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Chronic Effects of Kraft Mill Effluents and Endocrine Active Chemicals on *Daphnia magna*

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The environmental impact of bleached kraft mill (BKM) effluents is due to suspended solids, organic matter, color and toxicity (Pokhel and Viraragvan 2004). The main source of the effluents toxicity was the AOX (Adsorbable Organic Halogen) compounds coming from chlorine bleaching effluents (Vidal 1999). Presently, alternative bleaching processes are being introduced, such as elemental chlorine-free (ECF) and total chlorine free (TCF) bleaching (Thompson et al. 2001). As a result, acute toxicity is not detected in treated effluents (Diez et al. 2002), although chronic toxicity is still observed (Bailey and Young 1997). Endocrine active compounds (from extractive constituents) that are released during the pulping process produce part of the chronic toxicity. Although, part of these compounds is burned or recovered in a separate process, some sterols, triglycerides, resinic acids, etc. are dissolved in the effluent. In Chile, over half of the current total cellulose-production capacity (2 million tons/year) is located within the Biobio river Basin (8^{th} Region), which also provides drinking water to more than 2 million inhabitants (González et al. 1999). According to the estimation methodology used by Strömberg et al. (1996), Cook et al. (1997) and Mellanen et al. (1999), BKM could be discharging about 6 tons of sterols/year into the Biobio river. Moreover, studies on this river indicate reproductive and physiological effects on rainbow trout exposed to sediment from BKM discharges (Orrego et al. 2004). Similar effects had been observed on fishes exposed to BKM effluents in Canada and USA (Mattsson et al. 2001 and Larsson et al. 2002, respectively).

Daphnids (like *Daphnia* spp. and *Ceriodaphnia* spp.) are utilized in acute and chronic tests to assess water quality. Indeed, BKM-untreated effluents display acute toxicity with *D. magna* (LC_{50} 48h, 8 %) (Diez et al. 2002). However, acute toxicity can be eliminated after aerobic treatment (LC_{50} 48h, > 100 %) (Priha 1996, Diez et al 2002). Unfortunately, chronic toxicity based on daphnid fecundity is still present even after biological treatment of BKM effluents (Martel et al. 2004).

To study the hormonal effect on daphnids, Olmstead and LeBlanc (2000) have shown that endocrine active chemicals, such as diethylstilbestrol and androestenedione, produce a significant increase in the abdominal processes relative to body size in *D. magna* females.

The goal of the present study is to detect endocrine effects on *Daphnia magna* growth exposed to BKM effluents and endocrine active chemicals.

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MATERIALS AND METHODS

ECF-effluent samples were obtained from a local BKM, where effluents are primary treated in a settler tank to reduce fiber and total solids. Samples were transported on ice in insulated coolers to the laboratory and stored in the dark at 4 ± 1 °C.

Female *Daphnia magna* were obtained from in-house cultures. They were fed three times weekly with a suspension of baker's yeast, trout chow and alfalfa with an equivalent carbon content of 7.2 mg C/L on Monday and Wednesday, and 10.8 mg C/L on Friday. A culture medium was changed before feeding, and neonates were removed from a culture within 24 h (USEPA 1993). The culture medium was maintained at 20 °C with a 16 h light: 8 h dark photoperiod. The maximum culture water hardness was 250 mg $CaCO_3$/L and the pH ranged between 7.5 and 8.6 (NCh2083 1999).

Prior to starting the chronic toxicity assay, the acute toxicity due to BKM effluent on female *D. magna* (<24 h old) was evaluated at 24 - 48 h. Mortality was recorded at the end of exposure, where mortality was defined as a lack of organism mobility when the vessel was shaken. Five concentrations of BKM effluents (6.25, 12.5, 25, 50, 100%) and one control were evaluated. Four replicates of 30 mL (each one containing five organisms) were performed for each concentration and the control. The culture was not renewed during the test. Oxygen concentration, pH and conductivity were measured at the beginning and end of each test. The 24 and 48 h mean lethal concentrations were calculated using the Probit and the Spearman-Karber methods, as appropriate (Cooman et al. 2003).

The endocrine effect of the BKM effluent was assessed for female *D. magna* (<24 h old) throughout the 21-day exposure period (USEPA 1993, Olmstead and LeBlanc 2002). A control and three effluent concentrations (5%, 10% and 20%) were employed. Control water was reconstituted from moderately hard water prepared according to USEPA (1993). For each concentration and one control, 10 replicates of 50 mL (each one containing one organism) were performed. The assay medium was renewed every 2 d, and the exposure time was 21 d.

On day 7, 14 and 21, the daphnids were examined using a light microscope fitted with a photographic camera to assess their development. Daphnids were placed on a glass microscope slide, immobilized by removing the medium from the slide, and anatomical development was recorded in photographs. The dimensions of abdominal cavities (measured as the largest length of the lateral immobilized organism) were measured under 2x magnification. Total body length, defined as the distance from the top of a head capsule to the base of the shell spine, was also measured at this time. The daphnids were photographed just once and then immediately returned to the assay vessel. Each *Daphnia* was measured only once during the assay period. Consequently, there were three assays running together, and the *Daphnia* of one running assay were photographed on day 7. Then on day 14, a different assay was photographed, and on day 21, the organisms in the remaining assay were photographed.

Diethylstilbestrol (97%, Aldrich, CAS 56-53-1) and androstenedione (4- androstene-3,17-dione, 98%, Aldrich, CAS 63-05-8) were used as a positive control in the assays. Three different diethylstilbestrol (0.75, 1.5 and 3.0 μM) and androstenedione (6.25, 12.5 and 25μM) concentrations were employed. Endocrine active chemicals concentrations were determined according to Olmstead and Leblanc (2000). A control and 10 replicates (each vessel contains one organism) of 50 mL were performed for each assay concentration. Controls were supplemented with ethanol (0.02 %). There

were 10 replicates (each vessel contains one organism) of 50 mL as well. The assay medium was renewed every 3 d during a total of 9 days. The daphnids were photographed just once (on day 3, 6 and 9) using the method described above; after that procedure, the assay was thrown away.

To study the body length and abdomen cavity without the solvent effect, a lineal regression curve, body length vs abdomen cavity, was fitted for the diethylstilbestrol and androstenedione control assays following Olmstead and Leblanc (2000). On days 3, 6 and 9 of chemical exposure, the predicted sexual characteristics length (SCL_p) for each daphnid was calculated using the daphnid's measured body length and the pre-established linear equation. A reference sexual characteristic length (SCL_R) for each day was then determined. The SCL_R consisted of the mean SCLp for all daphnids analyzed on that day. This value represented the expected sexual characteristic length for the group of daphnids based upon the mean total length of the daphnids. The normalized sexual characteristic length (SCL_N) of each daphnid was then calculated by using the following equation where SCL_M represents the actual measured sexual characteristic length (Olmstead and Leblanc 2000):

$$SCL_N = SCL_M - SCLp + SCL_R$$

Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD_5) were measured according to Standard Methods (APHA 1985). Phytosterol concentration was determined by CG-MS in a HP 5890 chromatographer with mass selective detector HP5972 (detection limits of 1 μg/L) (Cook et al. 1997). Total phenolic compounds concentration was measured by UV absorbance at 215 nm in a 1-cm quartz cell (UV_{215}). Total phenolic concentration was expressed as mg/L of phenol. Color was measured by VIS absorbance at 440 nm in a 1-cm glass cell (VIS_{440}). The pH of both measurements was 9.1 (0.2 M KH_2PO_4 buffer). Samples for determining COD, BOD_5, VIS_{440}, UV_{215} concentrations were membrane filtered (0.45 μm).

RESULTS AND DISCUSSION

Table 1 presents the physicochemical characteristics of BKM effluents. The COD/BOD_5 ratio (2.9) indicates that high concentrations of recalcitrant compounds are present in BKM effluents. Compounds with high molecular weight (over 1000 Da), such as lignin, do not produce BOD_5; nevertheless COD and a dark color are found.

The phytosterol concentration values (0.17 ± 0.01 mg/L) agree with the values determined for pulp mills where softwood is used as feedstock (0.39 ± 0.67 mg/L) (Strömberg et al. 1996; Cook et al. 1997; Mattsson et al. 2001).

Table 1. Characteristics of the BKM effluent with ECF bleaching sequence.

Parameter	Value
pH	3.4 ± 0.17
COD (mg/L)	881.5 ± 24.3
BOD_5 (mg/L)	300.5 ± 9.5
Total phenolic compounds (mg/L)	271.9 ± 14.2
Phytosterols (mg/L)	0.17 ± 0.01
Color (VIS_{440}) (1x1 cm)	0.41 ± 0.01

For the first 24 h, BKM effluent (LC_{50} 24h, > 100%), does not present acute toxicity to *D. magna*; however for longer exposure times (48 h), BKM effluent displayed acute

toxicity (LC_{50} 48 h = 43.7 %). Consequently, BKM effluent was utilized in the 21-day exposure assays at dilutions 5, 10 and 20 %.

Figure 1 shows the chronic effects of BKM effluent on *D. magna* growth. The body length (Figure 1a) and the abdomen cavity (Figure 1b) were compared to the control. These anatomical traits of *D. magna* were found to increase in similar proportions. The length of the body and the abdomen cavity decreased about 1% relative to the control when exposed to 5% BKM effluent. Whereas, both body length and abdomen cavity increased by 20% and 2% when *D. magna* are exposed to BKM effluent concentrations of 10 and 20 %, respectively.

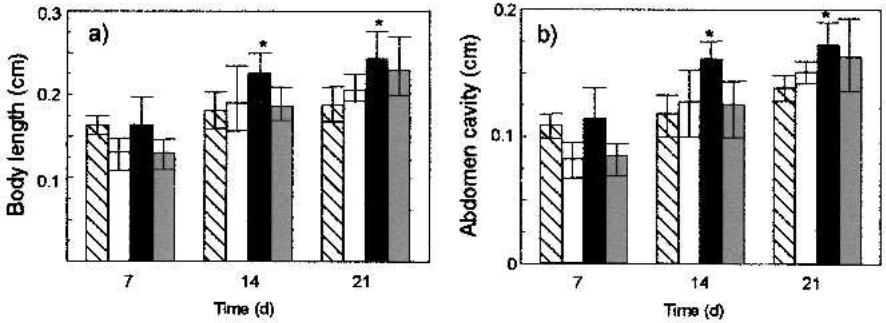

Figure 1. *D. magna* anatomical traits during 21 d of exposure to BKM effluent: a) body length and b) abdomen cavity. Assays: control (▨), 5% (□), 10% (■) and 20 % (▩)of effluent dilutions. An asterisk indicates a significant (<0.05) difference from the control (Dunnett's *t* test).

These results (Figure 1) disagree with those presented by Olmstead and LeBlanc (2000). They found that when female *D. magna* were exposed to endocrine hormonal chemicals like diethylstilbestrol, a stimulatory effect on the abdominal process, and a significant reduction in the body size was observed when with exposure to androstenedione. Still, in both cases, abdominal growth relative to body length presented differences, where growth differences indicated some hormonal response promoted by endocrine-active chemicals.

Table 2 shows *D. magna* body length and abdominal cavity for different hormonal compound and BKM effluent concentrations at day 6. Abdominal-N indicated normalized abdominal cavity length as was explained in the methodology.

As can be seen in Table 2, when *D. magna* is exposed to diethylstilbestrol (estrogenic), the body length and abdominal cavity continuously decreased as the concentration increased from 0.75 to 3.0 μM. On the other hand, androstenedione (androgenic) did not induce anatomical trait changes until 25 μM; at this concentration, body length and abdominal cavity decreased 17 %, approximately. In spite of that, effects of endocrine active chemicals on *D. magna* were not significantly different ($p>0.05$). These results disagree with those presented by Olmstead and LeBlanc (2000).

Table 3 shows comparative results between BKM effluent and endocrine active chemicals to which *D. magna* were exposed, presenting the growth rate between abdominal cavity/body lengths over time. The k value is the slope obtained from plotting the abdominal cavity/body length ratio over time for each studied concentration. Table 3 presents the averages of k and normalized k (k_N) for the

concentration range of each treatment. The k_N values were calculated using abdominal-N characteristics.

Daphnids exposed to BKM effluent experienced slower abdominal change relative to body length than those exposed to estrogenic and androgenic drugs.

Table 2. *D. magna* abdominal cavity and body length in different mediums at day 6.

Compounds/ Concentration		Length (cm)	
		Diethylstilbestrol	Androstenedione
Control[a]	Body	0.1133 ± 0.0124	0.1642 ± 0.0203
	Abdominal	0.0711 ± 0.0116	0.1094 ± 0.0187
	Abdominal-N[c]	0.0711 ± 0.0059	0.1094 ± 0.0045
C1[b]	Body	0.1136 ± 0.0117	0.1988 ± 0.0185
	Abdominal	0.0754 ± 0.0051	0.1244 ± 0.0192
	Abdominal-N	0.0752 ± 0.0061	0.1133 ± 0.0426
C2[b]	Body	0.1022 ± 0.0133	0.1674 ± 0.0093
	Abdominal	0.0663 ± 0.0109	0.1117 ± 0.0066
	Abdominal-N	0.0753 ± 0.0068	0.1087 ± 0.0113
C3[b]	Body	0.1100 ± 0.0020	0.1231 ± 0.0395
	Abdominal	0.0686 ± 0.0040	0.0903 ± 0.0309
	Abdominal-N	0.0712 ± 0.0024	0.0878 ± 0.0300

[a]Control were made with: ethanol (solution 0.02% v/v) for the hormonal tests. [b]C1, C2 and C3 were: (0.75; 1.5; 3.0 μM), (6.25;12.5;25 μM) for diethylstilbestrol, androstenedione, respectively. [c]Abdominal-N was normalized as explained in the methodology.

Otherwise, ANOVA tests showed no difference between k (or k_N) values of *D. magna* exposed to BKM effluent and those exposed to androgenic compounds (LSD, Ducan and Tukey tests). These results agree with the k values calculated from Olmstead and LeBlanc (2000). The k value (0.0071 1/d) for 5% BKM effluent was higher than the control k value (0.0048 1/d), which means that abdominal cavity change compared to body length change in *D. magna* is slower in the control than in 5% BKM effluent. However, this relationship was lower for 10% BKM effluents (k = 0.0014 1/d) and 20% BKM effluents (0.0039 1/d) in comparison with the control.

Table 3. Rate of abdominal cavity / body length (k) and normalized rate (k_N) relative to the time.

Treatment	Concentration	k (1/d)	k_N (1/d)
Diethylstilbestrol (μM)	0.75	0.0114	0.0103
	1.5	0.0055	0.0103
	3.0	-0.0013*	-0.0471
Androstenedione (μM)	6.25	0.0620	0.0125
	12.5	-0.0040	-0.0146
	25	0.0113	0.0003
BKM effluent (%)	5	0.0071	-[#]
	10	0.0014	-
	20	0.0039	-

*Negative values indicate that abdominal cavity/body length relate to the time decrease relative to the control. [#] Values were not normalized relative to the control (water).

In conclusion, diluted BKM effluent had a positive effect on *D. magna* growth. The selection of *D. magna* abdominal traits as the control parameter to determine the effects of endocrine active chemicals produced by BKM effluent was not effective. Nevertheless, the rate of abdominal cavity/body length over time is similar for BKM effluent and androstenedione compounds. To determine the influence of BOD_5 removal on *D. magna* growth, future studies should continue assessing BKM effluent after biological treatment.

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