Environmental Science & Technology

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Evaluating the Potential of Effluents and Wood Feedstocks from Pulp and Paper Mills in Brazil, Canada, and New Zealand to Affect Fish Reproduction: Chemical Profiling and In Vitro Assessments

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Supporting Information

ABSTRACT: This study investigates factors affecting reproduction in fish exposed to pulp and paper mill effluents by comparing effluents from countries with varying levels of documented effects. To explore the hypothesis of wood as a common source of endocrine disrupting compounds, feedstocks from each country were analyzed. Analyses included in vitro assays for androgenic activity (binding to goldfish testis androgen receptors), estrogenic activity (yeast estrogen screen), and neurotransmitter enzyme inhibition (monoamine oxidase and glutamic acid decarboxylase). Chemical analyses included conventional extractives, known androgens, and gas chromatograph index (GCI) profiles. All effluents and wood contained androgenic activity, particularly in nonpolar fractions, although known androgens were undetected. Effluents with low suspended solids, having undergone conventional biotreatment had lower androgenic activities. Estrogenic activity was only associated with Brazilian effluents and undetected in wood. All effluents and wood inhibited neurotransmitter enzymes, predominantly in polar fractions. Kraft elemental chlorine free mills were associated with the greatest neurotransmitter inhibition. Effluent and wood GCI profiles were correlated with androgenic activity and neurotransmitter enzyme inhibition. Differences in feedstock bioactivities were not reflected in effluents, implying mill factors mitigate bioactive wood components. No differences in bioactivities could be discerned on the basis of country of origin, thus we predict effluents in regions lacking monitoring would affect fish reproduction and therefore recommend implementing such programs.

INTRODUCTION

While many major environmental issues regarding pulp and paper effluent discharges, such as biochemical oxygen demand, suspended solids and nutrients management, and dioxins and furan releases, have largely been addressed, one major issue remains: the effects of mill effluents on fish reproduction. Documented reproductive effects have included delayed sexual maturity, decreased gonad size, reduced gonadal and circulating sex steroids, and reduced secondary sex characteristics in wild fish. Initially reported in Scandinavia,[1] effects have been reported throughout pulp producing nations, including Canada, the U.S.A., and New Zealand (reviewed in refs 2−4). Laboratory and in situ studies report a suite of effects with the most consistent related to androgenic and estrogenic modes of action.[5−7] The rapid onset of some effects (e.g., sex steroids

Received: September 26, 2011
Revised: December 18, 2011
Accepted: December 23, 2011
Published: December 23, 2011

dx.doi.org/10.1021/es203382c | Environ. Sci. Technol. 2012, 46, 1849−1858

in wild fish and egg production in laboratory fish), and their reversal following exposure cessation,[8−10] suggest that causative compounds for these responses are readily bioavailable and metabolized quickly. Evidence of these effects exists from both regulatory programs, such as the Canadian Environmental Effect Monitoring (EEM) Program,[11] and extensive research studies, such as in New Zealand.[3]

Despite the evidence, a relationship between mill process parameters, effluent treatment type and specific reproductive effects in fish remains unclear.[2] It has been hypothesized that a common origin of causative agents exists, including the wood furnish or additives. It is thought that these compounds survive or are possibly altered during pulping, bleaching, and effluent treatment.[2]

In Canada and New Zealand, the pulp and paper industry is well established, where mills have been retrofitted to address environmental concerns as they arose. Upgrades have included the implementation of elemental chlorine free (ECF) bleaching and biological treatment. Continual changes in both production and wastewater treatment have been shown to be beneficial for minimizing effluent-related effects, as evidenced at one mill in New Zealand.[12] The newest mills in the world are currently located in South America and have been constructed with all these latest process modifications included; however, there is little information regarding effluent effects. South American pulp-producing countries have not yet been mandated to conduct monitoring, and as such, the only available information is generated from recent research. This research shows that effluent from state-of-the-art mills in Chile can cause estrogenic effects in laboratory,[13,14] caged,[15] and wild fish.[16] This lack of information hinders the ability of mature pulp and paper sectors in other countries from implementing effective solution-oriented changes. Historically, comparisons between studies, and especially between different pulp producing nations, have been difficult. This is due to the varied approaches involving different fish species, experimental conditions, responses evaluated and study objectives.[17] A comprehensive evaluation of effluents using the same protocols and end points is therefore needed to address this knowledge gap.

New tools and approaches developed as part of a Canadian national initiative are offering leads toward minimizing effects on fish reproduction and the ability to compare mill effluents more consistently. These tools include a gas chromatographic index (GCI), which provides a measure of extractable organics, and for kraft mill effluents, correlates positively to BOD, correlates negatively to egg production in fathead minnows,[18] and is linked to effects in wild fish.[19] New evidence of impacts in the hypothalamus provides a plausible mechanism where rapidly manifested effects may be initiated through interactions with receptors and enzymes involved in neurotransmitter synthesis or metabolism.[20−22] The hypothalamus−pituitary−gonad reproductive axis is tightly regulated by the neurotransmitters gamma-aminobutyric acid (GABA) and dopamine in many teleost species.[23−25] Dopamine is a potent inhibitor of gonadotropin-releasing hormone (GnRH) in the brain and luteinizing hormone (LH) in the pituitary, thereby controlling a multitude of neurotransmitters and sex steroids. Conversely, GABA stimulates GnRH and blocks dopamine's inhibition of LH to cause LH release. Monoamine oxidase (MAO) catabolizes dopamine, while glutamic acid decarboxylase (GAD) catalyzes the decarboxylation of glutamate into GABA. Inhibition of these enzymes would lead to either increased levels of dopamine or decreased levels of GABA in the brain, either of which would be inhibitory to fish reproduction. Hypothalamic effects have been linked to spawning inhibition with a limited number of Canadian mill effluents.[22]

The objective of this study is to evaluate effluents from countries where reproductive effects and industries are well established (Canada, New Zealand), to countries where mills are state-of-the-art, with no effects data (Brazil). To explore the hypothesis of wood extractives as a source of endocrine disrupting compounds in final effluents, representative samples of wood feedstocks from each country were incorporated. Final mill effluents and feedstocks were evaluated for (i) androgenic and estrogenic activities, (ii) neuroactive substances affecting the neuroendocrine control of ovulation, and (iii) conventional effluent extractives, known androgens and GCI profiles to determine if any relationships exist between bioassay responses, feedstock and effluent chemistry, and mill operational parameters. Selected effluent extracts are being separately evaluated for their effect on reproduction in rainbow trout.

EXPERIMENTAL SECTION

All solvents were HPLC grade unless otherwise specified (Optima grade, Fisher, Ottawa, ON, Canada), gases were ultrahigh purity grade (Air Liquide, Hamilton, ON, Canada).

Mill Selection. Final effluents and wood feedstocks were collected from four Canadian mills, five Brazilian mills, and two mills from New Zealand representative of the pulping and bleaching processes, effluent treatment systems, and wood types used globally (Table 1). In addition to conventional mill metrics (biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), flow), information regarding other parameters (wood type switching, nonmill wastewaters, tertiary treatment) were obtained through a comprehensive questionnaire. To avoid any changes to effluent quality that may occur during handling and international shipment, all extractions were conducted within the country of origin using supplies and a protocol distributed by the host Canadian laboratory. The stability of extracted components on frozen SPE cartridges was verified prior to commencement of the study (Figure S1, Supporting Information).

Effluent Collections. Extraction and filtration supplies were sent to participating laboratories in Brazil (Aplysia, Vitória, ES) and New Zealand (Scion, Rotorua). Samples (6 L) of final treated effluent from each mill were collected as grab or 24-h composites in prewashed 1 L glass bottles. Extractions were performed on the same day (New Zealand), or within two days (stored at 4 °C, Brazil, Canada).

Effluent Extractions. Extraction methods were based on the approach of Orrego et al.[26] and modified by including extractions of filtered solids, use of a less selective SPE solid phase and separation of compounds by polarity. Room temperature effluent pH (∼7−8) was lowered using hydrochloric acid (3.0 M) to pH 4.0 (±0.2) and filtered through glass fiber filters (45 mm, 1.2 μm, Whatman GF-C, VWR, Mississauga, ON, Canada). Fouled filters were air-dried, wrapped in hexane-rinsed aluminum foil and sealed in polyethylene bags for shipment to Canada. To the pH-adjusted final effluent filtrate, 2% (v/v) methanol was added and eluted (500 mL) through solid phase extraction (SPE) cartridges (1 g, Oasis HLB, Waters, Mississauga, ON, Canada) preconditioned with 2 × 20 mL of dichloromethane, methanol, and water (pH = 4.0). Cartridges were washed with 10 mL water (pH = 4.0) and dried for 1 h. Loaded SPE cartridges and fouled filter

Table 1. Participating Mills Sampled in Canada, Brazil, and New Zealand[a]

										treatment efficiency			
		mill type	ECF bleaching sequence	wood type at sampling	wood type switch	storm water	other wastewater	treatment type	wastewater flow (m^3/ADMT)	% BOD removal	% COD removal	TSS % removal	tertiary
Canada	Mill A	ECF kraft	$DE_{op}D$	hardwood (aspen)	yes	yes	no	AS	44.5	98	74.3	94.1	no
	Mill B	TMP	na[b]	softwood mix (75% spruce/25% pine)	no	no	no	AS	45.9	99	93	91	no
	Mill C	TMP	na	softwood mix (spruce 85%/15% balsam fir)	no	small	yes[c]	ASB	30.6	74.6	np[h]	np	no
	Mill D	ECF kraft	$DE_{op}DED$	softwood mix	yes	no	no	novel[g]	23.8	92	41	np	no
Brazil	Mill A	ECF kraft	$D_oE_{op}DD$ D_oE_oPPP	hardwood (eucalypt)	no	yes	yes	ASB	28.8	89.9	62.5	75	no
	Mill B	kraft	unbleached	softwood mix (*Pinus taeda* and *elliotos*)	no	no	no	AS	52.4	93.8	88	93.2	no
	Mill C	ECF kraft	$D_{hot}O_pD$	softwood and hardwood (*Pinus radiata* and eucalyptus)	no	no	no	AS	44.1	94	80	np	yes[I]
	Mill D	ECF kraft	$OADE_{op}D$	hardwood (eucalyptus)	no	no	no	AS	30.6	>99	88.3	95	np
	Mill E	ECF kraft	$D_{hot}E_{op}DP$	hardwood (eucalyptus)	no	no	yes[d]	AS	21.8	97.2	81	86	np
New Zealand	Mill A	ECF kraft	$OODE_{op}D$	softwood (95%) and hardwood (5%) (*Pinus radiata* and eucalyptus)	no	yes	yes[e]	ASB	53.2	92.3	np	76	no
	Mill B	ECF kraft (49%) and TMP (51%)	D(EOP)PD and (EOP)(P)	kraft, hardwood (eucalyptus); TMP, softwood (*Pinus radiata*)	yes (kraft); no (TMP)	yes	yes[f]	ASB	74.7	89.7	np	np	no

[a]ADMT = air dried metric tonne, ECF-*K* = elemental chlorine free (ClO_2) bleached kraft pulp (*D* = chlorine dioxide, *E* = extraction stage, $_{op}$ = with oxygen and hydrogen peroxide, $_p$ = with hydrogen peroxide, $_o$ = with oxygen, *A* = acid hydrolysis), U−*K* = Unbleached kraft, TMP = Thermo-mechanical pulping, AS = activated sludge secondary treatment system, ASB = activated stabilisation basin (aerated lagoon) treatment system, BOD = biological oxygen demand, COD = chemical oxygen demand, TSS =total suspended solids [b]Not applicable. [c]Small volume from nearby oil refinery. [d]Sanitary sewer enters in to treatment system. [e]Small volume of underflow from adjacent landfills. [f]Whitewater from adjacent tissue mill, and leachate from adjacent landfill and from natural wetland system. [g]Novel treatment system: non-ASB or -AS. [h]Data not provided by mill. [I]Mill uses ultrafiltration on 40% of flow prior to discharge; sample for study was taken before tertiary treatment.

dx.doi.org/10.1021/es203382c | Environ. Sci. Technol. 2012, 46, 1849–1858

papers were shipped frozen to be eluted and extracted in Burlington, Canada, ON, alongside Canadian mill effluents shipped directly. Spiking experiments with phenolics, diterpenes, and sterols showed that frozen storage of loaded SPE cartridges up to two weeks produced adequate recoveries (Figure S1, Supporting Information).

Fouled filter papers were Soxhlet extracted for 18 h sequentially with dichloromethane and methanol producing nonpolar (**FP-DCM**) and polar (**FP-MeOH**) fractions. Loaded SPE cartridges were eluted sequentially using 40 mL solvent to produce two fractions: dichloromethane (**SPE-DCM**) and methanol (**SPE-MeOH**). All DCM fractions were dried using anhydrous Na_2SO_4, concentrated using rotary evaporation and evaporated under nitrogen to just-dryness and reconstituted for chemical (toluene) and biological (methanol) assessments at 1 L final effluent/mL solvent.

Wood Feedstock Extractions. Chips were sampled directly from mill stockpiles and shipped with effluents (Canadian mills) or loaded filters and SPE cartridges (Brazilian, New Zealand mills). Chips were air-dried and milled to ~1 cm^3 pieces, and Soxhlet extracted (24 h, 1 L) sequentially with hexane, dichloromethane, and acetone to ensure a best possible discrimination of bioactive compounds by polarity. After each solvent, chips were air-dried. Extracts were dried and concentrated as described above to 2.5 g/mL equivalents.

Gas Chromatography-Tandem Mass Spectrometry. All fractions were methylated using freshly prepared diazomethane (Diazald, Sigma Aldrich, Mississauga ON, Canada) in diethyl ether. Gas chromatography-tandem mass spectrometry (GC-MS-MS; Agilent 7890 GC, 7000B triple-quadrupole MS) analyses were performed as follows: HP-MS5 column (Agilent, 30 m × 0.25 mm, 0.25 μm), programmed 90 °C for 0.5 min, 5 °C/min to 300 °C for 10 min; splitless injection temperature 270 °C, helium carrier (1.3 mL/min). Positive ion electron impact full scan mass spectra (unit resolution, 50−500 m/z) were used to generate total ion chromatograms (TICs). Prior to injection, an internal standard (C19 alkane, Restek, Bellefont, PA, USA) was added to account for matrix effects. The GCIs were calculated by integrating the total peak areas (1.7−10 min) and adjusting to the response of the internal standard (area of C19 molecular ion m/z = 178; average relative response 0.96 ± 0.08; n = 43). Adjusted peak areas were then normalized against corresponding method blanks, each of which were assigned a GCI = 1.0 (modified from ref 8).

Effluent chemicals functioning as androgens (progesterone, androstenediene (AD), androstadienedione (ADD),[27−29] and manool[30]) were analyzed using the same GC conditions, using multiple reaction monitoring mode: progesterone (quantifier 313/191 and qualifier 313/299), AD (quantifier 285/244 and qualifier 285/124), ADD (quantifier 123/107 and qualifier 123/79), and manool (quantifier 272/257 and qualifier 272/135). Method detection limits were: progesterone (0.655 μg/L and 0.275 μg/g), AD (0.406 μg/L and 0.163 μg/g), ADD (5.5 μg/L and 2.2 μg/g) for effluent and wood extracts respectively, and quantifications were based on 6-point calibrations. Detection limits for phenolics, diterpenes, and sterols (Figure S1, Supporting Information) are reported elsewhere.[30] Resin acids (eight pimaric, sandaracopimaric, isopimaric, palustric, levopimaric, dehydroabietic, and abietic acids) and fatty acids (two linoleic and oleic acids) were quantified as previously described.[31]

Androgen Receptor Binding Assay. Androgen receptor (AR) ligands in fractions of wood and effluents were evaluated using a competitive binding assay with goldfish testis androgen receptors.[32] Briefly, androgen receptors were isolated from goldfish (*Carassius auratus*, 50−100 g) testes (2 pools from each of 7 males; 8 g total). Scintillation tubes contained 1% (v/v) methanol vehicle; previously shown to not affect results.[32] Dilutions were performed as required, to ensure specific binding and to obtain testosterone equivalents (TEq) from the linear portion of the standard curve. Each fraction was incubated in triplicate using both AR preparations. Method detection limits were 128 ng/L TEq effluent and 51 ng/g TEq wood.

Yeast Estrogen Screening Assay. Fractions of pulp mill effluents and feedstocks were evaluated for estrogen agonists using a yeast (*Saccharomyces cerevisiae*) estrogen screen (YES).[33] Results are represented as 17β-estradiol equivalents, with a method detection limit of 10.4 ng/L and 4.1 ng/g for effluent and wood, respectively.

Neurotransmitter Enzyme Assays. Effluent and wood fractions were evaluated for substances potentially affecting the neuroendocrine control of ovulation through the inhibition of MAO and GAD. For the MAO assay, whole goldfish brains were sonicated immediately after dissection in cold Na/K buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4) with 0.5% (v/v) Triton X-100. Sonicated tissue was centrifuged at 15 000 × g at 4 °C for 10 min, supernatants removed and stored (−80 °C). For the GAD assay, brains were homogenized in 1:10 (w/v) 10 mM phosphate buffer (pH 7.5) and stored (−80 °C).

MAO activity was measured according to Basu et al.,[20,34] with modifications, using the Amplex Red Monoamine Oxidase Assay Kit (Invitrogen, Burlington, ON, Canada). Fractions were preincubated with tissue (n = 4) for 30 min before reaction initiation at a final concentration of 2.5 μg/mL. The MAO inhibitor clorgyline (1 μM) was used as a positive control for MAO inhibition while 10 μM H_2O_2 was used as a positive control for resorufin production (λ_{ex} = 544 nm, λ_{em} = 590 nm) for 2 h, following reaction initiation using a SpectroMax M5 (Moleculare Devices, Sunnyvale, CA, U.S.A.). For background values, no protein was added to wells.

GAD activity was measured in triplicate by incubating 50 μg brain homogenate with fractions (25 μg/mL) in 10 mM phosphate buffer (60 μM pyridoxal-5′-phosphate, 120 μM dithiothreitol, pH 7.4) to a final volume of 400 μL.[35] 3-Mercaptopropionic acid was used as a positive control.

Percentage inhibition (expressed as percent of negative control) was determined by calculating enzyme activity in the presence and absence (vehicle only) of fractions. Methanol vehicle presented no statistically significant interference.

Statistics. Comparisons of biological activities were performed using Sigmaplot (V11.0, Systat Software, Chicago, IL, U.S.A.) using t tests, one-way ANOVAs (Tukey's post hoc), and nonparametric tests (Kruskal−Wallis). All correlations are Pearson Correlation Coefficients (r); 0.8−1 is classed as very strong, 0.6−0.8 strong, and <0.6 weak.

RESULTS

GC Indices and Extractives. Indices for each fraction from final effluents and wood feedstocks are presented in Figure 1. For mills from Brazil, New Zealand (NZ), and Canada Mill D, total GCIs ranged from 20 to 40. For Canada Mills A−C, a number of features standout. First, Canada Mills A and B have total GCIs lower than all other mills (~11), whereas Canada Mill C exhibited the highest total GCI value (57) of the mills surveyed. Apart from Canada Mill C, the highest GCIs for all fractions are associated with SPE-DCM, with SPE-MeOH

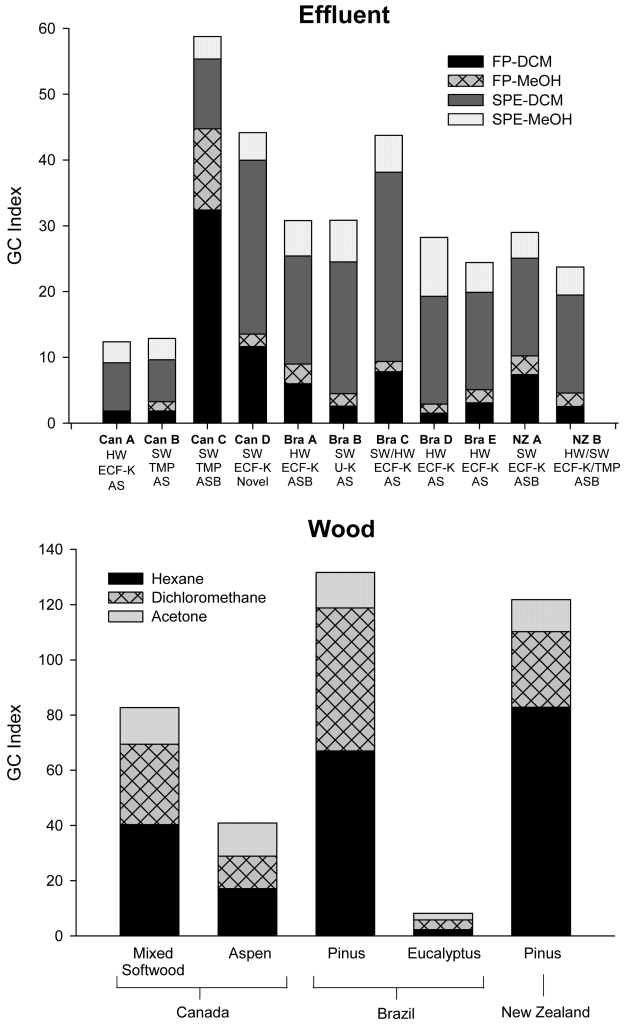

Figure 1. GC indices of extracted final effluents and extracted wood feedstocks from mills in Canada, Brazil, and New Zealand. HW = hardwood, SW = softwood, ECF-K = elemental chlorine free (ClO_2) bleached kraft pulp, TMP = thermo-mechanical pulping, U-K = unbleached kraft, AS = activated sludge secondary treatment system, ASB = aerated stabilization basin treatment system.

typically the next highest. For Canada Mill C, the highest GCI (31) was found with the FP-DCM fraction. For effluents, no obvious relationship between GCIs, wood type, production type, or treatment type was observed. However, GCI was correlated to BOD for kraft mills (0.834, p = 0.001, data not shown).

Fractions from softwood feedstocks produced the highest total GCIs (69−110), followed by the hardwood Canadian Aspen (36), while the lowest total GCI values were associated with Brazilian *Eucalyptus* (8; Figure 1). Within the softwoods examined, GCIs were consistently highest in hexane fractions, with corresponding decreases occurring with increasing polarity of the extraction solvent (dichloromethane followed by acetone).

Total resin acids for combined effluent fractions were <0.06 mg/L with the exception of Canada Mill C (0.36 mg/L), where the bulk (0.28 mg/L) were in the FP-DCM fraction. Total fatty acids for combined effluent fractions were <0.16 mg/L, except for Canada Mill C and NZ Mill A (0.58 and 0.23 mg/L respectively). Like resin acids, the majority of fatty acids were found in FP-DCM fractions (Figure S2, Supporting Information). Softwood chip extracts contained higher resin acids (955−3220 mg/L), while those from hardwoods were much lower (2.1−16.1 mg/L). In all effluents and feedstocks, when resin acids were detected, dehydroabietic acid predominated (effluents <0.08 mg/L except Canada Mill C 0.23 mg/L; softwood chips 575−1375 μg/g; hardwood chips 1.8−8.5 μg/g). The largest proportion of resin acids was present in the most nonpolar extraction solvent (DCM or hexane) for effluents and feedstocks (Figure S2, Supporting Information).

Concentrations of progesterone, AD and ADD, compounds with putative androgenic activity, as well as phenolics and sterols, were below detection limits for all samples. Manool was detected in effluents from Canada Mills C (total manool 10.0 μg/L) and D (29.2 μg/L) and not detected in wood. On the basis of the affinity of manool to the goldfish testis AR,[30] it accounted for <0.05% of the androgenic activities measured for those mills.

Androgenicity and Estrogenicity. All fractions from effluents and wood chips were evaluated for their contents of ligands for androgen (Figure 2) and estrogen (Figure S3, Supporting Information) receptors. All effluents contained some level of androgenicity, as measured by binding to goldfish

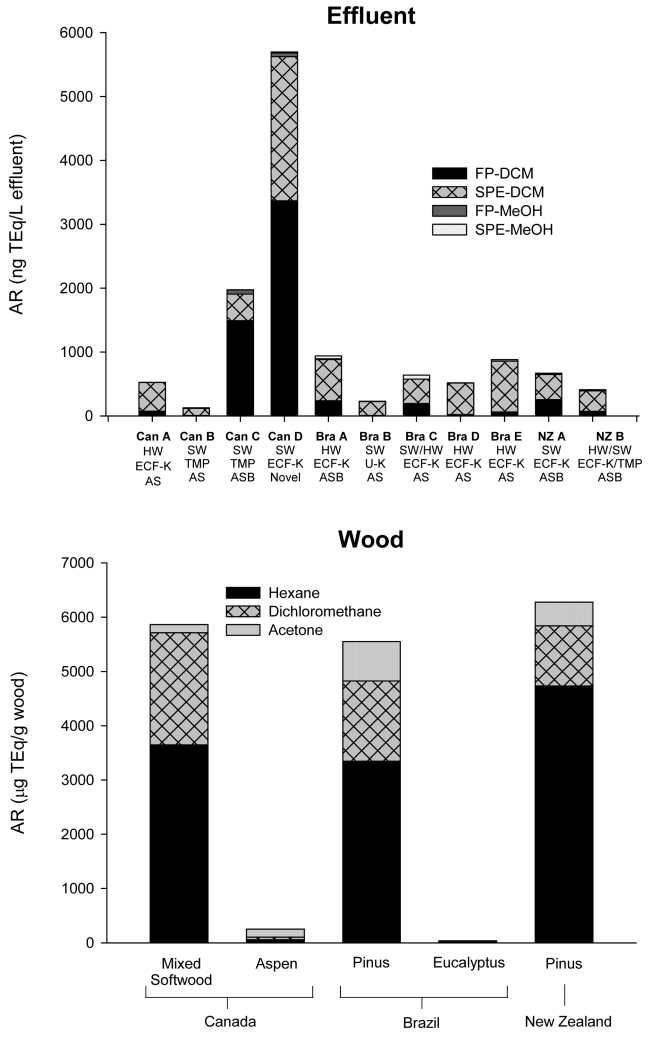

Figure 2. Testosterone equivalents obtained after incubations of final effluent and wood feedstock extracts with goldfish testis androgen receptors (AR). HW = hardwood, SW = softwood, ECF-K = elemental chlorine free (ClO_2) bleached kraft pulp, TMP = thermo-mechanical pulping, U-K = unbleached kraft, AS = activated sludge secondary treatment system, ASB = aerated stabilization basin treatment system.

dx.doi.org/10.1021/es203382c | Environ. Sci. Technol. 2012, 46, 1849−1858

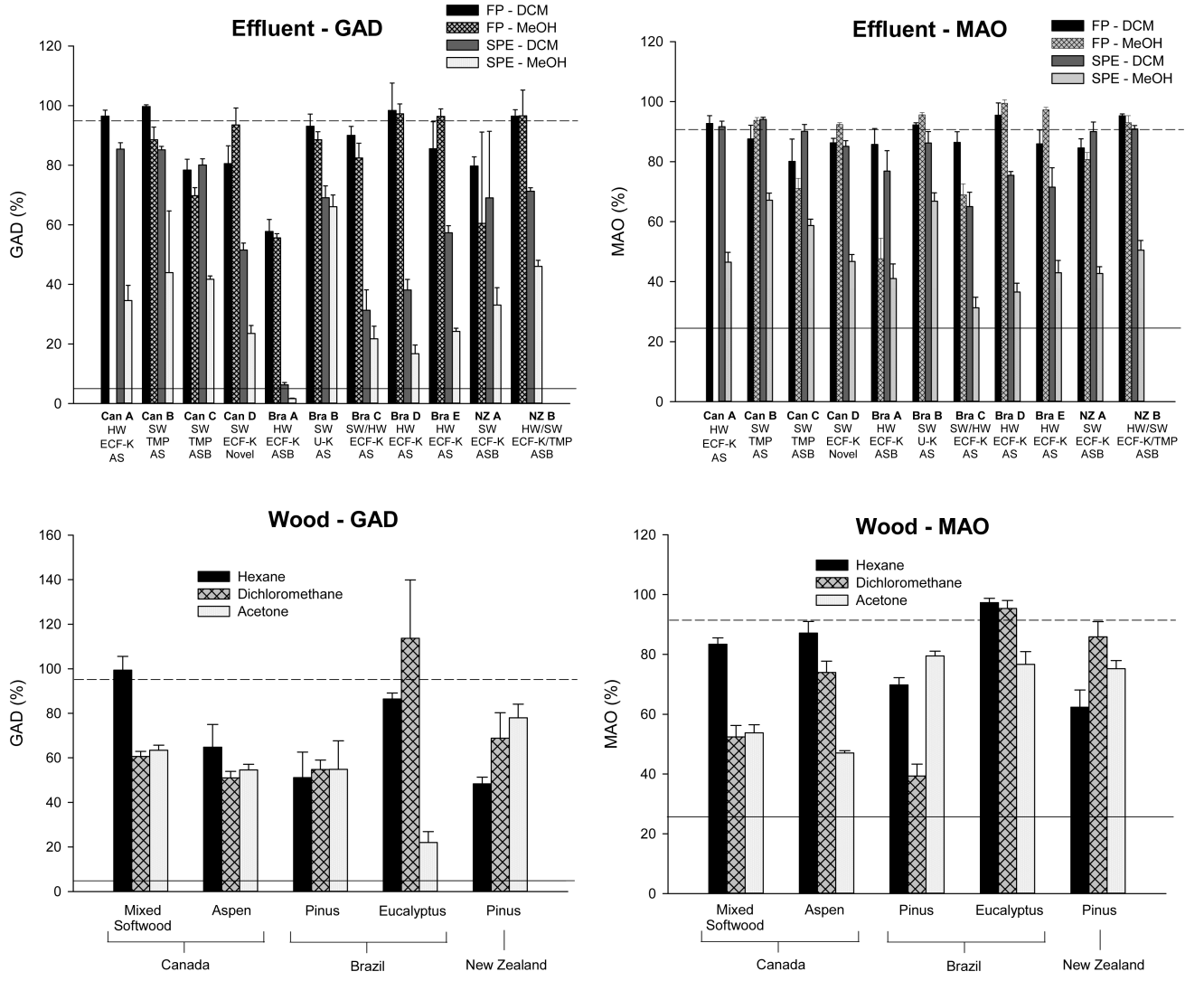

Figure 3. Glutamic acid decarboxylase (GAD) and monoamine oxidase (MAO) neurotransmitter inhibition expressed as a percentage of negative control following incubations with effluent and wood extracts. Dotted lines indicate average activity of method blanks, solid lines indicate average activity of positive control (GAD = 3-mercaptopropionic acid, MAO = clorgyline). All bars are mean ± standard deviation. HW = hardwood, SW = softwood, ECF-K = elemental chlorine free (ClO_2) bleached kraft pulp, TMP = thermo-mechanical pulping, U-K = unbleached kraft, AS = activated sludge secondary treatment system, ASB = aerated stabilization basin treatment system.

testis AR. Canada Mill D, which utilizes a novel treatment system, contained the greatest total AR activity (5697 ng TEq/L, $p < 0.001$), while Canada Mill C (1976 ng TEq/L, $p < 0.001$) had the next highest (Figure 2). For the remaining mills, totals ranged from 129 to 940 ng TEq/L. In all but two cases (Canada Mills C and D), the SPE-DCM fraction contained the highest level of androgenic activity (113−796 ng TEq/L, $p < 0.001$), while the FP-DCM fraction typically contained the majority of any remaining activity (0−255 ng TEq/L; Figure 2). Canada Mills C and D contained the largest activity in the FP-DCM fraction (1491 and 3366 ng TEq/L, respectively, $p < 0.001$), while the SPE-DCM contained lesser levels (417 and 2259 ng TEq/L, respectively, $p < 0.001$). No further relationship between wood, production or treatment type was found.

When comparing the androgenicity of wood fractions, fractions from the Brazilian hardwood *Eucalyptus* (37 μg TEq/g) and Canadian Aspen (252 μg TEq/g) contained much lower AR activity when compared to softwoods from all three countries (5551−6278 μg TEq/g; Figure 2). Androgenic activities were generally greatest in the most nonpolar feedstock fraction (Hexane), with decreased activity in more polar extraction solvents.

Very little estrogenic (ER) activity was evident using the YES assay, with nearly all results below detection limits (10.4 ng/L effluent; 4.1 ng/g wood; Figure S3, Supporting Information). Only three fractions showed any ER activity, and all were from Brazilian mills. Two were SPE-DCM fractions (Brazil Mills B and D), and one was a FP-MeOH fraction (Brazil Mill E). No ER activity was observed in any wood fractions.

Neurotransmitter Enzymes. All effluents and wood feedstocks were evaluated for their ability to inhibit two neurotransmitter enzymes, GAD and MAO, with every effluent and wood sample containing inhibitors of both enzymes (Figure 3). For all effluents, SPE-MeOH fractions caused the greatest inhibition for MAO, and in eight of the eleven mills for GAD ($p < 0.001$). For the remaining three (Brazil Mills A, B, and C), the highest GAD inhibition was contained in both SPE fractions ($p < 0.001$). Generally, FP fractions for all mills did not inhibit GAD or MAO. Brazil Mill A stands out by inhibiting GAD the greatest, where all fractions significantly interfered with the

dx.doi.org/10.1021/es203382c | Environ. Sci. Technol. 2012, 46, 1849−1858

enzyme, although this was not reflected with MAO. For both GAD and MAO, independent of country, wood feedstock, pulping process, and treatment type, the seven kraft ECF mills caused the greatest inhibitions ($p < 0.002$; Figure 4). In the case

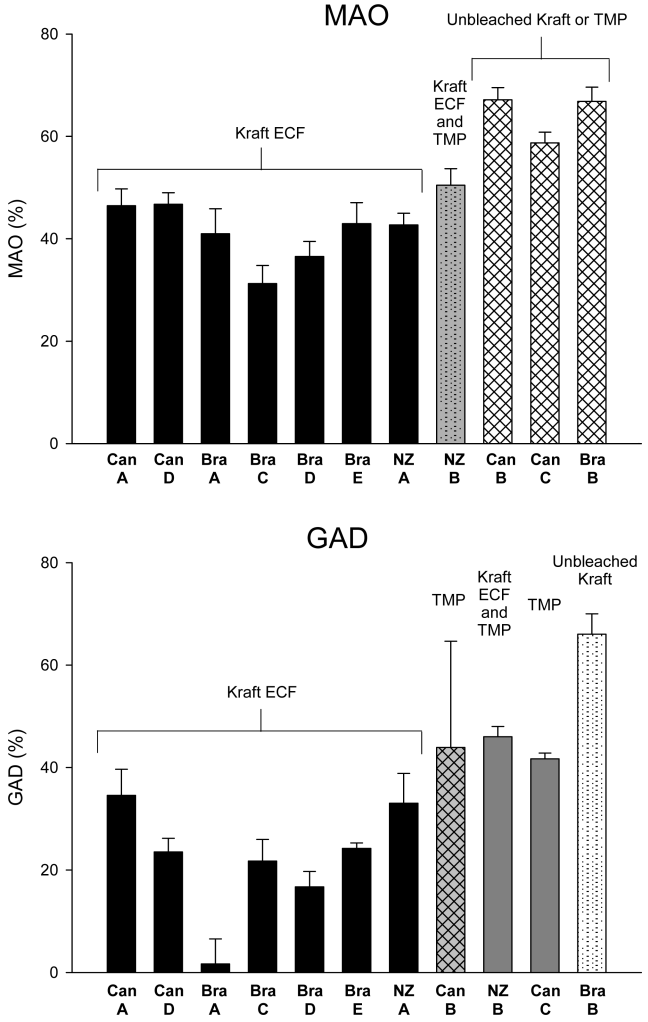

Figure 4. Monoamine oxidase (MAO) and glutamic acid decarboxylase (GAD) neurotransmitter inhibition expressed as a percentage of negative control following incubations with effluent SPE-MeOH fractions. All bars are mean ± standard deviation. Groupings are based on statistically significant differences (ANOVA, $p < 0.002$), except for GAD Can B vs kraft ECF ($p = 0.648$), NZ B and Can C ($p = 0.979$), Bra B ($p > 0.798$) because of an assay problem with Can C replicates. Can = Canada, Bra = Brazil, NZ = New Zealand.

of MAO, New Zealand Mill B, which produces ECF kraft and thermomechanical (TMP) pulp, showed an intermediate potency ($p < 0.001$), while the least potent mills of this study were the unbleached kraft and two TMP mills ($p < 0.002$). For GAD, New Zealand Mill B and Canada Mill C (TMP only) were of intermediate potency ($p < 0.001$), while the unbleached kraft mill, Brazil B, was the least potent ($p < 0.001$).

While neurotransmitter assays showed that all wood feedstocks contain GAD and MAO inhibitors (Figure 3), no trends between species, countries, and fractions are evident.

Parameters Related to In Vitro Activities. Relationships between bioactivities, effluent and wood chemistry, and production metrics were assessed using Pearson correlations (Tables S2 and S3, Supporting Information), with highlights detailed below.

Relationships with AR Activity. With effluents, the FP-DCM fractions produced a very strong correlation of 0.995 ($p < 0.001$), when the mill utilizing novel effluent treatment (Canada Mill D) was excluded (Table S2, Supporting Information). When the solvent types were pooled, a strong correlation of 0.784 ($p < 0.001$) was noted for DCM (Figure S5, Supporting Information), again when Canada Mill D was excluded. The MeOH extractions showed a weak significant correlation of 0.447 ($p = 0.042$).Since all effluent fractions, regardless of production, wood type and treatment (activated sludge or aerated lagoons), were highly correlated, the novel treatment system used by Canada Mill D is justified as an outlier. No correlations were observed between effluent total resin or fatty acids and AR binding.

The AR ligand contents of wood were also strongly correlated to wood GCIs (0.9210, $p < 0.001$), which was driven by fractions extracted from softwoods (Figure S5, Supporting Information, 0.890, $p < 0.001$). AR activity was also very strongly correlated to total resin acids (0.920, $p < 0.001$) and strongly to total fatty acids (0.792, $p < 0.001$; Table S3, Supporting Information).

Relationships with GAD and MAO Inhibition. Effluent MAO and GAD inhibition were strongly correlated with GCI when extractions were pooled by solvent type. Pooling all MeOH fractions (Table S2, Figure S6, Supporting Information), strongly significant negative correlations are observed between GCI and MAO (−0.741, $p < 0.001$), as well as between GCI and GAD (−0.753, $p < 0.001$), when one mill (Canada Mill C) is excluded. These correlations can be justified by the high effluent TSS of this mill (Supporting Information Table S1). With pooled DCM extractions (Table S2, Figure S6, Supporting Information), a strongly significant negative correlation between GCI and MAO inhibition (−0.657, $p < 0.001$), and GCI and GAD inhibition (−0.629) was noted. No relationship with MAO or GAD and effluent total resin or total fatty acids was observed. Additionally, no correlations were found with MAO and GAD inhibition and GCI, FA, or RA for wood feedstocks.

DISCUSSION

In this study, we sought to benchmark the current global potential of mill effluents and wood feedstocks to affect fish reproduction by comparing samples from three major pulp-producing nations, where each facility was in regulatory compliance. Mills included those that pulped hardwoods and softwoods, used TMP, ECF-, and unbleached kraft pulping, and employed aerated lagoons, activated sludge and one novel treatment system. We showed that treated effluents from all mills contained ligands for the goldfish testis AR and inhibitors of two neurotransmitter enzymes, however, little estrogenicity was detected. Factors found to influence in vitro activities included a novel effluent treatment system, high TSS, and pulping and bleaching processes. Extracts of wood feedstocks showed similar contents of biologically active substances, however, some large distinctions between hardwoods and softwoods were noted. A GCI was shown to predict AR ligand content, MAO inhibition, and to a lesser extent GAD inhibition, in effluents, and the AR ligand contents of softwoods.

The GCI used was modified from other studies[8,18] by distinguishing particulate bound from dissolved compounds, a range of polarities, and for the first time to profile wood extracts. Building on its ability to predict effects on fathead minnow egg production,[8] we sought to evaluate if the GCI

dx.doi.org/10.1021/es203382c | Environ. Sci. Technol. 2012, 46, 1849–1858

could also predict the in vitro activities of mill effluents. For kraft mill effluents, total GCI has been strongly correlated to BOD,[8] which was also the case when all kraft mills in this study were considered. Because of the complexity of effluent and wood extracts, a GCI further offers the ability to compare extracts from multiple sources without determining the identities of the actual constituents. In general, effluent SPE-DCM fractions had the highest GCIs, except Canada Mill C (FP-DCM). Canada Mill C also had the highest TSS (>12 fold, Supporting Information Table S1), which may have contributed to it having the highest GCI total of all mills (Figure 1). Overall, little distinction in GCI can be observed between wood type, pulping or bleaching types, or biological treatment type in effluent extracts.

Since very polar or very large compounds are not included in the GCI, the development of an index using liquid chromatography may help in predicting activity associated with these compounds. Such compounds would be found in methanol effluent extracts, which were darkly colored, yet exhibited generally low GCIs. It is worth noting, however, that GCIs were still predictive of MAO inhibition in these same extracts.

Because of their degradability, effluent resin and fatty acids were low, with the exception of Canada Mill C, which had the highest TSS, and would contain particulate-bound acids. Softwood chip extracts contained significantly higher resin and fatty acids and GCI values than for hardwoods, as expected.[36] It is noteworthy that these wood-derived differences are not reflected in effluents from hardwood versus softwood mills. It is likely that a large percentage of the detectable material by GC is significantly metabolized during biological treatment.[37]

Ligands for the AR were consistently detected in all final effluents and predominantly in softwood chip extracts (Figure 2). In contrast, very low YES activity was detected in only three Brazilian mills, and not wood chip extracts. In one of these mills, Brazil Mill A, sanitary waste from the site is also treated in the treatment system, which may account for some of the YES activity. The overall lack of estrogenic activity is surprising given the evidence for Canadian[38] and South American[13,15] mill effluents and experiments with stilbenes, plant sterols, and resin acids.[39−41] It is however, consistent with studies at both New Zealand mills[12] included in this study. That estrogenic activity was detected in three Brazilian mills (Supporting Information Figure S3) coincides with the strength of the in vitro activity noted recently in Chilean mill effluent extracts from both *Eucalyptus* and *Pinus*.[14,26] The lack of estrogenic response in this study may be related to the in vivo aromatization of AR ligands, which would not be detectable using the YES assay.[42] The estrogenic activity of selected fractions is currently being further examined using rainbow trout.

Excluding Canada Mills C and D, little overall difference in AR activity is exhibited between pulping and bleaching types, wood types, and biological treatment systems. Canada Mill D, which utilizes a novel treatment system, contained >8-fold the total average androgenicity of the other mills. Canada Mill C, which had the highest TSS levels, was the mill that contained the highest AR levels in the FP-DCM fraction (Figure 2). This is consistent with previous studies from New Zealand that showed filtering the solids removed androgenic activity.[5] These two mills illustrate the role that conventional biotreatment and subsequent solids removal has on reducing the overall androgenicity of final effluents.

We found very strong positive correlations of GCI to AR binding activity for both wood and effluent extracts. In all but two cases, effluent androgenicity was predominantly associated with the SPE-DCM fraction, with the majority of the remainder of activity in the FP-DCM fraction. This indicates AR ligands are intermediate to nonpolar, consistent with effects-directed studies of androgens in Canadian kraft chemical recovery condensates,[30] and bioaccumulation studies at Canadian mills.[43,44]

Similar to effluents, most of the androgenic activity for wood was in the nonpolar fractions. Unlike effluent fractions, wood vextracts showed a large difference between hardwoods and softwoods with hardwoods having negligible AR activity (Figure 2). This discrepancy was also reflected in the GCI (Figure 1) and total resin acid (Figure S2, Supporting Information) values. The lack of a difference in effluents between mills pulping softwoods or hardwoods suggests that (a) hardwood mills produce androgenic compounds at some point during pulping, bleaching, or subsequent biotreatment; (b) the androgenic compounds are significantly degraded in softwood mills; or (c) some androgenic compounds found in both types of wood are of similar nature and nondegraded.

Because of the complexity of final effluents, the actual causative androgens still remain elusive, despite many studies.[45] Androgens associated with wood and effluents, ADD, AD, and progesterone[27−29] were not detected in this study, and thus are not involved in the androgenic activities we observed. Recently, the diterpene manool was discovered as a ligand for the goldfish testis AR and a major contributor to the androgenicity of Canadian kraft chemical recovery condensates.[30] In this study, manool was only detected in Canada Mills C and D effluents, accounting for <0.05% of those mills' AR activities. Since the structure of manool is similar to resin acids, which are readily biodegradable,[37] it can be implied that effluent biotreatment is effective in the removal of manool and other structurally analogous AR ligands. Collectively, based on the results of this study, it appears that effluent biotreatment has beneficial effects toward reducing androgen discharges, especially for softwood mills.

Neuroendocrine activity, measured as MAO and GAD inhibition, was observed in all effluents and wood chips from the three countries studied. Effluents from the seven ECF kraft mills produced the greatest MAO and GAD inhibition relative to the one unbleached kraft and two TMP mills. While this suggests that kraft ECF bleaching generates more compounds affecting the neuroendocrine system, implications for the pulp and paper sector necessitate further examination. Contrary to AR activity, neuroendocrine activity was greatest in the most polar effluent extracts. Indeed, there was no correlation between AR and either MAO or GAD ($p > 0.05$, data not shown), confirming different classes of compounds are involved. This is not surprising, given the structural conformations required between these three end points, and that each represents a different mode of action affecting fish reproduction. Given that the most polar fractions for wood did not inhibit GAD or MAO differently from nonpolar extracts, it would appear that pulping, bleaching and effluent treatment play a role in the generation of neurotransmitter inhibitors, as evidenced above for ECF kraft.

Previous work[20] showed that for final treated effluent at a Canadian TMP mill, MAO inhibition (65%) remained associated with the water fraction following extraction using polyvinylpolypyrrolidone powder. In contrast, we show that it is possible to remove neuroactive compounds from effluents using SPE. Both TMP mills of this study showed similar MAO inhibition of

60−65% in the most polar fraction (SPE-MeOH). Interestingly, while no GAD inhibition was observed by Basu et al.,[20] we found it not only at both Canadian TMP mills, but in all mill effluents from Canada, Brazil, and New Zealand. Since we observed the most potent GAD inhibitors in SPE fractions, the polar compounds recovered by SPE were possibly retained on the polyvinylpolypyrrolidone powder used for effluent extractions.[20] These results all indicate that GAD and MAO inhibitors are very polar, water-soluble, and therefore, readily bioavailable. This supports the hypothesis of neurotransmitter involvement in the rapid onset and subsequent recovery of some reproductive end points, such as egg production[8] and sex steroid levels.[10]

In conclusion, this study determined the prevalence of androgenic and neuroactive compounds in all effluents and wood feedstocks sampled in Brazil, Canada, and New Zealand. No differences in bioactivities could be discerned on the basis of effluent or wood country of origin. Differences in bioactivities between hardwood and softwood chip extracts were not reflected in effluents generated from these feedstocks, implying that pulping, bleaching, and treatment mitigate bioactive wood components irrespective of locale. This highlights the difficulty in identifying causative agents, and does not discount the possibility that other process chemicals are important for effluent related effects. The difference in bioactivity between wood and effluents highlights the impact that mill operating conditions can have in final effluent biological activities. Effluent treatment type (novel vs ASB/AS) and effluent solids management were important in controlling androgenic compound releases, while ECF kraft was associated with increased inhibition of GAD and MAO. The GCI, incorporating solids extraction and differentiation of compound polarity, was predictive of the majority of in vitro activity. On the basis of this study, we would predict effluents where no monitoring is conducted, to also affect fish reproduction. It is therefore recommended that monitoring programs, analogous to the Canadian EEM program, be implemented in these jurisdictions.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Summary of effluent sample characteristics, recovery of effluent extractives, correlations for effluent fractions, correlations for wood extracts, total resin and fatty acid concentrations, estrogen receptor binding assay, gas chromatographic index, relationship between GCI and goldfish testis androgen receptor binding, and relationship between GCI and MAO. This information is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

The authors gratefully acknowledge the participating mills for providing samples and mill information, the staff in Aplysia (Brazil), Scion (New Zealand), FPInnovations (Canada), and Environment Canada. Jim Bennett is also acknowledged for his photo used in the Table of Contents and Abstract.

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