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EVALUATING THE POTENTIAL OF EFFLUENT EXTRACTS FROM PULP AND PAPER MILLS IN CANADA, BRAZIL, AND NEW ZEALAND TO AFFECT FISH REPRODUCTION: ESTROGENIC EFFECTS IN FISH

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Abstract: The authors examined the potential of pulp mill effluent from pulp-producing countries (Canada, Brazil, New Zealand) to affect fish reproduction. Specifically, the estrogenic effects in juvenile rainbow trout (*Oncorhynchus mykiss*) pulse-exposed to 11 different mill effluent extracts (intraperitoneal injections of solid-phase extraction–dichloromethane nonpolar fraction). The results indicated that effluent extracts were estrogenic in juvenile trout irrespective of the gender, as reflected by increasing level of plasma vitellogenin (VTG; Brazil > New Zealand > Canada). Despite the high variability observed among mills, differences in VTG levels were related to the type of mill process (kraft > elementary chlorine-free kraft > thermomechanical pulping). Moreover, effluent treatments did not appear to significantly decrease VTG induction. A consistent estrogenic effect was observed in those mills that process a combination of feedstocks (softwood and hardwood), with the highest increase in VTG related to eucalyptus feedstock. The results demonstrate significant estrogenic effects of pulp mill effluents on chronically exposed juvenile trout, suggesting that in vivo metabolic activation of precursors is necessary to cause the observed increases in VTG levels. This molecular estrogenic response provides a useful starting point for predicting population-level impacts through the adverse outcome pathway methodology. *Environ Toxicol Chem* 2017;36:1547–1555. © 2016 SETAC

Keywords: Pulp mill Intraperitoneal injection Fish Vitellogenin VTG Estrogenic Estrogen Effluent

INTRODUCTION

Effects of pulp and paper mill effluents on fish reproduction have been reported for more than 3 decades [1–3]. Various North American, Scandinavian, New Zealand, and Chilean studies have found that effluents from all types of mill processes, wood furnishes, and treatments are capable of impairing fish reproduction from the molecular to the individual or population level [4–6].

Although the body of research on the reproductive effects of such effluents in fish is 1 of the most extensive involving endocrine-disrupting substances in the environment [7], understanding of the actual causes of, and mechanisms involved with, such reproductive effects is still controversial and remains incomplete [6]. Despite significant effort, limited progress in identifying causative compounds of reproductive effects in fish and its sources has been made, mainly because of a combination of factors including the complexity and variability of effluents from pulp and paper mill industries, in addition to the variety of experimental techniques utilizing different fish species, environmental or laboratory conditions, and research objectives [6]. Furthermore, pulp and paper mill effluents are extremely complex mixtures, which are poorly described chemically, with detailed chemical analyses rarely provided in fish exposure experiments. Effluent characteristics depend on the wood feedstock type (softwood/hardwood), process (mechanical/kraft, unbleached/bleached), and treatment technology used [8].

A previous study simultaneously comparing effluent samples from Brazil, Canada, and New Zealand [4], using recently developed chemical measurements that are predictive of the effects of mill effluents on fish reproduction, investigated mills that employ a range of pulp production technologies, including those utilizing the best available technology. That study included extracts of wood feedstocks that are representative of those used globally in pulp production. Both filtration and solid-phase extraction (SPE) were utilized in series to broadly fractionate effluents based on polarity to determine the prevalence of androgenic effects (measured as ligands for goldfish testis androgen receptor [AR]). Such effects were derived from intermediate to nonpolar ligands associated almost exclusively with the nonpolar fractions, and inhibition of neurotransmitter enzymes (monoamine oxidase [MAO] and glutamic acid decarboxylase) related to the most polar effluent extracts [4], which may lead to increased levels of dopamine, affecting the hypothalamus–pituitary–gonad axis production of gonadotropin hormones, thereby decreasing the synthesis of steroids affecting the final fish vitellogenesis [9–12]. The prevalence of these effects was such that no differences were observed on the basis of wood feedstock or country. A decrease in androgenic effect was only associated with the type of effluent treatment, whereas relationships between pulping and bleaching processes and glutamic acid decarboxylase or MAO inhibition were observed. Furthermore, few estrogenic effects

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were observed by the in vitro yeast estrogen screening assay, and these were only observed in Brazilian mill nonpolar SPE extracts [4]. This lack of observed estrogenic effects differs from recent studies using in situ exposures [13] and previous studies using similar intraperitoneal injections of SPE extracted effluents where not only estrogenic effects were prevalent [5,8,14] but also the presence of compounds capable of inducing androgen aromatization enzymes leading to a final estrogenic effect was established [15–17]. The reasons for these discrepancies are unclear and need to be understood to formulate best management practices for mills [18] that result in effluents that have minimal or no effect on fish reproduction.

Previous research indicates that resin acids and phytosterols are consistently found in pulp mill effluents (despite effluent treatment improvements) and are capable of inducing liver mixed-function oxygenase enzymes, being also implicated in liver damage [14,19,20]. This hepatocellular injury can not only affect detoxification enzymes but also cause endocrine-disruption effects [21], representing another confounding factor with respect to determining the toxicity and estrogenicity of such effluents [14].

To address the discrepancy with results from previous studies and to investigate whether in vivo metabolic processes are involved, the present study evaluated the reproductive toxicity of mill effluent extracts (generated using SPE) from pulp-producing countries such as Canada and New Zealand, where the industry is well established and longtime efforts in minimizing impacts and continuous monitoring programs have been developed, compared with a South American producing country (Brazil) with no monitoring programs (Table 1). A chronic, multiple intraperitoneal injection protocol was used to enable international shipping and allow an evaluation of the primary short-term reproductive endpoints in the plasma vitellogenin (VTG) level and gonadosomatic index (GSI).

The present study builds on previous work from these countries [4] and from Chile [5,8] utilizing a number of molecular endpoints, including hepatic ethoxyresorufin-O-deethylase (EROD) activity induction and altered activity of liver respiratory-metabolic enzymes including lactate dehydrogenase (LDH) and citrate synthase to establish if inhibitory effects on hepatic aerobic and anaerobic metabolic capacity because of cellular liver damage from other components within mill effluent extracts act as confounding factors when assessing detoxification enzyme and endocrine-disruption effects. Finally, vitellogenesis was studied in detail as 1 of several examples where linkages exist via an adverse outcome pathway (AOP) between initiating events at the molecular level and effects at the individual and population levels. Such results can be used to predict population-level impacts of mill effluents in jurisdictions where effects-based monitoring programs are not in place.

MATERIALS AND METHODS

Effluent samples

Final mill-treated effluent samples (6 L) were collected either as grab samples or 24-h composite samples (Table 1) [4]. New Zealand samples were collected and extracted on the same day by SCION (New Zealand), whereas Brazilian samples were collected and extracted by APLYSIA (Environmental Research) within 2 d. Canadian mill samples were immediately shipped refrigerated overnight to the Canada Center for Inland Waters (Environment Canada) and extracted on arrival.

Effluent solid-phase extractions and extract selection

Final effluent samples were pH-adjusted (7–8) using concentrated hydrochloric acid (3 M) to 4 ± 0.2 and then 2% (v/v) methanol was added. These fortified effluents (500 mL) were extracted by SPE using a reverse-phase macroporous hyper-crosslinked copolymer resin cartridge (1 g, 20-cc cartridge, Oasis HLB; Waters) previously equilibrated with 2×20 mL of dichloromethane, methanol, and water (adjusted to pH 4). Extractions were undertaken under a slight vacuum (rate of 1–2 drops/s, ~15 mm Hg). Following complete elution, samples were washed in 10 mL of high-performance liquid chromatography–grade water (pH 4) and completely dried under full vacuum for 1 h. Forty milliliters of dichloromethane were added to elute the cartridge, producing the SPE–dichloromethane–extracted material fraction (medium polarity to nonpolar compounds) and 40 mL of methanol to produce SPE–methanol–extracted material fraction (polar compounds) [4].

Intraperitoneal injection

We utilized extracts and fractions generated from Milestone et al. [4]. When fraction compositions were analyzed by gas chromatography (GC)–tandem mass spectrometry, the highest GC index was calculated by integrating the detected peak to the response of the internal standard (C19 alkane; Restek) and normalized against corresponding method blanks. This provided a measure of extractable organics and is positively correlated to biochemical oxygen demand, which was observed in the SPE–dichloromethane fraction for all effluents. Thus, this fraction was selected for the intraperitoneal injections. A preliminary acute 96 h toxicity test was undertaken to determine nonlethal intraperitoneal doses [22] of mill extracts and steroid standards [8].

The SPE–dichloromethane extracts were concentrated using rotary evaporation followed by reduction to dryness by gaseous nitrogen, resuspended in 100 μL of acetone, and finally dissolved in a carrier solution (corn oil) until acetone was completely evaporated prior to fish intraperitoneal injection. Extract doses were calculated before injection, based on individual fish weights and a desired injection volume of 200 μL of effluent extracts (SPE–dichloromethane, 200 mL effluent equivalents per 100 g of trout body wt) using doses previously described [8].

In total, 420 immature hatchery-reared rainbow trout, *Oncorhynchus mykiss* (43 ± 12 g; Rainbow Springs Hatchery), were exposed by intraperitoneal injection (25 mg/kg wet wt) to 11 pulp mill effluent extracts over 28 d (Table 1): 5 extracts coming from Brazil (Bra A, Bra B, Bra C, Bra D, Bra E), 4 obtained from Canada (Can A, Can B, Can C, Can D), and 2 from New Zealand (NZ A, NZ B), plus 17 β-estradiol as a model estrogen standard (5 mg/kg wet wt) and testosterone (1 mg/kg wet wt) as a model androgen standard (Sigma–Aldrich) based on our previous experience using aromatizable androgens that cause a final estrogenic effect in vivo [5]. The control group was intraperitoneally injected with corn oil carrier (25 mg/kg wet wt). Previously, fish were acclimatized for 2 wk in 3×1000 L tanks (5.9 kg/m^3 of density at 12 ± 1 °C) in flow-through water (3 L/min) and daily fed until satiated (1.5 mm Silver Cup pellets; Martin Mills). Fish were anesthetized (100 mg/L, tricaine methanesulfonate) weighed, placed in a flow-through water holding tray system, and intraperitoneally injected using a precision animal health syringe 187 (SOCOREX model 187; Swiss) and 22-G disposable needles (BD P/N 305155). After

Table 1. Pulp and paper mill effluents description and solid-phase extracts main effects

	Bra A	Bra B	Bra C	Bra D	Bra E	Can A	Can B	Can C	Can D	NZ A	NZ B
Effluent description[a]											
Mill type	ECF Kraft	Kraft	ECF Kraft	ECF Kraft	ECF Kraft	ECF Kraft	TMP	TMP	ECF Kraft	ECF Kraft	ECF Kraft/TMP
Bleaching	$D_OE_{OP}DD$	U	$D_{HOT}O_PD$	$OADE_{OP}D$	$D_{HOT}E_{OP}DP$	$DE_{OP}D$	NA	NA	$DE_{OP}DED$	$OODE_{OP}D$	DEOPPD
Treatment type	ASB	AS	AS	AS	AS	AS	AS	ASB	NOVEL	ASB	ASB
Wood type	HW	SW	SW/HW	HW	HW	HW	SW	SW	SW	SW/HW	HW/SW
GCI	+++	+++++	++++	+++	+++	++	+	+	++++	+++	+++
In vitro effects[a]											
AR (activity)	++	+	+	+	++	++	+	+	++++	+	+
GAD (% Inhibition)	+++++	+	+++	+++	+++	+	+	+	++	+	++
MAO (% Inhibition)	+	–	++	+	++	–	–	–	+	–	–
YES (induction)	–	+	–	+	–	–	–	–	–	–	–
In vivo effects											
VTG (increase, ng/mL plasma)	++ (7)	+++	+++	++++	+++	+ (7,21,28)	+ (4,7,21,28)	++ (4)	+ (7,14,21)	++	+++
EROD (induction, pmol/min/mg of protein)	+ (4,7,14,21)	+++	+ (4,7,14,21)	– (4,7,14,21,28)	+ (4,7,14,21)	++ (4,7,21)	+++	++ (4,7,14,21)	++ (4,7,14)	+++	+++

[a]Milestone et al. [4].

+, ++, +++, and ++++ indicate that a statistically significant effect was observed (activity, percent inhibition, increase, induction, gas chromatographic index) and its level of intensity from multiple times effect evaluation; – means the effect was not observed during all those multiple times. For vitellogenin and ethoxyresorufin-O-deethylase, effects (numbers) indicate days without statistical significant increase or induction respectively (according to Figure 2).

ABS = aerated stabilization basin; AR = androgen receptor (ng toxic equivalency/g wood); AS = activated sludge; D = chlorine dioxide; E = caustic extraction; ECF = elementary chlorine-free; EROD = ethoxyresorufin-O-deethylase; GAD = glutamic acid decarboxylase; GCI = gas chromatographic index; HW = hardwood; MAO = monoamine oxidase; n or N = neutralization; NOVEL = reverse osmosis; o = oxygen; O = oxygen delignification; p or P = peroxide; SW = softwood; TMP = thermomechanical pulping; VTG = vitellogenin; YES = yeast estrogen screening assay; Z = ozone.

injection, 30 fish/treatment (14 treatments, 2 repetitions/treatment) were placed in 28 70 L tanks (4.1 kg/m^3 of density) under the same acclimatization conditions.

Tissue sampling and fish reinjection

Fish preexposure state of health was evaluated in 6 organisms euthanized before the experiment. After 4 d, 7 d, 14 d, 21 d, and 28 d of exposure, 6 fish per treatment (3 fish per replicate tank) were anesthetized (100 mg/L, tricaine methanesulfonate) and blood, gonad, and liver tissue sampled. Remaining fish were reinjected (after 7 d, 14 d, and 21 d) following the same treatment and dose protocol described above. Blood samples (1–3 mL) were obtained by caudal puncture using 4 mL heparinized Vacutainers (BD 367884) and then centrifuged at 3000 *g* for 10 min, and the plasma was stored at –20 °C. Fish were euthanized by exsanguination under anesthesia, and liver and gonad tissue samples were stored at –80 °C. Fish acclimatization and sampling procedures were in accordance with Canadian Council on Animal Care guidelines.

Physiologic and metabolic endpoints

Condition indexes. Condition factor (K = [total weight/length^3] × 100), liver somatic index (LSI = [liver weight/total organism weight] × 100), and GSI (= [gonad weight/total organism weight] × 100) were calculated based on morphometric information.

Induction of liver cytochrome P4501A1. Activity of cytochrome P4501A1 (CYP4501A1) enzyme was evaluated as EROD activity in floating postmitochondria (fraction S9) obtained from liver samples homogenized in 0.1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid grinding buffer (pH 7.8) and centrifuged at 10 000 *g* per 20 min (4 °C), following a previously described method [23] with modifications [15]. Briefly, for microplate (96-well nonbinding black surface polystyrene plates, COSTAR 3650; Corning) fluorescence detection of resorufin, we used a 530 nm and 590 nm excitation and an emission filter in a Synergy HT Multidetection Microplate Reader (Bio-Tek Instruments). Activity of EROD was expressed as picomoles per minute per milligram of protein, measured by the Bradford microplate method [24].

Liver aerobic/anaerobic capacity analyses. Both LDH and citrate synthase enzymatic activities were measured in liver samples homogenized in imidazole buffer (50 mM, pH 7.6) and centrifuged at 2300 *g* for 10 min at (4 °C).

Activity of LDH was measured as the rate of oxidation of reduced nicotinamide adenine dinucleotide to nicotinamide adenine dinucleotide [25], and citrate synthase activity was determined as the rate of reduction of 5,5′-dithiobis-2-nitrobenzoic acid [26]. Both assays were run in triplicate at 340 nm for 3 min using a microplate reader (Bio-Tek Synergy). Finally, LDH and citrate synthase enzymatic activities were expressed as micromoles per minute per milligram, using international units reported as specific activity by transformation (1 unit corresponds to 1 μmol of substrate converted to product per minute).

Vitellogenin. A previously described and modified enzyme-linked immunosorbent assay was used to measure plasma VTG concentrations [27]. Briefly, trout plasma samples were incubated in 96-well microplates (COSTAR 3690; Corning), blocked with bovine serum albumin (Sigma-Aldrich), and coated with a primary polyclonal antibody (rabbit anti–sea bream VTG; Biosense Laboratories), followed by a conjugated secondary antibody (goat antirabbit immunoglobulin G peroxidase; Sigma-Aldrich). Plasma concentrations of VTG were

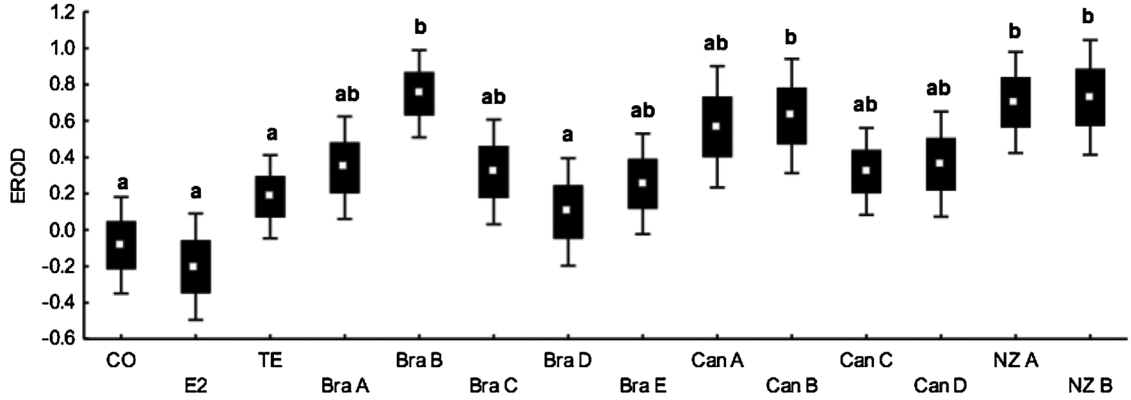

Figure 1. Liver 7-ethoxyresorufin-O-deethylase enzymatic activity (in picomoles per minute per milligram of protein) of immature rainbow trout (log base 2 scale) following multiple intraperitoneal injections of pulp and paper mill effluent extracts and steroid standards. Factorial 2-way analysis of variance ($p < 0.05$) of 7-ethoxyresorufin-O-deethylase means and variation of all 30 fish per treatment (3 fish per 2 replicates each sampling time) as a function of all 5 sampling times. Box (standard deviation) and whisker (2 × standard deviation), followed by Tukey's multiple post hoc tests (a, b). Bra = Brazil; Can = Canada; CO = corn oil; E2 = 17β-estradiol; EROD = 7-ethoxyresorufin-O-deethylase; NZ = New Zealand; TE = testosterone.

determined based on the method of Tyler et al. [28] at 490 nm using a Synergy HT Multidetection Microplate Reader (Bio-Tek Instruments), based on a standard calibration curve of 1 ng/mL to 1000 ng/mL plasma.

Statistical analyses

All statistical analyses were performed using STATISTICA 10 software (StatSoft). Normal distribution was evaluated by the Shapiro-Wilk test ($p < 0.05$), and GSI, EROD, VTG, LDH, and citrate synthase data were then log-transformed to achieve normality.

The rainbow trout used in this experiment was a sexually immature population (42 ± 12 g), which is why an unbalanced sex distribution of fish per treatment was not unexpected (3:1 female to male). Therefore, analysis of variance/covariance (ANOVA/ANCOVA) was initially developed to evaluate gender-related effects associated with continuous variables (covariates VTG, EROD, GSI, LDH, citrate synthase). The ANCOVA was determined by plotting the dependent variables versus covariate combinations (EROD—$[GSI/VTG]_{(slopes)} = 0.1311/0.1394$; VTG—$[GSI/EROD]_{(slopes)} = 0.1421/0.1399$). Levene's test was used for evaluation of the homogeneity of variances/covariances ($F_{0.05[3,961]} = 2.503 < F_{[critical]} = 2.741$) prior to the ANOVA/ANCOVA (all statistical assumptions were fully satisfied).

After the ANCOVA showed no significant influence of gender as a categorical predictor for any covariate combinations and because of the significant unbalanced gender proportion in our experiment, all statistical differences among treatments and controls were evaluated by pooling male and female data by 2-way ANOVA and then confirmed by a Tukey's post hoc multiple comparison test ($p < 0.05$).

RESULTS

Gender as variant/covariant

No mortality was observed, and statistically significant differences between experimental replicates were found during the experiment ($p \leq 0.05$). The ANOVA/ANCOVA indicated that gender did not influence any observed endpoints except for the expected effect in GSI (male gonads were always smaller than female gonads). This nonsignificant influence of gender as a categorical predictor was represented by least square means bidimensional graphs (Supplemental Data, Figure S1).

Condition indexes

In spite of the observed differences between male and female GSI, the 2-way ANOVA ($p \leq 0.05$) showed no statistically significant differences with the controls, indicating that all fish were in a similar immature state. Furthermore, no significant treatment or time differences were observed in either condition factor (K) or LSI during all of the exposure times, except Can B that presented a significantly lower LSI (Supplemental Data, Figure S2).

Metabolic enzyme analyses

Liver citrate synthase and LDH enzymatic activities in fish exposed to all treatments and standards were not significantly different from controls during the whole experiment (Supplemental Data, Figure S3).

Liver CYP4501A1

Induction of liver detoxification enzymes was measured as EROD activity (its specific temporal variation by country; Supplemental Data, Figure S4). Significant inductions were observed in fish injected with Bra B, Can B, NZ A, and NZ B extracts at all sample times (Figure 1). Induction of EROD was observed in all effluent treatments by the fourth injection (28 d of exposure), with the exception of Bra D mill, which was not statistically different from controls at any time point.

Yolk precursor protein (VTG)

Temporal trends in increased plasma VTG following intraperitoneal injections varied by country (Figure 2). Significant increases of plasma VTG levels were found in fish injected with 17β-estradiol, Bra B, Bra C, Bra D, Bra E, and NZ B extracts irrespective of the sampling time. Fish injected with Bra A extract showed increased plasma VTG levels throughout the experiment except day 7, whereas fish injected with NZ A increased VTG at all times except day 14. Fish injected with Can C extract showed a delayed increase of VTG levels from day 7 to day 28. The remaining Canadian mills, A, B, and D, showed increased VTG only on particular days. Control fish and fish injected with testosterone did not show significantly increased plasma VTG levels during all exposure times (Figure 2).

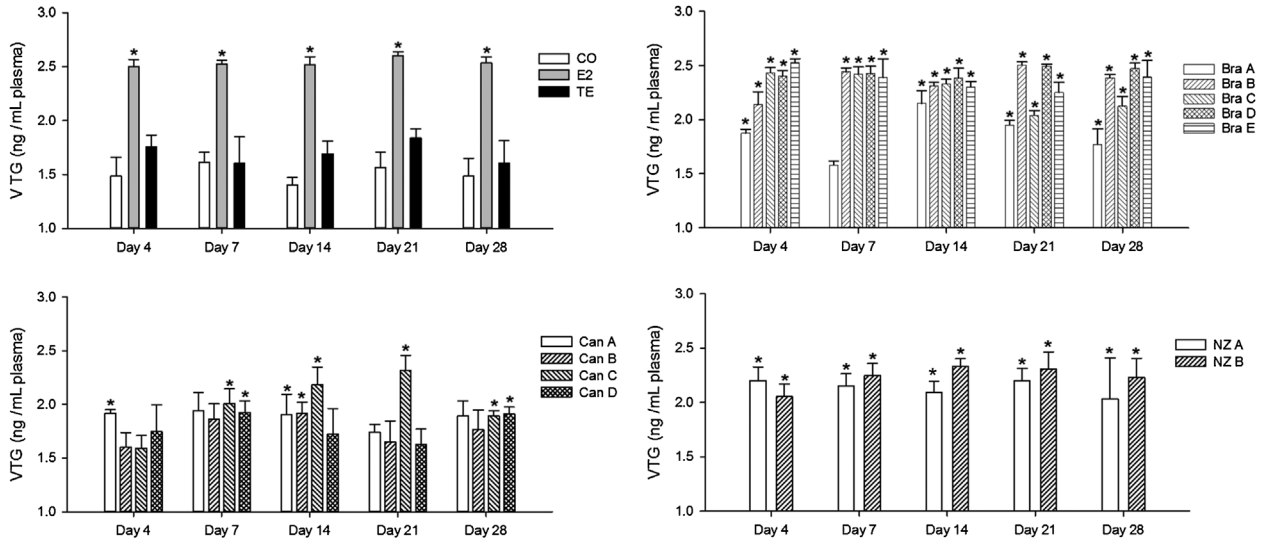

Figure 2. Liver plasma vitellogenin level of immature rainbow trout (log base 2 scale) following multiple intraperitoneal injections of pulp and paper mill effluent extracts and steroid standards. Bars represent mean ± standard error. Statistical differences between treatments and the corresponding corn oil control (*) were determined by 2-way analysis of variance ($p < 0.05$), followed by Tukey's multiple post hoc tests. Total of 30 fish per treatment (3 fish per 2 replicates and sampling time). Bra = Brazil; Can = Canada; CO = corn oil; E2 = 17β-estradiol; NZ = New Zealand; TE = testosterone; VTG = vitellogenin.

DISCUSSION

The present study examined the in vivo fish endocrine-disruption effects of pulp mill effluents, specifically looking at the estrogenicity in juvenile rainbow trout pulse-exposed to 11 different pulp mill effluent extracts (intraperitoneal injections of SPE–dichloromethane nonpolar fraction) of selected Canadian, New Zealand, and Brazilian pulp mills. The present study also investigated if any observed inhibitory effects on hepatic function could represent a confounding factor when interpreting plasma VTG levels as estrogenic effect biomarkers.

Activities of LDH and citrate synthase were not affected in any intraperitoneally injected trout during the experiment, suggesting an absence of cellular injuries and unaffected aerobic and anaerobic metabolic capacities of fish livers related to these pulp mill SPE–dichloromethane exposures. Thus, it was unlikely that any changes in the present results were the result of liver damage. This is consistent with the very low resin acid–type compounds described in our previous analysis of the SPE–dichloromethane fraction (total resin acids range, 0–0.034 mg/L) [4]. Similar to the present findings, unaffected LDH and citrate synthase enzymatic activities were previously reported in experiments using similarly extracted SPE effluent fractions from Chilean pulp mills [14] or 0.05 ppm of dehydroabietic acid intraperitoneally injected into juvenile trout [29].

As expected for pulp and paper mill effluent SPE–dichloromethane extract chronically intraperitoneally injected to fish [8,15], induction of hepatic EROD activity was observed in all mills after 28 d with the exception of Bra D (Supplemental Data, Figure S4). This induction does not appear to be clearly related to the type of mill, bleaching, or effluent treatment (Table 1). However, the induction seems to be linked with the softwood type, with Bra B, Can B, and NZ A (95% softwood + 5% hardwood [4]) groups' EROD induced during all sampling times. For the other mills that use a combination of wood sources, Bra C (softwood/hardwood) and NZ B (hardwood/softwood), no clear induction pattern could be established because of their different and unknown wood ratios

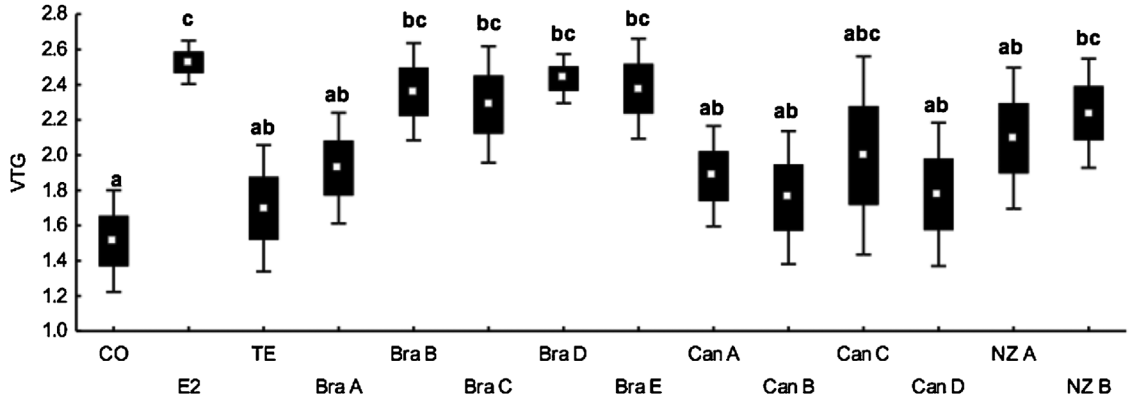

Figure 3. Plasma vitellogenin (VTG) level (in nanograms per milliliter of plasma) of immature rainbow trout (log base 2 scale) following multiple intraperitoneal injections of pulp and paper mill effluent extracts and steroid standards. Factorial 2-way analysis of variance ($p < 0.05$) of VTG means and variation of all 30 fish per treatment (3 fish per 2 replicates each sampling time) as a function of all 5 sampling times temporal trends, box (standard deviation) and whisker (2 × standard deviation), followed by Tukey's multiple post hoc tests (a–c). Bra = Brazil; Can = Canada; CO = corn oil; E2 = 17β-estradiol; NZ = New Zealand; TE = testosterone; VTG = vitellogenin.

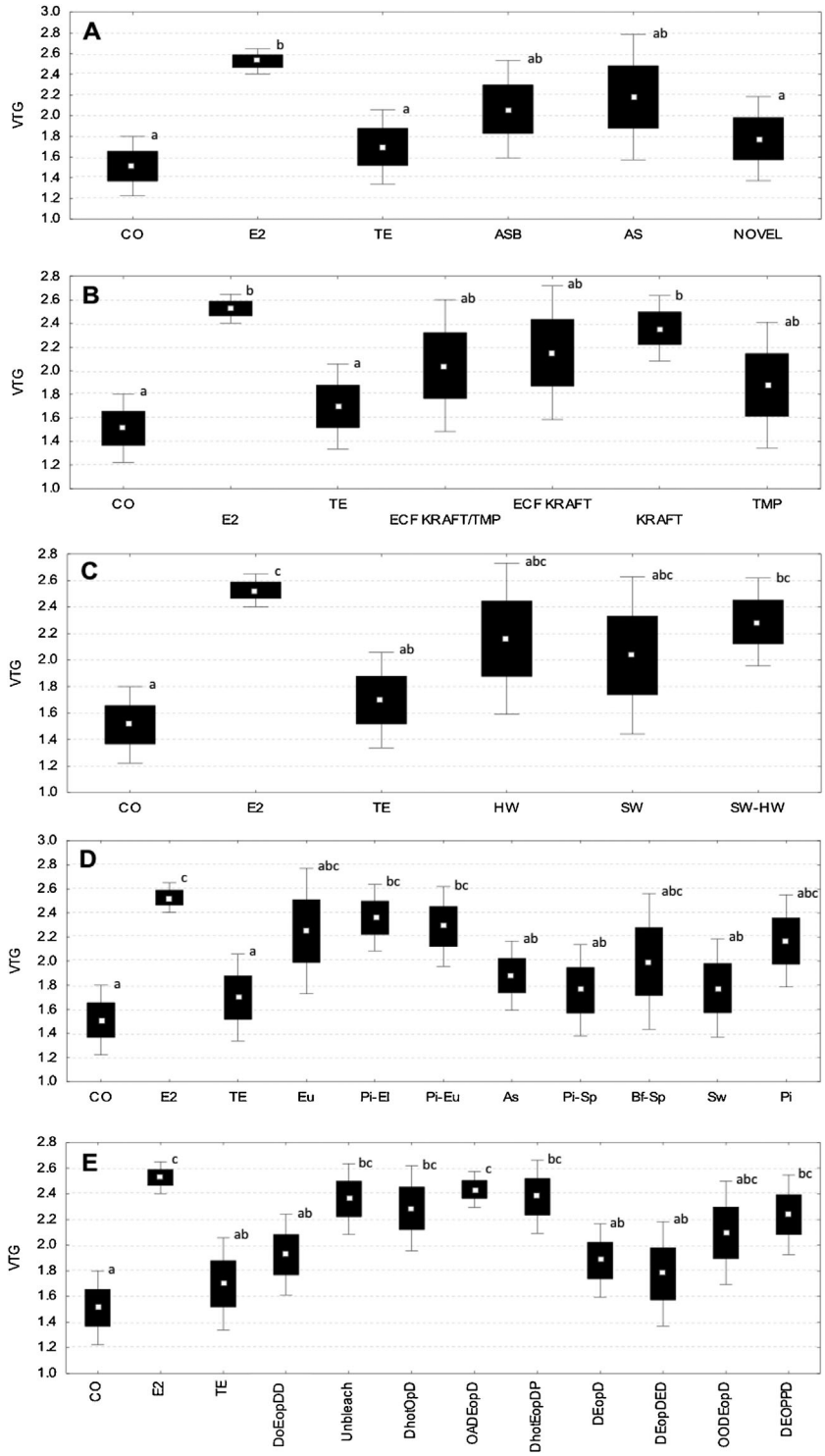

Figure 4. Plasma vitellogenin (VTG) level (in nanograms per milliliter of plasma) of immature rainbow trout (log base 2 scale) versus major grouping factors: (**A**) effluent treatment, (**B**) mill type, (**C**) major wood type, (**D**) wood type at sampling, (**E**) elementary chlorine-free bleaching sequence (see Table 1 for details). Two-way analysis of variance ($p < 0.05$) of VTG means and variation of all 30 fish per treatment (3 fish per 2 replicates each sampling time) as a function of all 5 sampling times. Box (standard deviation) and whisker (2 × standard deviation), followed by Tukey's multiple post hoc tests (a–c). ASB = aerated stabilization basin; AS = activated sludge; Bf = balsam fir; CO = corn oil; D = chlorine dioxide; E = caustic extraction; E2 = 17β-estradiol; ECF = elementary chlorine-free; El = elliotos; Eu = eucalyptus; HW = hardwood; n or N = neutralization; NOVEL = reverse osmosis; o = oxygen; O = oxygen delignification; p or P = peroxide; Pi = pinus; Sp = spruce; Sw = softwood; SW = softwood; TE = testosterone; TMP = thermomechanical pulping; Z = ozone.

used. Hardwood type seemed to be related to the low induction observed in Bra A and Bra E and to the absence of induction in Bra D. Even though our previous experience showed that the presence of resin acids can reduce EROD induction and produce an antiestrogenic effect by reducing the increase in VTG [15], as mentioned previously, very low resin acid concentrations were detected in the SPE–dichloromethane fraction injected in this experiment [4].

Previous work showed that this SPE–dichloromethane fraction had the highest GC indexes, which were positively

correlated to androgenic activity (AR binding activity) and to the inhibition of MAO neurotransmitter enzymes (greater in elementary chlorine-free kraft mills) [4]. Furthermore, very low estrogenic activities were detected in all samples, with only 3 fractions from Brazilian effluents showing a response, of which 2 were caused by the same fraction used in the present study [4]. That lack of estrogenic response differed from those found in the present in vivo study, where estrogenic effects were consistently detected, indicated by significant increases in plasma VTG levels of SPE–dichloromethane effluent extract intraperitoneally injected fish, predominantly from Brazilian and New Zealand mills (Figure 3).

The available literature describing effluent effects on VTG induction is highly variable and controversial and may relate to differences in exposure strategies (type/concentrations/time) and to differences in the sensitivity of the biological model used (different species, ages, gender, etc.) [6]. A negative impact on reproduction was expected in vivo from these extracts because of the observed in vitro inhibition of MAO and glutamic acid decarboxylase (previously tested using goldfish brain [4]) that should lead to increased dopamine levels and decreased gamma-aminobutyric acid levels with a subsequent influence on the release of gonadotropin-releasing hormone from the hypothalamus, pituitary gonadotrophins I and II, and gonadal sex steroids [30]. However, chronic exposure of juvenile trout to SPE–dichloromethane effluent extractives by multiple intraperitoneal injections utilized in the present study showed a clear estrogenic effect, suggesting that different mediated mechanisms are involved.

Similar experiments involving intraperitoneally injected juvenile trout with a series of SPE-extracted untreated or primarily and secondarily treated Chilean mill effluents indicated the simultaneous presence of estrogen receptor (ER) agonists or chemicals that induce changes leading to increases in endogenous estrogens [5]. That previous research demonstrated up-regulation of CYP19a gene expression in the ovary and increased circulating estrogens as well as VTG levels in juvenile triploid rainbow trout chronically exposed not only to elementary chlorine-free kraft effluent extracts but also to laboratory standards such as 17β-estradiol, aromatizable androgens (methyltestosterone, androstenedione), and phytosterols (β-sitosterol, often present in pulp mill effluents). Furthermore, secondary treatment processes similar to those applied to pulp mill effluents can reduce phytosterols such as β-sitosterol through bacterial transformation to androstenedione [31,32], a major aromatizable androgen which up-regulated CYP19a gene expression in similar experiments [5].

However, plant-derived substances such as phytosterols, flavonoids, and lignans (present in pulp mill effluents) are also reported to inhibit protein-level aromatase activity [16], suggesting that VTG induction via ER binding is a viable explanation [33].

Moreover, it has been recently shown that, as expected, exposure to the aromatizable androgens (i.e., methyltestosterone) induced the ovarian aromatase messenger ribonucleic acid in adult female mummichog (*Fundulus heteroclitus*); however, such exposure reduced liver VTG1 expression [34]. Furthermore, dehydrotestosterone (a nonaromatizable androgen) was also able to up-regulate VTG1 expression in males, suggesting a mechanism involving nonspecific binding to ER, as previously reported [35].

The discrepancy between the weak estrogenic activity observed using an in vitro yeast estrogen screening assay in our previous work [4] and the present results may also be explained by differences in our exposure model that would include metabolic processes that could be involved in the activation of precursors to estrogens in aromatization, the final step of the steroid biosynthetic pathway. Such a time-sensitive conversion process would be different in specific organs such as gonads or brain [36,37]. This androgen aromatization process would also require an adequate exposure period to measure responses [34]. Unfortunately, neither aromatase expression/activity nor ER binding assays were included as the target of the present study and therefore cannot be discounted as a mechanism of action of the observed estrogenic effects.

High variability was observed in effluent treatment types regarding the increase of VTG (activated sludge > aerated stabilization basin > reverse osmosis). The activated sludge–treated mill group showed the highest variability in VTG level, clearly associated with the high level of Brazilian mills compared with the low level of variability observed in Canadian mills (Table 1 and Figure 4A). The aerated stabilization basin–treated mills group showed a lower VTG induction level and variability (regardless of country). The reverse osmosis–treated mill (Can D) also showed low VTG increase relative to what was observed for Brazil and New Zealand mills. Despite the high variability observed among mills and countries (Figure 4A), the biological treatment (activated sludge/aerated stabilization basin) seems to not have the same effectiveness depending on the country. Some differences related to the type of mill process were observed, showing that the ability to increase plasma VTG levels varied with effluent type, with unbleached kraft > elementary chlorine-free kraft > thermomechanical pulping (Table 1 and Figure 4B). The high variability of VTG level in elementary chlorine-free kraft mills related to the high levels of variability observed in Brazilian mills, which was different from the low variability levels in the same types of Canadian and New Zealand mills. Differences in VTG level caused by wood types were not clearly present in the present study (Figure 4C); however, a consistent estrogenic effect was observed in those mills, which processed a combination of softwood and hardwood feedstock. Although there were differences in the wood types and wood combinations being pulped at the time of sampling (Figure 4D), the highest VTG increase was associated with a eucalyptus feedstock. Because of the variety of bleaching sequences used (Figure 4E), it becomes extremely difficult to establish a relationship among mills; however, it is possible to observe a similar VTG increase between mill effluent without bleaching and complex bleaching sequences, suggesting that the estrogenic effects observed would be related not to the type of bleaching but rather to the wood source and effluent treatment type.

The present results indicate that all extracts from all pulp mill effluents from pulp-producing countries are estrogenic in juvenile trout chronically exposed to specific SPE–dichloromethane extractive fractions, reflected by increasing levels of plasma VTG irrespective of gender (Brazil > New Zealand > Canada). This is consistent with previous intraperitoneal injection studies of Chilean mills pulping eucalyptus feedstocks [5,8,15] and recent waterborne exposure bioassays using tertiarily treated (*Eucalyptus globulus*, feedstock) pulp mill effluent [13], suggesting that such feedstocks are sources of substances that can be modified in vivo into estrogenic compounds.

The apparent role of metabolic activation in estrogenic responses could also be necessary to activate androgenic effects. A new class of diterpene ligands for the goldfish testicular AR

has been identified [38]; however, it remains unclear if these compounds are not simply potent ligands for the AR but outright androgens or precursors to androgens involved in well-documented masculinization studies at kraft mills in the United States [39], New Zealand [9], and Canada [40].

The value of mechanistic studies such as those employed in the present study (early changes in important biochemical endpoints), when coupled with long-term monitoring data sets, has been recognized in the AOP approach recently demonstrated [41], which provides a recommended method to estimate population impacts by inference using observed responses at lower levels of biological organization applicable to ecological risk assessment. Vitellogenesis was 1 of several examples found to link initiating events at the molecular level to adverse outcomes at the individual and population levels. Even though the decrease in the levels of VTG in female fish is the biomarker that has been established as the link to reproductive impairment using the AOP framework [41], the estrogenicity measured by this easily monitored tool (induction of plasma VTG levels particularly in male fish) should not be discarded as a possible link in reproductive effects observed at subindividual and individual levels. This could explain potential alterations at the population level by pulp and paper mill effluent exposure, especially in countries (e.g., Chile, Brazil) where effects-based monitoring programs are not in place. Nevertheless, estrogenicity and imposex under field semicontrolled and laboratory conditions using trout [5,8,13] and alteration at the hormonal level in native fish populations are being related to long-term reproductive changes attributable to pulp and paper mill effluent exposure in Chile [42]. The present study provides further evidence that the effects of mill effluents on fish reproduction that have been extensively documented in Canada and New Zealand would be expected to occur in Brazilian aquatic receiving environments.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3675.

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Data Availability—Data, associated metadata, and calculation tools are available from the corresponding author (Rodrigo.Orrego@uantof.cl).

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