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Chronic Effects of *Pinus radiata* and *Eucalyptus globulus* Kraft Mill Effluents and Phytosterols on *Daphnia magna*

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Abstract Two kraft pulp mill effluents were compared in terms of their chronic toxicity to *Daphnia magna*. One resulted from pulping *Pinus radiata* and the other came from a parallel processing of *Pinus radiata* and *Eucalyptus globulus* (mixed kraft pulp mill effluent). The concentration of phytosterols found in the mixed kraft pulp mill effluent was higher than in the effluent from *Pinus radiata*, with values of 0.1082 and 0.02 μg/L, respectively. The phytosterols *per se* are responsible for 12.9% and 8.1% of the deviation from the natural shape, while the kraft pulp mill effluents account for 25.6%–27.8% of shape deviation. The role of *β*-sitosterol and stigmasterol is discussed in relation to endocrine disruption.

Keywords Kraft pulp mill effluents · Endocrine disruption · *Daphnia magna*

In spite of innovation in processes and wastewater treatment in the wood pulp industry, effluent discharges from kraft pulp mills have been identified as a potential source of endocrine disruption in aquatic ecosystems (Orrego et al. 2009). Many different approaches using diverse strategic indicators have been considered. Exposure to pulp mill effluents has resulted in general physiological impacts as indicated by fish biomarkers, as well as specific alterations in reproduction of aquatic organisms (Xavier et al. 2005; Hewitt et al. 2006; Orrego et al. 2009). A great variety of organic compounds are still present in kraft pulp mill effluent (KPME), particularly phytosterols and other aromatic molecules with potential endocrine activity (Vidal and Diez 2005, Chamorro et al. 2005, 2010, Belmonte et al. 2006). Growth, reproduction and development of sex characteristics in daphnids are sensitive to chemical compounds with hormonal activity in the environment (Olmstead and Le Blanc 2000; Xavier et al. 2005).

Pine and eucalyptus are the main types of wood processed for pulp in Chile. Therefore, the goal of this study is to compare the endocrine-disruption capacity of KPMEs from different wood supplies on *Daphnia magna* and evaluate the endocrine effects due to exposure to *β*-sitosterol and stigmasterol, two phytosterols present in pine and eucalyptus pulping wastewater.

Materials and Methods

Wastewater samples were obtained from two local kraft pulp mills with an elemental chlorine free (ECF) bleaching system. One processed only *Pinus radiata* while the production of the other one was based on 50% *Pinus radiata* and 50% *Eucalyptus globulus*. The samples were taken after secondary treatment and were transported on ice to the laboratory where they were stored in the dark at 4 ± 1°C. The physico-chemical nature of the effluents is described in Chamorro et al. (2010).

The potential effects of pine KPME, mixed KPME, *β*-sitosterol (Carbiochem, 99%) and stigmasterol (Merck, 95%) on *D. magna* reproductive parameters were evaluated through 21-day exposures using 17α-ethynylestradiol (EE2) (Sigma-Aldrich, 98%) as positive control. They were

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dissolved in the Daphnia culture medium or effluent as shown in Table 1.

Female *D. magna* were obtained from in-house cultures and exposures were started with neonates (<24 h old) following standard procedures for chronic toxicity bioassays (USEPA 1994). Stock cultures and bioassays were maintained at 20 ± 2°C with a photoperiod of 16 h light, 8 h dark. The daphnids were fed the unicellular green algae *Selenastrum capricornutum* supplemented 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. The quality of test organisms was checked using acute toxicity testing with potassium dichromate as reference toxicant. The range of KPME concentration for chronic studies was established after acute toxicity testing (data not shown) to minimize test organism mortality over the 21 d test duration. Effluent samples were filtered through a 0.45-um membrane and their pH was adjusted to 7.0 ± 0.5 before the bioassays. Phytosterol concentration in chronic toxicity bioassays was based on the values found in KPMEs (0.35–3.2 mg L^{-1}) (Verta et al. 1996; van den Heuvel et al. 2002). Ten replicates of 70 mL (each containing one organism) were established for each sample, including the blank and control (see Table 1). The experimental solutions were renewed every 2 d during the test. Dissolved oxygen concentration (OD), pH and conductivity were measured at the beginning and end of each test. Growth, survival and reproduction were evaluated at 7, 14 and 21 d (USEPA 1994).

The potential of the different samples to exert a morphologic change on *D. Magna* was tested using a photograph procedure at 7, 14 and 21 d. The final objective was to determine variation in the proportion of body length to width – at the abdominal cavity – expressed as percentage of allometric growth rate (%AGR) (Olmstead and LeBlanc 2000; Xavier et al. 2005) and calculated as shown in Eq. 1.

$$\%\mathrm{AGR} = \left(\left(\frac{\text{Body Length}}{\text{Body Width}}\right) - 1\right) \times 100\% \quad (1)$$

where %AGR is the ratio of growth in body width to growth in body length, expressed in percentage.

Statistical processing involved first checking data for normality (χ^2 test) and the homogeneity of variances (Bartlett's test). The significance of differences in reproductive parameters and allometric growth was determined by ANOVA followed by Tukey's test, all from the statistical computer package TOXSTAT (USEPA 1994).

Table 1 Characteristics of samples used in this study

Samples	Characteristic
Blank (B)	Culture medium for sample dilution
Control (C)	17α-ethynylestradiol (EE2) (1 mg L^{-1})
P	*P. radiata* KPME, dilution: 75%
M	Mixed KPME, dilution: 25%
Pβ	*P. radiata* KPME, dilution: 75% + β-sitosterol (1 mg L^{-1})
PS	*P. radiata* KPME, dilution: 75% + stigmasterol (1 mg L^{-1})
PβS	*P. radiata* KPME, dilution: 75% + β-sitosterol (0.5 mg L^{-1}) + Stigmasterol (0.5 mg L^{-1})
Mβ	Mixed KPME, dilution: 25% + β-sitosterol (1 mg L^{-1})
MS	Mixed KPME, dilution: 25% + stigmasterol (1 mg L^{-1})
MβS	Mixed KPME, dilution: 25% + β-sitosterol (0.5 mg L^{-1}) + Stigmasterol (0.5 m L^{-1})
β	β-sitosterol (1 mg L^{-1})
S	Stigmasterol (1 mg L^{-1})
βS	β-sitosterol (0.5 m L^{-1}) + stigmasterol (0.5 mg L^{-1})

Mixed KPME: 50% *P. radiata* and 50% *E. globulus*

Results and Discussion

Table 2 shows the physicochemical characteristics of pine and mixed KPMEs. In all cases, pH was maintained within the neutral range (7.1–7.7) which is considered appropriate for the survival and development of most aquatic organisms. The concentration of phytosterols found in the mixed KPME was higher than in the effluent from *P. radiata*, with values of 0.1082 and 0.02 μg/L, respectively. These values are consistent with previous measurement of effluents using these plant species as raw materials (La Fleur 1996; Verta et al. 1996).

Table 2 Physicochemical characteristics of effluents

Parameter (units)	*P. radiata*	Mixed
pH	7.2 ± 0.2	7.7 ± 0.1
COD (mg L^{-1})	213.7 ± 8.3	202.0 ± 9.0
BOD_5 (mg L^{-1})	34.0 ± 11.0	16.0 ± 4.0
Total phenolic (mg L^{-1})	204.2 ± 17.0	164.0 ± 8.0
Color (unit 1×, 1 cm)	0.2 ± 0.3	0.4 ± 0.1
Nitrogen (NH_4^+–N) (mg L^{-1})	0.5 ± 0.3	0.7 ± 0.1
Total nitrogen (TN) (mg L^{-1})	1.7 ± 0.1	1.9 ± 0.1
Total phosphoruos (PO_4^{-2}–P) (mg L^{-1})	0.8 ± 0.3	0.9 ± 0.2
Phytosterols (μg L^{-1})	0.020	0.1082

COD chemical oxygen demand; *BOD_5* biological oxygen demand; *Mixed* mixed kraft mill effluent (50% *P. radiata* and 50% *E. globulus*)

Figure 1 shows the %AGR versus time. Samples with EE2 (positive control) show 17% and 27% reduction on the %AGR with respect to the blank at days 14 and 21 (Fig. 1c). In other words, the width of the abdominal region increased in relation to body length. A similar effect in relation to the blank was observed on *D. magna* exposed to pine KPME, both pure and enriched with phytosterols (P, Pβ, PS, PβS), and exposed to mixed KPME, both pure and enriched with the same phytosterols (M, Mβ, MS, MβS) ($p < 0.05$). However, when pine KPME was assayed alone (P) or enriched with specific phytosterols (Pβ, PS and PβS) the values of %AGR were lower than in pure mixed KPME (M) or its enriched combinations with phytosterols (Mβ,MS and MβS).

Although only the enriched combination with stigmasterol (PS vs MS) showed a statistical difference between both effluents ($p < 0.05$). The allometric effect of the tested phytosterols dissolved in culture medium was not the same. β-sitosterol was more effective than stigmasterol, and this difference was maintained when they were incorporated into the effluents.

Table 3 shows the first average brooding time, the brooding average and the average of total neonates during the assays. According to the results, the first brood average time is delayed when *D. magna* is exposed to either EE2, mixed KPME and also to phytosterols. The strongest effect was observed with mixed KPME alone (a delay of 12 d) compared to the blank (6.1 d). So, it seems that when the sample is more estrogenic, the first brooding time becomes longer. Thus, β-sitosterol (β: 10.9 $\pm$ 2.2 d), stigmasterol (S: 9.8 $\pm$ 1.6 d) or β-sitosterol + stigmasterol (βS: 10.0 $\pm$ 2.7 d) solutions in culture medium show values similar to the positive control and not too far from the mixed KPME (M). On the contrary, pine KPME assays (P, Pβ, PS and PβS) show a first brooding time which is closer to the blank but significantly different when β-sitosterol is involved ($p < 0.05$).

Similar trends were observed for the results for reproduction frequency and average total neonates (see Table 3). The total number of offspring for the positive control was 27.5 $\pm$ 9.5, which is 45% of the fecundity found in the normal condition (blank). Goto and Hiromi (2003) found a

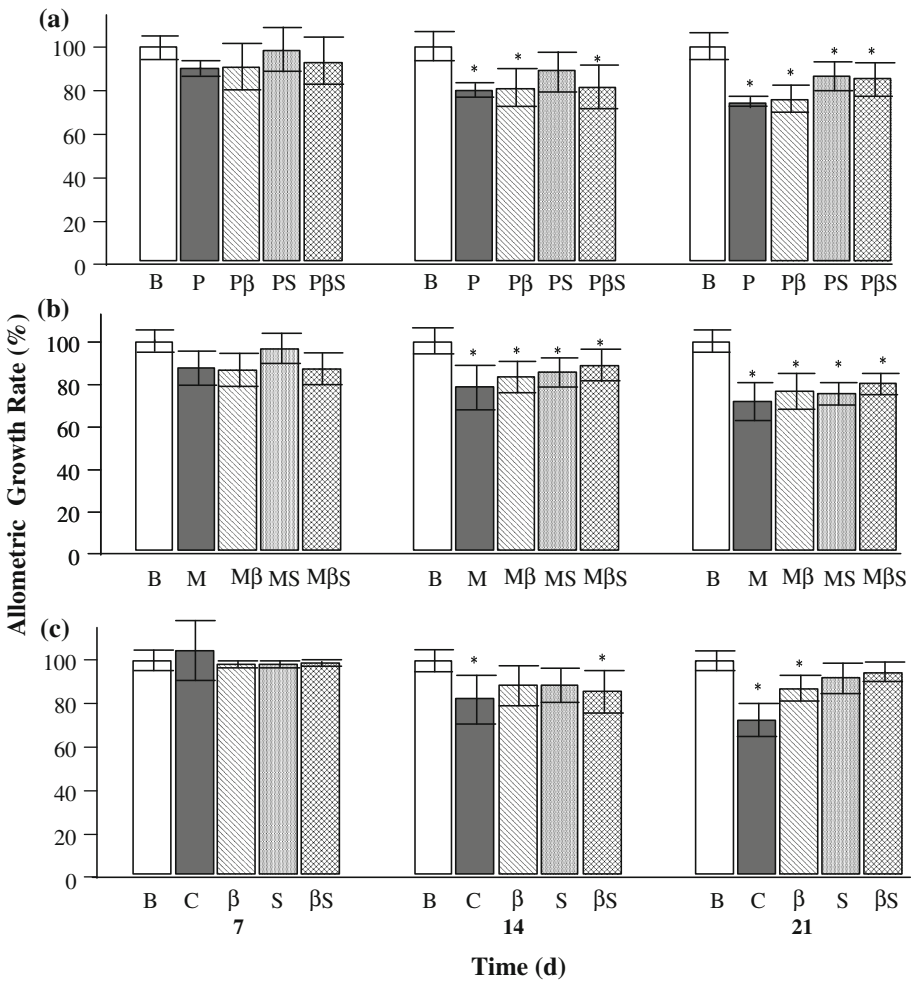

Fig. 1 *Daphnia magna* allometric growth rate at 7, 14 and 21 d with exposure to: **a** *P. radiata* kraft mill effluent (*P*); **b** mixed kraft mill effluent (*M*) and **c** water solution. Where for graphics *P*, *M* and Control (*C*) = *dark gray shaded bar*, effluents and water solution with β-sitosterol (β) = *striped bar*, with stigmasterol (*S*) = *light gray shaded bar* and β-sitosterol +stigmasterol (βS) = *crossed bar*; Blank (*B*) = *open bar*

Table 3 Brooding time and fecundity of *D. magna* exposed to kraft mill effluent and phythosterols

Sample	First average brooding time (d)	Reproduction frequency (n°)	Average of total neonates (n° offspring)
Blank	6.1 ± 0.3	6.4 ± 1.4	61.3 ± 3.5
Control	10.0 ± 0.3*	4.2 ± 1.0*	27.5 ± 9.5*
P	7.4 ± 0.5	5.6 ± 0.9	57.7 ± 8.4
M	12.6 ± 1.5*	4.1 ± 0.5*	25.3 ± 6.8*
Pβ	7.8 ± 1.0*	5.0 ± 0.4*	62.2 ± 4.6
PS	7.4 ± 0.8	5.4 ± 0.5	60.2 ± 5.6
PβS	8.0 ± 1.0*	5.4 ± 0.5	60.5 ± 3.6
Mβ	7.7 ± 2.3	3.6 ± 0.8*	25.6 ± 1.8*
MS	7.4 ± 0.8	3.1 ± 0.7*	23.4 ± 0.7*
MβS	9.2 ± 0.6*	3.4 ± 0.5*	22.5 ± 4.8*
β	10.9 ± 2.2*	3.5 ± 0.8*	26.3 ± 5.9*
S	9.8 ± 1.6*	4.3 ± 0.8*	24.2 ± 2.2*
βS	10.0 ± 2.7*	3.9 ± 0.8*	21.1 ± 1.3*

Data are presented as the mean ± SD; *significantly different from the blank $p \leq 0.05$

25% reduction in offspring with half the EE2 concentration used here. The same level of effect as EE2 at 1 mg L^{-1} was obtained in exposures to mixed KPME (M) and to phytosterols in the culture medium (β, S and βS) ($p > 0.05$). These findings corroborate the estrogenic potency of the mixed KPME. On the other hand, the results show no evidence of fecundity inhibition under pine KPME (P) exposure.

Adding phytosterols to the effluents did not significantly change (to the degree expected, at least) the exposure effects of the pure effluents on the three reproductive parameters recorded in this study. The insignificant effect of pine KPME on reproduction did not change with the incorporation of the estrogenic β-sitosterol and stigmasterol. The addition of phytosterols to the already estrogenic mixed KPME maintained its level of reproductive disruption on *Daphnia's* fecundity and reproductive frequency, or even the effect was significantly ($p < 0.05$) less intense than that of pure effluent, for example on the first brooding time (M > Mβ, MS, MβS). It has been found that the effect of dissolved toxic chemicals can be diminished by an increase in the content of organic matter (Nikkilä and Kukkonen 2001). The effluents' complex chemical composition, with a COD above 200 mg L^{-1}, is probably the cause of the exogenous phytosterols becoming non-bioavailable due to adsorption to the effluents' organic matter. But if the organic content of KPME inactivates exogenous phytosterols, the same would occur with the phytosterols carried by the effluent from the pulping process. This means that KPME phytosterols are not the main estrogenic molecules causing *D. magna* reproductive disruptions during exposure to KPMEs. This crustacean is sensitive to a variety of estrogenic compounds such as 17 β-oestradiol, diethylstilbestrol (DES), bisphenol A and 4-nonylphenol (Brennan et al. 2006) and KPMEs are rich in aromatic compounds with potential endocrine activity.

Our data support the previous determination of stronger estrogenic activity in eucalyptus-derived KPME than in pine KPME (Chamorro et al. 2010). The difference is probably based not on the total concentration of chemicals present in the effluents since pine KPME has higher values (Table 2), but on specific ones predominant in the eucalyptus effluent.

Effluent's factors capable of modifying the body proportion of *Daphnia* have the same power in pine and mixed KPME. Other molecules apart from β-sitosterol and stigmasterol must be contributing to the mentioned allometry. The phytosterols *per se* are responsible for 12.9% and 8.1% of the deviation from the natural shape, while the KPMEs account for 25.6%–27.8% of shape deviation.

The fact that an intense allometry was found in the exposure to pine KPME and that the same animals reached normal levels of reproduction suggest independence between both parameters. Therefore, the alteration of body shape apparently would not have consequences for the species perpetuation, although, such statement needs to be verified first through multigenerational studies in order to rule out longtime consequences on the population.

In conclusion, the estrogenic activity of KPMEs is dependent on the species processed for wood pulp. A mixture of *E. globulus* and *P. radiata* KPME has stronger effects on *D. magna* reproduction than *P. radiata* alone, but both kinds of effluent have an equally powerful effect in terms of distortion of body growth. Phytosterols, among other compounds, may interfere with body growth, but their role in mixed KPME reproductive alterations is less evident.

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References

Belmonte M, Xavier C, Decap J, Martínez M, Sierra R, Vidal G (2006) Improvement of the abietic acid biodegradation contained in ECF effluent due to biomass adaptation. J Hazard Mater 135:256–263

Brennan S, Brougham C, Roche J, Fogarty A (2006) Multigenerational effects of four selected environmental oestrogens on *Daphnia magna*. Chemosphere 64:49–55

Chamorro S, Xavier C, Vidal G (2005) Behaviour of aromatic compounds contained in the kraft mill effluents measurements by UV–VIS. Biotechnol Prog 21:1567–1571

Chamorro S, Hernández V, Monsalvez E, Becerra J, Mondaca MA, Piña B, Vidal G (2010) Detection of estrogenic activity from kraft mill effluents by yeast estrogen screen. Bull Environ Contam Toxicol 84:165–169

Goto T, Hiromi J (2003) Toxicity of 17a-ethynylestradiol and norethindrone, constituents of an oral contraceptive pill to the swimming and reproduction of cladoceran *Daphnia magna*, with special reference to their synergetic effect. Marine Pollut Bull 47:139–142

Hewitt LM, Parrott JL, McMaster ME (2006) A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: sources and characteristics of bioactive substances. J Toxicol Environ Health B 9:341–356

La Fleur LE (1996) Sources of pulping and bleaching derived chemical in effluents. In: Servos MR, Munkittrick KR, Carey JH, Van der Kraak GJ (eds) Environmental fate and effects of pulp and paper mill effluents. St Lucie Press, Delray Press, FL, USA, pp 21–31

Nikkilä A, Kukkonen JVK (2001) Effects of dissolved organic material on binding and toxicokinetics of Pyrene in the waterflea *Daphnia magna*. Arch Environ Contam Toxicol 40:333–338

Olmstead AW, LeBlanc GA (2000) Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. Environ Toxicol Chem 19:2107–2113

Orrego R, Guchardi J, Hernandez V, Krause R, Roti L, Armour J, Ganeshakumar M, Holdway D (2009) Pulp and paper mill effluent treatments have differential endocrine-disrupting effects on rainbow trout. Environ Toxicol Chem 28:181–188

USEPA (1994) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-91/002, Office of Research and Development, US Environmental Protection Agency, Washington, DC 206460

van den Heuvel M, Ellis R, Tremblay L, Stuthridge T (2002) Exposure of reproductive maturing rainbow trout to a New Zealand pulp and paper mill effluent. Ecotoxicol Environ Saf 51:65–75

Verta M, Ahtiainen J, Nakari T, Langi A, Talka E (1996) The effect of waste constituents on toxicity of TCF and ECF pulp bleaching effluents. In: Servos MR, Munkittrick KR, Carey JH, Van der Kraak GJ (eds) Environmental fate and effects of pulp and paper mill effluents. St Lucie Press, Delray Press, FL, USA, pp 41–51

Vidal G, Diez MC (2005) Methanogenic toxicity of wood processing effluents. J Environ Manage 74:317–325

Xavier C, Chamorro S, Vidal G (2005) Chronic effects of kraft mill effluents and endocrine active chemical on *Daphnia magna*. Bull Environ Contam Toxicol 75:670–676