.5 μg/L and in the whole PTE was 1.7 μg/L. Thus, the average reduction of CPs was 70% in STE compared to that in PTE. However, a great variation in the CP concentrations was found between the weekly composites. Predominant CPs detected in both the STE and the PTE were 4,5-dichloroguaiacol and 6-monochlorovanillin. The mean RA concentration in STE was 5 μg/L, whereas in PTE it was 1,100 μg/L. As with the CPs, the concentrations of the RAs varied considerably between the whole effluent composites. In the STE, the predominant RAs were abietic acid, dehydroabietic acids (DHAA), isopimaric acid (IPA), and palustric acid (PA). In the PTE, IPA and DHAA were dominant, although the concentrations of pimaric acid, PA, and neoabietic acid were almost at the same level. Based on the whole effluent concentrations of the CPs and RAs, the nominal concentration of the CPs in all the diluted secondary effluent treatments was <0.035 μg/L and in the diluted PTE was 0.06 μg/L. The RA concentration in all the diluted STE treatments was <0.3 mg/L and in the primary effluent treatment was 40 mg/L.

Accumulation of effluent constituents in the bile

The accumulation of the CPs (Fig. 1) and RAs (Fig. 1) in the bile of fish was used to assess the relative exposure to the effluent constituents. The bile concentrations of the CPs (free + conjugated) were low in fish exposed to STE concentrations of 1.3% (0.6 μg/ml, $n = 3$), 2.3% (0.3 μg/ml, $n = 3$), and 3.5% (0.17 μg/ml, $n = 3$), whereas at 7% STE the bile CP concentration was 1.5 μg/ml ($n = 3$, $p < 0.05$). Thus, the accumulation of the CPs in the bile was not dose related. In the PTE group, the bile CP concentration was 1 μg/ml ($n = 3$, $p < 0.05$). In the 7% STE group, the most abundant CPs were 2,4- and 2,4,6-chlorophenols, whereas in the PTE group 4,5-dichloroguaiacol (52%) and 6-monochlorovanillin (14%) were dominant. The ratio between the calculated concentration of ambient water CPs (free) and the bile CPs (free and conjugated) was $1:3 \times 10^4$ in 7% STE and in 3.5% PTE $1:1.7 \times 10^4$. The nominal water concentrations of the CPs were not correlated with the bile accumulation of the CPs ($r^2 = 0.59$, $p = 0.23$, $n = 5$).

The sum of free and conjugated RAs in the bile of fish exposed to 1.3, 2.3, and 3.5% STE was statistically indistinguishable from the control, whereas at 7% STE the accumulation was substantially higher (70 μg/ml, $p < 0.05$). Thus, as with the CPs, no dose dependence was found in RA accumulation. However, high RA accumulation (160 mg/L, $p < 0.05$) was measured in fish exposed to 3.5% PTE. In both the STE and PTE groups, DHAA, IPA, and PIA were the most abundant RAs. The ratio between the nominal concentration of ambient water RAs and the RAs in the bile was $1:9.3 \times 10^4$ in 3.5% STE, $1:2.4 \times 10^5$ in 7% STE, and $1:4 \times 10^3$ in 3.5% PTE. The correlation coefficient (r^2) between the nominal water concentrations of the RAs in the exposure and the bile accumulation of the RAs was 0.53 ($p = 0.27$, $n = 5$).

Hepatic monooxygenase activity

Compared to the control, STE caused a significant EROD increase (Fig. 2) in fish exposed to 3.5% (twofold, $p < 0.05$, $n = 15$) and 7% (twofold, $p < 0.05$, $n = 15$) effluent concentrations. In fish exposed to 1.3 and 2.3% STE, EROD activity was equal to the control group. There was no correlation between effluent volume dilution and liver EROD activity in the STE groups ($r^2 = 0.77$, $p = 0.12$). Although EROD induction remained low at the secondary effluent treatments, an 11-fold relative induction ($p < 0.05$, $n = 15$) compared to the control was observed in fish exposed to 3.5 vol % primary treated effluent. Similar to the EROD induction, liver PROD activity (Fig. 2) was significantly increased (1.6-fold, $p < 0.05$) in the 3.5 and 7% STE (1.8-fold) groups and in the 3.5% PTE group (ninefold) compared to the control. Additionally, the combined data from hepatic EROD and PROD activity exhibited a high correlation ($r^2 = 0.85$, $p < 0.01$, $n = 85$). By linear regression, no statistically significant relationships were found between the hepatic EROD and the other biomarkers measured.

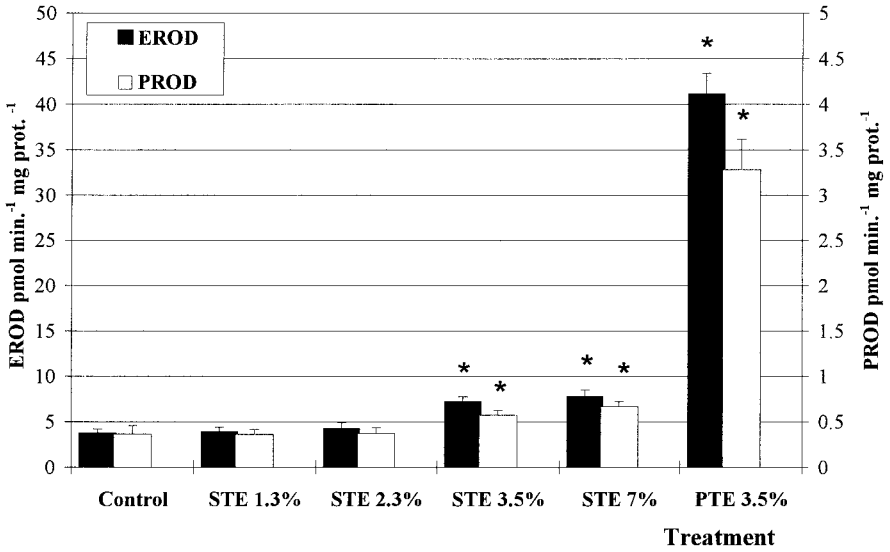

Fig. 2. The activity (pmol min^{-1} mg $prot.^{-1}$) of hepatic microsomal 7-ethoxyresorufin-*O*-deethylase and pentoxyresorufin-*O*-deethylase in whitefish exposed to different concentrations of biologically treated effluent and one concentration of primary treated effluent. Values: mean ± standard error of the mean (n = 14 or 15 fish per group). Asterisks (*) indicate significant differences ($p < 0.05$) between control and effluent exposed groups.

Reproductive steroids

Plasma concentration of 17β-estradiol (Fig. 3) decreased in fish exposed to both 3.5% STE (mean 1.2 nmol/L, 37%, $p < 0.05$, $n = 14$) and 3.5% PTE (1.5 nmol/L, 20%, $p > 0.05$, $n = 13$), relative to the control (1.9 nmol/L). Quite similarly, compared to the control (11.8 nmol/L) testosterone (Fig. 3) was reduced by 41% ($p < 0.05$, $n = 14$) in the 3.5% STE group (mean 7.0 nmol/L) and by 40% ($p < 0.05$, $n = 14$) in the 3.5% PTE group (7.1 nmol/L).

Blood and plasma biomarkers

Relative to the control, the levels of plasma IgMs of whitefish were unchanged in all the STE groups. Similarly, in the PTE group, the slight increase (7%) was not statistically significant (Table 3). Blood Hb concentration remained unchanged in all the groups (Table 3), as was Hct and plasma glucose (Table 3).

Growth and condition of fish during the experiments

No mortality was recorded in exposed animals, even in the primary effluent (3.5%). The highest CF was found within the control group (0.996 ± SD 0.054), but no statistical differences were found among the groups (the data not shown). During the 30-d exposure, the mean increase in weight of the fish in all groups, including the control, was 34% (data not shown).

DISCUSSION

Assessing the exposure of fish to effluents

In addition to causing a drastic decrease in such organochlorines as CPs, the changes implemented in 1992 in pulping,

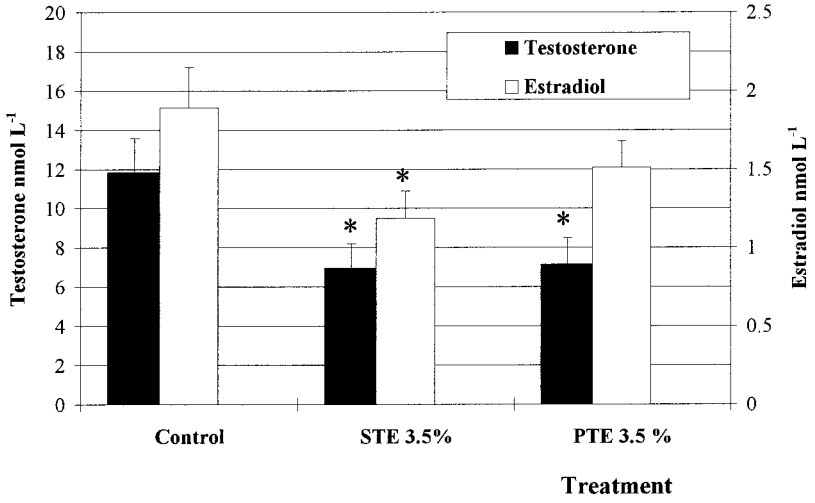

Fig. 3. Plasma 17β-estradiol and testosterone concentrations (nanomolar) in whitefish exposed for 1 month to 3.5% secondary treated effluent and primary effluent obtained from the mill producing elemental chlorine-free kraft pulp and printing paper. Values are expressed as means ± standard error of the mean (n = 13 to 15 fish per group). Significant difference ($p < 0.05$) between control and effluent exposed groups is indicated by asterisks (*).

Table 3. Hematological parameters measured in whitefish exposed for 1 month to the primary effluent and the following secondary treated effluent from the elemental chlorine-free bleached kraft pulp and paper mill[a]

Treatment	Immunoglobulin M (g/L)	*N*	Hematocrit (%)	*N*	Hemoglobin (g/L)	*n*	Glucose (mmol/L)	*n*
Control	1.26 (0.38)	15	34 (5)	15	48.9 (5.3)	14	2.06 (0.34)	15
SE 1.3%	1.24 (0.37)	15	34 (3)	14	47.6 (5.2)	15	2.06 (0.29)	13
SE 2.3%	1.35 (0.37)	14	34 (5)	14	50.4 (6.0)	14	1.73 (0.28)	13
SE 3.5%	1.19 (0.43)	15	34 (3)	15	52.7 (9)	15	1.9 (0.26)	15
SE 7%	1.31 (0.36)	15	34 (4)	15	48.7 (6.6)	15	1.99 (0.31)	15
PE 3.5%	1.55 (0.41)	15	34 (3)	13	49.4 (6.4)	13	2.06 (0.48)	13

[a] Mean (±SD); n = number of fish analyses.

bleaching, and wastewater treatment have also decreased the presence of wood-derived extractives, such as RAs and fatty acids in the effluent of the mill investigated. Compared to the situation before the changes, the total CPs decreased by 98 to 99% and RAs by 94% in the final whole effluent of the mill. As a consequence, the CP and RA concentrations present in the recipient water are nearly nondetectable by GC [2,22]. Thus, in relation to these chemical markers in BKME, the exposure of fish to the effluent constituents was low in the present experiments, particularly when compared to the earlier studies conducted before the mill alterations [6,16,17]. However, the effluent concentrations chosen for the exposures were environmentally relevant and representative of those prevailing in the receiving area of mill effluents [15,17,22]. In addition, to roughly predict the scope of ecotoxicological risks, one higher level of effluent dilution (7%) was also studied for comparison. Overall, in regard to the exposure of fish to the effluents in the wild, the present laboratory simulation was well comparable with the field circumstances. In this study, we examined responses in fish after a 30-d exposure period. However, since variations in the concentrations of effluent constituents, e.g., CPs and RAs, in the whole effluent composites were large, measurements performed at various times during exposure may have provided more information on the characteristics of the fish responses. This type of information would be useful in evaluating the significance of the responses in fluctuating and changing field circumstances.

Fish bile as a measure of exposure

Accumulation of water contaminants in the bile of exposed fish can be used as an estimate of the received internal dose of these constituents. Consequently, bile metabolites have been suggested as a sensitive and reliable biomarker of exposure to components of pulp and paper mill effluent [6,15,17]. Due to the low ambient CP and RA concentrations in the present exposures, the biliary accumulation of CPs and RAs in exposed fish was negligible at low (1.3–3.5%) BKME concentrations. Simultaneous field exposure of whitefish in the receiving water of the mill revealed a similar low biliary accumulation of CPs and RAs. Further, the rapid clearance of CPs and RAs by biliary elimination suggests minor effects on fish in these environmentally relevant effluent concentrations. When measurable, the bioconcentration factors (BCFs) between the bile and the ambient water were 10^4 for the CPs and 10^5 for the RAs, comparable to the BCFs reported earlier [17,23]. However, the fish exposed to the PTE showed a substantially lower BCF of the RAs (10^3), apparently indicating substantially lower bioavailability of the RAs by the fish in the PTE treatment. Due to the low levels of CPs and RAs analyzed in the commercial fish food used in the experiments, low accumulations of these constituents were also detected in the bile of the control fish. Moreover, due to the lack of mass-selective GC, the nonspecific background complicated the interpretation of the results.

Exposure of fish to pulp and paper mill effluents and monooxygenase induction

Several field and laboratory studies have reported that effluents from mills using different pulping and bleaching technologies have the capability of inducing hepatic CYP1A-dependent MOs in fish [7,17,24]. Consequently, MO induction is one of the most consistent biological responses measured in fish affected by pulp and paper effluent. It has been generally accepted that most pulp and paper mill effluents contain MO-inducing components, although the low inducing capacity of effluents seems to be predominant. This is well documented by an extensive Canadian study [25], in which only 33% of the 36 STEs, obtained from several types of mills (bleached-kraft, thermomechanical, chemothermomechanical, and ground wood) caused significant, more than twofold EROD induction in rainbow trout at a 10% effluent concentration. However, a wide range of effluent concentrations from 0.33 vol % (a mill with 50% ClO_2 substitution in bleaching and secondary treatment) to 9.1 vol % (unbleached pulp, aerated lagoons as secondary treatment) has observed the thresholds for MO induction for five mills tested [26]. Munkittrick et al. [7] concluded that the concentration threshold required for biological effects including EROD induction at some mills was less than 1 vol %. In our subchronic experiments, approximately twofold induction was measured in fish exposed to 3.5 and 7 vol % STE compared to the control. The threshold effluent concentrations for the MO induction were about 1% in the present laboratory and in the recent field exposures [15]. Although there is no distinct trend in MO induction regarding different pulping, bleaching, and treatment processes [25], varying levels of MO induction, in addition to many physiological and toxicological factors, reflect varying concentrations and structures of specific chemicals related to factors in production and treatment processes [11]. Although the CPs and RAs measured in this study were not representative of the EROD inducer itself, they may act as surrogates for the existence or dilution of other possibly inducing agents. However, the individual compounds responsible for MO induction were beyond the scope of the present experiments.

It is evident that many technological alterations in pulping, bleaching, and effluent treatment introduced in mills have resulted in improved quality of wastewaters, with a substantially lower impact on the aquatic ecosystems [27]. Apparently, the reduced potency of effluents for MO induction also reflects these improvements. In our previous studies, a notable decrease (80%) in EROD induction was seen in whitefish caged

in the receiving area of the mill under study in 1993 [2], after the shift to 100% chlorine dioxide substitution in bleaching and the implementation of a biological treatment, compared to the same recipient in 1991. However, we have previously observed that the EROD induction potency remained constant in fish exposed to low-chlorine ($C_{1–5\%}/D_{95–99\%}$) bleaching effluent compared to the sequence used formerly ($C_{5–45\%}/D_{55–95\%}$) [21], suggesting that a partial increase in ClO_2 in the bleaching does not reduce EROD activity in fish. However, lower-chlorinated compounds are more easily degraded during the biotreatment of effluents. Furthermore, increased substitution of chlorine dioxide for molecular chlorine in bleaching with secondary treatment reduced the levels of PCDDs/PCDFs found in fish exposed to the effluent [28]. In 1995, EROD induction in exposed whitefish in the recipient of the mill [15] was well correlated to the inductions in the present laboratory validation. Overall, MO inducing potency of the final effluent of the mill has been observed to remain approximately the same since 1993, after which the mill has introduced only small alterations in its processes.

Activity of PROD, considered as an indicator of CYP2B induction in mammals, was elevated also in the whitefish exposed to the mill effluents. Although piscine isoenzymes have been shown to possess homologues to the mammalian 2B family, these enzymes lack the inducibility of typical mammalian CYP2B inducers [29]. Whether the PROD induction indicates a CYP2B response remains unclear in the present study, but the distinct correlation between the EROD and PROD induction supports the theory that PROD activity only reflects the CYP1A induction, as also suggested earlier [20,30].

Currently, several findings strongly support the suggestion that polychlorinated dibenzo-*p*-dioxins and -furans are not, at least primarily, responsible for MO induction in fish exposed to BKME [28]. Recently, in regard to their chromatographic properties, compounds causing MO induction in fish are said to be moderately hydrophobic, likely planar, aromatic PAHs with low chlorine substitution [11]. These included an alkyl-substituted phenanthere (retene), which has been observed to induce EROD in rainbow trout [31]. It has been speculated that the MO inducers in BKME are generated primarily from lignin or humic material liberated and leached from cellulose in both the pulping and bleaching [11,26].

Effects of secondary treatment of bleached-kraft mill effulent on monooxygenase induction

Our laboratory experiments showed a prominent MO induction in whitefish exposed to the diluted PTE, whereas the induction of the subsequent biologically treated effluent from the same mill was one fifth of the former. This indicates that the biotreatment of wastewaters with the activated sludge process was effective in removing the induction potency of the effluent. In our previous laboratory validation before the introduction of the activated sludge plant (aerated lagoons as secondary treatment), a high EROD induction was observed in exposed fish, although the mill used the bleaching sequence with a high chlorine dioxide substitution (98–99%) [21]. High EROD induction in fish exposed to effluents with a substantially lower chloroorganic content is in accordance with the conclusions that chlorine is not essential for MO induction in fish exposed to BKME [9,24].

In accordance with our studies over the 1990s, it has been shown that optimized or enhanced secondary treatment could substantially reduce the EROD-inducing potential of the pulp mill effluent to negligible levels [9], although more work is required to understand the real role of secondary treatment on effluent quality [25]. It is evident that secondary treatment may reduce, but not abolish, the potential of an effluent to induce CYP1A activity in fish [32].

Effects of exposure on fish reproductive functions

Several studies have consistently reported the effects of BKME on fish reproductive functions. Exposed fish exhibited reduced circulating levels of reproductive steroid hormones, reduced gonadal growth, an increased age to sexual maturation, and reduced expression of secondary sexual characteristics [13,14,33]. A recent review on the in situ assessment of pulp mill effluents on reproductive parameters indicated that eight of the 10 populations showed increasing age to sexual maturity, and four of the six species studied had reduced gonad size, recorded in 14 of the 24 reported cases [34]. Consistent with earlier studies, we also observed significant changes in the levels of plasma steroid hormones 17β-estradiol and testosterone in fish exposed to both the STE and the PTE. However, simultaneous field studies gave contradictory results when caged juvenile whitefish did not display significantly decreased steroid levels [15], while feral female perch and roach in the same area had reduced levels [35]. In theory, in pulp mill effluents, some compounds, including β-sitosterol, exist that cause hormonal changes in fish [14], although the detailed mechanisms of the endocrine disruptions, as well as the compounds involved, are still largely unknown. Thus, further studies are needed in this respect. The CYP1A activity and the levels of the sex steroids did not correlate, which is consistent with several other reports failing to show direct correlation between induced MO activity and the hormone levels [7,8,36].

Exposure and physiological responses

Pulp and paper mill effluents include factors that have been found to affect the immune system in fish [37]. Reduced levels of IgM have been previously observed in whitefish exposed to the effluent from the same mill before the process and treatment changes [17,21]. The immunological system of whitefish, as indicated by IgM levels in blood plasma, was not affected by the pulp and paper mill effluents under the laboratory conditions. Similar results were, however, confirmed earlier in the field conditions [22]. It is thus evident that the alterations to the processes and in the wastewater treatment have had beneficial effects on fish immunological systems, which may decrease the susceptibility to microbial diseases or parasitic infections. As with the IgM, the other measured parameters, plasma glucose, Hct and blood Hb, were not affected in fish. Disturbances in metabolic dysfunctions including carbohydrate metabolism have previously been observed in fish exposed to BKME [38]. In all, the general additional physiological parameters examined in the present study showed no physiological disruptions or disturbances.

CONCLUSIONS

When compared to the field exposure, the responses in the present laboratory simulation were consistent with the field exposure, and the selected series of biomarkers proved to be relevant in quantifying the effluent exposure and effects on fish, both in the laboratory and in the field. However, in contrast to the field exposure, decreased levels of the reproductive steroids were observed in fish exposed even to the final effluent of the mill. On the other hand, the effluent treatment was seen

to substantially reduce the potency for MO induction in fish. Thus, it is evident that modernized mill processes and advanced wastewater treatment technology have substantially reduced the load of harmful constituents in BKME, consequently diminishing the biological impact on the receiving aquatic environment. However, there still exist some risks relative to the health and fitness of fish.

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