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Lack of Estrogenic and Endocrine Disrupting Effects in Juvenile Rainbow Trout Exposed to a New Zealand Pulp and Paper Mill Effluent

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Abstract – Previous studies have noted effects of pulp and paper effluents on the physiology of fishes including; smaller gonads, increased age to maturation, alterations in secondary sex characteristics, reduced plasma sex steroid levels, and the induction of vitellogenin (Vtg) in males and juveniles. A program to determine the potential impacts of a modern New Zealand pulp and paper mill effluent on fishes employed a combination of long- and short-term exposures of the juvenile (1+) rainbow trout, *Oncorhynchus mykiss*, to the effluent. Juvenile (1+ aged) rainbow trout were exposed to a mixed thermomechanical pulp/bleached kraft (TMP/BK) mill effluent at a range of concentrations from environmentally relevant (10%) to 70% (effluent by volume) in three exposure studies. During 21-, 56- and 320-day exposures to 10% and 30% (v/v) effluent, no significant impacts on circulating testosterone, and pregnenolone levels were observed. No significant induction of liver MFO activity was observed at any exposure concentration. Vitellogenin induction or expression of the estrogen receptor in juvenile males was not observed in fish from either experiment. High experimental mortality was observed in fish exposed to 70% (v/v) secondary treated effluent compared to a reference treatment during the 21-d exposure and was linked to an atypically high suspended solids load. Thus, the combined data from these experiments demonstrated a lack of estrogenicity or impacts on steroidogenesis following exposure to TMP/BK mill effluent. However, a parallel experiment using sexually mature rainbow trout did show elevated Vtg in males. Thus, the presence of estrogenic compounds cannot be ruled out, but appears to be infrequent. Results from parallel studies with mosquitofish have shown androgenic activity in the effluent under investigation.

Keywords - fish, estrogen, endocrine, reproduction, bleached kraft, thermomechanical

INTRODUCTION

The impacts of pulp and paper mill effluents on aquatic organisms has been extensively investigated since the 1960's and linked to a variety of toxic effects including reproductive impairment [1]. Reproductive effects have included: decreased egg and gonad size, delayed maturity [2,3,4], changes to secondary sex characteristics [5,6], and decreases in serum sex steroid levels [7]. The synthesis of plasma vitellogenin (Vtg) and

Vtg gene expression in the liver of male fish has been reported following bleached kraft mill effluent (BKME) exposure [8,9].

In recent years the international pulp and paper industry has committed considerable resources to environmental improvement through process modifications and the installation or upgrade of secondary treatment facilities [10]. However, despite some evidence of recovery [11], significant impacts on reproductive parameters continue to be recorded at sites where process and treatment modifications have been implemented [12,13]. Results have been conflicting, in that physiological changes have been observed at mills with and without elemental chlorine free (ECF) bleaching, secondary effluent treatment, and other treatment/technology upgrades in place. It has been suggested that reproductive endocrine impacts are not due solely to the bleaching process or to chemical additives of pulping, but could also be caused by naturally occurring plant compounds or metabolites thereof.

The aim of these studies was to assess potential reproductive endocrine effects in juvenile rainbow trout following exposure to a secondary treated pulp and paper mill effluent. This was accomplished by employing a combination of long- and short-term experiments with juvenile trout of varying developmental stages to a range of secondary treated effluent concentrations, from 10% to 70% (v/v).

METHODS AND MATERIALS

Mill description

The Tasman Mill is located adjacent to the Tarawera River in the Bay of Plenty region of the North Island, New Zealand. The mill is an integrated thermomechanical pulp/bleached kraft (TMP/BK) pulp and paper mill (i.e., uses both kraft and thermomechanical pulping processes producing 760 and 1010 air dried metric ton/d (ADMT), respectively). Mill furnish is primarily softwood (*Pinus radiata*) with occasional eucalypt production and has been ECF since April 1998. The TMP effluent is pretreated in-mill using an aerobic, moving-bed bioreactor system. This pretreated effluent and the effluent from the remainder of the mill operations are collected into a single drain and treated in an aerated-lagoon system with a 5- to 6-d retention time before release to the Tarawera River. The concentration of this effluent in the Tarawera River ranges between 5% and 12% (v/v). Detailed effluent chemistry has been reported elsewhere [13].

Experiment one: 320-d exposure

The first experiment was a 320-d laboratory exposure conducted at Forest Research, Rotorua, New Zealand. Rainbow trout eggs were stripped and pooled from three ripe females and fertilized on-site with sperm pooled from two males obtained from the Department of Conservation National Trout Hatchery trap situated on the Tongariro River. Eggs were left undisturbed for 15 min while water hardening occurred, then transported on ice back to the laboratory and immediately allocated to treatments and replicates. The experimental setup consisted of five PVC pots (15 cm diameter) with a total volume of 2 L. Eggs were housed in fine mesh baskets (5 cm deep), held at the top of each pot. Approximately 1500 eggs were divided among five replicates per treatment (300 eggs per pot). Final treatment effluent was continuously pumped using a peristaltic

pump into the effluent exposure mixing tank at a concentration of 15% (v/v). Temperature was maintained between 11.8 and 14°C during the exposure. Effluent was obtained from the Tasman Mill immediately before discharge into the Tarawera River and transported to the laboratory on a weekly basis. Dechlorinated city water was used as reference and diluent. When trout had reached swim-up stage they were transferred into larger hatchery rearing troughs with divided baskets (70 cm x 45 cm x 20 ccm). Baskets were divided into two sections with mesh screens and were suspended in the rearing troughs (3 m x 0.5 m x 0.25 m) that had a drain of 75 mm diameter at one end to allow continuation of flow-through exposure. Flow rate was maintained at 1 L/min for all experiments and the maximum fish loading rate was 0.66 g/L/d.

Following 320 d of exposure, fish were sacrificed with a blow to the head and measurements of length, weight and liver size were taken for calculations of somatic indices and condition factor. Blood samples were collected (via caudal puncture) and pooled, then placed on ice in heparinized collection tubes until centrifugation and storage of plasma at -80°C. Liver samples were removed and frozen in liquid nitrogen and stored at -80°C prior to Vtg and estrogen receptor analyses. Trout were fed commercial salmon food ad libidum daily until satiation. The size of pellet was adjusted as fish grew. Endpoints included fertilization and hatching success, length at hatch and swim-up, growth and development, induction of plasma Vtg and gene expression, sex ratios, liver somatic index (LSI), and condition factor. Results of fertilization, hatching success, length at hatch and swim-up, growth and development will be presented elsewhere.

Experiments two and three: 21- and 56-d experiments

Both experiments two and three were conducted at an on-site exposure/mesocosm facility at the Tasman Mill, Kawerau, New Zealand. Juvenile rainbow trout were obtained from the Eastern Fish and Game Ngongotaha Hatchery, Rotorua, New Zealand. Trout were age 1+ averaging 25 to 30 cm and 200 to 300 g. Trout were held under ambient light conditions at the on-site exposure facility and the exposure tanks were aerated continuously. Fish were fed commercial salmon food at a daily ration maintained at 1% of measured wet body weight. Fish exposure tanks were 7,500-L epoxy coated fiberglass tanks divided into three 2,500-L sections. The facility was roofed and fenced to provide shade during the summer months. Reference water was pumped directly from the Tarawera River upstream of any points of discharge. Secondary treated effluent was transported via road tanker on a weekly basis from a sample point immediately prior to discharge into the Tarawera River. Effluent was held in an 85,000-L concrete reservoir and continually recirculated using submersible pumps in order to prevent solids settling and effluent becoming anaerobic. Reference water and 100% effluent was gravity fed to mixing tanks where effluent dilution was controlled using valves. Flows were measured and adjusted on a daily basis.

Experiment two was undertaken in April 1999 and was a 56-d experiment whereby fish were exposed to either reference water or 10% secondary treated effluent for a period of 28 d. During this period, 20 fish per treatment were sampled at days 7, 14 and 28. Following day 28, effluent exposure ceased and was replaced with reference water. During the depuration period, 20 fish per treatment were again sampled on days 7, 14, and 28 following the cessation of effluent exposure. Each treatment was divided into triplicate tanks, with 40 fish per tank. Flow rates into each 2,500-L replicate were 4 L/min and mean fish weight at the end of the experiment was 299 g giving a loading rate

of 1.0 g/L/d. Experiment three commenced in February 2000, consisting of a 21-d exposure to 10%, 30% or 70% final treated effluent. There were two replicates per treatment containing 20 fish per replicate. Flow rates were maintained at 3 L/min per 2,500-L tank. The mean weight of trout at the end of the experiment was 214 g and thus the maximum fish loading rate was 0.99 g/L/d.

After both experiment two and three, fish were sacrificed by a blow to the head, measured, weighed and gender noted. Gonad, liver and spleen weights were also recorded. Blood samples were collected immediately via caudal puncture and blood was kept on ice in heparinized collection tubes until transported to the laboratory for 15 min centrifugation (500 g) and storage of plasma at -80°C. Liver tissue samples were divided and each half stored at -80°C for MFO and mRNA analyses. Endpoints for both experiment two and three included: circulating sex steroid levels, induction of Vtg at the protein and mRNA level, expression of the estrogen receptor, condition factor, spleen somatic index (SSI), LSI, gonadal somatic index (GSI), and EROD induction. Rainbow trout used as positive controls for Vtg analyses were juveniles injected intraperitoneally with 50 mg/kg 17β-estradiol and sacrificed after three weeks.

Biochemical analyses

The concentration of Vtg protein and the expression of Vtg mRNA were determined using plasma and liver samples in an ELISA or reverse transcription-polymerase chain reaction (RT-PCR), respectively.

Vtg concentration in plasma was determined using an enzyme linked immunosorbent assay (ELISA) provided by Biosense Laboratories, Quantitative Rainbow Trout Vtg ELISA kit (Vtg-102). Vtg induction at the mRNA level was determined by RT-PCR using a Primus 25/96 Thermocycler, according to methods adapted from Ren et al. [14], Pakdel et al. [15] and Van den Heuvel [16]. Expression of the estrogen receptor was also determined at the mRNA level using RT-PCR techniques described above.

Circulating sex steroid hormones were measured according to McMaster et al. [17]. Plasma samples were thawed and steroid hormones were extracted with diethyl ether. The plasma extract from males and females was analyzed for testosterone and pregnenolone using standard radioimmunoassay (RIA) procedures. MFO enzyme activity was estimated as EROD activity using a modification of the fluorescence plate-reader technique outlined by van den Heuvel et al. [18].

Statistical analyses

Condition factor, liver size, and body weight data were analyzed using analysis of covariance (ANCOVA). Data were log-transformed prior to using ANCOVA. It should be noted that although statistical comparisons using ANCOVA were completed on body and liver weight, data are presented as somatic indices for ease of comparison. Fulton's condition factor was calculated as body weight ÷ length^3 x 100, LSI as liver weight ÷ body weight x 100. Steroid, Vtg, EROD, and length data, were compared using ANOVA, with a Bartlett test for homogeneity of group variances, and a Tukey post-hoc test. Analysis of covariance and ANOVA statistical testing were completed using the SYSTAT® and GraphPad Prism 3® software pages. The critical level of statistical differences for all analyses in this paper was assessed at $\alpha = 0.05$.

RESULTS

Experiment one

Exposure of juvenile rainbow trout to 15% (v/v) treated effluent from fertilized egg stage to 10-months old had no effect on induction of Vtg (Fig. 1). No induction of Vtg mRNA or expression of the estrogen receptor (data not shown) was observed. Circulating pregnenolone and testosterone concentrations (not shown) were unchanged by exposure. There was no difference in the sex ratio of the juvenile trout with 62% and 64% males observed in the effluent and reference treatments, respectively.

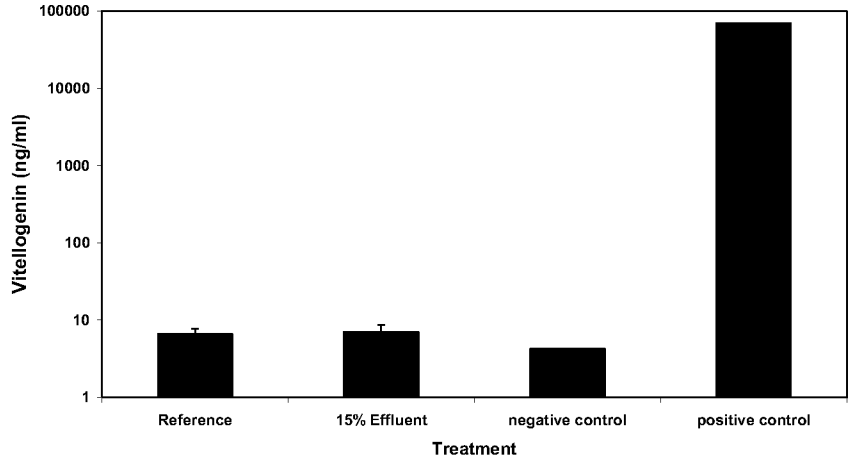

Fig. 1. Mean (± 95% CI) concentrations of plasma vitellogenin in juvenile rainbow trout exposed to reference water and 15% (v/v) secondary treated effluent. n= 3 for the negative and positive controls. Negative control represents hatchery reared adult male rainbow trout and the positive control represents estradiol injected trout. Reference and 15% effluent bars represent the mean value of 50 gender-pooled samples.

Experiment two

Exposure of juvenile rainbow trout to 10% (v/v) effluent had no effect on LSI, GSI, SSI, or condition factor in exposed individuals (not shown). Induction of Vtg was as measured at the protein and mRNA level (Fig. 2). Estrogen receptor expression (data not presented), and circulating pregnenolone and testosterone (Fig. 3) concentrations were unaffected by effluent exposure

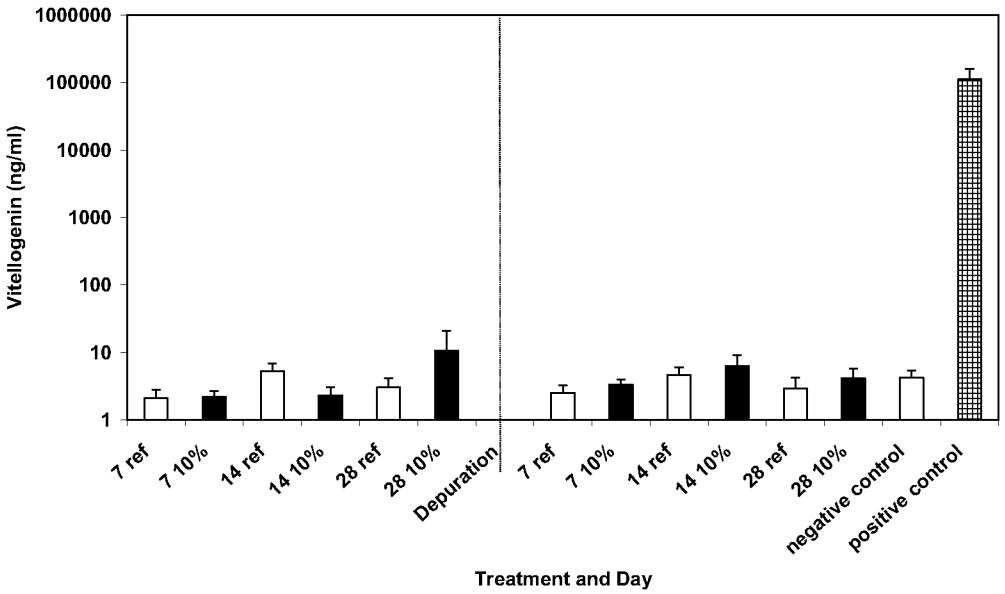

Fig. 2. Mean (± 95% CI) plasma vitellogenin concentrations in juvenile male rainbow trout exposed to reference water and 10% effluent in reference water for 28 d. Samples were taken on days 7, 14, and 28 and then following depuration sampled on day 7, 14, and 28.

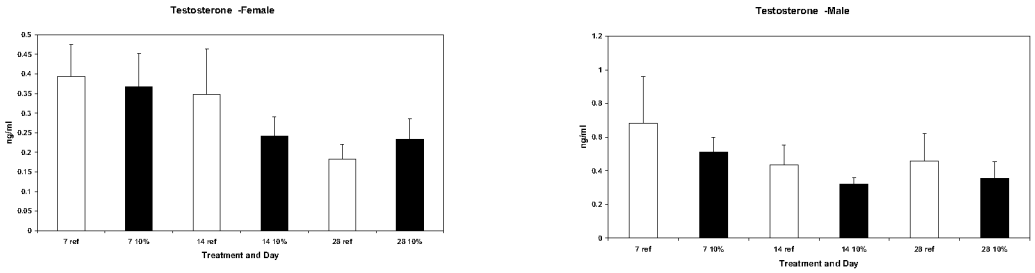

Fig. 3. Mean (± 95% CI) concentrations of plasma testosterone in juvenile males and females exposed to Tarawera River (reference) river water and 10% effluent in river water for 28 d.

Experiment three

Exposure to 10%, 30%, and 70% (v/v) effluent had no effect on GSI, LSI, SSI and condition factor. No significant induction of liver MFO enzyme activity (measured as EROD) (Fig. 4) was observed in exposed individuals, nor could induction of Vtg, measured at the protein (Western blot & ELISA) and Vtg mRNA (RT-PCR) level (Fig. 5) be measured in effluent exposed individuals. Exposure to effluent did not induce expression of the estrogen receptor (data not presented), nor alter circulating pregnenolone and testosterone concentrations (Fig. 6). High mortality (88% within first

four days of exposure) was observed in the 70% treatment, resulting in the termination of that treatment at day 5.

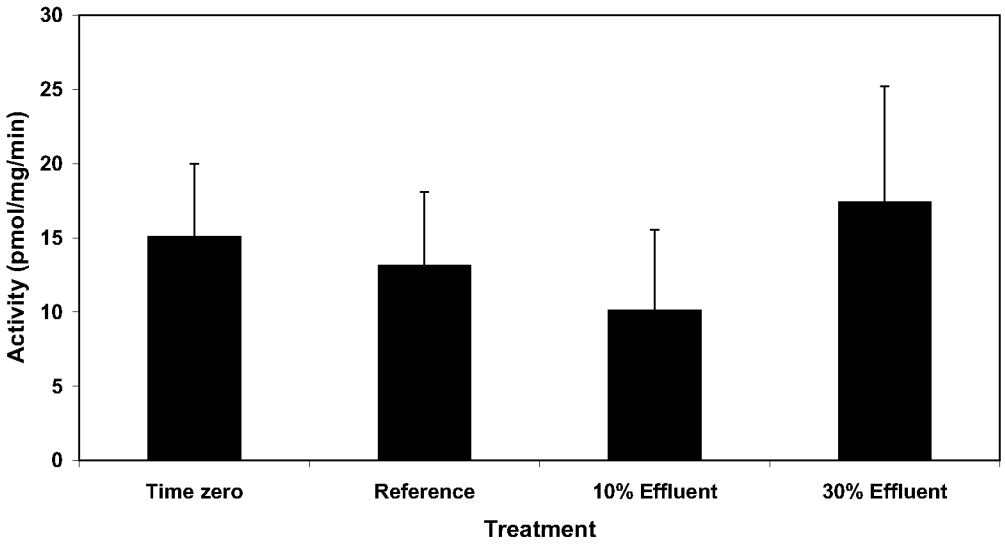

Fig. 4. Mean (± 95% CI) hepatic EROD activity in male and female rainbow trout exposed to reference water and 10 and 30% effluent. Each bar represents mixed sexes.

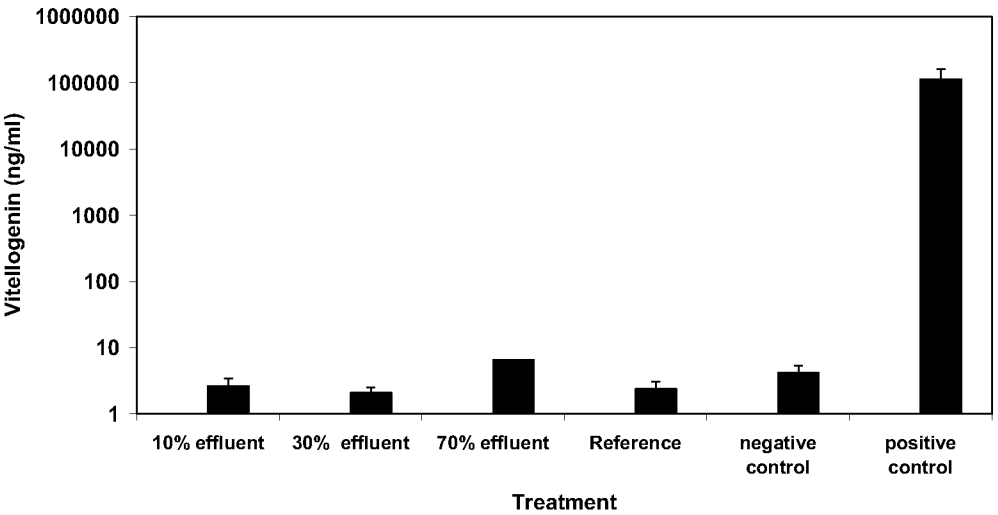

Fig. 5. Mean (± 95% CI) plasma vitellogenin concentrations in juvenile male rainbow trout exposed to reference water and 10, 30 and 70% bleached-kraft-mill effluent.

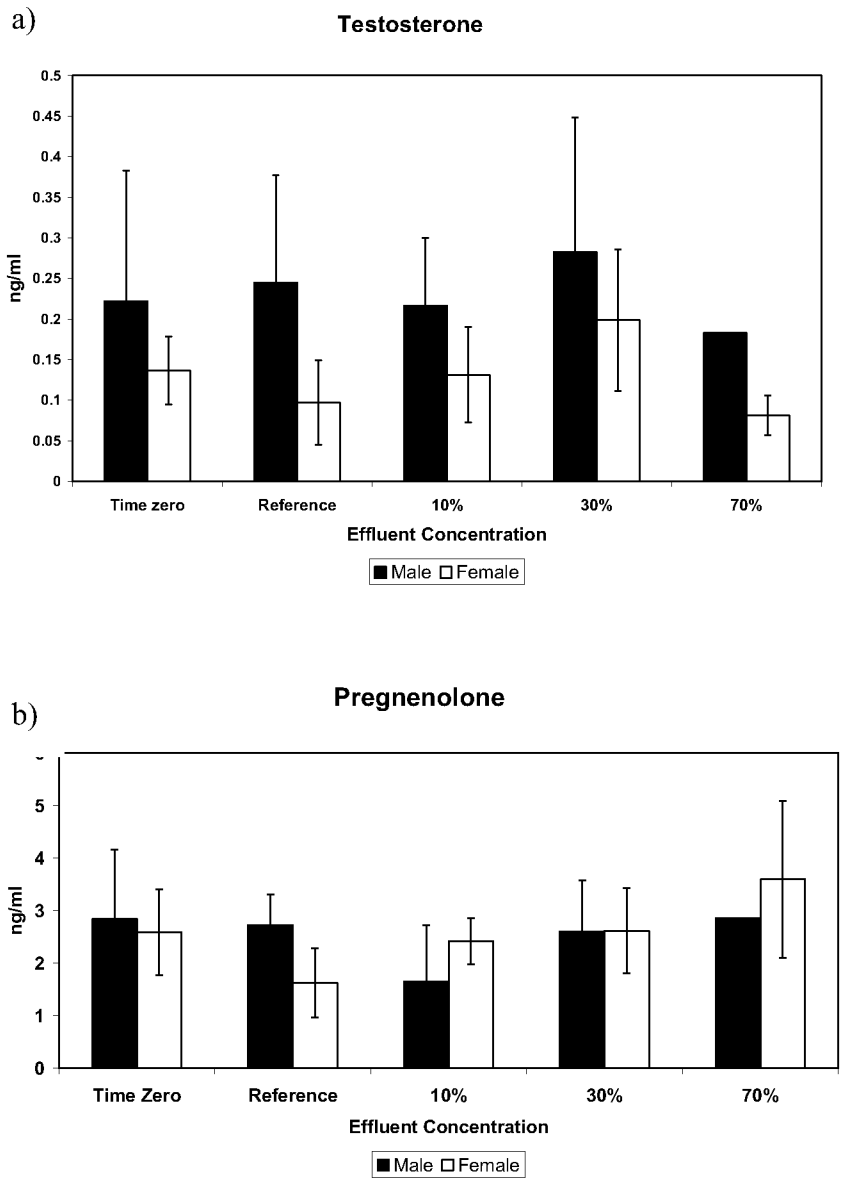

Fig. 6. Mean (± 95% CI) concentrations of plasma steroid sex hormones a) testosterone and b) pregnenolone in juvenile rainbow trout exposed to Tarawera (reference) river water and 10, 30 and 70% effluent in river water for 21 d.

DISCUSSION

The TMP/BK mill effluent in this experiment consistently failed to induce expression of the estrogen receptor in hepatic tissues or Vtg production at gene and protein levels in juvenile rainbow trout. Evidence of estrogenic activity in BKME and black liquor was first noted by Zacharewski et al. [19] using *in vitro* recombinant receptor-reporter gene assays. More recently, caging studies by Soimasuo et al. [20] and Mellanen et al. [21] observed Vtg gene expression in whitefish exposed to a Finnish BKME. Laboratory

exposures of juvenile rainbow trout demonstrated that a Canadian BKME was also capable of inducing the synthesis of plasma Vtg [9]. In the three mills studied by Mellanen et al. only one [21] was capable of inducing Vtg production. Effluent from the Tasman mill has been observed to induce Vtg in mature male trout in a longer-term experiment with blood samples taken shortly after the completion of experiment three presented here. This discrepancy in results would suggest that this mill might only periodically use or produce estrogenic compounds. Surfactants containing the precursors of weakly estrogenic compounds used either for cleaning or as process additives were measured in effluents and sludge in highly variable quantities [22]. However, natural plant compounds such as the known estrogen genistein has also been observed to be present in pulp mill effluents [23]. Phytosterols are consistently present in the effluent used for this experiment [24], but their ability to act in an estrogenic fashion is doubtful.

Recently, a pulp and paper mill effluent has been strongly implicated in increasing the male:female sex ratio in fish exposed during the early developmental periods in the field [12]. This appears to be an androgen dependent phenomenon. In experiment one, no such skewed sex ratio could be observed in the trout exposed from fertilization to 320 d. This is despite the fact that this effluent has been documented to cause androgen-like effects [5]. Sex ratio was skewed to male fish in both treatments. As fish were sexed visually, and not histologically, it is potentially possible that some females were identified as males in both treatments.

Depressions in circulating sex steroid hormones have been found in fish exposed to pulp and paper mills that implement a variety of treatment and process/production procedures (i.e., with/without chemical bleaching and with/without secondary treatment [2-4,13]. Laboratory exposures, similar to those performed here also documented sex steroid hormone reductions in juvenile rainbow trout [9]. The present studies failed to produce any evidence supporting decreases in circulating sex steroids following exposure to TMP/BK mill secondary treated effluent. Experiments run concurrently with the present studies, exposing adult female rainbow trout to identical secondary treated effluent, demonstrated subtle effects in fish exposed from eight months prior to spawning including several months during the pre-vitellogenic stage. The observed reproductive effects were paralleled by reduced circulating sex steroid levels [13].

MFO activity has frequently been used as an indicator of exposure to BKME. A previous study of the same effluent saw a 2.7-fold induction in EROD activity in male trout [13]. However, more recent data parallels this study in that EROD induction is no longer detectable [24]. The MFO induction potential of this effluent has always been weak, even prior to ECF bleaching and as gradual environmental improvements continue, this response no longer appears to be detectable.

During mesocosm exposures of juvenile rainbow trout to secondary treated effluent, concentrations ranging from 10% to 30% (v/v) failed to impact upon physiological or reproductive parameters including length, weight, condition factor, liver, and spleen size. However, exposure to 70% effluent resulted in high mortality, as well as significantly larger spleen and livers compared to the other treatments. High mortality observed in the 70% effluent concentration was unexpected as toxicity tests with this effluent have continually demonstrated that 100% treated effluent was not acutely toxic to fish. It is possible that toxicity was due to atypically high suspended solids loading during the experimental period (55 mg/L *cf* annual mean of 38.3 ± 4.4mg/L) which may have resulted in increased levels of ammonia or resin acids.

From these experiments, it can be concluded that the potential for this effluent to cause effects in rainbow trout through an estrogenic mechanism is low. Inconsistencies between these, and other experiments conducted with this effluent, likely reflect the high temporal variability in effluent composition, and that the effluent is of sufficient quality that biologically active compound are below the threshold of effects in rainbow trout.

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