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Estrogenic and anti-estrogenic effects of wood extractives present in pulp and paper mill effluents on rainbow trout

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ABSTRACT

Wood extractives present in pulp and paper mill effluents may cause reproductive disturbances in fish. A chronic-exposure toxicity experiment using immature rainbow trout (*Oncorhynchus mykiss*) was conducted in order to assess the endocrine disrupting effects of two Chilean pulp and paper mill specific extracts (solid phase extraction, SPE) obtained from primary and secondary treated effluents. The (anti)estrogenic potencies and toxicity of the wood extractives regularly present in pulp mill effluent such as dehydroabietic acid (DHAA), β-sitosterol (BS), and model estrogen 17β-estradiol (E2) were evaluated by analysis of plasma vitellogenin (VTG) levels, gonadal somatic index (GSI) and liver ethoxyresorufin-*O*-deethylase (EROD) activity, respectively. The protocol involved the use of multiple intra-peritoneal injections (1 injection every 7 days for a total exposure period of 28 days). Analysis of variance/covariance, demonstrated no differences associated with fish gender other than GSI. The phytosterol BS, E2 and both pulp mill effluent extracts showed significant inductions of EROD and increased VTG levels after 4, 7, 14, 21 and 28 of exposure. While fish injected with secondary treated effluent extract showed a delayed induction in VTG levels compared to primary effluent injected fish, no effects on VTG and EROD levels were observed in DHAA injected fish. Moreover simultaneous injection of DHAA + E2 reduced the VTG levels found in E2 injected fish, indicating a potential indirect anti-estrogenic effect of this resin acid. The results of this study indicate that Chilean pulp and paper mill effluent extracts are estrogenic in rainbow trout males and females.

1. Introduction

A vast amount of research on the impacts of pulp and paper mill effluents has pointed to its overall negative impacts on reproductive function in exposed fish (Hewitt et al., 2008). Specific responses observed in rainbow trout include induction of liver 7-ethoxyresorufin-*O*-deethylase (EROD) enzymatic activity, reduction in gonad size, depression of circulating sex steroids, increase in plasma vitellogenin (VTG) in immature fish (Tremblay and Van Der Kraak, 1999; Oakes et al., 2005), reduced ovarian development and increase of VTG in adult male trout (van den Heuvel and Ellis, 2002; Ellis et al., 2005) and anti-estrogenic effects of pulp mill extractives in trout primary hepatocytes (Marlatt et al., 2006). This research was mostly conducted in North America, Finland and New Zealand and partially differs from recent observations of Chilean pulp and paper mill effluent effects on their receiving environments.

Previous work showed that immature female and male rainbow trout caged in a Chilean river receiving pulp and paper mill effluents exhibited significant estrogenic effects including increased plasma VTG levels and increased female gonad maturation (Orrego et al., 2005, 2006). This estrogenic effect was then confirmed in a follow-up laboratory study testing the effect of specific pulp mill final effluent extractives in triploid female rainbow trout using a single intra-peritoneal injection exposure protocol (Orrego et al., 2009). An early increase in VTG levels was observed in all exposed fish irrespective of the effluent treatments, and subsequently related to increased endogenous 17β-estradiol and up-regulated expression of genes coding for enzymes involved in estrogen biosynthesis from androgens (cytochrome P45019 aromatase [CYP19a]) (Orrego et al., 2010). These results suggested that the observed estrogenic effect can be related to compounds that act as estrogen receptor (ER) agonists or induce changes leading to increased amount of endogenous estrogens (e.g. estrogens and aromatizable androgens). However, due to the singular intra-peritoneal injection protocol used, these early effects did not last during the experiment.

Pulp and paper mill effluents are a very complex mixture and may contain compounds with both androgenic and estrogenic properties (Ellis et al., 2003; Hewitt et al., 2003; Svenson and Allard, 2004). Wood extractives are natural wood constituents receiving significant attention due to the variety of toxicities they exert on

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aquatic biota. Most of the acute toxicity of wood industry effluents has been attributed to resin acids (a group of lipophilic di-terpene carboxylic acids present in softwood) and phytosterols (plant sterols structurally related to androgens and estrogens) which may be liberated from wood during pulp manufacturing processes (Christianson-Heiska et al., 2008). Such compounds potentially end up in the final discharge either unaltered or as a biotransformation metabolic product of microbial activity (Hewitt et al., 2008).

Phytosterols such as β-sitosterol, campesterol and stigmasterol are some of the most common extractable constituents of wood present in pulp mill effluents (Cook et al., 1997). β-Sitosterol is known to cause several endocrine effects in fish, including estrogenic activity such as VTG induction (Mellanen et al., 1996; Tremblay and Van Der Kraak, 1998; Orrego et al., 2009), alteration in plasma sex hormone levels and altered gonadal steroidogenesis (MacLatchy and Van Der Kraak, 1995; Orrego et al., 2010).

Resin acids accumulate in the liver and bile of fish (Merilainen et al., 2007) and elevated levels have been found in fish caged in pulp and paper mill receiving waters (Karels and Oikari, 2000). Dehydroabietic acid (DHAA) is one of the most common resin acids and has been implicated in a variety of disturbances at the cellular level in fish hepatocytes (Nikinmaa et al., 1999; Rissanen et al., 2003; Merilainen et al., 2007), such as decreased plasma cortisol levels in eel (*Anguilla anguilla*) (Teles et al., 2003), and recently an anti-estrogenic effect of decreased plasma VTG in zebrafish (*Danio rerio*) (Christianson-Heiska et al., 2008).

In spite of modern wastewater treatment technologies implemented by most of the pulp and paper mills industries during the last decades (e.g. activated sludge treatment), where most of the wood extractives are significantly reduced or removed from the effluents (>90%) (Lahdelma and Oikari, 2005), significant amounts of resin acids and phytosterols continue to be found in final treated effluents (Orrego et al., 2009) and their receiving environments (e.g. lake sediments) (Lahdelma and Oikari, 2005, 2006).

The aim of this study was to examine the reproductive effects of primary treated and secondary treated Chilean pulp and paper mill effluent extracts (solid phase extracted, SPE) along with two wood extractive standards previously detected in both effluents, dehydroabietic acid (DHAA) and β-sitosterol (BS), in a chronic multiple-injection experiment using immature rainbow trout and evaluating differences associated with fish gender. A subsequent objective was to investigate the potential anti-estrogenic effect of DHAA in trout by comparing the effects of a combined injection using 17β-estradiol (DHAA + E2).

2. Materials and methods

2.1. Effluent samples

Primary and secondary treated pulp mill effluent samples (primary clarifier and aerated extended lagoon/activated sludge basin, respectively) were obtained from mills using kraft pulping process with elementary chlorine free (ECF) bleaching technology (bleached kraft mill effluent, BKME). The primary treated mill processes equal amounts of pine and eucalyptus (50/50%) and has an annual white cellulose production of 550,000 tons. The secondary treated mill processes pine (60%) and eucalyptus (40%) and has an annual production of 850,000 tons.

2.2. Effluent solid phase extractions (SPE)

Effluent samples were extracted using reverse phase C-18 (non-polar) cartridges as previously described for the analysis of organic compounds derived from treated pulp mill effluents (Burnison et al., 1999; Orrego et al., 2009). Briefly, 3 L samples of each effluent were filtered through a Whatman binder-free glass microfiber filter (type GF/C: 4.7 cm in diameter and 0.3 μm particle retention) and extracted by SPE using ACCUBOND ODS C-18 reverse phase cartridges previously conditioned with two volumes of Milli-Q water and one volume of methanol. Extractions were performed under 4 mL min^{-1} of vacuum (2 cartridges were prepared for each sample).

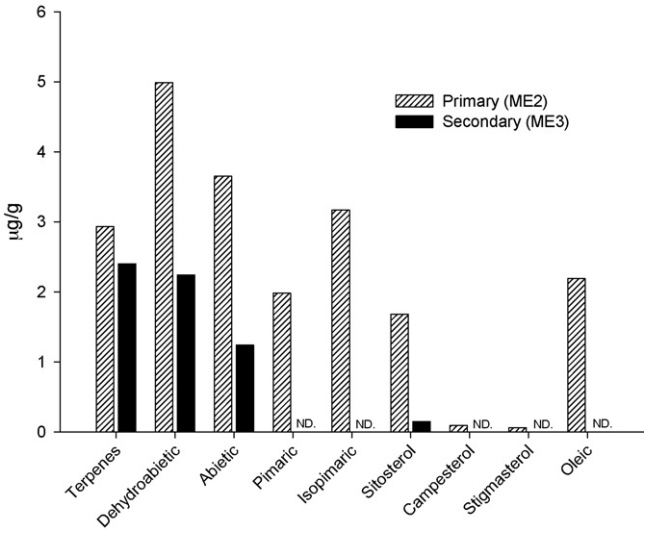

Fig. 1. Effluent composition for the three major groups of chemicals, terpenes, resin acids and phytosterols. ND: not detected.

Compositions of the effluents were analyzed from one of the cartridges previously eluted with two ethyl acetate volumes, two of methanol and then derivatized with diazomethane. The effluent composition for the three major groups of chemicals, terpenes, resin acids and phytosterols (Fig. 1) was previously reported (Orrego et al., 2009). The extracts were reconstituted in methanol and analyzed by gas chromatography mass detection (GC/MS) in a Hewlett-Packard 5890 II with HP 5972 detector series (Avondale, PA. USA) and a HP 5MS column (0.25 mm diameter and 0.25 μm thickness), using an injector and a mass detector temperature of 250 and 300 °C, respectively.

The second cartridges of each extract were eluted using a sequence of hexane, ethyl acetate, methanol and were then reduced by gaseous nitrogen. Finally the ethyl acetate and methanol elutriates were re-suspended together in 100 μl of acetone and dissolved in corn oil (carrier) for subsequent injection into the fish. The acetone was evaporated prior to intra-peritoneal injection.

2.3. Dose calculation and intra-peritoneal injection

A preliminary 96 h acute toxicity test was undertaken to estimate non-lethal intra-peritoneal doses (Rottmann et al., 2001) of steroids, phytosterol standards and pulp mill extracts (Orrego et al., 2009). Fish were finally injected with effluent extracts at a volume of 200 μl per 100 g of body weight.

A total of 165 sexually immature hatchery-reared (Linwood Acres Trout Farm, ON, Canada) rainbow trout *Oncorhynchus mykiss* (72 ± 20 g), were used in a 28-day chronic-exposure toxicity experiment. Fish were exposed by intra-peritoneal injection to two Chilean pulp and paper mill extracts obtained from primary and secondary treated effluents (ME2 and ME3, respectively; 25 mg/kg wet weight), and wood extractives dehydroabietic acid (DHAA; 5 mg/kg wet weight) and β-sitosterol (BS; 5 mg/kg wet weight), a model estrogen 17β-estradiol (E2, 5 mg/kg wet weight) and a combination of DHAA + E2 (5 and 5 mg/kg wet weight, respec-