Ecotoxicology and Environmental Safety 171 (2019) 256–263

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Assessing wild fish exposure to ligands for sex steroid receptors from pulp and paper mill effluents in the Biobio River Basin, Central Chile

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ARTICLE INFO

Keywords:
SPMDs
Estrogenic compounds
Wild fish
SSBP
AR
GSI
EROD

ABSTRACT

Bioactive substances in the Biobio River Basin in Chile were examined by deploying Semi Permeable Membrane Devices (SPMDs) upstream and downstream of 4 pulp mill effluent discharges. Androgenic and estrogenic activity of SPMD extracts were then evaluated using in vitro fish sex steroid receptor binding assays. The results indicated the occurrence of estrogenic type compounds associated with one of the mill discharges. A significant correlation among the presence of these compounds, an increase in gonadosomatic index GSI and induction of hepatic EROD activity of two native fish species was observed. However, no significant presence of mature oocytes in female gonads was detected. Although EROD induction was observed in sites impacted by mill effluents, an increase of its activity occurred towards the downstream areas, suggesting other non-mill sources. More research is needed to understand the environmental changes in context of the new technological improvements in treatment systems to MBBR (Moving Bed Biofilm Reactor) recently implemented by the pulp mill industries.

1. Introduction

The reproductive impacts of pulp and paper mill effluents on wild fish have been established thoroughly in countries such as Canada (Pollock et al., 2010; Bowron et al., 2009), Sweden (Pettersson et al., 2007), USA (Orlando et al., 2007; Fentress et al., 2006), New Zealand (Landman et al., 2008; West et al., 2006) and more recently in developing countries such as Chile (Chiang et al., 2011a, 2011b). The observed effects include decreased egg production, increased age to maturation, reduced levels of reproductive steroids, induction of estrogen and androgen-mediated proteins, intersexuality and effects on secondary sex characteristics. Although an average pattern of metabolic disruption downstream of mills has been observed in countries like Canada, whereby fish exhibit smaller relative gonad sizes, increased relative liver sizes and increased condition factor (e.g. Canadian Environmental Effects Monitoring (EEM) Program, Lowell et al., 2005), the causative remains unclear (reviewed in Hewitt et al., 2008).

Intensive research efforts are underway for identifying the sources, compounds, and remediation solutions for reproductive effects detected in wild fish exposed to mill effluents long-term. Bowron et al. (2009) synthesized 20 years of demonstrating that reproductive impacts persist following process changes, indicating that bioactive compounds are still being discharged.

Moreover, bioactive compounds appear to survive effluent secondary treatment systems. Chemical analyses of solid phase extracted (SPE) biotreated effluents still detect levels of resin acids (e.g. isopimaric, abietic, dehydroabietic), phytosterols (e.g. sitosterol, stigmasterol), phenolics, diterpenes, among others (Bowron et al., 2009; Orrego et al., 2009; Scott et al., 2011; Milestone et al., 2012). However, evidence indicates that well maintained effluent biotreatment systems and tightly run mill operations with good spill control and condensate handling contribute to improved overall performance in terms of effects on fish reproduction (Hewitt et al., 2008; Bowron et al., 2009). Indeed, correlations between effluent biological oxygen demand (BOD) and

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https://doi.org/10.1016/j.ecoenv.2018.12.092
Received 15 June 2018; Received in revised form 18 December 2018; Accepted 27 December 2018
Available online 03 January 2019
0147-6513/

effects on egg production in spawning pairs of fathead minnows have recently been established on a national basis in Canada (Martel et al., 2017). Minimizing the release of bioactive substances (using BOD as a surrogate) to treatment systems is a logical approach to dealing with this complex problem. The question of whether effluents from state of the art mills could cause effects on reproduction is highly relevant since Best Available Technology (BAT) is not universally applied and the effects on fish reproduction continues to be found worldwide (Milestone et al., 2012; Orrego et al., 2017).

The newest and most modern mills in the world are operating in South America (since the last decade of 20th century). However, information on their effects in aquatic receiving environments regarding effects in wild fish populations is scarce (Chiang et al., 2011a, 2011b). These mills operate with state of the art technology, their discharge limits of biological oxygen demand (BOD), total suspended solids (TSS) and chlorinated organics are among the lowest in the world. However, it is important to note that the new technologies employed at these mills were designed for targeted reductions in these parameters; not to address the effects of mill effluents on fish reproduction.

In Central Chile, and specifically in the Biobio River Basin, pulp and paper mill industries generate up to 83% of the total national pulp production (21.489.000 Air died metric tonnes per year. FAO, 2016:ISSN 0255-7665), with a continuous expansion since 2000. The Biobio River is a system of utmost importance for Chile, providing water for irrigation, drinking, and hydroelectric power generation. Pulp and paper mill industries each produce more than 1000 t/day of pulp from pine and eucalyptus in the region, discharging their treated effluents into the river. Although the Chilean pulp and paper industry has implemented effluent treatment systems (primary and secondary) during the last two decades, evidence of endocrine-disruption effects in fish was first reported in 2005 (Orrego et al., 2005a, 2005b). It is also important to note that areas affected by mill discharges in the Biobio were also associated with changes in wild fish abundance and diversity (Habit et al., 2006).

The initial evidence for effects of Chilean mill effluents on fish reproduction involves a series of laboratory and field studies where rainbow trout (*Oncorhynchus mykiss*) were caged in the Biobio River below mill outfalls (Orrego et al., 2005a, 2005b) and exposed to sediments collected in those areas (Orrego et al., 2006). The evidence indicated that mill effluents in Chile exhibit fairly strong estrogenic effects via a two-fold induction of plasma vitellogenin (VTG) in rainbow trout exposed for 29 d to sediments collected below the discharge of four Chilean mills (Fig. 1, Table 1). The increased VTG was also associated with increased gonad size and the presence of more mature ovarian follicles in females. Immature female trout caged for 21 d in the same locations where the sediments were collected, exhibited 4–5-fold increases in VTG levels that coincided with enhanced gonadal maturation (presence of vitellogenic oocytes) (Orrego et al., 2005a, 2005b). The ability of extracts of Chilean mill effluents to affect fish reproduction via pulse exposure using intraperitoneal injections was also examined, indicating estrogenic and anti-estrogenic effects associated with a specific group of wood extractives including phytosterols and resin acids (Orrego et al., 2009, 2010a, 2010b, 2011). These estrogenic effects on fish associated with pulp mill effluent discharges into the Biobio River were recently confirmed under laboratory and field conditions, also showing the appearance of intersex characteristic in juvenile male rainbow trout (Chiang et al., 2015).

The ability of pulp and paper mill effluent from Canada, New Zealand and Brazil to cause androgenic and estrogenic effects in fish has recently been demonstrated in vitro (Milestone et al., 2012) and in vivo (Orrego et al., 2017). However, no clear relationship with the type of wood, treatment, or bleaching process were established. Collectively, these results indicate a cause for concern regarding the potential of mill effluents to affect fish reproduction. Although Chiang et al. (2011a) demonstrated the potential for pulp mill effluent impacts at the endocrine, individual and population level in two native fish species in the Itata River, there are challenges associated with the absence of receiving environment monitoring requirements, the “protected” status of Chilean native freshwater fishes, and the absence of an accepted monitoring framework (Chiang et al., 2010) such as in the Canadian EEM Program.

The objective of this study was to examine two representative wild resident fish populations at sites above and below mill outfalls in the Biobio River in order to evaluate their reproductive performance in a study design application of the Canadian EEM approach. In an effort to link chemical exposure in situ with potential effects on fish populations, passive sampling devices (Semi-Permeable Membrane Devices, SPMDs) were deployed during fish collections to chemically profile hydrophobic substances at these sites and to also assess the exposure of compounds functioning as ligands for fish sex steroid receptors.

2. Material and Methods

2.1. Field fish sampling

Field fish samples were collected in spring-summer (November), in five sampling stations along the Biobío River both downstream pulp mill discharges: Negrete (PC), Nacimiento (NC), Santa Juana (SJ) and Concepción (PL), and an upstream site (PG) (see the main physicochemical properties in Table S1, Supplementary material). In addition, one reference site was located in the Laja River, a tributary of the Biobio River which does not receive any pulp mill effluent discharge (RL) (Fig. 1). Two species of fish (from a total of 37 existing species), were collected considering their abundance in the study areas, small size (not more than 10 cm) and relative limited mobility: *Trichomycterus areolatus* (common name Bagre) a benthic species, and *Percilia irwini* (Carmelita) a benthic–pelagic species inhabiting relatively shallow waters. Twenty fish were length selected for each species based in our previous studies to ensure maturity. Fish were sacrificed in the field (euthanasia by cervical dislocation subsequent to anesthesia, according to the Canadian Council of Animal Care protocols), sized, sex and length was measured immediately (Fig. S1. Supplementary material), and dissected tissues (liver) were stored in liquid nitrogen and then transported to the Biomarkers Laboratory of EULA-Center (University of Concepcion) for further analysis. Gonads were also extracted and stored in Bouin solution for histological analysis.

Physiological indexes of condition were calculated based on the morphometric information such as the condition factor (k) (100 *weight/length^3), gonadosomatic index (GSI) (100 *gonad weight /total weight of fish) and Liver somatic index (LSI) (100 *liver weight/ total weight of fish).

Cytochrome P450 isoform 1A1 enzyme activity was evaluated as 7-ethoxyresorufin-*O*-deethylase (EROD) (Lubert et al., 1985) in the floating fraction (S9) obtained from livers homogenized in a sucrose buffer (0.1 M, pH 7.5) and centrifuged at 9000*g* for 20 min at 4 °C. Its final value was expressed as pmol resorufin/min/mg of protein (total protein of liver tissue). Protein analysis was performed using a Bio-Rad Protein Kit (Hercules, CA, USA), which uses bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as a reference material.

The histological analyses were performed in the Department of Cellular Biology of the Faculty of Biological Sciences (University of Concepción). The gonads in Bouin solution (48 h), were then washed and dehydrated with a series of ethanol solutions (70–99%). Finally, they were embedded in liquid paraffin at 58 °C (24 h) for their later cutting (7 μm) and staining with haematoxylin and eosin (0.5%). Maturity states were assigned according to bibliographic references (Huaquin et al., 2002), using a 100X magnification (ZEISS AXIOPLAN 2, Digital NIKKON DXM 1200) and the proportion of cells in the distinct maturation stages (I, II, III and IV according to the scale of Chiang et al., 2012a, 2012b).

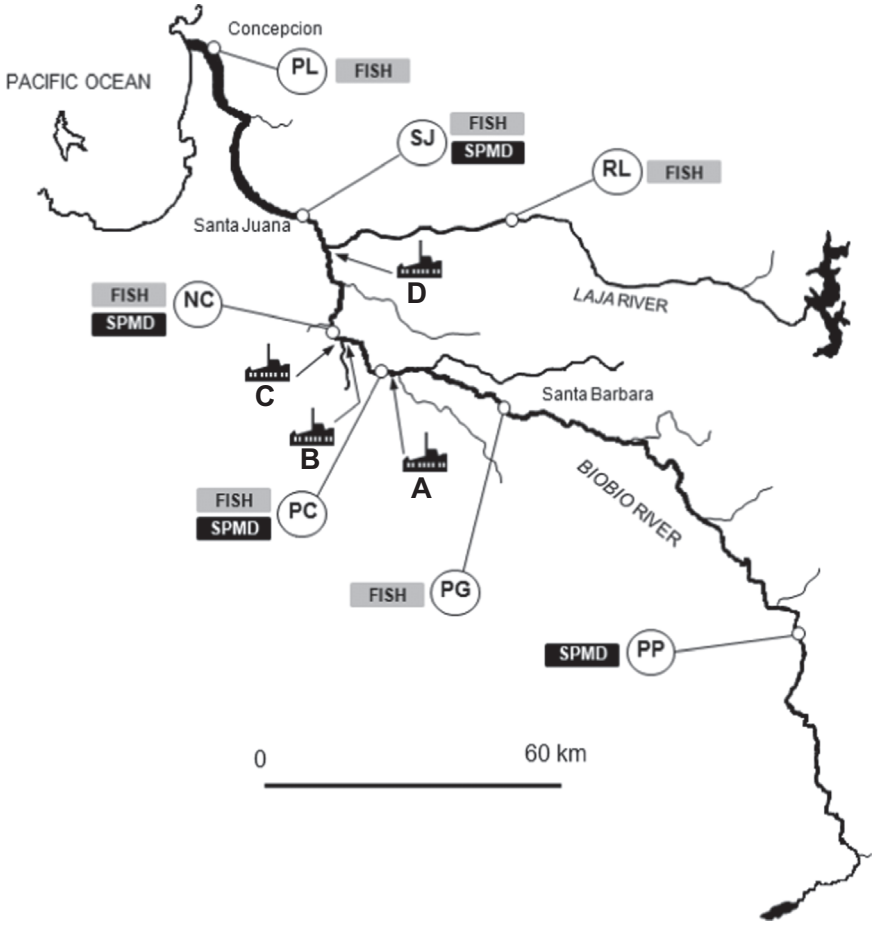

Fig. 1. Biobio River Basin showing pulp mill locations and sites where wild fish were collected and SPMDs deployed.

2.2. SPMD deployments in the Biobio river

The SPMDs (EST Laboratories, USA, lot#130803) were deployed at four sites for 21 d in the Biobio River. The first deployment occurred during spring-summer time (November 2003) at Nacimiento (NC. 37°30'14"S, 72°51'02"W) and Santa Juana (SJ. 36°43'13"S, 72°54'17"W); the second deployment was during summer time at La Pola (PP. 37°59'55"S, 71°30'25"W) and Negrete (PC. 37°28'34"S, 72°50'32"W) (Fig. 1). SPMDs were deployed in duplicate in stainless steel containment vessels at each site. To account for background accumulations, duplicate trip blanks for both periods were exposed during deployment and recovery procedures. After deployment, all SPMDs were gently wiped to remove surface biofilms and particulates, sealed in metal cans and frozen at −20 °C until dialysis. Details of dialysis procedures are described in Parrott et al. (1999). Residual triolein was removed from dialyzed extracts using HPLC equipped with a preparative gel permeation (GPC) column (21.2 × 350 mm Envirosep ABC, Phenomenex, CA, USA) that had been calibrated with triolein. Purified SPMD extracts were reduced in volume by rotary evaporation and brought up to 400 µL in methanol for incubations with fish sex steroid receptor preparations.

It is important to highlight that during the autumn-winter (high flow season), accessibility issues and significant increase of water flow speed makes fish sampling (electrofishing) difficult as well as water sampling. Hence the need to prove the effectiveness of SPMDs and in general of passive sampling methods as an alternative to address the issue of monitoring specially where biota sampling is not possible.

2.3. Binding assays with fish sex steroid receptors

SPMD extracts were tested for their potential to interact with fish sex steroid receptors using established protocols for goldfish testicular androgen receptors (AR; Wells and Van Der Kraak, 2000) and goldfish plasma sex steroid binding protein (SSBP; Van Der Kraak and Biddiscombe, 1999). Standard displacement curves for each receptor were performed using the same concentration of methanol (1% v/v) and this concentration did not affect hormone binding. For each assay, the specific binding of each SPMD extract was converted into hormone

Table 1
Principal characteristics of mill discharging into Biobio River (Data at SPMDs deployment time).

	Kraft type	Bleaching sequence	Wood type	Treatment type	Wastewater flow (m^3/ADMT)	Tertiary
Mill A	BSKP	DEopDD	SW	Aereated Lagoon	60	No
Mill B	BEKP	DEopD	HW	Activated Sludge	40	No
Mill C	TMP	unbleached	Paper	Activated Sludge	30	No
Mill D	BSKP/UKP	DEopDD	SW	Activated Sludge	100	No

BSKP = bleached softwood kraft pulp, BEKP = bleached eucalyptus kraft pulp, UKP = unbleached kraft pulp, SW = soft wood, HW = hard wood, D = chlorine dioxide, E = extraction stage, op = with oxygen and hydrogen peroxide).

equivalents (pg/μL extract) using the corresponding hormone standard curve. For the SSBP assay, the standard curve was generated by incubating varying amounts (0.1–20 nM) of unlabeled testosterone with a fixed amount of [3 H]-testosterone and pooled plasma preparations from a total of 6 goldfish. Blood was collected for plasma preparations from the caudal veins of each fish using heparinized 1 ml syringes and kept on ice in heparinized Eppendorf tubes until centrifugation at 4500 g for 15 min at 4 °C. Plasma preparations from individual fish were pooled and stored at −20 °C.

Prior to incubations with SPMD extracts, SSBP preparations were characterized by saturation analyses of [3 H]-testosterone to generate total binding and nonspecific binding values and a Scatchard plot (Van Der Kraak and Biddiscome, 1999). For each SPMD extract, two replicate competition assessments were conducted using the pooled preparations from 6 fish and phosphate buffer.

For the AR assay, the standard curve was generated by incubating varying amounts (0.1–20 nM) of unlabeled testosterone with a fixed amount of [3 H]-testosterone and pooled testicular preparations from a total of 6 male goldfish. Goldfish testicular preparations were prepared from freshly euthanized individuals (spinal severance), and excised tissues were pooled and kept on ice until homogenization and cytosolic preparation. Tissues were homogenized in three volumes of buffer H (50 mM Tris-HCl, 1 mM NaEDTA, and 30% glycerol; pH 7.5 at 48 C) using a Potter-Elvehjem Teflont-glass homogenizer (Fisher Scientific, Nepean, ON, Canada), then centrifuged at 1000*g* for 15 min at 48 °C using a Sorvall RC 5C Plus Centrifuge (Sorvall Products, Newtown, CT, USA). The supernatant was centrifuged at 100,000 g for 60 min at 48 °C using a Beckman Optima L preparative ultracentrifuge (Beckman Instruments, Fullerton, CA, USA). Next, the cytosolic fraction was charcoal-stripped to remove endogenous steroids. First, a charcoal pellet was obtained by centrifuging buffer C (50 mM Tris-HCl, 1 mM NaEDTA, 10% glycerol, 1% Norit A-charcoal, 0.1% Dextran T-70t [Sigma Chemical]; pH 7.5 at 48 °C) at 10,000 g for 10 min at 48 °C and removing the supernatant. The gonad cytosolic fractions were added to the charcoal pellet (for a final concentration of 0.35% charcoal), vortexed, incubated for 5 min on ice, and then centrifuged at 10,000 g for 10 min at 48 °C. The supernatant was collected and stored at -80C until incubations with SPMD extracts.

Prior to incubations with SPMD extracts, testicular AR preparations were characterized by saturation analyses of [3 H]-testosterone to generate total binding and nonspecific binding values and generate a Scatchard plot (Wells and Van Der Kraak, 2000). For each SPMD extract, two replicate competition assessments were conducted using the pooled preparations from 6 fish.

2.4. Gas chromatography-mass spectrometry analyses of SPMD extracts

Aliquots of final SPMD extracts (1 quarter of final volume or 100 μL) were reduced to dryness and re-dissolved in toluene for profiling by gas chromatography-mass spectrometry (GC-MS). The GC-MS system consisted of a Hewlett-Packard 6890 GC with a HP 7683 Series injector and a HP 5973 MSD (Avondale, PA, USA). Separations were performed using a 30 m HP 5MS 0.25 mm i.d., 0.25 mm phase thickness column. The GC temperatures were programmed as follows: 80 °C for 2 min, 38 °C/min to 240 °C, 15 °C/min to 285 °C, which was then held for 10 min. The MSD utilized positive ion electron impact ionization and was used in full scan mode (m/z 50–500) to characterize compounds accumulated by SPMDs from the Biobio water column. Mass spectra generated for resolved components were compared against the NIST 2011 Mass Spectral library for tentative structural assignment groupings of pulp and paper derived components and non-mill derived compounds were made.

2.5. Data analysis

Continuous data were examined for normal distribution (Shapiro–Wilks test) and, if necessary, log transformed to avoid non-normality. Except for the GSI, testosterone equivalents generated from binding studies (SSBP and AR), and EROD activity, values were log transformed prior to analysis. One-way ANOVA (SPSS. 1998; SYSTAT for Windows, Ver 9. Evanston, IL, USA) was used to evaluate statistical significant differences between sampling areas for GSI, SSBP and AR. Two-way ANOVA (STATISTICA ver 7.1. StatSoft, Tulsa, OK, USA) were then employed to determine differences in EROD activity among the different sample sites and between species. In both analysis differences were confirmed by a multiple comparison Tukey post hoc test ($p < 0.05$). Factorial ANOVA (STATISTICA, ver 7.1. StatSoft, Tulsa, OK, USA) indicated no significant differences (length, K and GSI) associated with gender irrespective of fish species. Results were represented using a Sigma-restricted parametrization as coding strategy for gender and by least square means bi-dimensional (Fig. S2. Supplementary material). Due to the unbalance gender proportion observed (Fig. S1) and the non-significant influence of gender observed respect of GSI, their statistical analysis was carried out pooling females and males together.

3. Results

3.1. Fish population reproductive performance

No statistical significant differences were observed in the K and LSI in all the sampling sites along the Biobio River. The GSI result showed significant differences between sample sites in both species *P. irwini* (Fig. 2) and *T. areolatus* (Fig. 3). Fish captured downstream of the first pulp mill effluent discharge at site PC showed increased GSI. Additionally, in both species the gonad development analysis indicates that even though those fish showed the highest GSI, oocytes in the late development stage of maturation (IV) were not observed. This indicates the presence of large gonads in female fish but without maturation detected. Mature oocytes were observed in gonads of both fish species collected in both reference sites (RL and PG) and downstream pulp and paper mill sites (SJ and PL) but in very low number.

3.2. Induction of liver CYP4501A1 enzymes

Significant EROD induction was observed downstream of the reference station within the Biobio River in both fish species (Fig. 4). The similar pattern indicates an increase in fish captured in PC, decreasing in NC (showing no significant differences with the references sites, RL and PG), and increased again in SJ and PL. The last site showed the highest increase detected, although no pulp mill effluent was discharged into the river at this site. This indicates either a downstream effect or the presence of other potential emissions that are able to induce EROD activity in fish livers. Interestingly, when comparing EROD inductions patterns between species, *T. areolatus* (benthic species) always showed the highest EROD induction compared with *P. irwini*, and statistical significant differences were observed in fish captured downstream of the pulp and paper mill discharges (SJ and PL).

3.3. Binding assays with fish sex steroid receptors

SPMDs deployed in the Biobio River showed accumulated compounds that interacted with goldfish SSBP and AR (Fig. 5A and B). No statistical difference in affinities for extracts to goldfish SSBP were found between site PP (upstream reference site) and sites NC (pulp mills influence area) and SJ (downstream site). However, significantly higher hormone equivalents were accumulated by the SPMDs only at site PC, the site below the uppermost mill discharge in the Basin ($p < 0.05$; Fig. 5A). A significant increase in hormone equivalents by androgenic type compounds was also observed at site PC following extract incubations with goldfish testicular AR ($p < 0.05$; Fig. 5B), however the magnitude of the increase relative to the reference site was lower (2-

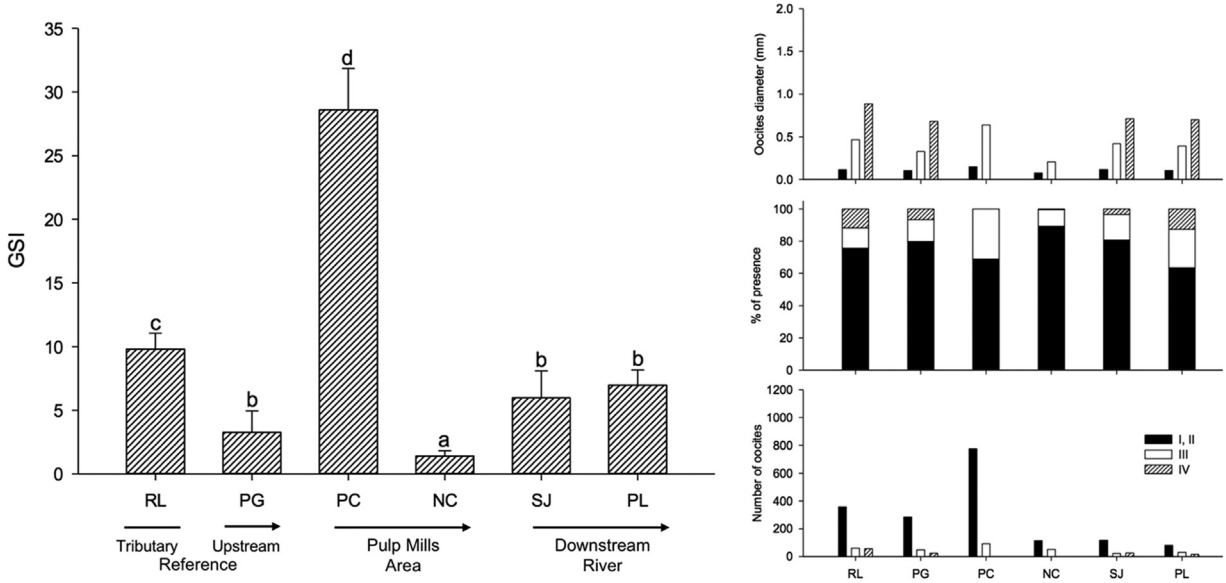

Fig. 2. *GSI and Gonad histology analysis of female P. irwini.* Letters (a-d) indicate statistically different groups (One-way ANOVA, $p < 0.05$), confirmed by a multiple comparison Tukey post hoc test ($p < 0.05$).

fold vs. 5-fold).

3.4. GC-MS analyses of SPMD extracts

Extracts of SPMDs from each site were also profiled by full scan GC-MS and the total ion chromatograms are provided in (Fig. 6). Tentative identities were assigned to compounds accumulated by SPMDs during their deployments by comparisons of their mass spectra (background subtracted) against those of library spectra (NIST 2011). Compounds detected in laboratory blanks were typical of those detected using SPMD passive samplers. The large fronted peak in each of the chromatograms contains residual fatty acids derived from the fish lipid triglyceride triolein remaining in the extracts following SPMD dialysis and GPC. Similarly, other compounds detected are typical of laboratory blanks such as phthalate esters, and siloxated organics typical of GC column bleed. Many of the pulp and paper mill-derived compounds were confirmed using authentic standards that are commercially available and included resin acids, chlorinated phenolics. Most of the compounds detected of pulp and paper mill origin were detected at site PC (Fig. 3), that included sulphur (S_8) and other fatty acids associated with mill effluents including tetradecanoic acid and pentadecanoic acid. There was also evidence of non-mill anthropogenically-derived alkylated polycyclic aromatic hydrocarbons (PAHs) at site PC, whose origin has been established mainly from pyrogenic sources (Barra et al., 2009).

4. Discussion

This study shows documented evidence of reproductive effects in two wild fish species exposed to pulp and paper mill effluents in the Biobio River (Central Chile) and the ability of SPMDs to accumulate endocrine disrupting substances at these sites. Despite in vitro evaluation of SPMD extract indicated the occurrence of estrogenic compounds associated with a spatial gradient, similar to the pattern found in

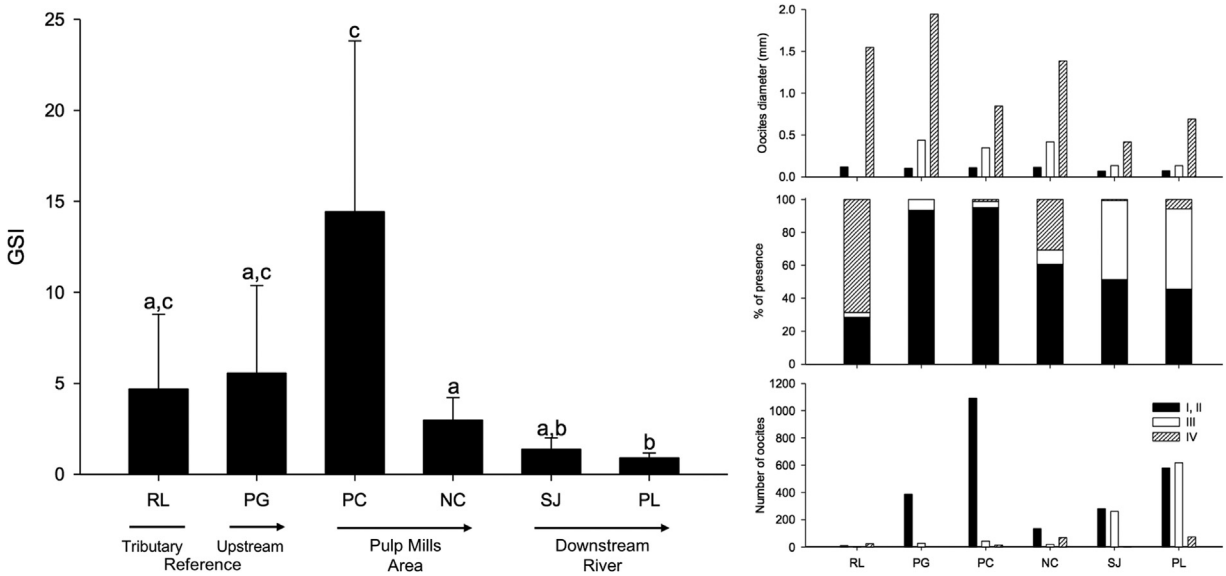

Fig. 3. GSI and Gonad histology analysis of female *T. areolatus*. Letters (a-c) indicate statistically different groups (One-way ANOVA, $p < 0.05$), confirmed by a multiple comparison Tukey post hoc test ($p < 0.05$).

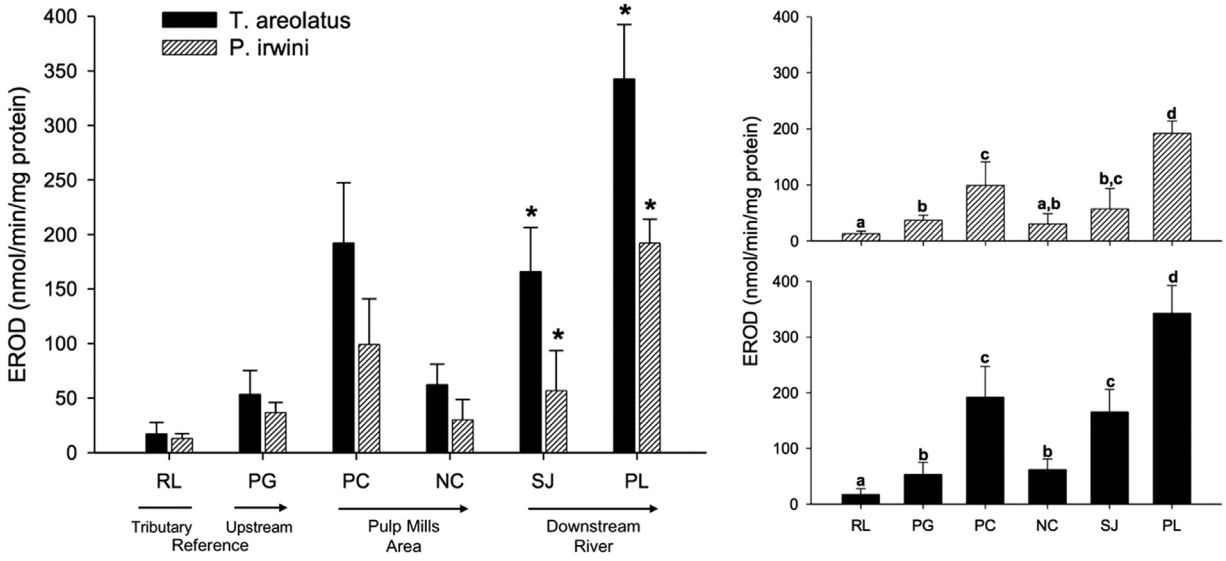

Fig. 4. Hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) enzymatic activity of *P. irwini* and *T. areolatus*. Letters (a-d) indicates statistical significant differences between groups (One-way ANOVA, $p < 0.05$) and * indicates statistical significant differences between species (Two-ways ANOVA, $p < 0.05$), both analysis of variance confirmed by a multiple comparison Tukey post hoc test ($p < 0.05$).

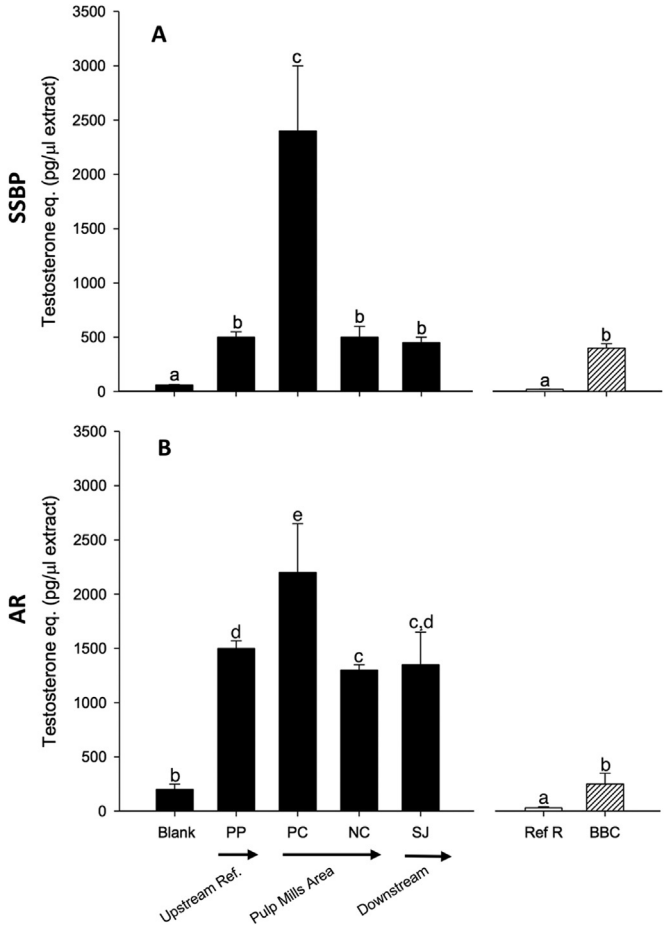

Fig. 5. Testosterone equivalents associated with binding of SPMD extracts to goldfish sex steroid receptors (black bars). A: Plasma Sex Steroid Binding Protein (SSBP) and B: Testicular Androgen Receptors (AR). Letters (a-e) indicate statistically different groups (One-way ANOVA, $p < 0.05$), confirmed by a multiple comparison Tukey post hoc test ($p < 0.05$). Activities for SPMDs from the Biobio River are compared with those from a 7d deployment during May 2003 in Blackbird Creek, ON Canada (BBC; 50% bleached kraft effluent) and a reference river (Ref R; Little Gravel River, ON Canada).

molecular (EROD) and individual (GSI) markers measured in fish, no direct cause-effect relationship was clearly established. Significant differences between both fish species were related to their habitat (benthic vs demersal). Furthermore, chemical profiling of SPMD extracts revealed the presence of compounds typical of mill effluents but also revealed some PAH derivatives not associated with anthropogenic activities. The pattern of PAHs observed in sediments associated with the pulp and paper mill industrial activities in the middle area of the Biobio river was established as pyrogenic origin probably due to biomass combustion (Barra et al., 2009).

The increase in GSI observed coincides with the increase detected in previous studies on caged fish downstream pulp mill discharges (Orrego et al., 2006). Although gonad sizes significantly increased in site PC (after the first pulp mill discharge) in both species, the highest percentage of oocytes were mostly immature (state I and II). Compared to previous research in reproductive endpoints of these two wild fish species (Chiang et al., 2011b), this observed effect could be related to an anticipated spawning (normally occurring during late spring - early summer season) forced by the presence of hormonally active substances detected in the area.

Results of EROD activity in both species shows statistical differences between sites relative to those in fish collected in both reference sites (main tributary RL and upstream site PG). A clear induction is observed in fish collected after the first pulp mill discharge, similar to previous studies on caged fish and sediment exposure trials (Orrego et al., 2005a, 2005b, 2006). However, the EROD activity drops to reference values below the second discharge and finally increases in sites downstream towards the river mouth, being highest near Concepción city (the most populated urban site in the basin). This high EROD induction observed near the mouth of the river has been previously described by our group (Fossi et al., 1995; Barra et al., 2001; Orrego et al., 2005b; Inzunza et al., 2006) and it was explained by a high content of PAHs (associated mainly with sediments) that have shown a petrogenic pattern (Barra et al., 2001, 2009). Comparing both species, *T. areolatus* always showed the higher activities, probably due to their benthic behavior. Furthermore, it has also been demonstrated that EROD activity of females gradually decline towards the onset of ovulation and then rise again during the postspawning period being associated to peaks in sex steroid production (Chiang et al., 2012b)., though there is the possibility that steroid-like compounds could be inducing CYP450 protein levels (as EROD activity) downstream effluent discharges as well as the presence

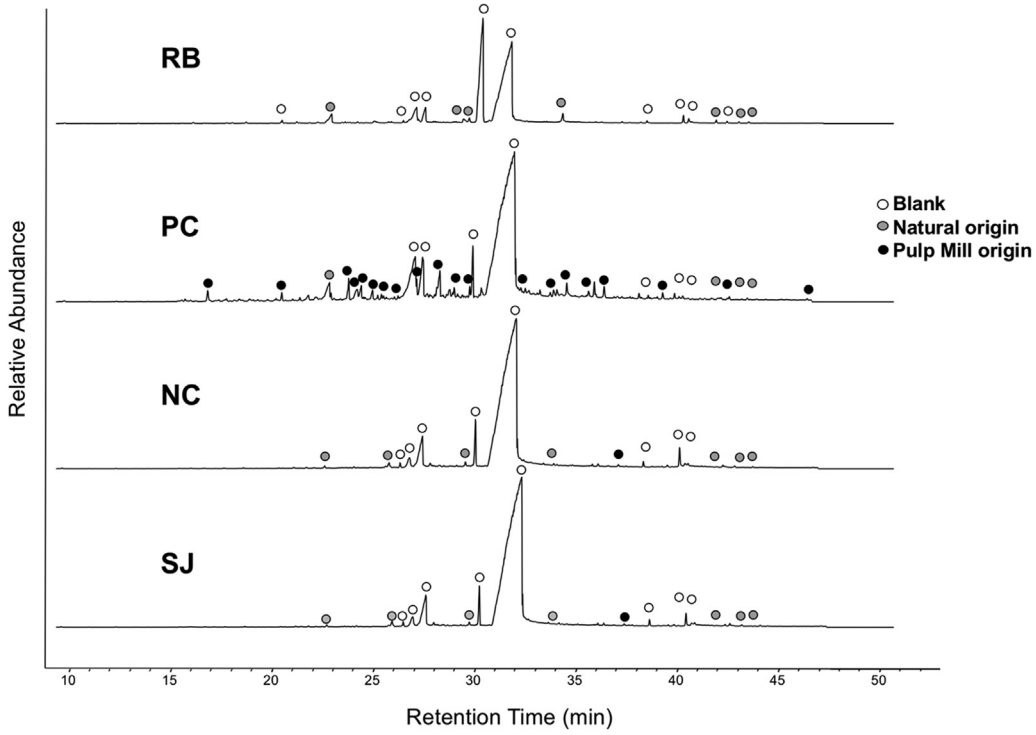

Fig. 6. Full scan GC-MS profiles of SPMD extracts. Constituents within SPMD extracts are indicated from their mass spectra and retention times to be derived from laboratory blanks, naturally occurring in a river system or from pulp and paper mill effluents according with the NIST 2011 Mass Spectral Library.

of other xenobiotics.

SPMDs deployed in the Biobio River downstream of the pulp and paper mill at PC (Fig. 1) for 28 d accumulated compounds functioning as ligands for goldfish plasma SSBP and testicular AR. The magnitude of the difference in hormonal activities (PC vs PP-NC-SJ, Fig. 5) measured by AR (androgens affinity) compared to SSBP (androgens and estrogens affinity) indicates estrogens are the main contributor to the total hormone activities measured by SSBP, which binds both hormone types equally. For comparison purposes, hormone equivalents derived from the same receptor assays incubated with extracts of SPMDs deployed for 7d in Blackbird Creek and a reference site (Little Gravel River) ON Canada are also shown (insets, Fig. 5A and B). Blackbird Creek is the recipient of bleached kraft mill effluent from a mill located in Terrace Bay ON and at the time of the deployments, the effluent concentration was approximately 50% (v/v). The relative hormonal accumulations between both sites (Ref-R vs BBC) were 13-fold for SSBP and 10-fold for the AR, indicating most of the activity in this mill effluent is derived from androgens.

It is not clear why the SPMDs deployed below the mill at PC were the only ones that exhibited accumulations of hormonally active substances able to react with AR and SSBP receptors. Possible reasons for this include deployments in other sites were not located in the effluent plume, or the mill effluent from site PC is the only effluent active substances. The mill located above site PC is an elemental chlorine free (ECF) bleached kraft mill constructed during the early 1990s, utilizes *Pinus radiata* softwood as a feedstock and has a conventional biotreatment system with an aerated lagoon, while the other mills in the River have activated sludge secondary treatment systems (Table 1). While PC is a relatively modern facility, other emerging evidence suggests that the same two species of wild fish captured below a newly constructed ECF kraft mill in the Itata River of the Biobio system also exhibited different aspects of reproductive dysfunction (Chiang et al., 2011a, 2011b). It was recently demonstrated in an international survey comparison project that the androgenicity of pulp mill effluents, evaluated as AR were detected in almost all ECF-Kraft mills, and it was related with the nonpolar fraction (extracted by Hexane) and generally greatest in softwood processing industries (Milestone et al., 2012). Sampling at the SJ site below the other softwood mill, Mill D (Table 1) was conducted farther downstream from the discharge point, so dilution may be a factor and explain the intermediate SPMD extract estrogenic activity and lesser number of mill-derived compounds detected in the extracts.

Estradiol and testosterone bind with equal affinity to SSBP, so we can conclude that estrogens present in the Biobio River below site PC are responsible for the increased activity associated with the SPMD extract at that site, consistent with previous studies showing estrogenic responses in fish caged in the Biobio River and fish exposed to sediment collected from the Biobio River (Orrego et al., 2005a, 2005b, 2006). While it is not presently known which compounds may be functioning as androgens in Canadian effluents, it suggests that Chilean pulp mill effluents are exerting effects on fish reproduction through different mechanisms than effluents from some Canadian mills. Current evidence in Canada indicates that components of wood furnish released during digestion and bleaching are the key agents causing reproductive effects in fish and are discharged as part of organic loadings measured as BOD. If wood components are also eliciting effects on fish reproduction in Chile, it then follows that different compounds, originating from different wood species (e.g. Eucalyptus and especially *Pinus radiata*) could affect reproduction via different mechanisms.

Caged fish studies (Orrego et al., 2006) and laboratory studies with sediment collected below mill outfalls (Orrego et al., 2005a, 2005b), indicates the presence of estrogenic chemicals in areas impacted by mill effluents in Biobio system. This persistent estrogenic effect was then corroborated by intraperitoneal injecting triploid rainbow trout with Chilean primary and secondary treated pulp and paper mill effluents SPE extracted (Orrego et al., 2009), and could be associated to the presence of estrogens that act as estrogen receptor agonists, or androgens that increase endogenous estrogens by inducing aromatase expression/activity (Orrego et al., 2010a, 2010b). Furthermore, recent research indicates that in spite of the potential androgenic effects of pulp mill effluents demonstrated by in vitro analysis (e.g. binding assays with goldfish testicular AR), may cause in vivo metabolic activation that can lead to a final estrogenic effect demonstrated by increased

level of plasma VTG (Orrego et al., 2017), gonad alterations and intersex in juvenile male trout (Chiang et al., 2015).

Further work is necessary to evaluate endocrine disruption in the Biobio basin, in order to distinguish pulp mill industry-derived bioactive compounds from those derived from municipal sewage effluents and to determine the extent and magnitude of the effects observed especially in wild fish communities. Recently, industry in the Biobío has modernized the treatment systems to MBBR (Moving Bed Biofilm Reactor. Odegaard, 2006), therefore an assessment on the impacts of these changes on the receiving environment in a long term spatial-temporal monitoring program such as Canadian EEM program, is warranted. Our single temporal investigation was able to detect changes in wild fish that were associated with hormonal active compounds detected by SPMDs, therefore validates this EEM-approach for use in Chile.

Acknowledgments

This work was possible through the financial support of Environment Canada, the Canada Research Chair Program and the Research Directorate of the University of Concepcion. R Barra thanks the support of the CONICYT/FONDECYT 1180063 and CONICYT/FONDAP/CRHIAM 15130005.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.12.092.

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